African Journal of Microbiology Research Vol. 5(32) pp. 5882-5888, 30 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.831

Full Length Research Paper

Cloning, expression and characterization of a glucose dehydrogenase from *Bacillus* sp. G3 in *Escherichia coli*

Xuejiao Chen[1,2], Haitao Ding[1], Yiqing Du[1], Hui Lin[1], Zeli Li[1] and Yuhua Zhao[1]*

[1]Institute of Microbiology, College of Life Science, Zhejiang University, Hangzhou 310058, China.
[2]Hangzhou Wahaha Group Co., Ltd, Hangzhou 310000, China.

Accepted 17 October, 2011

The glucose dehydrogenase gene (*gdh*), cloned from *Bacillus* sp. G3, was composed of 786 bp nucleotide and the deduced protein molecular mass of one subunit was 28.1 kDa. The recombinant glucose dehydrogenase (rGDH-G3) was functionally expressed in *Escherichia coli*. The results revealed that expressed rGDH-G3 had a high specific activity of 371.9 U/mg at 25°C and pH 8.0, with oxidized nicotinamide adenine dinucleotide (NAD^+) as the cofactor. The enzyme was optimally active at 40°C and pH 9.0. The enzyme displayed broad specificity for other sugars such as D-galactose or maltose. The catalytic efficiency of the rGDH-G3 would be improved 4 times when oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) was used as cofactor instead of NAD^+.

Key words: *Bacillus* sp. G3, enzymatic property, glucose dehydrogenase, inverse polymerase chain reaction (IPCR), optimal pH.

INTRODUCTION

Glucose dehydrogenase (GDH, EC 1.1.1.47), a member of the short-chain family of alcohol dehydrogenase, consists of four identical subunits (30 kDa) (Pauly and Pfleiderer, 1975), it catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone in the presence of cofactor oxidized nicotinamide adenine dinucleotide (NAD^+) or oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), and it posses the property of dual cofactor specificity. It has been shown that the GDH plays an important role in spore germination, and is a marker enzyme synthesized at sporulation stage (Nakatani et al., 1989). In recent years, GDH has been widely studied and used in many fields including biofuel cells (Okuda-Shimazaki et al., 2008), clinical tests (Du et al., 2008), and as a catalyst for coenzyme regeneration in large-scale chiral synthesis (Lin et al., 1999; Wong and Drueckhammer, 1985) .

In order to meet the increasing demands for the above applications, the glucose dehydrogenase gene (*gdh*) of microorganism have been cloned and over-expressed in *Escherichia coli* from sporulating cells of *Bacillus megaterium* (Heilmann et al., 1988; Nagao et al., 1992) and *Bacillus subtilis* (Vasantha et al., 1983). The characteristics of GDH from *B. megaterium* (Makino et al., 1989a; Mitamura et al., 1989; Nagao et al., 1992) and *B. subtilis* (Fujita et al., 1977) have been investigated in detail. In this paper, we reported the results of a study that was aimed at isolating the *gdh* from *Bacillus* sp. G3, expressing in *E. coli* BL21 (DE3), as well as purifying and

*Corresponding author. E-mail: yhzhao225@zju.edu.cn. Tel: 86-571-88208557. Fax: 86-571-88206995.

Abbreviations: ***gdh*,** Glucose dehydrogenase gene; **GDH,** glucose dehydrogenase; **GDH-G3,** glucose dehydrogenase from *Bacillus* sp. G3; **PCR,** polymerase chain reaction; **IPCR,** inverse PCR; **ORF,** open reading frame; **rGDH-G3,** recombinant glucose dehydrogenase; **NAD^+,** oxidized nicotinamide adenine dinucleotide; **$NADP^+$,** oxidized nicotinamide adenine dinucleotide phosphate; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Oligonucleotide primers used for *gdh* isolation, DNA amplification and cloning.

Primer	Sequence
dG3F	5'-GAYRTNATGATHAAYAAYGC-3'
dG3R	5'-ATRTANCCCATNGGDATCAT-3'
iG3F	5'-AATCGCTTCACGGCTTCC-3'
iG3R	5'-AGGGCGGATTGAAACTAA-3'
GDH3F	5'-GGAATTCCATATGTATAGTGATTTAGAAGGA-3'
GDH3R	5'-CGGGATCCTATTACCCACGCCCAGC-3'

[a]Underlined bases are restriction sites (*Nde*I in GDH3F and *Bam*HI in GDH3R).

characterizing this new enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmid and chemicals

The strain *E. coli* DH5α and *E. coli* BL21 (DE3) were used, respectively for cloning and expression. *Bacillus* sp.G3 used as the source of *gdh* was cloned and identified by our laboratory. Plasmids pMD19-T (Takara, Dalian, China) and pET28 (a+) (Invitrogen, Shanghai, China) were used as vectors for the cloning and expression of the *gdh*, respectively. Restriction enzymes and other modification enzymes, Taq DNA polymerase, and T4 DNA ligase were purchased from Takara, Dalian, China. Primers were synthesized by Invitrogen, Shanghai, China. Ni-NTA-resin was purchased from Invitrogen, Shanghai, China. DNA gel extraction kit was purchased from Axygen, Shanghai, China. NAD^+ and $NADP^+$ were purchased from Alfa Aesar, Tianjin, China. All other chemicals and solvents used were of analytical grade and available commercially.

Enzyme activity assays

The activity of glucose dehydrogenase from *Bacillus* sp. G3 (GDH-G3) was assayed by measuring the increase in absorbance of nicotinamide adenine dinucleotide (NADH) at 340 nm. The standard reaction mixture contained 100 mM sodium phosphate buffer (pH 8.0), 200 mM glucose, and 1 mM NAD^+ with a final volume of 1 ml. One microlitre (1 µl) diluted enzyme solution was added to the assay mixture and incubated at 25°C for 5 min. The apparent extinction coefficient of NADH was 6220 M^{-1} cm^{-1}. One unit of GDH activity was defined as the amount of enzyme required to release 1 µM of NADH per minute at 25°C and the pH of 8.0. All assays were repeated three times.

Cloning of the glucose dehydrogenase gene (*gdh*)

To clone a fragment of *gdh* from *Bacillus* sp.G3, a polymerase chain reaction (PCR) strategy with degenerate primers were used. Primers dG3F and dG3R (Table 1) were designed based on the conserved amino acid sequence (D V/I MINNA and M V/I PMGYI) of GDHs from different species of *Bacillus*. The PCR was conducted under the following conditions: 94°C for 5 min; 5 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 30 s; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a terminal extension at 72°C for 2 min. After separation and purification, a 400 bp PCR product was sequenced. The complete GDH-G3 gene was obtained by using the inverse PCR (IPCR) technique (Ochman et al., 1988). The genomic DNA from *Bacillus* sp.G3 was digested with several endonucleases, and then self-ligated by using T4 DNA ligase at 16°C overnight. The ligation products were used as template for IPCR. A pair of primers iG3F and iG3R (Table 1) used were designed based on the above product using Primer Premier 5.0. And the reaction was conducted for 30 cycles: 94°C for 30 s, 55°C for 30 s and 72°C for 3 min. The resultant fragment was sequenced, and then the complete GDH-G3 gene was assembled according to overlapping sequences from the two fragments.

Construction of expression plasmid

Recombinant DNA techniques were carried out according to standard methods described by Sambrook and Russell (2001). The open reading frame (ORF) of GDH-G3 was amplified by PCR with the genomic DNA of *Bccillus* sp. G3 as the template, and GDH3F and GDH3R (Table 1) as primers, respectively. The purified PCR product was digested with *Nde*I and *Bam*HI, and ligated with the pET-28a (+) that was linearized with the same enzymes, forming a new ORF that encoded an N-terminal His_6-tag. The recombined plasmids were transformed into *E. coli* DH5α competent cells for amplification.

Expression and purification of recombinant glucose dehydrogenase (rGDH-G3)

To express 6his-tagged *gdh*, the recombinant vector was transformed into *E. coli* BL21 (DE3) competent cells using $CaCl_2$-heat shock method (Sambrook and Russell, 2001). The positive transformants were cultured at 37°C in Luria–Bertani (LB) medium containing 50 µg ml^{-1} kanamycin to an OD_{600} = 0.6. After induction with 0.1 mM IPTG at 25°C for 16 h, the culture was harvested. The cell pellet was washed twice with Buffer A (25 mM NaH_2PO_4, 250 mM NaCl, pH 8.0) and lysed by the ultrasonic disruption, followed by centrifugation at 14000 rpm for 30 min at 4°C. The supernatants were loaded onto a Ni-NTA-resin (Invitrogen, Shanghai, China) column pre-equilibrated with Buffer A. After washed with the Buffer B (25 mM NaH_2PO_4, 250 mM NaCl, 20 mM imidazole, pH 8.0), the rGDH-G3 was eluted with 10 ml Buffer C (50 mM NaH_2PO_4, 250 mM NaCl, 250 mM imidazole, pH 8.0), and then supplemented with 20% (v/v) glycerol. The purified enzyme fractions were dialyzed and stored at 4°C. The protein concentration was determined by the method of Bradford with BSA as the standard (Bradford, 1976). The purity and molecular mass of the enzyme was analyzed by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and stained with 0.05% Coomassie brilliant blue R-250 (Varghese and Diwan, 1983).

Characterization of the recombinant glucose dehydrogenase (rGDH-G3)

The optimal temperature of rGDH-G3 was studied at various

temperatures between 25 and 65°C under standard conditions. To determine the thermostability, the enzyme solution was preincubated in sodium phosphate buffer (25 mM, pH 8.0) at 25–65°C for 60 min, respectively. To investigate the optimum reaction pH range for rGDH-G3, four buffers were used and they include 100 mM citrate buffer (pH 4.0–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0), 100 mM Tris/HCl (pH 7.0–9.0), and 100 mM Gly/NaOH (pH 8.5–10.5). The pH stability was determined by pre-incubating diluted enzyme in the above buffers at 25°C for 60 min. For kinetic studies, the reaction rates were measured for a variety of substrates concentrations and the kinetic parameters were evaluated by Lineweaver–Burk plots method.

RESULTS AND DISCUSSION

Isolation and sequence analysis of *gdh* from *Bacillus* sp.G3

A BLAST search in the GenBank database showed that the fragment was highly identical to other *gdh* in the database (Table 1). Based on the conserved DNA sequence, a pair of gene-specific primers iG3F and iG3R (Table 1) for IPCR were designed. The digested genomic DNA from *Bacillus sp*.G3 was self-ligated, and was used as template for IPCR to clone the flanking sequence of the GDH-G3 gene. A notable 1500 bp band was amplified only from *NdeI*-digested genomic DNA. It was sequenced and then the complete GDH-G3 gene was assembled according to overlapping sequences of the two fragments. The DNA sequence of GDH-G3 gene showed significant homology (97-78% identities) with other *gdh* sequences present in NCBI.

The nucleotide sequence of the gene and the deduced amino acid sequence were deposited in GenBank (Accession no. GQ402830). The gene contained a 786 bp ORF encoding a subunit of 261 residues with a predicted molecular mass of 28.1 kDa.

The deduced amino acid sequence of GDH-G3 showed 99% (maximum) identity with GDH subunit from *B. cereus* BDRD-ST26 (Accession no. ZP_04269989) and *B. cereus* H3081.97 (Accession no. ZP_03238186). In addition, it showed more than 80% sequence homology with the GDHs from most species of *Bacillus*. Sequence alignment of the deduced amino acid sequence of the GDH-G3 with other GDH sequences in GenBank database revealed the presence of highly conserved regions (Figure 1).

Expression and purification of glucose dehydrogenase (GDH)

After transformation and induction, the recombinant plasmid was successfully expressed in heterologous host strain *E. coli* BL21 (DE3). The recombinant protein was further purified by using Ni^{2+}-chelating affinity chromatography. With the His_6-tag at the N terminus, which facilitated strongly binding of the protein to the Ni-NTA matrix, most unbound proteins were washed away by the wash buffer. As shown in Figure 2, portion of the enzyme was expressed in a highly soluble form and the purified enzyme revealed a single protein band corresponding to approximately 28 kDa on SDS-PAGE, which was agreed with the predicted 28.1 kDa molecular mass. After being purified 20-fold, the specific activity of the enzyme was 371.9 U/mg (25°C, pH 8.0, with NAD^+ as the cofactor).

Effects of pH and temperature on enzyme activity

The rGDH-G3 had optimal activity at 40°C (Figure 3A) and pH 9.0 (100 mM Tris/HCl, Figure 3B), respectively. The enzyme had more than 60% of the maximum activity in a pH range of 7.5–9.5 and a temperature range of 30-50°C. The assays of optimal temperature indicated that the activity increased in an almost linear fashion from 25-40°C, but decreased once the temperature was above 45°C. The activity was hardly detected at temperature higher than 70°C. It was observed that the activity of rGDH-G3 was affected by the buffers used. The recombinant enzyme in Tris/HCl performed much better than in Gly/NaOH buffers, while sodium phosphate buffer was better than Tris/HCl at the same pH.

As shown in Figure 3B, the pH/activity profile of GDH-G3 was similar to GDH-I, GDH-II and GDH-Iwg3 (Mitamura et al., 1989), with the optimal activity shifted a little towards a higher value. Optimal rGDH-G3 activity occurred at pH 9.0 while that of others from *B. megaterium* (Mitamura et al., 1989), *B. thuringiensis* M 15 (Boontim et al., 2004), *B. subtilis* (Fujita et al., 1977) occurred at pH 8.0, but the optimal pH in this study was lower than pH 9.5 for LsGDH from *L. sphaericus* G10 (Ding et al., 2010). Figure 1 showed the amino acid residues in GDH-G3 that are different from the other GDHs. Maybe the surrounding residues near the acid/base catalytic center affect the protonation and then effect a change in optimal pH (Shibuya et al., 2005).

Effects of pH and temperature on enzyme stability

The assays of enzyme thermostability indicated that the enzyme was stable below 40°C. After 60 min incubation, there was 72% of enzyme activity remained with 40°C treatment, whereas only 29% of the activity remained at 45°C (Figure 4A). The thermostability of GDH-G3 was similar to its homologous counterparts, except GDH-III from *B. megaterium* IAM1030. Nagao et al. (1992) have reported that the two alterations, Leu-167 to Gln and Ala-258 to Thr, weakened the intersubunit interaction of the tetramer of GDH-III (Nagao et al., 1992). Therefore, it could be presumed that, maybe, the replacement of Gln-167, Thr-258 of GDH-III with the Leu and Aln could have promoted the thermostability of GDH-G3. And it is

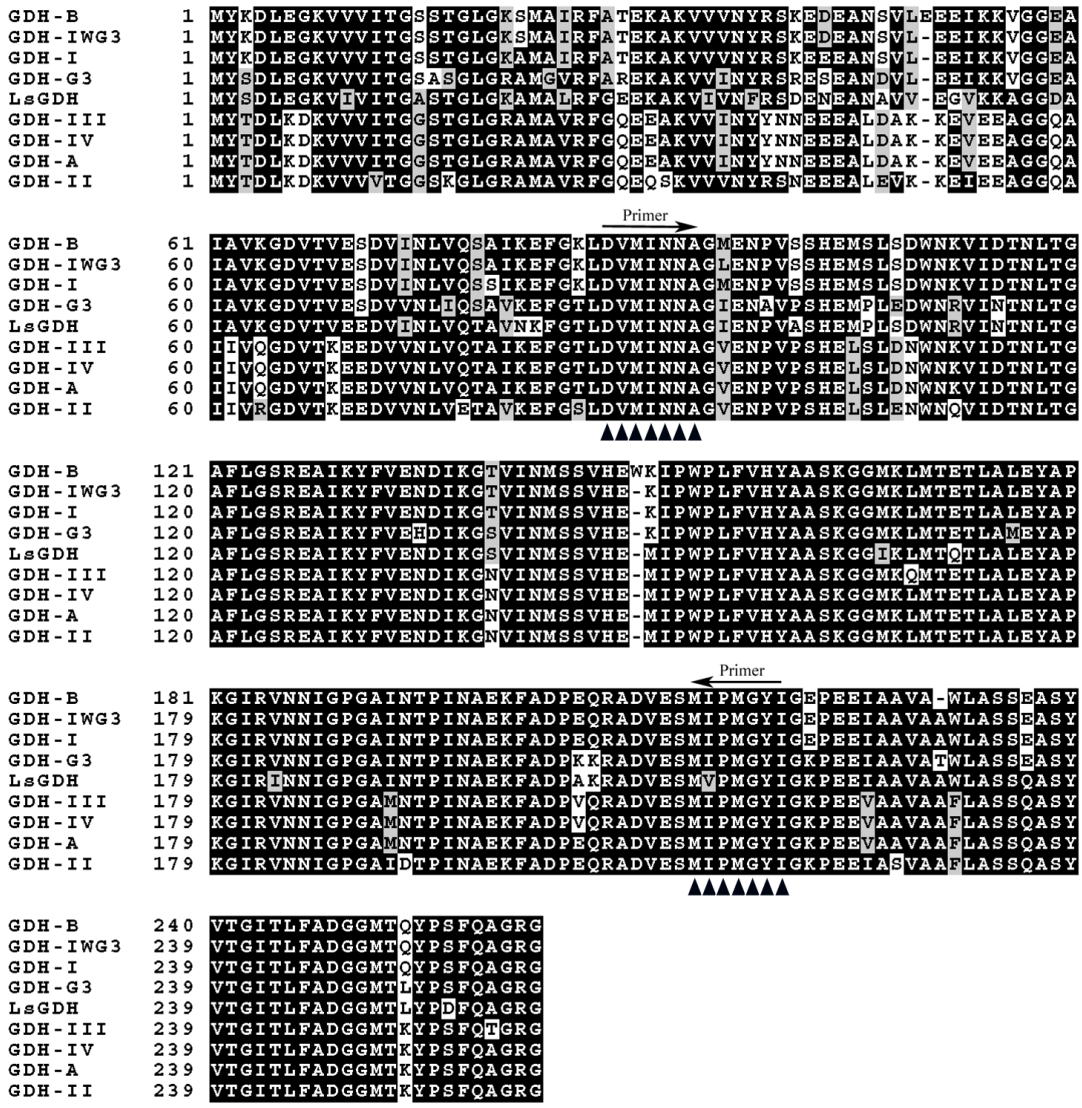

Figure 1. Sequence alignment of glucose dehydrogenases of *Bacillus* sp.G3 and other species of *Bacillus*. Sequences were aligned using Clustalx1.83. Identical residues and conserved substitutions are shaded black and gray by BOXSHADE 3.21 (K. Hofmann and M. Baron), respectively. GDH-I (Accession no. BAA14098.1), GDH-II (Accession no. BAA14100.1), GDH-III (Accession no. BAA01475.1), and GDH-IV (Accession no. BAA01476.1) were cloned from *Bacillus megaterium* IAM1030. GDH-Iwg3 (Accession no. 1RWB_A) was cloned from *Bacillus megaterium* Iwg3. GDH-G3 (Accession no.ACU78107) was cloned from *Bacillus* sp. G3.

possible that the presence of Leu-252 instead of Lys increased the heat resistance of GDH-G3, as described in previous reports (Makino et al., 1989b; Mitamura et al., 1989).

The pH-stability of GDH was examined by incubation at 25°C at various pH for 60 min and measurement of residual activity taken. The rGDH-G3 preserved its activity at the pH range between 4.0 and 9.0 (Figure 4B). Almost all of the GDHs from *Bacillus*, either wide type or mutant, were stable in the range of pH 6.0-7.5, particularly at 6.0 or 6.5, but the rGDH-G3 was more stability at pH 7.0. The GDH-G3 and GDH-Iwg3 are similar in the sequence (88% identity), and stable in the acidic range although GDH-Iwg3's stability was comparatively higher. The observed stability of the clones could be attributed to the effects of replacements as earlier reported by Mitamura et al. (1989).

Substrate specificity and enzyme kinetics

Table 2 illustrated that the rGDH-G3 possessed broad substrate specificity toward aldose sugars and disaccharides than others, particularly for D-galactose,

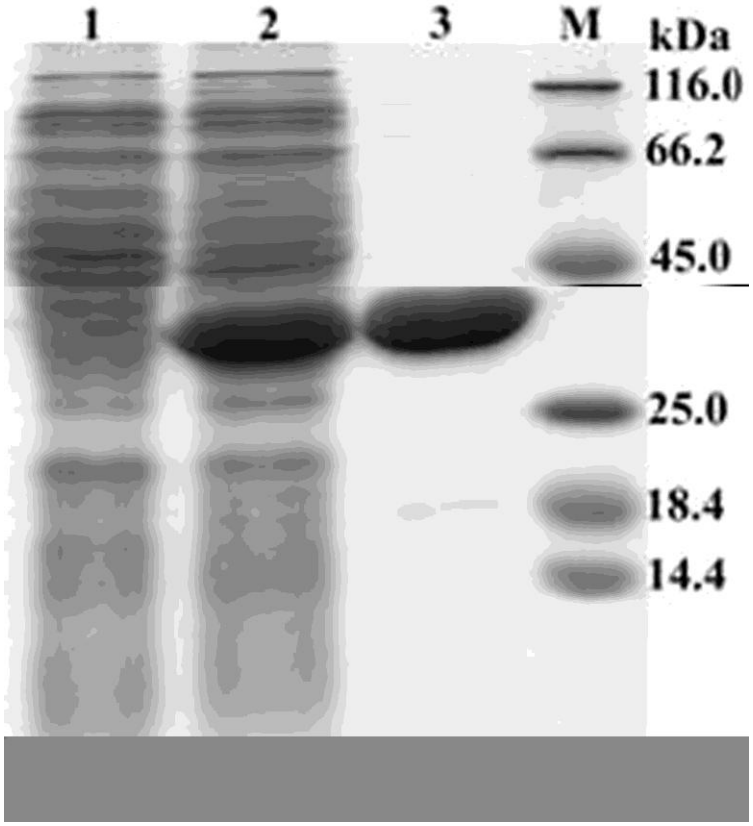

Figure 2. SDS-PAGE analysis of the rGDH-G3 from *E. coli* BL21 (DE3). **Lane 1,** Uninduced cellular extract; **Lane 2,** induced protein sample; **Lane 3,** the recombinant GDH (corresponds to 28 kDa) purified by Ni^{2+}-NTA; **Lane M,** standard protein molecular weight markers.

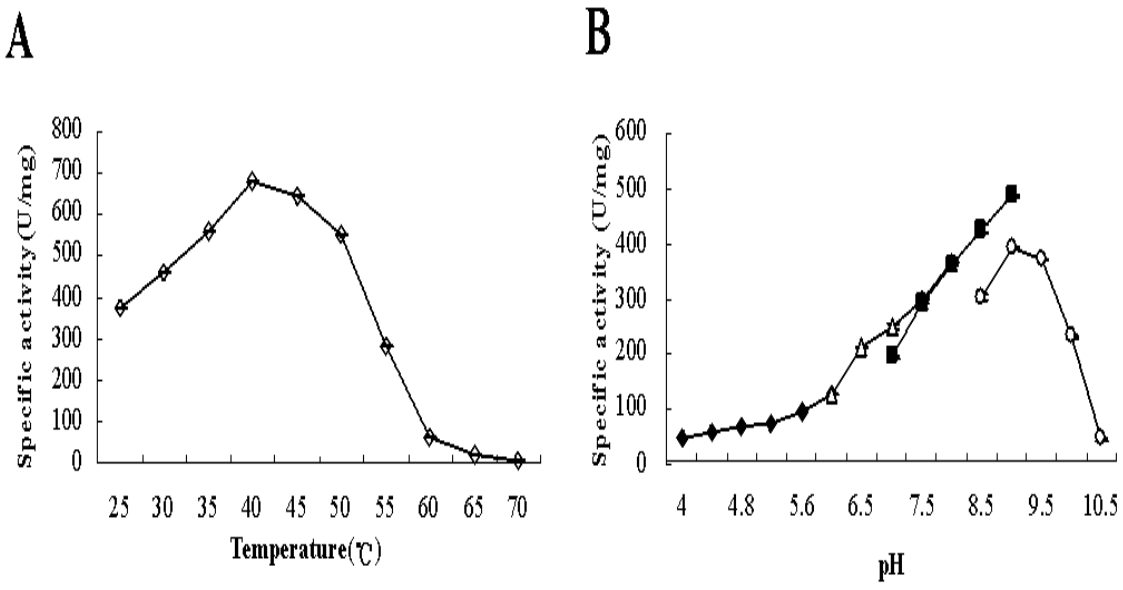

Figure 3. Effects of pH and temperature on enzyme activity. **A,** Effect of temperature on enzyme activity of the rGDH-G3 from 25-65°C in 100 mM sodium phosphate buffer (pH 8.0); **B,** effect of pH on enzyme activity of the rGDH-G3. pH range from 4.0-10.5 was used with the following buffers: ◆**,** 100 mM citrate buffer (pH 4.0–6.0); ▲**,** 100 mM sodium phosphate buffer(pH 6.0–8.0); ■**,** 100 mM Tris/HCl (pH 7.0–9.0); ○**,** 100 mM Gly/NaOH (pH 8.5–10.5).

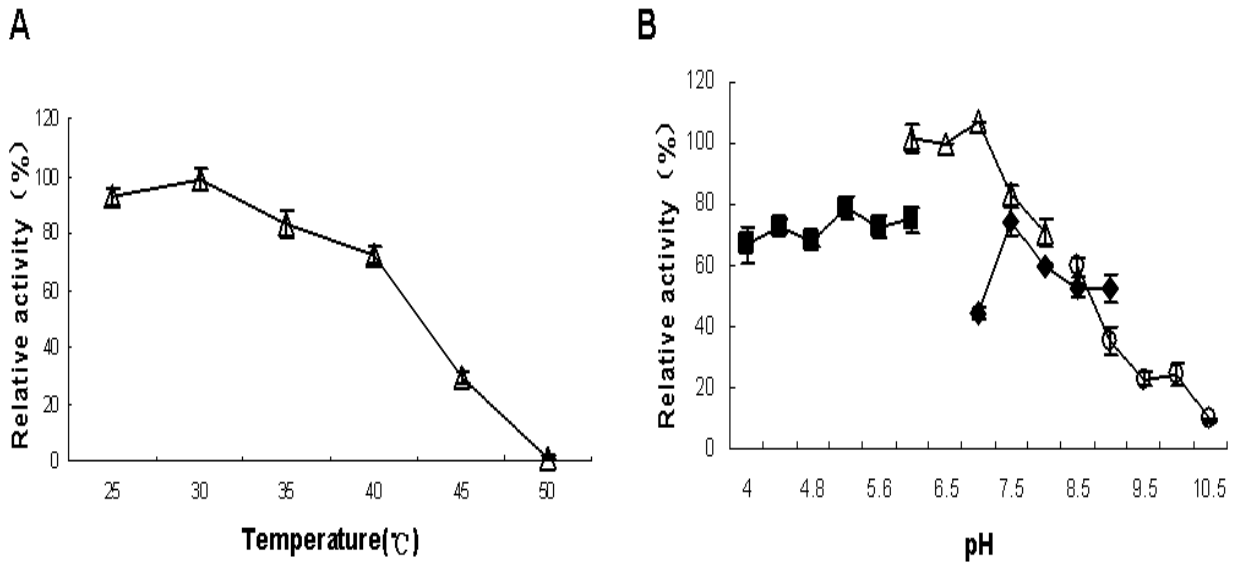

Figure 4. Effects of pH and temperature on enzyme stability. **A,** Thermostability of the rGDH-G3. After exposure for 60 min to the indicated temperature in sodium phosphate buffer (25 mM, pH 8.0). The activity of untreated rGDH-G3 was defined as 100% (319.8 U/mg protein); **B,** effect of pH on the stability of the rGDH-G3. The diluted enzyme pre-incubated in the different buffers (pH 4.0-10.5) at 25°C for 60 min. ■**,** 100 mM citrate buffer (pH 4.0–6.0); ▲**,** 100 mM sodium phosphate buffer(pH 6.0–8.0); ◆**,** 100 mM Tris/HCl (pH 7.0–9.0); ○**,** 100 mM Gly/NaOH (pH 8.5–10.5). The activity at pH 6.5 was defined as 100% (224.9 U/mg protein).

Table 2. Substrate specificity of rGDH-G3.

Substrate (0.2M)	Relative activity (%)[a]								
	GDH-G3	LsGDH	GDH-lwg3	GDH-I	GDH-II	GDH-IV	GDH-III	GDH-A	GDH-B
D-Glucose	100	100	100	100	100	100	100	100	100
D-Mannose	7.1	7.6	13	16	5.4	11	2.6	2	1
D-Galactose	22	17.3	3	5.8	1.8	3.8	0.9	0	0
D-Fructose	0.6	0.5	1.5	1.7	0.4	0.6	0.1	0	0
D-Arabinose	0.2	0	<0.1	<0.1	<0.1	<0.1	<0.1	ND[b]	ND
D-Xylose	6.4	22.5	9.9	12	3.5	7.1	1.8	ND	ND
Sucrose	6.3	6.3	ND	ND	ND	ND	ND	ND	ND
Lactose	2.6	4.2	ND	ND	ND	ND	ND	ND	ND
Maltose	13	22.4	ND	ND	ND	ND	ND	ND	ND
References	This study	(Ding et al., 2010)	(Mitamura et al., 1989)			(Nagao et al., 1992)		(Heilmann et al., 1988)	

[a]Initial rates were determined as described in Materials and Methods, except that D-glucose was replaced with various sugars. The activities were related to that of D-glucose (347 U/mg protein) as 100%. For comparison, the values of other GDHs were also given, and all used NAD^+ as cofactor.
[b] None detected.

the C4 epimer of glucose, which reached 22% of the activity with D-glucose. This property broadens the application fields of the enzyme. It catalyzed a range of saccharide substrates, but almost no activity was detected towards fructose and arabinose. As reported by Bonete et al. (1996), any configuration change of glucose at C2, C3 and C4 positions markedly decrease the enzymes affinity for the substrate.

In order to investigate the kinetic parameters of rGDH-G3, the initial rate of enzyme reaction was measured under different substrates concentrations. The results in Table 3 showed that the K_m, and K_{cat} values for NAD^+ were remarkably larger than that for $NADP^+$, whereas the K_{cat} / K_m values for $NADP^+$ was approximately fourfold higher than that for NAD^+. Due to the different affinity for NAD^+ and $NADP^+$, the catalytic efficiency of the rGDH-G3 was higher when $NADP^+$ was used as coenzyme, which was consistent with GDH-I, GDH-II and GDH-lwg3

Table 3. The kinetic analysis of the recombinant GDH.

	K_m (mM) [a]	V_{max}(μM s^{-1} mg^{-1})	K_{cat} (s^{-1}) [b]	K_{cat}/K_m (mM $^{-1}$ s^{-1})
D-glucose	31.8±0.4	0.82±0.05	23±1	0.73±0.06
NAD^+	0.210±0.001	5.131±0.005	144±0.1	687.5±3.6
$NADP^+$	0.0095±0.0018	1.20±0.02	33.8±0.6	3687±629

[a]The kinetic parameters were determined as described in Materials and Methods. The K_m of glucose was determined in the range of 10-200 mM, with a fixed NAD^+ 1 mM. The K_m for NAD^+ (0.05–1 mM) and $NADP^+$ (0.005–0.1 mM) was determined with a fixed glucose concentration of 200 mM.
[b] The values of K_{cat} were calculated for one subunit.

(Mitamura et al., 1989). However, the amino acid residues involved in the kinetic constants are still unknown. Therefore, further studies based on directed evolution, site-directed mutagenesis and crystallography is necessary to unravel the exact relationship between structure and function of GDH-G3.

ACKNOWLEDGEMENTS

This study was supported by the National Hi-Tech Research and Development Program (863) of China (No. 2007AA06Z329), National Natural Science Foundation of China (31070079), the Science and Technology Project of Zhejiang Province (2008C13014-3), and the International Cooperation Project in Science and Technology of Zhejiang Province (No. 2008C14038).

REFERENCES

Bonete MJ, Pire C, FI LL, Camacho ML (1996). Glucose dehydrogenase from the halophilic archaeon *Haloferax mediterranei*: enzyme purification, characterisation and N-terminal sequence. FEBS Lett., 383(3): 227-229.

Boontim N, Yoshimune K, Lumyong S, Moriguchi M (2004). Purification and characterization of D-glucose dehydrogenase from *Bacillus thuringiensis* M15. Ann Microbiol, 54(4): 481-492.

Bradford MM (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Ding HT, Du YQ, Liu DF, Li ZL, Chen XJ, Zhao YH (2010). Cloning and expression in E. coli of an organic solvent-tolerant and alkali-resistant glucose 1-dehydrogenase from Lysinibacillus sphaericus G10. Bioresour. Technol., 102(2): 1528-1536.

Du P, Wu P, Cai C (2008). A glucose biosensor based on electrocatalytic oxidation of NADPH at single-walled carbon nanotubes functionalized with poly(nile blue A). J. Electroanal. Chem., 624(1-2): 21-26.

Fujita Y, Ramaley R, Freese E (1977). Location and properties of glucose dehydrogenase in sporulating cells and spores of *Bacillus subtilis*. J. Bacteriol., 132(1): 282-293.

Heilmann HJ, Magert HJ, Gassen HG (1988). Identification and isolation of glucose dehydrogenase genes of *Bacillus megaterium* M1286 and their expression in *Escherichia coli*. FEBS J., 174(3): 485-490.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680-685.

Lin SS, Miyawaki O, Nakamura K (1999). Continuous production of L-carnitine with NADH regeneration by a nanofiltration membrane reactor with coimmobilized L-carnitine dehydrogenase and glucose dehydrogenase. J. Biosci. Bioeng., 87(3): 361-364.

Makino Y, Ding JY, Negoro S, Urabe I, Okada H (1989a). Purification and characterization of a new glucose dehydrogenase from vegetative cells of *Bacillus megaterium*. J. Ferment. Bioeng., 67(6): 374-379.

Makino Y, Negoro S, Urabe I, Okada H (1989b). Stability-increasing mutants of glucose dehydrogenase from *Bacillus megaterium* IWG3. J. Biol. Chem., 264(11): 6381-6385.

Mitamura T, Urabe I, Okada H (1989). Enzymatic properties of isozymes and variants of glucose dehydrogenase from *Bacillus megaterium*. FEBS J., 186(1-2): 389-393.

Nagao T, Mitamura T, Wang XH, Negoro S, Yomo T, Urabe I, Okada H (1992). Cloning, nucleotide sequences, and enzymatic properties of glucose dehydrogenase isozymes from *Bacillus megaterium* IAM1030. J. Bacteriol., 174(15): 5013-5020.

Nakatani Y, Nicholson WL, Neitzke KD, Setlow P, Freese E (1989). Sigma G RNA polymerase controls forespore-specific expression of the glucose dehydrogenase operon in *Bacillus subtilis*. Nucleic Acids Res., 17(3): 999-1017.

Ochman H, Gerber AS, Hartl DL (1988). Genetic applications of an inverse polymerase chain reaction. Genetics, 120(3): 621-623.

Okuda-Shimazaki J, Kakehi N, Yamazaki T, Tomiyama M, Sode K (2008). Biofuel cell system employing thermostable glucose dehydrogenase. Biotechnol. Lett., 30(10): 1753-1758.

Pauly HE, Pfleiderer G (1975). D-Glucose Dehydrogenase from Bacillus-Megaterium M-1286-Purification, Properties and Structure. H.S. Z. Physiol. Chem., 356(10): 1613-1623.

Sambrook J, Russell DW (2001). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, New York, pp. 1.84-1.88, 1.116-1.119.

Shibuya H, Kaneko S, Hayashi K (2005). A single amino acid substitution enhances the catalytic activity of family 11 xylanase at alkaline pH. Biosci. Biotechnol. Biochem., 69(8): 1492-1497.

Varghese G, Diwan AM (1983). Simultaneous staining of proteins during polyacrylamide-gel electrophoresis in acidic gels by countermigration of coomassie Brilliant blue R-250. Anal. Biochem., 132: 481-483.

Vasantha N, Uratani B, Ramaley RF, Freese E (1983). Isolation of a developmental gene of Bacillus subtilis and its expression in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A., 80(3): 785-789.

Wong CH, Drueckhammer DG (1985). Enzymatic synthesis of chiral hydroxy compounds using immobilized glucose dehydrogenase from *Bacillus cereus* for NAD(P)H regeneration. Bio-Technol, 3(7): 649-651.