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ORIGINAL PAPER

Genetic surface-display of methyl parathion hydrolase on *Yarrowia lipolytica* for removal of methyl parathion in water

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Abstract In this study, the *mph* gene encoding methyl parathion hydrolase from *Pseudomonas* sp. WBC-3 was expressed in *Yarrowia lipolytica* and the expressed methyl parathion hydrolase was displayed on cell surface of *Y. lipolytica.* The activity of methyl parathion hydrolase displayed on the yeast cells of the transformant Z51 was 59.5 U mg^{-1} of cell dry cells (450.6 U per mL of the culture) in the presence of 5.0 mM of Co^{2+}. The displayed methyl parathion hydrolase had the optimal pH of 9.5 and the optimal temperature of 40 °C, respectively and was stable in the pH range of 4.5–11 and up to 40 °C. The displayed methyl parathion hydrolase was also stimulated by Co^{2+}, Cu^{2+}, Ni^{2+} and Mn^{2+}, and was not affected by Fe^{2+}, Fe^{3+}, Na^{+}, K^{+}, Ca^{2+} and Zn^{2+}, but was inhibited by other cations tested. Under the optimal conditions (OD_{600nm} = 2.6, the substrate concentration = 100 mg L^{-1} and 40 °C), 90.8 % of methyl parathion was hydrolyzed within 30 min. Under the similar conditions, 98.7, 97.0, 96.5 and 94.4 % of methyl parathion in tap water (pH 9.5), tap water (pH 6.8), seawater (pH 9.5) and natural seawater (pH 8.2) were hydrolyzed, respectively, suggesting that the methyl parathion hydrolase displayed on the yeast cells can effectively remove methyl parathion in water.

Keywords Methyl parathion hydrolase · Surface display · *Yarrowia lipolytica* · Methyl parathion detoxification · Pesticide pollution

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Introduction

Methyl parathion (MP) is a member of organophosphorus pesticides which are being widely used in agriculture, plant protection and chemical warfare agents and considered to be extremely toxic (Theriot and Grunden 2011). It would cause fatal effects on vertebrate such as fishes and mammals at high concentration for its roles of acetylcholinesterase (AchE) inhibitor and would lead to various chronic clinical effects to humans when they exposed to it at a low concentration for a long time (Theriot and Grunden 2011). MP can be frequently detected in both terrestrial and aquatic environments and is classified as both a persistent organic pollutant and environmental endocrine-disrupting chemical (Liu et al. 2007). Therefore, its degradation and effective removal from the polluted environments have received considerable attention (Pakala et al. 2007). Current techniques for detoxifying organophosphate pesticides include harsh chemical treatment, incineration, and landfills. These processes also lead to serious environmental pollution.

Methyl parathion hydrolase (MPH) has the ability to hydrolyze MP to ρ-nitrophenol (PNP) and dimethylthiophosphoric acid which fail to inhibit acetyl cholinesterase (AchE) and, thus, have reduced toxicity towards higher animals with well-developed nervous systems by 120-fold (Theriot and Grunden 2011). It has been found that some bacteria such as *Plesiomonas* sp. M6 (Fu et al. 2004), *Pseudomonas pseudoalcaligenes* (Wu et al. 2004), *Pseudomonas* sp. strain WBC-3 (Liu et al. 2005), *Serratia* sp. strain DS001 (Pakala et al. 2007), *Acinetobacter radioresistens* USTB-04 (Liu et al. 2007), *Stenotrophomonas* sp. SMSP-1 (Shen et al. 2009), and *Burkholderia* sp. FDS-1 (Zhang et al. 2006) can produce MPH, and the genes encoding these hydrolases have been cloned and expressed in *P. pseudoalcaligenes, Escherichia coli*, *Streptomyces lividans* and *Pichia pastoris* (Wu et al. 2004; Fu et al. 2004; Yu et al. 2009; Shen et al. 2009). However, it has not been found that yeasts isolated from natural environment can produce MPH. In addition, MPH has many promising applications, including the therapy for organophosphorus poisonings, bioremediation of the polluted environments, biodegradation of organophosphorus residues, and development of biosensors for organophosphates (Yang et al. 2008a). However, most of the bacteria-derived MPH are located in the cells. The transport limitation of organophosphates across the cell membrane of bacteria is a barrier to whole-cell degradation efficiency, and it becomes a bottleneck in this biodegradation process (Yang et al. 2008c). In order to use MPH in practical environment bioremediation e.g. MP polluted water or soil disposal, this enzyme should be very handy and stable during its use and could be recovered for the second-chance recycle (Yang et al. 2008c).

In recent years, microbial surface displayed enzymes have received increasing attention as the enzymes can be regenerated according to the activation of the promoter, and they are 'naturally' immobilized on the cell surface and can be reused for several times (Ueda and Tanaka 2000). In order to anchor the heterologous proteins to cell surface of yeast cells, it is necessary to fuse the proteins to the *C*-terminal half regions of glycosylphosphatidylinositol (GPI)-cell wall protein (CWP) of the yeast cell wall (Ueda and Tanaka 2000). Like *Saccharomyces cerevisae*, the structure of GPI-CWP in the cell wall of *Yarrowia lipolytica* is composed of a secretion signal region, an active region, a support region rich in serine and threonine, and a putative GPI anchor-attachment signal (Yue et al. 2008). Therefore, the surface display vector pINA1317- YlCWP110 for protein display on the yeast *Y. lipolytica* was constructed using *C*-terminal anchor domain of YlCWP1 from *Y. lipolytica* in our laboratory (Yue et al. 2008). The vector has many advantages over any other surface display vectors (Yue et al. 2008). Although organophosphorus hydrolase including MPH has been displayed on the surface of *E. coli* (Li et al. 2008; Yang et al. 2008c) and *Pseudomonas putida* JS44 (Yang et al. 2008b), MPH has not yet been displayed on the yeast cells for hydrolysis of MP so far.

Pseudomonas sp. strain WBC-3 could produce efficient MPH and its encoding gene (*mph* gene) has already been cloned (Liu et al. 2005). Therefore, in this study, the *MPH* gene from *Pseudomonas* sp. strain WBC-3 was cloned into the surface display vector pINA1317-YlCWP110 and expressed in the cells of *Y. lipolytica* and the recombinant MPH was displayed on the yeast cells. The displayed MPH was characterized and used for removal of MP in water.

Materials and methods

Strains, plasmids and media

The yeast *Y. lipolytica* Po1h (*MatA, ura3-302, xpr2-322, axp1-2*, *ΔAEP, ΔAXP, Suc*$^+$) (Madzak et al. 2004) was used as a host for cell surface display; The surface display vector pINA1317-YlCWP110 which contains the *C*-terminal end of *YlCWP1* from *Y. lipolytica* was constructed in this laboratory (Yue et al. 2008); the pET20b vector carrying MPH gene cloned from *Pseudomonas* sp. WBC-3 was kindly donated by Professor Xian-en Zhang from Wuhan Institute of Virology. The signal peptide sequence of this gene has already been eliminated through PCR. The pMD19-T was purchased from TaKaRa (Japan). The yeast strain was grown in YPD medium (g L^{-1}) (10.0 yeast extract, 20.0 bacto-peptone, 20.0 glucose). The yeast transformants were selected on YNB medium (1.7 g L^{-1} yeast nitrogen base without amino acids and ammonium sulfate, 10.0 g L^{-1} glucose, 5.0 g L^{-1} ammonium sulfate). The PPB medium was used to cultivate the yeast transformants for production of the displayed enzyme (Jolivalt et al. 2005). The

Table 1 The primers used in this study

P1s	5′-GCCGCACCGCAGGTGCGCACCTCGG-3′
P1a	5′-TCGGCCGG**GTCC**GTGGGTCCGAACAGGC-3′ (the underlined bases are the shared bases with P2s and the bold and underline bases are the mutated bases)
P2s	5′-GACAAG**CCTG**GGTGCCTGGGCCGGCTG GC-3′ (the underlined bases are the shared bases with P1a and the bold and underline bases are the mutated bases)
P2a	5′- TCACTTGGGGTTGACGACCGAGTAGTTCACCGGCACG-3′
mph1	5′-GGCCGTTCTGGCCGCCGCACCGCAGGTGCG-3′ (the bases underlined encode *SfiI* restriction site)
mph2	5′-GGATCCTCAGT*GATGGTGATGGTGATGCTTGG*GGTTGACGACCGAGTAG -3′ (the bases underlined encode *BamHI* restriction site and the bases *italicized* encode 6-His tag)

E. coli strain DH5α [*F* − *endA1 hsdR17(rK* − */mK* +) *supE44 thi-1λ* − *recA1 gyr96ΔlacU169(80lacZΔ M15)*] used for plasmid recovery and cloning experiments was grown in Luria broth (LB). The *E. coli* transformants were grown in LB medium with 30 μg mL^{-1} of kanamycin.

Site-directed mutagenesis of the *MPH* gene

After analysis of the DNA sequence of the *mph* gene used in this study, it was found that the gene had the same *Sfi*I restriction site as in the surface display vector pINA1317-YlCWP110 (Yue et al. 2008). In order to guarantee the subsequent construction of the recombinant surface-display vector, the *Sfi*I restriction site inside this *mph* gene needed be mutated on the condition that the corresponding protein sequences must not be changed. Therefore, in order to change the original four bases GGCC into GGAC to achieve the mutation, the primer pairs P1s and P1a and the primer pairs P2s and P2a (Table 1) were designed according to the *mph* gene (GenBank accession No. AY251554), respectively for PCR. The PCR amplification was carried out using the primers pairs P1s and P1a and the primer pairs P2s and P2a and the plasmid pET20b as the template, respectively. The PCR products from the two PCR amplifications were mixed, denatured, allowed to anneal using the shared sequence of the primers and subjected to a fusion PCR amplification, using the flanking primers P1s (5′) and P2a (3′) as described above. The amplification products were separated by electrophoresis on a 10.0 g L^{-1} agarose gel and the expected 1,908 bp fragments were extracted using TaKaRa Agarose Gel DNA Fragment Recovery Kit Ver.2.0 (Invitrogen), and sequenced to verify the correct *mph* gene. The recovered fragments were ligated into the plasmid pMD19-T simple vector and the resulting plasmid carrying the fragments was designated as pMD19-T simple-mphM. All the primers used in this study are shown in Table 1.

Construction of the recombinant plasmid for display of the MPH on cells of *Y. lipolytica*

According to the mutated *mph* gene sequence as described above and the multiple cloning site of the surface display vector pINA1317-YlCWP110 (Yue et al. 2008), a pair of primers mph1 and mph2 (Table 1) were designed in order to make the bases encoding 6-His tag be ligated into 3′-end of the site-mutated *mph* gene. PCR amplification was processed with pMD19-T simple-mphM as a template and the PCR reaction system was composed of 5.0 μL of 10 × Ex Taq buffer (TaKaRa, Japan), 5.0 μL of 2.5 mM dNTP, 1.0 μL of 20.0 μM of each primer, 1.0 μL of the plasmid DNA, 0.5 μL of La Ex DNA polymerase, and 36.5 μL of double-distilled water. PCR cycle parameters were 94 °C 3 min; 94 °C 30 s, 70 °C 30 s, 72 °C 1 min, 30 cycles; final extension at 72 °C for 10 min. The PCR products were separated in agarose gel and recovered using TaKaRa Agarose Gel DNA Fragment Recovery Kit Ver.2.0. The recovered PCR products were ligated into pMD19-T simple vector and transformed into the competent cells of *E. coli* DH5α. The transformants were selected on the LB plates with ampicillin. The plasmids were extracted with TIANprep Mini Plasmid Kits [TIANGEN BIOTECH (Beijing) CO., LTD.] and sequenced; the resulting recombinant plasmid was named pMD19-T simple-mph and applied to the subsequent experiments.

The MPH display on the yeast cells of *Y. lipolytica* was performed as described by Yue et al. (2008). pMD19-T simple-mph was digested with *Sfi*I at 50 °C

overnight, followed by *BamH*I digestion at 37 °C for 3 h. The digests containing the *mph* gene were recovered using TaKaRa Agarose Gel DNA Fragment Recovery Kit Ver.2.0. The recovered digest products were ligated into the surface display vector pINA1317-YlCWP110 digested with the same enzymes (Yue et al. 2008) and the resulting plasmid carrying the *mph* gene was designated as pINA1317-YlCWP110-MPH (Fig. 1). The plasmid was transformed into *E. coli* DH5α. The transformants were selected on the LB plates with kanamycin. The plasmids in the transformants were extracted and purified as described above. Then, pINA1317-YlCWP110-MPH extracted from *E. coli* transformants was digested with *Not*I. The linear fragments carrying YlCWP110-MPH were recovered using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0.

Transformation and selection

The recovered linear fragments as described above were transformed into *Y. lipolytica* Po1h by lithium acetate method (Xuan et al. 1988). The transformants were spread on the YNB plates and grown at 28 °C for 96 h. *Y. lipolytica* Po1h only carrying fragment YlCWP110 without the *mph* gene was used as a control. The putative positive transformants were verified on the YNB plates supplemented with 20 mg L^{-1} MP (Dr. Ehrenstorfer GmbH, 10 mg mL^{-1}, stock in methanol) to observe the yellow halos around the colonies. Furthermore, after the different positive transformants were grown at 28 °C in 50.0 mL of PPB liquid medium in 250-mL flask by shaking at 180 rpm for 4 days, respectively, the MPH activity of different positive transformants was determined as described below. Cell density (OD_{600nm}) of the cultures was determined at 600 nm with spectrophotometer.

Enzyme activity assay

MPH activity was detected using a spectrophotometric assay that measured the appearance of PNP (Yu et al. 2009). The cells in the cultures obtained above were washed three times with phosphate-buffered saline (PBS) buffer (pH 7.2) by centrifugation at 6,000×*g* and 4 °C for 5 min. The pellets obtained were resuspended in 40.0 mM sodium phosphate (pH 8.0) of the same volume. The assay mixture consisted of 944.0 μL of 40.0 mM glycine–NaOH (pH 9.5), 6.0 μL of 10.0 mg mL^{-1} MP as the substrate and 50.0 μL of the

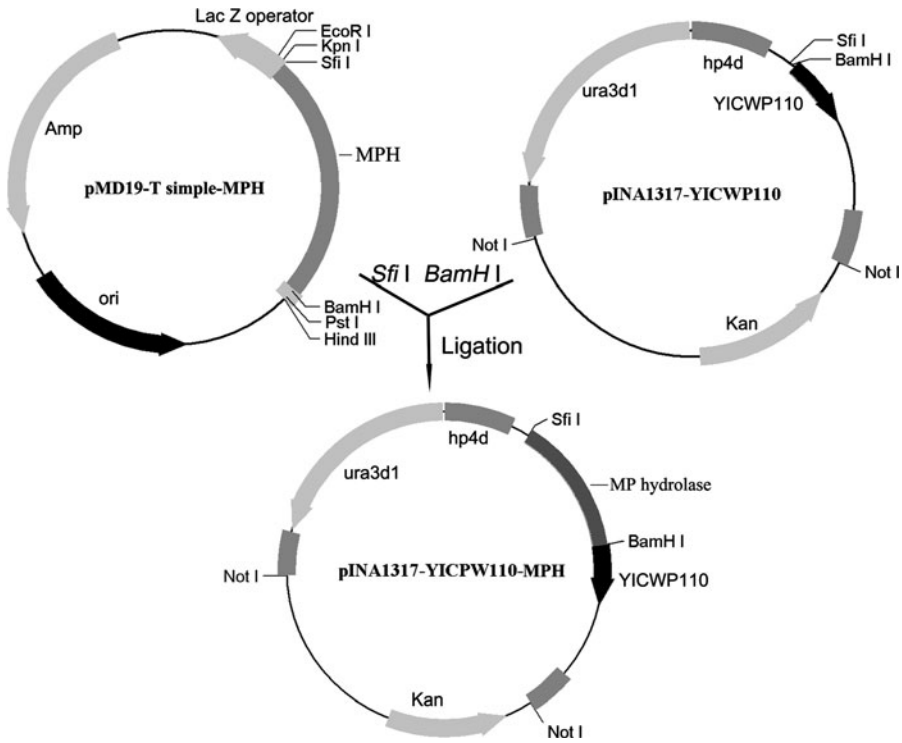

Fig. 1 Construction of the plasmid pINA1317-YlCWP110-MPH for MPH surface display on *Y. lipolytica* cells

washed cells (4×10^8 cells mL^{-1}). The reaction was run at 35 °C for 10 min and then terminated by the addition of 200.0 μL of 10 % HNO_3. After that, 500.0 μL of the reaction mixture was mixed with 5.0 mL of 0.5 M glycine–NaOH buffer (pH 10.0) to develop the color. Calibration curve was prepared using the solution of different concentrations of PNP which served as a standard. One unit (U) of MPH activity is defined as the amount of the displayed enzyme that produces 1.0 μM of PNP in 1 min at 35 °C. Finally, it was found that the transformant Z51 had the highest MPH activity. Therefore, the transformant Z51 was used in the subsequent investigations.

Confirmation of integration of the target gene into *Y. lipolytica* genome

To certify that DNA fragments carrying YlCWP110-MPH have been integrated into the genome of the transformant Z51, the genomic DNA from the corresponding transformant was extracted and used as the template for PCR. The forward primer and the reverse primer used for this checking were mph1 and mph2 as described above, respectively. PCR amplification was performed as described above. The sizes of the PCR products were evaluated using the automated gel documentation and analysis system (Gene-Genius, USA) and the PCR products were sequenced by Shanghai Sangon Company.

Immunofluorescence microscopy

The immunofluorescence microscopy was conducted as described by Adams et al. (1998) using 6 × His monoclonal antibody produced by Clontech (USA) as the primary antibody, and immunoglobulin G/fluorescein isothiocyanate (IgG/FITC) as the secondary antibody produced by ZSGB-BIO (China). *Y. lipolytica* Po1h and the transformant Z51 were grown in PPB liquid medium for 104 h, respectively. The cells were collected and washed three times by centrifugation with PBS buffer. The pellets obtained were suspended in 3.7 % formaldehyde and incubated overnight at 28 °C with shaking. The yeast cells were suspended in the buffer containing 250 μL PBS and 1.0 mg/mL BSA. Then, 1.0 μL of the 6 × His monoclonal antibody was added to the cell suspension and the mixture was incubated for 30 min. The treated cells were washed with PBS buffer by centrifugation. The washed yeast cells were resuspended in the buffer containing 250.0 μL PBS and 1.0 mg/mL BSA. Then, 1.0 μL of IgG/FITC was added to the cell suspension and the mixture was incubated in the dark and at 0 °C for 30 min. Finally, the labeled cells were observed under the fluorescence microscope and photographed.

Proteinase accessibility assay

Five milliliters of the transformant Z51 cells harboring pINA1317-mph were centrifuged and resuspended in 1.0 mL of 150.0 g L^{-1} sucrose, 15.0 mM Tris–HC1, and 0.1 mM EDTA, pH 7.8 and 5.0 mL of 20.0 mg mL^{-1} proteinase K was added to the cell suspension. The mixture was incubated at room temperature for 1 h. To inhibit further proteinase K activity, 10.0 mM phenylmethylsulfonylfluoride was added after the incubation. The proteinase K-treated and untreated cells were assayed for MPH activity as described above.

Effects of pH and temperatures on the displayed MPH activity and stability

The effect of pH on the displayed MPH activity was determined by incubating the transformant Z51 cells (the final concentration of the yeast cells was 2×10^7 cells mL^{-1}) displaying MPH along with the substrate MP in the buffers in the pH range of 3.0–12.0 using the enzyme assay conditions as described above. Its pH stability was tested by pre-incubating the yeast cells displaying MPH in the buffers in the pH range of 3.0–12.0 for 24 h at 4 °C and the displayed MPH activity was measured immediately after the incubation using the method as described above. The buffers used were 40.0 mM Na_2HPO_4–citric acid buffer (pH 3.0–8.0) and 40 mM glycine–NaOH buffer (pH 8.5–12.0).

The optimal temperature for the displayed MPH activity was determined at temperatures from 20 to 70 °C in 40 mM glycine–NaOH buffer (pH 9.5). The temperature stability of the displayed MPH was tested by pre-incubating the transformant Z51 cells displaying MPH in 40.0 mM glycine–NaOH buffer (pH 9.5) at temperatures from 20 to 70 °C for 60 min, and the residual activity was measured immediately as described above. The relative MPH activity of the pre-incubated sample at 4 °C was considered to be 100 %.

Effects of different metal ions on the displayed MPH activity

The different metal ions (the final concentration of 5.0 mM) were added to 200.0 μL (4×10^8 cells mL^{-1}) of the transformant Z51 cells displaying MPH and the mixtures were incubated at 4 °C for 60 min and the residual activity was determined after the incubation. The relative activity without metal ion treatment was defined as 100 %. The metal ions tested were Zn^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}, Ca^{2+}, K^+, Mn^{2+}, Hg^{2+}, Li^+, Fe^{2+}, Ag^+, Na^+, Ba^{2+}, Ni^{2+}, and Co^{2+}.

MP hydrolysis

Effect of different MP concentrations on its hydrolysis was studied by varying its concentrations from 20.0 to 120 μL of 10.0 mg mL^{-1} in 10.0 mL of the reaction mixture containing 40.0 mM glycine–NaOH buffer (pH 9.5) and 500 μL of the washed cells (4×10^8 cells mL^{-1}). The mixtures were incubated at 40 °C for 10 min, followed by the measurement of the released PNP as described above. Effect of different cell densities on hydrolysis was investigated by varying the yeast cell concentrations from $OD_{600nm} = 0.4$ to $OD_{600nm} = 4.0$ in 10.0 mL of the reaction mixture containing 40.0 mM glycine–NaOH buffer (pH 9.5) and 100 μL of 10.0 mg mL^{-1} of the substrate. The mixtures were incubated at 40 °C for 10 min, followed by the measurement of the released PNP as described above.

After the optimal conditions for the MP hydrolysis were fixed, the mixtures containing MP and yeast cells were incubated at 40 °C and the sample was withdrawn at time internal of 5 min and the released PNP was determined as described above.

At the same time, the MP hydrolysis in tap and sea water with different pHs was also performed. 500.0 mL of the water containing 20 μg mL^{-1} of MP and 2×10^7 cells mL^{-1} of the yeast cells displaying MP hydrolyase was incubated at 40 °C by shaking at 180 rpm for 40 min. The water containing 20 μg mL^{-1} of MP and distilled water were used the controls. The released PNP was determined as described above. After the first reaction was finished, the yeast cells were collected and washed by centrifugation. Then, the pellets obtained were used for the second reaction and so on.

Results

Surface display of the MPH on *Y. lipolytica*

As stated in "Materials and methods" above, in order to ligate the *mph* gene to pINA1317-YlCWP110, the *Sfi*I site in the *mph* gene must be mutated. After the PCR reaction as described in Materials and methods, the GGCC in the *mph* gene was changed to the GGAC (data not shown). At the same time, the bases encoding 6-His tag were also ligated into the 3′-end of the site-mutated *mph* gene as shown in Table 1. Then, the site-mutated *mph* gene was ligated into the surface display vector pINA1317-YlCWP110 and the resulting pINA1317-YlCWP110-MPH was lineared with *Not*I as shown in Fig. 1. After the lineared DNA fragments carrying the *mph* gene were transformed into the competent cells of *Y. lipolytica* Po1h, the MPH activity of the different transformants (over 200 transformants) obtained was determined as described in Materials and methods. The results in Fig. 2 showed that the transformant Z51 among them had the highest MPH activity (36.5 U mg^{-1} of cell dry weight and 450.6 U per mL of the culture) while *Y. lipolytica* Po1h only carrying fragment YlCWP110 without the *mph* gene (control) had no MPH activity.

After the transformant Z51 and *Y. lipolytica* Po1h only carrying fragment YlCWP110 without the *mph* gene were grown on the plates with methyl parathion, only halo zone was formed around the colony of the transformant Z51 because of the production of PNP (data not shown). After the genomic DNAs of the transformant Z51 and *Y. lipolytica* Po1h were used as the templates and the mph1 and mph2 (Table 1) were used as the primers for PCR, only PCR products from

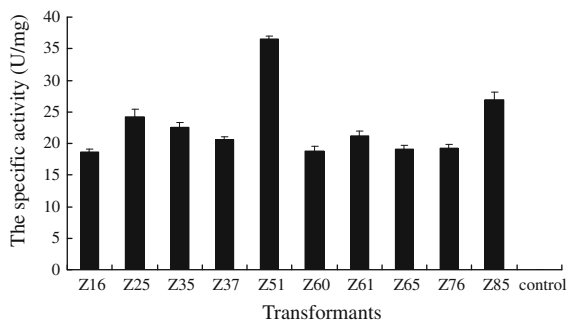

Fig. 2 The MPH activity of the different transformants. The control: *Y. lipolytica* Po1h only carrying fragment YlCWP110 without the *mph* gene. Data are given as means ± SD, $n = 3$

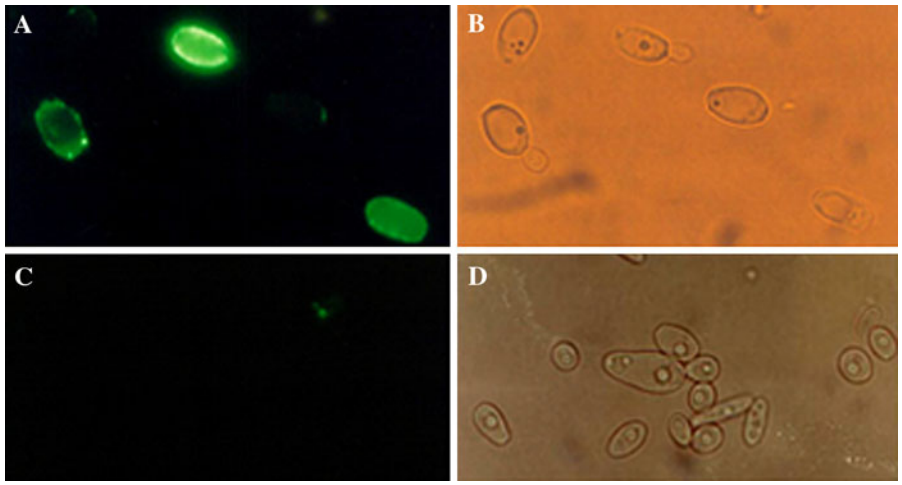

Fig. 3 The immunofluorescent labeling of the transformed *Y. lipolytica* cells using 6 × His monoclonal antibody as the primary antibody and IgG/FITC as the secondary antibody. The microphotographs were taken under visible light (**b**, **d**), and immunofluorescence microphotographs were taken under emission at 550 nm (**a**, **c**). **c**, **d**: *Y. lipolytica* control cells harboring YlCWP110; **a**, **b**: *Y. lipolytica* cells harboring YlCWP110-MPH. Magnification 40 × 10

the genomic DNA of the transformant Z51 were obtained (data not shown). After sequencing of the PCR products, they contained the whole sequence of the mutated *mph* gene, suggesting that the mutated gene indeed has been integrated into the genomic DNA in the transformant Z51.

In order to confirm the presence of the 6 × His-MPH-YlCWP110 fusion protein on the yeast cell surface, the immunofluorescence labeling of the yeast cells was performed with 6 × His monoclonal antibody as the primary antibody and IgG/FITC as the second antibody as described in Materials and methods. It can be clearly seen from the results in Fig. 3 that after the *Y. lipolytica* cells displaying 6 × His-MPH fusion protein were labeled by IgG/FITC, the green fluorescence on the yeast cells was observed, while no fluorescence was observed on the control cells in which only had YLCWP110 integrated into the genome. These results clearly demonstrated that the 6 × His-MPH- YlCWP110 fusion protein was displayed on the cell surface, allowing its recognition by the antibodies. It can also be noticed from the results in Fig. 3 that 100 % of the observed cells displayed MPH.

Proteinase accessibility assay

The proteinase accessibility assay showed that 10.4 % of the MPH activity was detected after the cells were treated with proteinase K (Table 2). This meant that 89.6 % of the MPH activity on the cell surface was removed after the proteinase K treatment. This again demonstrated that the recombinant MPH was indeed displayed on the cell surface of *Y. lipolytica* Po1h.

Cell growth of *Y. lipolytica* Po1h and the transformant Z51

During cell growth of *Y. lipolytica* Po1h and the transformant Z51 in PPB medium, their cell density (OD_{600nm}) was measured. The results in Fig. 4 revealed that the cell density of *Y. lipolytica* Po1h was a little higher than that of the transformant Z51, especially after 18 h of the cultivation. This suggested

Table 2 The results of proteinase accessibility assay

Differently treated yeast cells	Proteinase K-untreated yeast cells	Proteinase K-treated yeast cells
Specific activity (U/mg of cell dry weight)	36.5 ± 0.4	3.8 ± 0.4

Data are given as means ± SD, $n = 3$

that the cell growth of *Y. lipolytica* Po1h was negatively affected after the MPH was displayed on its cell surface. After the statistical analysis using the software SPSS, the results showed that there were significant differences ($P < 0.05$) between the cell growths of *Y. lipolytica* Po1h and the transformant Z51.

Effects of different temperature and pH on the activity and stability of the displayed MP hydrolase

After the activity of the surface displayed MPH was determined at temperatures from 20 to 70 °C, the results in Fig. 5a showed that the activity was the highest at 40 °C. The thermo-stability was investigated by pre-incubating the recombinant cells displaying the MPH in the temperature range of 20–90 °C in the same buffer as described in the "Materials and methods" section for 120 min and the remaining activity was determined. As shown in Fig. 5a, the enzyme was stable up to 40 °C, keeping over 95.2 % activity of the control, but inactivated rapidly at temperatures above this.

After the surface displayed MPH activity was measured in the pH range of 3–12 in buffers with the same ionic concentrations, it could be observed from data in Fig. 5b that its maximum activity occurred at pH 9.5. To investigate pH stability of the displayed MPH, the recombinant cells displaying the MPH were incubated at 4 °C for 24 h in the buffers with the pH range of 3–12 that had the same ionic concentrations and the residual activity was measured. The results in Fig. 5b indicated that the displayed MPH kept stable between pH 3–11.5 in which about 70 % relative activity was remained. However, its activity was lost rapidly when the pH was over 11.5.

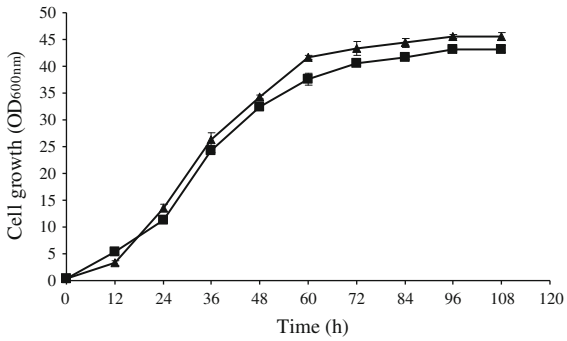

Fig. 4 Cell growth of *Y. lipolytica* Po1h (*filled triangle*) and the transformant Z51 (*filled square*). Data are given as means ± SD, $n = 3$

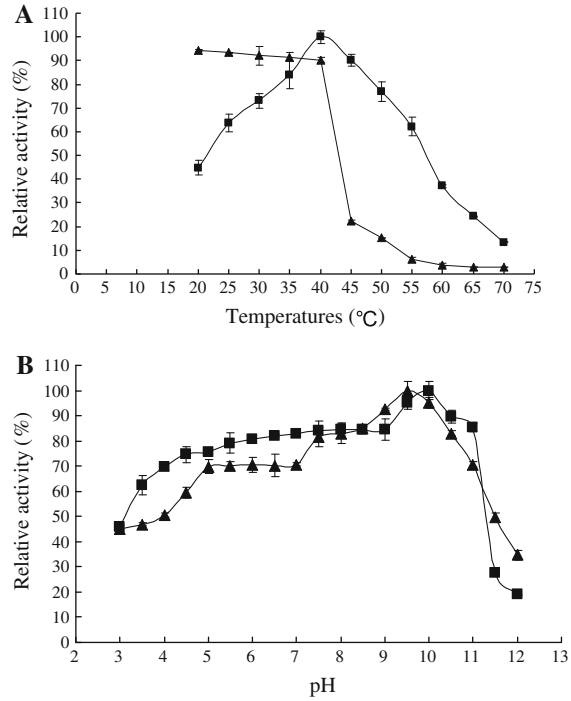

Fig. 5 Effects of different temperatures (**a**) and pHs (**b**) on activity and stability of the displayed methyl-parathion hydrolase. The surface displayed MPH activity was measured at temperatures from 20 to 70 °C (**a**) and in the pH range of 3 to 12 in buffers with the same ionic concentrations (**b**) and The relative MPH activity was calculated. The relative MPH activity of the pre-incubated sample at 4 °C and pH 9.5 was considered to be 100 %, respectively. Data are given as means ± SD, $n = 3$

Effects of different cations on activity of the surface displayed MPH

It has been reported that organophosphate hydrolase is the metal-dependent enzyme and contains two Zn^{2+} or Co^{2+} ions per subunit and the enzyme in which the Zn^{2+} or Co^{2+} ions are replaced by Cd^{2+}, Mn^{2+} or Ni^{2+} also inhibits catalytic activity (Efremenko and Sergeeva 2001). The Co^{2+}-containing enzyme shows the highest catalytic activity with respect to phosphotriester (Efremenko and Sergeeva 2001). Therefore, it is very important to know the effects of different cations on activity of the surface displayed MPH. As shown in Table 3, Co^{2+} (5.0 mM) indeed significantly elevated the activity of the displayed MPH by more than one fold, and Ni^{2+}, Cu^{2+} and Mn^{2+} could slightly

Table 3 Effect of different cations (5.0 mM) on the recombinant methyl-parathion hydrolase activity

Cations	Relative activity (%)
Control	100 ± 1.1
Zn^{2+}	99.6 ± 4.8
Mg^{2+}	93.1 ± 1.5
Ca^{2+}	95.1 ± 1.5
Na^{+}	94.4 ± 1.5
Hg^{2+}	77.9 ± 3.7
Cu^{2+}	131.4 ± 3.6
Mn^{2+}	113.8 ± 2.0
Fe^{3+}	97.3 ± 0.8
Fe^{2+}	95.9 ± 1.1
Ba^{2+}	61.6 ± 3.1
K^{+}	97.8 ± 2.0
Co^{2+}	161.6 ± 1.6
Ag^{+}	50.4 ± 2.8
Ni^{2+}	102.3 ± 4.0
Li^{+}	92.8 ± 1.9

The concentration of the cations in the control was 0. After ANOVA analysis, it was found that there were no significant differences ($P > 0.05$) between the relative activities in the presence of Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, Fe^{2+}, Fe^{3+} and K^{+}. Data are given as means ± SD, $n = 3$

stimulate the activity. However, Li^{+}, Ag^{+}, Ba^{2+} and Hg^{2+} inhibited the activity. The results in Table 3 also showed that the rest of the cations listed in Table 3 had no significant effect on the displayed MPH activity because after ANOVA analysis, it was found that there were no significant differences ($P > 0.05$) between the relative activities in the presence of Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, Fe^{2+}, Fe^{3+} and K^{+}.

Methyl-parathion hydrolysis

In order to use the displayed MPH to remove the MP in environments, it is necessary to optimize the conditions for hydrolysis of methyl parathion. Therefore, effects of different MP concentrations and different cell density of the yeast transformant Z51 in 10.0 mL of the reaction mixture on MP hydrolysis were carefully investigated as described in Materials and methods. It was found that when the MP concentration was 100 mg/L, 46.5 % of MP in the reaction system was hydrolyzed and when the enzyme activity reached 2372.5 U mg^{-1} of the substrate, 61.7 % of MP in the reaction system was hydrolyzed (data not shown). Under such optimal conditions, 90.8 % of the added MP was hydrolyzed within 30 min (Fig. 6). After MP in seawater and tap water with different pHs was hydrolyzed by the displayed MPH, it was found that the pHs and different water had significant influence on MP hydrolysis (Fig. 7). The results in Fig. 7 revealed that 98.7, 97.0, 96.5 and 94.4 % of MP in tap water (pH 9.5), tap water (pH 6.8), seawater (pH 9.5) and natural seawater (pH 8.2) were hydrolyzed within 40 min, respectively and our results also indicated the MPH displayed on the yeast cells could be reused for several times (data not shown). However, the results in Fig. 7 showed that MP hydrolysis in seawater was worse than that in tap water, suggesting that some cations in the seawater may have inhibitory effect on the MPH activity.

Discussion

After the mutated *mph* gene was transformed into *Y. lipolytica* Po1h, the transformant Z51 obtained had the highest MPH activity (36.5 U mg^{-1} of cell dry weight and 450.6 U per mL of the culture) (Fig. 2). The crude activity of MPH from wild-type *Pseudomonas* sp. strain WBC-3 could reach to 6.9 U mg^{-1} protein and the recombined enzyme activity from *E. coli* BL21 was 12.7 U mg^{-1} protein (Liu et al. 2005). Another study of the *mph* gene expression in *P. patoris* revealed that the recombined enzyme activity could come to 5.1 U mL^{-1} (Yu et al. 2009). This demonstrated that the MPH gene from *Pseudomonas* sp. strain WBC-3 had already been over-expressed in *Y. lipolytica* Po1h.

After the mutated *mph* gene with the bases encoding 6 × His tag was integrated into the genomic DNA

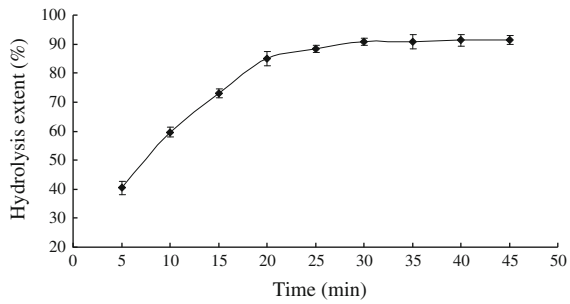

Fig. 6 The effects of different reacting time on the hydrolysis of methyl parathion. Data are given as means ± SD, $n = 3$

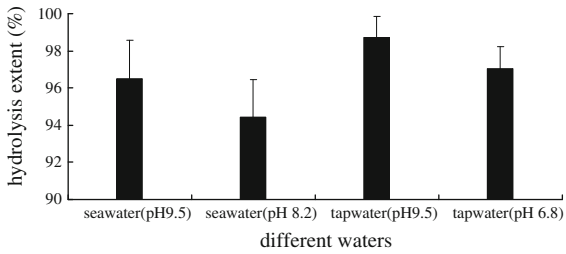

Fig. 7 Hydrolysis of MP in different waters and pHs. After ANOVA analysis, there were significant differences ($P < 0.05$) between hydrolysis extents in seawater and tap water (pH 9.5). However, there were no significant differences ($P > 0.05$) between hydrolysis extents in other cases. Data are given as means ± SD, $n = 3$

of *Y. lipolytica* Po1h and expressed in it (Fig. 3), 100 % of the observed cells displayed MPH (Fig. 4). In our previous studies (Yue et al. 2008; Ni et al. 2009), it was also found that 100 % of the *Y. lipolytica* cells displayed enhanced GFP, hemolysin, and alkaline protease using the same glycosylphosphatidyl inositol-anchor-fusion expression system and the *Y. lipolytica* cells displaying hemolysin and alkaline protease exhibited hemolytic activity toward erythrocytes from flounder and proteolytic activity against all the proteins tested, respectively. However, after OPH (organophophorus hydrolase) gene from *Flavobacterium* species was expressed in the cells of *S. cerevisiae*, only 10 % of the total population expressed OPH on the cell surface and a substantial decline in the number of plasmid-bearing cells with only 50 % of the cells containing the plasmid after 48 h cultivation in the SD-W medium was observed (Takayama et al. 2006). This meant that the surface display technique developed in this study was better than any other techniques.

It has been reported that OPH and GFP are co-displayed on the cell surface of *E. coli* (Yang et al. 2008c). The MPH and enhanced green fluorescent protein (EGFP) fusions were surface-displayed on *E. coli* by employing the Lpp-OmpA chimera as an anchoring motif and it was found that the engineered *E. coli* with surface-expressed MPH-GFP has two major advantages over the same strain expressing cytosolic MPH-GFP, including 7-fold higher whole-cell activity and 2-fold stronger fluorescence (Yang et al. 2008d). However, as *E. coli* is an opportunistic pathogen, any products from it can not be accepted by the people. MPH also has been displayed on the surface of *Pseudomonas putida* JS444 using only *N*- and *C*-terminal domains of the ice nucleation protein (INPNC) from *Pseudomonas syringae* INA5 as an anchoring motif (Yang et al. 2008b). However, expression of MPH in *E. coli* and *P. putida* must be induced with IPTG and this will limit their applications on a large scale. Although the parathion hydrolase encoded by the *OPH* gene from *Flavobacterium* species could be displayed on *S. cerevisiae* MT8-1, the recombinant yeast cells must be grown in the SD-W medium containing adenine sulfate, L-histidine-HCl, L-leucine, and uracil and the medium must be buffered with HEPES at pH 7.0 (Takayama et al. 2006). In contrast, the plasmid pINA317 used in this study uses a strong recombinant growth-phase-dependent promoter, hp4d which makes the expression free from special induction (Madzak et al. 2004). Therefore, when the transformant Z51 was grown in the PBB medium in which the components were simple, no inducer was added (Fig. 2). The vector carries zeta elements, which allow them to integrate genomic DNA in *Y. lipolytica* strains which are generally regarded as safe. Therefore, the cloned *mph* gene was integrated into the genomic DNA of the transformant Z51 (data not shown). Furthermore, only a "yeast expression cassette" was used for the transformation of the recipient strain. The resulting strain is devoid of bacterial DNA, retaining generally regarded as safe status and avoiding the spread of antibiotic resistance genes in the environment. Therefore, the plasmid and the recipient strain had many merits and the transformant obtained was suitable for application in environments.

The activity of the displayed MPH was the highest at 40 °C and the enzyme was stable up to 40 °C (Fig. 5a). The native MPH produced by *Pseudomonas* sp. WBC-3 also displayed relatively broad optimal temperature around 40 °C and was stable at the temperatures from 40 to 60 °C (Chu et al. 2003). This meant that the optimal temperature and stability of the displayed MPH were similar to those of the native one.

The maximum activity of the displayed MPH occurred at pH 9.5 and the displayed MPH kept stable between pH 3–11.5 (Fig. 5b). However, its activity was lost rapidly when the pH was over 11.5. The activity of the native MPH was stable in the pH range from 7 to 11 and the optimal pH was 11.0 (Chu et al. 2003). This meant that some properties of the MPH displayed on the yeast cells were different from those

of the native one. This may be due to the glycosylation of the recombinant MPH in the yeast cells and its fusion to the anchoring motif.

The OPHC2 produced by *P. pseudoalcaligenes* has optimum activity for the reaction at 65 °C and pH 9.0 with MP as a substrate and it also shows good thermal and pH stability (Wu et al. 2004). The maximum activity of the parathion hydrolase displayed on the cells of *S. cerevisiae* was obtained when the yeast cells were grown in a synthetic dextrose medium lacking tryptophan at pH 7.0 at 20 °C (Takayama et al. 2006). A novel organophosphorus pesticide hydrolase produced by *Penicillium lilacinum* BP303 had a pI of 4.8, and the enzyme activity was optimal at 45 °C and pH 7.5 (Liu et al. 2004). This suggests that the organophosphorus hydrolases from different sources have different optimal pH and temperatures.

The results in Table 2 showed Co^{2+} indeed significantly elevated the activity of the displayed MPH, and Ni^{2+}, Cu^{2+} and Mn^{2+} could slightly stimulate the activity. However, Ag^{+}, Ba^{2+} and Hg^{2+} inhibited the activity. It has been reported that Cr^{2+}, Mn^{2+}, Li^{+} and Fe^{2+} could enhance the catalytic efficiency of the native MPH produced by *Pseudomonas* sp. WBC-3 while Hg^{2+}, Zn^{2+}, $A1^{3+}$ and Bi^{3+} showed inhibition effect (Chu et al. 2003). The activities of a novel organophosphorus pesticide hydrolase produced by *P. lilacinum* BP303 were strongly inhibited by Hg^{2+}, Fe^{3+}, while Cu^{2+} slightly activated the enzyme (Liu et al. 2004). Most metal ions including Co^{2+}, Cr^{2+}, Ca^{2+}, Ni^{2+}, K^{+}, Li^{2+} and Mg^{2+} have no effect on the activity of OPHC2 produced by *P. pseudoalcaligenes* (Wu et al. 2004). This again demonstrated that some properties of the displayed MPH were different from those of the native one and any other organophosphorus pesticide hydrolases. However, the maximum activity of the parathion hydrolase was also obtained in the presence of cobalt chloride at 0.1 mM (Takayama et al. 2006).

The results in Figs. 6 and 7 indicated that the MPH displayed on the yeast cells can effectively remove MP in water and could be used for several times (data not shown). It has reported that MP at an initial concentration of 1,200 mg L^{-1} could be totally biodegraded by *A. radioresistens* USTB-04 as the sole carbon source <4 days in the presence of phosphate and urea as phosphorus and nitrogen sources, respectively and MP at an initial concentration of 130 mg L^{-1} was completely biodegraded in 2 h in the presence of cell-free extract (Liu et al. 2007). Although OPH could be displayed on the cell surface of *S. cerevisiae* MT8-1, the displayed OPH was not used for removal of OP in the environments (Takayama et al. 2006). Like other enzymes, all the results mentioned above showed that the MPH displayed on cell surface of *Y. lipolytica* may be used for the detoxification of organophosphorus pesticides in the environments.

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