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Full Length Research Paper

A high potential organophosphorus pesticide-degrading enzyme from a newly characterized, *Pseudomonas aeruginosa* NL01

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Twenty seven bacterial isolates were obtained from diazinon-treated agricultural soils in North of Iran and amongst them, an isolate was found to grow in the mineral salt agar plates supplemented with diazinon at concentration of 200 mg L^{-1} as the sole carbon source. The bacterial isolate was identified by 16S rRNA gene sequence as *Pseudomonas aeruginosa* NL01. A 36 kDa organophosphorus hydrolase (OPH) enzyme, designated as OPH NL07, was purified by DEAE sepharose column chromatography. The purified enzyme showed a single band on SDS-PAGE. The Michaelis constant (Km) and maximal velocity (Vmax) values of enzyme for paraoxon as substrate were 10 µM and 66.67 µM/min, respectively. The effect of pH and temperature on enzyme activity indicated that the OPH NL07 had the highest activity at pH 8 and was active at a range of temperatures from 25 to 60°C, although, optimum temperature for enzyme activity was 37°C. Enzyme activity was enhanced by 1.28 and 1.09 fold in the presence of $CoCl_2$ and $CaCl_2$, each at 2 mM, while addition of $ZnCl_2$, $MgCl_2$, $FeCl_3$, SDS and EDTA, each at 2 mM inhibited the enzyme by 0.93, 0.92, 0.51, 0.06 and 0.00 fold, respectively. The OPH NL07 seems to be suitable as biocatalyst for practical use in bioremediation of organophosphorus pesticides (OPs)-contaminated soils.

Key words: Diazinon, organophosphorus pesticides, organophosphorus hydrolase, *Pseudomonas aeruginosa.*

INTRODUCTION

Organophosphorus pesticides (OPs) belong to a group of very potent neurotoxins that are commonly used as insecticides, pesticides and chemical warfare agents (Kang et al., 2005). OPs intoxicate thousands of humans, and cause much death in the world every year (Bird et al., 2008). These compounds inhibit acetyl cholinesterase (AChE) in the nervous system, leading to a subsequent loss of nerve function and eventually death (Yu et al., 2009). After usage, these compounds remain in soil, water, agricultural products, aquatic products (Fu et al., 2004). One of the main ways to resolve the problem of OPs residue is to bioremediate these compounds (Ang et al., 2005). OPs contain three phosphoester bonds and, hence, are often termed posphotriesters. In general, hydrolysis of only one of the phosphoester bonds (P-O, P-S, P-F, and P-CN) can reduce significantly the toxicity of an OP. For example, in the case of parathion, this hydrolysis results in a 100-fold reduction in toxicity (Lan et al., 2006).

Organophosphorus hydrolase (OPH, EC 3.1.8.1) is a homodimeric and metal dependent enzyme with the molecular weight of 72 kDa which catalyses the hydrolysis of phosphoester bonds in organophosphorus neurotoxins

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at a remarkable rate (Efremenko and Sergeeva, 2001). OPH hydrolyses a large variety of OPs by producing less toxic products. It has applications in detoxification and decontamination of OPs containing agricultural field and chemical weapons stock. This makes OPH a suitable element for biodegradation of these compounds (Li et al., 2008). A variety of organophosphorus pesticide-degrading bacteria, including *Pseudomonas diminuta* MG (McDaniel et al., 1988), *Flavobacterium* sp. (Mulbry et al., 1986), *Arthrobacter* sp. (Ohshiro et al., 1999), *Penicillium lilacinum* BP303 (Liu et al., 2004), have been isolated from environment that has come in contact with these compounds. These bacteria are able to grow in OPs containing soils and use the organophosphorus compounds as a carbon source. As mentioned, *P. diminuta* MG and some other *Pseudomonas* species such as *Pseudomonas* sp. WBC-3 (Dong et al., 2005) and *Pseudomonas putida* (Rani and Lalithakumari, 1994) possess the ability to degrade phosphotriesters.

This paper describes a comprehensive study on the isolation and characterization of efficient OPs degrading bacterial strains from different types of diazinon-treated farm soils. Among different bacterial isolates obtained from various sources, one isolate that effectively degrades OPs such as diazinon and paraoxon was finally selected for further studies. The selected isolate was identified by morphological, biochemical and 16S rRNA gene sequence analysis as *Pseudomonas aeruginosa* NL01. The purification and characterization of Organophosphorus hydrolase (OPH NL07) was investigated as well.

MATERIALS AND METHODS

Sample collection and screening of OPs degrading bacteria

Soil samples were collected from farm lands of Damavand, North of Iran, which had an almost 10-year history of diazinon use in pest and insect control. The soil samples were then transferred to the laboratory, and 5 g of the samples were suspended in 500-ml flasks containing 100 ml of mineral salt medium (MSM) enriched with diazinon (200 mgL^{-1}) as a carbon source. The MSM had the following composition in (gL^{-1}): $(NH_4)_2HPO_2$, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.001; K_2HPO_4, 0.1; Co $(NO3)_2.4H_2O$, 0.01. The flasks were incubated at 180 rpm for 10 days at 32°C. At each 24 hr intervals, a loop full of samples from the flasks were streaked onto mineral agar plates supplemented with 200 mgL^{-1} diazinon and the plates were incubated at 32°C for 3 days. The single bacterial colonies were picked up and sub cultured onto mineral agar plates containing diazinon of the same concentration until pure cultures were obtained. The hydrolysis zone (area) resulting from diazinon degrading were observed in 12 out of 27 strains and only one strain with a higher ratio of the clearing hydrolysis zone diameter to colony size was selected for further analysis (Liu et al., 2004; Mulbry and Karns, 1989; Nazarian and Amini, 2008).

Biochemical and molecular identification of the selected isolate

The bacterial isolate grown on diazinon-containing agar plate was subjected to biochemical and morphological analysis. Biochemical identification of selected isolate was done using standard biochemical test (API 20E kit, bioMérieux, Mercy, France). Antibiogram test was done with different antibiotic disks (9 various antibiotics) and assessed according to the NCCLS guidelines (NCCLS. Wayne, Pennsylvania,1998). For molecular identification, the selected bacterial isolate was referred for 16S rRNA gene sequence analysis (Dubnau et al., 1965). Chromosomal DNA extraction was performed from 2 ml bacterial culture collected at the mid-exponential growth phase using DNA extraction kit (Roche Applied science, Germany) according to the instructions of manufacturer. PCR amplification of 16S rDNA with universal eubacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') was conducted in a reaction mixture containing 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM of each primer, and 1 U of Taq DNA polymerase. After denaturation of chromosomal DNA at 98°C for 3 min, 16S rDNA was amplified during 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 s, and extension at 72ºC for 1 min (Shourian et al., 2009). The 1436 bp PCR product was purified using a PCR product purification kit (Roche Applied science, Germany). Sequencing of the PCR product was carried out with a ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA) using mentioned primers. The National Center for Biotechnology Information BLAST tool was employed for identification of the species closed to the sequence of strain NL01. The phylogenetic tree was constructed using unweighted pair group method with arithmetic mean (UPGMA) by Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0 based on 16S rRNA sequence data (Tamura et al, 2007).

Extraction and purification of OPH from selected isolate

Bacterial cells were cultured in MSM supplemented with diazinon (200 mgL^{-1}) overnight. The cells were harvested by centrifugation at 10000 ×g for 10 min and resuspended in 50 mM Tris-HCl (pH8) buffer containing 1 mM phenyl methylsulphonyl fluoride (PMSF). The Bacterial cells were disrupted by sonicating for 15 times in 10s-periods at 15s intervals by using a sonifier (Hielscher UP 400, Germany).

The cell suspension was centrifuged at 40000 ×g for 30 min. The pellet was resuspended in 25ml Tris Hcl (50 mM, pH8) buffer containing 2.5% Triton X100 and incubated at 4°C for 16hr. The cell lysate was centrifuged at 40000×g for 120 min. The supernatant was precipitated with 60% ammonium sulphate. The pellet was dissolved in a minimal volume of Tris Hcl (50 mmol/L, pH=8) buffer and dialyzed against the same buffer at 4°C overnight. The dialyzed solution was loaded onto DEAE sepharose CL-6B column (GE healthcare, USA) that had been equilibrated with Tris HCl (50 mmol/L, pH 8) buffer. The column eluted with a 10 to 1000 mM NaCl gradient solution (Liu et al., 2004). The fractions collected for enzyme activity assay.

Biochemical characterization of purified OPH

Polyacrylamide gel electrophoresis

The purity and molecular mass of OPH were determined by SDS-PAGE (12%) using a mini-PROTEAN® 3cell (BIO-RAD). The molecular mass of the enzyme was estimated using β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), Lactate dehydrogenase (35,000 Da), REase Bsp981 (25, 000 Da) and β-lactoglubulin (18.400Da) as standards (Sigma). The protein bands were stained with Coomassie Brillian Blue G-250.

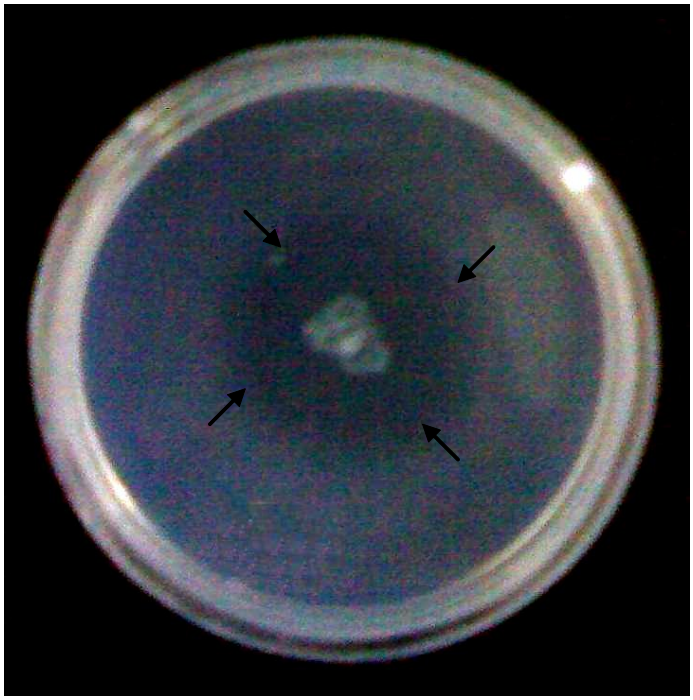

Figure 1. Hydrolysis bond arised from degrading of diazinon in MSM.

Enzyme activity assay

The release of *p*-nitro phenol from hydrolysis of paraoxon (organophosphate compound) was used to measure the enzyme activity. Two hundred micro liter of enzyme solution was added to 780 µL of a 50 mM Tris–HCl (pH 8.0) assay buffer containing 20 µL of 40 mM paraoxon as a substrate, and incubated at 37°C for 10 min. The rate of paraoxon hydrolysis was measured by monitoring the change of absorbance (410 nm) using a unico UV/Vis 4802 spectrophotometer. One unit of OPH activity is defined as the amount of enzyme liberating 1 µmol of *p*-nitro phenol per minute at 37°C (Wu et al., 2004).

Determination of kinetic parameters

Kinetic studies on OPH were carried out in 50 mmol/L Tris HCl (pH8) assay buffer containing various concentrations of paraoxon (10, 20, 30, 40, 50 µM) as substrate at 37°C for 10 min. The correlation of enzyme activity with substrate concentration could be fitted to Michaelis-Menten kinetics using nonlinear regression analysis. For Km and Vmax studies, the initial velocity of the enzyme was measured.

Effects of temperature, pH and metal ions on enzyme activity

Optimum temperature and pH for purified enzyme were determined by measuring the activity after 10 min incubation at different temperatures (25, 37, 45, 60, 70 and 80°C) and pH range between 2 to 12. The effect of pH on enzyme activity was determined by running reactions in various buffers (100 mM) including sodium acetate (pH 2, 3, 4 and 5), sodium phosphate (pH 6 and 7), Tris-HCl (pH 8 and 9), and glycine-NaOH (pH 10, 11 and 12) under the same conditions described before. The effects of temperature and pH on the stability of the enzyme were studied by measuring the residual activity after incubating the enzyme solutions for 90 min at mentioned temperatures and pHs. The effect of metal cations on OPH enzyme was studied by running enzyme reactions in Tris–HCl (pH 8.0) assay buffer containing 2 mM of metal ions and chemical agents including $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CoCl_2$, $MnSO_4$, and $Fecl_3$.

RESULTS AND DISCUSSION

Biochemical and molecular characterization of the selected isolate

Diazinon degrading bacteria was successfully isolated by using an enrichment technique. After 3 days a clear hydrolysis zone around colony showed that an isolated bacterium was able to hydrolyse diazinon as a carbon source in MSM agar containing diazinon (Figure 1). Morphological and biochemical tests showed that the selected isolate, designated NL01, was Gram negative, rod-shaped and aerobic. Biochemical tests showed that the selected isolate was catalase, oxidase, gelatinase, arginine dihydrolase positive, and urease and glucose negative. The bacterium was found to metabolize glucose and citrate and capable of growth at temperatures ranging from 20 to 40°C. Both standard tests and API 20NE analysis (Table 1) showed that strain NL01 was likely to be *Pseudomonas aeruginosa*. In antibiogram test, from 9 various antibiotics, the strain was resistance to streptomycin and tetracycline (Table 1). By comparing its morphological, physiological, and biochemical characteristics in API 20NE tests with the reference strain *P. aeruginosa* (ATCC 27853^T), the strain was identified as *P. aeruginosa*. Following 16S rRNA sequencing, the strain was identified and confirmed as *Pseudomonas aeruginosa* and deposited in GenBank under the accession number *JF331665*. The 16S rRNA analysis demonstrated a rather close relationship to the DNA sequences of *P. aeruginosa* (98% similarity with the reference strain, ATCC 27853^T). This close relationship was also confirmed by the phylogenetic clustering of strain NL01 with other strains of *P. aeruginosa* in 16S based phylogenetic tree (Figure 2). As Thompson et al. pointed out 16S sequence similarities of ≥97% should be considered as the same species, strain NL01 was thus identified as *P. aeruginosa* at the molecular level (Thompson et al., 2004).

Purification of the OPH NL07

The enzyme activity was detected at the late log phase and reached to the maximum level at 5 days after cultivation. Enzyme was purified from crude extract by ammonium sulfate precipitation, dialyze and DEAE-sephrose CL-6B chromatography and designated as OPH NL07. The purification steps and results are summarized in Table 2. The enzyme was purified 59.01-fold to a specific activity of 22.52 U/mg protein from the

Table 1. Biochemical characteristics and antibiotic susceptibility of diazinon-degrading strain NL01 isolated from farm lands (Damavand, North of Iran).

Character	Strain NL01
Morphology	**Gram-negative (bacillus-like)**
Reduction of nitrate	+
Indole production	+
Fermentation of glucose	-
Pyocyanin	+
Arginine dihydrolase	+
Urease	-
β-Galactosidase	-
Cytochrome oxidase	+
Hydrolysis of:	
Esculin	-
Gelatin	+
Oxidation of:	
Glucose	+
Arabinose	-
Mannose	-
Mannitol	+
N-Acetyl-glucosamine	+
Maltose	-
Potassium gluconate	+
Capric acid	+
Adipic acid	+
Malic acid	+
Trisodium citrate	+
Susceptibility to antibiotics	
Penicillin G	S
Cefamezin	S
Kanamycin	S
Gentamicin	S
Chloramphenicol	S
Ampicillin	S
Streptomycin	R
Amikacin	S
Tetracycline	R

R; Resistance, S; sensitive, different sensitivities of strain NL01 to an antibiotic were assessed according to the NCCLS guidelines.

crude extract with a yield of 12.73%.

Biochemical characterization of the OPH NL07

The purified enzyme gave a single band in SDS–PAGE. This indicated that the purified sample was electrophoretically homogeneous under the dissociating conditions. The molecular weight of the purified enzyme estimated by SDS–PAGE analysis was approximately 36 kDa (Figure 3). The molecular weight of purified enzyme was similar to OPH from *P. diminota* MG (McDaniel et al., 1988) and *Flavobacterium* sp. strain ATCC27551 (Harper et al., 1988). With paraoxon as the substrate, the kinetic parameters of the purified enzyme were determined by Line weaver–Burk plot. The Km value of paraoxon was 10 µM and the Vmax was about 66.67 µM/min (Figure 4). Low Michaelis constant (Km) value represented very

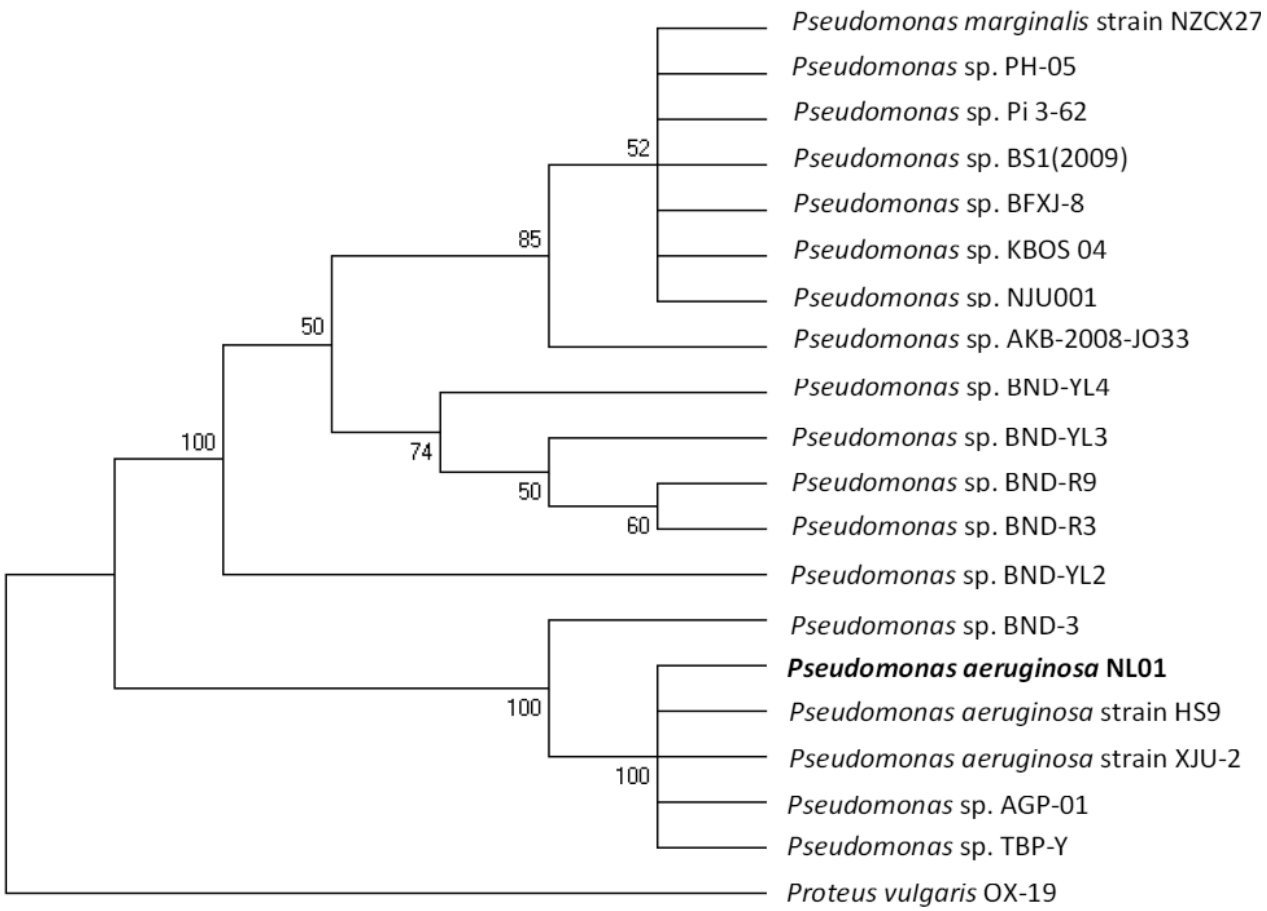

Figure 2. Phylogenetic relationship of *Pseudomonas aeruginosa* NL01 based on partial 16S rRNA sequence.

Table 2. Purification of OPH NL07 that is monitored through its activity.

Step	Volume (ml)	Total protein (mg/ml)	Enzyme activity (U)	Specific activity (U/mg)	Recovery of activity (%)	Purification fold
Crude	27	508.88	196.33	0.385	100	1
Supernatant (After Treat with Triton X100)	25	308.22	192.33	0.624	97.96	1.62
Precipitation with ammonium sulphate 40%	37	99.43	111.33	1.119	56.74	2.9
Precipitation with ammonium sulphate 60%	15	39.12	91.66	2.34	46.68	6.08
Enzyme fraction of chromatography line exchanging ionic DEAE	6	1.11	25	22.52	12.73	59.01

high activity for paraoxon hydrolysis in comparison with Km of *Pseudomonas diminuta* OPH (Dumas et al., 1989) and *Pseudomonas pseudoalcaligenes* OPHC2 (Wu et al., 2004) that have reported 90 and 64.6 µM, respectively.

The temperature profile of OPH activity was studied in a range of 20 to 80°C. This profile showed that the activity increased to a maximum of 25 U with gradual increase up to 37°C. At higher temperatures, the activity decreased

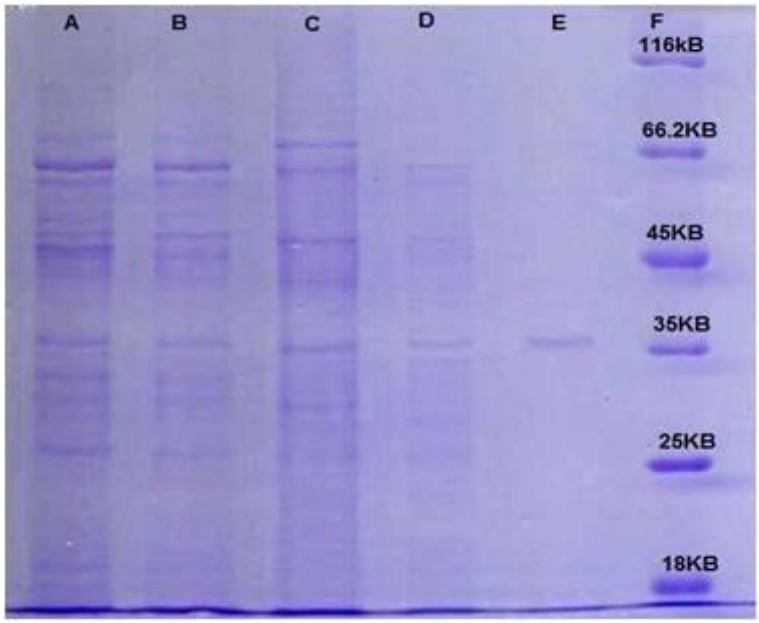

Figure 3. SDS–PAGE analysis of the crude and purified OPH NL07 (A: crude extract; B: after treat with triton X100; C: $(NH_4)_2SO_4$ 40% precipitation; D: $(NH_4)_2SO_4$ 60% precipitation; E: purified enzyme; F: protein markers).

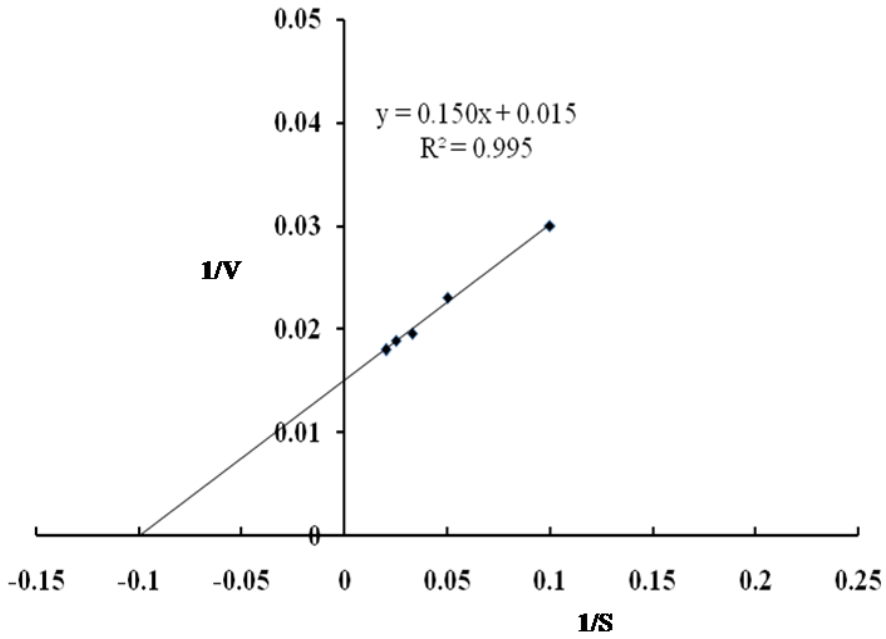

Figure 4. Kinetics plot of hydrolysis reaction of purified OPH NL07 with paraoxon as substrate.

with a sharp drop when temperature was increased from 45 to 70°C. The optimal temperature for the enzyme activity was 37°C. The enzyme was fairly stable at 45°C and completely inactivated at 80°C (Figure 5). The pH-activity profile of the enzyme was bell-shaped, with maximum values at pH 8 (Figure 6). As shown in Figure 6, the enzyme was found to be stable in the pH range between 7.0 to 9.0. The temperature profile of purified

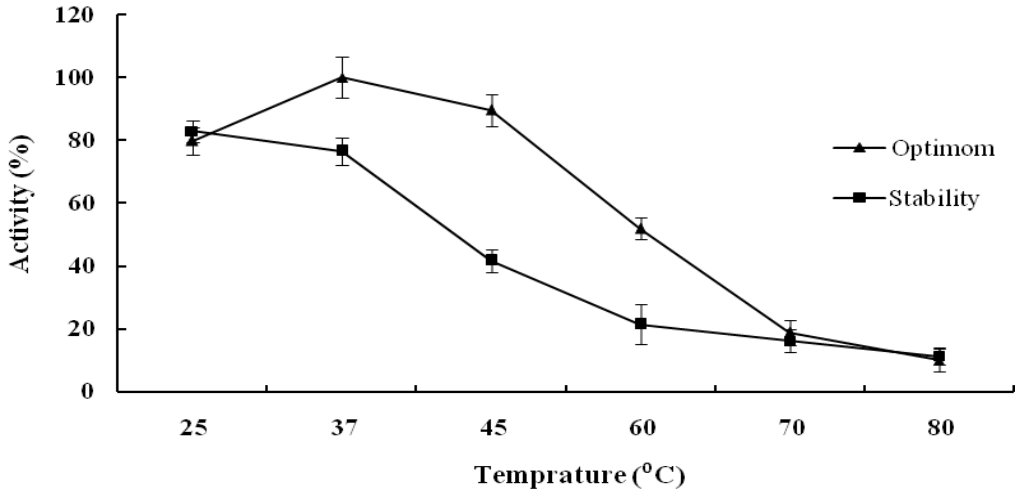

Figure 5. The temperature-activity profile of the purified OPH NL07.

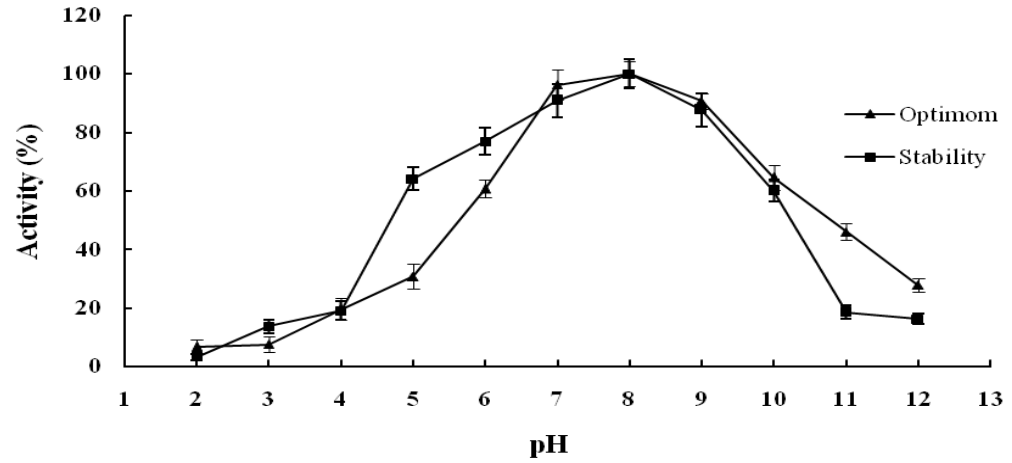

Figure 6. The pH-activity profile of the purified OPH NL07.

enzyme was similar to *P. diminuta* OPH and *Penicillium lilacinum* OPH (Liu et al., 2004), but was different from *P. pseudoalcaligenes* OPHC2 that its optimum temperature was 65°C (Wu et al., 2004).

The effect of Co^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+} and Fe^{3+} (2 mM) on Organophosphorus hydrolase activity of NL01 strain was studied at pH 8 and 37ºC. The activity of the enzyme in standard reaction mixture without these cations was taken to be 100%. Under the described reaction conditions, Co^{2+} remarkably improved the OPH activity up to 128% (Table 3). In the presence of the other metal ions, the activity of the enzyme was in the following order: Ca^{2+}> Zn^{2+}> Mg^{2+}> Fe^{3+}. The activity of OPH in the presence of SDS (2 mM) and EDTA (2 mM) was decreased to 6% and 0%, respectively. We found that Co^{2+} and Ca^{2+} moderately increased the purified enzyme activity whereas Fe^{3+} decreased the enzyme activity. Also, chelating agent ethylenediamine tetra acetate (EDTA) remarkably decreased the enzyme activity (down to 0%). These results indicated that the OPH NL07 is sensitive to divalent cations, in contrast to *Penicillium lilacinum* OPH (Liu et al., 2004) and *Pseudomonas pseudoalcaligenes* OPHC2 (Wu et al., 2004) that EDTA do not have any effect on their activity. Diazinon, parathion and methyl parathion are common organophosphates that cause very dangerous environmental pollution. However, after hydrolysis they produce less toxic or nontoxic compounds. Because OPs compounds are synthetic and not fully degradable by many of soil organisms, these compounds and their toxic products remain in soil, water, and agricultural products. Only some organisms can initially detoxify these OPs contaminants. For example, hydrolysis of diazinon and other OPs by organophosphorus hydrolase contain organisms reduce toxicity (McDaniel et al., 1988; Mulbry et al., 1986; Ohshiro et al., 1999; Liu et al., 2004). Accordingly, the

Table 3. The effect of metal ions and chemical agents on the OPH NL07 activity.

Metal ions and chemicals	Relative activity (%)
Normal	100
Zn^{2+}	93
Co^{2+}	128
Ca^{2+}	109
Mg^{2+}	92
Fe^{3+}	51
SDS	6
EDTA	0

engineering or finding of new organisms that are able to degrade OPs will be important for bioremediation objects (De la Pena Mattozzi et al., 2006; Shimazu et al., 2001; Walker and Keasling, 2002).

Conclusion

In this report, the isolated strain contains OPs hydrolyzing enzyme had a high potential to detoxificate diazinon and paraoxon. Low Km value and good pH and temperature stability could make the purified enzyme an attractive biocatalyst for applied bioremediation. In addition, this new organism with high potential activity to hydrolyze the organophosphorus compounds is a very useful tool for application in biofertilizers for efficient biodegradation and bioremediation. Future studies need to determine the enzyme modulation, cloning and expression, and its stabilization and immobilization.

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