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ORIGINAL PAPER

High-level expression of recombinant glucose dehydrogenase and its application in NADPH regeneration

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Abstract Two glucose dehydrogenase (E.C. 1.1.1.47) genes, gdh223 and gdh151, were cloned from *Bacillus megaterium* AS1.223 and AS1.151, and were inserted into pQE30 to construct the expression vectors, pQE30-gdh223 and pQE30-gdh151, respectively. The transformant *Escherichia coli* M15 with pQE30-gdh223 gave a much higher glucose dehydrogenase activity than that with the plasmid pQE30-gdh151. Thus it was used to optimize the expression of glucose dehydrogenase. An proximately tenfold increase in GDH activity was achieved by the optimization of culture and induction conditions, and the highest productivity of glucose dehydrogenase (58.7 U/ml) was attained. The recombinant glucose dehydrogenase produced by *E. coli* M15 (pQE30-gdh223) was then used to regenerate NADPH. NADPH was efficiently regenerated in vivo and in vitro when 0.1 M glucose was supplemented concomitantly in the reaction system. Finally, this coenzyme-regenerating system was coupled with a NADPH-dependent bioreduction for efficient synthesis of ethyl (*R*)-4-chloro-3-hydroxybutanoate from ethyl 4-chloro-3-oxobutanoate.

Keywords *Bacillus megaterium* · Glucose dehydrogenase · NADPH regeneration · Enantioselective reduction · Ethyl 4-chloro-3-oxobutanoate

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Introduction

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 is one of the industrially important enzymes and useful in glucose determination kits and glucose biosensors. In addition, GDH has an increasing application in many bioreduction reactions for coenzyme regeneration. The asymmetric reduction of many chiral synthons catalyzed by oxidoreductases may prove especially promising [1–3]. Most of the oxidoreductases require nicotinamide cofactors (NADH or NADPH) to provide the reduction equivalents when catalyzing the enantioselective reduction of prochiral ketone [4, 5]. Normally yeast cells are good catalysts for bioreduction since they are capable of high regeneration of NADPH or NADH. However, when they catalyze the chiral compound synthesis, mixtures of stereoisomeric alcohols are also produced in many cases [6, 7]. In order to asymmetrically reduce β- keto esters to chiral alcohols efficiently, a commonly applied strategy is to clone and express the corresponding exogenous aldehyde-ketone reductases in *Escherichia. coli* [8]. However, this new strategy is always subject to a lower efficiency of nicotinamide cofactor generation and regeneration in *E. coli* than that in yeast strains. Since these nicotinamide cofactors are too expensive to be directly added in large-scale bioprocesses, it is necessary to develop some processes to generate and regenerate $NADP^+$ cofactor effectively in *E. coli* system.

Some previous work has been done to produce NADPH by using cytoplasmic enzymes, such as glucose

6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), malic enzyme (ME), and NADP-dependent isocitrate dehydrogenase (NADP-IDH), etc. [9]. Among them, GDH or G6PDH is preferred for the construction of a low-cost NADPH-regenerating system because it catalyzes the oxidation of cheap D-glucose to D-glucono-δ-lactone with concomitant reduction of $NADP^+$. For example, GDH from *Bacillus megaterium* IAM1030 was used to assist the regeneration of NADPH efficiently and has been employed in many chiral reduction reactions [10]. GDHs are produced by various bacterial species. The purified enzymes from *Bacillus cereus*, *B. megaterium*, and *Bacillus subtillis* have been characterized. Amino acid sequence alignment has indicated that $NAD(P)^+$-dependent GDHs from the *Bacillus* species have more than 80% homology. Since the GDH activity in these natural strains cannot meet the demand of its increasing applications, some heterogous expressions of GDH in *E. coli* were reported [11, 12]. Although the GDH activity was improved significantly by recombinant *E. coli* strategy, further increase of the expression was still necessary. In our laboratory, *B. megaterium* AS1.223 and *B. megaterium* AS1.151 were screened out with higher bioactivity of GDH. In this work, GDH genes were cloned from AS1.223 and AS1.151, respectively. The bioactivities of recombinant GDHs using these two genes were compared. The results showed that one new gdhα gene from AS1.223 could lead to higher level expression of GDH in *E. coli*; and thus was employed to regenerate NADPH for the asymmetric biosynthesis of ethyl (*R*)-4-chloro-3-hydroxybutanoate from ethyl 4-chloro-3-oxobutanoate, which was catalyzed by an NADPH-dependent aldehyde reductase (ALR, EC1.1.1.2) expressed in another recombinant *E. coli* strain.

Materials and methods

Chemicals

Ethyl 4-chloro-3-oxobutanoate (COBE) was purchased from New Jersey J&K CHEMICA, NJ, USA. G6PDH (*Leuconostoc masenteroides*) was supplied by Worthington Co., Lakewood, NJ, USA. Ethyl (*R*)-4-chloro-3-hydroxybutanoate ((*R*)-CHBE) was a product from Sigma Co., St. Louis, MO, USA. β-NADPH-Na_4 and $NADP^+$ were obtained from CALBIOCHEM Co., Windsor, ON, Canada and AMRESCO, Solon, OH, USA, respectively. All of other chemicals were in analytical grade and commercially available.

Strains, plasmids, and enzymes

Bacillus megaterium AS1.223 and AS1.151 were obtained from Institute of Microbiology, Chinese Academy Science (IMCAS). Recombinant *E. coli* M15 (pQE30-ALR) was constructed to express ALR and preserved in our laboratory [13]. *E. coli* TG1 and *E. coli* M15 were used as the host strains for cloning and expression, respectively. pGEM-T (Gary Kobs Promega Corporation, Madison, WI, USA) and pQE30 (Qiagen GmbH, Hilden, Germany) were used as cloning and expressing vectors, respectively. All the restriction endonucleases and T4 ligase were purchased from TaKaRa (Dalian) Co. Ltd. (Okayama-Shi, Okayama, Japan).

Cloning of the GDHα gene and construction of the expressing plasmid

Genomic DNA of *B. megaterium* AS1.223 and AS1.151 was prepared according to standard procedures [14]. The GDHα gene was amplified by PCR with the primers of GGAATTCGGATCCATGTA TACAGATTTAAAAGA and AACTGCAGAAGC TTTAGCT TTATTTATTAACCT. Each primer contained *Eco*RI/*Bam*HI and *Pst*I/*Hin*d III restriction sites, respectively. The condition of PCR amplification was 35 cycles of 30 s at 94 °C, 30 s at 48 °C and 2 min at 72 °C. The amplified 825 bp DNA fragments were recovered by a QIAquick Gel extraction kit (Qiagen), and cloned in pGEM-T to obtain the cloning vectors pTgdh-223 and pTgdh-151, and then transformed into *E. coli* TG1. The sequences of these two gdh genes were sequenced, and gdh223 (GenBank accession no. AY930464) was named for the gene from AS1.223 and gdh151 (GenBank accession no. AY930465) for the gene from AS1.223.

The above cloning vectors were extracted from the cell culture and cleaved by *BamH*I/*Hind* III. The smaller fragment of each cleaved mixture was recovered and inserted into pQE30 to obtain pQE30-gdh223 and pQE30-gdh151, respectively (Fig. 1); and the plasmids were transformed into *E. coli* M15 to obtain *E. coli* M15 (pQE30-gdh223) and M15 (pQE30-gdh151), respectively.

Medium and cultivation

Bacillus megaterium AS1.223 and *B. megaterium* AS1.151 were cultivated at 35 °C in a medium containing 1% trypone, 0.3% beef extract, and 0.5% NaCl. The recombinant strains were cultivated at 37 °C in a Luria-Bertani (LB) medium with 100 μg/ml ampicillin

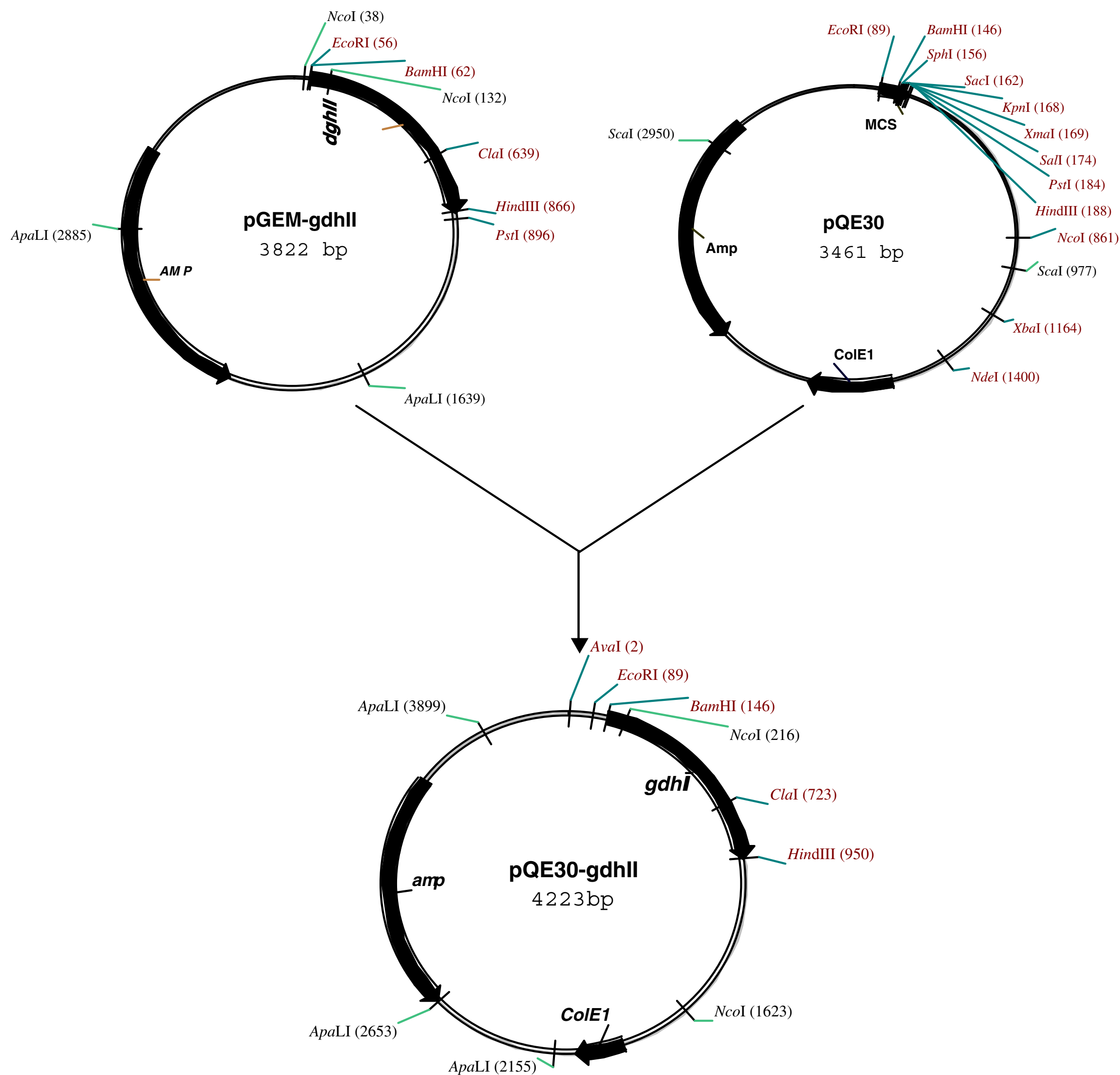

Fig. 1 A schematic description for the construction of expression vector pQE30-gdhII. When gdh223 and gdh151 were used as gdhII gene, the expression vectors, pQE30-gdh223 and pQE30-gdh151, were constructed, respectively

and 30 μg/ml kanamycin. When the cell density reached OD_{600} 2.0, 0.5 mM isopropyl β-D-thiogalacto-pyranoside (IPTG) was added to induce the expression of recombinant enzyme. After 3-h expression, the cells were harvested by centrifugation (4,000*g*, 10 min), washed with 100 mM potassium phosphate buffer (KP_i pH 6.0), and then stored at 4 °C for future use.

In order to improve recombinant GDH activity, a rich medium (MMBL) was employed to support higher growth of recombinant cells. The MMBL medium is consisted of: glucose, 20 g/l; tryptone, 20 g/l; yeast extract, 10 g/l; K_2HPO_4, 5 g/l; KH_2PO_4, 3.5 g/l; $(NH_4)_2HPO_4$, 3.5 g/l; NaCI, 5 g/l; Mg_2SO_4·7HO, 1 g/l; ampicillin, 100 μg/ml; and kanamycin, 30 μg/ml [15, 16]. Various conditions for cell growth and induction were tested to optimize the production of the recombinant enzyme by the recombinant *E. coli* M15.

Assay of enzyme activity and protein concentration

After cultivation, cells of recombinant *E. coli* M15 were harvested by centrifugation at 5,000*g* for 15 min, and were sonicated on ice (30 × 10 s pulses with 10-s intervals) using Ultrusonic Cell Disrupter (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China) in 100 mM potassium phosphate buffer (pH 7.0). The debris was removed by centrifugation at 13,000*g* for 10 min to obtain a cell-free extract. The GDH activity of cell-free extract was determined at 30 °C with an assay mixture containing 75 mM Tris–HCl (pH 8.0), 2.0 mM $NADP^+$, and 0.1 M D-glucose. One unit of enzyme activity was defined as the amount of producing 1 μmol NADPH per min. The reactions were monitored by measuring the increase of A_{340}.The protein concentration of the cell-free extract was determined

by the method of Bradford (Bio-Rad, Richmond, CA, USA), using bovine serum albumin as the standard. The bioactivities of GDH in *B. megaterium* AS1.223 and AS 1.151 were determined by using the same procedure.

GC and HPLC conditions

Ethyl acetate was used to extract COBE and CHBE from the reaction mixture. After mixing and centrifugation, the top phase (organic layer) was assayed to determine the yield and optical purity of (*R*)-CHBE. The concentrations of COBE and CHBE were determined by gas/liquid chromatography. The optical purity of (*R*)-CHBE was measured by high-performance liquid chromatography on a Chiralcel OB packed column (4.6 × 250 mm; Daicel Chemical Industries, Tokyo, Japan) at 217 nm with an eluent of n-hexane/2-propanol (9:1, v/v) at the flow rate of 1 ml/min. Under these conditions (*R*)- and (*S*)-CHBE were eluted at 10.5 and 11.6 min, respectively.

Results and discussion

Cloning and homological analysis of the GDHII gene

Two GDH coding genes, gdh223 and gdh151, were obtained by PCR from *B. megaterium* AS1.223 and *B. megaterium* AS1.151, respectively. The nucleotide sequences of gdh233 and gdh151 compared. The results showed that both gdh223 and gdh151 had high homology (94.7%) with the reported sequence of gdhα in *B. megaterium* IAM1030; and that the deduced amino acid sequences of GDH223 and GDH151 had very high homology (98.5%) with the reported sequence of GDH. Further analysis (Fig. 2) indicated that the only difference between GDH 223 and GDH 151 was one amino acid: Arg39 was found in GDH223, while Ser39 was found in GDH151. Because the previously reported gdhα gene belongs to the family of short-chain GDHs [17], accordingly, these two new GDHα genes (gdh223 and gdh151) should be derived from the same GDH family.

Comparison of the bioactivity of glucose dehydrogenase in recombinant *Escherichia coli* M15

Based on the two cloning plasmids, two expression vectors (pQE30-*gdh*223) and (pQE30-*gdh*151) were constructed and transformed into *E. coli* M15, respectively. As shown in Table 1, under the same culture conditions in LB medium, higher specific enzyme activity (2.16 U/mg protein) was achieved with M15 (pQE30-gdh223) after 3-h induction using 0.5 mM IPTG; while a lower specific bioactivity (0.19 U/mg protein) was achieved with M15 (pQE30-gdh151). Both the expressed target proteins in M15 (pQE30-gdh223) and M15 (pQE30-gdh151) were soluble. The specific enzyme activity from M15 (pQE30-gdh223) was about ten times higher than that from M15 (pQE30-gdh151) and three times higher than the highest specific GDHα activity in recombinant *E. coli* reported in the literature to date (ca. 0.70 U/mg protein) [17]. Compared to native *B. megaterium* AS1.223, the specific activity of GDHα in M15 (pQE30-gdh223)

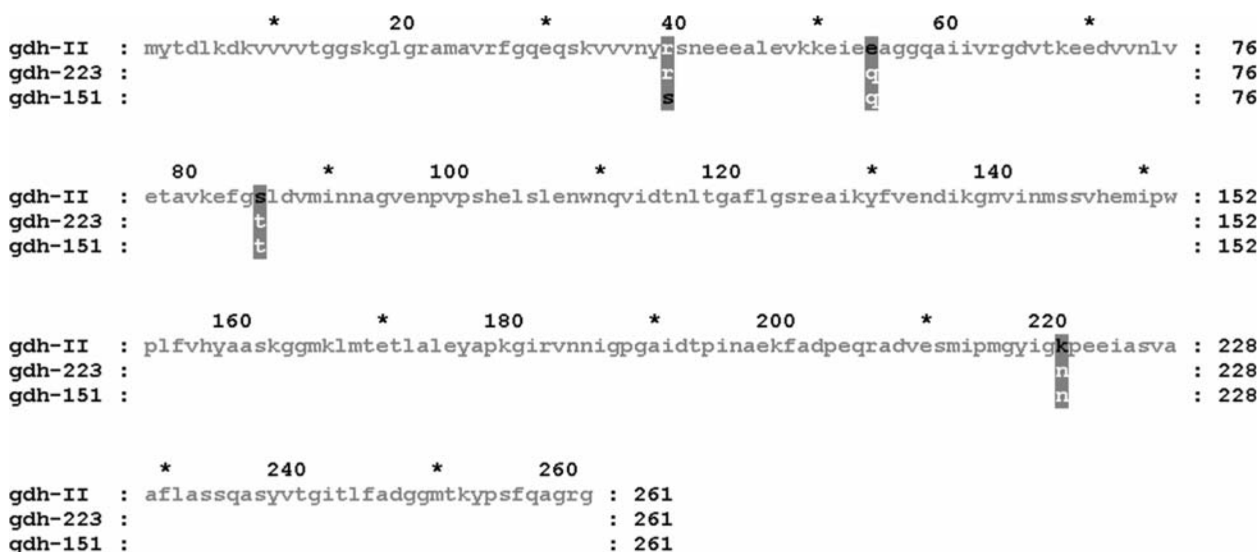

Fig. 2 Homology alignment of amino acid sequences of three glucose dehydrogenases. The amino acid sequences of GDHα, GDH151, and GDH223 were deduced from the corresponding nucleotide sequences

Table 1 Comparison of specific GDH activities among different strains

	Escherichia coli M15	*Bacillus megaterium* AS1.223	*Escherichia coli* M15 (pQE30-gdh223)	*Bacillus megaterium* AS1.151	*Escherichia coli* M15 (pQE30-gdh151)
Specific GDH activity (U/mg protein)	0.06	0.16	2.08	0.11	0.21

was increased 50 times. As aforementioned, the difference in amino acid sequence between GDH233 and GDH151 was only in the 39th amino acid (arginine (Arg, R) in GDH223 and serine (Ser, S) in GDH151). The GDHα consists of four identical subunits (28.2 × 4kDa), which are similar to other short-chain dehydrogenases (SDRs) in their overall folding and subunit architecture. One of the basic residues is well-conserved arginine among $NADP^+$-preferring SDRs, and only Arg39 was found around the adenine ribose moiety of SDRs [18]. Unlike Arg39 in GDH223, the 39th amino acid in GDH151 is Ser39 which might lead to negative change in coenzyme specificity and eventually reduced the enzyme bioactivity. This explains the different expression levels of gdh223 and gdh151 in the same host. Since the reported GDHII still retains Arg39 in its amino acid sequence, its specific GDH activity was higher than those expressed by gdh151. However, three amino-acid differences between gdh223 and the GDHII gene resulted in the lower bioactivity of recombinant GDH expressed by the GDHII gene than that expressed by the gdh 223 gene. Because of its high specific GDH activity, *E. coli* M15 (pQE30-gdh223) was employed as a coenzyme (NADPH) regenerator in the following experiments.

Improvement of glucose dehydrogenase production by optimization of expression conditions

In order to increase the productivity of recombinant GDH, a rich medium (MMBL) was used for the growth of *E. coli* M15(pQE30-gdh223) at 37 °C. In MMBL medium, the maximal OD_{600} reached ca. 11.0; while in LB the maximal OD600 was about 4.0. As a result, the recombinant enzymatic activity was improved from 5.6 to 30.0 U/ml when using MMBL instead of LB medium (Fig. 3). In the process of recombinant enzyme production in *E. coli*, IPTG induction is the turning point between cell growth and recombinant protein biosynthesis. The effect of induction timing was evaluated by adding 0.5 mM IPTG at various stages of exponential growth and stationary phases. As shown in Table 2, the highest values of GDH activity and specific GDH activity were observed when 0.5 mM IPTG was added at late stage in exponential growth. When IPTG was added at this growth stage, the optimal IPTG concentration was 0.3 mM. The optimal expression time was determined by taking samples at 2-h intervals till 8 h after induction. After 6-h induction the specific GDH activity reached 4.55 U/mg protein, and the highest volumetric productivity of GDH was up to 58.7 U/ml. Compared with the GDH activity in LB, about tenfold increase in GDH activity was achieved by the optimization of culture and induction conditions in MMBL. It was reported previously that the highest productivity of GDH in recombinant *E. coli* was 261 U/g wet cells [12]. In the present study, about 1,000–1,300 U/g wet cells was achieved under the optimized expression conditions. Moreover, higher biomass (OD600 12.5) and productivity could be attained simultaneously by inducing the culture at late exponential growth phase. It was also found that the specific activity of recombinant GDH after IPTG induction changed with expression conditions, especially induction time (Table 2). The reason could be that the solubility and refolded conformation of recombinant enzyme were influenced greatly by induction conditions, and some post-translated time was needed to get a mature protein with high enzymatic activity.

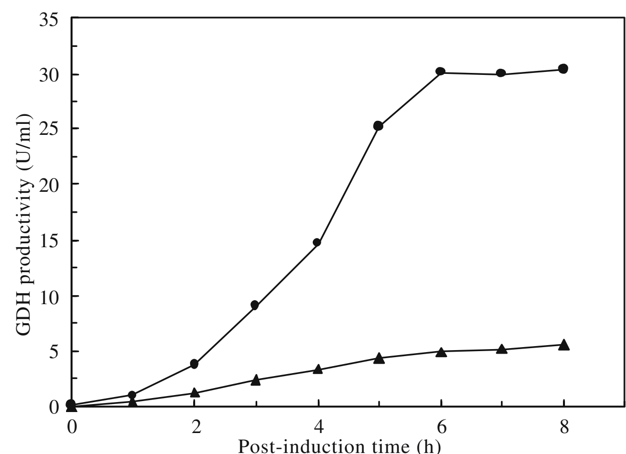

Fig. 3 Time course studies on GDH activities when recombinant M15 (pQE30-gdh223) was cultivated in LB (*filled triangle*) or MMBL (*filled circle*). All the tests were carried out in 250 ml flasks at 37 °C. For LB medium, 0.5 mM IPTG was added aseptically when the cell grew to 2.0 (OD600); for MBL medium, 0.5 mM IPTG was added aseptically when the cell grew to 5.0 (OD600)

Table 2 Effects of induction conditions on specific GDH activity with *Escherichia coli* M15 (pQE30-gdh223)

	Induction timing[a]				IPTG concentration (mM)[b]				Post-induction time (h)[c]			
	Early	Middle	Late	Stationary	0.1	0.3	0.6	0.9	2	4	6	8
GDH bioactivity (U/ml)	9.87	36.5	53.2	52.6	49.6	57.9	54.6	53.8	35.3	48.1	58.7	55.3
Specific GDH bioactivity (U/mg protein)	3.46	3.78	4.13	3.54	3.93	4.45	4.27	4.18	2.72	3.86	4.55	4.32

All the tests were carried out in 250 ml flasks at 37 °C. The post-induction expression time is 5 h besides the tests for the post-induction time

[a] Culture volume is 30 ml; 0.5 mM IPTG was added at various stages of the exponential growth phase: early (OD_{600} 2.0), middle (OD_{600} 6.0), late (OD_{600} 9.0), stationary (OD_{600} 11.0)

[b] Culture volume is 30 ml; IPTG was added at late stage of exponential growth phase

[c] Culture volume is 30 ml; 0.3 mM IPTG was added at late stage of exponential growth phase

Efficient regeneration of NADPH with this recombinant *Escherichia coli* M15

The effect of the recombinant GDH on coenzyme regeneration was evaluated by determining the profiles of NADPH in a bioreaction system. *E. coli* M15 (pQE30-gdh223) cells were harvested by centrifugation (4,000*g*, 10 min, 4 °C). The cells were washed with 100 mM potassium phosphate buffer (pH 7.0), and resuspended in the same buffer solution to determine the extracellular concentration of NADPH. As shown in Fig. 4, the extracellular NADPH concentration increased greatly when using *E. coli* M15 (pQE30-gdh223) instead of *E. coli* M15 in the reaction system. When the biomass of M15 (pQE30-gdh223) increased from 0.5 g (DCW)/l to 1.0 g (DCW)/l, the extracellular NADPH concentration was increased from 9.5 to 12.5 mM. The above results suggested that high intracellular GDH activity produced high concentration of NADPH, and the extracellular NADPH also increased by the enhanced driving force of cross-membrane mass transfer of NADPH. Therefore, whole cells of *E. coli* M15 (pQE30-gdh233) can be used to intensify the regeneration of extracellular NADPH.

The crude GDH enzyme of M15 (pQE30-gdh233) was prepared according to the above procedure and used to compare the ability of regenerating coenzyme with commercially G6PDH. When commercial G6PDH was used, high conversion rate of NADPH from $NADP^+$ (>99.0%) was attained after 5-h reaction when 0.1 or 0.5 mM $NADP^+$ and 0.1 M glucose were initially added in the cell-free reactive system (Fig. 5). High conversion rates of NADPH from $NADP^+$ (>99.0%) were also obtained when commercial G6PDH was replaced with 0.1 mg/ml crude GDH enzyme in the same cell-free system. This suggested that the crude GDH enzyme from *E. coli* M15 (pQE30-gdh223) was very effective in regenerating the coenzyme (NADPH).

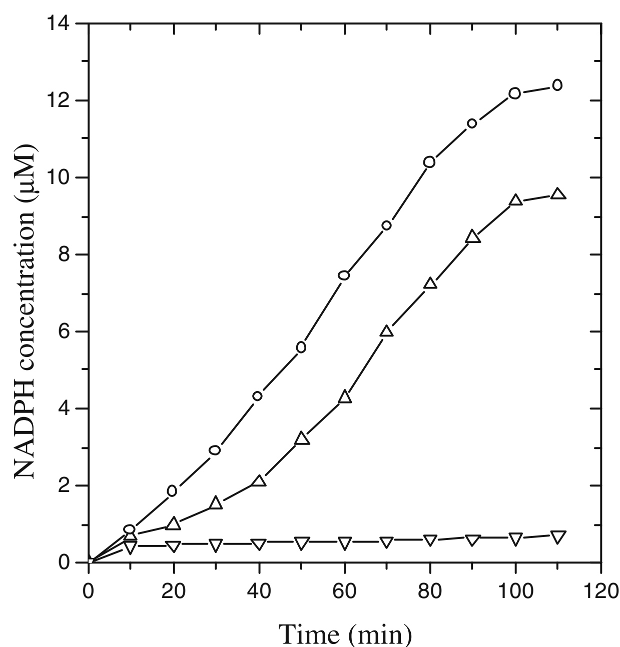

Fig. 4 The profiles of extracellular NADPH concentration with *Escherichia coli* M15 (pQE30-gdh233) or *Escherichia coli* M15. NADPH concentration was determined at 340 nm, with the 100 mM potassium phosphate buffer (pH 6.0) as the blank. The molar absorption coefficient of NADPH at 340 nm is 6,300/M/cm. All the tests were carried out in 3 ml KP_i buffer (100 mM, pH 7.0), including 100 mM glucose at 37 °C. *Open circle*, 1 mg/ml wet cells of M15 (pQE30-gdh223); *Open triangle*, 0.5 mg/ml wet cells of M15 (pQE30-gdh223); *Open inverted triangle*, 1 mg/ml wet cells of M15

Practical application of M15 (pQE30- gdh223) in (*R*)-CHBE synthesis

Many bioreduction reactions are coupled with the regeneration of coenzyme (NADH/NADPH). In order

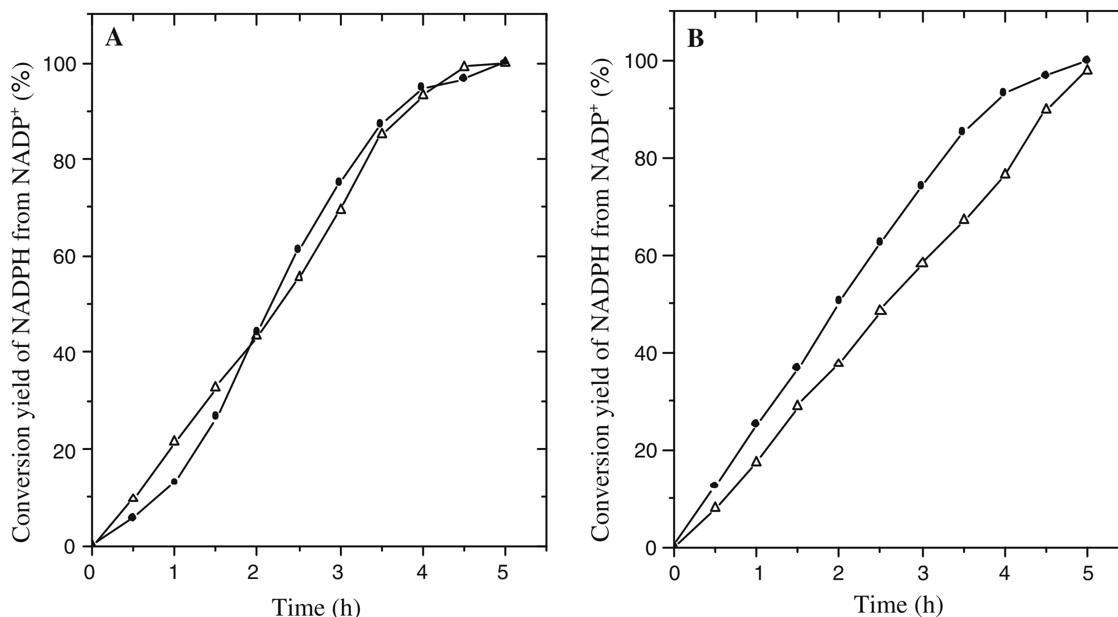

Fig. 5 The profiles of NADPH conversion from $NADP^+$ using crude GDH enzymes from M15 (pQE30-gdh233) (**a**) or G6PDH (**b**). NADPH concentration was determined at 340 nm, with the 100 mM potassium phosphate buffer (pH 6.0) as the blank. The molar absorption coefficient of NADPH at 340 nm is 6,300/M/cm. All the tests were carried out in 3 ml KP_i buffer (100 mM, pH 7.0) containing 100 mM glucose at 37 °C. **a** 0.1 mg/ml crude GDH enzyme from M15 (pQE30- gdh223); **b** 1 U/ml G6PDH. *Open triangle*, 0.1 mM $NADP^+$; *Filled circle*, 0.5 mM $NADP^+$

to demonstrate the efficiency of regenerating NADPH in a bioreduction process, whole cells of M15 (pQE30-gdh223) was employed in the asymmetric bioreduction of ethyl 4-chloro-3-oxobutanoate (COBE) to (*R*)-4-chloro-3- hydroxybutanoate ethyl ((*R*)-CHBE), which was catalyzed by ALR. The ALR-expressing *E. coli* M15 (pQE30-ALR) was constructed in our laboratory [13]. The commercial G6PDH or whole cells of M15 (pQE30-gdh223) were initially added into the same bioreduction system in order to maintain the regeneration of the coenzyme (NADPH). As shown in Fig. 6, when neither G6PDH nor M15 (pQE30-gdh223) was added in the reaction system, the yield of (*R*)-CHBE was only about 27.3%. However, when whole cells of M15 (pQE30-gdh223) were added, the yield of (*R*)-CHBE reached 100% with an enantioselectivity >99% after 4.5 h. This result was similar to or even better than those obtained when commercial G6PDH was used in the same system. The results revealed that M15 (pQE30-gdh223) could be used to replace commercially available G6PDH to generate NADPH effectively for this stereo-selective reduction reaction.

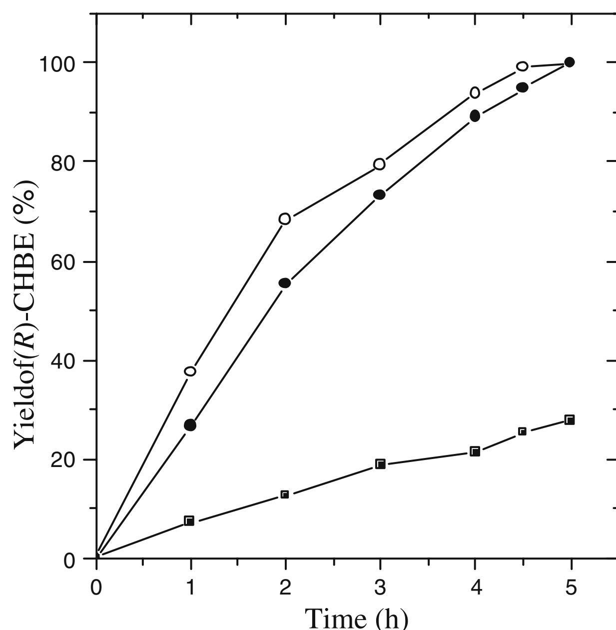

Fig. 6 Comparison of the bioreduction from COBE to (*R*)-CHBE among different reaction systems. All the tests were carried out in 10 ml KP_i buffer (100 mM, pH 6.0), including 6 mg/ml COBE and 100 mM glucose at 37 °C. *Open circle*, 40 mg/ml wet cells of M15 (pQE30-ALR) and 10 mg/ml wet cells of M15 (pQE30-gdh223); *Filled circle*, 40 mg/ml wet cells of M15 (pQE30-ALR), 10 U/ml G6PDH and 10 mM $NADP^+$; *Filled square*, 40 mg/ml wet cells of M15 (pQE30-ALR)

Conclusion

Two different GDH genes in *B. megaterium* were cloned and sequenced. The amino acid sequence of GDH223 is only one amino acid different with that of GDH151 (Arg39 in GDH223 and Ser39 in GDH151). The specific GDHα activity with *E. coli* M15 (pQE30-gdh223) was 10.4 times higher than that with *E. coli* M15

(pQE30-gdh151) and three times than that reported in literature. By using M15 (pQE30-gdh223), the productivity of GDH could be as high as 58.7 U/ml by optimizing the expression conditions. The expressed GDH enzyme could regenerate NADPH effectively no matter whether it was utilized as crude enzyme or whole cells. The whole cells of M15 (pQE30-gdh223) could be used to replace commercial G6PDH to regenerate NADPH for the asymmetrical biosynthesis of (*R*)-CHBE. This study could be helpful in establishing an efficient and cost-effective method to regenerate coenzyme (NADPH) for similar enantioselective reduction of prochiral ketones.

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