Available online at www.sciencedirect.com
SCIENCE @ DIRECT®
ELSEVIER
Enzyme and Microbial Technology 36 (2005) 648–653
ENZYME and MICROBIAL TECHNOLOGY
www.elsevier.com/locate/enzmictec

Purification and some characteristics of recombinant insecticide-resistant mosquito carboxylesterase B1 expressed in *Escherichia coli*

Jian-Liang Zhang, Chuan-Ling Qiao*, Wen-Sheng Lan

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China

Received 21 January 2004; accepted 2 July 2004

Abstract

A recombinant insecticide-resistant mosquito carboxylesterase B1 was purified to homogeneity from an *Escherichia coli* expression system. After non-denaturing electrophoresis, active carboxylesterase B1 bands were identified using fast blue RR. Lineweaver–Burk plots of the crude and purified CaE B1 indicate that this enzyme obeys Michaelis–Menten kinetics with K_m value for malathion of 39.3 and 67.4 mM. The V_m of purified enzyme is approximately 17-folds of the value determined in crude homogenate. Carboxylesterase B1 detoxification of parathion had a major limitation which is the 1:1 stoichiometry. To improve the effectiveness of enzymatic detoxification, we developed an approach in which the catalytic activity of organophosphorus compound-inhibited carboxylesterase B1 was restored by having sufficient amounts diacetylmonoxime. It was demonstrated that repeated addition of 25 times the molar concentration of parathion to carboxylesterase B1 in the presence of 4 mM diacetylmonoxime every 2 h did not result in significant inhibition of the enzyme. Consequently the stoichiometry of enzyme detoxification is higher than 64: 1 for parathion.
© 2004 Elsevier Inc. All rights reserved.

Keywords: Insecticide-resistant mosquito carboxylesterase B1; Purification; Kinetics; Detoxification

1. Introduction

As the demand for agriculture produce increases, so inevitably does the need for pesticides. It is estimated that 4 million ton of pesticides are applied to world crops annually for pest control, but less than 1% of the total applied pesticides generally get to the target pests [1]. Due to environmental concerns associated with the accumulation of these pesticides in food products and water supplies, there is a great need to develop safe, convenient and economically feasible methods for pesticide detoxification.

Currently bioremediation seems to be a promising approach for degradation of environment pollutants. The term bioremediation in the context of the environmental sciences is defined as the use of biological agents to reduce or eliminate hazardous substances from the environment [2]. This can range from simply allowing indigenous organisms to degrade toxic substances under natural conditions, to the active augmentation of this process by artificial means. Moreover, with the contemporary revolution in biotechnology in which recombinant organisms or proteins can be produced, numerous novel approaches of bioremediation are possible and should be investigated.

Insects are able to survive a wide variety of physical and biological conditions due to their amazing adaptability. The ability to survive the defenses of their hosts has required that insects evolve unique metabolic mechanisms for detoxification of a wide variety of compounds. Presently more than 500 insect species have demonstrated resistance to certain pesticides, including the organochlorines, organophosphates, carbamates, pyrethroids and newer Bt biopesticides [3,4]. Since the resistant insects can in vivo detoxify many kinds of pesticides, the enzymes encoded by resistant genes of the insects must be very useful to degrade the pesticide pollutants in the environment. If we can produce large quantities of enzymes in a form which can be sprayed or applied in the field, residues of the harmful chemicals can also be broken down [5].

* Corresponding author. Tel.: +86 10 62553369; fax: +86 10 6256 5689.
E-mail address: qiaocl@ioz.ac.cn (C.-L. Qiao).

0141-0229/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2004.07.020

Many insecticide-resistance associated esterases, a kind of hydrolase, have been purified and characterized from several insect species [4–6]. In our laboratory it was showed that the organophosphate resistance of *Culex* is mainly conferred by the overproduction of esterase B1, which is the result of gene amplification. Now this gene has been cloned into the pET(28a) [7,8]. The transformant was observed to express caboxylesterase B1 (CaE B1). In the present paper we report the expression, purification, and some detoxification characteristics of the CaE B1 from *E. coli*. The enzyme is now available in sufficient amounts for detailed structural and degradation studies.

2. Materials and methods

2.1. Cell culture and medium

The transformed *E. coli* BL21 (DE3) with plasmid pET-28a-B1 was established and stored at −70 °C. Inocula was prepared by transferring the spores into a culture tube containing 1–2 mL Luria–Bertani (LB) medium (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; kanamycin, 50 mg; per liter distilled water, pH 7.0). After incubating for 18 h, this culture was inoculated into 20 mL of the same medium in a Erlenmeyer flask. These precultures were used to inoculate a 5 L fermentor (BIOTECH-2001, Shanghai, China) containing 2.5–3 L liquid medium. When the fed-batch fermentation was performed, air supply was increase from 1 to 2.2 L/min slowly, agitation speed from 600 to 1200 rpm. The pH was controlled at 7.0 by adding ammonia.

2.2. Cell fraction

Bacterial cells were collected from the culture medium by centrifugation at 5000 × *g* for 20 min. Cells were resuspended in 0.2 M phosphate buffer (pH 7.0) and incubated with 50 mg/L lysozyme for 20 min at 30 °C. The samples were then lysed using sonication, applying to each 10 mL volume 10 circles of 15 s, alternating with 45 s cooling periods. Nucleic acids and other cell debris were then removed by centrifugation at 12000 × *g* for 15 min. The supernatant was designed “crude enzyme”.

2.3. Protein determination

Protein concentration was estimated using the method of Bradford with bovine serum albumin as standard [9].

2.4. Carboxylesterase B1 assays

CaE B1 activity was assayed with β-naphthyl acetate (β-NA) as substrate. The buffer system used was 0.2 M phosphate buffer, pH 7.0. Substrate solutions were pre-equilibrated at 37 °C. 0.5 mL diluted enzyme solution was added to a final volume of 3 mL. The samples mixed and absorbance measured in a Beckman Du-650 (USA) spectrophotomer. Absorbance was measured at 555 nm for β-NA and one unit of activity (U) was defined as 1 μmol substrate hydrolyzed per minute per milligram protein under the stated conditions [10].

2.5. Purification procedure

Using metal chelation chromatography, the purification procedure was performed. The buffer systems used in this experiment were as followings: 8× binding buffer, 40 mM imidazole, 4 M NaCl, 160 mM Tris–HCl, pH 7.9; 8× wash buffer, 480 mM imidazole, 4 M NaCl, 160 mM Tris–HCl, pH 7.9; 4× elute buffer, 4 M imidazole, 2M NaCl, 80 mM Tris–HCl, pH 7.9; 4× strip buffer, 400 mM EDTA, 2 M NaCl, 80 mM Tris–HCl, pH 7.9; 8× charge buffer, 400 mM $NiSO_4$.

All purification steps were performed at 4 °C. When the column was charged and equilibrated, namely, when the binding buffer drained to the top of the column bed, the “crude enzyme” was loaded on to the column. A flow rate of about 25 mL prepared extract per hour is optional for efficient purification. Then wash the column with 25 mL of 1× binding buffer. After the binding buffer drained to the top of the column bed, 15 mL of 1× wash buffer were used to wash the column. Finally elute the bound protein with 15 mL of 1× elute buffer. The elution solution containing protein (determined by Coomassie Brilliant Blue G250) was collected and concentrated. The concentrated protein was stored at −70 °C in glycerol 50% (v/v) for subsequent analysis.

2.6. Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a mini gel apparatus (BIO-RAD, MINI2-D), using the Tris–glycine discontinuous system previously described [11] with 5% stacking and 8% resolving gels. Activity staining was performed on gels from which SDS had been omitted. After non-denaturing electrophoresis, gels were stained with fast blue RR [12,13].

2.7. Detoxification of malathion by CaE B1

The rates of hydrolysis of malathion by CaE B1 was monitored. The spectrophotometric method of Talcott [14] was used to assay CaE B1 activity. In brief, the release of ethanol from hydrolysis of the substrate was coupled to the reduction of INT with alcohol dehydrogenase and NADH diaphorase. The incubation mixture contained enzyme solution (16 μg protein/mL) in 0.2 M phosphate buffer, pH 7.0, containing 437 μg/mL INT, 25 U/mL alcohol dehychogenase, 1.8 mM NAD, and 0.1 U/mL NAD diaphorase. Reactions were started by injecting 5 μl of 150 mM malathion (in acetone) into the cuvette, making the final malathion concentration 300 μM. This concentration was sufficient to saturate the carboxylesterase assayed in the studied.

2.8. Detoxification of parathion by CaE B1 with diacetylmonoxime (DAM)

The detoxification method of Caranto et al. [15] was used. The incubation mixture in 0.2 M phosphate buffer, pH 7.0, contained 0.1 μM CaE and variable concentrations of parathion with or without DAM. Residual enzyme activity was determined after incubation at room temperature for different intervals of time. In these studies two protocols were used: (1) various concentrations of parathion were added at $t = 0$ to the reaction mixture to constitute different parathion/CaE molar concentrations. At different time intervals, residual activity of CaE was assayed; (2) to a series of reaction mixtures, equal amounts to parathion were added at $t = 0$. After 2 h, the first reaction mixture was discarded after its residual activity was determined. To each of the remaining reaction mixtures the same amount of parathion was added and enzyme activity was determined in the second tube after another 2 h. This procedure was repeated numerous times until the enzyme activity decreased to about 50% of its original value. Residue CaE activity was plotted against the cumulative concentration of parathion added to each reaction mixture.

3. Results and discussion

3.1. Expression of carboxylesterase B1 in E. coli

In order to achieve high-level expression of CaE B1 in *E. coli*, fed-batch cultures of *E. coli* were used. It resulted in high cell densities and large amounts of recombinant proteins (data not shown). This conclusion was ascertained by the appearance of clear band at a position corresponding to a protein of approximately 62 kDa (Fig. 1). Moreover, the target protein displayed activity by activity-stained non-denaturing electrophoresis with β-naphthyl acetate substrate (Fig. 2).

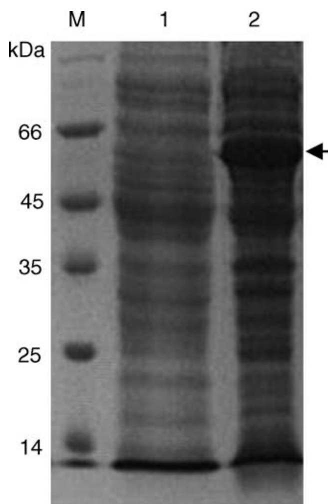

Fig. 1. Expression of the carboxylesterase B1 in *E. coli*. Gel electrophoresis of the crude extracts from cells without pET-28a-B1 (lane 1) and with pET-28a-B1 (lane 2).

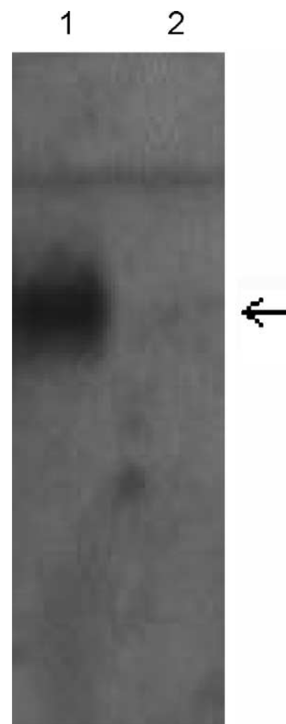

Fig. 2. Activity-stained non-denaturing gels of extracts from *E. coli* cells used for expression.

3.2. Purification

The purification protocol developed in this study took advantage of metal chelation chromatography. The His-Tag sequence binds to Ni^{2+} cations, which are immobilized on the His-Bind resin using the charge buffer. After unbound proteins are washed away, the target protein is recovered by elution with imidazole. This procedure resulted in rapid one-step purification of proteins to homogeneity (Fig. 3). Previous

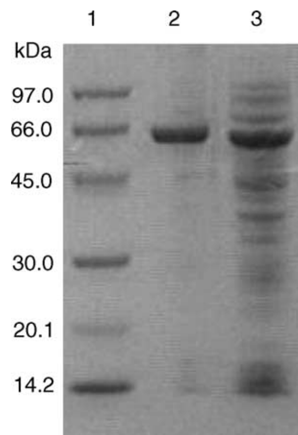

Fig. 3. SDS-PAGE of crude and purified CaE B1 from *E. coli* cells used for expression (lane 1: marker; lane 2: purified enzyme; lane 3: crude enzyme).

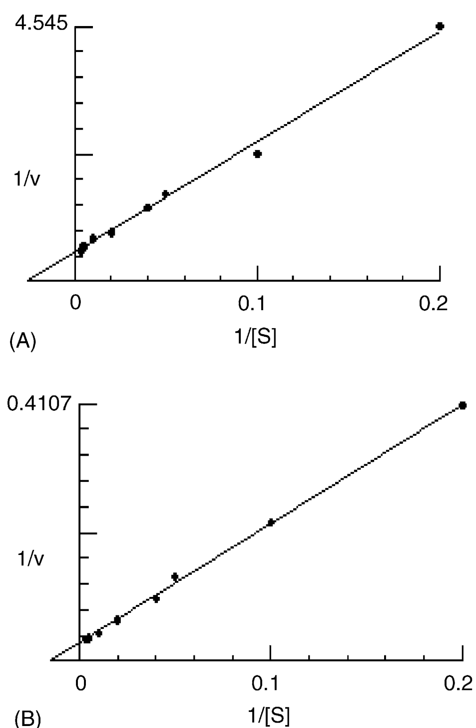

Fig. 4. Determination of K_m and V_{max} of CaE B1 using malathion as a substrate: (A) crude enzyme; (B) purified enzyme.

studies had suggested that this enzyme had a MW of 62 kDa [16]. This experiment confirmed the findings for the purified protein.

3.3. Detoxification characteristics

For organophosphorus compounds containing carboxyl ester bond, such as malathion, the enzyme hydrolysis by CaE B1 is based on the reversible acylation of a serine residue within the active center of the protein. In this work the kinetics tests were conducted with use of malathion as the organophosphate reactant. Lineweaver–Burk plots of the crude and purified CaE B1 (Fig. 4) indicate that this enzyme obeys Michaelis–Menten kinetics with K_m value for malathion of 39.3 and 67.4 mM. The V_m of purified enzyme is approximately 17-folds of the value determined in crude homogenate.

However, for many those organophosphorus compounds containing no carboxyl ester bond, such as parathion, the CaE B1 was firstly phosphorylated by the compounds. In this case, the phosphorylated enzyme cannot be reactivated by water and it is impossible to regenerate the free active enzyme [17]. Fig. 5 shows the results of inhibition of CaE B1 by parathion (the extraploted amount of parathion needed to completely inhibited this amount of enzyme would be 0.1 nmol).

To improve the enzymatic detoxification of parathion, one possible approach is to use a combination of enzyme with a specific uncharged oxime, such as diacetylmonoxime (DAM). Fig. 6 demonstrated that CaE in the presence of

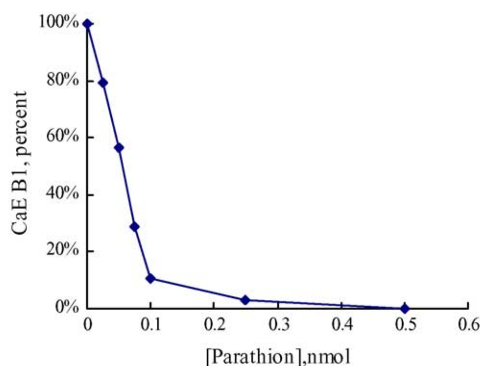

Fig. 5. Inhibition of CaE B1 by parathion. The incubation mixture contained 0.1 nmol CaE B1 and increasing amounts of paraoxon in a final volume of 1 mL (0.2 mM phosphate buffer, pH 7.0). The mixture was incubated at room temperature for 2 h, then residual CaE activity was measured.

4 mM DAM continued to neutralize repetitive additions of parathion. A total of 2.5 nmol of parathion was neutralized by 0.1 nmol of CaE B1 in the presence of 4 mM DAM (approximately 25 times more than the expected amount). This continuous neutralization of parathion was further evident, when progressively large than molar excesses of parathion were added to CaE in the presence of DAM (Fig. 7). When the total amount of parathion exceeded the molar concentration of enzyme by greater than 60, about 50% enzyme activity was inhibited.

4. Discussion

The combination of genetics engineering and microbial cultivation method has become a powerful tool for producing recombinant gene products. In addition to genetic manipulation, optimization of cultivation condition can also lead to significant improvements in the production of heterologous proteins from *E. coli*. Fed-batch cultures of *E. coli* are often used to attain high cell densities and large amounts of

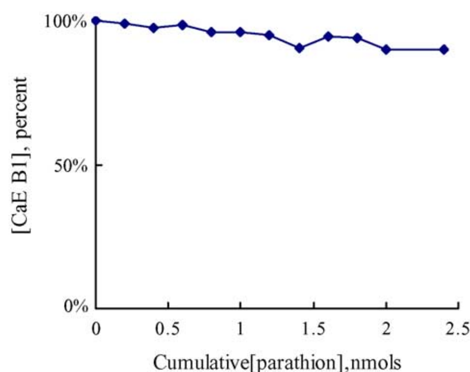

Fig. 6. Reactivation of CaE in the presence of DAM after repeated additions of parathion. The reactivation of CaE B1 (0.1 nmol) by DAM (4 mM) was carried out in the phosphate buffer (0.2 mM, pH 7.0) after repeated additions of parathion at 2 h intervals. The residual CaE B1 activity was measured, 2 h after each addition of parathion.

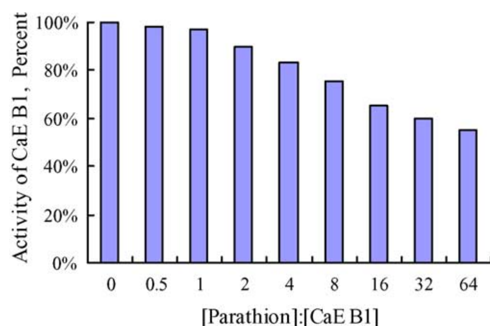

Fig. 7. Reactivation of parathion-inhibited CaE B1 by DAM. The incubation mixture contained 0.2 mM phosphate buffer, pH 7.0, 4 mM DAM, 0.1 nmol CaE B1, and the indicated molar excess of parathion in a final volume of 1 mL. The mixture was incubated at room temperature for 4 h, then residual CaE activity was measured.

recombinant proteins [18]. The success of a fed-batch cultivation to achieve high cell density depends on conditions like feeding of essential nutrients in a suitable concentration range, sufficient oxygen supply and relatively low cultivation temperature, etc. [18,19].

The purification protocol developed in this study took advantage of metal chelation chromatography. Consequently the crude enzyme was purified to electrophoretic homogeneity which would be used for more detailed kinetic investigation and detoxification research.

The use of enzyme to hydrolyze organophosphates has been considered by other workers [20,21]. For organophosphorus compounds containing carboxyl ester bond, such as malathion, the enzyme hydrolysis by CaE is based on the reversible acylation of a serine residue within the active center of the protein. This acylation causes the release of the alcohol moiety and of the corresponding covalently acylated enzyme. This acylated intermediate is hydrolysed by nucleophilic attack of water that releases the corresponding carboxylic acid moiety, plus the free active enzyme again ready to initiate a new catalytic cycle. On the other hand, for many those organophosphorus compounds containing no carboxyl ester bond, the first step is the phosphorylation of the enzyme. However, in this case, the phosphorylated enzyme cannot be reactivated by water and it is impossible to regenerate the free active enzyme. In this work we examined the kinetics of CaE B1 obtained from the fermentation. The low K_m observed in this study indicates that the crude enzyme preparation and the purified enzyme retain significant affinities for malathion. But CaE B1 detoxification of parathion had a major limitation which is the 1:1 stoichiometry. To improve the enzymatic detoxification of organohosphorus compounds containing no carboxyl ester bond, namely increase the parathion: enzyme stoichiometry, one possible approach is to use a combination of enzyme with a specific reactivator drug as a pretreatment. In general the organophosphate-inhibited CaE can be reactivated rapidly by proper oxime so long as it has not undergone aging. In this paper, it was demonstrated that the addition of diacetylmonoxime amplifies the effectiveness of CaE B1 to detoxify organophosphate. This is possible because the OP-inbibited CaE was reactivated in the presence of diacetylmonoxime.

With the contemporary revolution in biotechnology and the current ease in which recombinant organisms can be produced, numerous novel approaches to bioremediation are possible and should be investigated. With industrialization, and increasing world population, and increased demands or chemicals, the reliance on natural degradative processes will no longer be adequate for the preservation of a healthy ecosystem. With recombinant technology, insect metabolic enzymes can be used in bioremediation, either as single isolated gene products or more likely in combination with existing bioremediation organisms.

Acknowledgements

This work was supported by NSFC Grant (No. 3014001-4291) and National 863 project (Nos. 2002AA649300 and 2002AA601160).

References

[1] Pimentel D. Effects of pesticides on the environment. In: Proceedings of the 10th International Congress on Plant Protection, vol. 2, 1983. p. 685–91.
[2] Roe RM, Hodgson E, Rose RL, Thompson DM, Devorshar C, Anspaugh DD, et al. Basic principles and rationale for the use of insect genes in bioremediation: esterase, phosphotriesterase, cytochrome P450 and epoxide hydrolase. Rev Toxicol 1998;2:169–78.
[3] Gould F, Anderson A, Reynolds A, Bumgarner L, Moar W. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera:Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J Econom Entomol 1995;88:1545–59.
[4] Sparks TC, Graves JB, Leonard BR. Insecticides resistance and the tobacco budworm: past, present and future. In: Roe RM, Kuhr RJ, editors. Reviews in pesticide toxicology. Raleigh, NC: Toxicology Communications Inc.; 1993. p. 339–78.
[5] de Vries GE. Insect enzymes for bioremediation. Trends Plant Sci 2000;5:9.
[6] Anspaugh DD, Kennedy GG, Roe RM. Purification and characterization of a resistance-associated esterase from the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Pestic Biochem Physiol 1995;53:84–95.
[7] Yan YC, Qiao CL, Shang HY. Express of the detoxifying gene B1 in *Escherichia coli* and *Synechococcus*. Entomol Sin 2000;7:161–8.
[8] Huang J, Qiao CL, Li X, Xing JM. Cloning and fusion expression of detoxifying gene in *Escherichia coli*. Acta Genet Sin 2001;28:583–8.
[9] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[10] Asperen KV. A study of housefly esterases by means of a sensitive colorimetric method. J Ins Physiol 1962;8:401–16.
[11] Laemmli JK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[12] Erickson RP, Steers EJ. Comparative study of isoenzyme formation of bacterial β-galactosidase. J Bacteriol 1970;102:79–84.
[13] Pisani FM, Rella R, Raia CA, Rozzo C, Nucci R, Gambacorta A, et al. Thermostable β-galactosidase from the archaebacterium Sulfolobus solfataricus—purification and properties. Eur J Bio-Chem 1990;187:321–8.

[14] Talcott RE. Hepatic and extrahepatic malathion carboxylesterase. Assay and localization in the rat. Toxicol Appl Pharmacol 1979;47:145–50.

[15] Caranto GR, Waibel KR, Asher JM, Larrison RW, Brecht KM, Schutz MB, et al. Amplification of the effectiveness of acetylcholinesterase for detoxification of organophosphorus compounds by bis-quaternary oximes. Biochem Pharmacol 1994;47:347–57.

[16] Leng XF, Tang ZH, Wang YC. Molecular toxicology of insecticides and insect resistance. Beijing: Chinese Agriculture Press; 1996. pp. 23–24.

[17] Sogorb MA, Vilanova E. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol Lett 2002;128:215–28.

[18] Riesenberg E, Schulz V, Knorre WA, Pohl HD, Korz D, Sanders EA, et al. High cell density cultivation of *Escherichia coli* at controlled specific growth rate. J Biotechnol 1991;20:17–28.

[19] MacDonald L, Nesay JO. Effects of medium quality on the expression of human interleukin-2 at high cell density in fermentor cultures of *Escherichia coli* K-12. Appl Environ Microbiol 1990;56:640–64.

[20] Cho CM, Mulchandani A, Chen W. Bacterial cell surface display of organohosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. Appl Environ Microbiol 2002;68:2026–30.

[21] Zhang JL, Qiao CL. Novel approaches for remediation of pesticide pollutants. Int J Environ Pollut 2002;18:423–43.