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Quantitative analysis on inactivation and reactivation of recombinant glycerol dehydratase from *Klebsiella pneumoniae* XJPD-Li

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ABSTRACT

The genes encoding glycerol dehydratase were cloned and characterized by genomic DNA from *Klebsiella pneumoniae* XJPD-Li, and the assigned accession number EF634063 was available from the GenBank database. The DNA sequence analysis showed that the clone included three ORFs (*dha*B, *dha*C and *dha*E, encoding α, β and γ subunit of glycerol dehydratase, respectively). Among three subunits of glycerol dehydratase, amino acid residues H_{13}, S_{193}, N_{359}, E_{407}, and M_{515} of α subunit, N_{47}, L_{150}, V_{189} of β subunit are different with what had been reported. Subsequently, the expression vector was constructed and transformed into *E. coli* BL21, and the colony carried genes of glycerol dehydratase were selected. SDS-PAGE examination showed that the three subunits were well expressed. The specific activity of recombined glycerol dehydratase reached to 0.299 U mg^{-1}, which was about 3 times comparing with that of the wild strain. The research also displayed that both glycerol and O_2 could inactive the glycerol dehydratase expressed in *E. coli* quickly in 10 min. The inactivated glycerol dehydratase could be effectively reactivated under the system as follows: the concentration of ATP, Mg^{2+} and coenzyme B_{12} were 50 mM, 10 mM and 3 μM, respectively, when the ratio (W/W) of glycerol dehydratase to reactivation factor was 4:1. The O_2-inactivated and glycerol-inactivated dehydratase could be reactivated to 97.3% and 98.9% of initial activity in 10 min in above-mentioned conditions, respectively. The reactivation factor together with ATP was considered as the "ON/OFF" reactivating condition.

1. Introduction

Glycerol dehydratase (glycerol hydrolyase, EC 4.2.1.30, GDHt) catalyzes the adenosylcobalamin (coenzyme B_{12})-dependent conversion of glycerol to the corresponding aldehyde. Some genera of *Enterobacteriaceae*, such as *Klebsiella pneumoniae* [1–4], *Klebsiella oxytoca* [5], and *Clostridium butyrium* [6,7], etc., could express glycerol dehydrate and produce 1,3-propanediol (1,3-PD), a monomer for a novel polymer polytrimethylene terephthalate (PTT) [8,9]. It is well understood that 1,3-PD is produced through 3-hydroxypropionaldehyde (3-HPA) when bacteria grow anaerobically in a medium containing glycerol [10,11]. The glycerol dehydratase ($\alpha_2\beta_2\gamma_2$), encoding with *dha*BCE [12], is known to proceed a radical mechanism involving coenzyme B_{12} as an essential cofactor [13]. Although glycerol is the essential substrate for the production of 1,3-propanediol, it was advocated that the glycerol dehydratase underwent concomitant irreversible inactivation by glycerol during catalysis [14,15]. The mechanism-based inactivation was demonstrated and summarized by the irreversible cleavage of the Co—C bond of coenzyme B_{12}, forming 5′-deoxyadenosine and a cobalamin-like species [16–18]. Holoenzyme of glycerol dehydratase also undergoes inactivation by O_2 in the absence of substrate. The formation of OH-CbI upon inactivation suggests that inactivation is caused by reaction of the activated Co—C bond of the coenzyme with O_2 [19].

Nevertheless, it is desirable that inactivated enzymes in permeabilized cells (in situ) of *K. pneumoniae* and *K. oxytoca* were found to be reactivated by exchanging the modified coenzyme with intact adenosylcobalamin in the presence of ATP and Mg^{2+} [20]. A molecular chaperone-like reactivating factor which participated in reactivation of the inactivated enzyme in vitro was found critically important [18,21–23]. The factor not only reactivates glycerol-inactivated and O_2-inactivated holoenzymes but also activates the enzyme CN-CbI complex in the presence of free AdoCbI, ATP, and Mg^{2+} [18,24]. Cloning, identification and expression of GDHt and the reactivating factor have been reported and

Abbreviations: 1,3-PD, 1,3-propanediol; GDHt, glycerol dehydratase; *K. pneumoniae*, *Klebsiellla pneumoniae*; IPTG, isopropyl-β-D-thiogalactoside; CTAB, *n*-cetyl trimethylammonium bromide.

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analyzed in detail [25–29]. More recently, the structure information and the molecular basis for specificities of reactivating factor are available by researchers. It is found that come to consistence that the reactivating factor is essential to the reactivation of inactivated-glycerol dehydratase, which has been utilized in the reactivation process [12,30–33]. The contributing degrees of reactivation factor and other ingredients, however, have not been mentioned in articles reported and the construction of the effective and economic reactivation system has not yet been discussed in detail.

In the previous paper, we have reported that one new strain identified and named as *K. pneumoniae* XJPD-Li (GenBank accession number EF634066) [4]. Taking the catalytic efficiency into account, the glycerol dehydratase produced by *K. pneumoniae* XJPD-Li exhibited good properties for the production of 1,3-propanediol from glycerol, such as higher fermentation temperature (40 °C) and higher pH (8.0), and fast glycerol consuming with higher molar yield of 1,3-PD, comparing with other reported 1,3-propendiol production bacteria [1,3,9,34]. The reason why glycerol dehydratase kept in high activity drew our attention to the catalysis system in this strain. Further research then was carried out in this article. Herein we describe cloning and sequence analysis of the *dha*BCE gene encoding glycerol dehydratase from *K. pneumoniae* XJPD-Li. High level expression system for the glycerol dehydratase gene in *E. coli* is also reported. The inactivation and reactivation process of the recombined glycerol dehydratase was then investigated quantitatively in vitro. At the same time, the contributions of factors involved were determined in the reactivating process. It was hoped that quantitative analysis of this process is making for the well control of the reactivation of inactivated-glycerol dehydratase.

2. Materials and methods

2.1. Bacterial strains and plasmids

K. pneumoniae XJPD-Li stored in our lab was used as the glycerol dehydratase gene source for the cloning experiment. The plasmids pKRX-T (SBS Genetech, Beijing, China) and PET-28a (+) (Novagen, Germany) were used for the cloning. Expression experiments were conducted with *E. coli* strain Top10 (Tiangen Biotechnology Co., Ltd. Beijing, China) or BL21 (Invitrogen, Germany).

2.2. Media and growth conditions

E. coli was routinely grown in Luria-Bertani (LB) medium at 37 °C, which was supplemented with ampicillin (50 mg/ml) or kanamycin (50 mg/ml) when necessary.

2.3. Construction of the PKRX-T-dhaBCE cloning vector

The genomic DNA from *K. pneumoniae* XJPD-Li used as a template was extracted with *n*-cetyl trimethylammonium bromide (CTAB) method [35]. Primers were designed by based on the known glycerol dehydratase sequence from *K. pneumoniae* (GenBank accession number U30909): f, 5′-CCGGAATTC ATGAAAAGATCAAAACGATTTGC-3′ (introduced *Eco*R I restriction site underlined) and d, 5′-CATGAGCTCTTAGCTTCCTTTACGC-AGCTTATG-3′ (introduced *Sac* I restriction site underlined). These primers were subsequently applied in a PCR reaction to amplify the glycerol dehydratase gene (*dha*BCE). The reaction mixture used included a 200 μM concentration of each of the four deoxynucleoside triphosphates (dNTP), a 0.2 μM concentration of each of the primers, 0.2 μL of 5 U *Taq plus* DNA polymerase, 2.5 μL of 10× buffer (Mg^{2+}), and 100–200 ng of the genomic DNA as the template in the system of 25 μL. The reaction were initiated at 94 °C (4 min), followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 68 °C (30 s), and extension at 72 °C (3 min), and ended with final extension at 72 °C for 10 min. The PCR product of the expected size was gel-purified, ligated into the cloning vector PKRX-T at 4 °C overnight and transformed into *E. coli* Top10 (competent cells). Positive clones were selected and identified by colony PCR and restriction enzyme digestion. DNA sequence was carried out by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, PR China).

Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast) and DNAMAN 5.2.2 were employed for GenBank search, identity assessment and protein domain determination. Deduced amino acid sequences were obtained from EMBL and GenBank databases.

2.4. Construction of expression plasmid for glycerol dehydratase genes

The plasmid PKRX-T bearing the gene of the *dha*BCE from *K. pneumoniae* XJPD-Li was double digested with *Eco*R I and *Sac* I for further construction. The resulting 2.7 kb fragment containing the intact *dha*BCE genes was inserted into pET-28a (+) vector which had been linearized with the same enzymes. Transformation of the ligation mixture (pET-28 (+) *dha*BCE) into *E. coli* BL21 (DE3) was carried out by heat shock.

The selected recombinant *E. coli* was aerobically grown at 37 °C in LB medium containing kanamycin (50 mg L^{-1}). Isopropyl-β-D-thiogalactoside (IPTG) was added to a concentration of 0.4 mmol L^{-1} for induction when OD_{600} reached 0.4–0.6. The cells were harvested by centrifugation at 10,000 rpm and 4 °C for 5 min, washed twice with 100 mM potassium phosphate buffer (pH 8.0), after incubated at 25 °C for further 5 h.

2.5. Enzyme and protein assays

Cell-free extracts prepared by ultrasonication was assayed for glycerol dehydratase activity by the 3-methyl-2-benzothiazolinone hydrazine (MBTH) method according to Toraya et al. [36]. The resulting azine derivatives were determined spectrophotometrically. Since glycerol serves as substrate and suicide inactivator for glycerol dehydratase, 1,2-propanediol (0.2 mol L^{-1}) 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 at 40 °C.

Protein concentration was determined using the method of Bradfoard with crystalline bovine serum albumin (BSA) as a standard. Specific activity is expressed as units per mg of protein.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% polyacrylamide gel on a vertical mini gel apparatus at 120 V for1.5 h. Molecular weight marker was purchased from Dingguo Biotechnology Co., Ltd., Beijing, China. Proteins were stained with Coomassies Brilliant Blue R-250 strain.

2.6. Inactivation of glycerol dehydratase

Washed cells were suspended in 2 or 3 ml of potassium phosphate buffer (mentioned above), and disrupted by ultrasonic. The cell free extract was cleared by centrifugation as above mentioned. The changes of enzymatic activity along the time course were determined under the supplementation of different concentration of glycerol (0.0, 0.10, 0.15, 0.20, 0.30 mol L^{-1}) or aeration, according to Toraya et al. [16,24]

2.7. Reactivation of inactivated glycerol dehydratase

Different concentrations of ATP, Mg^{2+}, and CoB_{12} and reactivation factor of glycerol dehydratase were applied into the reaction

Table 1
Factors and levels for $L_9(3^4)$ orthogonal experimental design

Levels	Factors			
	A (mmol L^{-1})	B (mmol L^{-1})	C	D (μmol L^{-1})
1	30	5	8	3
2	50	10	6	7.5
3	80	20	4	12

(A) ATP concentration; (B) Mg^{2+} concentration; (C) GDHt/GDHt reactivation factor (W/W); (D) CoB_{12} concentration.

system with O_2 or glycerol-inactivated glycerol dehydratase. To settle the levels of orthogonal experiment design, the concentration of reaction ingredients was determined according to Toraya et al. [16,20,24] and single-factor experiments were done previously. The design of orthogonal experiment was shown in Table 1. (The reactivation factor was cloned and over-expressed with detected activity by one member in our lab and the GenBank accession numbers of the genes (*dha*FG) for reactivation factor big subunit and small subunit are EF634064 and EF634065, respectively. The manuscript is under preparation.) Enzymatic activity and aldehyde was examined to calculate the extent of reactivation of the inactivated enzyme.

3. Results and discussion

3.1. Cloning and sequence analysis of the glycerol dehydratase gene

Genomic DNA of glycerol dehydratase from *K. pneumoniae* XJPD-Li was extracted with CTAB method according to Molecular Cloning: A Laboratory Manual. The extraction was examined by agarose gel electrophoresis where a single bright band around 23 Kbp was visualized in Fig. 1. The genomic DNA from *K. pneumoniae* XJPD-Li was then utilized as template for the cloning of glycerol dehydratase gene. PCR amplification was carried out under the conditions described in Section 2 (Section 2.3). It could be seen from Fig. 2 that one bright band of approximately 2700 bp was isolated in the agarose electrophoresis. That is, the PCR product of the gene (*dha*BCE) encoding glycerol dehydratase was correctly

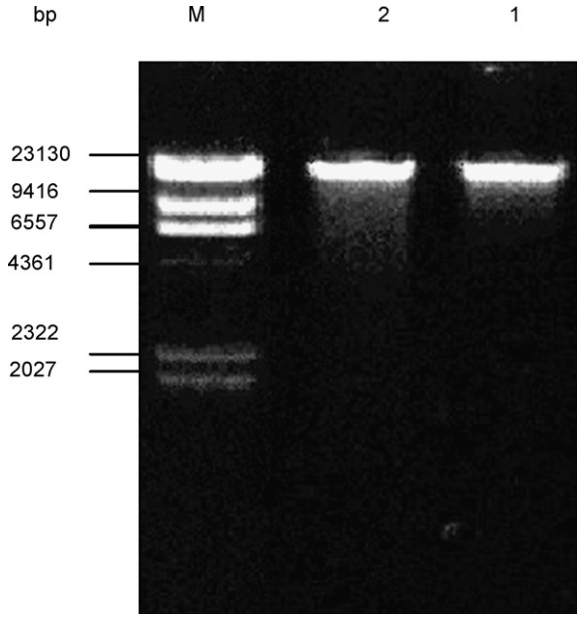

Fig. 1. Agarose gel electrophoresis of genomic DNA of glycerol dehydratase from *K. pneumononiae* XJPD-Li. *Note*: M, Marker, 1 & 2, genomic DNA.

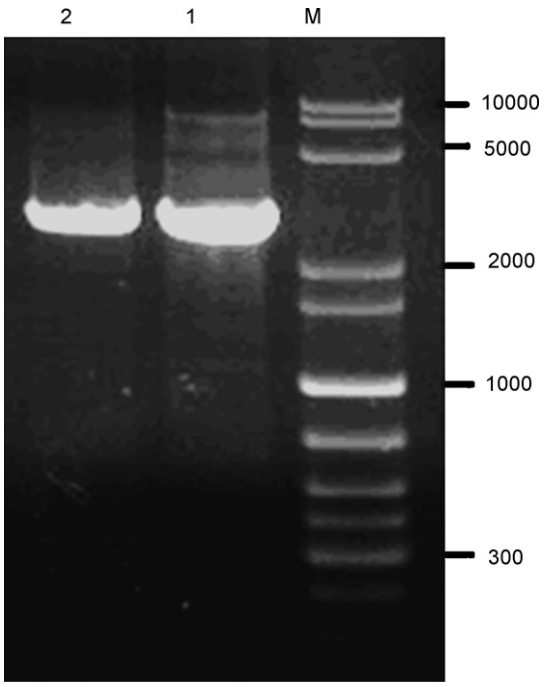

Fig. 2. Agarose gel electrophoresis of PCR amplification of gene for glycerol dehydratase from *K. pneumononiae* XJPD-Li. *Note*: M, Marker, 1 & 2, products of PCR.

amplified by primers designed from genomic DNA from *K. pneumoniae* XJPD-Li as expected. The purified PCR product was then ligated to lineared PKRX-T vector. Afterwards the corrected ligation mixture was transformed into competent cell by heat shock. Ampicillin resistant transformants were identified by colony PCR and double enzymatic digestion, and positive clone was send to sequence. The assigned accession number EF634063 was available from the GenBank database after submitting the sequence of *dha*BCE.

Gene sequence alignment showed that glycerol dehydratase from *K. pneumoniae* XJPD-Li had a significant level of identity with that from *K. pneumoniae* (U30909) (99.26% identity). Three complete ORFs, *dha*B, *dha*C and *dha*E, were identified as the *dha* codon. The *dha*B gene (1668 bp, from 1 to 1,668) encodes α subunit with 555 amino acids. It was immediately followed by the *dha*C gene (584 bp base pairs 1681 to 2265) encoding the β subunit (194 aa). In addition, the *dha*B terminal codon (TAA) was separated from the *dha*C terminal codon (GTG) by 13 base pairs, and the *dha*E gene encoding γ subunit (141 aa) was coded by base from 2268 to 2693, following the *dha*C gene. The calculated molecular weight of three subunits were 60,702 Da, 21,322 Da and 16,101 Da, respectively. The subunits glycerol dehydratase from *K. pneumoniae* XJPD-Li exhibited significant homologies with GDHt from *K. pneumoniae* (U30909) (99.10% identity). Among three subunits of glycerol dehydratase from *K. pneumoniae* XJPD-Li, amino acid residues H_{13}, S_{193}, N_{359}, E_{407}, and M_{515} of α subunit, N_{47}, L_{150}, V_{189} of β subunit are different [37], comparing with protein sequences in the EMBL and GenBank database.

In Table 2, the deduced amino acid sequences of the *dha*BCE coding regions of *K. pneumoniae* were compared with those of the corresponding subunits of *K. pneumoniae* XJPD-Li glycerol dehydratase, *Citrobacter freundii* glycerol dehydratase [27], *Clostridium pasteurianum* glycerol dehydratase [38], *K. oxytoca* diol dehydratase [39], and *K. pneumoniae* diol dehydratase [28]. It could be found that the calculated molecular masses of the corresponding gene products were very similar, especially α subunit, and the glycerol dehydratase from *K. pneumoniae* and *C. freundii* showed significant homology among those of the protein listed.

Table 2
Sequences homologous to the *Klebsiella pneumoniae dha*BCE genes

Organism	Gene name			Gene length (bp)			Protein molecular mass (Da)			Amino acid identity (%)		
	L	M	S	L	M	S	L	M	S	L	M	S
Klebsiella pneumoniae	*gld*A	*gld*B	*gld*C	1668	585	426	60,621	21,310	16,094	100	100	100
Klebsiella pneumoniae XJPD-Li	*dha*B	*dha*C	*dha*E	1668	585	426	60,702	21,322	16,101	99.1	98.4	100
Citrobacter freundii	*dha*B	*dha*C	*dha*E	1668	585	429	60,433	21,487	16,121	94	89	86
Clostridium pasteurianum	*dha*B	*dha*C	*dha*E	1665	540	441	60,813	19,549	16,722	78.1	68.8	66.7
Klebsiella oxytoca	*pdd*A	*pdd*B	*pdd*C	1662	672	519	60,348	24,113	19,173	71	58	54
Klebsiella pneumoniae	*pdd*A	*pdd*B	*pdd*C	1662	684	522	60,379	24,401	19,489	69	<64	<59

The *dha*BCE genes of *K. pneumoniae* XJPD-Li (this study), *C. freundii*, and *C. pasteurianum* and the *gld*ABC genes of *K. pneumoniae* encode coenzyme B_{12}-dependent glycerol dehydratases. The *pdd*ABC genes of *K. oxytoca* and *K. pneumoniae* encode coenzyme B_{12}-dependent diol dehydratases. The values for amino acid identity are given with respect to the *Klebsiella pneumoniae* sequence. L, large subunit; M, medium subunit; S, small subunit.

3.2. Construction and identification of expression vector pET-28 a (+)-dhaBCE

The construction of expression vector of pET-28a (+)-*dha*BCE described in Section 2 is shown in Fig. 3. Agarose gel electrophoresis indicated that the expression vector for gene of *dha*BCE was constructed successfully. The products of expression vector is transformed into a host strain *E. coli* BL21 (DE3) (competent cell) for protein production.

Induced by IPTG, recombinant *E. coli* BL21 (pET-28a (+)-*dha*BCE) was cultivated in LB medium containing the corresponding antibiotic (kanamycin). Harvest cells were washed and subjected to SDS-PAGE analysis. A commercial low molecular-mass calibration kit of standard proteins served as subunit molecular mass standards. Protein bands were visualized in gels by staining with Coomassie brilliant blue. Thick protein bands corresponding to the α, β and γ subunits of glycerol dehydratase, with apparent molecular masses of 60.1 kD, 21.3 kD, 16.1 kD, respectively, were observed in Fig. 4. The 0.299 U mg^{-1} of special enzymatic activity of recombined glycerol dehydratase was determined by cell free extract, which was about 3 times comparing with that of wild strain. The molecular masses of the three subunits were in the same range as described for the corresponding subunits of glycerol dehydratase from other organisms [27,39].

3.3. Inactivation of recombined glycerol dehydratase in vitro

The glycerol dehydratase underwent inactivation by the physiological substrate glycerol during catalysis [14]. The enzyme activity

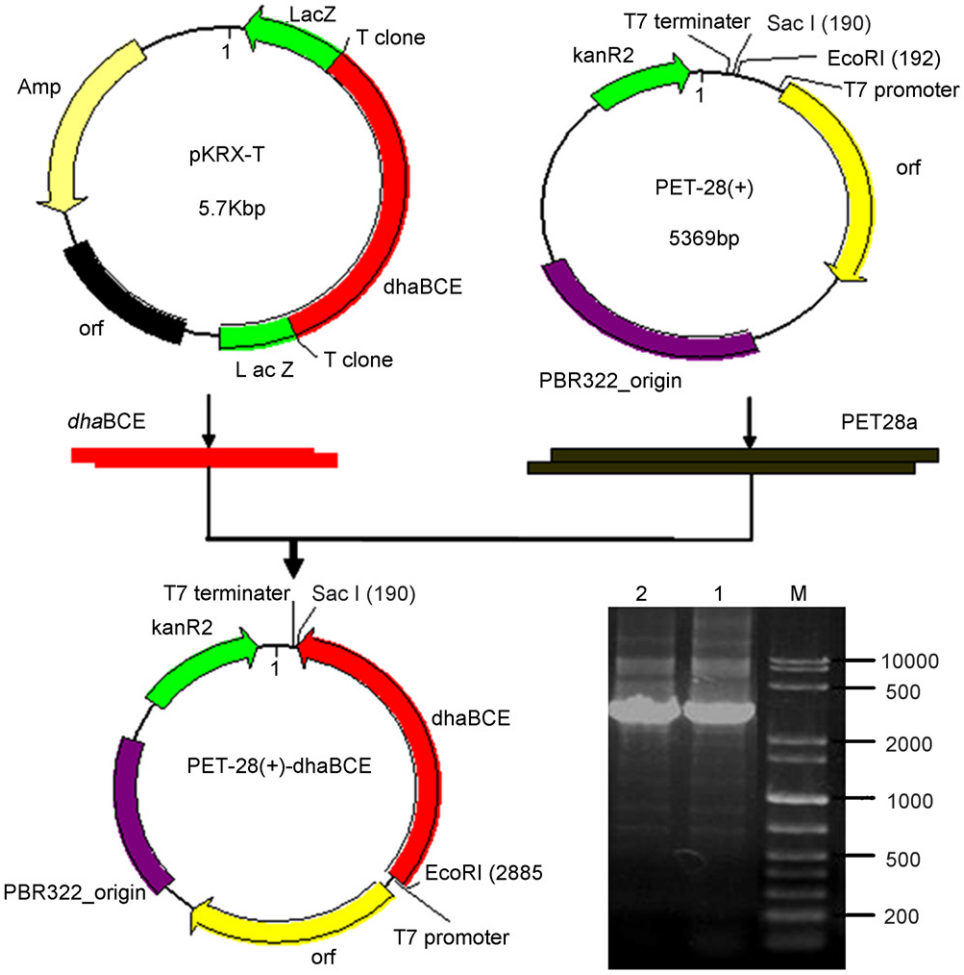

Fig. 3. Construction of PET-28a (+)-*dha*BCE plasmids and agarose gel electrophoresis of PCR products. *Note*: M, Marker, 1 & 2, products of PCR.

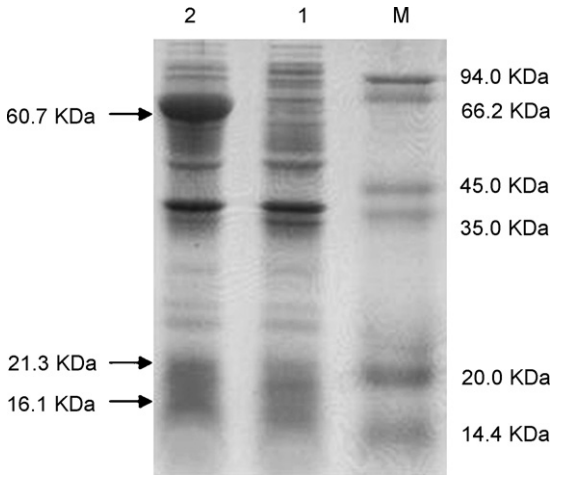

Fig. 4. SDS-PAGE analysis of expression products by *E. coli* BL21 (DE3) (PET-28a (+)-*dha*BCE). *Note*: M, Marker; 1, without IPTG; 2, induced by IPTG.

was determined when oxygen or glycerol was supplied into the enzyme catalysis system. As depicted in Fig. 5, without glycerol or O_2, the recombined glycerol dehydratase from *K. pneumoniae* XJPD-Li could keep almost total active in 60 min. With the co-incubation with different concentration of glycerol, the glycerol dehydratase activity decreased along the time course. About 60% and 30% enzyme active remained when the concentration of glycerol dehydratase were 0.1 and 0.15 mol L^{-1}, respectively. The activity of enzyme decreased to 50% of the initial enzyme activity after 5 min with the glycerol concentration of 0.2 mol L^{-1}, and the remaining activity was near to zero after 60 min. Under higher concentration, the recombined glycerol dehydratase was inactivated much more quickly. Moreover, oxygen also played a role of inactivated factor. It was found that oxygen could inactive the enzyme activity faster than glycerol (0.3 mol L^{-1}). The activity of enzyme was 22% remained in 5 min, and slowly decreased in the following.

It is evident that glycerol dehydratase underwent the glycerol-suicide and sensitive to aeration. The relative activity decreased quickly at the initial stage and almost linearly with time of incubation. The inactivated-glycerol dehydratase was also prepared by Toraya et al. They found that the dehydration of glycerol by the toluene-treated *K. pneumoniae* ATCC 25,955 cells (in situ glycerol dehydratase) with added adenosylcobalamin was accompanied by

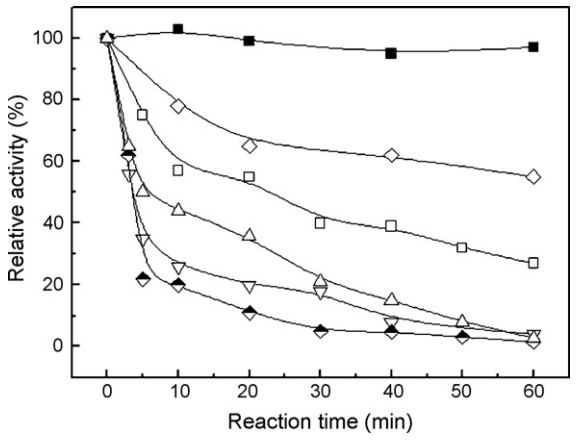

Fig. 5. Inactivation of recombined glycerol dehydratase by glycerol or O_2. (■) Without glycerol and O_2; (◆) inactivated by O_2; (◊) inactivated by glycerol 0.10 mol L^{-1}; (□) inactivated by glycerol 0.15 mol L^{-1}; (△) inactivated by glycerol 0.20 mol L^{-1}; (▽) inactivated by glycerol 0.15 mol L^{-1}.

Table 3
The design matrix and experimental data for optimizing the reactivation system of recombined glycerol dehydratase

Run	Factors				Results	
	A	B	C	D	Special activity (U mg^{-1})	Reactive degree (%)
1	1	1	1	1	0.1913	63.8
2	1	2	2	2	0.2015	67.2
3	1	3	3	3	0.2072	69.1
4	2	1	2	3	0.2033	67.8
5	2	2	3	1	0.2938	98.9
6	2	3	1	2	0.2211	73.8
7	3	1	3	2	0.2341	78.1
8	3	2	1	3	0.2048	68.3
9	3	3	2	1	0.2183	72.8
Reactive degree ($k_{average}$)						
k_1	66.7	69.9	68.6	78.5		
k_2	80.2	78.1	69.3	73.0		
k_3	73.1	71.9	82.0	68.4		
R	13.5	8.2	13.4	10.1		
Q	A_2	B_2	C_3	D_1		

Reactive degree (%) = $\frac{\text{special activity after reactivation}}{\text{special activity before inactivation}} \times 100\%$.

concomitant inactivation and ceased almost completely with 3 min, as did that by the in vitro enzyme [20]. The inactivated-glycerol dehydratase was utilized as the starting materials for the reactivation system.

3.4. Quantitative analysis reactivation process of inactivated glycerol dehydratase

Factors involved in the reactivation of inactivated glycerol dehydratase from *K. pneumoniae* XJPD-Li were quantitatively analyzed. Toraya etc. had reported that the reactivating factor, free AdoCbl, ATP, and Mg^{2+} were absolutely required for both reactivation of the glycerol-inactivated holoenzyme and activation of the enzyme CN-Cbl complex [18]. According to what had been reported [28] and results of single factor experiments (data not shown), the orthogonal test L_9 (4^3) was designed to optimize the reactivation system of inactivated glycerol dehydratase. The effects on the reactivation system were investigated in the orthogonal experimental design study: (A) ATP concentration; (B) Mg^{2+} concentration; (C) GDHt/GDHt reactivation factor (W/W); (D) CoB_{12} concentration. Fixed levels of these four variables were given in Table 1. The matrix design with levels and results of the special activity and reactive degree of inactivated glycerol dehydratase are shown in Table 3. It was indicated that from the Range analysis that the effects of the factors on the reactivation system of glycerol dehydratase in order of importance were ATP concentration and GDHt/GDHt reactivation factor (W/W), CoB_{12} concentration, Mg^{2+} concentration. The optimum condition were that the ratio (W/W) of glycerol dehydratase to reactivation factor was 4:1; concentration of ATP was 50 mmol L^{-1}; concentration of coenzyme B_{12} and Mg^{2+} were 10 mmol L^{-1} and 3 μmol L^{-1}, respectively. By comparison of the maximum slope with the control, the O_2-inactivated and glycerol-inactivated dehydratase could be effectively reactivated to 97.3% and 98.9% of initial activity under the optimum condition in 10 min, respectively. It was relatively faster than reported, where the extent of reactivation increased with time of incubation and reached at least 71% at 20 min by the factor in the presence of AdoCbl, ATP, and Mg^{2+} [24].

In order to better describing the reactivation progress, the contribution extent of factors was determined for the reactivation of inactivated glycerol dehydratase. The enzyme activity in the optimized reactivation system mentioned above was chose as the control, being considered as 100%. It was found that all these con-

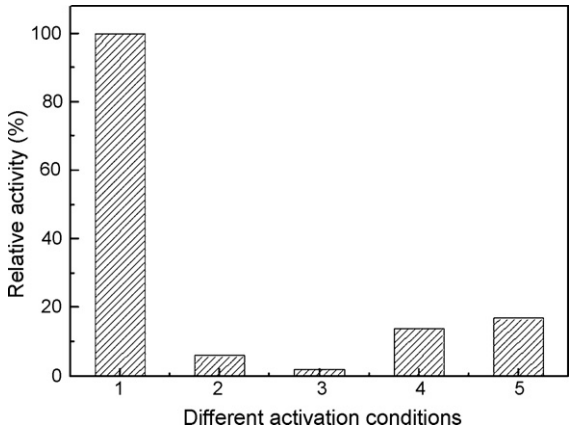

Fig. 6. Contribution extent of factors for reactivation of inactivated glycerol dehydratase. *Note*: 1, all four factors; 2, without ATP; 3, without reactivation factor; 4, without CoB_{12}; 5, without Mg^{2+}.

ditions were important for the reactivating process. Without any factors, the relative activity dropped less than 20% of initial activity (Fig. 6). Meanwhile, reactivation factor and ATP played much critical role in the reactivating process. The reactivation was described taking place in two steps by Toraya et al. that ADP-dependent cobalamin release firstly and ATP-dependent dissociation of the resulting apoenzyme-reactivating factor complexes [22,24,26]. It was consistent with the report that reactivated holoenzymes took place in the ATP/ADP-switching mechanism [29].

The concentration of aldehyde was also determined along the time course in the optimized reaction system. It could be clearly seen from Fig. 7 that both glycerol and O_2 inactivated-GDHt from *K. pneumoniae* XJPD-Li could be reactivated and the activity of the former was relatively easier to recover than that of the latter. The activity of glycerol dehydratase could be recovered quickly in the first 10 min and turn to be stable. On the contrary, the production of propionaldehyde was close to nil in the reactivation system without reactivation factor and ATP (Fig. 7), which were critical for the reactivation of inactivated glycerol dehydratase. The reactivation factor together with ATP was considered as the "ON/OFF" reactivating condition.

Under the optimum reactivation system, both glycerol and O_2 inactivated-GDHt could be reactivated rapidly with high extent. It could be proposed that the quantitative construction reaction system will be necessary for enzyme reactivating effectively and economically. The proper reactivation factors ratio ensured the good fermentation properties of *K. pneumoniae* XJPD-Li.

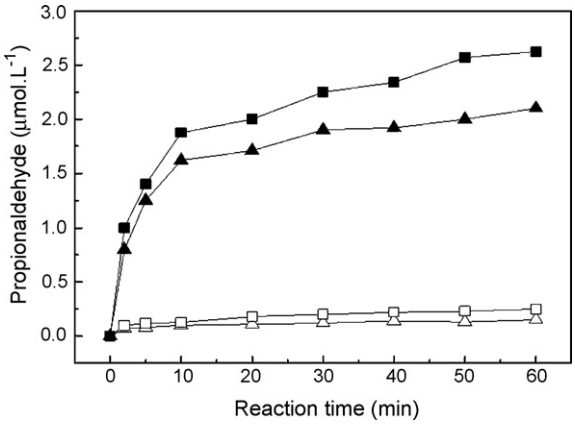

Fig. 7. Process curve of reactivation of inactivated glycerol dehydratase. (■) Glycerol-inactivated glycerol dehydratase; (□) glycerol-inactivated glycerol dehydratase without ATP and reactivation factor; (▲) oxygen-inactivated glycerol dehydratase; (△) oxygen-inactivated glycerol dehydratase without ATP and reactivation factor.

4. Conclusions

The gene of glycerol dehydratase from *K. pneumoniae* XJPD-Li was cloned and over-expressed in *E. coli* BL21 (DE3). The GenBank accession number was assigned by EF634063. Three complete ORFs, *dha*B (1668 bp, from 1 to 1668), *dha*C (585 bp base pairs 1681 to 2265) and *dha*E (426 bp, from 2268 to 2693), encode α, β and γ subunit. The calculated molecular weight of three subunits was 60,702 Da, 21,322 Da and 16,101 Da, respectively. The gene and amino acid sequence of glycerol dehydratase from *K. pneumoniae* XJPD-Li has a significant level of identity with GDHt from *K. pneumoniae* (99.26% and 99.10% identity, respectively). Among three subunits of recombined glycerol dehydratase, amino acid residues H_{13}, S_{193}, N_{359}, E_{407}, and M_{515} of α subunit, N_{47}, L_{150}, V_{189} of β subunit are different with those from *K. pneumoniae* (GenBank accession number U30909). The over-expressed glycerol dehydratase showed higher enzyme activity than that of wild strain.

The recombined glycerol dehydratase underwent inactivating process with either O_2 or glycerol. The reactivation system of recombined glycerol dehydratase was optimized. When the concentration of ATP, Mg^{2+} and coenzyme B_{12} were 50 mM, 10 mM and 3 μM, respectively, and in ratio (W/W) of the glycerol dehydratase to reactivation factor was 4:1, the O_2-inactivation and glycerol-inactivation dehydratase could recover 97.3% and 98.9% of initial activity in 10 min, respectively. The reactivation factor and ATP were critical to the reactivation of glycerol dehydratase. Relatively, the activity of glycerol inactivated GDHt was easier to be recovered than that of O_2-inactivated GDHt.

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