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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization and application of fusidane antibiotic biosynethsis enzyme 3-ketosteroid-Δ^1-dehydrogenase in steroid transformation

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Abstract Microbial Δ^1-dehydrogenation is one of the most important transformations in the synthesis of steroid hormones. In this study, a 3-ketosteroid-Δ^1-dehydrogenase ($kstD_F$) involved in fusidane antibiotic biosynthesis from *Aspergillus fumigatus* CICC 40167 was characterized for use in steroid transformation. $KstD_F$ encodes a polypeptide consisting of 637 amino acid residues. It shows 51% amino acid identity with a kstD from *Thermomicrobium roseum* DSM 5159. Expression of $kstD_F$ in *Escherichia coli* and *Pichia pastoris* showed that all $kstD_F$ activity is located in the cytoplasm. This indicates that it is a soluble intracytoplasmic enzyme, unlike most kstDs from bacteria, which are membrane-bound. The expression of $kstD_F$ was performed in *P. pastoris*, both intracellularly and extracelluarly. The intracellularly expressed protein displayed good activity in steroid transformation, while the extracellularly expressed protein showed nothing. Interestingly, the engineered *P. pastoris* KM71 ($KM71_I$) and GS115 ($GS115_I$) showed different transformation activities for 4-androstene-3,17-dione (AD) when $kstD_F$ was expressed intracellularly. Under the same conditions, $KM71_I$ was found capable of transforming 1.0 g/l AD to 1,4-androstadiene-3,17-dione (ADD), while $GS115_I$ could transform 1.5 g/l AD to both ADD and boldenone (BD). The production of BD is attributed to a 17β-hydroxysteroid dehydrogenase in *P. pastoris* $GS115_I$, which catalyzes the reversible reaction between C17-one and C17-alcohol of steroids. The conversion of AD by $GS115_I$ and $KM71_I$ may provide alternative means of preparing ADD or BD. In brief, we show here that $kstD_F$ is a promising enzyme in steroid Δ^1-dehydrogenation that is propitious to construct genetically engineered steroid-transforming recombinants by heterologous overexpression.

Keywords 3-Ketosteroid-Δ^1-dehydrogenase · Steroidal antibiotic fusidanes · Steroid transformation · 4-Androstene-3,17-dione · 1,4-Androstadiene-3,17-dione · Boldenone

Introduction

Microbial transformations play irreplaceable roles in the synthesis of steroid hormones. Among these, Δ^1-dehydrogenation is one of the most crucial transformations. The introduction of C1,2 double bonds to some steroid hormones, such as adrenocortical hormones, may greatly increase their activities and decrease their side effects (Abul-hajj 1972). The Δ^1-dehydrogenation of steroids by organisms is carried out by 3-ketosteroid-Δ^1-dehydrogenases (kstDs) [4-ene-3-oxosteroid: (acceptor)l-ene-oxidoreductase, EC 1.3.99.4]. These are flavoproteins functioning in the conversion of 4-ene-3-oxosteroid to 1,4-diene-3-oxosteroid by trans-axial elimination of the Cl and C2 hydrogens (Itagaki et al. 1990). The transformation of 4-androstene-3,17-dione (AD) to 1,4-androstadiene-3,17-dione (ADD) is one example of this.

In general, isolated microorganisms with good kstD activity are bacteria, mainly actinobacteria. The physiological role of kstDs in these microorganisms involves the use of steroids as carbon sources. They initiate cleavage of the nucleus of steroids by the introduction of a double bond into

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ring A (Chiang et al. 2008; Florin et al. 1996). Therefore, these microorganisms are usually not good biocatalysts of steroids Δ^1-dehydrogenation due to their powerful steroid-degradation capacities as well as the other side reactions (Chiang et al. 2008; Donova 2007; Szentirmai 1990). This is why molecular characterization and heterologous expression of kstDs have attracted such attention as means of creating recombinant kstD bacteria (Choi et al. 1995; Li et al. 2007; Morii et al. 1998; Plesiat et al. 1991). KstDs have been isolated and characterized from many actinobacteria, including *Pseudomonas* sp., *Nocardia* sp., *Rhodococus* sp., *Mycobacterium* sp., and *Arthrobacter* sp. So far, however, all of the kstDs found in bacteria have been membrane-bound and localized in the inner membrane fraction by two hydrophobic transmembrane domains. These include kstDs from *Arthrobacter simplex*, *Pseudomonas testosterone*, *Rhodococcus erythropolis*, and *Mycobcterium* sp. (Li et al. 2007; Molnar et al. 1995; Plesiat et al. 1991; Wagner et al. 1992). Many attempts have been made to express these kstDs heterologously in *Escherichia coli*, *Streptomyces lividans*, and *Bacillus subtilis*, and some experiments have achieved a 30 to 100-fold increases in kstD production over the original strains (Choi et al. 1995; Li et al. 2007; Morii et al. 1998; Plesiat et al. 1991). However, these expressions did not show themselves to be successful upon application. Expressed in *E. coli*, these bacterial kstDs were found to consist mainly of inclusion bodies, and only a trace activity was detected in the membrane (Li et al. 2007; Plesiat et al. 1991; Wagner et al. 1992). The secretory overexpression systems of *S. lividans* and *B. subtilis* were also employed to produce a kstD from *A. simplex* extracellularly. The results of this attempt indicated that the *A. simplex* kstD is produced intracelluarly instead of extracellularly (Li et al. 2007; Choi et al. 1995). The expression of the *A. simplex* kstD in *B. subtilis* showed itself to be the only version of the protein suitable to steroid conversion in practice. It could transform 1.0 g/l AD to ADD at a 45.3% conversion rate (Li et al. 2007). As well known, membrane-bound proteins are not easy to overexpress in their functional forms in heterologous hosts. Therefore, the membrane-bound attribute of these bacterial kstDs is likely to be what limits their heterogenous overexpression so seriously (Wagner et al. 1992; Freigassner et al. 2009; Linares et al. 2010).

In the course of our research on fusidane antibiotic biosynthesis in *Aspergillus fumigatus*, we discovered a soluble kstD ($kstD_F$) responsible for Δ^1-dehydrogenation in steroidal fusidane antibiotic biosynthesis. This may be a better kstD than bacterial kstDs for heterologous expression and application in steroid transformation. $KstD_F$ showed no obvious hydrophobic transmembrane domains, and it can be expressed as a soluble enzyme in *E. coli* and *Pichia pastoris*. The recombinant *P. pastoris* with overexpressed $kstD_F$ is promising in the conversion of AD to ADD or boldenone (BD).

Materials and methods

Strains, plasmids, media, and chemicals

A. fumigatus CICC 40167 was obtained from the China Center of Industrial Culture Collection (CICC, Beijing, China). *E. coli* BL21 (λDE3) and pET-28a(+) were from Merck Biosciences (Germany). *P. pastoris* GS115, *P. pastoris* KM71, and expression vectors pPIC3.5 k and pPIC9k were from Invitrogen (USA). Restriction endonucleases, T4 DNA ligase, and DNA polymerase were from Takara (Japan). Steroids including 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione, testosterone, and boldenone were from Sigma (USA). *E. coli* strains were cultured in Luria-Bertani (LB) medium (Difco, Detroit, MI, USA). Buffered gylcerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY) were used for the growth and heterologous protein expression of *P. pastoris* strains. The BMGY was composed of 2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base (w/o amino acid (AA)), 0.4 μg/ml biotin, and 1% glycerol. The BMMY was composed of 2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base (w/o AA), 0.4 μg/ml biotin, and 0.5% methanol.

Heterologous expression of $kstD_F$

According to the available genome sequence of *A. fumigatus* Af293 (GenBank AAHF00000000), primers *kstD*-U_E (GAATTCCCTGATGCTGGAGATGGG) and *kstD*-D_E (AAGCTTGACCTTGCCGTGGTTGTT) were designed to amplify the entire open reading frame (ORF) of $kstD_F$ from *A. Fumigatus* CICC 40167. The PCR product was ligated into the pMD19-T vector according to the manufacturer's instructions (Takara). Then, the $kstD_F$ gene was digested with *Hind*III/*Eco*RI and ligated into the expression vector pET-28a(+), creating pET-28a(+)-$kstD_F$. The resulting recombinant plasmid was then used to transform *E. coli* BL21 (λDE3) according to the manufacturer's instructions, creating BL21-pET28a-$kstD_F$.

The entire ORF of $kstD_F$ with the *Eco*RI/*Not*I sites was cloned with primers *kstD*-U_P (GAATTCATGGCCGCAA GACAGCTC) and *kstD*-D_P (GCGGCCGCCTATACATGCT CAGAAGCAATAT). The resulting fragment digested with *Eco*RI and *Not*I was ligated into the pPIC3.5 K and pPIC9K, resulting in the recombinant pPIC3.5 K-$kstD_F$ and pPIC9K-$kstD_F$. Both pPIC3.5 K-$kstD_F$ and pPIC9K-$kstD_F$ were linearized with *Sal*I and then electrotransformed (2.5 kV, 25 μF, 1000Ω) into *P. pastoris* KM71 and GS115 cells. High-level expression transformants were screened from YPDS plates containing Geneticin at a final concentration of 0.5 g/l. These were named $KM71_I$, $GS115_I$, $KM71_E$, and $GS115_E$.

The expression of kstD$_F$ in *E. coli* BL21(λDE3) was performed in 30 ml LB per 250 ml Erlenmeyer flask at 37 °C with shaking at 200 rpm. When the optical density at 600 nm (OD_{600}) reached a value of 0.5–0.6, IPTG was added to a final concentration of 0.5 mM. The cells were then grown for another 8 h with shaking at 200 rpm at different induction temperatures: 20 °C, 30 °C, and 37 °C. Thirty milliliters of culture was collected, centrifuged for cells, and resuspended in 50 mM Tris-HCl (pH 7.0) to adjust OD_{600} to 4.0. Resuspendant was prepared for the subsequent sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) using *E. coli* BL21(λDE3) transformed with pET-28a(+) as a control (BL21-pET28a).

The expression of kstD$_F$ in *P. pastoris* was conducted according to the protocol described by Handumrongkul et al. (1998). The intracellular expression cell lysate and the extracellular expression culture supernatant were analyzed separately by SDS-PAGE.

Whole-cell steroid biotransformation

The cultures of BL21-pET28a-*kstD$_F$* grown in LB medium and induced at 20 °C, 30 °C, and 37 °C were incubated with AD at a final concentration of 0.1 g/l for 4 h at 30 °C with shaking at 200 rpm. The reaction mixture was extracted three times with the same volume of ethyl acetate. The organic phases were collected and detected by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) using BL21-pET28a as a control.

KM71$_I$ and GS115$_I$ were cultured for 20–24 h at 30 °C in BMGY medium (30 ml per 250 ml flask) with shaking at 200 rpm. The cells were harvested and resuspended in BMMY medium (30 ml per 250 ml flask) for protein induction. After 6 days, AD dissolved in methanol was added into the BMMY medium to a final concentration of 0.25 g/l. The reaction mixtures were incubated at 30 °C for 2 h and 4 h, respectively, and then extracted for TLC and HPLC analysis. *P. pastoris* KM71 and GS115 were used as controls.

The culture process for KM71$_E$ and GS115$_E$ was identical to that used for KM71$_I$ and GS115$_I$. After 6 days of induction in BMMY medium, the whole cell and the culture supernatants of KM71$_E$ and GS115$_E$ were collected, respectively, and used to transform AD. AD dissolved in methanol was added to a final concentration of 0.25 g/l, and phenazine methosulfate (PMS) (dissolved in 50 mM Tris-HCl buffer, pH 7.0) as an exogenous electronic acceptor for kstD$_F$ was added to final concentrations of 0.013 g/l and 0.039 g/l for KM71$_E$ and GS115$_E$, respectively. The reaction mixtures were incubated at 30 °C for 4 h and then extracted for TLC and HPLC analysis. The culture supernatants of *P. pastoris* KM71 and GS115 were used as controls.

To test the AD conversion capabilities of KM71$_I$ and GS115$_I$, AD dissolved in methanol was added to the BMMY cultures with substrate concentration gradients of 1 g/l, 1.5 g/l, 2 g/l, and 2.5 g/l. The reaction mixtures were incubated at 30 °C for 6 days and then extracted three times with the same volume of ethyl acetate. Samples were treated as described previously for TLC and HPLC analysis. *P. pastoris* KM71 and GS115 were set as controls.

Analytical methods

Steroid transformation was analyzed by TLC and HPLC. TLC was performed on a TLC plate (HSGF254, Qingdao Marine Chemical Factory, China), developed by petroleum ether/ethyl acetate (6:4 *v*/*v*), and visualized by spraying with 20% H_2SO_4 and heating at 100 °C for about 5 min until the colors developed.

HPLC was carried out with an Agilent 1100 instrument (Agilent Technologies, USA). The chromatographic conditions were as follows: an Agilent XDB-C18 column (4.6× 250 mm) with a constant temperature of 40 °C, a mobile phase of methanol/water (70:30), and a flow rate of 1 ml/min. Analyses were performed simultaneously with UV detection at 254 nm. Liquid chromatography–mass spectrometry (LC–MS) was performed with an Agilent 1100 LC/MSD apparatus (Agilent Technologies, USA) with conditions as follows: electron spray impact; ion spray voltage, 30 v; ion source temperature, 100 °C; and desolvation temperature, 300 °C.

Sequence analysis and multiple sequence alignments were performed using Clustal X2.1 (Larkin et al. 2007). Analysis with basic local alignment search tool (BLAST) was conducted on a NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). The transmembrane regions and orientation were predicted by TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). The theoretical molecular mass and isoelectric point (pI) of the protein were calculated using the ProtParam tool (http://us.expasy.org/tools/protparam.html).

Isolation of subcellular fractions and localization of the soluble kstD$_F$

The cellular distribution of kstD$_F$ in BL21-pET28a-kstD$_F$, KM71$_I$, and GS115$_I$ was determined by measuring the kstD activity in different subcellular fractions. Cells were collected by centrifugation at 4 °C (10 min, 5,000×*g*). Precipitates were then dissolved in 50 mM Tris-HCl (pH 7.0). *E. coli* cells were disrupted by sonication for 5 min, while *P. pastoris* cells were broken by high-pressure cracking. The cell debris were removed by centrifugation at 4 °C (30 min, 10,000×*g*). Membranes were sedimented by high-speed centrifugation (1 h, 100,000×*g*) and subsequently resuspended in 50 mM Tris-HCl (pH 7.0). Proteins in the 100,000×*g* supernatant were defined as soluble proteins. Each of these fractions was then assayed for the kstD

activity, which was dissolved in Tris-HCl buffer and cultivated with substrate AD and electronic acceptor PMS at final concentrations of 0.013 g/ml and 0.039 mg/ml, respectively, at 30 °C for 4 h. Equal volumes of ethyl acetate were added three times to extract the steroids. The phase of ethyl acetate was collected and used for TLC and HPLC analyses.

Purification and characterization of $kstD_F$

To determine the kinetic parameters of $kstD_F$ in *E. coli*, Ni^{2+}-chelating sepharose column was used to purify the targeted protein. BL21-pET28a-$kstD_F$ cells were lysed by sonication. The cell lysate was centrifuged at 10,000×*g* for 20 min, and the supernatant was filtered through 0.45-μm membranes. The flow-through was then applied to a 15-ml Ni^{2+}-chelating Sepharose Fast Flow column (Amersham Biosciences, USA) which had been pre-equilibrated with buffer A (5 mM imidazole, 0.5 M NaCl, 20 mM phosphate buffer, pH=8.0). The protein was eluted with buffer B (200 mM imidazole, 0.5 M NaCl, 20 mM phosphate buffer, pH=8.0), followed by a 5-ml Hitrap desalting column (Amersham Biosciences) to remove salts. The desalting column was used according to the manufacturer's instructions. The concentration of $kstD_F$ protein was estimated by UV absorption and the bandscan method.

To determine the kinetic parameters of $kstD_F$ expressed in *P. pastoris*, the $kstD_F$ protein was purified by hydrophobic interaction chromatography column with conditions as follows: HiPrep 16/10 Phenyl FF (high sub) column, AKTA explorer (Amersham Biosciences); mobile phase, 20 mM Tris-HCl (pH 7.0) containing 1 M $(NH_4)_2SO_4$; and elution, 20 mM Tris-HCl (pH 7.0) containing 0.2 M $(NH_4)_2SO_4$.

The $kstD_F$ enzyme activity assay was performed as described previously (van der Geize et al. 2002). One unit is defined as the amount of enzyme capable of transforming of 1 mM AD to ADD in 1 min.

Determination of the derivatives of AD and ADD

Two derivatives of AD and ADD were detected in the fermentation broth of $GS115_I$, when $GS115_I$ was added with 2.5 g/l AD. Under the 254 nm UV light, we located the two steroids on silica gel TLC plates, scraped them from silica gel TLC plates, and then extracted them with ethyl acetate. The extract was evaporated and resuspended in methanol, which was analyzed by HPLC and LC–MS.

Results

$KstD_F$ from *A. fumigatus* CICC 40167

Antibiotic fusidanes are a category of steroidal secondary metabolites. They have been detected in about 20 fungal species of the *Pezizomycotina*. Helvolic acid is a typical fusidane. It has a C1,2-double bond and *A. fumigatus* is a known producer. In 2009, a gene cluster encoding helvolic acid biosynthesis in *A. fumigatus* Af293 was discovered by two separate labs. This cluster includes the gene Afu4g14850, which is speculated to encode a kstD responsible for the C1,2-double bond in helvolic acid (Lodeiro et al. 2009; Mitsuguchi and Seshime 2009).

It has been demonstrated that the clusters of helvolic acid and the other fusidanes are distinguished for lack of introns (Lodeiro et al. 2009). Thus, the complete ORF of kstD (*$kstD_F$*) (gene accession number: XM_746255.1) was directly cloned from *A. fumigatus* CICC 40167 according to the sequence of Afu4g14850 from the genome sequence of *A. fumigatus* Af293 (Nierman et al. 2005). The *$kstD_F$* is 99% identical to Afu4g14850, which consists of 1,791 nucleotides and encodes a deduced protein of 597 amino acids. The molecular weight of $kstD_F$ was estimated to be 64.37 kDa, and the pI value was calculated to be 7.81. A BLASTp search indicated that the deduced amino acid sequence of *$kstD_F$* shared the highest identity, 51%, with the kstD from *Thermomicrobium roseum* (Genbank P12676). No other kstD from fungi had been deposited in GenBank. The BLASTp search also showed that the deduced amino acid sequence of *$kstD_F$* shared lower similarities with well-defined bacterial kstDs from *Rhodococcus rhodochrous* (GenBank AB007847), *A. Simplex* (GenBank D37969.1), and *M. neoaurum* (GeneBank GQ228843) (34%, 36%, and 44%, respectively) (Molnar et al. 1995; Morii et al. 1998; Wei et al. 2010). Sequence comparison of $kstD_F$ with these bacterial kstDs (Fig. 1) revealed that residues 299 through 320 are an active center, and the putative N-terminal flavin adenine dinucleotide (FAD)-binding motif in $kstD_F$ is consistent with the sequence previously described: GSG(A/G)(A/G)(A/G)X17E (Wierenga et al. 1986). In the active site, the conserved residues Arg-320 in $kstD_F$ (Arg-242 in the *A. simplex* kstD) have been implicated to be involved in substrate binding, while the highly conserved residues Glu-318 (Glu-240 in the *A. simplex* kstD, Glu-239 in the *R. rhodochrous* kstD and Glu-282 in the *M. neoaurum* kstD) regarded as electrophilic zones are substituted by Gly-318 in $kstD_F$ (Molnar et al. 1995).

Bacterial kstDs were reported to be membrane-bound proteins localized in the inner membrane fraction by two hydrophobic transmembrane domains (Plesiat et al. 1991; Molnar et al. 1995; Wagner et al. 1992). A kstD from *A. simplex* was found to have two long hydrophobic stretches that form transmembrane domains (amino acids 193–214 and 379–401) (Fig. 1) (Molnar et al. 1995). The kstD from *M. neoaurum* NwIB-01 was predicted to possess two transmembrane domains by TMpred program analysis (amino acids 11–31 and 544–561) (Wei et al. 2010). For a kstD from *R. rhodochrous*, the hydrophobic amino acids, residues

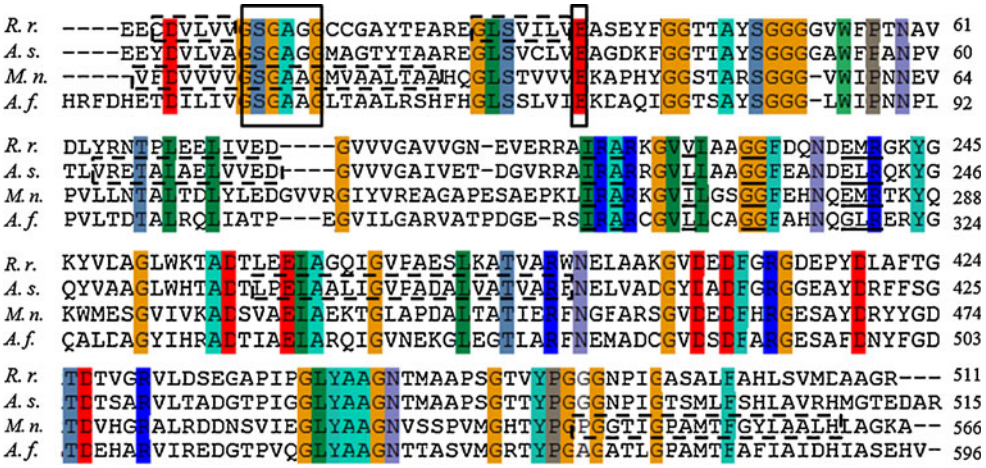

Fig. 1 Sequence alignment analysis of kstD$_F$ against well-defined kstDs. The consensus amino acid residues for FAD binding and the active center are shown in the *solid line box* and *underline*, respectively. Hydrophobic stretches that may form transmembrane domains are shown in the *dotted line box*. *A. fumigatus* (*A. f.*), *R. rhodochrous* WO3338 (*R. r.*), *A. simplex* IFO 12069 (*A. s.*), and *M. neoaurum* NwIB-01 (*M. n.*)

8–13, and residues 30–36 were found to form the β/α/β hydrophobic structure (Morii et al. 1998). However, the TMpred program predicted that kstD$_F$ would contain neither typical signal-anchor sequences nor obvious hydrophobic transmembrane stretches, implying that kstD$_F$ may be not a membrane-bound protein.

Expression and characterization of kstD$_F$ in *E. coli* BL21(λDE3)

To characterize kstD$_F$, the expression of kstD$_F$ was first carried out in *E. coli* BL21(λDE3) by the expression vector pET-28a(+) to generate the expression strain BL21-pET28a-*kstD$_F$*. SDS-PAGE results showed an obvious protein band close to the estimated molecular weight of kstD$_F$ in the protein profile of BL21-pET28a-*kstD$_F$* (Fig. 2a). Compared to *E. coli* BL21(λDE3) with pET-28a(+), BL21-pET28a-*kstD$_F$* displayed good kstD activity and was capable of transforming AD to ADD (Fig. 2b). The expression and activity of kstD$_F$ in BL21-pET28a-*kstD$_F$* were affected by culture temperatures. As shown in Fig. 2, the expression level of kstD$_F$ increased progressively as the temperature increased from 20 °C to 30 °C to 37 °C (Fig. 2a). However, the activity of kstD$_F$ decreased progressively as temperature increased (Fig. 2b). The cell culture induced and cultured at 20 °C was capable of transforming 0.015 g/l AD to ADD at a 100% conversion rate in 4 h. In the same conditions, although the amount of kstD$_F$ induced and cultured in 37 °C was many times higher than that induced and cultured at 20 °C, the conversion rate of 0.015 g/l AD to ADD by the cell culture induced and cultured at 37 °C was no more than 50%. Therefore, low induction temperature was beneficial to the expression and maintenance of kstD$_F$ activity, and excessive expression of kstD$_F$ at higher culture temperatures could result in the inactivation of most of the kstD$_F$. This implied that *E. coli* BL21(λDE3) was not an optimal host for kstD$_F$ expression.

Given that the activity of kstD$_F$ expressed in *E. coli* BL21 (λDE3) suffered great losses as culture temperature increased, the kstD$_F$ expressed at 20 °C was selected for characterization. The kstD$_F$ protein was purified by Ni^{2+}-chelating chromatography. The Michaelis constant (K_m) of kstD$_F$ for AD was determined to be 191 μM.

Almost of all the kstD$_F$ activity of BL21-pET28a-*kstD$_F$* could be sedimented by centrifugation. This indicated that kstD$_F$ was located intracellularly in BL21-pET28a-*kstD$_F$*. To determine whether kstD$_F$ might be a membrane-bound protein, the activity distribution of kstD$_F$ in BL21-pET28a-*kstD$_F$*

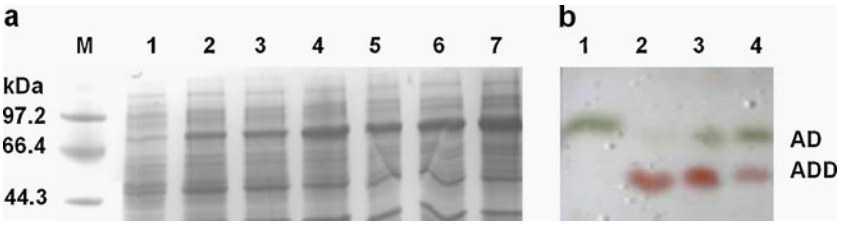

Fig. 2 Protein expression and kstD$_F$ activity for AD transformation by BL21-pET28a-*ksdD$_F$*. **a** SDS-PAGE profile of protein. *Lane 1*: *E. coli* BL21(λDE3) with pET-28a(+) as a control incubated and cultured at 37 °C for 8 h. *Lanes 2–3*: BL21-pET28a-ksdD$_F$ induced and cultured at 20 °C for 8 h. *Lanes 4–5*: BL21-pET28a-ksdD$_F$ induced and cultured at 30 °C for 8 h. *Lanes 6–7*: BL21-pET28a-ksdD$_F$ induced and cultured at 37 °C for 8 h. The upload volume of samples *1–7* was 10 μl. **b** TLC chromatogram of AD transformation for 4 h. *Lane 1*: AD transformed by *E. coli* BL21(λDE3) with pET-28a(+) as a control. *Lane 2*: AD transformed by BL21-pET28a-ksdD$_F$ induced and cultured at 20 °C. *Lane 3*: AD transformed by BL21-pET28a-ksdD$_F$ induced and cultured at 30 °C. *Lane 4*: AD transformed by BL21-pET28a-ksdD$_F$ induced and cultured at 37 °C. Samples *1–4* were all 0.015 g/l AD transformed by equal volumes of cell culture

cell was investigated by separating subcellular fractions and determining the kstD$_F$ activity. The cell extract prepared from BL21-pET28a-*kstD$_F$* was fractionated by fractional centrifugation, and the distribution of the kstD$_F$ in different fractions was examined. Of the total kstD activity, a major part (97%) resided in the cytosolic fraction, while only 3% was present in the membrane fraction (Table 1), indicating that kstD$_F$ is a soluble protein. Significantly, the activity distribution of kstD$_F$ in the *E. coli* expression host was entirely different from those of kstDs from *P. testosterone* and *N. restrictus* (Plesiat et al. 1991; Sih and Bennet 1962) (Table 1). This confirmed the TMpred prediction that kstD$_F$ would be soluble rather than membrane-bound.

Expression and characterization of kstD$_F$ in *P. pastoris*

The soluble expression of kstD$_F$ in *E. coli* implied that the kstD$_F$ may be more suitable for heterogenous expression than bacterial kstDs. For this reason, eukaryotic hosts *P. pastoris* strain KM71 and strain GS115 were then chosen to overexpress the fungi kstD$_F$. pPIC3.5 K, a powerful intracellular expression plasmid, was firstly selected. The recombinant kstD$_F$ strains were designated as KM71$_I$ and GS115$_I$. The expression profiles of KM71$_I$ and GS115$_I$ showed that the expressed kstD$_F$ was larger than 66 kDa (Fig. 3a). To test kstD$_F$ activity, KM71$_I$ and GS115$_I$ were cultured for 6 days and selected to transform AD. In both cases, 0.25 g/l AD was entirely transformed to products for no more than 4 h (Fig. 3b). This indicated a more powerful transformation capacity than BL21-pET28a-*kstD$_F$*.

As shown in Fig. 3b, KM71$_I$ and GS115$_I$ transformed AD to different products. This may be attributed to the original AD transformation capacity of *P. pastoris* GS115 (Fig. 3b) and KM71 (data not shown). One AD product produced by *P. pastoris* GS115 and KM71 developed as celadon spots on TLC and was later identified as testosterone (TS) by LC–MS and an authentic sample. Another product, produced by GS115$_I$, developed as brick-red spots on TLC in AD transformation and was identified as boldenone (BD). The AD transformed by GS115$_I$ was further analyzed by HPLC. The HPLC profiles of the samples transformed by GS115$_I$ for 2 h showed four chemical peaks: ADD (t=7.5 min), boldenone (t=10.1 min), AD (t=11.2 min), and testosterone (t=14.2 min) (Fig. 3c). The HPLC profiles of the samples transformed by GS115$_I$ for 4 h showed no remaining TS. The HPLC profiles also showed that the transformation of AD by KM71$_I$ produced mainly ADD without BD, and only trace TS was detected in the sample after 2 h, none after 4 h.

Structurally, biotransformation from AD to TS is usually carried out by 17β-hydroxysteroid-dehydrogenases (17Hsd), which are widespread in microorganisms such as yeasts. The 17Hsds have been characterized as reversible redox enzymes that can transform the reversible reduction/oxidation between C17-one and C17-alcohol of steroids (Donova et al. 2005). Therefore, the transformation relationship between AD, ADD, TS, and BD by GS115$_I$ can be described as shown in Fig. 3d. This was further verified by the transformation of ADD and TS to BD by GS115$_I$.

Considering that kstD$_F$ was well expressed intracellularly in *P. pastoris*, the extracellular expression of kstD$_F$ in *P. pastoris* becomes more interesting because it might be more powerful for steroid transformation. pPIC9K, a derived plasmid of pPIC3.5 K that expresses proteins extracellularly, was then used to express kstD$_F$ in *P. pastoris* KM71 and GS115. The recombinant kstD$_F$ strains were designated as KM71$_E$ and GS115$_E$, respectively. The expression profiles of KM71$_E$ and GS115$_E$ were consistent with those of kstD$_F$ expressed in KM71$_I$ and GS115$_I$. Surprisingly, KM71$_E$ and GS115$_E$ showed no kstD$_F$ activity for AD transformation. To further confirm the solubility of kstD$_F$ in *P. pastoris* KM71$_I$ and GS115$_I$, cells of KM71$_I$ and GS115$_I$ were fractionated, and each of these fractions was assayed for kstD$_F$ activity. The results of AD transformation demonstrated that both kstD$_F$s from KM71$_I$ and GS115$_I$ were indeed active as soluble proteins: the distribution of kstD$_F$ activity in the cytosolic fraction accounted for 98% and 99% of the total kstD$_F$ activity for KM71$_I$ and GS115$_I$, respectively. The distinct performances of intracellular and extracellular kstD$_F$ expressed in *P. pastoris* indicated that kstD$_F$ is not suitable for extracellular expression in *P. pastoris*.

To further characterize the activity of kstD$_F$ expressed in *P. pastoris*, kstD$_F$ was purified from GS115$_I$ by hydrophobic interaction chromatography. Its activity was determined to be 51.68 U/mg. Its K_m value was determined to be 47 μM, lower than the K_m value (82 μM) of kstD1 from *R. erythropolis* (van der Geize et al. 2002). The K_m value of kstD$_F$ from *P. pastoris* is only a quarter of that from *E. coli*, indicating that *P. pastoris* is the more suitable host for kstD$_F$ expression.

Application of KM71$_I$ and GS115$_I$ in the transformation of AD

Steroids are minimally water-soluble. This seriously retards the efficiency of steroid biotransformation because of

Table 1 Activity distribution of different 3-ketosteroid-Δ^1-dehydrogenases expressed in *E. coli*

Cellular fractions	Enzyme activities (%)		
	kstD$_F$ from *A. fumigatus*	kstD from *P. testosteroni*[a]	kstD from *N. restrictus*[b]
Cytosolic soluble proteins	97	3.3	10
Membrane fraction	3	95	90

[a] Reported by Plesiat et al. (1991)

[b] Reported by Sih and Bennet (1962)

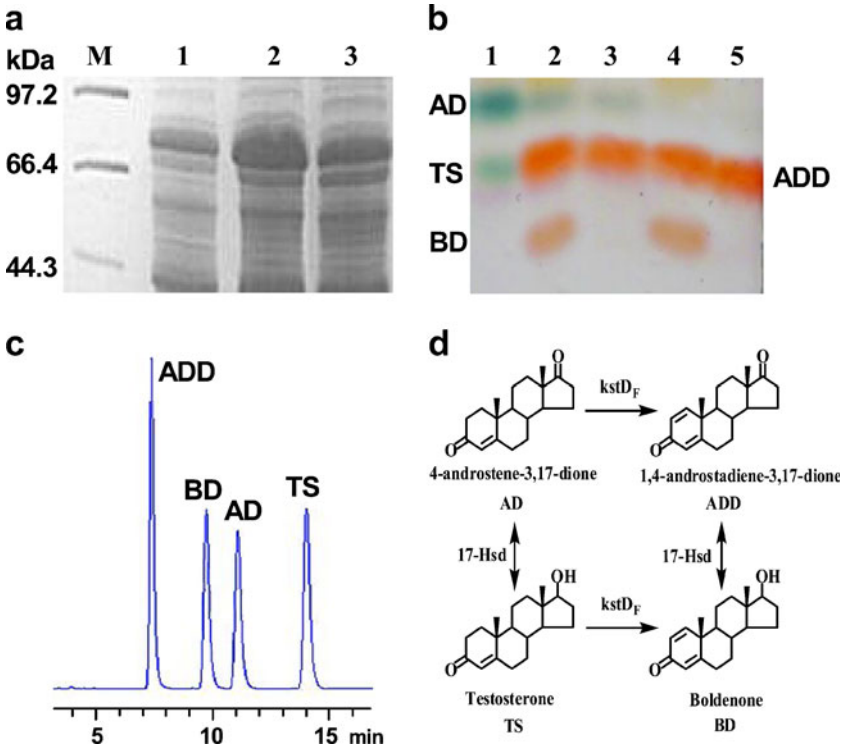

Fig. 3 Protein expression and kstD$_F$ activity for AD transformation by KM71$_I$ and GS115$_I$. **a** SDS-PAGE protein profile. *Lane 1*: wild-type *P. pastoris* KM71 cultured for 6 days after induction. *Lane 2*: KM71$_I$ cultured for 6 days after induction. *Lane 3*: GS115$_I$ cultured for 6 days after induction. **b** TLC chromatogram of AD transformation. *Lane 1*: AD transformed by wild-type GS115 for 2 h. *Lane 2*: AD transformed by GS115$_I$ for 2 h. *Lane 3*: AD transformed by KM71$_I$ for 2 h. *Lane 4*: AD transformed by GS115$_I$ for 4 h. *Lane 5*: AD transformed by KM71$_I$ for 4 h. **c** HPLC analysis of AD conversion by GS115$_I$ for 2 h. **d** Putative transformation relationship between AD, ADD, TS, and BD as assessed by GS115$_I$

inadequate mass transfer. Therefore, strategies need to be developed to improve their dispersity and solubility in reaction media (Malaviya and Gomes 2008). *P. pastoris* is a methylotrophic yeast that can use menthol as a carbon source. In addition, menthol is the powerful inducer for kstD$_F$ overexpression under the control of methanol-inducible promoter in pPIC3.5 K. In this study, therefore, menthol can be just used as a good solvent to facilitate the addition of AD to the reaction medium. In combination with the classical cultivation process of *P. pastoris*, the transformation process of AD by KM71$_I$ or GS115$_I$ was designed as follows: KM71$_I$ and GS115$_I$ were precultivated at 30 °C, 200 rpm for 20–24 h in BMGY medium. Then AD dissolved in methanol was added (final methanol concentration, 0.5%). Over the following 6 days, the same amount of methanol (0.5% final concentration) was added every other day.

KM71$_I$ and GS115$_I$ showed different AD conversion capacities. In shake flask fermentation process (30 ml medium per 250 ml flask), KM71$_I$ could transform 1.0 g/l AD into ADD at a 100% conversion rate, while GS115$_I$ could completely transform 1.5 g/l AD to produce ADD and BD simultaneously (Fig. 4a). Although TS was produced from 1.0 g/l AD transformation by *P. pastoris* strain GS115 (Fig. 4b) and strain KM71 (data not shown), both the 1.0 g/l AD transformation by KM71$_I$ and the 1.5 g/l AD transformation by GS115$_I$ showed no TS production (Fig. 4b). Further analysis indicated that the transformation of AD by KM71$_I$ did not foster the accumulation of TS as an end product, though little TS was detected on the first day after the addition of AD. In contrast, TS was detected when above 1.5 g/l AD was transformed by GS115$_I$ (Fig. 4b).

BD is an important steroid. To enhance the proportion of BD in the end products of AD transformation by GS115$_I$, glucose as a provider of reducing power was added to the GS115$_I$ fermentation process. Moderate concentrations of glucose were added after 4 days of induction by methanol. Then the GS115$_I$ culture was continued for another 3 days. The results indicated that glucose significantly enhanced the transformation of AD to BD. It showed that 3% glucose was optimal for BD production, resulting in 75% BD in the end products (Fig. 5). In the glucose-free control, BD made up of only 41%.

Discussion

Generally, bacterial kstDs are known as key enzymes functioning in the initial stage of steroid catabolism. These are widespread in the microorganisms that can utilize steroids as carbon and energy sources. The bacterial kstDs, especially those from actinobacteria such as *Mycobacterium* sp., *Rhodococcus* sp., *Arthrobacter* sp., and *Nocardia* sp., have been studied in terms of molecular, catalytic, and physiological properties, as well as their applications in steroid transformation (Donova 2007). In this study, we revealed a fungal kstD from *A. fumigatus* (kstD$_F$) and investigated its value to steroid transformation. KstD$_F$ and its orthologous counterparts in fungi are important tailoring enzymes involved in the biosynthesis of steroidal fusidane antibiotics, such as

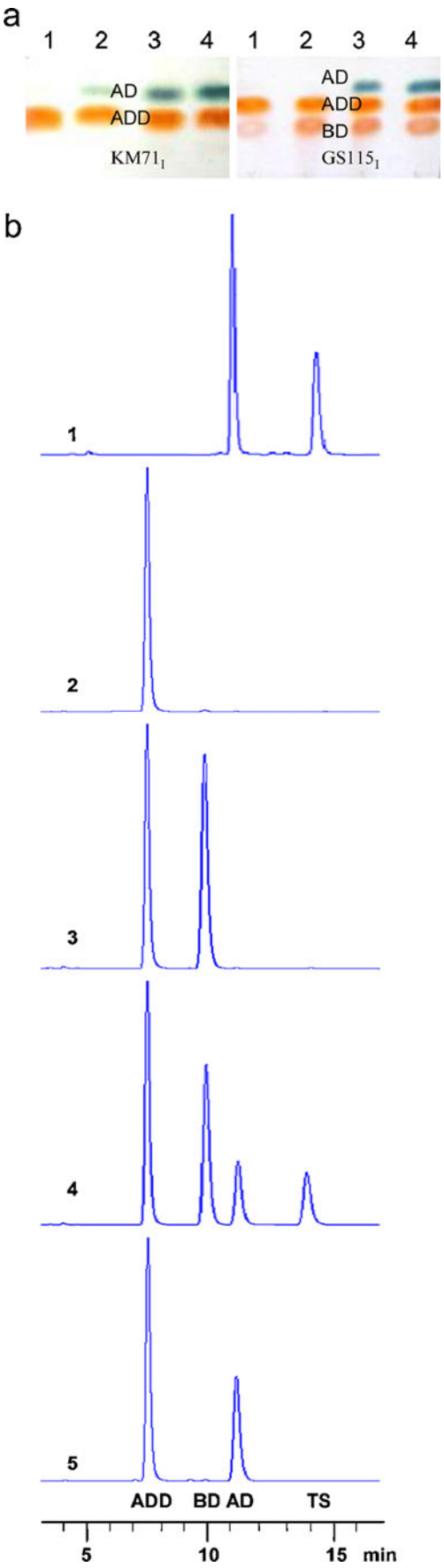

Fig. 4 Properties of AD conversion by $KM71_I$ and $GS115_I$. **a** TLC chromatogram of AD transformation by $KM71_I$ and $GS115_I$ for 6 days. $KM71_I$: *lane 1*, 1.0 g/l AD; *lane 2*, 1.5 g/l AD; *lane 3*, 2.0 g/l AD; and *lane 4*: 2.5 g/l AD. GS115: *lane 1*, 1.0 g/l AD; *lane 2*, 1.5 g/l AD; *lane 3*, 2.0 g/l AD; and *lane 4*, 2.5 g/l AD. **b** HPLC profile of AD transformation for 6 days. *Graph 1*: 1.0 g/l AD transformed by *P. pastoris* GS115. *Graph 2*: 1.0 g/l AD transformed by $KM71_I$. *Graph 3*: 1.5 g/l AD transformed by $GS115_I$. *Graph 4*: 2.5 g/l AD transformed by $GS115_I$. *Graph 5*: 2.5 g/l AD transformed by $KM71_I$

helvolic acid (Lodeiro et al. 2009; Mitsuguchi and Seshime 2009). Therefore, they are distinctly different from bacterial kstDs in physiological function. In addition, the stereochemistry of steroidal fusidanes is not typical of common steroids—ring B adopts a boat conformation instead of a chair conformation. However, $kstD_F$ showed good activity for the common steroid AD. Its K_m value was determined to be 47 μM, which is lower than that of kstD1 from *R. erythropolis* (82 μM). $KstD_F$ also showed good activity for other steroids, such as progesterone and testosterone. In this study, we used AD as a type substrate to investigate the properties of $kstD_F$.

Based on molecular property analysis of $kstD_F$ by TMpred program prediction and determination of the subcellular activity distribution in heterologous expression hosts, we supposed $kstD_F$ to be a soluble protein because it had no detectable hydrophobic transmembrane domains, and most of its activity was found in the cytosol. In this way, $kstD_F$ is distinct from the well-defined membrane-bound kstDs found in bacteria. The difference in molecular properties between $kstD_F$ and bacterial kstDs may be associated with their physiological roles. As well known, steroids are minimally water-soluble compounds. Concentrations usually remain below 0.1 mM. The uptake and degradation of steroids by microorganisms are closely associated with the direct contact of the cell membrane and steroid particles (Atrat et al. 1991; Fernandes et al. 2003; Rajkhowa et al. 2000). Bacterial kstDs are the initial enzymes in steroid degradation. It may therefore be required for them to bind to the cellular membrane tightly by hydrophobic transmembrane domains to make it easier to contact with steroids in cellular membrane. Obviously, the biosynthesis of steroidal antibiotic fusidanes in fungi occurs intracelluarly, which may not require the $kstD_F$ to bind tightly with the cellular membrane. Nevertheless, the extracellular expression of $kstD_F$ in *P. pastoris* showed no activity, which may be because of misfolding or of some required intracelluar components to support its active state. The physiological properties of $kstD_F$ merit further investigation.

For many steroid hormones, the Δ^1 double bonds are a pivotal moiety for pharmacological activity. Therefore, the development of engineered microorganisms with powerful kstD activity is important to the pharmaceutical industry. Nevertheless, well-characterized kstDs from actinobacteria

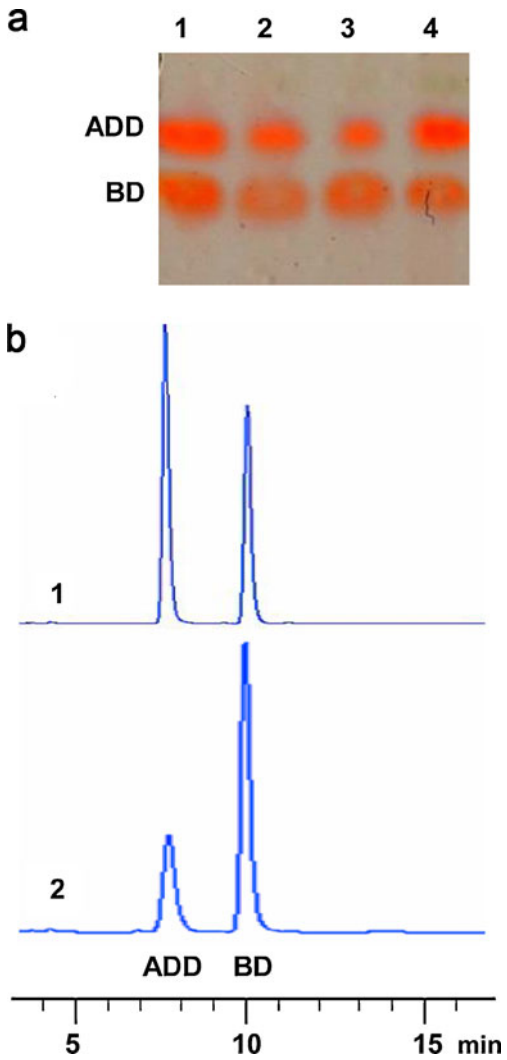

Fig. 5 Effects of glucose on the transformation of AD by $GS115_I$. **a** TLC profile. *Lane 1*: without glucose. *Lane 2*: 2% glucose. *Lane 3*: 3% glucose. *Lane 4*: 4% glucose. **b** HPLC profile. *Graph 1*: without glucose. *Graph 2*: 3% glucose

were all found to be membrane-bound enzymes (Donova 2007). The construction of genetically engineered recombinant strains for bacterial kstDs has not yet been very successful (Choi et al. 1995; Li et al. 2007; Morii et al. 1998; Plesiat et al. 1991; Molnar et al. 1995; Wagner et al. 1992). The expression of kstD of *A. simplex* in *B. subtilis* was found to be the only case that was in any way effective in practice with regard to steroid conversion. It could transform 1.0 g/l AD to ADD at a 45.3% conversion rate (Li et al. 2007). In this study, $kstD_F$ was identified as a soluble enzyme. It was successfully expressed intracellularly in *E. coli* and *P. pastoris*. The engineered recombinant *P. pastoris* strains, $KM71_I$ and $GS115_I$, were found to be more powerful under actual use than recombinant *B. subtilis* reported by Li et al. (2007). In the shake flask fermentation (Fig. 4a), $KM71_I$ was found capable of transforming more than 1.0 g/l AD into ADD at a 100% conversion rate, and $GS115_I$ was found capable of transforming 1.5 g/l AD to ADD and BD at a 100% conversion rate. This indicates that these two strains may be more promising than other engineered recombinant kstD strains in steroid Δ^1-dehydrogenation. Especially, strain $KM71_I$ showed good properties in preparing ADD without by-products. Fermentation optimization may further enhance the AD transformation efficiency of $KM71_I$ and $GS115_I$.

P. pastoris $GS115_I$ was found to be capable of transforming AD to both ADD and BD, and in some case, TS. The transformation of AD to ADD, TS, and BD is certainly the result of synergism between exogenous $kstD_F$ and endogenous 17Hsd (Fig. 3d). Both *P. pastoris* strains, KM71 and GS115, could transform AD to TS, indicating that both KM71 and GS115 contain the gene for 17Hsd. 17Hsd in yeast and other microorganisms are well defined as reversible redox enzymes (Donova et al. 2005). The different performances of AD transformation seen in $KM71_I$ and $GS115_I$ should be attributed to the differences in physiological redox status between strain $KM71_I$ and strain $GS115_I$. As shown in Fig. 3d, conversion of steroidal C17-one to C17-alcohol is a reduction reaction, which may be promoted by reducing power. Glucose is often used as a provider of reducing power in the microbial transformation process. Here, to enhance the proportion of BD in the end products, glucose was added to the $GS115_I$ fermentation process to regulate the redox status of $GS115_I$. The addition of glucose in the late stages of AD transformation by $GS115_I$ suggested that the relative amount of end product BD can be greatly enhanced. Thus, it can be expected that the transformation of AD to BD or ADD by $GS115_I$ can be regulated by optimizing the physiological redox status of $GS115_I$. In optimal redox status, the conversion of AD by $GS115_I$ may provide an alternative means of preparing BD.

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