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ORIGINAL PAPER

# Biodegradation of methyl parathion and *p*-nitrophenol by a newly isolated *Agrobacterium* sp. strain Yw12

**Shenghui Wang · Chen Zhang · Yanchun Yan**

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**Abstract** Strain Yw12, isolated from activated sludge, could completely degrade and utilize methyl parathion as the sole carbon, phosphorus and energy sources for growth in the basic salt media. It could also completely degrade and utilize *p*-nitrophenol as the sole carbon and energy sources for growth in the minimal salt media. Phenotypic features, physiological and biochemical characteristics, and phylogenetic analysis of 16S rRNA sequence showed that this strain belongs to the genus of *Agrobacterium* sp. Response surface methodology was used to optimize degradation conditions. Under its optimal degradation conditions, 50 mg $l^{-1}$ MP was completely degraded within 2 h by strain Yw12 and the degradation product PNP was also completely degraded within 6 h. Furthermore, strain Yw12 could also degrade phoxim, methamidophos, chlorpyrifos, carbofuran, deltamethrin and atrazine when provided as the sole carbon and energy sources. Enzymatic analysis revealed that the MP degrading enzyme of strain Yw12 is an intracellular enzyme and is expressed constitutively. These results indicated that strain Yw12 might be used as a potential and effective organophosphate pesticides degrader for bioremediation of contaminated sites.

**Keywords** Methyl parathion · *p*-Nitrophenol · Degradation · *Agrobacterium* sp. · Response surface methodology

S. Wang · Y. Yan (✉)
Graduate School, Chinese Academy of Agricultural Sciences (GS of CAAS), 12 Zhongguancun Nandajie, Beijing 100081, China
e-mail: yanyanchun@caas.net.cn

S. Wang
State Key Joint Laboratory of Environmental Simulation and Pollution Control, Department of Environmental Science and Engineering, Tsinghua University, Beijing 100084, China

C. Zhang
College of Resources and Environmental Sciences, China Agricultural University, Beijing 100091, China

## Introduction

Organophosphate pesticides, such as methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenol phosphorothioate, MP) and methamidophos, are extremely toxic compounds, which are still commonly used in some countries for crop protection. Since 2007 in China the five high-toxic organophosphorous pesticides, methamidophos, parathion, methyl parathion, monocrotophos, phosphorus amine, have been banned for commercial agricultural use, but contamination still exists due to their wide used. Recently, a research report showed that surface waters of China are moderately contaminated by six organophosphorous pesticides, though their concentrations were low and rarely exceed the environment quality standard (Gao et al. 2009). The contamination could have negative

effects on ecological environment and their potential neurotoxicity to the non-target organisms including humans have raised serious concern (Tripathi and Agarwal 1998; Singh and Walker 2006).

Microbial degradation is generally considered to be a safe and effective technique for insecticide elimination. So far, many strains with the ability to degrade methyl parathion have been isolated worldwide, such as *Pseudomonas* sp. A3, *Plesiomonas* sp. M6, *Pseudomonas* sp. strain WBC-3, *Ochrobactrum* sp. B2, *Serratia* sp. strain DS001, *Stenotrophomonas* sp. SMSP-1 (Ramanathan and Lalithakumari 1999; Zhongli et al. 2001; Liu et al. 2005; Qiu et al. 2006; Pakala et al. 2007; Shen et al. 2010). *p*-Nitrophenol (PNP) is one of the major degradation product of the OP pesticides, which is considered as a priority pollutant by the Environmental Protection Agency (EPA) of USA (Munnecke and Hsieh 1976; Chaudhry et al. 1988; Rani and Lalithakumari 1994). PNP concentrations in natural waters generally are less than 10 mg $l^{-1}$ (Kulkarni and Chaudhari 2006; Zhang et al. 2009), and its fate in the environment has been studied extensively (Bhatti et al. 2002; Singh and Walker 2006). Mineralization of PNP by microorganisms have been reported previously (Spain and Gibson 1991; Bhushan et al. 2000). Several PNP degrading bacteria also have been isolated from different geographical regions, including *Arthrobacter* sp. Y1, *Serratia* sp. DS001, *Rhodococcus* sp. CN6, *Moraxella* sp. and *Bacillus sphaericus* (Spain and Gibson 1991; Kadiyala and Spain 1998; Li et al. 2008; Zhang et al. 2009). It was reported that MP or PNP was hardly degradable and persistently toxic in the environment (Rehman et al. 2007) and isolates that can simultaneously degrade both compounds are scarce (Rani and Lalithakumari 1994; Ramanathan and Lalithakumari 1999; Liu et al. 2005).

The aims of this study were to isolate and characterize a new MP and PNP degrading bacterium, optimize its degrading conditions, examine its biodegradation ability and investigate its degrading enzyme.

## Materials and methods

### Media and chemicals

MP (>86% purity), chlorpyrifos (>98% purity), methamidophos (>71% purity), phoxim (>90% purity), carbofuran (>96% purity), deltamethrin (>98% purity), and atrazine (>80% purity) were obtained from Shandong Huayang Technology Co., Ltd., China. PNP (>98% purity) was purchased from Aldrich-Sigma Chemicals Co. Acetone and dichloromethane were of HPLC purity grade. All other reagents used in this study were of analytical reagent grade.

Enrichment medium (EM) that contained (in gram per liter), 10 g tryptone, 1.0 g NaCl, 1.0 g $KH_2PO_4$, 1.0 g glucose was used to culture strains. The carbon-free and phosphate-free basic salt medium (BSM) that contained (in gram per liter) 0.9 g $NaNO_3$, 0.225 g KCl, 0.009 g $MnSO_4$, 0.018 g $CaCl_2$, 0.225 g $MgSO_4{\cdot}7H_2O$ supplemented with MP was used to isolate MP degrading strains. The mineral salt medium (MSM) that contained (in gram per liter) 1.0 g $NH_4NO_3$, 0.5 g NaCl, 0.5 g $KH_2PO_4$, 1.5 g $K_2HPO_4$, 0.5 g $(NH_4)_2$ $SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$ was used to determine MP and PNP degradation. MP and PNP stock solutions were prepared aseptically by rationing into sterile media.

### Isolation of a MP-degrading strain by domesticated culture

The OP-contaminated sludge was collected from Huayang pesticides manufacturer of Shandong, China. Activated sludge samples (5.0 g) were added to 100 ml BSM medium containing 100 mg $l^{-1}$ MP and incubated at 30°C with shaking at 180 rpm in the dark. 10 ml of the culture broth was transferred weekly to 100 ml of fresh BSM medium with a stepwise increase in MP concentration to 800 mg $l^{-1}$. Dilutions of the sequential culture broth were plated on BSM agar plates containing 800 mg $l^{-1}$ MP, and incubated at 30°C for 3 dys. All isolates were screened based on the formation of degrading haloes as described previously (Zhang et al. 2005). Potential isolates were selected for further study.

### Identification of isolated MP degrader

The isolate was characterized and identified based on its morphological, biochemical properties and 16S rRNA gene sequence analysis (Holt et al. 1994). The 16S rRNA gene was amplified by PCR with Forward primer (AGAGTTTGATCCTGGCTCAG)

and Reverse primer (GGCTACCTTGTTACGACT). PCR products were cloned into a pMD18-T vector (TaKaRa) and sequenced. The determined 16S rRNA partial sequence was aligned with those available in the GenBank database. Phylogenetic analysis of the 16S rRNA were conducted using the neighbor-joining methods and were performed with ClustalX and MEGA 4.0 (Tamura et al. 2004; Tamura et al. 2007).

### The optimization of the MP degrading condition

The important parameters which significantly influenced MP biodegradation selected for the optimal degrading conditions were temperature, pH and the initial inoculum biomass. A series of experiments were carried out with different degradation initial inocula biomass (0.05–0.30 g $l^{-1}$), incubation temperature (20–40°C), and pH (5.0–9.0) (Xinghui Qiu et al. 2007). Response surface methodology (RSM) based on the Box-Behnken design of experiment was used to optimize these parameters and their interaction which significantly influenced MP biodegradation by strain Yw12 (Mohana et al. 2008; Guo et al. 2009). Strain Yw12 was inoculated into MSM medium containing 50 mg $l^{-1}$ MP as the sole carbon source and the samples were collected at the first hour for detecting MP residues. Cultures without inoculation were used as abiotic controls. All the experiments were performed in triplicate. A three-variable Box-Behnken design with three replicates at the center point was applied in this experiment. The experimental design is shown in Table 1. Equation 1 shows the second-order polynomial equation.

$$Y = \beta_o + \sum_{i-1}^{k} \beta_i X_i + \sum_{i-1}^{j-1} \sum_{j-1}^{k} \beta_{ij} X_i X_j + \sum_{i-1}^{k} \beta_{ii} X_i^2$$

$Y$ is the predicted response, $X_i$ and $X_j$ are variables, $\beta_o$ is the constant, $\beta_i$ is the linear coefficient, $\beta_{ii}$ is the quadratic coefficient, and $\beta_{ij}$ is the interaction coefficient.

### Degradation experiments

Degradation studies were carried out under the optimum conditions in MSM medium containing 50 mg $l^{-1}$ MP or 50 mg $l^{-1}$ PNP. Strain CPK was pre-cultured in EM medium to exponential phase and was then transferred to MSM medium containing 50 mg $l^{-1}$ MP or 50 mg $l^{-1}$ PNP, respectively. Uninoculated media with the same concentration of MP or PNP were used as controls. Cultures were regularly checked for bacterial growth, the degradation of MP or PNP. The bacterial growth was measured turbidometrically by a spectrophotometer (SCINCO S-3100, Korea) at 600 nm. Cultures were run in triplicate.

The MP residues were analyzed with GC-FPD. Cultures were regularly harvested and extracted twice with equal volume of dichloromethane. The average recovery rate of MP was 98%. The lower liquid was analyzed by GC. The separation parameters were as follows: nitrogen was used as the carrier gas (80.0 ml $min^{-1}$), and the compounds were eluted on an RTX-1301 capillary column, with the temperatures of the injector, detector and column set at 210, 250 and 190°C, respectively. The injection volume was 2 µl.

The concentration of PNP was measured by a spectrophotometer (SCINCO S-3100, Korea) at 397 nm, as described previously (Zhang et al. 2009). Cultures were regularly collected and centrifuged at 8,000×$g$, 4°C for 10 min. The supernatant fluid of each sample was filtered through a 0.45 µm filter before being analyzed by spectrophotometry.

### Substrate range

Cross-feeding studies with other contaminants were also performed. The liquid MSM medium was supplemented with methamidophos, phoxim, chlorpyrifos, carbofuran, deltamethrin, atrazine at 50 mg $l^{-1}$, respectively. Uninoculated media with the same concentration of pesticides were used as controls. The growth and pesticides residues were measured as previously described (Tse et al. 2004; Yang et al. 2005). All of the treatments were replicated 3 times.

### Cellular location and expression characteristic of MP-degrading enzyme

Strain was pre-cultured in EM medium to exponential phase and transferred to EM medium containing 100 mg $l^{-1}$ MP. After full growth, the samples were divided into two parts. One part was used to prepare cytoplasm, cell inner membrane and outer membrane fractions as previously described (Myers and Myers

**Table 1** Box-Behnken experimental design with three independent variables

| Run | $X_1$ | $X_2$ | $X_3$ | Responses (residues of MP, mg $l^{-1}$) |
|---|---|---|---|---|
| 1 | −1 | −1 | 0 | 8.05 |
| 2 | 1 | −1 | 0 | 4.96 |
| 3 | −1 | 1 | 0 | 6.59 |
| 4 | 1 | 1 | 0 | 4.4 |
| 5 | −1 | 0 | −1 | 6.22 |
| 6 | 1 | 0 | −1 | 3.88 |
| 7 | −1 | 0 | 1 | 6.15 |
| 8 | 1 | 0 | 1 | 3.74 |
| 9 | 0 | −1 | −1 | 6.17 |
| 10 | 0 | 1 | −1 | 2.71 |
| 11 | 0 | −1 | 1 | 6.03 |
| 12 | 0 | 1 | 1 | 2.87 |
| 13 | 0 | 0 | 0 | 2.53 |
| 14 | 0 | 0 | 0 | 3.09 |
| 15 | 0 | 0 | 0 | 2.62 |

$X_1$ temperature, −1 (25°C), 0 (29°C), 1 (33°C); $X_2$ media pH, −1 (7), 0 (8), 1 (9); $X_3$ biomass amount −1 (0.1 g $l^{-1}$), 0 (0.15 g $l^{-1}$), 1 (0.2 g $l^{-1}$)

The data were analyzed using statistical analysis system (SAS) software

All the values were averages of three replicates from three independent experiments

1992). The other part collected by centrifugation was washed twice with 0.2 M phosphate buffer (pH 7.0) and then was resuspended. The whole cell protein of strain Yw12 was obtained by low-temperature ultrasonic treatment. After removal of the cell debris, the supernatants were separated by SDS-PAGE and native-PAGE.

The MP degrading enzyme activity was assayed in reactions containing 0.1 ml of the crude enzyme solution, 0.9 ml of 50 mM Tris HCl buffer (pH 8.0) and 50 mg $l^{-1}$ MP. The mixture was incubated at 37°C for 10 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. And then 1 ml of 10% sodium carbonate solution was added for displaying color. The absorbance was measured at 397 nm. Enzyme activity could be calculated according to the amount of PNP. MP-degrading activity was expressed as nanomoles of PNP formed per minute per milligram of protein. All enzyme assays were run in triplicate.

## Results and discussion

### Isolation and identification of MP degrading bacteria

Several bacterial strains with the ability of degrading MP were isolated from the activated sludge samples. One strain designed as Yw12, capable of utilizing MP and PNP as sole carbon, phosphorus and energy sources for growth and producing the largest yellow halo on the BSM solid plates, was selected and identified.

This bacterium is non-spore-forming, polarly flagellated, aerobic and Gram-negative rod shaped. It shows nitrate reductase-positive, catalase-negative, hydrolyzation of starch and glutin-negative, indole production-negative. It also utilizes maltose, D-galactose, sucrose, xylose, D-glucitol, mannitol, L-cystine as sole carbon sources for growth. A phylogenetic tree based on the Yw12 strain 16S rRNA gene sequence was constructed (Fig. 1). Its 16S rDNA partial sequence showed very high similarity with *Agrobacterium* sp. Based on the above phenotypic characteristics and phylogenetic analysis, strain Yw12 was identified as *Agrobacterium* sp. The accession number was DQ468100.

Microorganisms with the ability to degrade organic pollutants are mostly obtained by enrichment culture (Zhang et al. 2006; Pakala et al. 2007; Kim and Ahn 2009). This study tried to replace enrichment culture with domesticated culture to isolate strains. Enrichment medium and enrichment culture procedures were not introduced in the process of domesticated culture. The activated sludge samples were directly added to inorganic carbon and phosphate free basic salt medium, then it was subcultured and continuously increased selection pressure. Finally, strains with high degrading ability were selected. During the process, strains with low degrading ability were eliminated at the beginning while the effective degrading strains continued to improve its degrading ability under selection pressure with gradual increase of pesticide concentration. However, results showed that it is a rapid and effective method for isolating pesticide degrading bacteria.

### Impact factors of MP degradation by *Agrobacterium* sp. strain Yw12

The Box-Behnken design was used to explore the independent variables including temperature, pH of

medium and initial inoculum biomass which significantly influenced the biodegradation of MP by strain Yw12. The design of independent variables and the experimental results are listed in Table 1. A total number of 15 runs were required for optimization of the three variables, in which three replicates were conducted at the center point. Based on the data (Table 1), the statistical analysis was accomplished by using the PROC RSREG program of SAS software. After removing the non-significant interaction coefficients ($P > 0.05$), the following second-degree polynomial equations Eq. 2 was obtained to explain MP biodegradation by strain Yw12:

$$Y_{Yw12} = 2.747 - 1.254X_1 - 1.080X_2 - 0.024X_3 + 1.903X_1^2 + 1.350X_2^2 + 0.348X_3^2 \quad (2)$$

$Y_{Yw12}$ is the predicted MP residues. $X_1$, $X_2$ and $X_3$ are the coded values for the temperature, pH of media and initial inoculum biomass, respectively. The model was significant ($P < 0.05$) and $R^2 = 0.9345$.

From the results of regression analysis, the significant terms ($P < 0.05$) were temperature and pH, while, inoculum size ($X_3$) ($P > 0.05$) was not significant. Under the fixed value of inocula biomass at 0.15 g $l^{-1}$, the response surface displays the effects of temperature and pH of media on the MP biodegradation. As shown in Fig. 2, optimal conditions for MP degradation by strain Yw12 were determined to be 30°C and pH 8.4.

## Biodegradation of MP and PNP by strain Yw12

The degradation patterns of strain Yw12 were studied in MSM media with MP or PNP as the sole carbon and energy sources (Figs. 3, 4). Under the optimum conditions (temperature: 30°C; pH: 8.4; inocula biomass: 0.15 g dry wt $l^{-1}$), about 95% degradation was observed within 1 h and 50 mg $l^{-1}$ MP was completely degraded by strain Yw12 within 2 h (Fig. 3). The degradation of MP was accompanied by bacterial growth and transient accumulation of PNP. The cell density of Yw12 increased from 0.115 to 0.186 ($OD_{600}$ nm) in 6 h. Then, PNP was degraded rapidly and disappeared finally within 6 h (Fig. 3). Furthermore, strain Yw12 also completely degraded 50 mg $l^{-1}$ PNP within 8 h, when PNP was provided as sole carbon and energy sources, while cell density of Yw12 increased from 0.084 to 0.18 ($OD_{600}$ nm) in 8 h (Fig. 4). The data indicated that strain Yw12 utilized MP and PNP as the sole carbon source for growth and degradation.

To date, many isolated microorganisms could only degrade a limited number of pesticides (Feng et al. 1997; Qiu et al. 2006; Zhang et al. 2006; Kim and Ahn 2009; Li et al. 2009; Wang et al. 2010; Zhang et al. 2010). However, in practice, a variety of different pesticides are used to control different pests during agricultural production. So, it is a mixed pattern of pesticide contamination. There are two ways to resolve the problem. One way is to combine isolates which could degrade different pesticides to

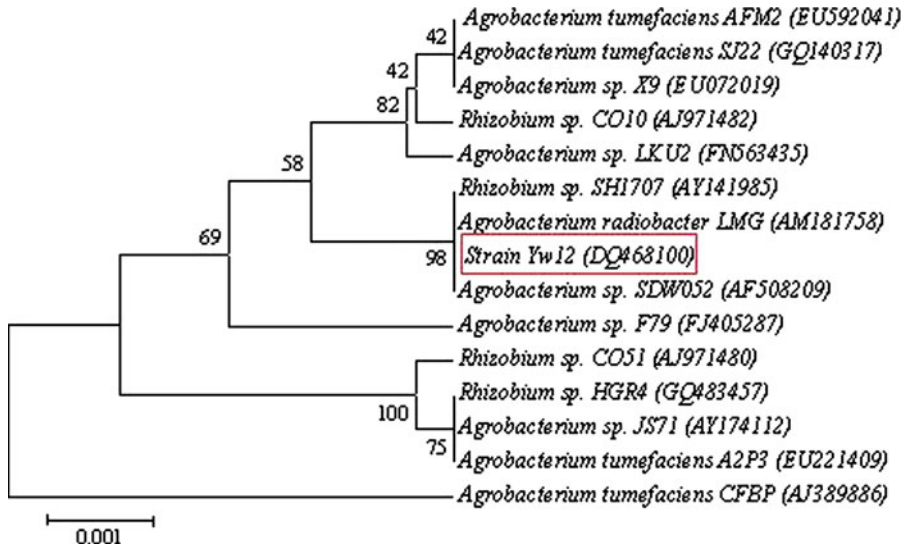


**Fig. 1** Phylogenetic tree of strain Yw12 based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 1000 repetitions are indicated as percentages at all branches

eliminate this pollution (Xu et al. 2007; Krishna and Philip 2008). Another way is to isolate the effective bacteria or construct super-bacteria which could degrade a number of different pesticides. Recently, a genetically engineered microorganism (GEM) was successfully constructed and it could simultaneously degrade methyl parathion (MP) and carbofuran (Jiang et al. 2007). In this study, strain Yw12 could also degrade and utilize methamidophos, phoxim, chlorpyrifos, carbofuran, deltamethrin, and atrazine as the sole carbon source for growth and degradation (Table 2). Strain Yw12 also could degrade other non organophosphorus pesticides. Some possible reasons may be responsible for this. The bacterial phosphotriesterases were reported to be the most promiscuous of all enzymes (Scott et al. 2008). Generally, they have a broad substrate range, being able to hydrolyse a number of related compounds. In addition to the hydrolysis of P–O bonds in phosphotriesters, they also could catalyze the hydrolysis of P–S bonds (Lai et al. 1995), P–F bonds (Dumas et al. 1990; Watkins et al. 1997), P–CN bonds (Raveh et al. 1992), and C–O bonds in esters and lactones (Roodveldt and Tawfik 2005b; Roodveldt and Tawfik 2005a). Both carbofuran and deltamethrin contain C–O bonds, so the organophosphorus hydrolase of strain Yw12 could recognize the similar substrate and degrade them. Another reason is the evolution of catabolic genes or the acquisition of specific enzymes by genetic exchange between strains in environment (Kellogg et al. 1981; van der Meer 1994), which could make strain Yw12 have the ability to degrade atrazine. Thus, this isolate will be potentially useful in bioremediation of a variety of pesticides contaminated soils.

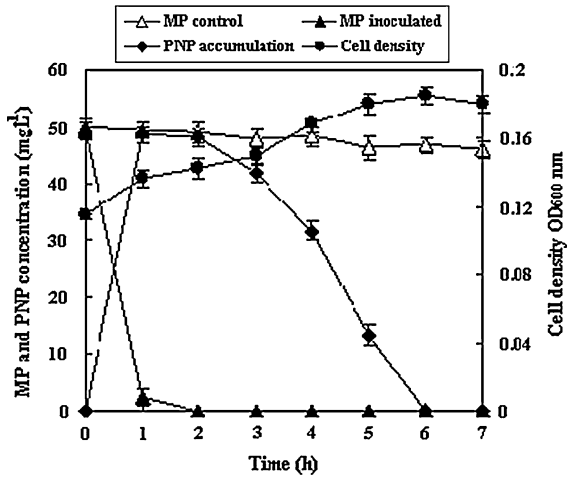


**Fig. 3** Degradation of MP by *Agrobacterium* sp. strain Yw12 and bacterial growth monitored by measuring OD at 600 nm. MP control (*open triangle*); MP inoculated (*closed triangle*); PNP accumulation (*closed diamond*); $OD_{600}$ nm (*closed circle*). Values are means ± SD of three replicates

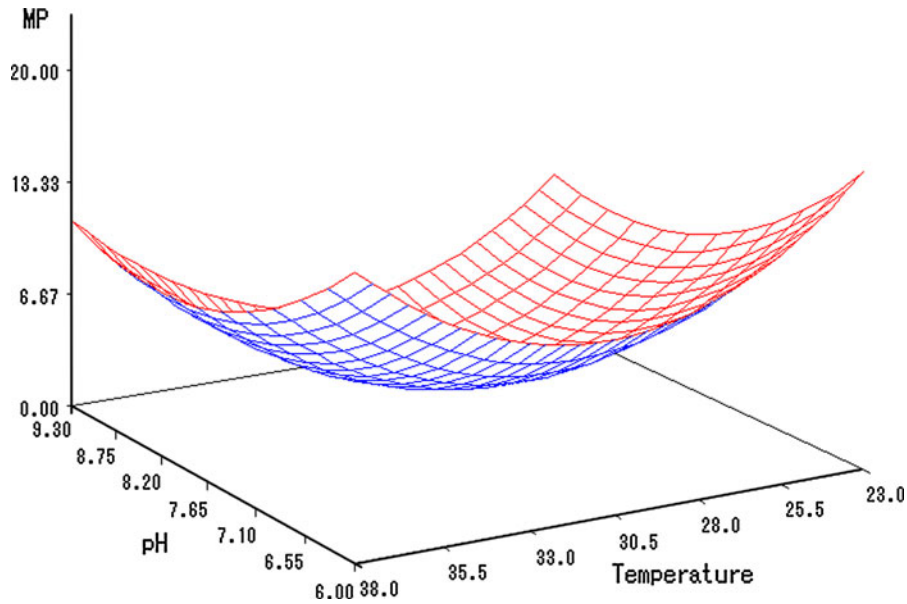


**Fig. 2** Response surface curves showing the effects of temperature and pH of media on MP biodegradation with total biomass amount of strain Yw12 at 0.15 g dry wt $l^{-1}$

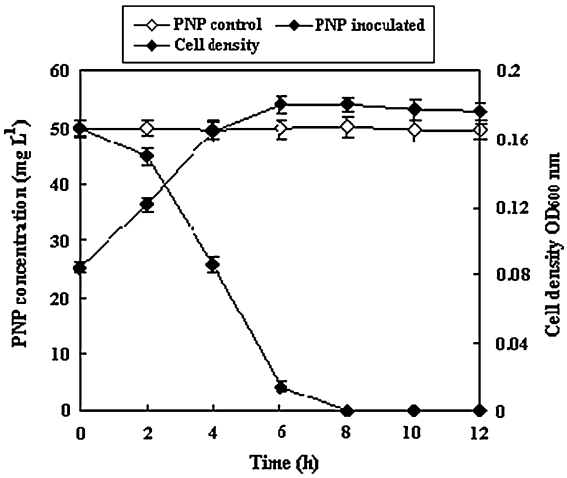


**Fig. 4** Degradation of PNP by *Agrobacterium* sp. strain Yw12 and bacterial growth monitored by measuring OD at 600 nm. PNP control (*open square*); PNP inoculated (*closed diamond*); $OD_{600}$ nm (*closed circle*). Values are means ± SD of three replicates

**Table 2** Degradation of other pesticides (50 mg $l^{-1}$) by strain Yw12 in 96 h

| Substrate | $OD_{600}$nm | Degraded amount (%) |
|---|---|---|
| Methamidophos | 0.326 ± 0.012 | 43.21 ± 0.19 |
| Phoxim | 0.379 ± 0.014 | 48.63 ± 0.22 |
| Chlorpyrifos | 0.249 ± 0.007 | 33.77 ± 0.16 |
| Carbofuran | 0.138 ± 0.003 | 12.52 ± 0.11 |
| Deltamethrin | 0.146 ± 0.005 | 14.02 ± 0.09 |
| Atrazine | 0.122 ± 0.002 | 12.33 ± 0.07 |

### Cellular location of strain Yw12 MP-degrading enzyme

The MP-degrading activities in cytoplasm, cell inner membrane and outer membrane fractions of strain Yw12 were investigated. It was found that almost half of the MP degrading enzyme activity was located in the cytoplasm (50%) and another half was located on cell inner membrane (42%). Only a small quantity of enzyme activity was detected in cell outer membrane (0.08%). The majority of organophosphorus hydrolase activity is located in the cytoplasm and cell inner membrane, implying that the MP degrading enzyme belongs to intracellular enzymes. And this is consistent with previous reports that some organophosphorus hydrolase were membrane-associated in other organisms (Brown 1980; McDaniel et al. 1988).

### The expression patterns of strain Yw12 MP-degrading enzyme

The essence of microbial degradation of pesticides is enzymatic reaction. In most of the studies on microbial degradation of organophosphorus pesticides, the first reaction was hydrolysis of the phosphotriester P=S or P=O bond by organophosphorus hydrolase (Mulbry et al. 1986). In this study, strain Yw12 could metabolize MP by cleavage of the phosphotriester (P=O) bond by organophosphorus hydrolase to yield diethylthiophosphate (DETP) and PNP, then completely mineralize it.

In microorganisms, many enzymes are inducible (De Crombrugghe et al. 1969; Bourassa and Vadeboncoeur 1992) and it was reported that bacterial organophosphorus hydrolase is constitutively expressed (Mulbry and Karns 1989). The protein patterns of the crude extracts from strains under pesticide induced and non-induced conditions could be investigated by native-PAGE (Gangming Xu et al. 2008). The whole cell protein of Yw12 strain was analyzed by SDS and native-PAGE, in order to study the expression patterns of the MP degrading enzyme in strain Yw12. The protein patterns of the crude extracts from Yw12 strain cultivated under EM media, EM media containing 100 mg $l^{-1}$ MP and EM media containing 100 mg $l^{-1}$ PNP were investigated. The whole cell protein of Yw12 strain from EM media was as the control. The whole cells protein cultivated in EM media containing MP or PNP had no specific protein band compared with cells grown in other two control media (Fig. 5). It implied that no specific protein was induced by MP. These results indicated that the organophosphorus hydrolase of strain Yw12 might be constitutively expressed.

## Conclusions

In conclusion, an effective MP and PNP degrading bacterium *Agrobacterium* sp. Yw12 has been isolated from the activated sludge samples by domesticated culture, which could utilize MP or PNP as the sole carbon, phosphorus and energy sources for growth. This strain has a broad degradation spectrum, it not only has very strong MP and PNP degrading enzyme

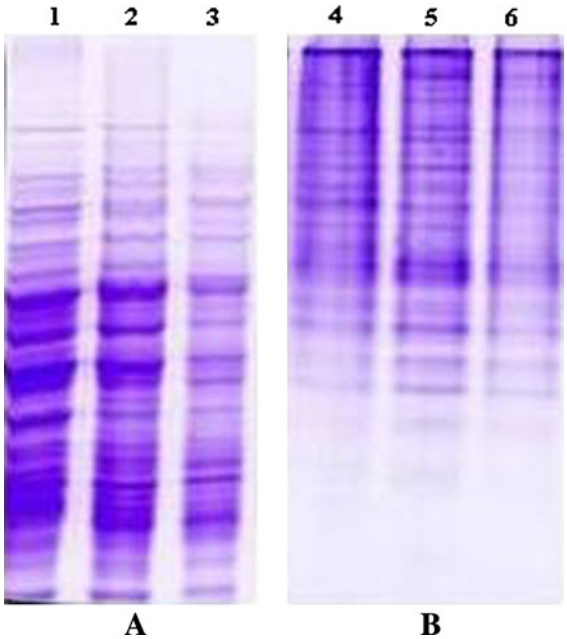


**Fig. 5** SDS-PAGE (**a**) and Native-PAGE (**b**) analysis of the expression patterns of MP-degrading enzyme in strain Yw12 under induced and non-induced conditions. Cells were cultured in EM media with different carbon sources. *Lane 1,4* control; *lane 2,5* with 100 mg $l^{-1}$ MP; *lane 3,6* with 100 mg $l^{-1}$ PNP

activity but also degrade other pesticides. The MP degrading enzyme of strain Yw12 belongs to intracellular enzymes that might be constitutively expressed. These results show that strain Yw12 is an efficient degrader to clean-up of contaminated pesticide waste in the environment and enriched the resource of methyl parathion degrading bacteria in contaminated soil which offered the foundation for bioremediation of organophosphorus pesticide pollution. Additional work is aimed at identifying new genes and enzymes involved in the degradation process.

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