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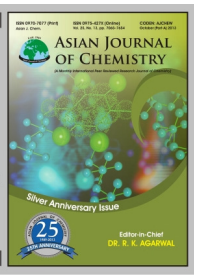

Coenzyme B_{12}-Dependent Glycerol Dehydratase Deficiency in *Klebsiella pneumoniae*

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The physiology and fermentation properties of coenzyme B_{12}-dependent glycerol dehydratase deficient mutant *Klebsiella pneumoniae* B1.9131 were investigated. Compared with that of wild type strain, glycerol dehydratase activity decreased by 80 %. Activities of *dha* regulon coded two enzymes are influenced by inactivation of GDHt. 1,3-Propanediol oxidoreductase activity decreased by 62 % while glycerol dehydrogenase activity increased by 36 %. Fed-batch fermentation showed that more of the metabolic flux of glycerol was directed to lactate and ethanol in the mutant. Instead, lactate other than 1,3-propanediol was the main product and its final concentration increased from 41 to 76 g L^{-1} with mutant isolate. The concentration of 1,3-propanediol decreased from 91 to 40 g L^{-1}. The results demonstrate that GDHt is not indispensable in glycerol metabolism but is crucial for the efficient synthesis of 1,3-propanediol.

Key Words: *Dha* regulon, Dehydratase, Fermentation, Glycerol, *Klebsiella pneumoniae*, 1,3-Propanediol.

INTRODUCTION

1,3-Propanediol (1,3-PD) is one of the platform chemicals in the chemical industry and can be synthesized either from petrochemical or renewable resources. The value of 1,3-propanediol lies predominantly in its use in polymers prepared from 1,3-propanediol and terephthalic acid, demand of which is estimated to be one to two billion pounds per year in 10 years[1]. The microbial approach to 1,3-propanediol production becomes an emerging area of intellectual endeavor and industrial practice with great promise in recent years[2].

1,3-Propanediol is excreted in a growth-associated manner with glycerol by *Klebsiella pneumoniae* relying on *dha* regulon[3]. 1,3-propanediol synthesis is carried out in two enzymatic steps. Glycerol dehydratase (EC 4.2.1.30, GDHt) removes a water molecule from glycerol in presence of coenzyme vitamin B_{12} to form 3-hydroxypropionaldehyde (3-HPA)[4]. Subsequently, 1,3-propanediol oxidoreductase (EC 1.1.1.202, PDR) transfers a reducing equivalent from NADH to 3-hydroxypropionaldehyde, giving 1,3-propanediol. The isofunctional dehydratase diol dehydratase (EC 4.2.1.28, DDHt) in *K. pneumoniae* could also convert glycerol, 1,2-propanediol and 1,2-ethanediol to 3-hydroxypropionaldehyde, propionaldehyde and acetaldehyde, respectively[5]. GDHt is associated with the *dha* regulon, while DDHt is associated with the pdu regulon. Both GDHt and DDHt have a subunit composition of $\alpha_2\beta_2\gamma_2$[6] and are coenzyme B12-dependent, except for the diol dehydratase of *Clostridium butyricum*[7]. They may work individually or together in several microorganisms, such as *Bacillus*, *Clostridia*, *Citrobactor*, *Lactobacillus* and *Klebsiella*[8].

In order to investigate its metabolic role in *K. pneumoniae* CGMCC 1.9131, GDHt mutant *K. pneumoniae* was constructed *via* knocking out *dha*B-α. we obtained a GDHt mutant isolate and used it to study glycerol metabolism with fed-batch fermentation. The time profiles of fermentation and phsiological change will be studied in this work.

EXPERIMENTAL

Klebsiella pneumoniae CGMCC 1.9131 was isolated from campus soil in Tsinghua University. The strain is stored in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China).

Construction of gene-specific mutants: An 1000-bp segment of truncated GDHt gene *dha*B-α of *K. pneumoniae* was polymerase chain reaction (PCR)-amplified with oligonucleotides primer1 (5'- CGAATTCGTGATGGCGCAGATGAAC GT-3') and primer 2 (reverse 5'- GTCTAGACGCATCTCGT TGCTGCCGT-3'). The segment was cloned into pGEM-T easy vector (Promega, USA) and then transferred to suicide vector pGPCm after being digested with EcoRI and XbaI, resulting in vector pGP-Ba. The pGP-Ba vector was then transformed into *E. coli* SM10[9] and the resulting strain was used as donor

in conjugation with *K. pneumoniae*. Transconjugants were selected for both chloromycetin resistance from pGPCm inserting and ampicillin resistance because the *K. pneumoniae* strain is resistive to ampicillin while the *E. coli* SM10 is sensitive.

Growth conditions: The basic culture media contained (L^{-1}): Glycerol 30 g, yeast extract 1.5 g, $(NH_4)_2SO_4$ 4.0 g, K_2HPO_4 0.69 g, KH_2PO_4 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, trace element solution 1 mL and Fe^{2+} solution 1 mL. The trace element solution contained (L^{-1}): $MgSO_4 \cdot 4H_2O$ 100 mg, $ZnCl_2$ 70 mg, $Na_2MoO_4 \cdot 2H_2O$ 35 mg, H_3BO_3 60 mg, $CoCl_2 \cdot 6H_2O$ 200 mg, $CuSO_4 \cdot 5H_2O$ 29.28 mg, $NiCl_2 \cdot 6H_2O$ 25 mg and HCl (37 %) 0.9 mL.

The fermentable cultivation was conducted in a 5 l fermentor (B. Braun, Germany) with a working volume of 4 l. The fed-batch fermentation was carried out at 37 ºC, pH 6.8, 250 rpm and 0.5 vvm air flow. The initial glycerol is 25 g L^{-1}. The pure glycerol was fed when glycerol concentration is below 5 g L^{-1}.

Substrate and products analysis: Dry cell mass was computed from the optical density calibration curved determined at 650 nm (OD_{650}). Glycerol, 1,3-propanediol, 2,3-butanediol (2,3-BD), lactate, succinate, acetate and ethanol were determined by a Shimadzu 10AVP HPLC system using an Aminex HPX-87H column (300×7.8mm) (Bio-Rad, Palo AHO, Ca, USA) as described[10]. 3-HPA was determined using the colorimetric method described by Circle *et al.*[11].

Measurement of enzyme activities: GDHt and DDHt activities were determined by the 3-methyl-2-benzothiazolinone hydrazone method[12], in which glycerol or 1,2-propanediol was used as the respective substrates. PDR and glycerol dehydrogenase (GDH) activities were determined according to the method of Xu *et al.*[13].

RESULTS AND DISCUSSION

Construction and assay of GDHt deficient mutants: The *dha* regulon enables microorganisms such as *Klebsiella pneumoniae* or *Citrobacter freundii* to grow on glycerol. Four enzymes are encoded by this regulon: glycerol dehydrogenase (GDH), dihydroxyacetone kinase (DHK), GDHt and PDR. GDHt and PDR are naturally under the control of two different promoters and are transcribed in different directions. An 1000-bp DNA fragment from the GDHt-encoding region of *K. pneumoniae* was amplified by PCR. The resulting sequence was cloned into the suicide vector to construct pGP-Bα harboring chloromycetin and ampicillin resistance. pGP-Bα was then introduced by conjugational transfer into *K. pneumoniae*, giving rise to chloromycetin-resistant isolate. Four GDHt deficient isolates, *K. pneumoniae* B1.9131-1,2,3 and 4, were obtained. The occurrence of an insertion event was checked by PCR as mentioned in experimental section. The 1160-bp DNA fragment was observed when genomic DNA from B1.9131-1, 2, 3 and 4 was used as a template (Fig. 1, lane 1, 2, 3 and 4) whereas no fragment was amplified with genomic DNA from the wild strain B1.9131 (lane CK). The occurrence of the insertion in *dha*B-α was further confirmed by the GDHt enzymatic activity assay. As shown in Table-1, GDHt activities of 4 isolates samples decreased by

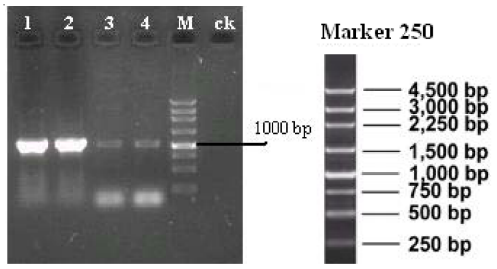

Fig. 1. PCR detection of *K. pneumoniae* B1.9131 and CGMCC 1.9131. Lanes 1-4: *K. pneumoniae* B1.9131-1,2,3,4; M: Marker; ck: K. pneumoniae CGMCC 1.9131. The mutants were identified by PCR, with forward (5'-GTCGACGGATCCCAAGCTT-3') and reverse (5'-AGATCCTCCACCACGTTAC-3') primers

TABLE-1
ACTIVITY OF GLYCEROL DEHYDRATASE (GDHt) AND DIOL DEHYDRATASE (DDHt) IN *K. pneumoniae* CGMCC 1.9131 AND THE MUTANTS

Strains	GDHt activity	DDHt activity
	Protein (U mg^{-1})	Protein (U mg^{-1})
CGMCC 1.91316	0.93 ± 0.03	0.25 ± 0.01
B1.9131-1[a]	0.19 ± 0.01	0.18 ± 0.01
B1.9131-2	0.22 ± 0.01	0.18 ± 0.002
B1.9131-3	0.17 ± 0.01	0.14 ± 0.01
B1.9131-4	0.19 ± 0.01	0.15 ± 0.003

Bacteria were cultivated in the basic culture media as described in "Growth conditions" section at 37 ºC and 150 rpm for 12 h. Enzyme assays were determined according to the method described by Xu *et al.* [13]. The average data of three individual experiments are presented. [a]B1.9131-1, 2, 3 and 4 are four GDHt-deficient mutant isolates.

76 to 82 % in comparison with that of its wild type. DDHt may contribute to the residual activities. Mutant B1.9131-3 had the lowest GDHt activity amongst the four mutants. This isolate was selected for the subsequent fermentation work.

Effect of GDHt deficiency on PDR and GDH expression: Activities of PDR and GDH from CGMCC 1.9131 and B1.9131-3 were determined (Table-2). Compared with that of wild strain, the specific activities of GDHt and PDR decreased by 79.36 and 61.74 %, respectively, with *K. pneumoniae* B1.9131, whereas the specific activity of GDH increased by 36.45 %.

TABLE-2
EFFECT OF KNOCKING OUT THE GLYCEROL DEHYDRATASE (GDHt) GENE ON THE EXPRESSION OF 1,3-PROPANEDIOL OXIDOREDUCTASE (PDR) AND GLYCEROL DEHYDROGENASE (GDH)

Strains	Activity (U mg^{-1})		
	GDHt	PDR	GDH
CGMCC 1.9131	0.93 ± 0.04	1.1 ± 0.03	1.4 ± 0.06
B1.9131-3	0.19 ± 0.01	0.43 ± 0.01	1.9 ± 0.02

Bacteria were cultivated in the basic culture media as described in "Growth conditions" section at 37 °C and 150 rpm. The samples were obtained in 9 h. Enzyme assays were determined according to the method described by Xu *et al.* [13]. The average data of three individual experiments are presented.

1,3-Propanediol was not a substrate for the reactions catalyzed by coenzyme B_{12}-dependent dehydratases but manifested a competitive inhibition towards the substrates dehydrated by GDHt. The activities of GDHt were determined in the presence of increasing 1,3-propanediol concentrations.

GDHt activity decreased by 44.84 and 88.64 % in the presence of 1.0 and 2.0 mol L^{-1} 1,3-propanediol (Fig. 2), respectively which was similar to our previous studies with *K. pneumoniae* CGMCC 1.6366[14].

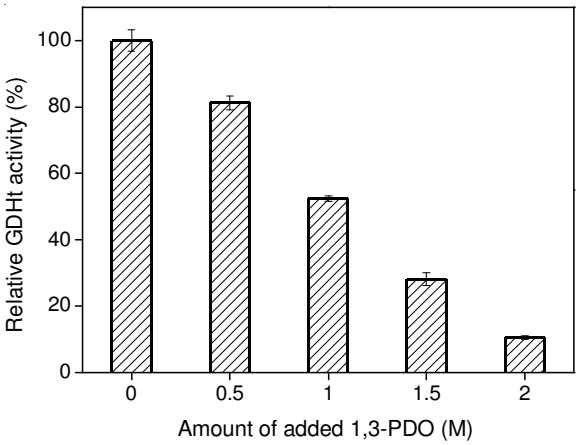

Fig. 2. Effect of 1,3-propanediol (1,3-PD) on the activity of glycerol dehydratases. The glycerol dehydratase (GDHt) activity was measured in the presence of 0-2 M 1,3-propanediol with MBTH method as described by Xu *et al.*[13]

3-HPA accumulation in fed-batch fermentation: 3-HPA is identified as a toxic intermediary metabolite in the 1,3-propanediol synthesis pathway[14,15]. High level of 3-HPA in the culture may cause the cessation of 1,3-propanediol production and cell growth. 3-HPA_{max} of 8.94 and 1.23 mM was detected in the batch fermentations with CGMCC 1.9131 and B1.9131, respectively (Fig. 3). It was found that a high activity ratio of GDHt over PDR contributed to 3-HPA accumulation in the early period of *K. pneumoniae* fermentation in our previous works[10,13]. The low level of 3-HPA agreed with the enzymatic activities (Table-2). However, the carbon flux to 1,3-propanediol pathway decreases with isolate B1.9131, although the fermentation could be sustained at higher glycerol concentration.

1,3-Propanediol production with CGMCC 1.9131 and B1.9131: The batch fermentations were conducted by CGMCC 1.9131 and 4 isolates in the shaker flasks to investigate the product spectrum (Table-3). As expected, 1,3-propanediol synthesis decreased remarkably with 4 mutant isolates. It is mentioned that the ethanol production was enhanced in all mutants cultures. The fed-batch fermentations in the 5 l fermentor were performed with CGMCC 1.9131 and B1.9131(Fig. 4). Although GDHt was deficient, 40 g 1,3-propanediol L^{-1} was obtained in cultures with B1.9131, confirming the expression of DDHt during the fermentation as mentioned above in

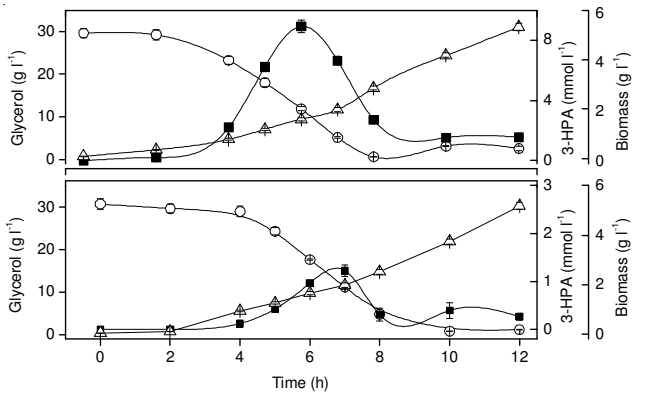

Fig. 3. Accumulation of 3-HPA in the early phase of batch fermentation by *K. pneumoniae* CGMCC 1.9131 (top entry) and B1.9131 (bottom entry): glycerol (open circles), cell mass (open triangles), 3-HPA (filled squares). Fermentation was carried out in a 5 l fermentor at 37 ºC, pH 6.8, 250 rpm and 0.5 vvm air flow. The initial 25 g glycerol L^{-1} was used in cultures. The average data of three individual experiments are presented

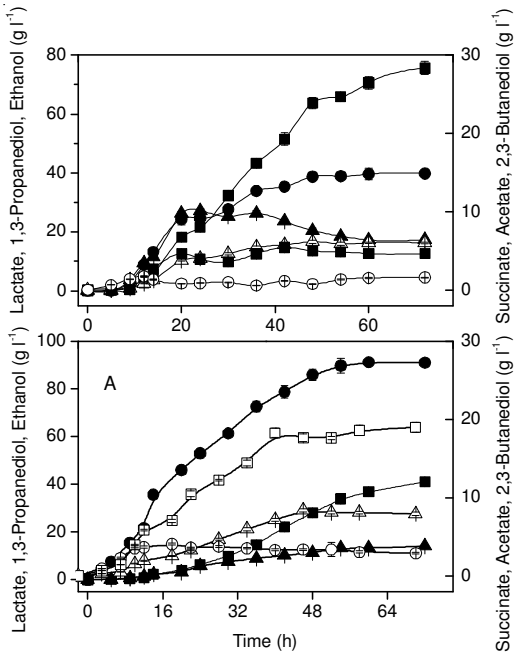

Fig. 4. Time course of fed-batch fermentation by *K. pneumoniae* CGMCC 1.9131 (bottom entry) and B1.9131 (top entry): 1,3-propanediol (filled circles), lactate (filled squares), ethanol (filled triangles), succinate (open triangles), acetate (open circles), 2,3-butanediol (open squares). Fed-batch fermentation was carried out in a 5 l fermentor at 37 ºC, pH 6.8, 250 rpm and 0.5 vvm air flow. The initial 25 g glycerol L^{-1} was used in betach cultures. The addition of pure glycerol into the fed-batch cultures was started when the glycerol concentration in the broth was below 5 g L^{-1}. The glycerol level in subsequent cultures was controlled at approximately 5-10 g L^{-1}. The average data of three individual experiments are presented

TABLE-3
FERMENTATIONS BY *K. pneumoniae* CGMCC 1.9131 AND THE MUTANTS

Strains	Product (g L^{-1})					
	Succinate	Lactate	Acetate	1,3-Propanediol	2,3-Butanediol	Ethanol
CGMCC 1.9131	0.06 ± 0.01	0	0.58 ± 0.02	13 ± 0.3	2.2 ± 0.08	1.5 ± 0.06
B1.9131-1	0.29 ± 0.01	2.5 ± 0.04	1.1 ± 0.05	4.2 ± 0.09	3.9 ± 0.17	3.8 ± 0.13
B1.9131-2	0.17 ± 0.004	0	0.55 ± 0.01	6.4 ± 0.23	1.9 ± 0.01	6.5 ± 0.08
B1.9131-3	0.19 ± 0.01	1.9 ± 0.05	0.54 ± 0.02	1.8 ± 0.08	3.0 ± 0.13	2.5 ± 0.08
B1.9131-4	0	1.3 ± 0.05	1.3 ± 0.02	2.8 ± 0.09	1.5 ± 0.07	4.8 ± 0.11

Products assay ware carried out as described in "Materials and methods" section. The average data of three individual experiments are presented.

Table-1. The fermentations with wild and mutant isolates shared the similar cell growth and glycerol uptake pattern (Figs. 3 and 4), indicating that knocking out of GDHt resulted in the redistribution of the carbon flux. *K. pneumoniae* has the sophisticated mechanism to maintain the substrate uptake and cell growth. The product spectra may change under the different genetic or chemical conditions. 75.61 g lactate L^{-1} was obtained in fed-batch with B1.9131, whereas only 40.99 g L^{-1} lactate was produced with CGMCC 1.9131. Ethanol formation was also greatly enhanced in the first 24 h. Succinate production remained almost at the same level, while 1,3-propanediol and 2,3-butanediol production decreased by about 56 and 75 %, respectively.

The *gdh* and *gdhr* genes encode GDHt and DDHt in *K. pneumoniae*, respectively. *K. pneumoniae* ATCC 25955 can produce both GDHt and DDHt, which are distinguishable *in vitro*, whereas *K. pneumoniae* ATCC 8724 forms only DDHt[5]. Although the two types of dehydratases have high sequence homology, GDHt is a soluble cytosolic enzyme, whereas DDHt is a low-solubility enzyme associated with carboxysome-like polyhedral organelles[6]. To broaden knowledge on the diversity of glycerol dehydratases, comprehensive sequence and molecular modelling analyses of these enzymes were performed[16]. The sequence analysis showed that GDHt and DDHt are not related, suggesting that they evolved from different ancestors. A gene fusion event occurred between α and β subunits of GDHt in several bacteria during enzyme evolution. This is confirmed by characterizing of GDHt expressed relying on fusion of α- and β-subunits[17]. The fusion protein GDHALB/C had the greatest catalytic activity.

Some researchers concentrate to comparison of glycerol dehydratases. GDHt and DDHt are isofunctional enzymes and undergo mechanism-based inactivation by a physiological substrate glycerol during catalysis. Inactivated holoenzymes are reactivated by their own reactivating factors that mediate the ATP-dependent exchange of an enzyme-bound, damaged cofactor for free adenosylcobalamin through intermediary formation of apoenzyme[18]. DDHt-reactivating factor cross-reactivates the inactivated GDHt, GDHt-reactivating factor did not cross-reactivate the inactivated DDHt. Interestingly, GDHt-based chimeric enzymes which carried N-terminal portions of the β and γ subunits of DDHt in the corresponding subunits of glycerol dehydratase was constructed, resulting change of solubility of the enzyme[6]. Recently, quantum mechanical/molecular mechanical calculations of GDHt catalysis considered the electrostatic coupling between the quantum-mechanical and molecular-mechanical subsystems and two alternative mechanisms, demonstrating the importance of electrostatic catalysis and hydrogen-bonding in enzyme-catalyzed radical reactions[19].

However, roles of GDHt and DDHt in 1,3-propanediol production was less studied. GDHt mutant *K. pneumoniae* was constructed via knocking out *dha*B-α. GDHt mutant isolate is used to study glycerol uptake and 1,3-propanediol synthesis in fed-batch cultures. The enzymatic analysis indicated both GDHt and DDHt were involved in the fermentation of glycerol. This is confirmed by the occurrence of 1,3-propanediol formation in GDHt-deficient mutant cultures.

Conclusion

This work attempts to understand GDHt's role in glycerol consumption and 1,3-propanediol synthesis with a GDHt-deficient mutant. GDHt-deficiency influence the activities of other two *dha* regulon coding enzyme, 1,3-propanediol oxidoreductase and glycerol dehydrogenase. 1,3-Propanediol oxidoreductase activity deceased by 62 % in cultures with mutant isolate, while the glycerol dehydrogenase activity increased by 36 %. This is accompanied by that more carbon is redistributed to lactate and ethanol formation. Cell growth and glycerol uptake were not influnced during the culture with GDHt-deficient isolate, suggesting *K. pneumoniae* metabolic pathway is flexible to adapt genetic perturbation. However, 1,3-propanediol and 2,3-butanediol production decreased by 56 and 75 %, respectively. Therefore, GDHt is not indispensable in glycerol metabolism but is crucial for 1,3-propanediol efficient production. Blocking of lactate and ethanol formation may be a plausible approach to promoting 1,3-propanediol production.

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REFERENCES

1. J. Kurian, *J. Polym. Environ*., **13**, 159 (2005).
2. M. Emptage, S.L. Haynie, L.A. Laffend, J.P. Pucci and G. Whited, Process for the Biological Production of 1,3-Propanediol with High Titer, US Patent 6514733 (2003).
3. A.P. Zeng, H. Biebl, H. Schlieker and W.D. Deckwer, *Enzym. Microbiol. Technol*., **15**, 770 (1993).
4. T. Toraya, *Cell. Mol. Life Sci*., **57**, 106 (2000).
5. R. Daniel, T.A. Bobik, G. and Gottschalk, *FEMS Microbiol. Rev*., **22**, 553 (1998).
6. T. Tobimatsu, T. Nishiki, M. Morimoto, R. Miyata and T. Toraya, *Arch. Microbiol*., **191**, 199 (2009).
7. J.R. O'Brien, C. Raynaud, C. Croux, L. Girbal, P. Soucaille and W.N. Lanzilotta, *Biochemistry*, **43**, 4635 (2004).
8. T. Sakai, A. Yamasaki, S. Toyofuku, T. Nishiki, M. Yunoki, N. Komoto, T. Tobimatsu and T. Toraya, *J. Nutr. Sci. Vitaminol*., **53**, 102 (2007).
9. V.L. Miller and J.J. Mekalanos, *J. Bacteriol*., **170**, 2575 (1988).
10. Z.M. Zheng, Y.Z. Xu, H.J. Liu, N.N. Guo, Z.Z. Cai and D.H. Liu, *Biotechnol. Bioeng*., **100**, 923 (2008).
11. S.J. Circle, L. Stone and C.S. Boruff, *Ind. Eng. Chem. Anal. Ed*., **17**, 259 (1945).
12. T. Toraya, K. Ohashi and S. Fukui, *Biochemistry*, **14**, 4255 (1975).
13. Y.Z. Xu, N.N. Guo, Z.M. Zheng, X.J. Ou, H.J. Liu and D.H. Liu, *Biotechnol. Bioeng*., **104**, 965 (2009).
14. Z.M. Zheng, K.K. Cheng, Q.L. Hu, H.J. Liu, N.N. Guo and D.H. Liu, *Biochem. Eng. J*., **39**, 305 (2008).
15. J. Hao, W. Wang, J. Tian, J. Li and D. Liu, *J. Ind. Microbiol. Biotechnol*., **35**, 735 (2008).
16. Y. Liu, A.A. Gallo, R.K. Bajpai, A. Chistoserdov, A.T. Nelson, L.N. Segura and W. Xu, *Int. J. Bioinf.. Res. Appl*., **6**, 484 (2010).
17. X.D. Wang, X.E. Zhang, Y.C. Guo, Z.P. Zhang, Z.A. Cao and Y.F. Zhou, *Biotechnol. Lett*., **31**, 711 (2009).
18. H. Kajiura, K. Mori, N. Shibata and T. Toraya, *FEBS J*., **274**, 5556 (2007).
19. Y. Liu, A.A. Gallo, J. Floria´n, Y.S. Liu, S. Mora and W. Xu, *J. Phys. Chem. B*, **114**, 5497 (2010).