Biodegradation (2008) 19:831–839
DOI 10.1007/s10532-008-9186-2

ORIGINAL PAPER

# Overexpression of methyl parathion hydrolase and its application in detoxification of organophosphates

**Jijian Yang · Chao Yang · Hong Jiang · Chuanling Qiao**

Received: 11 December 2007 / Accepted: 18 March 2008 / Published online: 30 March 2008


**Abstract** The coding region of *mpd* gene corresponding to mature methyl parathion hydrolase (MPH) was heterologously overexpressed in *Escherichia coli* BL21 (DE3) by using pET expression system. The lactose-induced expression yield of MPH is increased 2-fold compared with IPTG as inducer. Furthermore, it was found that specific activity of MPH increased 48% by reducing the induction temperature to 22°C. The addition of 25 mM lactose at 22°C, the MPH activity of fermentation broth had a specific activity of $1.4 \times 10^4$ U/mg protein. Plasmid was no significant decrease in the modified medium. The optimal pH and temperature of MPH were 8.0 and 30°C, respectively. Over a period of 5 months, the dried cells showed no significant decrease in the activity of the detoxifying enzymes. The crude enzymes in 50 mM citrate-phosphate buffer (pH 8.0) were able to degrade about 98% of the organophosphate pesticides sprayed on cabbage. The detoxification efficiency was superior to that of the treatments of water, detergent, and a commercially available enzyme product. Additionally, the products of pesticide hydrolysis generated by treatment with the enzyme extract were determined to be virtually nontoxic.

**Keywords** Decontamination · Environmental health · Fermentation · Lactose · Methyl parathion hydrolase

J. Yang · C. Yang · H. Jiang · C. Qiao (✉)
State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, Da Tun Lu, Beijing 100101, P.R. China
e-mail: qiaocl@ioz.ac.cn

J. Yang · C. Yang
Graduate School of the Chinese Academy of Sciences, No. 19, Yu Quan Lu, Beijing, P.R. China

## Introduction

Organophosphorus pesticides (OPs) such as parathion and methyl parathion (MP) have been used extensively in agriculture and household as pesticides, fungicides and herbicides. They are acute neurotoxins by virtue of their potent inhibition of acetylcholinesterase (AChE) (Singh and Walker 2006). These compounds cause enormous damage to non-target organisms because AChE is present in all vertebrates (Zhang et al. 2005).

The microbial degradation of OPs has become the focus of many studies because it is economical and effective. Methyl parathion-degrading gene (*mpd*) has been intensively researched (Cui et al. 2001; Fu et al. 2004; Rani and Lalithakumari 1994; Yang et al. 2006; Zhang et al. 2006). We previously cloned *mpd* gene from chlorpyrifos-degrading *Stenotrophomonas* sp. and the intact *mpd* gene was expressed in *E. coli* (Yang et al. 2006).

In *E. coli* expression systems, IPTG is generally used as the inducer for foreign gene expression;

however, the main drawback of this compound is its expense and toxicity to humans. The natural inducer of the lac operon, lactose, is therefore employed as an alternative inducer instead of IPTG, especially in large scale fermentations (Neubauer and Hofmann 1994; Monteiro et al. 2000). However, disadvantages still exist, such as the formation of inclusion bodies in the cytoplasm. In many instances, the solubility of the desired expressed proteins can be improved at lower induction temperatures (Schein and Noteborn 1988).

The engineered *E. coli* strains with the intracellular MPH or surface-expressed MPH have been applied for detoxification of organophosphates (Yang et al. 2006, 2008). Although the applications of MPH-expression *E. coli* in detoxification of OPs have been well documented, there are no reports on the optimal conditions of large-scale production and on the detoxification of agricultural products based on a biological strategy. In the present study, we report the construction of a genetically engineered *E. coli* overexpressing MPH, the optimal conditions of large-scale production, and the applications of lyophilized cell powder in detoxification of OP residues on vegetables.

## Materials and methods

### Chemicals, bacterial strains, and medium

*E. coli* BL21 (DE3) (Novagen) was used as a heterologous expression host. Plasmid pET30a (Novagen) was used for overexpression of recombinant MPH. Plasmid pMDQ (Yang et al. 2006) was used as the source of the *mpd* gene. Enzymes for molecular cloning were purchased from Promega. Lactose was purchased from Beijing Chemical Co. All other chemicals were of high purity commercially available (Sigma).

The compositions of LB medium were as follows (per liter): 5 g yeast extract, 10 g tryptone and 10 g NaCl. The modified fermentation medium for batch culture contained (per liter): 20 g glycerol, 15 g tryptone, 20 g yeast extract, 2.5 g $K_2HPO_4 \cdot 12H_2O$, 0.16 g $KH_2PO_4$, 0.5 g NaCl and 0.25 g $MgSO_4 \cdot 7H_2O$. Strains harboring plasmids were grown in LB medium with 50 μg/ml kanamycin or fermentation medium supplemented with 25 μg/ml kanamycin.

### Plasmid construction for overexpression of MPH in *E. coli*

The coding region for mature MPH that lacks the signal peptide was subcloned in pET30a by PCR amplification. The following primers were used: forward, 5′-CATATGGCCGCACCGCAGGTGCGCACCTCG-3′, and reverse, 5′-CTCGAGCTTGGGGTTGACGACCG-3′ (the *Nde*I and *Xho*I sites, respectively, are underlined). The PCR products were ligated with *Nde*I-*Xho*I-digested pET30a to yield pETM, which was transformed into *E. coli* BL21 (DE3). Positive clones were screened by PCR and restriction analysis. Positive recombinant plasmid was sequenced to ensure that no mutation had been incorporated during the PCR. *E. coli* BL21 (DE3) cells harboring pETM were induced with 1 mM IPTG for 3 h at 30°C when cells were grown to an $OD_{600} = 0.6$. The expressed products were determined by 12% SDS-PAGE.

### High density batch fermentation

Laboratory-scale fermentations were performed in a 5 l (working volume 3 l) BIOTECH-5B series fermenter. The fermenter containing the fermentation medium and antifoam was sterilized by autoclaving at 121°C for 20 min. Filter-sterilized kanamycin was added to the fermentation medium prior to inoculation. After cooling to 37°C and adjusting the pH to 7, the vessel was inoculated with *E. coli* culture broth grown overnight at 5% inocula. The vessel was aerated with 2 l/min air. The pH was controlled at $7 \pm 0.05$ by the addition of 25% (w/w) $NH_4OH$ or HCl. Dissolved $O_2$ was controlled at 20–30% saturation by control of both airflow and stirrer speed. Foam was controlled by the addition of silicon-antifoaming reagent. For protein expression, strain was allowed to grow at 37°C to mid-log phase ($OD_{600} = 4$) prior to induction. IPTG was added at a final concentration of 1 mM. The stock solution, of lactose was prepared by dissolving the lactose in the distilled water. Lactose was added to the cultures to give the desired concentrations. For all experiments the temperature was reduced to 30 or 22°C at the time point of induction. *E. coli* cells harboring plasmid pETM were harvested after 6–8 h of induction. During the cultivation process, culture samples were taken from different systems at different time points

for the measurement of plasmid stability and the target protein yield. Harvesting times were chosen to optimize active protein yields from the lactose cultures, at which time the cells were harvested from the culture medium by centrifuging at 5,000 g for 10 min. The wet cells were then dried at 20°C under vacuum, followed by freezing with liquid $N_2$ and grinding to a powder to release the enzymes from the cells.

### Plasmid stability

The percentage of plasmid-free cells was measured by replica plating on agar plates containing growth media with or without 50 mg/l kanamycin. Cell samples were taken from the fermentation and diluted in 50 mM phosphate buffer so that 50–100 colony forming units per plate were achieved. The samples were then spread onto the two different types of agar plates mentioned above and incubated overnight at 37°C.

### Preparation of enzyme solution

The fresh biomass was harvested, and resuspended in 0.2 M $Na_2HPO_4$–0.1 M citric acid buffer (pH 8.0). Cell lysate was prepared by addition of 10 µl of lysozyme (10 mg/ml) and incubated on ice for 1 h. The cells were disrupted by sonication in an ice-water bath for six periods of 10 s with 10 s intervals, after which cell debris was removed by centrifugation at 12,000 g for 15 min at 4°C, and the resulting supernatant was regarded as the crude enzyme solution and used for the enzyme activity assays.

Cell powder (0.12 g) was mixed with 1 ml of 0.2 M $Na_2HPO_4$–0.1 M citric acid buffer, pH 8.0, followed by centrifugation for 5 min at 12,000*g* to separate the cells. Protein concentrations were determined according to the method of Bradford (1976) using BSA as standard. The supernatant was kept at 4°C for the activity assay and insecticide bioassay.

### SDS-PAGE analysis

SDS-PAGE analysis was performed in the Mini-Protein system (Bio-Rad, USA). The progress of the expression and the amount of MPH in the soluble fraction after cell disruption was monitored for selected samples from each experiment according to the method of Laemmli (1970).

### Enzyme activity assay

The release of *p*-nitrophenol from 0.2 mM MP in 10 min was used to measure the MPH activity in 0.2 M $Na_2HPO_4$–0.1 M citric acid buffer (pH 8.0) at 30°C. To 2.92 ml of buffer, 60 µl of 10 mM MP preincubated at 30°C and 20 µl of the crude enzyme solution were added, and the rate of MP hydrolysis was measured by monitoring the change of absorbance (405 nm) using a Beckman DU800 spectrophotometer. A solution without the crude enzyme was used as a negative control. Enzymatic activity was expressed as units (µmol of *p*-nitrophenol formed per minute) per milligram of protein ($\varepsilon 405 = 17{,}700\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol).

### Detoxification of OP residues on vegetable by MPH

The test samples were prepared as follows: Ten grams of vegetable (cabbage) was sprayed with a known amount of pesticide followed by drying. The contaminated sample was suspended in 50 ml of enzyme or detergent solution (water for control) and incubated on an incubator shaker at 30°C and 150 rpm for 30 min. The vegetable was recovered and washed with water three times followed by drying at room temperature for 30 min. The vegetable leaves were then chopped into fine pieces and shaken vigorously with 30 g of anhydrous $Na_2SO_4$ and 0.2–0.8 g of activated carbon in a 250-ml glass vessel to dehydrate and remove the color. Subsequently, the residual pesticides on the leaves were extracted with 50 ml of $CH_2Cl_2$ for 30 min at 200 rpm followed by filtration, drying, and reconstitution by acetone and, finally, they were analyzed by gas chromatography (GC).

An HP-5890 Series II gas chromatograph equipped with an electronic capture detector and a fused silica capillary HP-1 column (25 m long, 0.32 mm i.d., 0.25 µm film thickness) was used for analysis of the pesticides. Methyl parathion was detected using nitrogen as a carrier gas at a flow rate of 1 ml/min. The injector, column, and detector temperatures were set at 300, 230, and 310°C, respectively.

### Bioassay

A mosquito larvae test was used to determine the detoxification efficiency and the toxicity of the degraded products following the method described by Raymond and Marquine (1994). Depending on the number of available larvae, two doses and three replications per dose were used with each insecticide or enzyme products according to mosquito's $LD_{50}$. Organophosphate insecticides, methyl parathion, parathion and malathion, were used in ethanol solutions.

## Results and discussion

### Plasmid construction for overexpression of MPH in *E. coli*

Recombinant plasmid pETM coding for mature MPH was obtained by insertion of the truncated *mpd* gene into pET30a and expression of recombinant MPH was under the control of T7 promoter. After the recombinant plasmid pETM was expressed in *E. coli* BL21 (DE3), a clear IPTG inducible band corresponding to about 32 kDa was observed in SDS-PAGE (Fig. 1). The molecular mass of the expression product was identical to the calculated mass of MPH. This protein accounted for 17.7% of total cell proteins based on densitometry of the gel.

The intact MPH contained a signal peptide-like sequence comprised of a 35-amino acid sequence at amino terminus, which was not necessary for the enzyme activity (Fu et al. 2004). In our lab, an effective chlorpyrifos-degrading bacterium was isolated from the sludge of the wastewater treating system of an OPs manufacturer. The *mpd* gene was cloned from the bacterium and expressed successfully in *E. coli* (Yang et al. 2006). In *Stenotrophomonas* sp. strain YC-1, the expression and posttranslational processing of the wild-type MPH may be well regulated. In the recombinant *E. coli* BL21, the high-level expression of the full-length MPH driven by the strong T7 promoter from plasmid pET30a resulted in the accumulation of the large amounts of unprocessed proteins. Since the initially synthesized proteins could not be effectively processed to produce active MPH, most of them were present in the form of an insoluble inclusion body.

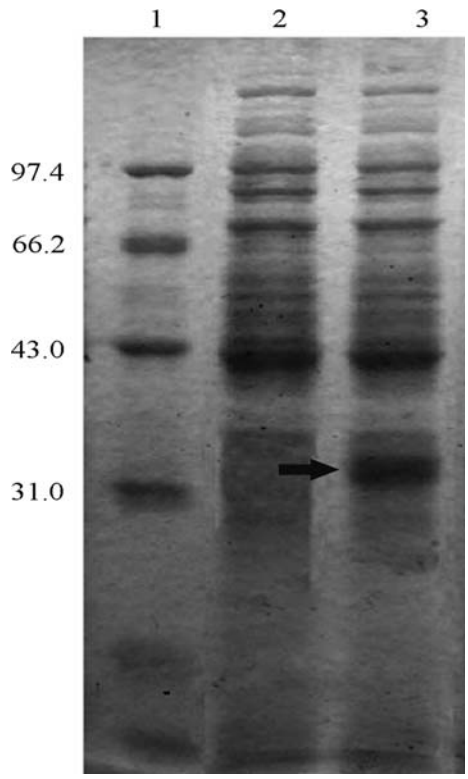


**Fig. 1** SDS-PAGE analysis of expression of BL21 (DE3) with or without plasmid pETM by IPTG induction. Lane 1: protein molecular weight standards (kDa), lane 2: protein of IPTG-induced without pETM, lane 3: protein of IPTG-induced with pETM. Arrows showed the new protein (32 kDa) bands expressed in the recombinant *E. coli* strains

In this study, the mature MPH without a signal peptide was subcloned into pET30a and expressed in *E. coli* BL21 (DE3). The expression of mature MPH in *E. coli* favored the posttranslational processing and reduced the formation of insoluble inclusion bodies, which led to the production of much active enzymes. These results indicated that the expression of a truncated *mpd* gene encoding mature MPH enhanced the yield of active MPH.

### Overexpression of methyl parathion hydrolase using lactose as inducer

In *E. coli* expression systems, the natural inducer of the *lac* operon, lactose, is employed as an alternative inducer instead of IPTG, especially in large-scale fermentations (Neubauer and Hofmann 1994). Therefore, we tested whether the high-level expression of MPH was achieved in *E. coli* BL21 (DE3) cells using

lactose as the inducer. In this study, the use of different concentrations of lactose (6–30 mM) as the inducer obtained higher total activity than with 1 mM IPTG induction (Fig. 2). IPTG yielded 1.2 g/l while peak expression at 25 mM lactose gave 2.4 g/l, a 2-fold increase in active MPH. These results indicated that lactose could induce expression of active MPH in the BL21 (DE3) cell line, and that expression levels could exceed those achieved with IPTG. However, high concentrations of lactose (150 mM) inhibited protein expression. Since there was no change in cell density or cell weight at harvest in these samples, this decrease in MPH expression is not due to cell death but is assumed to be a result of lactose or a by-product of its metabolism.

As shown in Fig. 3, the highest specific MPH activity was found when the *mpd* gene was induced by 25 mM lactose, at 22°C. It yielded $1.4 \times 10^4$ U of specific enzyme activity at 22°C for 8 h, however, it only yielded $9.4 \times 10^3$ U of specific enzyme activity at 30°C for 8 h. It was increased 48% compared to that from the same period at 30°C. At the same time, no significant decrease was found in cell density between 22 and 30°C. At lower induction temperature the rate of protein synthesis declines, which may be favorable to the formation of properly folded proteins (Knappik et al. 1993). Lower growth temperatures were able to reduce substantially the amount of inactive MPH in inclusion bodies, as described in many reports about expression of recombinant proteins in *E. coli* (Schein and Noteborn 1988). The result of MPH production supports the

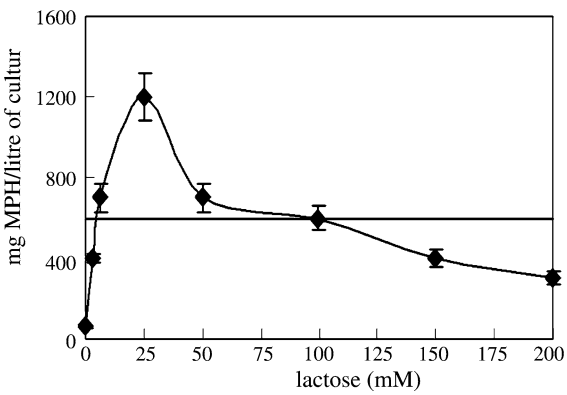


**Fig. 2** Expression of MPH using the pET30a vector in the BL21 (DE3) cell line using 0–200 mM lactose or 1 mM IPTG (dashed line). Data are the mean ± SD from three independent experiments

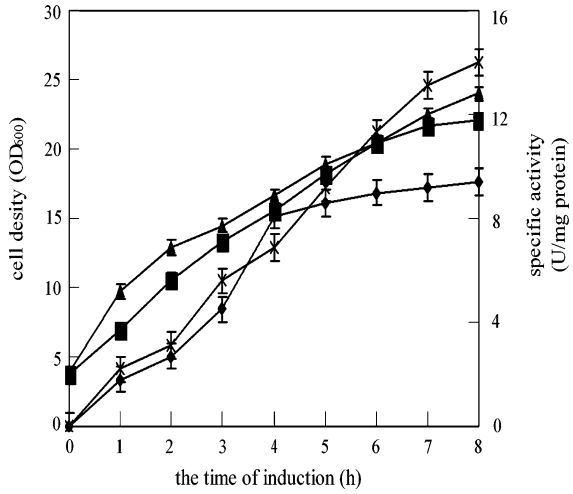


**Fig. 3** Growth and specific activity time profiles by lactose induction with 25 mM at 22 and 30°C (■, cell density at 22°C; ▲, cell density at 30°C ♦; specific activity at 30°C; ×, specific activity at 22°C). Data are the mean ± SD from three independent experiments

view that lowering temperature in fermentation conditions might yield higher levels of enzyme activity, as formation of properly folded proteins. In addition, proteolysis, both ATP-dependent and ATP-independent, is shown to decrease at lower induction temperature (Kosinski and Bailey 1991). These reasons might account for the increase in protein yield and enzyme activity with decreasing induction temperature.

IPTG is the most expensive component of expression experiments and use of 1 mM concentration results in a cost of $1.1/l. In a large-scale fermentation (e.g., 200 l) the cost of IPTG would be $234. For the pET30a/*mpd* expression systems utilizing 25 mM lactose for optimum expression the cost for lactose would be $0.08/l ($16/200 l), 13.8-fold less than IPTG and no longer the most expensive part of expression. If one considers that lactose results in increased MPH expression and low temperature results in higher specific enzyme activity the expense of expression is even further reduced.

## Plasmid stability

The instability of cell cultures containing plasmid vectors is a major problem in the commercial exploitation of molecular cloning techniques. The high activity of T7 RNA polymerase has made the T7

RNA polymerase-based expression system very powerful for high-level expression of recombinant protein. However, the overactivity of T7 RNA polymerase would also bring about negative effects on plasmid stability and protein production, especially when expressing a toxic protein. If the latter role is dominant, it is necessary to adopt some measures to attenuate the activity or the amount of T7 RNA polymerase in the cells. Apart from the stringent regulation by inserting some genes reducing the amount or the activity of T7 RNA polymerase into plasmids, optimizing the culture conditions would be another way. In this work, we have studied the effects of culture medium, selective pressure and culture temperature on the plasmid stability (Fig. 4). The results have indicated that adding antibiotic after induction has little effect in increasing plasmid stability, but inducing expression at low temperature and adding glycerol to the medium improved the plasmid stability (Matsui et al. 1990).

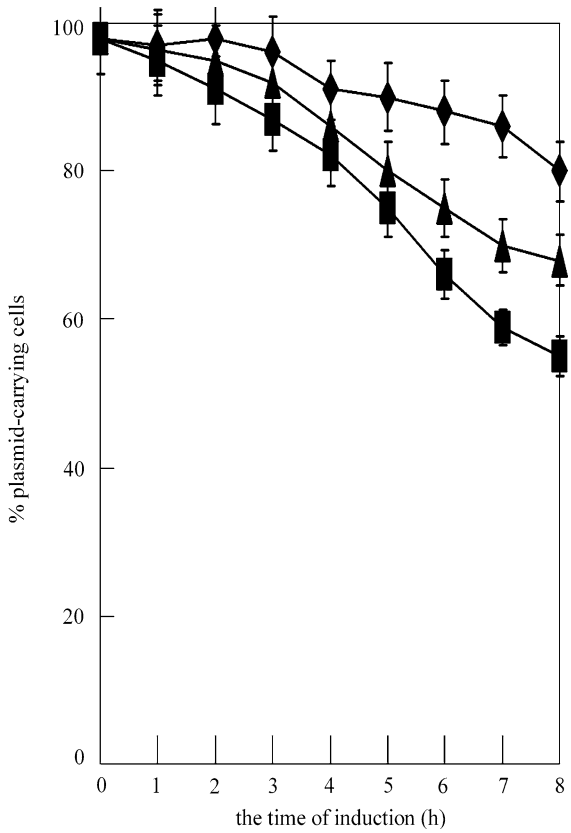


**Fig. 4** Plasmid stability of different culture systems at various induction times. No. 1 system (■): LB added 50 μg/ml kanamycin, at 30°C; No. 2 system (▲): modified medium added 25 μg/ml kanamycin, at 22°C; No. 3 system (♦): modified medium added 25 μg/ml kanamycin at 30°C. Data are the mean ± SD from three independent experiments

## Effects of pH and temperature on the specific activity and storage stability

The pH and temperature profiles were shown in Fig. 5. The optimal pH and temperature of MPH were 8.0 and 30°C, respectively. More than 90% of the maximum activity was retained in the pH range from 7 to 9 and in the temperature range from 25 to 35°C. The specific activity of MPH was determined monthly, and the cell powder was stored at room temperature and 4°C. As shown in Fig. 6, the specific activity was virtually unchanged during a 5-months

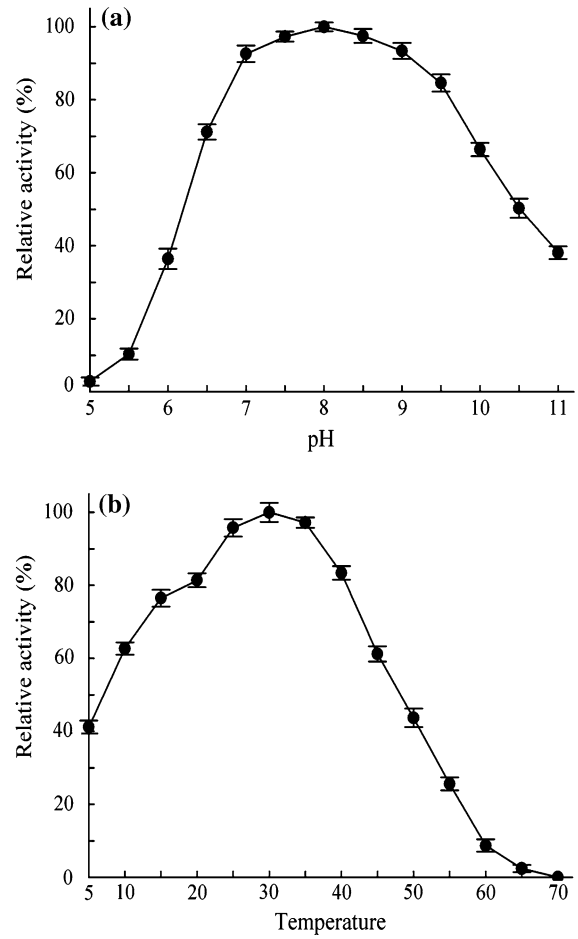


**Fig. 5** Effect of pH (**a**) and temperature (**b**) on the activity of MPH. The enzyme activity (U/mg) measured was defined as 100%. Data are the mean ± SD from three independent experiments

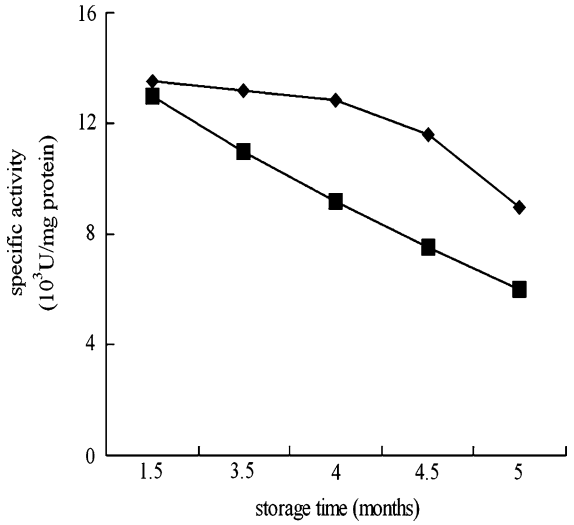


**Fig. 6** Stability of special activity (♦, cell powder at room temperature; ■, suspension in buffer at 4°C)

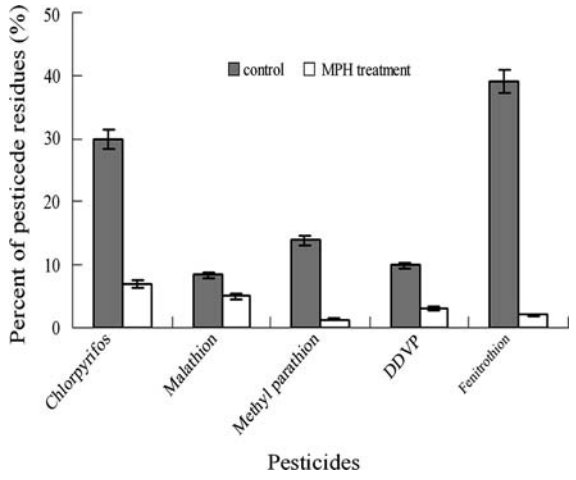


**Fig. 7** Efficiency of removal of organophosphate residues on vegetables as percentage of amount sprayed when washed with water and enzyme solution. Data are the mean ± SD from three independent experiments

period. Additionally, once suspended in buffer, the suspension retained the MPH activity for up to 45 days at room temperature and 3 months when stored at 4°C. The excellent long-term stability of MPH in cells when stored either as dry powder or as liquid suspension at 4°C is very promising for application in detoxification of organophosphates.

## Detoxification of pesticides

To evaluate the promise of MPH for detoxification of organophosphate pesticides, the enzyme extract of dried cells in pH 8.0 phosphate buffer was applied to degrade methyl parathion, fenitrothion, chlorpyrifos, DDVP, malathion, sprayed on vegetables, and the degradation efficiency was compared with that achieved by washing the vegetables with the detergent shuguoqing (product 1) and enzyme jiankangxiguan (product 2) purchased from the market. As shown in Table 1, washing the methyl parathion-contaminated vegetables with the enzyme extract of the cell powder with MPH activity produced the best detoxification, 98%, compared with 94–86% achieved by washing with commercially available enzyme product, detergent, or water. The addition of acid and base to the extract (in product 3) inhibited the activity of the MPH, probably owing to the loss of or decrease in the activity of the enzymes (details in Table 1).

MPH has a broad substrate specificity and is able to degrade various dimethyl and diethyl OPs (Cui et al. 2001; Yang et al. 2006). We observed similar effective degradation of methyl parathion, parathion, chlorpyrifos, DDVP and fenitrothion sprayed on vegetables when the extract of dried cell powder harboring MPH enzymes was used in comparison with washing with water alone (Fig. 7). Although

**Table 1** Efficiency of MPH cell powder extract and other products in degrading methyl parathion-contaminated cabbage

| MPH or detergent | Quantity of MPH or detergent | Methyl parathion (added) (mg) | Methyl parathion residue (%) |
|---|---|---|---|
| Control | 0 | 1.9 | 13.90 ± 1.41 |
| Product 1: shuguoqing | 60 μl | 1.9 | 12.80 ± 1.30 |
| Product 2: jiankangxiguan | 0.1 g | 1.9 | 8.00 ± 0.83 |
| Cell powder (add acid or base) | 0.1 g (0.17/0.67) | 1.9 | 6.22 ± 0.76 |
| Cell powder (MPH) | 0.1 g | 1.9 | 1.32 ± 0.25 |

**Table 2** Bioassay of mosquito larvae exposed to enzyme-hydrolyzed products of pesticide

| Organophosphate | Organophosphate concentration (ppm) | Cell powder (g) | Surviving larvae after MPH treatment (%) | Surviving larvae without treatment (%) |
|---|---|---|---|---|
| MP | 0.004 | 0.0025 | 86 ± 8.2 | 0 |
| MP | 0.004 | 0.0030 | 98 ± 10 | 0 |
| MP | 0.003 | 0.0025 | 97 ± 9.3 | 9 ± 0.8 |
| Malathion | 0.9 | 0.0100 | 5 ± 0.8 | 5 ± 0.8 |
| DDVP | 0.3 | 0.0025 | 100 | 43 ± 4.7 |

effective in degrading pesticides with P–O bonds, washing of the vegetables sprayed with the thiolester organophosphate insecticide malathion with the enzyme did not yield any improved degradation.

Bioassays

To demonstrate further the efficacy of enzyme-based detoxification of organophosphates, we performed bioassays on Beijing Chaoyang mosquitoes. The bioassay test involved determining the percentage of mosquito larvae surviving exposure to organophosphates before and after treatment with *E. coli* cell powder. As shown in Table 2, most of the larvae were killed on exposure to as low as 3 ppm of parathion. By contrast, >97% larvae were alive when exposed to a solution of 3 and 4 ppm of parathion treated with 2.5 and 3 mg of cell powder, respectively. Similar results were observed for the pesticide DDVP, with the survival percentage increasing from 43% to 100% for 300 ppm of DDVP treated with 2.5 mg of dry cell powder. The high percentage of larvae surviving exposure to hydrolyzed products suggests that these products were not toxic/poisonous and, hence, safe for insects. As expected, there was no change in the survival percentage for malathion, because MPH has no effect on the degradation of this thiolester organophosphate pesticide.

## Conclusion

The results presented in this study demonstrated that MPH was overexpressed in genetically engineered *E. coli* by lactose induction at low temperature, which would be suitable for laboratory-scale preparation or industrial-scale production. The cell suspension was effective in decontaminating organophosphates sprayed on vegetables and the hydrolyzed products were not toxic.

**Acknowledgements** This work was supported by grants from the 863 Hi-Tech Research and Development Program of the People's Republic of China (No. 2007AA06Z335) and the Innovation Program of the Chinese Academy of Sciences (No. KSCX2-YW-G-008). We appreciate valuable comments and suggestions of reviewers.

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