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Identification of ketone reductase *Ch*KRED20 from the genome of *Chryseobacterium* sp. CA49 for highly efficient *anti*-Prelog reduction of 3,5-bis(trifluoromethyl)acetophenone

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ABSTRACT

A strain of *Chryseobacterium* sp. CA49 was isolated to perform efficient *anti*-Prelog reduction of 3,5-bis(trifluoromethyl)acetophenone (**1a**) to enantiopure (*R*)-3,5-bis(trifluoromethyl)-1-phenylethanol ((*R*)-**1b**), a key intermediate for the chiral drug Aprepitant. The draft genome sequencing of the strain revealed 27 putative short chain dehydrogenases/reductases of COG1028. Their activity and stereoselectivity were assayed after expression in *Escherichia coli* as recombinant proteins, and the key enzyme *Ch*KRED20 was identified with excellent activity and stereoselectivity. The lyophilized powder of the crude recombinant enzyme was applied to generate (*R*)-**1b** with >99% conversion and >99.9% enantiomeric excess at a substrate concentration of 150 g/l within 24 h by using 2-propanol as the co-substrate. The results indicate great potential for industrial-scale application of *Ch*KRED20.

1. Introduction

Biocatalytic ketone reduction is a powerful approach for the production of optically active alcohols, which are widely used as versatile and fundamental intermediates for pharmaceuticals, agrochemicals and other products with chiral centers [1–6]. In the last decade, successful application of ketone reductases (KRED) either as whole-cells or isolated enzymes has risen rapidly. In particular, several processes using isolated/crude recombinant enzymes coupled with efficient recycling systems have been developed into industrial-scale production with excellent stereoselectivity and economic advantage [7–12]. The use of recombinant enzymes greatly facilitates the scale-up with high volumetric productivity and the absence of side reactions [2,13,14].

Aprepitant is a chiral drug used for the treatment of chemotherapy-induced nausea and vomiting as a NK-1 receptor antagonist, with an annual sale of approximately 387 million dollars in 2010 under the brand name Emend®. A key intermediate in the synthesis of Aprepitant is (*R*)-3,5-bis(trifluoromethyl)-1-phenylethanol ((*R*)-**1b**), which is obtained mainly through the transitional metal-catalyzed asymmetric reduction of the corresponding ketone **1a** (Fig. 1) [15]. Although the large-scale biocatalytic production of the other enantiomer (*S*)-**1b** has been well established by using a recombinant alcohol dehydrogenase from *Rhodococcus erythropolis* by Merck Research Laboratories [16], the biocatalytic production of (*R*)-**1b** has remained difficult mainly because the process requires a catalyst with excellent *anti*-Prelog stereoselectivity, which is relatively rare since it is well recognized that most microorganisms/enzymes catalyze ketone reduction that takes place following Prelog's rule [4,14,17]. Homann et al. performed a systematic screening of ~300 microbes for the ability to reduce **1a**, but only five of them were found to be (*R*)-selective with a maximum enantiomeric excess (ee) of 92% [17]. Recently, four strains of *Lactobacillus kefir* [18], *Penicillium expansum* [19], *Leifsonia xyli* [20], and *Microbacterium oxydans* [21] that are able to reduce **1a** to the corresponding (*R*)-**1b** with >99% ee have been reported by us and other groups, and conversion rates of 31–95% were achieved at a substrate concentration of ~5 g/l. In a recent work by Ouyang et al. [22], whole cells of *Leifsonia xyli* CCTCC M 2010241 were applied to reduce 200 mM (51.2 g/l) **1a**. A conversion rate of 91.8% was achieved in 30 h with a high cell concentration of 300 g/l, and no further conversion could be achieved with prolonged reaction time of up to 50 h. In contrast to most academic practices, a typical industrial-scale biotransformation catalyzed with KREDs requires significant substrate concentration of at least 100 g/l, a substrate-to-enzyme ratio of >50 to facilitate post-processing, as well as a high

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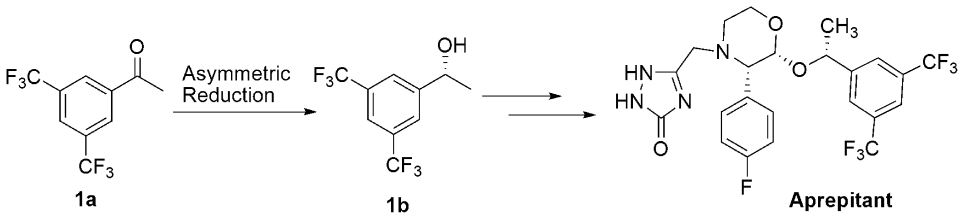

Fig. 1. Reduction of 3,5-bis(trifluoromethyl)acetophenone (**1a**) to (*R*)-3,5-bis(trifluoromethyl)-1-phenylethanol (**1b**).

conversion rate of >95% to facilitate product purification [2,7,8]. Therefore, novel KRED enzymes or KRED-producing stains with more robust characteristics are in high demand for the *anti*-Prelog reduction of **1a**.

Here, we report the isolation of a novel strain of *Chryseobacterium* sp. CA49 able to tolerate 50 g/l ketone **1a** to produce enantiopure (*R*)-**1b**. The key enzyme *Ch*KRED20 was identified through whole-genome sequence analysis and subsequent biotransformation, together with transcriptional analysis. The resulting reaction system, which was catalyzed with the lyophilized powder of the crude recombinant *Ch*KRED20 by using 2-propanol as the co-substrate, could well tolerate a substrate concentration of 200 g/l with high activity and excellent *anti*-Prelog stereoselectivity.

2. Materials and methods

2.1. Materials

The substrate 3,5-bis(trifluoromethyl)acetophenone (**1a**) was purchased from Alfa-Aesar (Tianjin, China). Racemic alcohols (**1b**) were prepared by reducing the ketones with sodium borohydride. Oligonucleotides were synthesized by Invitrogen Life Technologies (Shanghai, China) in PAGE-purified grade. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). DNA sequencing was carried out at Invitrogen (Shanghai, China). All other reagents were obtained from general commercial suppliers and used without further purification.

2.2. Enrichment and screening of microorganisms

One gram of soil sample collected from an orchard in the suburbs of Chengdu, China, was added to 50 ml of enrichment medium (pH 7.0) containing 0.2% (w/v) Na_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.04% (w/v) NH_4Cl, 0.04% (w/v) $MgCl_2$, and 0.2% (v/v) **1a** in a 250-ml flask. The mixture was incubated at 30 °C for 7–10 days with shaking at 230 rpm. Then, 0.5 ml of the mixture was transferred to 15 ml fresh enrichment medium in a 100-ml flask, and incubated at 30 °C for 7–10 days with shaking at 230 rpm. After 7–10 days, a loopful of the broth was properly diluted and plated on the same enrichment medium supplemented with 0.05% (w/v) yeast extract (Oxoid), 1.5% (w/v) agar powder, and 0.1% (v/v) **1a**. The plates were incubated at 30 °C for 1–3 days.

Each of the resulting single colonies was inoculated into 1 ml fermentation medium (pH 7.0) in 24-well plates containing 0.5% (w/v) tryptone (Oxoid), 0.15% (w/v) beef extract, 0.15% (w/v) yeast extract (Oxoid), 0.5% (w/v) NaCl, 1% (w/v) glucose, and 0.2% (v/v) **1a**, and incubated for 24 h at 30 °C with shaking at 230 rpm. Cells were harvested by centrifugation (1800 × *g* for 10 min at 4 °C), washed twice with potassium phosphate buffer (100 mM, pH 7.0), and resuspended in 1 ml of the same buffer containing 1% (w/v) glucose and 5% (v/v) 2-propanol. Bioreduction was then carried out at 30 °C with the addition of **1a** at a concentration of 1 g/l. After 36 h at 270 rpm, the reaction was terminated by extraction with ethyl acetate, and the conversion rate and optical purity were determined by gas chromatography on a Fuli GC9790 equipped with a flame ionization detector by using a CP-ChiraSil-DEX CB column (Varian, USA).

2.3. Classification of Chryseobacterium sp. CA49

Partial segment of 16S rDNA was amplified by PCR with two universal primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (27f) and 5′-GGTTACCTTGTTACGACTT-3′ (1492r), and the PCR products were directly sequenced at Invitrogen (Shanghai, China). The sequence was deposited at GenBank under accession number KC479152. The strain was deposited at China Center for Type Culture Collection (Wuhan, China) under the acquisition number CCTCC M 2012484.

2.4. Draft genome sequencing of Chryseobacterium sp. CA49 and gene annotation

Draft genome sequencing was performed according to a previously reported procedure [23]. Briefly, 10 μg genomic DNA was sent to BGI-Shenzhen (Shenzhen, China) to construct a PCR-free library. A short-insert (350 bp) genomic DNA library was constructed and paired-end sequenced on Genome Analyzer II (Illumina). Sequence data from the library were verified and low-quality sequences, base-calling duplicates, and adaptors were removed. Paired-end sequence data from the genomic DNA libraries were assembled using SOAPdenovo. All paired-end reads were aligned to contigs to construct scaffolds. Each nucleotide position in the final assembly was assessed for accuracy by aligning all filtered reads to the scaffolds by using SOAPaligner (http://soap.genomics.org.cn/soapdenovo.html). The open reading frame of each gene was predicted and annotated by KEGG/COG/SwissProt databases.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR)

The total RNA was isolated from freshly harvested cells of *Chryseobacterium* sp. CA49 by using RNAiso Plus kit (Takara Shuzo Co., Ltd, Dalian, China) and was used to synthesize cDNA with PrimeScript RT-PCR kit following the supplier's instructions (Takara Shuzo Co., Ltd, Dalian, China). The cDNA was used as a template to amplify target genes by using PCR with sequence-specific primers for *Ch*KRED08, *Ch*KRED19, and *Ch*KRED20 (Supplementary data, Table S1). The amplified PCR product was ligated into the pMD19-T vector (Takara Shuzo Co., Ltd, Dalian, China), and the sequence was verified by DNA sequencing at Invitrogen Life Technologies (Shanghai, China).

2.6. Cloning, heterologous expression, and purification of putative KREDs

Sequences of the 27 putative KREDs homologs were verified by direct cloning from genomic DNA. DNA fragments encoding each putative ketoreductase were amplified by PCR using specific primers (Supplementary data, Table S1) and ligated into pMD19-T vector (Novagen, Madison, WI, USA). After digestion with