RECOMBINANT GLYCEROL DEHYDRATASE FROM *Klebsiella pneumoniae* XJPD-LI: INDUCTION OPTIMIZATION, PURIFICATION AND CHARACTERIZATION

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Glycerol dehydratase (GDHt) is the rate limiting enzyme in the biosynthesis of 1,3-propanediol from glycerol. The optimization of inducting process for recombinant GDHt from *Klebsiella pneumoniae* XJPD-Li carried out to increase specific activity and ratio of soluble form. The optimum condition was inducing under the isopropyl-β-D-thiogalactoside concentration of 0.8 mM and the temperature of 20°C for 3 h. Homogeneity of GDHt then was obtained by affinity chromatography, resulted in 2.11-fold purification and an overall yield of 47.5%. The optimum pH and reaction temperature of GDHt were pH 8.0 and 45°C, respectively. The K_m for glycerol, 1,2-propanediol, 1,2-ethanediol and coenzyme B_{12} were 0.48, 1.43, 3.07 mM, and 10.03 nM, respectively. The GDHt showed relatively stable even under temperature of 40°C and a bit blunt to oxygen. The thermo-inactivation kinetic models were fit linear under different temperatures.

Glycerol dehydratase (EC 4.2.1.30, GDHt) catalyzes the conversion of glycerol and 1,2-propanediol (1,2-PDO) to 3-hydroxy-propinaldehyde (3-HPA) and propionaldehyde, respectively [1]. This reaction is the rate limiting step for biotransformation from glycerol to 1,3-propanediol (1,3-PDO), which is widely used for many synthesis reactions, in particular as a monomer for polycondensations to produce polyesters, polyethers and polyurethanes [2]. Interest in the microbial production of 1,3-PDO has been lasting for over thirty years. *Klebsiella (K. pneumoniae)*, *Citrobacter (C. freundii)*, and *Clostridia* of the *C. butyricum* groups have been found produce 1,3-PDO under anaerobic condition from glycerol fermentation [3–5]. They are industrially focused because of their appreciable substrate tolerance, yield and productivity in the production of 1,3-propanediol.

GDHt, as a key enzyme in the biosynthesis of 1,3-PDO from glycerol, was firstly purified to a stable, inactive, homogeneous complex with hydroxocobalamin from *Aerobacter aerogenes* by Zekon et al. in 1970 [6]. Todd et al. [7] obtained purified GDHt from *Lactobacillus reuteri* with molecular weight of approximately 200000, while only one subunit was examined clearly [7]. The composition of GDHt subunits was not clear until large number of researches were carried out on these strains. The affinity chromatography was the most effective method to obtain homogeneity from recombinant [8, 9]. It was found that GDHt utilized adenosylcobalamin (AdoCbl, CoB_{12}) as a cofactor and exhibits a $(\alpha\beta\gamma)_2$ structure,

where α, β and γ represented the large, medium and small subunits of the protein, respectively [10]. Substrate binding site triggers the formation of catalytic free radical through Co−C bond homolysis in the AdoCbl-dependent GDHt. This enzyme undergoes mechanism-based inactivation by glycerol during catalysis [11].

One excellent potential industrial strain named *K. pneumoniae* XJPD-Li was screened by Zhang et al. [12] which could fast consume glycerol with high productivity of 3.35 g l^{-1} h^{-1} and molar yield of 0.75 (1,3-PDO to glycerol) under the temperature of 40°C and the pH of 8.0. It was found that *K. pneumoniae* XJPD-Li could also produce 1,3-PDO under aerobic condition with high final concentration of 1,3-PDO (65.26 g l^{-1}), satisfied yield (0.56 mol mol^{-1}) and excellent productivity (3.16 g l^{-1} h^{-1}) [12, 13]. The gene (*dha*BCE) of rate-limiting enzyme, GDHt from *K. pneumoniae* XJPD-Li, was obtained to discover its good catalysis characterization [14]. The sequence alignment of gene and amino acid was performed between GDHt (U30903) from *K. pneumoniae* ATCC 25955 and GDHt from *K. pneumoniae* XJPD-Li. As a result, several amino acid residue differences were found in the non-active site. For the great interest of the GDHt from *K. pneumoniae* XJPD-Li, the optimization of inducing conditions were carried out to overexpress the recombinant GDHt with energetic activity and stability. After performing facile purification procedures, the catalysis properties of homogeneity were examined subsequently.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions. The gene (EF634063) of GDHt from *K. pneumoniae* XJPD-Li has been cloned and sequenced [14]. The plasmid pET-28a (+) (Novagen, Germany) and *E. coli* BL21 (DE3) (Invitrogen, Germany) were used as expression vector and host, respectively. *E. coli* was aerobically grown in Luria-Bertani (LB) medium at 37°C, containing 1,2-PDO (0.1%) and kanamycin (50 mg ml^{-1}), which were added when their transformants with plasmids were cultured.

Optimization of inducting conditions. Gene of GDHt amplified from *K. pneumoniae* XJPD-Li was ligated to expression vector pET-28a (+) directly, after digested by *EcoR* I and *Sac* I. The pET-28a-*dha*BCE was constructed and transformed into *E. coli* BL21 (DE3). When the culture reached an OD$_{600}$ of approximately 1.0 to 1.5, isopropyl-β-D-thiogalactoside (IPTG) with the concentration of 0.4, 0.8, or 1.2 mM was added into cultural medium, respectively. The cell growth and enzyme activity were examined every one or 2 h under different cultural temperatures. Bacterial growth was monitored by determining the optical density at 600 nm of the cultures using a spectrometer. Cells were harvested by centrifugation (12000 g, 5 min), and washed twice with phosphate buffer (containing 2% 1,2-PDO).

Purification of recombinant GDHt. Harvested cells (2 g) were suspended in the loading buffer (pH 8.0, containing 50 mM phosphate, 2% 1,2-PDO, 200 mM KCl and 2 mM phenylmethanesulfonyl fluoride (PMSF)) and disrupted by ultrasonication in an ice-bath for 10 min at 50% of the output (750W) with an Ultrasonic Liquid Processors (SONICS US). The cell debris was removed by centrifugation at 12000 g for 20 min from the disrupted solution. The cell-free supernate was decanted and centrifuged again at 12000 g for 10 min.

The cell-free supernate was applied to a column of Ni-nitrilotriacetate (Ni-NTA) agarose gel (Qiagen GmbH, Germany), equilibrated with loading buffer previously. The loading buffer was not changed to washing buffer (loading buffer with 20 mM imidazole), until all the unbinding protein was washed out. The elution buffer (loading buffer with 150 mM imidazole) was then applied to the column. The elution peak was collected to ultra-filtration concentration system (filtration membrane 100 kDa). The fraction harboring homogeneity proteins were pooled and used for the following experiments. All operations were carried out at 4°C. The samples of each step were applied to SDS-PAGE.

Enzyme and protein assays. GDHt activity was determined by improved 3-methyl-2-benzothiazolinone hydrazine (MBTH) method according to Zhang, et al. [12]. The resulting azine derivatives were determined spectrophotometrically at 305 nm (A$_{305}$). Since glycerol serves as substrate and suicide inactivator for GDHt, 1,2-PDO (0.2 M) was used as a substrate for routine assay of the enzyme. One unit of GDHt is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde per minute under the assay conditions. Specific activity is expressed as units per mg of protein. Protein concentration was determined using the method of Bradford with crystalline bovine serum albumin (BSA) as a standard.

SDS-PAGE was performed with a 12% polyacrylamide gel on a vertical mini gel apparatus at 120 V for 1.5 h. Molecular weight marker was purchased from Tiangen Biotechnology Co., (Beijing, China). Proteins were stained with coomassies brilliant blue R-250. The GEL-IMAGING system (Bio-Rad, USA) was utilized to analysis the band of SDS-PAGE, quantitatively.

Determination of properties. Assays to determine the optimum pH and temperature were performed in different buffer and water bath, respectively. The pH values of the buffer were adjusted by 3.0 M hydrochloric acid or 3.0 M potassium hydroxide. The optimum pH value was calculated by non-linear regression to the Bell-Shaped Double *pKa* equation by use of the curve fit feature of the Originpro 7.5 (USA).

Measurement of kinetic parameters. Purified enzyme was used for measuring K_m values for 1,2-PDO, 1,2-EDO, glycerol and coenzyme. One-minute assay was employed for measurement of K_m for glycerol, as glycerol induces suicide inactivation of the enzyme. The apparent K_m values were expressed in mM or nM and calculated by non-linear regression to the Michaelis−Menten equation by use of the nonlinear curve fitting programme (Originpro 7.5).

Thermostability of GDHt. The purified GDHt was incubated under the temperature of 37, 40 or 45°C. The measurement of residual enzyme activity was carried out in due course.

Oxygen inactivation of GDHt. In one case, the purified GDHt was incubated with CoB$_{12}$ (50 μM) at 37°C, according to Toraya, et al. [15]. The residual enzyme activity was examined after incubated 30 min. In the other case, the purified GDHt was incubated with CoB$_{12}$ (50 μM) at 37°C, and was aerated with air (0.05 v/v min) at the same time. The residual enzyme activity was examined every certain minute.

RESULTS AND DISCUSSION

Effect of inducing conditions on the expression of recombinant GDHt. First of all, *E. coli* BL21 (DE3) including the pET-28a (+)-*dha*BCE was induced under various concentrations of inducing agent. The SDS-PAGE of the whole cell sample showed that the concentration of IPTG 0.8 mM was much favored to the production of recombinant GDHt, where the quantity of recombinant GDHt did not increase with the concentration of inducer in a direct ratio (Fig. 1). The bands of target protein were indicated by arrows on the left of the figures. It was showed in SDS-PAGE that apparent molecular weights of GDHt were a bit larger than calculated ones, which was possibly lead by the changing of protein electrophoresis behavior with alkaline His-tag (6 Histidine) [16]. The concentra-
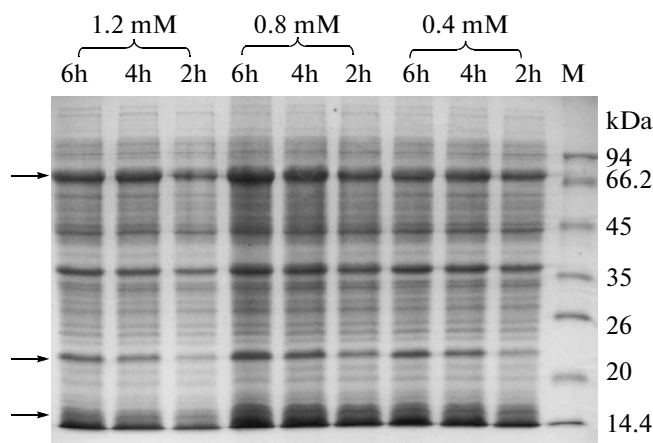
Fig. 1. The effect of induction time and the concentration of IPTG on the overexpression of GDHt from *E. coli* BL21 (DE3) (PET-28a (+)-dhaBCE).
M – standard protein weight. The arrows showed the large, medium and small subunits of GDHt from the top.

tion of 0.8 mM (IPTG) was then chosen according to the inducing performance.

According to Catherine et al. [17], it was founded that several proteins were all in aggregates when produced in genetically engineered strains of *E. coli* grown at 37°C. However, at a growth temperature of 23–30°C, 30–90% of the recombinant protein was soluble [17]. It is desirable

to express recombinant GDHt in its soluble, active form. The specific activity (U mg^{-1}), the total activity (U) of GDHt (crude enzyme) and the biomass of recombinant strains (OD$_{600}$) were then examined under various inducing temperatures. It could be described from Fig. 2 that the biomass of strain was enhanced along with the increasing of temperature, and the total activities of GDHt approached to the same level after inducing for 3 h under different temperature. However, the highest specific activity of GDHt (71.4 U mg^{-1}) was obtained under induction temperature of 20°C for 3 h.

In most cases, solubility is not an all-or-none phenomenon. In order to obtain the efficient inducing strategy, supernatant and cell debris of recombinant were examined further under the inducing temperature of 20 and 30°C (Fig. 3). The SDS-PAGE described that the ratio of soluble to insoluble protein was improved from 0.73 (30°C) to 1.34 (20°C) (Fig. 3). Prior to high inducing temperature, the production of soluble GDHt under the temperature of 20°C was higher than that under the temperature of 30°C. It was identical as the results in Fig. 2. So the temperature of 20°C was selected for the inducing condition. Under such condition, the recombinant GDHt from *K. pneumoniae* XJPD-Li was over expressed effectively with impressive specific activity which was higher than reported by other research groups [18].

Purification of recombinant glycerol dehydratase. The summary of the purification results for recombinant GDHt was shown in Table 1. Approximately 2.11-fold
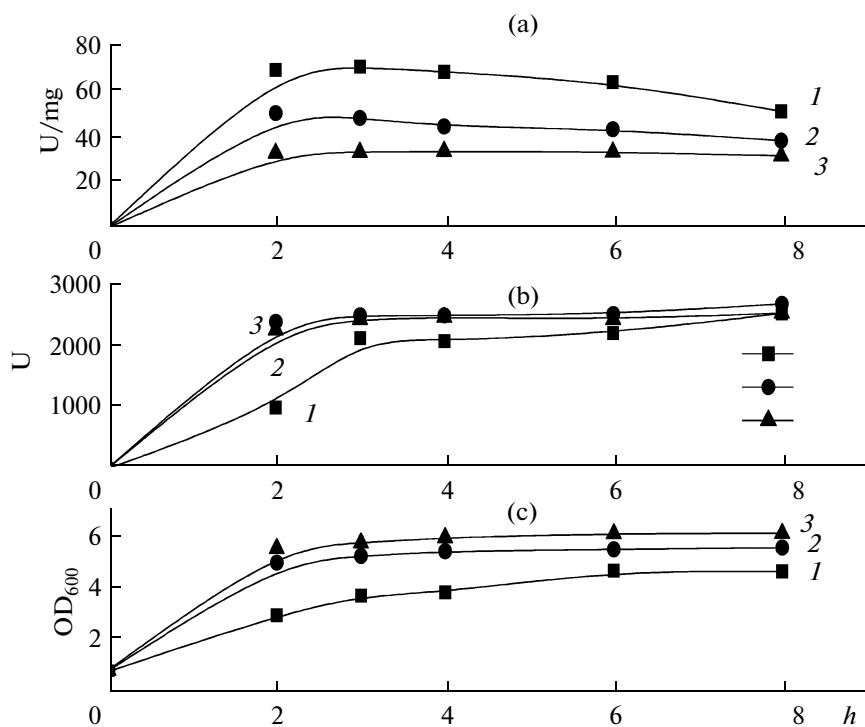

Fig. 2. Effect of inducing temperature on the overexpression of recombinant GDHt:
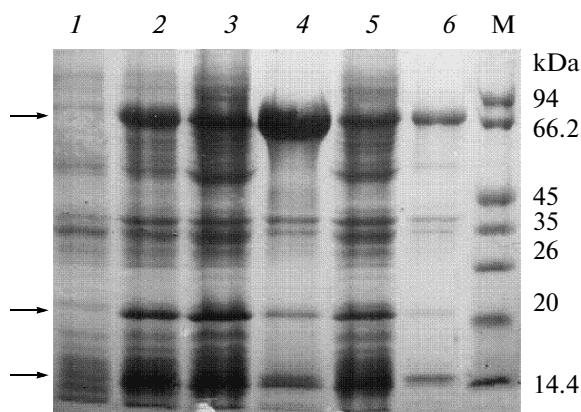a – specific activity; b – total activity; c – biomass; *1* – 20; *2* – 30; *3* – 37°C.

Fig. 3. Effect of inducing temperature on the ratio of soluble to insoluble quantity of recombinant GDHt:

1 − non-induced whole cell; *2* − induced whole cell; *3* − cell-free supernatant/crude enzyme (30°C, 3 h); *4* − cell-free debris (30°C, 3 h); *5* − cell-free supernatant/crude enzyme (20°C, 3 h); *6* − cell-free debris (20°C, 3 h); M − standard protein weight. The arrows showed the large, medium and small subunits of GDHt from the top.
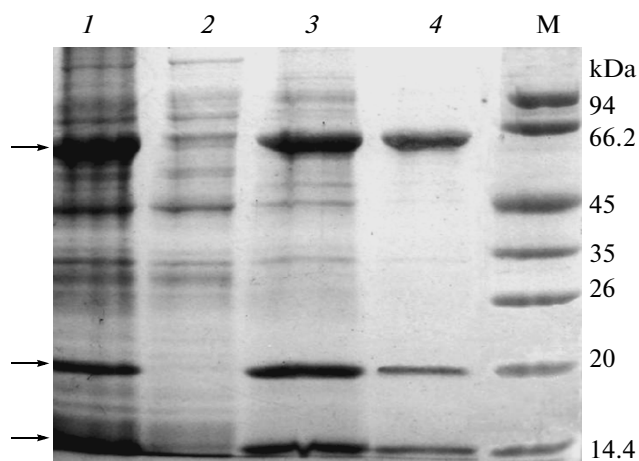

Fig. 4. SDS-PAGE analysis of the purification of recombinant GDHt:

M − marker; *1* − whole cell (induced by IPTG); *2* − unbinding peak; *3* − cell-free supernatant/crude enzyme; *4* − elution peak (150 mM imidazole). The arrows showed the large, medium and small subunits of GDHt from the top.

purification was achieved in an overall yield of 47.5% and final specific activity was 114.2 U mg^{-1}. The Ni-NTA affinity chromatography was the key step for the purification of recombinant GDHt from *E. coli* BL21 (DE3) harboring pET-28(a)-*dha*BCE. Samples from each step of the purification process were examined by SDS-PAGE. It was showed clearly in the Fig. 4 that few target protein was eluted in unbinding peak and the GDHt was purified to homogeneity almost in one affinity step. The ultra-filter step subsequently was benefit to move part lower molecular weight impurities and the high concentrated imidazole, the latter of which could disturb the measurement of activity for its strong absorbance at 315 nm. In addition, concentrated enzyme solution from this step was of convenience to be examined in following researches. Both affinity chromatography and ultra filtration could carry out feasibly and quickly, which was essential to avoid the loss of enzyme activity.

Characterization of GDHt. According to the catalysis characterization of GDHt reported, it showed that the optimum catalysis temperature and pH was normally around 30°C and 7.0. And most GDHt experienced the inactivity process with the existence of glycerol or oxygen [6, 7, 9]. Fig. 5 described the optimum temperature and pH for the recombinant GDHt catalyzing the conversion of 1,2-PDO to propionaldehyde, respectively. The deter-

mination of the optimum temperature for recombinant GDHt was performed by using a temperature range from 30 to 55°C and the optimum result was observed at 45°C. According to Toraya et al. [8, 15], the temperature of 30 or 37°C was close to the optimum condition. Normally, pH was also important for its influence on the diol conversion to propionaldehyde. A range of pH from 5 to 10 was tested and the highest activity was obtained between 7.5 and 8.5 with a maximum at pH 8.0. The further kinetic experiments were then performed under the optimized reactive conditions, pH (8.0) and temperature (45°C).

GDHt activity was also monitored by the conversion of various substrates. The K_m of the GDHt for the three preferential substrates (1,2-PDO, 1,2-EDO, glycerol) and coenzyme B$_{12}$ were determined. The highest substrate affinity was obtained for glycerol with a K_m of 0.48 mM followed by 1,2-PDO (K_m: 1.43 mM) and 1,2-EDO (K_m: 3.07 mM). It was obviously that glycerol was the optimum substrate among them. The K_m for glycerol in this research was lower than what was reported [8]. Affinity for the coenzyme B$_{12}$ was considerably higher with a K_m of 10.03 nM. It showed that the recombinant GDHt from *K. pneumoniae* XJPD-Li had higher affinity with coenzyme B$_{12}$ and glycerol than that of GDHt from various strains [18].

The thermostability of recombinant GDHt was determined by the original raw relative activity data

Table 1. Purification of recombinant glycerol dehydratase of *E. coli* (DE3)/pET 28a (+)-*dha*BCE

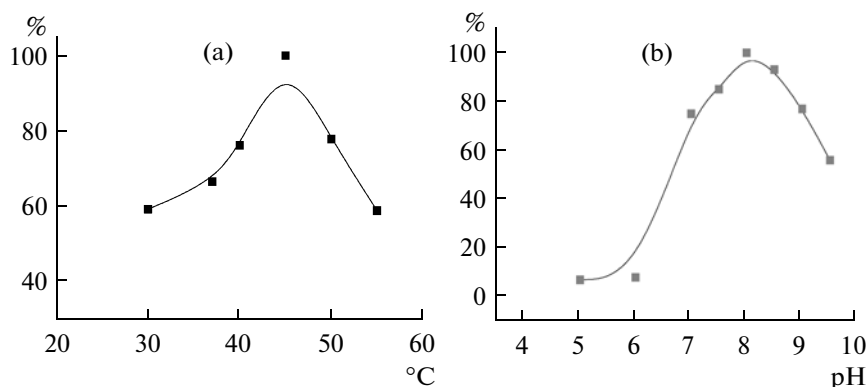| Purification Step	Protein, mg	Activity, U	Specific activity, U mg^{-1}	Yield, %	Purification fold
Crude enzyme	38.1	2068	54.4	100	1
Affinity (Ni-NTA)	10.1	998.2	98.9	48.3	1.82
Ultra-filtration	8.6	982.7	114.2	47.5	2.11

Fig. 5. Effect of temperature (a) and pH (b) on the activity of the purified GDHt.

transformed by log natural function. Results (lines) of regression analysis of experimental data (symbols) by the one-step two states showed that the thermo-inactivation reaction of GDHt was accorded with first order kinetic equation (Fig. 6). It could be observed from the equations that the GDHt here performed the excellent thermostability under the temperature of 37 and 40°C. The activity of GDHt did not reduce obviously until the reaction temperature increased to 45°C. The trend line equations shown in Table 2 indicated the good linear character of Log-Transformed data. That is, the inactivation kinetic models were fit linear under different temperatures.

As what reported before, oxygen was one of the key reasons to inactivate GDHt. Among the coenzyme B_{12}-dependent GDHts from various strains, they were found to be sensitive when exposure to oxygen [15, 19]. In addition, coenzyme B_{12}-independent GDHt from *C. butyricum* was founded extremely oxygen sensitive [20]. The sensitivity of oxygen by recombinant GDHt from *K. pneumoniae* XJPD-Li was also determined in this research according to Toraya et al. [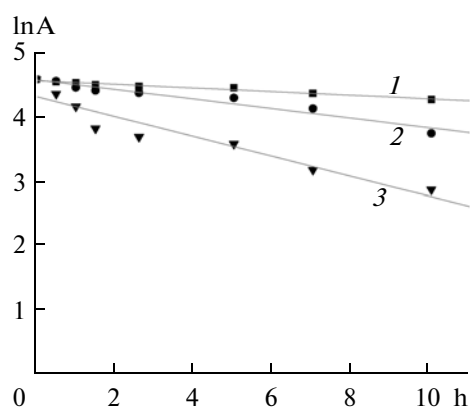15]. On contrast, GDHt in this research was not as sensible as what was reported. When the similar oxygen inactivation system was provided for recombinant GDHt from *K. pneumoniae* XJPD-Li, it was found that the enzyme showed less declination of activity after 30 min. Once aeration was applied in this system, the drop of activity of GDHt was clear. When substrate-free apoenzyme was incubated with CoB_{12} in the water bath of 37°C, aerated with air (0.05 v/v min), about 40% percent original activity of GDHt remained for 30 min (Fig. 7). The mechanism for inactivation of GDHt by glycerol has been explained by the loss of the intermediate radical from the active site, leaving catalytically incompetent cofactor (cobalamin and 5'-deoxyadenosine) tightly bound in the active site. Though it was common that oxygen was harmful for the anaerobic bioprocesses, such as the catalysis of nitrogenase [21], the inactivation mechanism of key enzyme by oxygen was not yet obtained.

The results obtained in present research demonstrate that the recombinant GDHt from *K. pneumoniae* XJPD-Li is one good biocatalyst with high activity and catalyzing
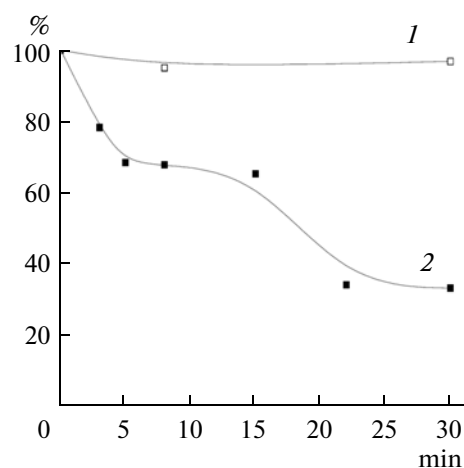

Fig. 6. Thermo-inactivation model of the recombinant GDHt.
lnA: A — relative activity. *1* — 37; *2* — 40; *3* — 45°C.

Fig. 7. Oxygen inactivation of the recombinant GDHt: *1* — purified GDHt incubated with coenzyme B_{12} (50 μM) at 37°C was aerated with air (0.05 v/v min); *2* — purified GDHt incubated with coenzyme B_{12} (50 μM) at 37°C.

Table 2. Linear regression analysis the thermal inactivation*

T, °C	Trend line equation Y = A + BX	R	SD	N	P
45	Y = 4.33456 − 0.15631X	−0.9518	0.19405	8	2.69936E-4
40	Y = 4.59774 − 0.07529X	−0.97408	0.06736	8	<0.0001
37	Y = 4.58469 − 0.02859X	−0.98662	0.0182	8	<0.0001

* R-relative coefficient; SD − standard deviation; N − number; P − probability.

condition under relatively high temperature and soft alkaline condition. It also shows thermostability and oxygen-blunt during catalyzing which was important for industrial enzyme.

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