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Culture and characteristics of recombinant protein production of an *Escherichia coli* strain expressing carboxylesterase B1

C.L. Qiao[a,*], B.C. Shen[a], J.M. Xing[a,b], J. Huang[a], J.L. Zhang[a], D.X. Zhao[c], B. Yang[a]

[a]*State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Academia Sinica, Beijing 100080, China*
[b]*Institute of Process Engineering, Academia Sinica, Beijing 100080, China*
[c]*Institute of Botany, Academia Sinica, Beijing 100093, China*

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Abstract

High-density culture was achieved through controlling specific growth rate by limiting glucose concentration to $<0.2\,g\,L^{-1}$. Carboxylesterase B1 capable of hydrolyzing organophosphate esters was purified from *Escherichia coli* strain BL21 carrying a cloned esterase B1 gene from mosquito. The recombinant strain BL21 carrying pET-ESTB1 was used for the fermentation. Product formation was induced by either a temperature shift from 30 to 42 °C or by feeding a mixture of glucose and lactose. Cell growth and production of detoxifying enzyme were affected by oxygen availability. The maximum biomass of *E. coli* BL21 (pET-ESTB1) increased from 14.9 to 31.5 g dry cell weight l^{-1}. Using the host strain *E. coli* BL21 (DE3), detoxifying enzyme was over-expressed at a biomass level of up to 31.5 g dry weight l^{-1}. The enzyme had a molecular mass of 64 kDa, its optimum temperature was approx. 37 °C; at pH 7 the relative activity after 3 h was 85.9% at 28 °C, 64.9% at 34 °C, and 4.5% at 40 °C. The enzyme activity of cells grown at lower temperatures was much higher; at 18 °C it almost twice than at 20 or 22 °C.

Keywords: High-density culture; *E. coli*; Carboxylesterase B1; Recombinant; Gene expression

1. Introduction

The unique evolutionary history of insect–plant relationships under insecticide selection in agricultural systems has produced novel detoxifying metabolic mechanisms in some insects that have possible applications in bioremediation of chemical contamination. With recombinant biotechnology, insect metabolic enzymes can be used in bioremediation (Anspaugh et al., 1995; Roe et al., 1998).

Carboxylesterase (CAE, EC 3.1.1) is an enzyme class that catalyzes the hydrolysis of organophosphates, organochlorides and carbamate ester bonds. CAE B1 is a recombinant protein produced in a *Escherichia coli* strain expressing CAE B1 and it has been used to hydrolyze selectively organophosphate insecticide residues in polluted water and/or in vegetables (Qiao et al., 2003). In order to improve the production of recombinant proteins, increasing attention has been paid to high cell density culturing techniques. High cell density culturing is a highly desirable process in industrial scale-up for production. Fed-batch culture has often been used to achieve high cell density, and various feeding strategies have been reported. A cell density of 61.2 g dry weight L^{-1} has been achieved in a total culture time of 24 h by continuously supplying a high concentration of glucose (Yee and Blanch, 1992).

Cellular concentrations (dry biomass) of 5–10 g L^{-1} are typical yields of batch cultures and feed-batch techniques can be used to achieve concentrations $>50\,g$ dry biomass L^{-1}. Feeding strategies for fed-batch culture include feed-back control as well as predetermined feeding profiles. The volumetric yield of recombinant products can be improved by controlling the specific growth rate and the substrate concentration (Nakagawa et al., 1995). High-cell-density culture requires sufficient oxygen supply, continual feeding of substrates such as glucose, and minimal formation of inhibitory by-products. Growth of *E. coli* can be inhibited by organic acids accumulating in the culture medium under aerobic conditions and a supply of excess sugar carbon source, or anaerobic conditions

*Corresponding author. Tel.: +86 10 62553369; fax: +86 10 62565689.
E-mail address: qiaocl@panda.ioz.ac.cn (C.L. Qiao).

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(Robbins and Taylor, 1989; Yee and Blanch, 1992; Nakagawa et al., 1995). *E. coli* is the most widely used organism for high-cell-density culturing. In the present study, high-level expression of mosquito esterase gene B1 in *E. coli* was achieved in high-cell-density culture, and enhancement of CaE B1 production and CaE B1 stablity was also attempted. The effects of environmental conditions and characteristics of the CaE B1 were also examined.

2. Materials and methods

2.1. Microorganism and culture conditions

The host vector system used in this study was *E. coli* BL21 carrying pET-ESTB1 (Huang et al., 2001). The pET-ESTB1 encodes the carboxylesterase induced by IPTG and kanamycin resistance.

The culture medium for batch culture was based on M9 medium (Zhou et al., 2003) with additional glucose ($4\,g\,L^{-1}$) at pH 7.0. For feed-batch culture the medium (pH 7.0) contained (L^{-1}): peptone 40 g; yeast extract 20 g; KH_2PO_4 8 g; Na_2HPO_4 15 g; $CaCl_2$ 0.08 g; NH_4Cl 0.4 g; $MgSO_4$ 0.8 g; and glucose 5 g.

Culture was initiated in a 2-L fermentor (L.E. Marabishi Co., Tokyo, Japan) containing 1.2 L liquid medium or in a 5-L fermentor (Biotech-2001, Shanghai, China) containing 2.5–3 L medium. The first active culture was established by transfer from a plate culture to a test tube containing 5 ml Luria-Bertani (LB) medium (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; and kanamycin, 50 mg; distilled water 1 L; pH 7.0) incubated at 30° C on a shaker operating at 220 rpm for 8 h. A 3-ml aliquot of the active culture was then transferred to a 500-ml Erlenmeyer flask containing 50 ml production medium and was cultivated at 30 °C and 220 rpm for 18 h to provide inoculum for the fermentor. This culture medium was transferred to the fermentor and incubated at 18, 20 and 22 °C. During the fed-batch fermentation air supply was increased from 1 L to $2.2\,L\,min^{-1}$ gradually, at an agitation rate of 600–1200 rpm. When the glucose concentration was $<0.1\,g\,L^{-1}$, glucose/lactose solution (glucose 200 g, lactose 40 g, distilled water 400 ml) was trickled in, with the concentration of glucose controlled $<0.2\,g\,L^{-1}$ and pH 7.0 maintained by adding NaOH.

2.2. Cell fractionation and protein determination

Bacterial cells were collected from the culture medium by centrifugation at 12,000*g* for 20 min. The cell pellet was resuspended in 0.2 M phosphate buffer (pH 7.0) and incubated with lysozyme ($50\,mg\,L^{-1}$) for 20 min at 30 °C. The samples were then sonicated, applying to each 10-ml volume 10 cycles of 15 s, alternating with 45-s cooling periods. Nucleic acids and other cellular debris were then removed by centrifugation at 12,000*g* for 15 min. The supernatant was retained as the crude enzyme. Protein concentration was estimated using the Bradford method with bovine serum albumin (Sigma Co., St Louis, MO) as standard (Bradford, 1976).

2.3. Enzyme purification

His-Bind kits (Novagen) were used for metal chelation chromatography, the buffer systems being: 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, 2 M 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. After the column was charged and equilibrated when the binding buffer drained to the top of the column bed, the crude enzyme was loaded onto the column. A flow rate of about 25 ml prepared extract h^{-1} was tested for efficient purification and then washed with 25 ml 1 × binding buffer. After the binding buffer drained to the top of the column bed, 15 ml 1 × wash buffer was used to wash the column. Finally, the bound protein was eluted with 15 ml 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 50% glycerol (v/v) for subsequent analysis.

2.4. Effect of temperature on activity

To study the thermal stability of the enzyme CaE B1, β-naphthylacetate (β-NA) was used as a substrate to determine the enzyme activity at different temperatures. Relative enzyme activity was assayed according to the method described in van Asperen (1962). To study the effect of temperature on the degradation of β-NA, a series of 6-mL portions of phosphate buffer (pH 7.0) containing 3×10^{-4} M β-NA and 3 mL crude enzyme were incubated at 28, 34 and 40 °C. At regular intervals, 3-mL samples were taken and mixed with 0.5 mL diazoblue laurysulphate solution. Absorbance of the resultant red product was immediately determined at 550 nm with a Beckman spectrophotometer.

2.5. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a mini-gel apparatus (Bio-Rad, Mini2-D), using the tris-glycine discontinuous system (Laemmli, 1970) with 5% stacking and 8% resolving gels. A low molecular weight SDS calibration kit containing proteins (14.4–97.0 kDa) was used to provide molecular weight standard markers. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

3. Results

3.1. Effect of glucose on growth

In batch fermentor experiments, the initial glucose level ($4\,g\,L^{-1}$) was observed to enhance cell growth relative to glucose absence (results not shown). Since preliminary experiments indicated that glucose supplementation stimulated cell growth and significant pH changes resulted from the acidification of the medium that occurred during glucose utilization, a glucose-fed fermentation was investigated. In this experiment the medium was as described in 3.1. Cells were grown in a fermentor with pH 7.0 maintained, and glucose controlled at $\leqslant 0.2\,g\,L^{-1}$ when the initial glucose was depleted. Fig. 1 shows that in this glucose-fed culture, cell production was nearly 15-fold that in the batch culture. Thus, continuous feeding of glucose at a relatively low level and pH controlled at 7.0 are essential for improved biomass production. (Fig. 2).

3.2. Effect of air supply on growth

To examine the pattern of growth as affected by temperature, agitation speed and air flow rate, three different protocols were applied (Table 1). The maximum dry biomass in fed-batch culture at 18 °C with the agitation speed changing from 300 to 800 rpm (MD-211) was $56.75\,g\,L^{-1}$ (Table 2), while at 20 °C and 500–1100 rpm (MD-212) it was higher, $85.25\,g\,L^{-1}$ (Table 2). In protocol MD-213, identical to MD-212 except for the temperature being 22 °C, the maximum dry mass was only $46.75\,g\,L^{-1}$.

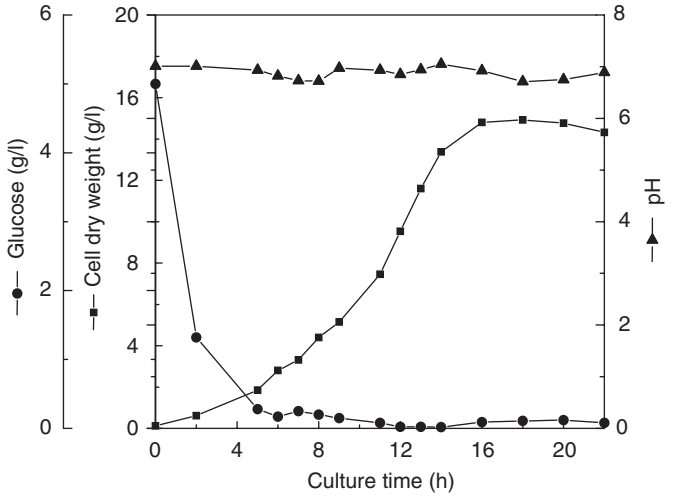

Fig. 1. Fermentation profiles in a glucose-fed fermentor operating at 18 °C under protocol MD-211 (see Table 1) on medium of initial composition described in 2.1 (●, g glucose L^{-1}; ■, g dry biomass L^{-1}; ▲, pH).

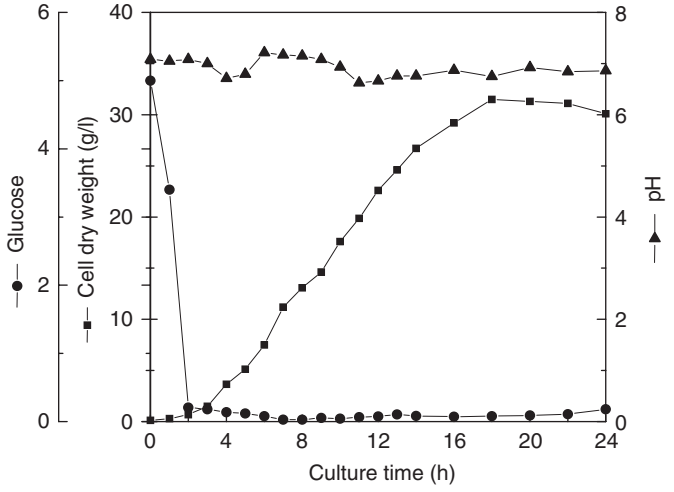

Fig. 2. Fermentation profiles in a glucose-fed fermentor operating at 20 °C under protocol MD-212 (see Table 1) on medium of initial composition described in 2.1(●, g glucose L^{-1}; ■, g dry biomass L^{-1}; ▲, pH).

Cultivation at the lower temperature, agitation speed and air flow rate (MD-211) resulted in the highest CaE B1 activity (Table 2), nearly twice that at either of the other two regimes, MD-212 and MD-213 (Table 1).

3.3. Purification

The enzyme produced at 18 °C was purified to homogeneity (Fig. 3) using metal chelation chromatography, in which the His-Tag sequence binds to Ni^{2+} cations, that are immobilized on the His-Bind resin using the charge buffer, and the proteins desorbed by washing, before the target protein is recovered by elution with imidazole. This resulted in rapid one-step purification of the enzyme CaE B1 to homogeneity (Fig. 3), and the enzyme was stable under the conditions described. Previous studies suggested that this enzyme had a MW of 62 kDa (Huang et al., 2001) and electrophoretic analysis confirmed this.

Table 1
Agitation and air flow rates of three glucose-fed fermentation protocols, MD-211–MD-213

Time (h)	Fermentor agitation rate (rpm)			Air supply rate (L min^{-1})		
	MD-211	MD-212	MD-213	MD-211	MD-212	MD-213
0	300	500	500	1	0.8	0.8
1	300	600	600	1	1.2	1.2
2	300	600	600	1	1.2	1.2
5	400	800	800	1	1.4	1.4
6	600	800	800	1.6	1.4	1.4
7	600	1000	1000	1.6	1.6	1.6
9	600	1000	1000	1.6	1.6	1.6
11	600	1100	1100	1.6	1.8	1.8
12	800	1100	1100	1.6	1.8	1.8
14	800	1100	1100	1.6	1.8	1.8
16	800	1100	1100	1.6	1.8	1.8
18	800	1100	1100	1.6	1.8	1.8

3.4. Characteristics of CaE B1 enzyme

Although CaE B1 was capable of hydrolyzing a wide array of related organophosphate compounds (data not shown), tests were conducted using malathion as the organophosphate reactant. The activity of purified CaE B1 for β-NA was greatest at 37 °C, although at 31 °C and 40 °C the activity was still more than three-quarters of the maximum (Fig. 4). Investigation of the thermal stability of the crude enzyme revealed that after 3 h at 28 °C inactivation was limited (Fig. 5), with >85% of the initial activity being retained. The corresponding residual activity after exposure to 34 °C was approx. 65% of the initial level. However, the enzyme was inactivated at 40 °C, with <5% of its initial activity remaining after 3 h.

4. Discussion

The combination of genetic engineering and microbial cultivation has provided a powerful tool for producing recombinant gene products. In addition to genetic manipulation, optimization of culture conditions has also led to significant improvements in the production of heterologous proteins by *E. coli*. Fed-batch cultures of *E. coli* are often used to attain high cell densities and large amounts of recombinant proteins (Riesenberg et al., 1991). The success of a fed-batch culture system to achieve high cell density depends on several parameters, including the feeding rate of essential nutrients, oxygen supply and culture temperature (MacDonald and Nesay, 1990; Riesenberg et al., 1991).

It is known that higher initial glucose level suppresses both growth and enzyme production, and leads to lower pH. For example, when the yeast *Phaffia rhodozyma* grew under fermentative conditions with limited oxygen or high

Table 2
Expression of CaE B1 in *E. coli* grown at different temperatures

Batch	Temperature (°C)	Dry biomass ($g\,L^{-1}$)	Units mg^{-1} biomass	Total units	Units L^{-1} medium
MD-211[a]	18	113.5	97.3	296188	98729
MD-212	20	170.5	39.5	180155	72062
MD-213	22	93.5	44.8	128176	64088

[a]Grown at lower agitation and airflow rate (see Table 1).

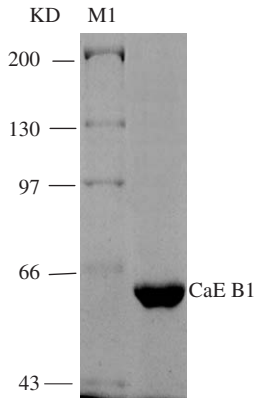

Fig. 3. SDS-PAGE of purified CaE B1 from *E. coli* used for expression (Marker, molecular weight; Lane 1, purified enzyme).

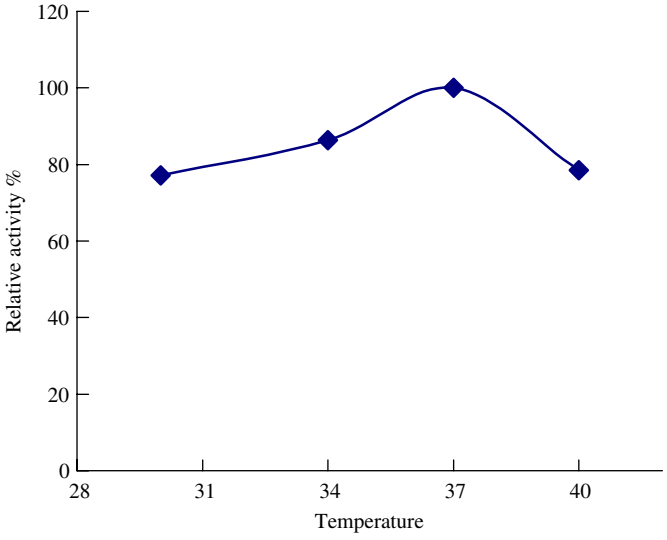

Fig. 4. Effect of temperature on the activity of purified CaE B1.

concentrations of glucose, astaxanthin production rate decreased remarkably (Yamane et al., 1997). It is possible that the effects seen in the present study are a consequence of acidification of the medium that occurs during glucose utilization. The fed-batch approach not only improves production, but also results in more efficient production of CaE B1 with respect to nutrient consumption. As indicated in the present work, fed-batch cultures enhance growth. This enhanced metabolic efficiency may be due to a decrease in the conversion of substrates to acid inhibitory to growth. Oxygen supply is also one of the important

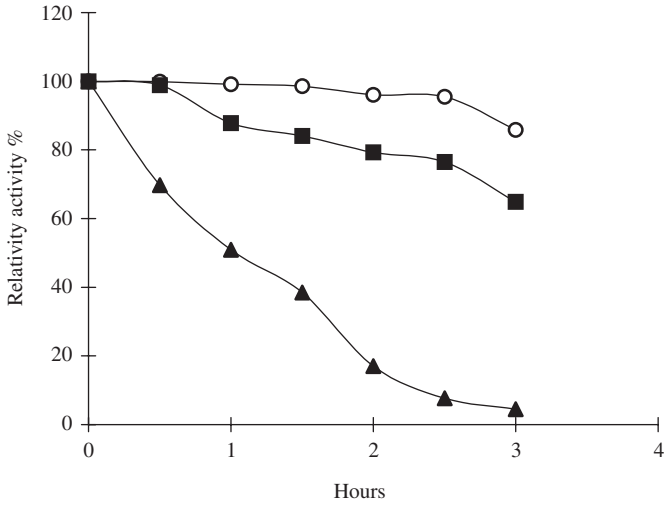

Fig. 5. Thermal stability of crude CaE B1 at 28 (○), 34 (■) and 40 °C (▲), assessed as residual activity (see Section 2.4).

parameters for high-density fermentation. Dissolved oxygen concentration plays an important role in the cell growth and expression of recombinant protein. Nakagawa et al. (1995) reported that only when the dissolved oxygen is maintained well above a critical concentration (20–25%) does the engineered bacterium grow well and express the target protein continuously. Lowering the growth temperature is probably the easiest method of preventing inclusion body formation (Schein, 1991). In consequence, low temperature incubation of the transformed cells in the present work resulted in a high yield of the protein product in soluble form. The rate of transformation and transcription is low and the protein has more time to reach its final folded configuration during culture at low temperature. In short, the solubility of recombinant protein is favored by growth at lower temperature. This is a temperature-dependent phenomenon reported for several other proteins (Kane and Hartley, 1988; Seeger et al., 1995).

The practical use of enzymes to hydrolyze organophosphates has been proposed (Zhang and Qiao, 2002), and in the work described here the kinetics and stability of an organophosphate hydrolyzing carboxylesterase, CaE B1, obtained from a fermentation were examined. The thermal stability of CaE B1, indicates that for improved performance it may be desirable to use this enzyme at temperatures $<20\,^{\circ}C$. For liquid-phase wastewater, it may be desirable to immobilize the enzyme on or within a solid support.

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