Bioresource Technology 147 (2013) 492–498

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Simultaneously improving stability and specificity of cell surface displayed glucose dehydrogenase mutants to construct whole-cell biocatalyst for glucose biosensor application

Bo Liang [1], Qiaolin Lang [1], Xiangjiang Tang, Aihua Liu *

Laboratory for Biosensing, Qingdao Institute of Bioenergy & Bioprocess Technology, and Key Laboratory of Bioenergy, Chinese Academy of Sciences, 189 Songling Road, Qingdao 266101, China

HIGHLIGHTS

- Wide type GDH was mutated by site-directed mutagenesis.
- Mutated GDH displayed bacteria showed excellent wide pH and thermo stability.
- Mutated GDH displayed bacteria exhibited high D-glucose specificity.
- Sensitive and stable electrochemical glucose biosensor developed.

ARTICLE INFO

Article history:
Received 13 July 2013
Received in revised form 13 August 2013
Accepted 14 August 2013
Available online 23 August 2013

Keywords:
Glucose dehydrogenase mutant
Surface display
Enzyme stability
Substrate specificity
Glucose biosensor

ABSTRACT

The improved stability and substrate specificity of cell surface displayed glucose dehydrogenase (GDH) mutants by replacing four amino acids from *Bacillus subtilis* by using site-directed mutagenesis was systematically investigated. A series of mutated GDHs including E170R/Q252L, V149K/E170R/Q252L, E170R/Q252L/G259A and V149K/E170R/Q252L/G259A, were fused to the ice nucleation protein for displaying on cell surface of *Eschericia coli*. Q252L/E170R/V149K, Q252L/E170R/G259A and Q252L/E170R/V149K/G259A variants were found stable at a wide pH range and shown excellent thermostability. Especially, the Q252L/E170R/V149K/G259A mutant showed half-life of ~3.8 days at 70 °C. Q252L/E170R/V149K/G259A variant exhibited the narrowest substrate specificity for D-glucose. The whole cell displayed GDH mutant could be cultured in a large scale with excellent enzyme activity and productivity. In addition, a sensitive and stable electrochemical glucose biosensor can be prepared using the GDH-mutant bacteria modified electrode. Thus, the whole cell biocatalysts are promising candidates for exploitation in a wide range of industrial applications.

1. Introduction

Glucose dehydrogenase (GDH) (EC 1.1.1.47) from *Bacillus* sp. is a member of short-chain dehydrogenase/reductase family (Jornvall et al., 1995), which widely exists in various organisms and involves in glucose metabolic pathways. *Bacillus subtilis* produces GDH in the forespore compartment during sporulation, which can react with D-glucose with NAD^+ or $NADP^+$ as its cofactor and produce D-gluconate (Fujita et al., 1977). Owing to the universality and high efficiency of enzyme reaction, GDH has been widely used in food and medicine industry fields as an important part of biofuel cell (Wang et al., 2012; Zhu et al., 2012), clinical blood glucose monitoring sensor (Bilen et al., 2007; Yoo and Lee, 2010) and NAD(P)H regeneration system (Schewe et al., 2008; Xu et al., 2006).

The crystal structure of glucose dehydrogenase (GlcDH) from *Bacillus megaterium* IWG3 has been determined (Zhang et al., 2009). It is a tetrameric protein composed of four identical subunits with a molecular weight of about 28 kDa (Nagao et al., 1992). A comparison of the amino acid sequences between the GDH from *B. subtilis* and GlcDH from *B. megaterium* revealed an identity above 80%, indicating parallel evolution of two species. The contact area between subunits A and B of GlcDH is significantly smaller than other short-chain dehydrogenase, and AB subunits lack of salt bridge, leading to weak interactions between them (Yamamoto et al., 2001; Zhang et al., 2009). The tetramer could rapidly dissociate into inactive monomers under the high temperature and alkaline condition (Pauly and Pfleiderer, 1977). Therefore, the poor stability of GDH from *Bacillus* sp. is the bottleneck in its practical industrial applications. Previous studies have

* Corresponding author. Tel.: +86 532 80662758; fax: +86 532 80662778.
E-mail address: liuah@qibebt.ac.cn (A. Liu).
[1] These authors contributed equally to this work.

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.08.088

focused on GlcDH from *B. megaterium* for improving its stability. Some mutants have been obtained using chemical mutagenesis and DNA rearrangement technology, such as E96A, Q252L, and E170K, etc. (Baik et al., 2003, 2005; Makino et al., 1989; Nagao et al., 1989). However, few studies have reported on the detailed mechanism and the variant with a better improved stability of GDH from *B. subtilis*. Moreover, wild type GDH could catalyze other saccharides with similar structure to glucose, such as D-maltose, D-xylose, D-galactose and D-mannose (Liang et al., 2013; Nishioka et al., 2012). Its low specificity has become the critical limit in the area of glucose biosensor development and other industrial applications. Although the structure-guided mutagenic study showed that substitution amino acid residues of C-terminal region could greatly affected the specificity of BmGlcDH-IV from *B. megaterium* IAM 1030 (Nishioka et al., 2012), little research has been conducted on the substrate specificity of GDH from *B. subtilis*.

Quikchange site-directed mutagenesis technique is a fast, easy and efficient method to obtain mutant gene by PCR reaction using the plasmid harboring the whole sequence as the template and primers containing the mutant sites. The technique has been successfully used to construct plasmids of various mutants (Olafsen et al., 2006).

The microbial cell surface display system enables foreign proteins to interact with substrates easily in external environment by fusing with appropriate anchoring motifs, such as OmpA, ice nucleation protein (INP) and a-agglutinin (Jahns and Rehm, 2012). The method could not only simplify laborious purification steps, but also improve stability of displayed proteins (Chen et al., 2011; Jahns and Rehm, 2012). To date, microbial surface display has been applied in many fields, such as enzyme library screening (Binder et al., 2010; Boder and Wittrup, 1997), vaccines (Majander et al., 2005; Samuelson et al., 2002) and biocatalysts to improve biomass production (Jin et al., 2013; Matano et al., 2013), biosensors (Li et al., 2012, 2013; Liang et al., 2012, 2013; Wang et al., 2013) as well as biofuel cells (Fishilevich et al., 2009; Xia et al., 2013).

Eschericia coli surface-displayed GDH using INP as an anchor motif had been constructed in our previous study (Liang et al., 2013). Unfortunately, the fusion protein was unstable when pH was above 10 and temperature rose to 40 °C. Additionally, the substrate specificity was not so satisfactory, because it also responded to D-maltose, D-mannose and D-xylose (Liang et al., 2013). Poly[(R)-3-hydroxybutyrate] depolymerase mutants were displayed on the surface of *E. coli* to form a whole-cell catalyst (Tan et al., 2013), and mutants with improved *p*-nitrophenyl butyrate activity were effectively screened. In the present study, the mutant GDH genes from *B. subtilis* generated through QuikChange technique were expressed as displayed enzymes on the surface of *E. coli*, and GDH mutants with excellent activity, stability and substrate specificity were obtained. Moreover, the scale-up of whole cell and its application in glucose biosensor were also investigated. Thus, the present research may provide a perfect whole cell biocatalyst for bioenergy, bioanalysis, and bio-industry.

2. Methods

2.1. Bacterial strain, plasmid, and culture conditions

E. coli DH5α (F^-φ80 lacZΔM15Δ (lacZYA-argF) U169 *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44λ-*thi*-1 *gyr*A96 *rel*A1 *pho*A) and BL21 (DE3) (F^- *omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3)) were used as the hosts for plasmid construction and enzyme expression, respectively. *E. coli* strains were grown in Luria–Bertani (LB) medium and incubated by rotary shaking at 200 rpm and 37 °C. When required, ampicillin at 100 μg/ml and kanamycin at 30 μg/ml were used for the corresponding recombinant *E. coli* selection.

2.2. Construction of expressed plasmids harboring GDH mutant-encoding genes

Plasmid pTInaPbN-Gdh harboring the genes of wild type GDH and N-terminal domain of INP from *Pseudomonas borealis*, which constructed in our previous study (Liang et al., 2013), was used as a template for further mutagenesis study. Site-directed mutagenesis via PCR was carried out by using QuikChange method. All primers used in this study are listed in Table 1. PCR products were digested with DpnI restriction enzyme and transformed into *E. coli* BL21 (DE3) by heat shock transformation. Transformants were selected on LB agar plates containing kanamycin (30 μg/ml). Four mutants, Q252L/E170R, Q252L/E170R/V149K, Q252L/E170R/G259A and Q252L/E170R/V149K/G259A were obtained.

2.3. Cell surface display of GDH wild type and mutants

Plasmids carrying mutant genes of Q252L/E170R, Q252L/E170R/V149K, Q252L/E170R/G259A and Q252L/E170R/V149K/G259A were transformed into *E. coli* BL21 (DE3). Strains were inoculated in LB liquid medium, and incubated by rotary shaking at 200 rpm until absorbance (OD_{600}) of ~0.6 at 37 °C. Expression of INP fused with GDH mutants were induced after isopropyl-β-D-thio galactopyranoside (IPTG) was added to the medium to a final concentration of 0.5 mM at 25 °C.

2.4. Assay of GDH activity, stability and substrate specificity

The whole cells displayed targeted proteins were washed twice with 75 mM Tris–HCl buffer (pH 8.0). The activities of wild-type and its variants were assayed spectrophotometrically by measuring the rate of NADH formation at 340 nm. The reaction solution consisted of 75 mM Tris–HCl buffer (pH 8.0), 5 mM D-glucose, 3 mM NAD^+ and whole cells. One unit of activity was defined as the amount of enzyme necessary for the production of 1 μmol NADH per minute.

To determine the thermal stability of the wild type and mutant enzymes, the whole cells were incubated in the 75 mM Tris–HCl buffer (pH 8.0) at 50–70 °C for 1 h. The residual activity was measured as described above. Besides, the whole cells with mutant GDH were incubated until no enzyme activity could be detected at 60 °C and 70 °C. Activity assay of the same amount of cells was performed at regular intervals. The pH stability was evaluated by incubating each of wild-type and its variants in a buffer solution at a pH between 3.5 and 10.5 (20 mM citrate–NaOH buffer, pH3.5–5.5; 20 mM phosphate buffer, pH5.5–7.5; 20 mM Tris–HCl buffer, pH7.5–8.5; 20 mM glycine–NaOH buffer, pH8.5–10.5) in the absence of NaCl for 1 h. The residual activity was determined as described above.

In order to determine the substrate specificity of the wild type GDH and its mutants, D-xylose, cellobiose, D-fructose, D-sucrose, D-maltose, D-mannose, D-galactose, D-ribose, L-arabinose (each

Table 1
PCR primers used in this study.

Primer	Sequence (5′ → 3′)
Q252L For	GACGGCATGACACTATATCCTTCATTCCAG
Q252L Rev	CTGGAATGAAGGATATAGTGTCATGCCGTC
E170R For	ATAAAGCTGATGACAAGAACATTGGCGTTGGA
E170R Rev	TCCAACGCCAATGTTCTTGTCATCAGCTTTAT
V149K For	TCCAGTGTGCACGAAAAGATTCCTTGGCCGTTA
V149K Rev	TAACGGCCAAGGAATCTTTTCGTGCACACTGGA
G259A For	CCTTCATTCCAGGCAGCCCGCGGTTAA
G259A Rev	TTAACCGCGGGCTGCCTGGAATGAAGG

100 mM) or D-glucose (5 mM) was added to the cell reaction buffer, respectively, and UV absorption values at 340 nm were measured.

2.5. The scale-up production of GDH-INP mutant protein by E. coli

The scale-up production and whole cell enzyme activity determination were performed in 3-L fermentor (BioFlo/CellGen115 Fermentor/Bioreactor, New Brunswick Company, USA). *E. coli* BL21 (DE3) harboring GDH mutant Q252L/E170R/V149K/G259A was used as the seed solution. Cells were cultured in M9 minimum medium at 37 °C and pH 7.0 with dissolved oxygen (DO) of 20%. When the cells growth reached the log phase, 0.5 mM IPTG as inducer were added into the fermentor and the temperature was reduced to 25 °C for induction of fusion protein. The culture was harvested after 24 h of fermentation. The dry weight and GDH activity of whole cell were determined.

2.6. Preparation of Nafion/GDH-mutant/MWNTs composite film modified electrode

The glassy carbon electrode (GCE) was polished to a mirror successively using 1.0, 0.3, 0.05 μm alumina slurry, followed by thoroughly washed by sonication in anhydrous ethanol and ultrapure water. The electrode was rinsed with ultrapure water and allowed to dry at room temperature. Multi-walled carbon nanotubes (MWNTs) suspension (2 mg/ml, 10 μL) was dropped on the surface of GCE and dried in air. Next, 10 μL of GDH mutant Q252L/E170R/V149K/G259A (GDH-mutant-bacteria) aqueous dispersion was dropped on the inverted GCE and dried over night at room temperature. Before use, 10 μL of Nafion solution (0.05 wt%) was syringed to the electrode surface. The modified GCE (Nafion/GDH-mutant-bacteria/MWNTs/GCE) was immersed in PBS to remove the loosely adsorbed bacteria and was stored at room temperature when not in use. Electrochemical measurements were performed using a CHI660D potentiostat (CH Instruments, Chenhua, Shanghai, China). The electrochemical response was measured in a conventional three-electrode system using a chemically modified GCE as working electrode, a saturated calomel electrode (SCE) as reference electrode and Pt wire electrode as auxiliary electrode.

3. Results and discussion

3.1. Site-directed mutagenesis of GDH genes

Several amino acid residues that affect the stability of wild type GlcDH from *B. megaterium* IMG3 have been investigated in previous studies (Baik et al., 2005; Vázquez-Figueroa et al., 2007). Among them, two mutations could greatly influence pH and thermostability of GlcDH. On one hand, Glu-170 of wild type GlcDH interacts with Lys-149 between subunits, and the anion-anion repulsion effect would reduce the connection between subunits at pH > 9. Therefore, the stabilization of the quaternary structure could be realized by the E170R substitution (Baik et al., 2005; Vázquez-Figueroa et al., 2007). On other hand, the Q252L mutation would strengthen the interaction between subunits via the formation of hydrophobic interaction. A comparison of the amino acid sequences between the GDH from *B. subtilis* in this study and GlcDH from *B. megaterium* IMG3 revealed an identity of 81.6%. As shown in Fig. 1, amino acid at position 170 is Glu in both GDH and GlcDH. However, amino acid at position 149 is valine in GDH rather than lysine in GlcDH, which would probably bring about some changes to the stability of GDH. In the aspect of substrate specificity, C-terminal region residues of BmGlcDH-IV from *B. megaterium* IAM 1030 were mutated, of which the G259A variant showed the narrowest substrate specificity without damaging its catalytic activity and thermostability (Nishioka et al., 2012). Therefore, in view of the high homology of amino acid sequences and positive results of improving stability and specificity of GDH from *Bacillus* sp., four residues were selected for site-directed mutagenesis in the present study, including Gln-252, Glu-170, Val-149 and Gly-259.

In order to obtain the mutant enzyme avoiding laborious purification steps and improving the stability of its structure, INP-based bacteria surface display system, which showed considerable enzyme activity in our previous study (Liang et al., 2012), was used in this study. The N-terminal of InaPb protein from *P. borealis* DL7 was used as the surface-anchoring motif. The plasmid pTInaPbN-Gdh (Liang et al., 2013) harboring the wild type GDH gene was used as the template for site-directed mutagenesis. Four mutants were obtained via QuikChange technique. The resulting vectors were transformed into *E. coli* BL21 (DE3), and enzymes were expressed in LB medium with kanamycin and IPTG at 25 °C.

3.2. Enzyme activity of displayed surface displayed mutated GDH

The whole cell was harvested and washed with 75 mM Tris–HCl buffer (pH 8.0). Firstly, enzymatic activity of wild type and mutant GDH were examined in order to investigate whether the mutant sites would have harmful influence on the catalytic activity. The results indicated that four variants showed activity toward D-glucose comparable to wild type at the same conditions (Table 2). However, the other three mutants showed slightly decreased GDH activity compared with Q252L/E170R variant, of which whole cell-GDH activity of Q252L/E170R/V149K/G259A variant was 39% lower than that of wild type, indicating that Gly-259 mutated probably led to the transformation of activity-site structure. These data are consistent with results reported by other directed evolution enzyme studies (Baik et al., 2005; Nishioka et al., 2012). It was confirmed that the catalytic efficiency of GlcDH had not been affected by the mutation of E170K/Q252L, because these two mutations were far from the activity site according to the three-dimensional structure (Baik et al., 2005). However, the specific activity of the G259A mutant was slightly lower, resulting from the change of C-terminal region of BmGlcDH-IV. The sugar-binding selectivity and catalytic activity could be altered by introducing mutation at this region (Nishioka et al., 2012). In addition, whole cell-GDH activity of Q252L/E170R/V149K variant was 13% lower than that of wild type, demonstrating that Val-149 may also bring some unfavorable changes to the structure of activity domain, which will be studied by structural analysis in the following work.

3.3. Stability of displayed forms of mutant GDH

The thermal stability was assessed by using *E. coli* BL21 (DE3) whole cells expressing wild-type GDH and its mutants after incubation at 50–70 °C for 1 h. As shown in Fig. 2A, mutants Q252L/E170R/V149K, Q252L/E170R/G259A, and Q252L/E170R/V149K/G259A retained its original enzyme activity even after being treated at 70 °C for 1 h. However, the wild type GDH lost almost all activity at 50 °C. The half-life of the Q252L/E170R/V149K/G259A mutant was ~21 days at 60 °C and ~3.8 days at 70 °C (Fig. 2B and C), which was slightly higher than that of K170/L252 mutation from *B. subtilis* (~3.5 days at 65 °C) in previous study (Vázquez-Figueroa et al., 2007). The results supported our hypothesis that was obtained through combining the amino acid sequence of GDH in this study and the thermostability of glucose dehydrogenase from *Bacillus* sp. reported previously (Baik et al., 2003, 2005; Makino et al., 1989; Vázquez-Figueroa et al., 2007). Besides the substitutions of Q252L/E170R, the excellent thermostability was also attributed to the other two replacements of amino acids (V149K and G259A) as well as the INP-based cell surface display system.

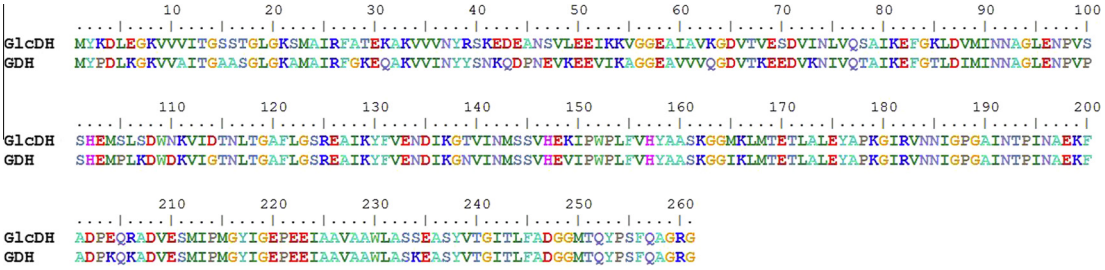

Fig. 1. Amino acid sequence alignment of GDH from *B. subtilis* in this study and GlcDH from *B. megaterium* IMG3.

Table 2
Catalytic activity of whole cells displayed wild type and mutant GDH.

Strain	GDH activity (U/mg dry cell)
Wild type	1.84 ± 0.06
Q252L/E170R	1.75 ± 0.05
Q252L/E170R/V149K	1.6 ± 0.05
Q252L/E170R/G259A	1.56 ± 0.05
Q252L/E170R/V149K/G259A	1.12 ± 0.03

The pH stability of wild-type GDH and its mutants was examined by incubating them in buffers of various pH values without NaCl. As shown in Fig. 3, except Q252L/E170R mutant and wild type, other variants did not show reduced enzyme activity in the absence of NaCl at alkaline pH, even increased activity was apparent at pH > 10. These results proved the assumption that E170R and V149K mutations would enhance the stability of the quaternary structure by reducing the electrostatic repulsion effect between subunits at pH values above 9.

3.4. Substrate specificity of cell displayed mutants GDH

The wild type BmGlcDH-IV from *B. megaterium* IAM 1030 exhibited broad substrate specificity (Nishioka et al., 2012). The G259A variant greatly altered this character and its activity to D-xylose, D-mannose and D-galactose was not detected at pH 8.0 (Nishioka et al., 2012). In our previous study, the cell-displaying wild type GDH could catalyze D-maltose, D-mannose and D-xylose. Here, G259A mutation was introduced into GDH to investigate the variation of substrate specificity. The whole cells were incubated with several different saccharides, including D-xylose, cellobiose, D-fructose, D-sucrose, D-maltose, D-mannose, D-galactose, D-ribose, L-arabinose (each 100 mM) or D-glucose (5 mM). As shown in Table 3, in contrast to the wild type, no absorbance peak at 340 nm was detected when whole cell of Q252L/E170R/V149K/G259A mutant was mixed with D-maltose, D-xylose, D-sucrose and D-mannose, separately. Although each of the four substitutions has influence on substrate specificity of GDH, G259A substitution greatly improved this character especially when D-maltose was used as substrate. In summary, Q252L/E170R/V149K/G259A mutant exhibited the narrowest substrate specificity with excellent stability whilst retaining comparable activity.

3.5. The scale-up of Q252L/E170R/V149K/G259A variant by E. coli

The preliminary study on the scale-up of whole cell was carried out in 3-L fermentor and M9 minimum medium using IPTG as an inducer. During the whole process, pH was maintained at 7.0 and DO at 20%. The whole cell was weighed and enzyme activity was assayed. The results showed that the dry weight of whole cells from fermentor was 4.87 ± 0.11 g/L, increasing about four times more than that of cells cultured in shaking flasks. Besides, the GDH activity of whole cell from fermentor was nearly 1.2 times

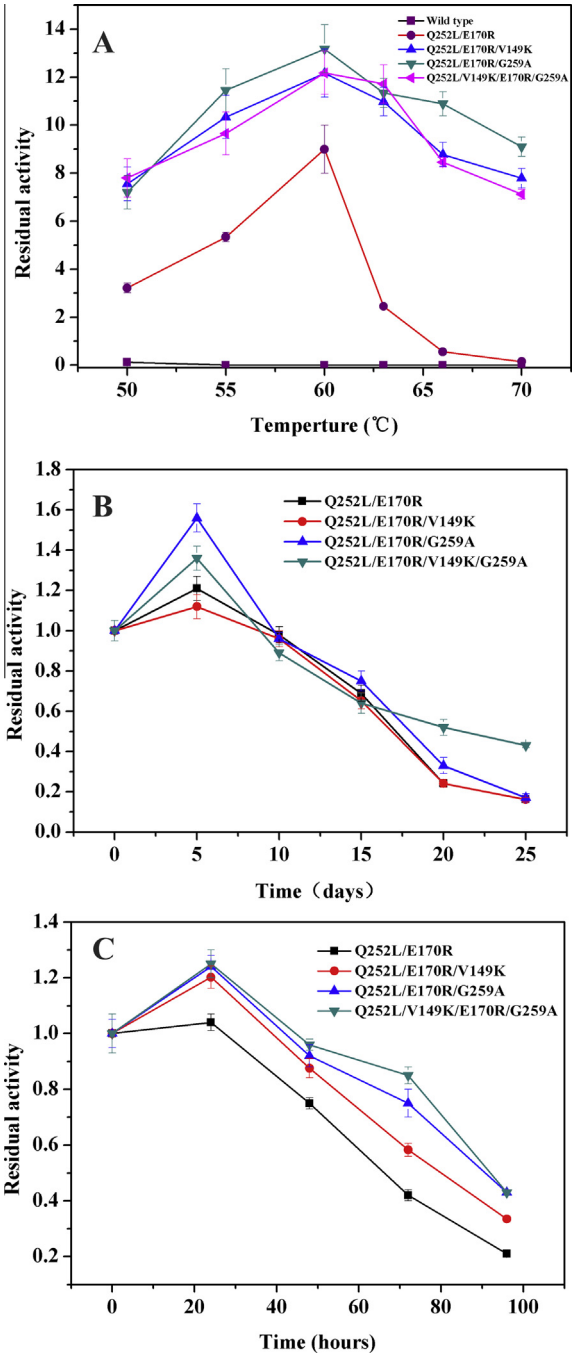

Fig. 2. The thermal stability of displayed forms of mutant GDH.

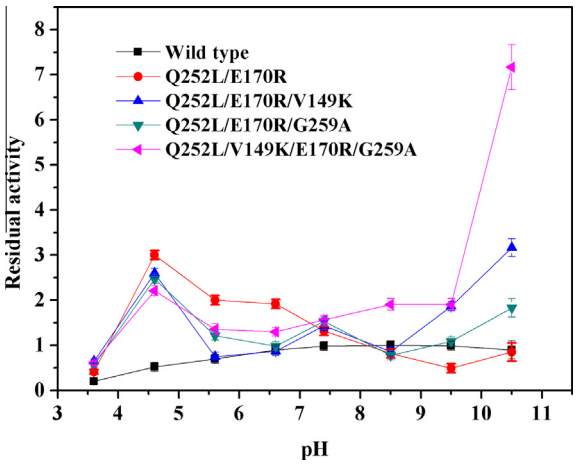

Fig. 3. pH stability of displayed forms of mutant GDH.

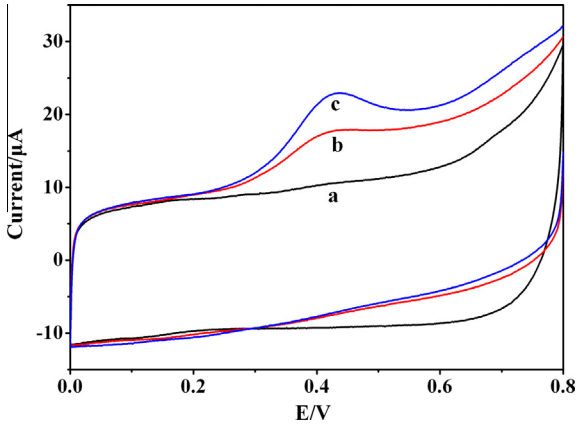

Fig. 4. Cyclic voltammograms of Nafion/GDH-mutant-bacteria/MWNTs/GCE in 0.1 M PBS (curve a); in 4 mM glucose + 2 mM NAD^+ (curve b) and in 6 mM glucose + 2 mM NAD^+ (curve c).

higher than that of from shaking flasks. All of the results indicated that the whole cell displayed GDH mutants could be cultured in a large scale with excellent activity and productivity.

3.6. Electrochemical glucose biosensor prepared using the GDH-mutant bacteria modified electrode

3.6.1. Electrochemical characterization of the Nafion/GDH-mutant-bacteria/ MWNTs/GCE

The electrochemical oxidation of glucose at Nafion/GDH-mutant-bacteria/MWNTs/GCE was investigated by cyclic voltammetry (Fig. 4). An anodic peak at 0.45 V (*vs.* SCE) which indicated the electrocatalytic oxidation process of NADH to NAD^+ was observed in the 4 mM and 6 mM glucose solution containing 2 mM NAD^+. No anodic peak was found in the 0.1 M PBS solution without glucose. The anodic peak current increased with the rise of glucose concentration, which demonstrated that the GDH-mutant had a well catalytic performance to glucose. Such results were comparable with the wild-type GDH modified GCE (Liang et al., 2013). It should be noted here that the proposed Nafion/GDH-mutant-bacteria/MWNTs/GCE was dried at room temperature, while the wild-type GDH modified GCE needed to dry at 4 °C at refrigerator (Liang et al., 2013), suggesting the thermostability of the enzyme for real application.

3.6.2. Amperometric determination of glucose using the glucose biosensor

The current–time curve was measured in varying glucose concentration with Nafion/GDH-mutant-bacteria/MWNTs/GCE by amperometry at an applied potential of +0.43 V (Fig. 5). The current increased rapidly after addition of glucose solution and reached 95% steady-state value within 3 s (Fig. 5). The current response was linear with the glucose concentration between 10 μM and 4 mM (Fig. 5, inset plot), which was much wider than the wild-type bacteria modified GCE (50–800 μM) (Liang et al., 2013). The linear regression equation is $y = 0.2847 + 2.7662x$ with the coefficient $R = 0.996$. The limit of detection (LOD) was estimated to be 3 μM glucose (S/N = 3), which was lower than the wild-type one. The Nafion/GDH-mutant-bacteria/MWNTs/GCE presented a much boarder linear range than those obtained with GDH/chitosan/CNTs/ionic-liquid-modified electrode (0.02–1 mM) (Bai et al., 2012), GDH/PPS/carbon-nanotube-modified electrode (50–700 μM) (Saleh et al., 2012) and GDH/CNTs/chitosan–glutaricdialdehyde film-modified electrode (5–300 μM) (Zhang et al., 2004). Again, the GDH-mutant whole cell can be used to improve the analytical performance of the prepared glucose biosensor.

3.6.3. Operational stability of the biosensor

A continuous measurement of glucose by amperometry at an applied potential of 0.43 V was performed over a period of 1 h to investigate the operational stability of the Nafion/GDH-mutant-bacteria/MWNTs/GCE (Fig. 6). The amperometric response drifted less than 7% (Fig. 6), demonstrated the good stability of proposed biosensor. Compared with the wild-type one (~10% signal drift during 25 min recording) (Liang et al., 2013), the displayed forms of mutant GDH based biosensor showed better operational stability.

Table 3
Substrate specificity of cell displayed mutants GDH.

Substrate	Specificity of cell surface displayed GDH (%)				
	Wild type	Q252L/E170R	Q252L/E170R/V149K	Q252L/E170R/G259A	Q252L/E170R/V149K/G259A
D-Glucose	100	100	100	100	100
D-Maltose	15.6 ± 0.2	13.2 ± 0.2	9.1 ± 0.1	1.6 ± 0.06	ND
D-Sucrose	5.9 ± 0.1	4.1 ± 0.1	ND	ND	ND
D-Xylose	9.6 ± 0.15	7.5 ± 0.1	ND	ND	ND
D-Mannose	3.6 ± 0.1	1.4 ± 0.05	ND	ND	ND
D-Fructose	ND	ND	ND	ND	ND
D-Galactose	ND	ND	ND	ND	ND
D-Ribose	ND	ND	ND	ND	ND
L-Arabinose	ND	ND	ND	ND	ND

ND, not detectable.

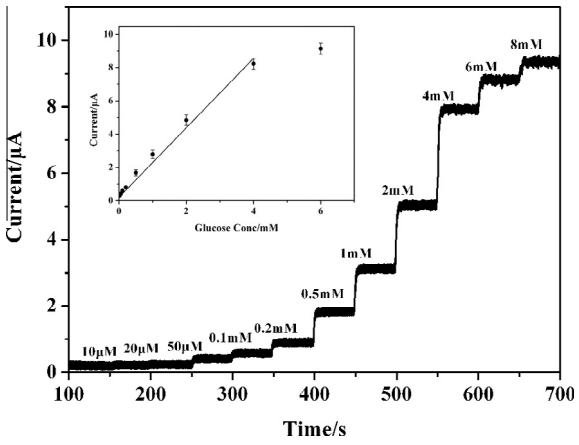

Fig. 5. Current–time curve obtained at the biosensor on the successive addition of glucose in 0.1 M phosphate buffer (pH 7.4) containing 2 mM NAD^+, on which the glucose concentration denoted the glucose concentration in the buffer solution. Applied potential, +0.43 V vs. SCE. Inset: typical calibration graph of the biosensor.

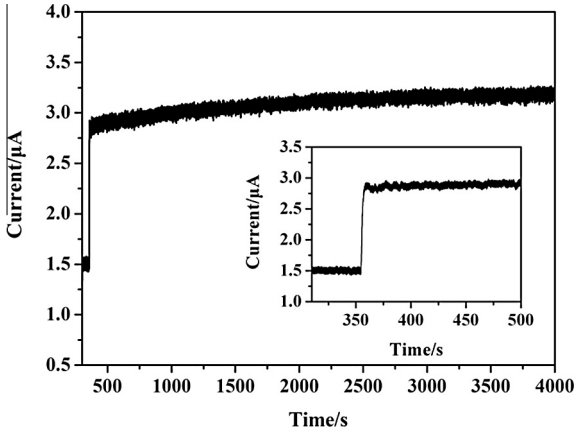

Fig. 6. Current–time response of the biosensor in 0.4 mM glucose solution. Inset: magnified view of current step upon spiking of 0.4 mM glucose.

4. Conclusions

In summary, the present study reported the improved stability and substrate specificity of mutants of cell surface displayed GDH from *B. subtilis*. The Q252L/E170R/V149K/G259A mutant showed the narrowest substrate specificity with significantly enhanced pH and thermal stability, while retaining a considerable activity toward D-glucose. The study of whole cell fermentation confirmed that the mutant is promising to realize massive production. Moreover, a stable and sensitive glucose biosensor using the GDH-mutant bacteria was developed. Therefore, the whole cell displayed GDH mutants could be used as industrial biocatalysts for wide range of applications, such as glucose sensor, biofuel cell, NAD(P)H regeneration system and biomass production.

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (Nos. 91227116, 31200598 and 21275152) and the Hundred-Talent-Project (No. KSCX2-YW-BR-7) and the Knowledge Innovation Project in Biotechnology (No. KSCX2-EW-J-10-6), Chinese Academy of Sciences. We thank Prof. Virginia K. Walker in Department of Microbiology and Immunology, Queen's University, Canada for providing INP. We are also grateful to Dr. Gebo Pan in Suzhou Institute of Nan-Tech and Nano-Biomics, Chinese Academy of Sciences for gifting multi-walled carbon nanotubes.

References

Bai, L., Wen, D., Yin, J., Deng, L., Zhu, C., Dong, S., 2012. Carbon nanotubes-ionic liquid nanocomposites sensing platform for NADH oxidation and oxygen, glucose detection in blood. Talanta 91, 110–115.

Baik, S.-H., Michel, F., Aghajari, N., Haser, R., Harayama, S., 2005. Cooperative effect of two surface amino acid mutations (Q252L and E170K) in glucose dehydrogenase from *Bacillus megaterium* IWG3 on stabilization of its oligomeric state. Appl. Environ. Microbiol. 71, 3285–3293.

Baik, S.H., Ide, T., Yoshida, H., Kagami, O., Harayama, S., 2003. Significantly enhanced stability of glucose dehydrogenase by directed evolution. Appl. Microbiol. Biotechnol. 61, 329–335.

Bilen, H., Kilicaslan, A., Akcay, G., Albayrak, F., 2007. Performance of glucose dehydrogenase (GDH) based and glucose oxidase (GOX) based blood glucose meter systems at moderately high altitude. J. Med. Eng. Technol. 31, 152–156.

Binder, U., Matschiner, G., Theobald, I., Skerra, A., 2010. High-throughput sorting of an anticalin library via EspP-mediated functional display on the *Escherichia coli* cell surface. J. Mol. Biol. 400, 783–802.

Boder, E.T., Wittrup, K.D., 1997. Yeast surface display for screening combinatorial polypeptide libraries. Nat. Biotechnol. 15, 553–557.

Chen, I., Dorr, B.M., Liu, D.R., 2011. A general strategy for the evolution of bond-forming enzymes using yeast display. Proc. Natl. Acad. Sci. USA 108, 11399–11404.

Fishilevich, S., Amir, L., Fridman, Y., Aharoni, A., Alfonta, L., 2009. J. Am. Chem. Soc. 131, 12052–12053.

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

Jahns, A.C., Rehm, B.H.A., 2012. Relevant uses of surface proteins-display on self-organized biological structures. Microb. Biotechnol. 5, 188–202.

Jin, Z., Han, S.Y., Zhang, L., Zheng, S.P., Wang, Y., Lin, Y., 2013. Combined utilization of lipase-displaying *Pichia pastoris* whole-cell biocatalysts to improve biodiesel production in co-solvent media. Bioresour. Technol. 130, 102–109.

Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalezduarte, R., Jeffery, J., Ghosh, D., 1995. Short-chain dehydrogenases reductases (SDR). Biochemistry 34, 6003–6013.

Li, L., Liang, B., Li, F., Shi, J.G., Mascini, M., Lang, Q.L., Liu, A.H., 2013. Co-immobilization of glucose oxidase and xylose dehydrogenase displayed whole cell on multiwalled carbon nanotube nanocomposite films modified-electrode for simultaneous voltammetric detection of D-glucose and D-xylose. Biosens. Bioelectron. 42, 156–162.

Li, L., Liang, B., Shi, J., Li, F., Mascini, M., Liu, A., 2012. A selective and sensitive D-xylose electrochemical biosensor based on xylose dehydrogenase displayed on the surface of bacteria and multi-walled carbon nanotubes modified electrode. Biosens. Bioelectron. 33, 100–105.

Liang, B., Li, L., Mascin, M., Liu, A., 2012. Construction of xylose dehydrogenase displayed on the surface of bacteria using ice nucleation protein for sensitive D-xylose detection. Anal. Chem. 84, 275–282.

Liang, B., Li, L., Tang, X., Lang, Q., Wang, H., Li, F., Shi, J., Shen, W., Palchetti, I., Mascini, M., Liu, A., 2013. Microbial surface display of glucose dehydrogenase for amperometric glucose biosensor. Biosens. Bioelectron. 45, 19–24.

Majander, K., Korhonen, T.K., Westerlund-Wikstrom, B., 2005. Simultaneous display of multiple foreign peptides in the FliD capping and FliC filament proteins of the *Escherichia coli* flagellum. Appl. Environ. Microb. 71, 4263–4268.

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

Matano, Y., Hasunuma, T., Kondo, A., 2013. Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing. Bioresour. Technol. 135, 403–409.

Nagao, T., Makino, Y., Yamamoto, K., Urabe, I., Okada, H., 1989. Stability-increasing mutants of glucose dehydrogenase. FEBS Lett. 253, 113–116.

Nagao, T., Mitamura, T., Wang, X.H., 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, 5013–5020.

Nishioka, T., Yasutake, Y., Nishiya, Y., Tamura, T., 2012. Structure-guided mutagenesis for the improvement of substrate specificity of *Bacillus megaterium* glucose 1-dehydrogenase IV. FEBS J. 279, 3264–3275.

Olafsen, T., Kenanova, V.E., Wu, A.M., 2006. Tunable pharmacokinetics: modifying the in vivo half-life of antibodies by directed mutagenesis of the Fc fragment. Nat. Protoc. 1, 2048–2060.

Pauly, H.E., Pfleiderer, G., 1977. Conformational and functional aspects of the reversible dissociation and denaturation of glucose dehydrogenase. Biochemistry 16, 4599–4604.

Saleh, F.S., Mao, L., Ohsaka, T., 2012. A promising dehydrogenase-based bioanode for a glucose biosensor and glucose/O_2 biofuel cell. Analyst 137, 2233–2238.

Samuelson, P., Gunneriusson, E., Nygren, P.A., Stahl, S., 2002. Display of proteins on bacteria. J. Biotechnol. 96, 129–154.

Schewe, H., Kaup, B.A., Schrader, J., 2008. Improvement of P450(BM-3) whole-cell biocatalysis by integrating heterologous cofactor regeneration combining glucose facilitator and dehydrogenase in *E. coli*. Appl. Microbiol. Biotechnol. 78, 55–65.

Tan, L.-T., Hiraishi, T., Sudesh, K., Maeda, M., 2013. Directed evolution of poly[(R)-3-hydroxybutyrate] depolymerase using cell surface display system: functional importance of asparagine at position 285. Appl. Microbiol. Biotechnol., DOI:10.1007/s00253-012-4366-8.

Vázquez-Figueroa, E., Chaparro-Riggers, J., Bommarius, A.S., 2007. Development of a thermostable glucose dehydrogenase by a structure-guided consensus concept. ChemBioChem 8, 2295–2301.

Wang, H., Lang, Q., Li, L., Liang, B., Tang, X., Kong, L., Mascini, M., Liu, A., 2013. Yeast surface displaying glucose oxidase as whole-cell biocatalyst: construction, characterization, and its electrochemical glucose sensing application. Anal. Chem. 85, 6107–6112.

Wang, J.-Y., Nien, P.-C., Chen, C.-H., Chen, L.-C., Ho, K.-C., 2012. A glucose bio-battery prototype based on a GDH/poly(methylene blue) bioanode and a graphite cathode with an iodide/tri-iodide redox couple. Bioresour. Technol. 116, 502–506.

Xia, L., Liang, B., Li, L., Tang, X., Palchetti, I., Mascini, M., Liu, A., 2013. Direct energy conversion from xylose using xylose dehydrogenase surface displayed bacteria based enzymatic biofuel cell. Biosens. Bioelectron. 44, 160–163.

Xu, Z., Jing, K., Liu, Y., Cen, P., 2006. High-level expression of recombinant glucose dehydrogenase and its application in NADPH regeneration. J. Ind. Microbiol. Biotechnol. 34, 83–90.

Yamamoto, K., Kurisu, G., Kusunoki, M., Tabata, S., Urabe, I., Osaki, S., 2001. Crystal structure of glucose dehydrogenase from *Bacillus megaterium* IWG3 at 1.7 Å resolution. J. Biochem. 129, 303–312.

Yoo, E.-H., Lee, S.-Y., 2010. Glucose biosensors: an overview of use in clinical practice. Sensors 10, 4558–4576.

Zhang, M., Smith, A., Gorski, W., 2004. Carbon nanotube–chitosan system for electrochemical sensing based on dehydrogenase enzymes. Anal. Chem. 76, 5045–5050.

Zhang, Q., Peng, H., Gao, F., Liu, Y., Cheng, H., Thompson, J., Gao, G.F., 2009. Structural insight into the catalytic mechanism of gluconate 5-dehydrogenase from *Streptococcus suis*: crystal structures of the substrate-free and quaternary complex enzymes. Protein Sci. 18, 294–303.

Zhu, Z., Sun, F., Zhang, X., Zhang, Y.H.P., 2012. Deep oxidation of glucose in enzymatic fuel cells through a synthetic enzymatic pathway containing a cascade of two thermostable dehydrogenases. Biosens. Bioelectron. 36, 110–115.