J Ind Microbiol Biotechnol (2011) 38:633–641
DOI 10.1007/s10295-010-0809-3

ORIGINAL PAPER

Synthesis of optically pure *S*-sulfoxide by *Escherichia coli* transformant cells coexpressing the P450 monooxygenase and glucose dehydrogenase genes

Jian-Dong Zhang · Ai-Tao Li · Hui-Lei Yu · Tadayuki Imanaka · Jian-He Xu

Received: 25 March 2010 / Accepted: 7 August 2010 / Published online: 19 August 2010

Abstract A cytochrome P450 monooxygenase (P450SMO) from *Rhodococcus* sp. can catalyze asymmetric oxygenation of sulfides to *S*-sulfoxides. However, P450SMO-catalyzed biotransformations require a constant supply of NAD(P)H, the expense of which constitutes a great hindrance for this enzyme application. In this study, we investigated the asymmetric oxygenation of sulfide to *S*-sulfoxide using *E. coli* cells, which co-express both the P450SMO gene from *Rhodococcus* sp. and the glucose dehydrogenase (GDH) gene from *Bacillus subtilis*, as a catalyst. The results showed that the catalytic performance of co-expression systems was markedly improved compared to the system lacking GDH. When using recombinant *E. coli* BL21 (pET28a-P450-GDH) whole cell as a biocatalyst, NADPH was efficiently regenerated when glucose was supplemented in the reaction system. A total conversion of 100% was achieved within 12 h with 2 mM *p*-chlorothioanisole substrate, affording 317.3 mg/L *S*-sulfoxide obtained. When the initial sulfide concentration was increased to 5 mM, the substrate conversion was also increased nearly fivefold: *S*-sulfoxide amounted to 2.5 mM (396.6 mg/L) and the *ee* value of sulfoxide product exceeded 98%. In this system, the effects of glucose concentration and substrate concentration were further investigated for efficient biotransformation. This system is highly advantageous for the synthesis of optically pure *S*-sulfoxide.

Keywords Coexpression · P450SMO · Glucose dehydrogenase · NADPH regeneration · D-glucose · Sulfoxidation

J.-D. Zhang · A.-T. Li · H.-L. Yu · T. Imanaka · J.-H. Xu (✉)
Laboratory of Biocatalysis and Bioprocessing,
State Key Laboratory of Bioreactor Engineering,
East China University of Science and Technology,
130 Meilong Road, Shanghai 200237,
People's Republic of China
e-mail: jianhexu@ecust.edu.cn

T. Imanaka
Department of Biotechnology, College of Life Sciences,
Ritsumeikan University, Shiga 525-8577, Japan

Introduction

Enantiomerically pure sulfoxides play an important role in asymmetric synthesis, either as chiral building blocks or as stereodirecting groups [1]. Many organic sulfoxides also exhibit biological activities as therapeutic agents displaying anti-ulcer (proton pump inhibition), antibacterial and antifungal properties. Furthermore, they can be used as psychotonics and vasodilators [2]. Asymmetric oxidation of prochiral sulfides is the most straightforward and economical method for the synthesis of enantiomerically pure sulfoxides, and a great number of reagents are available for this reaction [2]. Both isolated enzymes and whole cells have been used in the stereoselective oxidation of prochiral sulfides, in processes carried out in water under mild reaction conditions [3–6]. This makes a biocatalytic approach environmentally friendly and appealing for industrial applications.

Cytochrome P450s (CYPs) are hemoproteins encoded by a superfamily of genes converting a broad variety of substrates and catalyzing a variety of interesting chemical reactions such as carbon hydroxylation, dealkylation, epoxidation, heteroatom (N, S, P) oxidation, aromatic hydroxylation, reduction and dehalogenation [7]. Oxidation of sulfides by cytochrome P450-dependent monooxygenases have been studied mainly in the context of drug metabolism

by mammalian liver microsomes. The selectivity for sulfoxidation among these enzymes has been investigated, producing mainly (*S*)-sulfoxides with low selectivity [8]. Unfortunately, bacterial P450s such as P450BM3, P450Cam and P450terp also present relatively low stereoselectivity [9]. Recently, we have cloned a new P450 monooxygenase (P450SMO) gene from *Rhodococcus* sp. ECU0066 which displays a sulfide sulfoxidation activity with high selectivity [10]. The high sequence homology (73%) of P450SMO to P450RHF from a *Rhodococcus* sp. NCIMB 9784 indicates that the P450SMO belongs to class IV of P450 monooxygenase postulated by Roberts et al. [11]. These monooxygenase mediate catalysis in the absence of additional redox proteins [12, 13]. However, P450SMO catalyzes substrate oxidation through a stoichiometric consumption of the expensive cofactor NADPH, the high cost of which has been a great hindrance for cell-free application of this enzyme. Various approaches have been studied for cofactor regeneration in process development, such as chemical, electrochemical, photochemical, enzymatic methods [14]. However, these methods are still inefficient and expensive. Whole-cell reaction systems have been studied as an alternative to designing in vitro reaction systems by integrating an additional enzyme reaction for in vitro cofactor regeneration. Recombinant strains including baker's yeast (*Saccharomyces cerevisiae*) and *E. coli* expressing cyclohexanone monooxygenase from *Acinetobacter* sp. NCIB 9871 have been used as whole-cell biocatalysts for oxidations of several sulfides to the corresponding sulfoxides. These cells provide a constant supply of the enzyme and the NADPH cofactor, which dramatically simplifies the process [15]. However, normal *E. coli* cells can not regenerate NADPH sufficiently [16]. Zhang et al. showed that recombinant *E. coli* harboring the P450SMO gene can produce high optically pure (*S*)-sulfoxide, but productivity was very low unless NADPH was added [10].

Glucose dehydrogenase (GDH) (EC 1.1.1.47) catalyzes the oxidation of D-glucose in the presence of cofactor NAD^+ or $NADP^+$ and forms D-glucono-δ-lactone and NAD(P)H. The enzyme can be used in many reduction reactions for coenzyme regeneration [16, 17]. In this study, we successfully co-expressed the P450SMO gene from *Rhodococcus* sp. and the GDH gene from *Bacillus subtilis* in a recombinant *E. coli* cell. This construct allowed significant improvement of the production of asymmetrically oxidized sulfides without addition of expensive NADPH cofactor.

Materials and methods

Chemicals

Tryptone and yeast extract were obtained from Oxoid (Shanghai, China), and restriction enzymes were provided by TaKaRa (Dalian, China). *p*-Chlorothioanisole was purchased from Lancaster (Morecambe, UK). All other chemicals are of analytical grade and are commercially available.

Bacterial strains, vectors and culture conditions

E. coli DH5α supercompetent cells were obtained from Tiangen (Shanghai, China), *E. coli* BL21 (DE3) was from Invitrogen Life Technologies (Shanghai, China). Both strains were routinely grown in Luria–Bertani (LB) medium at 37°C unless stated otherwise. Ampicillin (100 μg/mL) and kanamycin (50 μg/mL) were used for the selection of recombinant *E. coli* strains. The pMD18-T plasmid for the direct cloning of PCR products was purchased from TaKaRa (Dalian, China). The pET20b and pET28a (+) plasmid for the heterogeneous expression study were obtained from Novagen (Shanghai, China).

Construction of plasmid vectors for coexpression of the P450SMO and GDH gene

Plasmids used in this study are listed in Table 1. The nucleotide sequences of the polymerase chain reaction (PCR) primers used are listed in Table 2.

The glucose dehydrogenase gene was amplified via PCR with primers 1 and 2 using genome DNA from *Bacillus subtilis* as the template. The PCR amplifications were performed with *Pfu* DNA polymerase (TaKaRa, Dalian, China), with initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 2 min and followed by a final extension at 72°C for 10 min. The resulting

Table 1 Plasmids used in this study

Plasmid	Promoter	Antibiotic for plasmid selection	Reference
pET20b-GDH	T7	Ampicillin (100 μg/mL)	This work
pET28a-P450SMO	T7	Kanamycin (50 μg/mL)	Zhang et al.
pET20b-GDH-P450SMO	T7	Ampicillin (100 μg/mL)	This work
pET28a-P450SMO-GDH	T7	Kanamycin (50 μg/mL)	This work

Table 2 Primers used for PCR amplification of DNA sequences

Number	Name	Sequence (5′-3′)
1	F-GDH	CGCGCATATGTATCCGGATTTAAAAGGAAAAGTCGT
2	R-GDH	ATGTAAGCTTTTAACCGCGGCCTGCCTGGAATG
3	F-PPG	CCCTCCAGCTGTGA***AAAGGAGATATACAT***ATGTATCCGG
4	R-PPG	***TCGAGTGCGGCCGCA***TTAACCGCGGCCTGCCTGGAATG
5	F-PGP	CAGGCCGCGGTTA***AAAAGGAGATATACC***ATGGGCAGC
6	R-PGP	***TGGTGGTGGTGGTGC***TCACAGCTGGAGGGTCAGCT

Restriction endonuclease sites in the primer sequences are underlined. Non-coding regions overhang 5′-addition of the primers is indicated in bold italic. Primers 3 to 6 were designed from original plasmids (pET28a-P450 and pET20b-GDH) according to the instruction of CloneEZ kit. The PCR template was pET20b-GDH or pET28a-P450

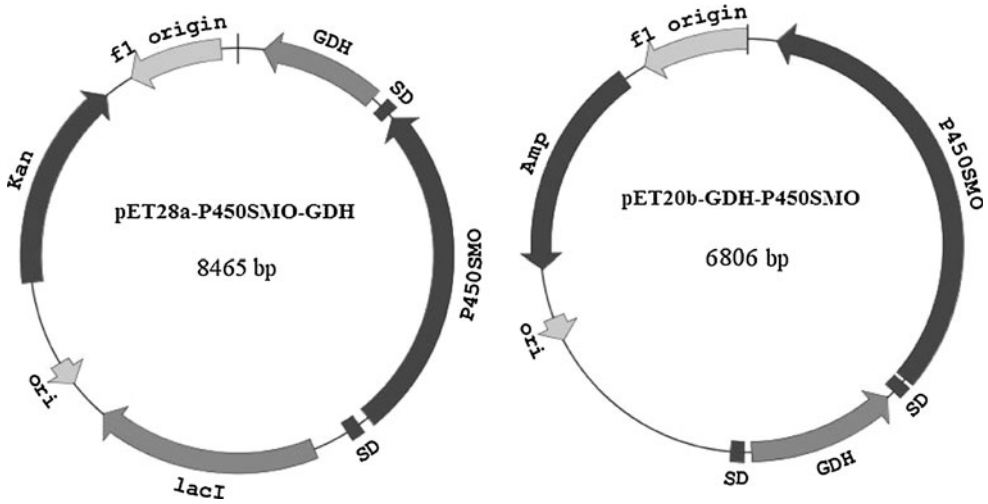

Fig. 1 Structure of expression plasmids

786 bp fragment was digested with *Nde*I and *Hin*dIII and then ligated into pET20b which was digested with the same restriction enzymes, generating the construct pET20b-GDH. Successful ligation into pET20b was confirmed by restriction analysis and determination of glucose dehydrogenase activity in cell-free extracts after recombinant expression in *E. coli* BL21 (DE3). The transformed strain was abbreviated as BL21 (pET20b-GDH).

Coexpression of P450SMO gene and GDH gene was performed in three ways. First, a two-plasmid system was used in which each gene was cloned into a different plasmid with the same origin of replication, i.e., P450SMO gene into pET28a (+) [10] and GDH into pET20b. A similar approach using two incompatible plasmids had been successfully used [18, 19]. The same *E. coli* strain transformed with both the pET28a-P450SMO and the pET20b-GDH was abbreviated as BL21 (pET28a-P450SMO/pET20b-GDH). The other two methods involved a single plasmid system in which the two genes were arranged in tandem in pET28a or pET20b. Coexpression plasmids, pET28a-P450SMO-GDH and pET20b-GDH-P450SMO were constructed as depicted in Fig. 1. For construction of pET28a-P450SMO-GDH, the gene encoding GDH was amplified by PCR with primers 3 and 4 using pET20b-GDH as the DNA templates. The PCR amplifications were performed with *Pfu* DNA polymerase (TaKaRa, Dalian, China), with initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and followed by a final extension at 72°C for 10 min. The amplified DNA fragment was cloned into the pET28a-P450SMO vector by CloneEZ™ kit (GenScript, Nanjin, China). For construction of pET20b-GDH-P450SMO, the gene encoding P450SMO was amplified by PCR with primers 5 and 6 using pET28a-P450SMO as the DNA template. The PCR amplifications were performed with *Pfu* DNA polymerase (TaKaRa, Dalian, China), with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 3 min and followed by a final extension at 72°C for 10 min. The amplified DNA fragment was cloned into the pET20b-GDH vector using a CloneEZ™ kit. As for the Shine–Dalgarno (SD) sequence, P450SMO used SD of pET28a and GDH included the SD of pET20b. All the expression plasmids were introduced into *E. coli* BL21.

Cultivation of recombinant *E. coli* cells and preparation of the resting cells

The verified clones were grown at 37°C in Luria–Bertani medium (per liter deionized water: 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl). Kanamycin 50 μg mL^{-1} was included for growth of *E. coli* BL21 (pET28a-P450SMO-GDH), and ampicillin 100 μg mL^{-1} was added for the strain containing pET20b-GDH-P450SMO. For two-plasmid systems, 50 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin were added. When the cultures were grown at 37°C with an OD_{600} of 0.6, 0.5 mM $FeCl_3$ was added (to improve the P450SMO production) and then induced with IPTG at a final concentration of 0.5 mM. The growth of the culture was continued at a lower temperature of 25°C, with vigorous shaking (150 rpm) for 18 h. Cells were harvested by centrifugation at 8,500*g* for 5 min at 4°C, washed twice with sodium phosphate buffer (50 mM, pH 7.5) and then resuspended in the same buffer. Resting cells obtained were employed as the whole cell biocatalysts and were stored at 4°C prior to use.

Carbon monoxide (CO) difference spectra analysis

Resting cells were resuspended and disrupted by sonication (To disrupt the cells, the cell suspension was put on ice and sonicated 90 times at 400 W for 4 s with 4 s of interval) in sodium phosphate buffer (50 mM, pH 7.5). The lysates were centrifuged (8,500*g* for 20 min at 4°C) to remove insoluble fractions. The supernatant was employed for spectroscopic measurement. The CO difference spectra were measured as follows: the crude extracts were reduced by adding several grains of sodium dithionite. Samples were dispensed into two cuvettes and a baseline spectrum was spectrophotometrically recorded (UV–Vis Spectrophotometer UVmini-1240, Shimadzu, Kyoto, Japan) in the range of 350–500 nm. One cuvette was then gently gassed with carbon monoxide for 1 min, after which a difference spectrum was immediately monitored in the same spectral range and subsequently baseline-corrected. The concentration of P450SMO in the aqueous solution was determined by the CO difference spectrum with a molar extinction difference of 91 mM^{-1} cm^{-1} between 450 and 490 nm [20].

Determination of enzyme activities

Resting cells were resuspended and disrupted by sonication in sodium phosphate buffer (50 mM, pH 7.5). The lysates were centrifuged to remove insoluble fractions. The crude extracts were dialyzed against sodium phosphate buffer (pH 7.5). Finally, the samples were concentrated fourfold by ultrafiltration (Centriprep 30,000 molecular weight cut-off concentrators, Millipore) and stored at −80°C with 20% glycerol until further use.

GDH activity was determined by measurement of the increase in absorbance at 340 nm due to the formation of NADPH. Therefore, 870 μL of 50 mM sodium phosphate buffer (pH 7.5) were mixed with 10 μL of cell-free extract and 10 μL of 10 mM $NADP^+$ in 50 mM sodium phosphate buffer (pH 7.5). Measurement was started by adding 100 μL of 1 M glucose in sodium phosphate buffer (pH 7.5). Molar extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}) was used to calculate enzyme activities. P450SMO activity was determined using the *p*-chlorothioanisole as substrate, as previously described [10]. Bradford protein assay was used for quantitative analysis of proteins with bovine serum albumin as the standard. The assay was based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs [21].

In vitro cofactor regeneration system

The function of the catalytic redox cycle of NADPH regeneration was investigated in vitro using sulfoxidation assay with mixtures of purified P450SMO [10] and GDH or cell-free extracts of each of the three coexpression systems. Reaction mixtures contained *p*-chlorothioanisole (1 mM), P450SMO (1 μM), $NADP^+$ (1 mM), D-glucose (50 mM), and GDH (10 U), in 1 mL of 50 mM sodium phosphate buffer pH 7.5. Reaction mixtures containing *p*-chlorothioanisole (1 mM), 1 μM P450SMO, and 1 mM of NADPH were used as control samples. Sulfoxide products and final NADPH concentration were assayed after incubation of the substrate with the enzyme for 2 h. The samples (0.5 mL) were saturated with NaCl and extracted with 0.3 mL ethyl acetate containing 2 mM of 4-nitroacetophenone as an internal standard. The organic layers were dried over anhydrous sodium sulfate, and subjected to GC analysis. All experiments were performed in duplicate.

Whole-cell biotransformation

The cells were washed twice with sodium phosphate buffer (50 mM, pH 7.5) and resuspended in the same buffer. Different amounts of sulfide and glucose were added to the reaction mixture. The biotransformations were carried out in 50 mL reaction vessel with 10 mL of resting cells (50 mg wet cells/mL reaction buffer) at 30°C for 12 h. Cells containing the same plasmid without a DNA insert were used as a negative control. At the scheduled times, aliquots of reaction mixture were withdrawn for product assay. All experiments were performed in duplicate.

Analytical methods

The product analysis was performed as described by Li et al. [22] with minor modifications. Briefly, GC (GC-14C, Shimadzu, Japan) with a column (AT•SE-54 30 × 0.25 mm × 0.33 μm) and an FID detector were used to determine the quantity of sulfoxide formed. GC analysis conditions were: injector 280°C; detector 280°C and oven 180°C. Enantiomeric excess (*ee*) of product was determined with HPLC (LC-10AT, Shimadzu, Japan) using a chiral column (Chiralcel OD-H, Daicel Co., Japan, 250 mm × Φ4.6 mm), which was eluted with hexane/isopropanol (93:7, v/v, 1.0 mL/min) and detected at 254 nm. Authentic standards for each enantiomer of sulfoxide were prepared by the method established previously [22].

Results

Heterologous expression of P450SMO and GDH genes

The P450SMO and GDH genes were cloned and heterologously expressed in *E. coli* BL21 (DE3) cells. With the analysis of the protein expression in *E. coli* BL21 (DE3) cells by SDS-polyacryl-amide gel electrophoresis (SDS–PAGE), two protein bands were evident, with a size of approximately 88 kDa (P450SMO) and 29 kDa (GDH) (Fig. 2). To check the expression of active P450SMO in the recombinant *E. coli* cells, the crude cell extracts of three coexpression systems were spectroscopically analyzed. The absorption spectrum of the crude cell extract of coexpressed enzymes showed a heme absorption with the maximum at 420 nm, which is typical of cytochrome P450 enzymes. The addition of dithionite led to a decrease of this shoulder due to reduction of the oxidized heme iron component. The addition of carbon monoxide to the dithionite-reduced protein resulted in a Soret band shift from 420 to 450 nm, as expected for a cytochrome P450 enzyme (data not shown).

Based on the data of the aqueous extracts, which were prepared under optimized conditions, the content of P450SMO was estimated to be about 131.8 nmol per liter of culture broth for BL21 (pET28a-P450SMO/pET20b-GDH), 127.5 nmol per liter of culture broth for BL21 (pET20b-GDH-P450SMO), and 248.4 nmol per liter of culture broth for BL21 (pET28a-P450SMO-GDH). The results showed that the P450SMO were actively expressed in three systems, and the content of P450SMO expressed in BL21 (pET28a-P450SMO-GDH) system was higher than in the other two systems. The GDH activity was estimated to be about 1.6 U/mg protein for BL21 (pET28a-P450SMO/pET20b-GDH), 1.5 U/mg protein for BL21 (pET20b-GDH-P450SMO), and 1.2 U/mg protein for BL21 (pET28a-P450SMO-GDH), respectively.

In vitro analysis of the recombinant intracellular cofactor regeneration system

After successful cloning of the respective genes and before using these for whole-cell biotransformations, the basic functioning of the cofactor regeneration system was investigated in vitro. As shown in Table 3, the sulfide conversion with the enzyme cofactor regeneration system was about twice that with no cofactor regeneration system, while the final concentration of NADPH in the cofactor regeneration system was about 4.5 times higher than that of the no-cofactor regeneration system. These data suggested that the

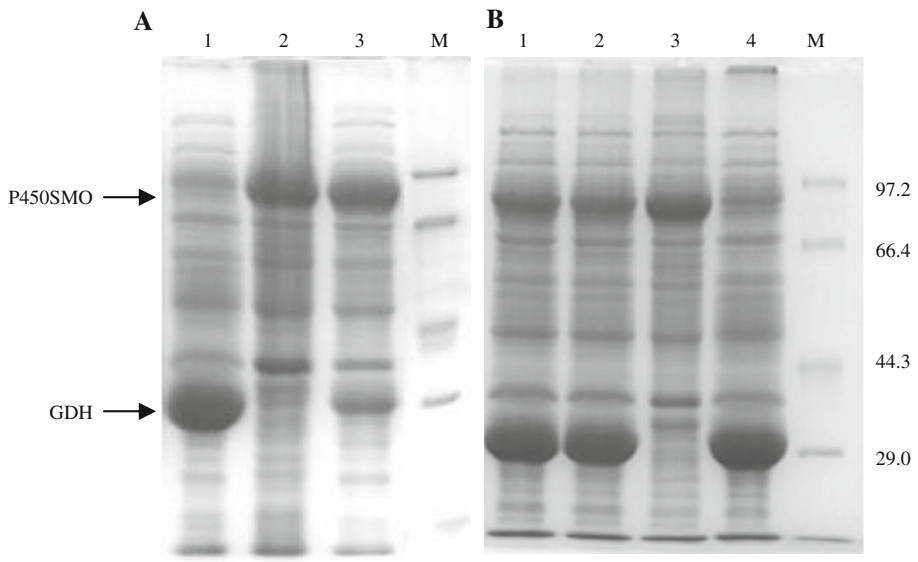

Fig. 2 SDS–PAGE analyses of the P450SMO and GDH co-expressed in *E. coli* BL21 (DE3). **a** *Lane M*, protein markers (in kDa); *Lane 1*, pET20b-GDH; *Lane 2*, pET28a-P450SMO; *Lane 3*, pET28a-P450SMO-GDH. **b** *Lane M*, protein markers (in kDa); *Lane 1*, pET28a-P450SMO/pET20b-GDH; *Lane 2*, pET20b-GDH-P450SMO; *Lane 3*, pET28a-P450SMO; *Lane 4*, pET20B-GDH. The P450SMO (*ca.* 88 kDa) and GDH (*ca.* 30 kDa) are indicated by the *arrows*

Table 3 In in vitro analysis of sulfide conversion and NADPH regeneration with different reaction systems

Reaction systems	Sulfide conversion (%)[a]	NADPH (mM)[c]
P450SMO (1 μM) + Sulfide (1 mM)	ND[b]	ND
P450SMO (1 μM) + NADPH (1 mM) + Sulfide (1 mM)	34.9	0.2
P450SMO (1 μM) + $NADP^+$ (1 mM) + GDH (10 U) + Glucose (50 mM) + Sulfide (1 mM)	71.8	0.9
BL21(pET28a-P450SMO-GDH) cell extracts + $NADP^+$ (1 mM) + Glucose (50 mM) + Sulfide (1 mM)	69.9	0.75
BL21(pET28a-P450SMO/pET20b-GDH) cell extracts + $NADP^+$ (1 mM) + Glucose (50 mM) + Sulfide (1 mM)	51.5	0.95
BL21(pET20b-GDH-P450SMO) cell extracts + $NADP^+$ (1 mM) + Glucose (50 mM) + Sulfide (1 mM)	50.3	0.95

P450SMO was purified from the recombinant cell constructed previously [10]. The concentration of P450SMO in the aqueous solution was determined by the CO difference spectrum with a molar extinction difference of 91 mM^{-1} cm^{-1} between 450 and 490 nm. P450SMO concentration calculated in BL21 (pET28a-P450SMO-GDH) cell extracts was 0.7 μM; in BL21 (pET28a-P450SMO/pET20b-GDH) cell extracts was 0.5 μM; in BL21 (pET20b-GDG-P450SMO) cell extracts was 0.5 μM. The actual NADPH concentration was calculated from the absorption data using the value of the extinction coefficient (6.2 mM^{-1} cm^{-1}). The reaction condition was in the "Materials and methods"

[a] Sulfide conversion was determined by GC analysis. [b] *ND* not detected. [c] final concentration of NADPH after 2 h reaction

NADPH was consumed by the P450 monooxygenase in cell-free extracts of BL21 (pET28a-P450SMO) and co-expression systems, and the NADPH regeneration catalyzed by GDH was forming a catalytic cycle.

Whole cell catalytic performance

To efficiently connect the two reactions, the GDH gene from *Bacillus subtilis* was coexpressed with the P450SMO gene in *E. coli* using three different coexpression styles. Resting cells of each transformant strain were prepared and subjected to reaction assay with 2 mM *p*-chlorothioanisole as the substrate in the presence of D-glucose (50 mM). As shown in Fig. 3, the highest production of sulfoxide was obtained with *E. coli* possessing pET28a-P450SMO-GDH, affording a conversion of 100% in 12 h, as compared to 53.3 and 67.6% conversions with *E. coli* possessing pET20b-GDH-P450SMO and pET28a-P450SMO/pET20b-GDH, respectively. Therefore, *E. coli* transformant cells possessing pET28a-P450SMO-GDH were used as a catalyst for the subsequent experiments.

The conversion efficiency of sulfide to sulfoxide using BL21 (pET28a-P450SMO-GDH) was further investigated. As shown in Fig. 4a, only about 25% conversion was achieved using the recombinant *E. coli* BL21 (pET28a-P450SMO) as a biocatalyst with an initial substrate concentration of 2 mM. When glucose was added to the reaction mixture, the sulfide conversion was increased to 36%. This result suggested that glucose was partially metabolized by *E. coli* enzymes and that a small amount of NADPH was regenerated. When the recombinant *E. coli* BL21 (pET28a-P450SMO-GDH) was used as a biocatalyst, better results were obtained. The highest conversion (100%) was achieved after 12 h reaction, and the substrate conversion increased nearly fourfold as compared to that without any

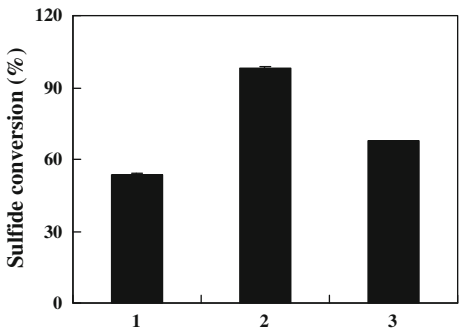

Fig. 3 Comparison of three recombinant *E. coli* coexpression systems. The reaction mixture (1 mL) was comprised of 2 mM substrate, 50 mM D-glucose, and 50 mg/mL of recombinant *E. coli* cells in 0.05 M sodium phosphate buffer (pH 7.5). Samples were incubated at 30°C for 12 h. Systems: *1* pET20b-GDH-P450SMO; *2* pET28a-P450SMO-GDH; *3* pET28a-P450SMO/pET20b-GDH. All experiments were performed in duplicate

energy source, affording 2 mM (317.3 mg/L) *S*-sulfoxide obtained. Due to the high cytotoxicity of sulfide, the efficiency of the reactions decreased when the initial sulfide concentration was increased to 5 mM (Fig. 4b). However, the conversion of sulfide was also increased nearly fivefold as compared to that without GDH being co-expressed in the system, and the product concentration reached 2.6 mM (396.6 mg/L). The *ee* value of sulfoxide product was higher than 98.0% (data not shown).

To further explain the effect of coexpression of GDH, the extracellular NADPH concentrations over the time course of sulfide oxidation in different reaction systems were measured. The actual NADPH concentration was determined at 340 nm using the value of the extinction coefficient (6.2 mM^{-1} cm^{-1}). As shown in Fig. 5, with the recombinant *E. coli* BL21 (pET28a-P450SMO) reaction system without GDH added, the profile of NADPH concentration

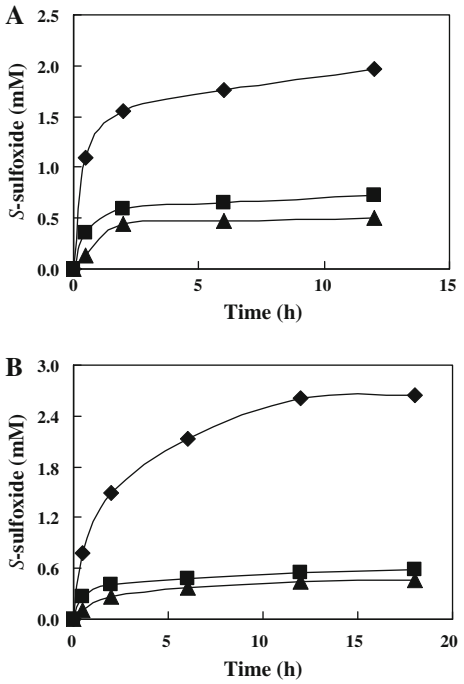

Fig. 4 Time courses of sulfide bioconversion. The reaction mixture (10 mL) was comprised of 50 mg/mL of *E. coli* cells in 0.05 M sodium phosphate buffer (pH 7.5). **a** 2 mM substrate, 50 mM D-glucose; **b** 5 mM substrate, 100 mM D-glucose. Samples were incubated at 30°C. (*filled diamond*) BL21 (pET28a-P450SMO-GDH); (*filled square*) BL21 (pET28a-P450SMO); (*filled triangle*): BL21 (pET28a-P450SMO) without D-glucose. All experiments were performed in duplicate

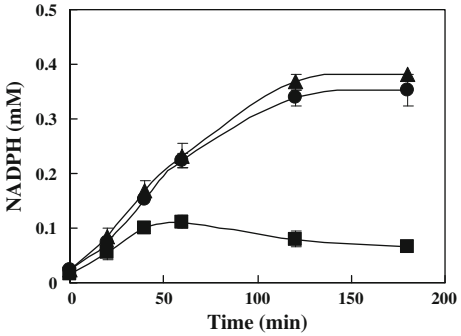

Fig. 5 Comparison of NADPH concentration in the reaction broth under different reaction systems. (*filled triangle*): BL21 (pET28a-P450SMO-GDH), 50 mM D-glucose and 2 mM sulfide substrate; (*filled circle*): BL21 (pET28a-P450SMO), 50 mM D-glucose, 0.1 mM $NADP^+$, 10 U/mL GDH and 2 mM sulfide substrate; (*filled square*): BL21 (pET28a-P450SMO), 50 mM D-glucose, 2 mM sulfide substrate

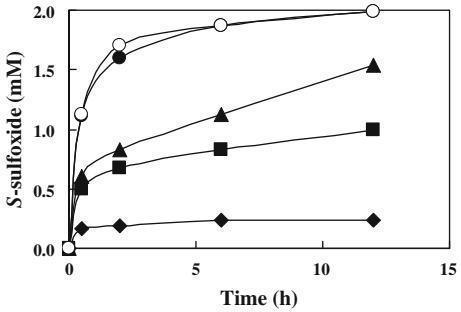

Fig. 6 Time courses of sulfide conversion with BL21 (pET28a-P450SMO-GDH) in the presence of different amounts of D-glucose. The reaction mixture (10 mL) comprised 2 mM substrate, 0–30 mM D-glucose and 50 mg/mL of *E. coli* cells in 0.05 M sodium phosphate buffer (pH 7.5). (*open circle*) 30 mM D-glucose; (*filled circle*) 20 mM D-glucose; (*filled triangle*) 10 mM D-glucose; (*filled square*) 5 mM D-glucose; (*filled diamond*) 0 mM D-glucose. All experiments were performed in duplicate

was kept at a low level. However, with the addition of glucose and GDH in the reaction system, the extracellular NADPH concentration increased obviously in the first 2 h, and then remained stable afterward. With the system catalyzed by recombinant *E. coli* BL21 (pET28a-P450SMO-GDH), the profile of extracellular NADPH concentration was quite similar to that in the GDH and glucose supplemented system. The results showed that GDH performed a key role for NADPH regeneration in the recombinant *E. coli* BL21 (pET28a-P450SMO-GDH) system, and the biotransformation of sulfide to *S*-sulfoxide could be efficiently completed with no exogenous GDH and $NADP^+$ addition. These results were consistent with the study by Xu [17], who used a recombinant *E. coli* expressing glucose dehydrogenase to efficiently regenerate NADPH.

Effect of glucose on sulfoxidation

The role of GDH on the BL21 (pET28a-P450SMO-GDH) system was further evaluated. We found that the concentration of the second substrate (D-glucose), used for regeneration of NADPH, significantly influenced the reaction rate. As shown in Fig. 6, the higher the D-glucose concentration, the higher the sulfide sulfoxidation rate. A high conversion rate and complete transformation could be achieved at 20 mM D-glucose within 12 h. With the BL21 (pET28a-P450SMO) as control, only 36% conversion could be achieved, and with more glucose added, the sulfide conversion did not increase (data not shown).

Effect of different amount of sulfide substrate on sulfoxidation

With the recombinant *E. coli* BL21 (pET28a-P450SMO-GDH) whole cell as biocatalyst, varying concentrations of substrate (1–10 mM) on sulfoxidation were evaluated. As shown in Fig. 7, when sulfide concentration was above 2 mM, the *S*-sulfoxide formation rate was evidently decreased. This result showed that high substrate concentrations (>2 mM) led to marked inhibition of the recombinant strain sulfoxidation.

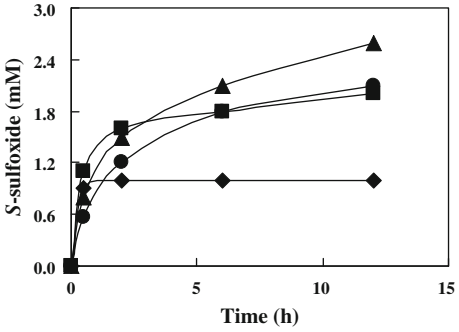

Fig. 7 Effect of different amounts of sulfide substrate on BL21 (pET28a-P450SMO-GDH) sulfoxidation. The reaction mixture (10 mL) comprised 1–10 mM substrate, 100 mM D-glucose and 50 mg/mL of recombinant *E. coli* cells in 0.05 M sodium phosphate buffer (pH 7.5). (*filled diamond*) 1 mM substrate; (*filled square*) 2 mM substrate; (*filled triangle*) 5 mM substrate; (*filled circle*) 10 mM substrate. All experiments were performed in duplicate

In addition, the product inhibition was also studied when a fixed concentration of sulfoxide (0–10 mM) was added in advance, and the results showed that the initial velocity of sulfoxide formation with each initial concentration of sulfoxide did not change (data not shown). Therefore, product inhibition did not seem to occur.

Discussion

Enantiopure sulfoxides are important building blocks in the organic synthesis of natural products because of their powerful stereodirecting ability in the carbon–carbon bond formation [23]. The activity of the sulfinyl group in the synthesis of drugs is exemplified by omeprazole, an antiulcer compound, and sulindac, a nonsteroidal antiinflammatory drug as well as an inhibitor of tumoral cell growth. Previous study has shown that P450SMO can accept a number of sulfide substrates, and has been applied for a wide variety of selective oxidation reactions resulting in formation of optically active sulfoxides [10]. However, the enzyme activity toward sulfide substrate was very low in in vitro biotransformation, and was also dependent upon the expensive cofactor, NADPH.

NADPH is an essential reducing agent in the P450 monooxygenase system. P450-catalyzed monooxygenation is initiated by electron transfer from NADPH to cytochrome P450 reductase; catalytic substrate oxidation will be terminated when NADPH in the reaction system is completely consumed. The use of *E. coli* whole cell biocatalysts coupled with a cofactor regeneration system was demonstrated to be a powerful strategy for enhancing the catalytic efficiency of oxidoreductases [24, 25]. In this study, three recombinant *E. coli* systems for co-expression of P450SMO and GDH gene were successfully established. The reaction seems to be substantially limited by the P450SMO activity, and an excess of P450SMO must be available, whereas an excess of GDH did not affect the total reaction rate. The results were similar to Weckbecker [26], who constructed two plasmid coexpression genes encoding alcohol dehydrogenase (ADH) and GDH. With the recombinant strain BL21 (pET28a-P450SMO-GDH) as biocatalyst, NADPH regeneration coupled with the coexpressed GDH resulted in a significant enhancement of sulfoxidation. However, like other monooxygenases [27, 28], the substrate inhibition was still a major drawback for P450SMO reaction. Further study on the two phase systems where substrate and product are stored in an organic layer, from which only small substrate quantities get into the enzyme-containing reaction buffer, may be a good choice to resolve this problem.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (grant Nos. 20506037 & 20672037&20902023), Ministry of Science and Technology, P.R. China (grant Nos. 2006AA02Z205 & 2007AA02Z225) and China National Special Fund for State Key Laboratory of Bioreactor Engineering (grant No. 2060204).

References

1. Mata EG (1996) Recent advances in the synthesis of sulfoxides from sulfides. Phosphorus Sulfur Silicon Relat Elem 117:231–286
2. Fernández I, Khiar N (2003) Recent developments in the synthesis and utilization of chiral sulfoxides. Chem Rev 103:3651–3705
3. Holland HL (1988) Chiral sulfoxidation by biotransformation of organic sulfides. Chem Rev 88:473–485
4. Holland HL (1992) Organic synthesis with oxidative enzymes. VCH, New York, p 255
5. Holland HL (2001) Biotransformation of organic sulfides. Nat Prod Rep 18:171–181
6. Holland HL, Brown FM, Kerridge A, Penkos P, Arensdor J (2003) Biotransformation of sulfides by *Rhodococcus erythropolis*. J Mol Catal B: Enzymatic 22:219–223
7. Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol 1:3003.1–3003.9
8. Hamman MABD, Haehner-Daniels SA, Wrighton AE, Rettie Hall SD (2000) Stereoselective sulfoxidation of sulindac sulfide by flavin-containing monooxygenases. Comparison of human liver and kidney microsomes and mammalian enzymes. Biochem Pharmacol 60:7–17
9. Fruetel J, Chang YT, Collins J, Loew G, Ortiz de Montellano PR (1994) Thioanisole sulfoxidation by cytochrome P450cam (CYP101): experimental and calculated absolute stereochemistries. J Am Chem Soc 116:11643–11648
10. Zhang JD, Li AT, Yang Y, Xu JH (2009) Sequence analysis and heterologous expression of a new cytochrome P450 monooxygenase from *Rhodococcus* sp. for asymmetric sulfoxidation. Appl Microbiol Biotechnol 85:615–624
11. Roberts GA, Grogan G, Greter A, Flitsch SL, Turner NJ (2002) Identification of a new class of cytochrome P450 from a *Rhodococcus* sp. J Bacteriol 184:3898–3908
12. Roberts GA, Celik A, Hunter DJ, Ost TW, White JH, Chapman SK, Turner NJ, Flitsch SL (2003) A self-sufficient cytochrome P450 with a primary structural organization that includes a flavin

domain and a [2Fe-2S] redox center. J Biol Chem 278:48914–48920
13. Liu L, Schmid RD, Urlacher VB (2006) Cloning, expression, and characterization of a self-sufficient cytochrome P450 monooxygenase from *Rhodococcus ruber* DSM 44319. Appl Microbiol Biotechnol 72:876–882
14. Itamar W, Daniel M (1989) Enzyme-catalyzed biotransformations through photochemical regeneration of nicotinamide cofactors. Enzyme Microb Technol 11:467–483
15. Chen G, Kayser MM, Milhovilovic MD, Mrstik ME, Martinez CA, Stewart JD (1999) Asymmetric oxidations at sulfur catalyzed by engineered strains that overexpress cyclohexanone monooxygenase. New J Chem 23:827–832
16. Xu ZN, Liu Y, Fang LM, Jiang XX, Jing KJ, Cen PL (2006) Construction of a two-strain system for asymmetric reduction of ethyl 4-chloro-3-oxobutanoate to (*S*)-4-chloro-3-hydroxybutanoate ethyl ester. Appl Microbiol Biotechnol 70:40–46
17. Xu ZN, Jing KJ, Liu Y, Cen PL (2007) High-level expression of recombinant glucose dehydrogenase and its application in NADPH regeneration. J Ind Microbiol Biotechnol 34:83–90
18. Yang W, Zhang L, Lu Z, Tao W, Zhai Z (2001) A new method for protein coexpression in *Escherichia coli* using two incompatible plasmids. Protein Expr Purif 22:472–478
19. Wang FH, Qu HJ, Zhang DW, Tian PF, Tan TW (2007) Production of 1, 3-propanediol from glycerol by recombinant *E. coli* using incompatible plasmids system. Mol Biotechnol 37:112–119
20. Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 239:2370–2378
21. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
22. Li AT, Zhang JD, Xu JH, Lu WY, Lin GQ (2009) Isolation of *Rhodococcus* sp. ECU0066: a new sulfide monooxygenase producing strain for asymmetric sulfoxidation. Appl Environ Microbiol 75:551–556
23. Mikolajczyk M, Drabowicz J, Kielbasinski P (1997) Chiral sulfur reagents; Applications in asymmetric and stereoselective synthesis. CRC Press, Boca Raton
24. Kataoka M, Kita K, Wada M, Yasohara Y, Hasegawa J, Shimizu S (2003) Novel bioreduction system for the production of chiral alcohols. Appl Microbiol Biotechnol 62:437–445
25. Ernst M, Kaup B, Müller M, Bringer-Meyer S, Sahm H (2005) Enantioselective reduction of carbonyl compounds by whole-cell biotransformation, combining a formate dehydrogenase and a (*R*)-specific alcohol dehydrogenase. Appl Microbiol Biotechnol 66:629–634
26. Weckbecker A, Hummel W (2005) Glucose dehydrogenase for the regeneration of NADPH and NADH. Methods Biotechnol 17:225–237
27. Zambianchi F, Pasta P, Carrea G, Colonna S, Gaggero N, Woodley JM (2002) Use of isolated cyclohexanone monooxygenase from recombinant *Escherichia coli* as a biocatalyst for Baeyer-Villiger and sulfide oxidations. Biotechnol Bioeng 78:489–496
28. Alphand V, Carrea G, Wohlgemuth R, Furstoss R, Woodley JM (2003) Towards large-scale synthetic applications of Baeyer-Villiger monooxygenases. Trends Biotechnol 21:318–323