Journal of Molecular Catalysis B: Enzymatic 97 (2013) 270–277

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Cloning, expression and characterization of a new enantioselective esterase from a marine bacterium *Pelagibacterium halotolerans* B2^T

Xiaolian Wei[a,1], Xiawei Jiang[b,1], Lidan Ye[a], Shenfeng Yuan[a], Zhirong Chen[a], Min Wu[c,**], Hongwei Yu[a,*]

[a] Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China
[b] State Key Laboratory for Diagnosis and Treatment of Infectious Disease, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China
[c] College of Life Sciences, Zhejiang University, Room 209, 866 Yuhangtang Road, Hangzhou 310058, China

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 26 August 2013
Accepted 2 September 2013
Available online 10 September 2013

Keywords:
Esterase
Marine
Enzyme biocatalysis
Methyl (*R*)-3-(4-fluorophenyl)glutarate
Pelagibacterium halotolerans B2^T

ABSTRACT

An esterase, designated as PE8 (219 aa, 23.19 kDa), was cloned from a marine bacterium *Pelagibacterium halotolerans* B2^T and overexpressed in *Escherichia coli* Rosetta, resulting an active, soluble protein which constituted 23.1% of the total cell protein content. Phylogenetic analysis of the protein showed it was a new member of family VI lipolytic enzymes. Biochemical characterization analysis showed that PE8 preferred short chain *p*-nitrophenyl esters (C2–C6), exhibited maximum activity toward *p*-nitrophenyl acetate, and was not a metalloenzyme. PE8 was an alkaline esterase with an optimal pH of 9.5 and an optimal temperature of 45 °C toward *p*-nitrophenyl acetate. Furthermore, it was found that PE8 exhibited activity and enantioselectivity in the synthesis of methyl (*R*)-3-(4-fluorophenyl)glutarate ((*R*)-3-MFG) from the prochiral dimethyl 3-(4-fluorophenyl)glutarate (3-DFG). (*R*)-3-MFG was obtained in 71.6% *ee* and 73.2% yield after 36 h reaction under optimized conditions (0.6 M phosphate buffer (pH 8.0) containing 17.5% 1,4-dioxane under 30 °C). In addition, PE8 was tolerant to extremely strong basic and high ionic strength solutions as it exhibited high activity even at pH 11.0 in 1 M phosphate buffer. Given its highly soluble expression, alkalitolerance, halotolerance and enantioselectivity, PE8 could be a promising candidate for the production of (*R*)-3-MFG in industry. The results also demonstrate the potential of the marine environment as a source of useful biocatalysts.

1. Introduction

Esterases (EC 3.1.1.1) are a class of versatile biocatalysts belonging to the family of hydrolase and are often applied in chemical synthesis processes due to their activity and stereospecificity toward chemicals and high stability in organic solvents [1]. They catalyze the cleavage and formation of ester bonds and are widely distributed in microorganisms from various environments [2]. Oceans constitute about 71% of the earth's surface. Such a huge habitat provides a vast pool of microorganisms, which become great donors of novel esterases. In addition, enzymes from marine bacteria may be expected to have unusual habitat-related properties such as salt tolerance, hyperthermostability, barophilicity, cold adaptivity, and often excellent region- and stereospecificity, which make them attractive for industrial application [3,4]. Furthermore, marine enzymes usually possess novel chemical and stereochemical characteristics, which increase their applicability in pharmaceutical and chemical fields [4].

Optically pure methyl (*R*)-3-(4-fluorophenyl)glutarate ((*R*)-3-MFG) is a pharmaceutically important precursor in the synthesis of the widely used antidepressant – (-)-paroxetine hydrochloride [5–8]. (*R*)-3-MFG can be synthesized from the prochiral dimethyl 3-(4-fluorophenyl)glutarate (3-DFG) by either enzymatic or non-enzymatic methods. The enzymatic desymmetrization of prochiral diester has been demonstrated to be an effective method for the synthesis of 3-substituted glutaric acid monoesters [5,8–10] (Table 1). The enantioselective aminolysis and ammonolysis of 3-DFG in organic solvent has been investigated in previous reports [9] with unsatisfactory yield, though high *ee* values were obtained. Yu et al. [5] employed porcine liver esterase (PLE) to conduct enantioselective hydrolysis of 3-DFG, which gave the undesired (*S*)-3-MFG in 95% *ee* and 86% yield. Recently, lipase B from *Candida antarctica*

Abbreviations: 3-DFG, dimethyl 3-(4-fluorophenyl)glutarate; (*R*)-3-MFG, methyl (*R*)-3-(4-fluorophenyl)glutarate; (*S*)-3-MFG, methyl (*S*)-3-(4-fluorophenyl)glutarate; 3-FGA, 3-(4-fluorophenyl)glutaric acid; IPE, isopropyl ether; MTBE, methyl tert-butyl ether; IPA, isopropanol; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide.

* Corresponding author. Fax: +86 571 8795 1873.
** Corresponding author. Fax: +86 571 8820 6261.
E-mail addresses: wumin@zju.edu.cn (M. Wu), yuhongwei@zju.edu.cn (H. Yu).
[1] These authors contributed equally to this work.

1381-1177/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.molcatb.2013.09.002

Table 1
Enantioselective hydrolysis of 3-DFG using different enzymes.

Enzyme	Yield (%)	*ee* (%)	Configuration	Major advantages	Major drawbacks	References
Porcine liver esterase (PLE)	86	95	(*S*)	Relatively high *ee* value	Undesired configuration(*S*);high cost of PLE	[5]
Lipase B from *Candida antarctica* (Novozym 435)	92.6	95.6	(*R*)0	Relatively high *ee* value and conversion	Long reaction time (64 h); high cost of the commercial enzyme; required large amount of the enzyme (20 g/l)	[8]
Esterase PE8 from *Pelagibacterium halotolerans* B2T	72.3	71.6	(*R*)	Highly soluble expression, easy preparation and low cost of the enzyme; short reaction time (36 h)	Moderate *ee* value and yield; should be further engineered to make it more suitable for industrial applications	Current study

(Novozym 435) was used in the preparation of (*R*)-3-MFG with high *ee* value and yield [8], but was not suitable for industrial application due to its high cost. The increasing demand of the pharmaceutical industry for the production of (-)-paroxetine hydrochloride requires new and efficient hydrolases for the preparation of (*R*)-3-MFG.

In the present study, an esterase (PE8) from the marine bacteria *Pelagibacterium halotolerans* $B2^T$ isolated from East China Sea [11] was cloned, heterologously expressed in *Escherichia coli* Rosetta in soluble forms and biochemically characterized. We also attempted to apply PE8 in the enantioselective hydrolysis of 3-DFG in an aqueous-organic phase (Scheme 1). Moreover, effects of various reaction conditions on the activity and enantioselectivity of PE8 were investigated and optimized.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Pelagibacterium halotolerans $B2^T$ was previously isolated by our lab [11], and cultivated in marine 2216 broth (BD, USA) at 30 °C. The *E. coli* strains DH5α and plasmid pET28b (+) (Novagen, Germany) were used as the host and vector, respectively, for cloning the esterase, and the plasmid pET28b (+) was used for gene cloning and sequencing. *E. coli* Rosetta (DE3) (Novagen, Germany) and pET28b (+) were used as the host and vector, respectively, for heterologous expression of the esterase. *E. coli* cells were grown in Luria-Bertani (LB) medium containing 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl at pH 7.2. Chloramphenicol (34 μg/ml) and/or kanamycin (20 μg/ml) was added to the medium when needed.

2.2. Reagents

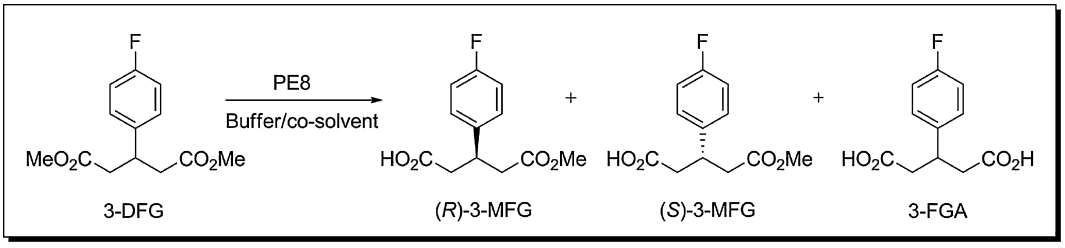
Restriction endonucleases, PrimeSTAR™ HS DNA Polymerase and T4 DNA ligase were purchased from Takara, China. Genomic DNA Purification Kit was obtained from Dongsheng, China. Gel extraction kit and Plasmid Miniprep kit were obtained from Axygen, China. Primers were synthesized by Invitrogen, China. Ni–NTA resins were purchased from GE Healthcare Bio-Science AB, Sweden. The *p*-nitrophenyl hexanoate was purchased from TCI, Japan, while other *p*-nitrophenyl esters and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Sigma. Isopropanol, n-hexane and trifluoroacetic acid were of HPLC grade. All other chemicals were also commercially available, with purity of analytical grade. 3-DFG (HPLC purity >99%) was synthesized as described previously [12].

2.3. Sequence analysis

A gene coding a putative esterase (PE8) was identified from the genome sequence of *Pelagibacterium halotolerans* $B2^T$ [13]. The similarity of deduced protein sequence was analyzed using the BLASTP program (http://blast.ncbi.nlm.nih.gov/). Phylogenetic and molecular evolutionary analysis were conducted using MEGA software version 5 [14].

2.4. Cloning, expression and purification of recombinant esterase

Genomic DNA was extracted from *Pelagibacterium halotolerans* $B2^T$ using genomic DNA Purification Kit described above. The gene encoding PE8 was amplified by PCR using PrimeSTAR™ HS DNA Polymerase with the following primers: PE8F-*Nde I*: 5′-AGGA*CATATG*ACCGAACCCGTAAAG-3′ and PE8R-*Hind III*: 5′-CGAT*AAGCTT*CTAGAGGATCTCGCG-3′. The digested PCR fragment was inserted between the *Nde* I and *Hin*d III sites of the pET-28b (+) vector, creating pET-28a-PE8, which was then transformed into chemically competent *E. coli* Rosetta (DE3) following standard protocols. Chloramphenicol (34 μg/ml) and kanamycin (20 μg/ml) were used as the selective pressure. Protein expression was induced in *E. coli* Rosetta cells carrying the pET-28a-PE8 vector by addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) to the culture when OD_{600} reached 0.6 followed by overnight cultivation at 25 °C. The cells were harvested by centrifugation at 4000 × *g*, 4 °C and washed twice in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.4). The cell pellet was then resuspended in Tris–HCl buffer (pH 8.0) and ruptured by ultrasonication. Cell debris was removed by centrifugation at 12,000 × *g* and 4 °C for 20 min, and the crude extract containing PE8 was purified by Ni-NTA affinity chromatography column following the



Scheme 1. Enzymatic enantioselective hydrolysis of 3-DFG in an aqueous-organic phase using PE8 as catalyst.

manufacturer's instructions. The elute obtained was dialyzed twice against 0.1 M Tris–HCl buffer (pH 8.0) before the determination of purity and apparent molecular mass by 12% SDS-PAGE. The relative quantities of the protein bands on the gels were analyzed by gel documentation unit assay (BioRad, USA).

2.5. Enzyme assays

Activity was measured using *p*-nitrophenyl acetate (C2) or other p-nitrophenyl esters (pNPEs) as substrates. The standard reaction mixture contained 1 mM substrate (dissolved in acetonitrile), 100 mM Tris–HCl buffer and the appropriate amount of enzyme in a total volume of 1 ml [15]. Unless otherwise specified, the enzyme reaction was performed for 3 min at 30 °C and the release of *p*-nitrophenol was measured at 405 nm using a UV–Visible spectrophotometer (WFZ-UV2800H, China). All samples were measured in triplicate and corrected for the autohydrolysis of the substrate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from *p*-nitrophenyl ester per minute. The substrate specificity was analyzed with the following *p*-nitrophenyl derivatives: acetate (C2), butyrate (C4), hexanoate (C6), octanoate (C8), decanoate (C10), laurate (C12), myristate (C14), and palmitate (C16). The effects of metal ions (Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Sr^{2+}, Mn^{2+}, Ni^{2+}, Ba^{2+}) and EDTA on the activity of PE8 were examined at a final concentration of 10 mM using *p*-nitrophenol acetate as substrate at 30 °C and pH 7.5. The optimal temperature for esterase activity was determined by measuring enzyme activity at temperatures ranging from 0 to 70 °C using *p*-NP acetate as substrate following the same protocol. The optimal pH was measured at 45 °C using *p*-NP octanoate as substrate over a pH range of 5.0–12.0. In this experiment, *p*-NP octanoate was used due to its high stability at alkaline pHs, at which pHs p-NP acetate was rapidly auto-hydrolyzed. The Michaelis-Menten constant (K_m), maximum velocity (V_{max}) and the turnover number (k_{cat}) were calculated by Lineweaver–Burk plot. Enzyme assay was carried out under optimal conditions with varying *p*-NP acetate concentrations (0.1–3 mM).

2.6. Enantioselective hydrolysis of 3-DFG using PE8

Enzymatic hydrolysis was performed in a 1.5 ml reaction vessel by suspending 3-DFG (40 mM) in phosphate buffer containing different amounts of organic co-solvents (v/v). The reaction mixture (0.5 ml) containing 5 mg of lyophilized crude PE8 (crPE8) were orbitally shaken at 20–40 °C, 200 rpm for 24 h. After that, the reaction was terminated by adjusting the pH to 2.0 with 5 M HCl and extracted with ethyl acetate (2 ml × 0.5 ml). Ethyl acetate was removed through vacuum drying and isopropanol (300 μl) was added to dissolve the residues, then the extracts were analyzed by high performance liquid chromatography (HPLC). All measurements were conducted in triplicate.

2.7. Product analysis

The determination of conversion, *ee*, and yield was performed by chiral HPLC analysis carried out on a HPLC system (Agilent 1100 Series) equipped with a UV detector and a system controller. Samples were run using a mobile phase of n-hexane (containing 0.1% trifluoroacetic acid) and isopropanol (95:5, v/v) at a flow rate of 0.5 ml/min on a Chiralpak AD-H column (250 mm × 4.6 mm) at 30 °C. The peaks were monitored at 266 nm, and the samples were analyzed in triplicate. The retention time of 3-DFG, (*R*)-3-MFG, (*S*)-3-MFG and 3-FGA were 19.4 min, 37.9 min, 43.9 min and 51.6 min, respectively. The conversion was calculated as follows: conversion = (A1 − A2)/A1, where A1 and A2 were the HPLC peak areas of 3-DFG before and after reaction. The *ee* values and yield were calculated according to methods previously reported [12].

2.8. Nucleotide sequence accession number

The nucleotide sequence of PE8 from *Pelagibacterium halotolerans* $B2^T$ was deposited in GenBank under accession number of KF544956.

3. Results and discussion

3.1. Sequence analysis

A putative ORF of 660 bp, encoding an esterase of 219 amino acids (PE8) with a theoretical molecular mass of 23.19 kDa and a deduced pI of 4.73 (http://web.expasy.org/protparam/) was identified from the genome sequence of *Pelagibacterium halotolerans* $B2^T$. BLASTP of the translated protein sequence showed maximum identity (46%) with the esterase from *Parvibaculum lavamentivorans* DS-1.

According to the phylogenetic analysis, PE8 and its closest relative protein could be classified into family VI esterases (Fig. 1). This family of esterases showed the smallest molecular mass among all esterases, in the range of 23 ± 26 kDa [2]. Due to the presence of the major conserved sequence motifs GFSQG including catalytic serine, PE8 was identified as a new member of family VI esterases.

3.2. Purification and characterization of PE8

To eliminate the possible influence of the remaining imidazole for the enzymatic assay, the enzyme was dialyzed after elution from the Ni-NTA affinity chromatography column. PE8 was 2.8-fold purified, with a specific activity of 48.4 U/mg and a yield of 93.8% (Table 2). The K_m, V_{max} and k_{cat} values for *p*-NP acetate were 0.83 mM, 288 μM/min, and 9.21 s^{-1}, respectively.

The purified PE8 was tested for lipase/esterase activity toward *p*-nitrophenyl esters with various acyl chain lengths (C2–C16). As shown in Fig. 2a, PE8 preferred short-chain *p*-nitrophenyl esters and the highest activity was obtained in the hydrolysis of *p*-nitrophenyl acetate (C2). However, the enzyme exhibited poor activities toward middle-chain and long-chain *p*-nitrophenyl esters. More specifically, no significant enzyme activity was observed for the substrates with a chain length ≥ C_{10}. Consequently, the enzyme can be classified as an esterase rather than a lipase.

Many hydrolases are known to require metal ions [16,17]. As shown in Fig. 2b, PE8 was stable in the presence of many metal ions, and it could retain more than 50% of activity with most of the ions except for Ni^{2+}, Zn^{2+} and Cu^{2+}. Moreover, the enzyme activity was totally inhibited by 10 mM $Zn^{2+}$and Cu^{2+}. The chelating agent EDTA had no obvious inhibition on enzyme activity, which indicated this esterase was not a metalloenzyme [17].

The activity of PE8 was measured over a temperature range of 0–70 °C and a pH range of 5.0–12.0 with *p*-nitrophenyl acetate or *p*-nitrophenyl octanoate as substrates. As shown in Fig. 2c, the optimal temperature for the PE8 activity was 45 °C, similar to that of another esterase (PE10) isolated from *Pelagibacterium halotolerans* $B2^T$ [18]. PE8 showed activity also at low temperatures. It can retain 27% relative activity at 4 °C, which might be related to the low temperature of the sea water, where *Pelagibacterium halotolerans* $B2^T$ was isolated [11]. Moreover, 53.6% of its maximum activity remained at 65 °C, making it feasible for a broad range of industrial applications. As shown in Fig. 2d, PE8 is an alkaline esterase with maximum activity toward *p*-nitrophenyl octanoate at pH 9.5, and still exhibited esterase activity at pH 11, which makes PE8 an attractive catalyst for future applications in industry.

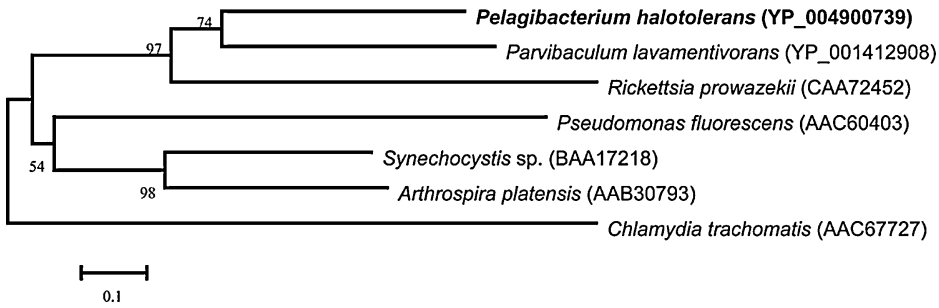


Fig. 1. Phylogenetic tree of PE8 from *Pelagibacterium halotolerans* B2^T (bold type) and other members of family VI lipolytic enzymes. The neighbor-joining tree was constructed using MAGE software. Bootstrap values were based on 1000 replications. Scale bar, 0.1 substitutions per amino acid position.

Table 2
Purification of the recombinant esterase PE8 after expression in *E. coli* Rosseta (DE3).

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
PE8 (crude extract)	9.23	161.36	17.48	100.00	1.00
PE8 (Ni-NTA)	3.13	151.38	48.43	93.81	2.77

3.3. Enantioselective hydrolysis of 3-DFG using PE8

Methyl (*R*)-3-MFG is a pharmaceutically important precursor, which has applications in the synthesis of the widely used antidepressant – (-)-paroxetine hydrochloride. Due to the excellent properties of PE8, the enzyme was tested for its activity in enantioselective hydrolysis of 3-DFG for the synthesis of methyl (*R*)-3-MFG. It turned out the enzyme had activity and enantioselectivity in 3-DFG hydrolysis, yielding products with a moderate *ee* value. To obtain a satisfactory *ee* value and conversion rate for industry applications, the reaction conditions were optimized.

3.3.1. Screening of organic co-solvents

Due to the poor solubility of 3-DFG in aqueous medium, a suitable organic solvent was required in which the substrate would be properly dissolved, meanwhile, the required activity and

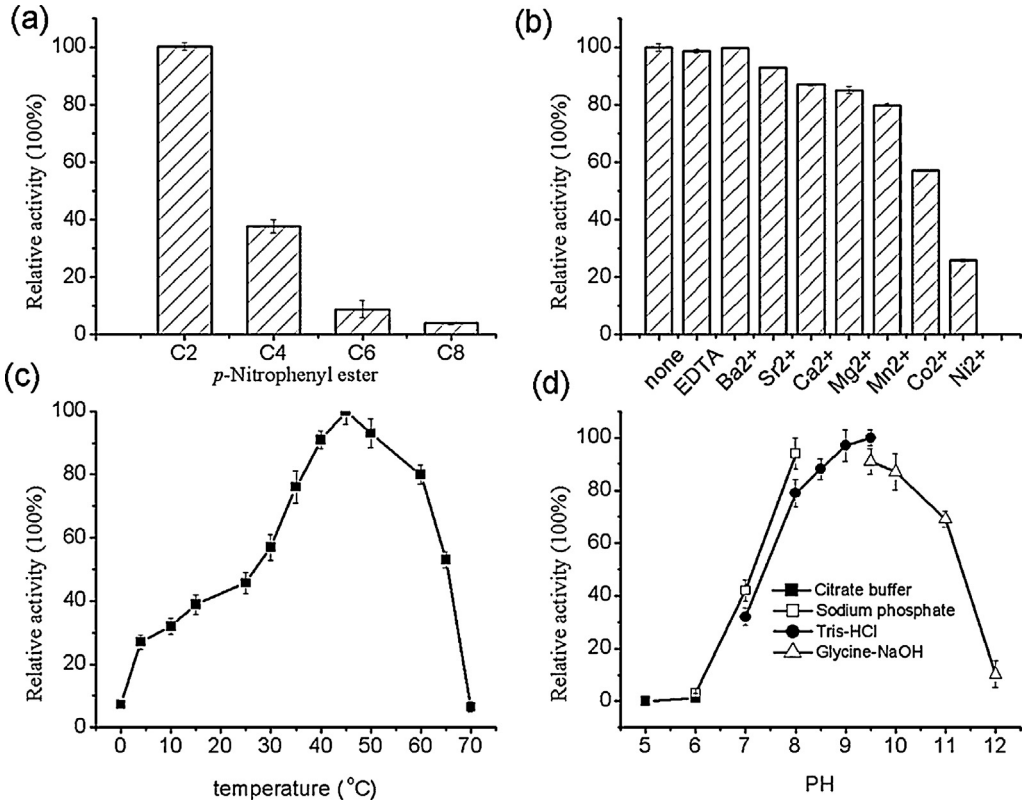


Fig. 2. Biochemical properties of PE8. (a) Substrate specificity of PE8. The esterase activity of the purified recombinant enzyme PE8 toward various chain lengths of *p*-NP esters was assayed at 30 °C with a pH 7.5. The highest level of activity with the C2 substrate was set as 100%. (b) Effect of metal ions (10 mM) on PE8 activity. Enzymatic assay was performed at 30 °C in 100 mM Tris–HCl buffer (pH7.5) with *p*-nitrophenol acetate as a substrate. The value obtained with no additives in the reaction mixture was taken as 100%. (c) Effect of temperature on PE8 activity. The activity was measured in the temperature range of 0–70 °C in 100 mM Tris–HCl buffer (pH 7.5) with *p*-nitrophenol acetate as substrate. The value obtained at 45 °C was taken as 100%. (d) Effect of pH on PE8 activity. The activity was determined at 45 °C over a pH range from 5.0 to 12.0 using *p*-nitrophenol octanoate as substrate. Buffers used were: 100 mM Citrate buffer (pH 5.0–6.0); 100 mM sodium phosphate buffer (pH 6.0–8.0); 100 mM Tris–HCl buffer (pH 7.0–9.5), and 100 mM glycine–NaOH buffer (pH 9.5–12). The values obtained using Tris–HCl at pH 9.5 was taken as 100%.

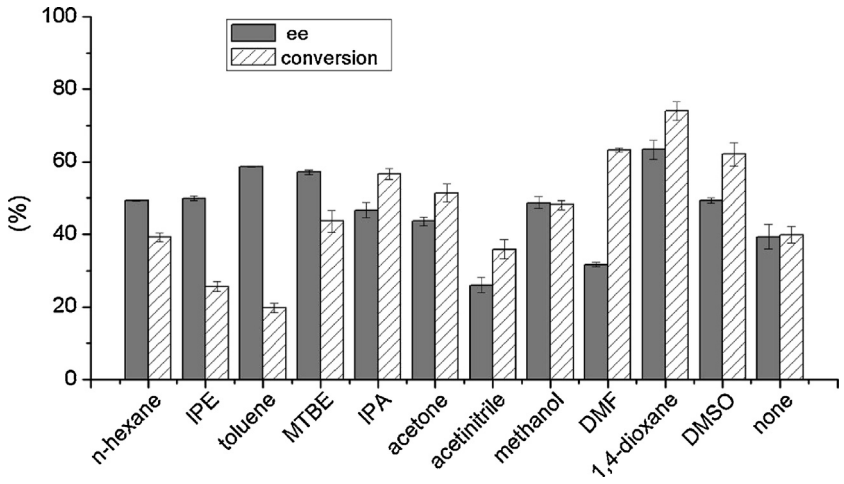


Fig. 3. Effect of co-solvents (10%, v/v) on enantioselective hydrolysis of 3-DFG. Reaction conditions: 5 mg crude PE8 (crPE8), 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (0.1 M, pH 8.0), containing 10% (v/v) various co-solvents, 200 rpm, 30 °C, 24 h.

enantioselectivity of the enzyme would not be negatively affected. The effect of different organic solvents on the esterase-catalyzed synthesis of (*R*)-3-MFG was investigated (Fig. 3). Hydrolysis was performed in various co-solvents at a concentration of 10% (v/v). The highest conversion (74%) and *ee* values (63%) were obtained after 24 h at 30 °C when 1,4-dioxane was used as co-solvent. In the absence of any organic co-solvent, the conversion and *ee* values were only 39% and 40%, respectively. The results demonstrate that the modification of a reaction medium by adding an appropriate organic co-solvent could improve the catalytic activity and enantioselectivity of an enzyme. The same phenomena have been reported for other esterases and lipases in previous studies [19–24]. The lowest conversion (25.6%) was determined in isopropyl ether. The results also demonstrate a correlation between the activity of PE8 and polarity of organic solvents. A good measure of polarity of an organic solvent is its log P value, defined as the logarithm of its partition coefficient in the standard n-octane/water two-phase system [25]. Furthermore, log *P* has been the most commonly used to describe the solvent's effect on the activity and/or stability of enzymes [26]. The activity of PE8 was profoundly enhanced in highly polar, water-miscible solvents such as DMSO (log $P = -1.3$), 1,4-dioxane (log $P = -1.1$) and DMF (log $P = -1.0$), as compared to the phosphate buffer without co-solvent. In contrast, the addition of water-immiscible solvents with low polarity, such as n-hexane (log $P = 3.1$), isopropyl ether (log $P = 2.2$) and toluene (log $P = 2.0$) greatly reduced the activity. This might be attributed to the fact that water plays an indispensable part in the hydrolysis of 3-DFG. The use of water-miscible solvents could increase the accessibility of water, thus enhancing the conversion of 3-DFG. These results indicate that the property of the solvent could significantly affect the catalytic efficiency of an enzyme. However, there is still no general understanding between the physicochemical properties of the solvent and the catalytic efficiency of the enzyme due to the diversity of substrates and enzymes used [26,27]. Therefore, taking both the *ee* value and conversion into consideration, in the subsequent experiments, 1,4-dioxane was selected as co-solvent for the enantioselective hydrolysis of 3-DFG.

3.3.2. Selection of co-solvent concentration

It is well known that an ideal biocatalyst needs to be fully enantioselective, producing the desired monoester without hydrolyzing the first product of the reaction. The final yield of (*R*)-3-MFG will depend on the activity, enantioselectivity (to produce mainly one enantiomer of the monoester) and the enantiospecificity (to hydrolyze the undesired (*S*)-3-MFG) of PE8. In our enzymatic reaction, three products were detected, namely, (*R*)-3-MFG, (*S*)-3-MFG, and 3-FGA. Therefore, in the following optimization, we will discuss the effects of various reaction parameters on the activity (conversion), enantioselectivity (*ee*) and enantiospecificity (yield of 3-FGA) during the hydrolysis of 3-DFG. Considering the fact that enzyme activity and enantioselectivity are profoundly influenced by the concentration of co-solvent [28,29], the influence of 1,4-dioxane concentration on the *ee* value of (*R*)-3-MFG and conversion of 3-DFG was investigated (Fig. 4a). When the concentration of 1,4-dioxane was relatively low ($\leq$17.5%, v/v), both the *ee* value and conversion increased with the addition of 1,4-dioxane, probably due to the increased solubility of 3-DFG caused by the addition of co-solvent. However, an excess of 1,4-dioxane resulted in the decrease of *ee* value as well as conversion. This may be attributed to the fact that high concentration of organic solvent molecules may alter the enzyme conformation by penetrating into the active center of the enzyme and consequently change the enzyme performance [30]. Taking both the *ee* value and conversion into consideration, although using 17.5% 1,4-dioxane, the *ee* value (66.8%) was by 0.26% lower than that using 15% 1,4-dioxane (67.1%), its conversion (98.9%) was much higher than that of 15% 1,4-dioxane (82.9%). Therefore, 17.5% was chosen as the optimal co-solvent concentration.

3.3.3. Effect of buffer pH

The efficiency of an enzyme is greatly influenced by the pH value of its surroundings [31,32]. The variation of pH could alter the ionic state of the substrate and the stereochemical configuration of enzyme in the neighborhood of active sites, which in turn influences the enzymatic activity and enantioselectivity [33,34]. The effect of buffer pH on the activity and enantioselectivity of PE8 toward 3-DFG was investigated using different buffered solutions with pH ranging from 4.0 to 12.0. As shown in Fig. 4b, the conversion was extremely low in acidic medium, with less than 20% conversion at pH 4.0, 5.0, and 6.0. The optimal pH for 3-DFG hydrolysis was 10, which is a little higher than that for *p*-nitrophenyl octanoate hydrolysis (pH 9.5). Nevertheless, the *ee* value at pH 8 (66.8%, sodium phosphate buffer) was much higher than that of pH 10 (59.2%, glycine–NaOH buffer). Considering the similar conversion (both ~98%) at the two pH values and the higher *ee* value at

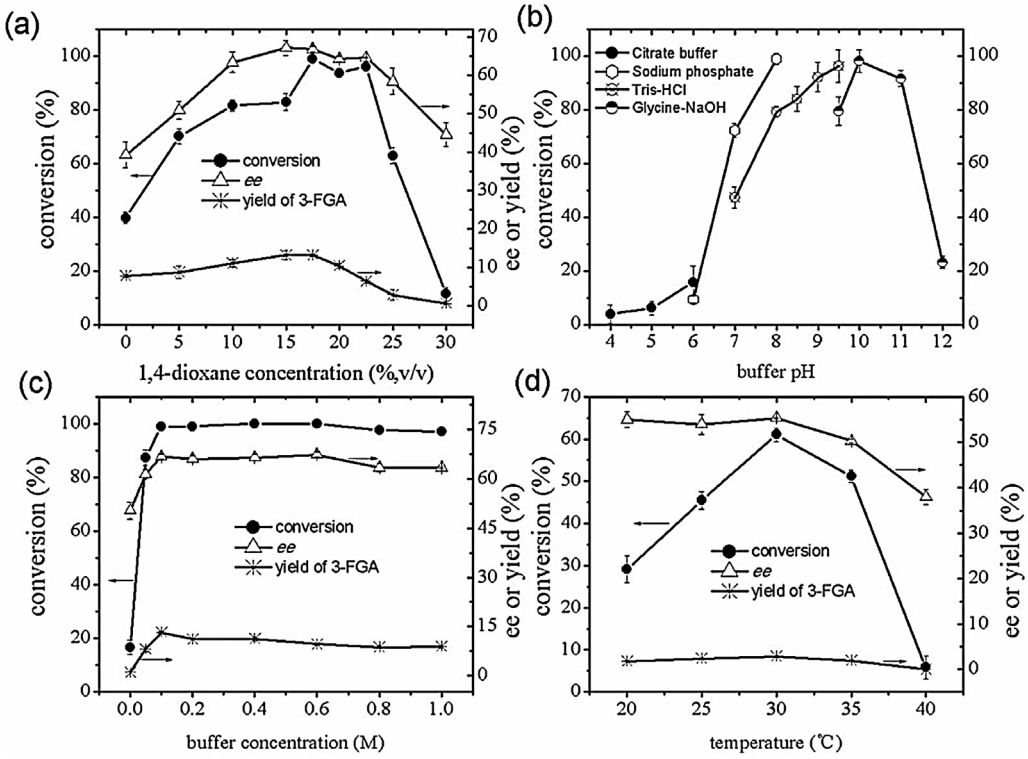


Fig. 4. Effect of 1,4-dioxane concentration, buffer pH, buffer concentration, and temperature on enantioselective hydrolysis of 3-DFG. (a) Effect of 1,4-dioxane concentration. Reaction conditions: 5 mg crPE8, 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (0.1 M, pH 8.0) containing different amount of 1,4-dioxane, 200 rpm, 30 °C, 24 h. (b) Effect of buffer pH. Reaction conditions: 5 mg crPE8, 0.02 mmol 3-DFG in 0.5 ml buffer (0.1 M) containing 17.5% (v/v) 1,4-dioxane with various pH values, 200 rpm, 30 °C, 24 h. Buffers used were: 100 mM Citrate buffer (pH 4.0–6.0); 100 mM sodium phosphate buffer (pH 6.0–8.0); 100 mM Tris–HCl buffer (pH 7.0–9.5), and 100 mM glycine–NaOH buffer (pH 9.5–12). (c) Effect of buffer concentration. Reaction conditions: 5 mg crPE8, 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (pH 8.0) containing 17.5% (v/v) 1,4-dioxane with different ionic strength, 200 rpm, 30 °C, 24 h. (d) Effect of temperature. Reaction conditions: 5 mg crPE8, 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (pH 8.0, 0.6 M) containing 17.5% (v/v) 1,4-dioxane, 200 rpm, 20 °C-40 °C, 24 h. Conversion (solid circle); *ee* values (empty triangle); yield of 3-FGA (star). Conversion (left *Y* axis); *ee* and yield of 3-FGA (right *Y* axis), indicated by the arrows beside the curves.

pH 8, pH 8 (sodium phosphate buffer) was selected as the optimal pH condition for this hydrolytic reaction.

3.3.4. Effect of ionic strength

Ionic strength is another parameter that might affect the activity and enantioselectivity of enzymes [35,36]. Therefore, the effect of buffer concentration (0–1 M) was tested at pH 8.0 (Fig. 4c). Both *ee* value and conversion increased sharply from 50.4% to 66.8% and 16.6% to 98.9%, respectively as the buffer concentration was increased from 0 to 0.1 M. With further increase of buffer concentration, the *ee* value was first leveled off and then decreased a little when the concentration exceeded 0.6 M. The highest *ee* value (67.4%) was achieved at 0.6 M. Conversion showed the same trend as *ee* value, while the yield of the undesired 3-FGA exhibited a different trend with the highest value of 13.2% observed at 0.1 M and the decreasing of the value with the further increasing of the buffer concentration. The results demonstrate that ionic strength plays a crucial role in activity and enantioselectivity of PE8. Similar

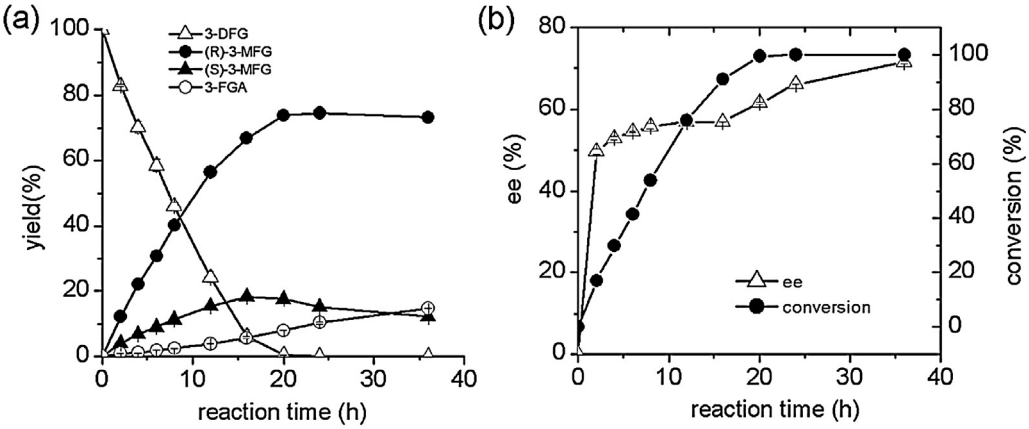


Fig. 5. Reaction time courses in the enantioselective hydrolysis of 3-DFG. Reaction conditions: 5 mg crPE8, 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (pH 8.0, 0.6 M) containing 17.5% (v/v) 1,4-dioxane, 200 rpm, 30 °C. (a) Developing of the yield of 3-DFG; (R)-3-MFG; (S)-3-MFG; and 3-FGA during the hydrolysis of 3-DFG. (b) Developing of *ee* value and conversion during the hydrolysis of 3-DFG.

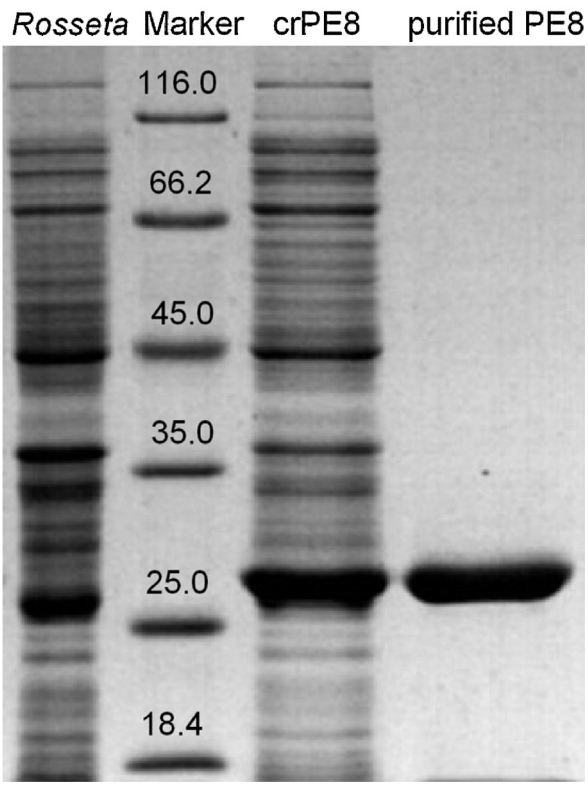


Fig. 6. SDS-PAGE of Rosseta (DE3), crPE8 and purified PE8. Lane 1, Rosseta (DE3) grown overnight at 37 °C; lane 2, marker (beta-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), beta-lactoglobulin (18.4 kDa)); lane 3, 5 mg crPE8 enzyme powder dissolved in 1 ml phosphate buffer (pH 8.0); lane 4, 2 mg purified PE8 powder dissolved in phosphate buffer (pH 8.0).

to most halophilic enzymes from halophilic archaea, which are inactive at low ionic concentrations [37], PE8 also exhibited low activity under low ionic strength. Furthermore, PE8 was stable in high ionic strength buffers. Since PE8 was cloned from *Pelagibacterium haoltolerants* B2^T, which was isolated from sea water, it is capable of growing in the presence of 0–13% (0–2.22 M) NaCl [11], so it is reasonable that its enzymes have significant halotolerance. Halophilic archaea established an osmotic balance with their high salt environment by maintaining internal levels of salt that are isotonic with the exterior [38]. Therefore it could be understood that relatively high level of ionic strength should be more favorable for the enzymes from the halophilic archaea.

3.3.5. Effect of reaction temperature

In enzyme-catalyzed reactions, temperature also has a significant impact on the activity, enantioselectivity and stability of a biocatalyst and the thermodynamic equilibrium of the catalyzed reaction [39]. To investigate the effect of temperature on the activity and enantioselectivity of PE8 for 3-DFG, five different temperatures were employed, ranging from 20 °C to 40 °C (Fig. 4d). It was observed that the conversion first increased with temperature, plateaued of 100% at 30 °C after 24 h, and then decreased rapidly with further elevation of temperature. Similar results were also found in the production of (*S*)-1-phenyl-1,2-ethanediol using a *Bacillus subtilis* esterase as catalyst [33]. The highest *ee* value was also achieved at 30 °C. It is noteworthy that the optimal temperature of PE8 for 3-DFG hydrolysis (30 °C) was much lower than that for *p*-NP acetate hydrolysis (45 °C). PE8 exhibited poor activity toward 3-DFG at 40 °C. It is well known that the temperature optimum of an enzyme differs for different substrates. Since 3-DFG has much larger steric hindrance than *p*-NP acetate, the poor activity for 3-DFG hydrolysis at elevated temperatures might be caused by the decreased size of the binding pocket of PE8 at relatively high temperatures. Furthermore, the enantioselectivity was lower at relatively high temperature. This phenomenon has been reported in a number of cases and a rational understanding of this phenomenon has also been proposed [8,40,41]. Taking above into consideration, 30 °C was chosen as the optimal temperature for further reactions.

3.3.6. Time course of yield, ee and conversion in the hydrolysis of 3-DFG

A time course of the reaction was monitored under the optimized conditions. Fig. 5a showed the evolution of yield of all products during the hydrolytic reaction. At beginning, the enzyme mainly hydrolyzed 3-DFG, producing (*R*)-3-MFG much more rapidly than (*S*)-3-MFG. However, after depletion of the diester, the enzyme tended to utilize the monoester, hydrolyzing the minority (*S*)-3-MFG. As shown in Fig. 5, *ee* value increased from 56.8% (16 h) to 71.6% (36 h) while the yield of (*R*)-3-MFG decreased from 74.5% to 73.2%. Although extending reaction time lowered the yield of (*R*)-3-MFG, it enabled the enzyme to generate a product with higher *ee*.

As shown in Fig. 5, it is apparent that both the yield and *ee* were time-dependent. The 24 h time point was selected to calculate the conversion yield and *ee* by taking into consideration the optimal conditions for the organic co-solvent, co-solvent concentration, buffer pH, buffer concentration, and the temperature. The activity of the enzyme was selected as the first metric for optimizing the reaction conditions. Therefore, after 24 h of reaction, those conditions with low conversion were excluded. When the conversion rates were approximately the same (~90%), reaction conditions were optimized based on the more favorable *ee* value.

3.3.7. Effect of protein purification

To determine whether purification could affect the enantioselective hydrolysis of 3-DFG, the crude PE8 (crPE8) was further purified by Ni-NTA affinity chromatography column (Fig. 6), and both the crPE8 and the purified PE8 were used in the hydrolysis of 3-DFG. As shown in Table 3, the *ee* value and the conversion

Table 3
Effect of purification on enantioselective hydrolysis of 3-DFG.

	Yield (%)				*ee* (%)	Conversion (%)
	3-DFG	(*R*)-3-MFG	(*S*)-3-MFG	3-FGA		
Before purification[a]	1.1 ± 0.2	71.4 ± 0.4	14.2 ± 0.3	13.2 ± 0.3	66.8% ± 0.7	98.9 ± 0.2
After purification[b]	1.2 ± 0.1	74.4 ± 0.9	14.9 ± 0.1	9.4 ± 0.7	66.6% ± 0.6	98.8 ± 0.1

Results are presented as means ± standard deviation (n = 3).

[a] Reaction conditions: 5 mg crPE8 powder (containing 0.487 mg PE8), 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (pH 8.0, 0.1 M) containing 17.5% (v/v) 1,4-dioxane, 200 rpm, 30 °C, 24 h.

[b] Reaction conditions: 1.1 mg purified PE8 (containing 0.487 mg PE8), 0.02 mmol 3-DFG in 0.5 ml phosphate buffer (pH 8.0, 0.1 M) containing 17.5% (v/v) 1,4-dioxane, 200 rpm, 30 °C, 24 h.

showed no significant difference between the two. Therefore, crPE8 could be directly used for biocatalytic reaction without further purification.

4. Conclusion

In this study, an esterase gene (*pe*8) from the marine bacterium *Pelagibacterium halotolerans* B2^T was successfully cloned and expressed in *E. coli* Rosseta (DE3). PE8 was assigned into family VI of lipolytic enzymes based on the phylogenetic analysis. A substrate specificity study showed that PE8 preferred short chain *p*-nitrophenyl esters, and exhibited maximum activity toward *p*-nitrophenyl acetate. PE8 was stable under a variety of metal ions, and was found not to be a metalloenzyme. Biochemical characterization of PE8 showed that it was an alkaline esterase with an optimal pH of 9.5 and an optimal temperature of 45 °C toward *p*-nitrophenyl acetate. Also it exhibited high activity and stability under highly alkaline conditions and in high salt solvents. (*R*)-3-MFG was obtained in 71.6% *ee* and 73.2% yield after a 36 h reaction under optimized conditions (0.6 M phosphate buffer (pH 8.0) containing 17.5% 1, 4-dioxane under 30 °C). To the best of our knowledge, PE8 is the first marine-derived esterase reported as a potential biocatalyst for the production of (-)-paroxetine. Nevertheless, the *ee* value was still not satisfactory for fine chemistry. In the future, PE8 will be further engineered to make it more suitable for industrial applications. Finally, the results also demonstrate the potential of marine environment as a source of useful biocatalysts.

Acknowledgements

This work is financially supported by National High-tech R&D Program (2011AA02A209), China National Natural Science Foundation of China (21176215/21176102), the outstanding Young Scholar of Zhejiang Province (R4110092), and the Program of Zhejiang Leading Team of S&T innovation (2011R5007). We are grateful for all editors and reviewers for their helpful advice. And we thank all the other member of Prof. Yu's group and Prof. Wu's group.

References

[1] U.T. Bornscheuer, FEMS Microbiol. Rev. 26 (2006) 73–81.
[2] J.L. Arpigny, K.E. Jaeger, Biochem. J. 343 (1999) 177.
[3] B. Sana, D. Ghosh, M. Saha, J. Mukherjee, Process Biochem. 42 (2007) 1571–1578.
[4] A. Trincone, Recent Pat. Biotechnol. 6 (2012) 134–148.
[5] M.S. Yu, I. Lantos, Z.Q. Peng, J. Yu, T. Cacchio, Tetrahedron Lett. 41 (2000) 5647–5651.
[6] L.T. Liu, P.-C. Hong, H.-L. Huang, S.-F. Chen, C.-L.J. Wang, Y.-S. Wen, Tetrahedron: Asymmetry 12 (2001) 419–426.
[7] C. De Risi, G. Fanton, G.P. Pollini, C. Trapella, F. Valente, V. Zanirato, Tetrahedron: Asymmetry 19 (2008) 131–155.
[8] W.M. Liu, Y. Hu, L. Jiang, B. Zou, H. Huang, Process Biochem. 47 (2012) 1037–1041.
[9] M. Lopez-Garcia, I. Alfonso, V. Gotor, Tetrahedron: Asymmetry 14 (2003) 603–609.
[10] Z. Cabrera, G. Fernandez-Lorente, J.M. Palomo, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microbiol. Technol. 43 (2008) 531–536.
[11] X.W. Xu, Y.Y. Huo, C.S. Wang, A. Oren, H.L. Cui, E. Vedler, M. Wu, Int. J. Syst. Evol. Microbiol. 61 (2011) 1817–1822.
[12] J. Perregaard, E.K. Moltzen, E. Meier, C. Sanchez, J. Med. Chem. 38 (1995) 1998–2008.
[13] Y.-Y. Huo, H. Cheng, X.-F. Han, X.-W. Jiang, C. Sun, X.-Q. Zhang, X.-F. Zhu, Y.-F. Liu, P.-F. Li, P.-X. Ni, J. Bacteriol. 194 (2012) 197–198.
[14] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, Mol. Biol. Evol. 28 (2011) 2731–2739.
[15] H.-J. Park, J.H. Jeon, S.G. Kang, J.-H. Lee, S.-A. Lee, H.-K. Kim, Protein Exp. Purif. 52 (2007) 340–347.
[16] R.B. Labuschagne, A. vanTonder, D. Litthauer, Enzyme Microb. Technol. 21 (1997) 52–58.
[17] L.J. Yu, Y. Xu, X.W. Yu, J. Mol. Catal. B: Enzym. 57 (2009) 27–33.
[18] X.W. Jiang, Y.Y. Huo, H. Cheng, X.Q. Zhang, X.F. Zhu, M. Wu, Extremophiles 16 (2012) 427–435.
[19] P.A. Fitzpatrick, A.M. Klibanov, J. Am. Chem. Soc. 113 (1991) 3166–3171.
[20] R.A. Wahab, M. Basri, M.B.A. Rahman, R. Rahman, A. Salleh, T.C. Leow, Int. J. Mol. Sci. 13 (2012) 11666–11680.
[21] D.T. Zhao, E.N. Xun, J.X. Wang, R. Wang, X.F. Wei, L. Wang, Z. Wang, Biotechnol. Bioprocess Eng. 16 (2011) 638–644.
[22] A.M. Klibanov, Nature 409 (2001) 241–246.
[23] C. Pilissao, P.D. Carvalho, M.D. Nascimento, Process Biochem. 44 (2009) 1352–1357.
[24] S. Wen, T. Tan, M. Yu, Process Biochem. 43 (2008) 1259–1264.
[25] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81–87.
[26] N. Doukyu, H. Ogino, Biochem. Eng. J. 48 (2010) 270–282.
[27] M. Graber, R. Iragne, E. Rosenfeld, S. Lamare, L. Franson, K. Hult, BBA-Proteins Proteom. 1774 (2007) 1052–1057.
[28] Y. Chen, J.H. Xu, J. Pan, Y. Xu, J.B. Shi, J. Mol. Catal. B: Enzym. 30 (2004) 203–208.
[29] H. Wakabayashi, M. Wakabayashi, W. Eisenreich, K.H. Engel, J. Agric. Food Chem. 51 (2003) 4349–4355.
[30] A.L. Serdakowski, J.S. Dordick, Trends Biotechnol. 26 (2008) 48–54.
[31] H.P. Dong, Y.J. Wang, Y.G. Zheng, J. Mol. Catal. B: Enzym. 66 (2010) 90–94.
[32] P. Jin, X.L. Pei, P.F. Du, X.P. Yin, X.L. Xiong, H.L. Wu, X.L. Zhou, Q.Y. Wang, Bioresour. Technol. 116 (2012) 234–240.
[33] X. Tian, G.W. Zheng, C.X. Li, Z.L. Wang, J.H. Xu, J. Mol. Catal. B: Enzym. 73 (2011) 80–84.
[34] G.W. Zheng, H.L. Yu, J.D. Zhang, J.H. Xu, Adv. Synth. Catal. 351 (2009) 405–414.
[35] M. Guillen, M.D. Benaiges, F. Valero, Biochem. Eng. J. 54 (2011) 117–123.
[36] A. Salis, D. Bilanicova, B.W. Ninham, M. Monduzzi, J. Phys. Chem. B 111 (2007) 1149–1156.
[37] Y. Cao, L. Liao, X.W. Xu, A. Oren, C. Wang, X.F. Zhu, M. Wu, Extremophiles 12 (2008) 471–476.
[38] D.J. Kushner, Can. J. Microbiol. 34 (1988) 482–486.
[39] A. Wolff, L. Zhu, Y.W. Wong, A.J.J. Straathof, J.A. Jongejan, J.J. Heijnen, Biotechnol. Bioeng. 62 (1999) 125–134.
[40] R.S. Phillips, Trends Biotechnol. 14 (1996) 13–16.
[41] K. Dabkowska, K.W. Szewczyk, Biochem. Eng. J. 46 (2009) 147–153.