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Review

A review on the detoxification of organophosphorus compounds by microorganisms

Yongliang Zheng, Lifang Long, Yuanyuan Fan, Jianping Gan, Jianping Fang and Weibin Jin*

Hubei Key Laboratory of Economic Forest Germplasm Improvement and Resources Comprehensive Utilization, Huanggang Normal University,No.146 Xingang II Road, Huangzhou, 438000, Hubei Province, China.

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Organophosphorus compounds (OPs) are widely used as pesticides and chemical warfare agents (CWAs). These synthetic compounds reside in the environment and cause serious pollution problems. Organophosphorus compounds can be detoxified rapidly by hydrolysis on exposure to environment, which are liable to be influenced by abiotic and biotic factors. Microorganisms isolated from different niches are the predominant entities in the biosphere with an exceptional ability to metabolize various OPs for their growth. The focus of this work is organophosphorus hydrolase (OPH, E.C. 8.1.3.1), which catalyzes the hydrolysis of many organophosphorus compounds and greatly reduces the toxicity and even can completely mineralize them. Several OPH enzymes, including o-phenylenediamine dihydrochloride (OPD), methyl parathion hydrolase (MPH) mevalonate pyrophosphate decarboxylase (MPD) etc, have been identified which accomplish hydrolysis for specific classes of organophosphorus compounds. The functional gene encoding OPH protein have been cloned, expressed and purified in both prokaryotic and eukaryocyte expression vector. To increase the enzyme activity or enhance their broad-spectrum property, the wild activated OPH was used to modify activated sites by the chemical modification of specific amino acid residues with the use of appropriately designed coenzyme analogs. The applications of the functional strains and OPH enzymes in bioremediation of OPs pollutants included: (i) Bioremediation: the technologies can be generally classified as *in situ* or *ex situ*; (ii) immobilization: an immobilized enzyme is the OPH that is attached to an inert, insoluble material such as calcium alginate and agar; immobilized whole cell, the target cell is the functional strains that is capable of degrading special OPs; (iii) construction of genetically engineering bacteria. A versatile genetically engineering bacterium that gained more functional genes from different sources was constructed by gene engineering and enzyme engineering, showing a creative and promising application in OPs detoxification.

Key words: Organophosphorus compounds, biodegradation, bioremediation, oph, genetically engineering bacteria.

INTRODUCTION

Organophosphorus compounds (OPs) are most widely used around the world and have been used as pesticides and chemical warfare agents in agriculture and other fields. OPs have the general structure with a terminal oxygen atom (or sulphur atom) connected to phosphorus by a double bond, that is a phosphoryl group, and two lipophilic groups as well as a leaving group bonded to the phosphorus (Figure 1). Usually, R1 and R2 are aryl or alkyl groups that are bonded to the phosphorus atom either directly (forming phosphinates), or through an oxy-

*Corresponding author. E-mail: wbjin@yangtzeu.edu.cn, Tel: 86-713-8833606.

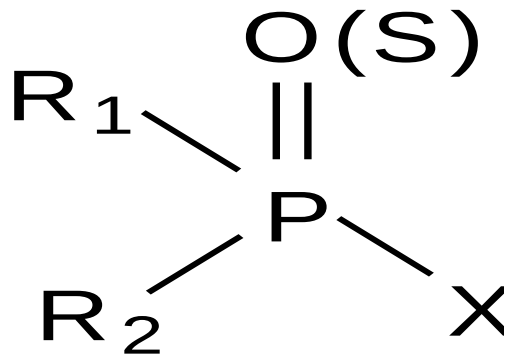

Figure 1. General chemical structure of an organophosphorus compounds.

gen or sulphur atom (forming phosphates or phosphorthioates). The X group, also binding to the phosphorus atom through an oxygen or sulphur atom is called 'leaving group'.

Although OPs play important roles in protecting agricultural crops from insect pests and weeds, and in controlling disease-transmitting vectors, they cause serious environmental pollution problems (Musa et al., 2011). Organophosphorus pesticides (as well as sarin and VX nerve agent) irreversibly inactivate acetylcholinesterase (AChE), which is essential to nerve function in insects, humans, and many other animals. Inhibition of acetylcholinesterase results in an accumulation of the neurotransmitter acetylcholine and the continued stimulation of acetylcholine receptors, and they could case acute or chronic poisoning in human and harm to our health (Ragnarsdottir, 2000).

Organophosphorus compounds can be detoxilized rapidly by hydrolysis on exposure to environment, such as sunlight, air, and soil, although small amounts can be detected in drinking water and food (Musa et al., 2011). Enzymatic hydrolysis of OPs can greatly reduce the toxicity and even completely mineralize them (Hsu et al., 2008; Harper et al., 2006). Various microorganisms capable of biodegrading OPs have been isolated from pollution environment, which is able to use OPs as the sole of carbon, nitrogen or energy source. The most widely characterized phosphotriesterase is the bacterial organophosphorus hydrolase (OPH, E.C.8.1.3.1), which had been isolated from *Pseudomonas putida, Flavobacterium, Arthrobacter,* etc. It is one of the most crucial enzymes in the detoxification of organophosphorus compounds, such as paraoxon, parathion, methyl parathion, coumphos and diazion. Organophosphorus hydrolase gene (oph, mpd and opd, etc,) were cloned, expressed and purified (Harper et al., 2006; Somara et al., 2002; Gao et al., 2002). Engineered microorganisms, with high efficiency, broad-spectrum substrates and more safe, were constructed in an attempt to completely detoxify OPs.

In this review, the main enzymes properties of organophosphorus biodegradation were summarized and the genetically modified and application prospects are put into a perspective involved in organophosphorus hydrolase.

MICROORGANISMS CAPABLE OF DEGRADING OPs

Although incineration and chemical hydrolysis have been widely used to destroy organophosphorus compounds (Lamoreaux et al., 1997), microorganisms are the predominant entities in the biosphere with an exceptional ability to exploit various OPs for their growth. Therefore, biodegradation is considered to be a reliable cost-effective technique and less secondary pollutants for pesticides abatement and a major factor determining the fate of organophosphorus pesticides in the environment. During the last few decades, much research has resulted in isolation and character of microorganisms capable of degrading kinds of OPs. Though these OPs are relatively alien for the microbes, they have evolved novel degradation enzyme and pathway(s) for their metabolism. However, development of these novel enzyme and pathway(s) in the evolutionary time scale is an extremely slow process. They are empowered to inhabit various ecological niches and pursue unusual metabolic and physiological activities (Timmis et al., 1994). OPs were used in their growth as the sole carbon, nitrogen, phosphorus and (or) energy source (Mattozzi et al., 2006; Liu et al., 2004; Bhushan et al., 2000). These microorganisms included bacteria, fungi and algae. Table 1 summarizes the performance of various microorganisms capable of detoxifying OPs.

Organophosphorus hydrolase (oph)

A variety of microorganisms can detoxify organophosphorus compounds by hydrolyzing them using organophosphorus acid anhydrases (Table 1). These OPs-degrading microbes have been isolated from environment polluted by some organophosphorus compounds for a long period (Allard and Neilson, 1997). They commonly initiate organophosphorus compounds degradation with special functional enzyme by cleaving P-O or P-S bond reaction, and these enzymes have a broad substrate range with a close similarity structure or a chemical functional group, and can hydrolyze a number of organophosphorus compounds. While one of these enzymes or some variant can allow the initial detoxification of an organophosphorus contaminant, the organism may not degrade the hydrolysis products, some of which are toxic and inhibit bacterial growth (Hong et al., 2001; Walker and Keasling, 2002). The OPs are mainly detoxified through oxidation and hydrolysis. Organophosphorus hydrolase (OPH, EC8.1.3.1) is a bacterial enzyme that has been shown to degrade a wide range of organophosphorus pesticides and nerve agents (Sogorb and

Table 1. Microorganisms capable of detoxifying Organophosphorus compounds.

Microorganism	Organophosphorus compound	Function	Reference
Bacteria			
Flavobacterium sp. ATCC27551	Parathion, Diazinon	Decomposed diazinon as sole carbon source and hydrolyzed to 2-isopropyl-6-methyl -4-hydroxy-pyrimidine and then converted to carbon dioxide	Sethunatha and Yoshida, 1973; Kawahara et al., 2010
Plesiomonas sp. M6	Methyl parathion, *p*-Nitrophenol	Hydrolyze methyl parathion to *p*-nitrophenol and able to use benzoic acid and phenylacetic acid as sole carbon and energy source	Cui et al.,2001
Bacillus sp.	Parathion	Hydrolyzed parathion to *p*-nitrophenol and liberated nitrite from the hydrolysis product *p*-nitrophenol	Siddaramappa et al., 1973
Pseudomonas sp. WBC-3	Methyl parathion, *p*-Nitrophenol	Utilises methyl parathion (MP) or *p*-nitrophenol (PNP) as the sole source of carbon, nitrogen, and energy	Liu et al., 2005
Pseudomonas aeruginosa MCMB-427	Dimethoate	Degradation dimethoate with plasmid-mediated	Deshpande et al., 2001
Azospirillum	Ethion	Aerobic degradation of ethion in minimal salts medium	Foster et al., 2004
Pseudomonas aeruginosa HS-D38	Methyl parathion, *p*-Nitrophenol	degraded methyl parathion and *p*-nitrophenol as the sole source of carbon, nitrogen and energy	Zheng et al., 2009
Pseudomonas putida	Paraoxon, Dimethoate, Parathion	Use as a sole carbon source , phosphorus source and(or) energy source	Mattozzi et al., 2003; Nazarian, 2007; Walker and Keasling, 2002
Burkholderia sp. NF100	Fenitrothion	Used as a sole carbon source, hydrolyzed an organophosphorus bond of fenitrothion, forming 3-methyl-4-nitrophenol	Hayatsu et al., 2000
Micrococcus sp.	Malathion, Chlorpyriphos	Degrade malathion and chlorpyriphos for growth as the sole carbon source by plasmid-harboring strain	Guha et al.,1997
Arthrobacter sp. B-5	Isoxathion	Utilize isoxathion as a sole nitrogen source and producted 3-hydroxy-5-phenylisoxazole and diethylthiophosphoric acid	Ohshiro et al., 1999
Acinetobacter sp.	Dimethoate	Degraded Dimethoate through cometobolism; Dichlorvos, methamidophos and parathion can also be degraded	Wang et al., 2001
Flavobacterium	Dichlorvos	Utilize dichlorvos as the sole source of phosphorus; Chlorpyrifos and phoxim could also be degraded as the sole phosphorus source.	Ning et al., 2012
Brevibacillus sp. KB2	Malathion	Biodegradation malathion under aerobic and energy-limiting conditions. The metabolites mal-monocarboxylic acid and mal-dicarboxylic acid were identified	Singh et al., 2012
Fungi			
Aspergillus flavus	Pirimiphos-methyl, Pyrazophos, Malathion	Used as phosphorus sources or carbon sources	Hasan, 1999
Trichoderma viride	Malathion	Breakdown malathion through the action of a carboxylesterase(s)	Matsumura, 1974
Penicillium waksmani	Parathion	Tolerated parathion at concentrations as high as 1000 ppm and converted it to aminoparathion	Rao and Sethunathan, 1974
Penicillium lilacinum BP303	Methyl parathion, Parathion, Paraoxon	Degrade various organophosphorus pesticides by cleaving P-O in the phosphotriesters bond and P-S linkage in the phosphothiolesters effectively	Liu et al., 2004
Saccharomyces rouxii WY-3	Methamidophos	Utilizing methamidophos as sole nitrogen and phosphorus sources, and also capable of utilizing methylamine, ethylamine and ammonium sulfate as nitrogen sources except nitrate and hydroxylamine	Liu et al., 2001
Others			
Nocardia sp.	Coumaphos	Coumaphos was used as a carbon source and hydrolyzed to diethylthiophosphoric acid and chlorferon	Shelton,1988
Microalgae	Methyl parathion	Utilized methyl parathion as a source of phosphorus	Megharaj et al., 1994
green algae	Fenamiphos	Transformed fenamiphos to fenamiphos sulfoxide (FSO), then to fenamiphos sulfoxide phenol (FSOP)	Cáceres et al., 2008

Table 2. Characteristics of organophosphorus hydrolases isolated from microorganisms.

Name	Accession Number	Source	Length (AA)	Mass (≈kD)	Reference
OPD	AAV39527.1	*Flavobacterium* sp. ATCC 27551	365	35	Mulbry and Karns, 1989
OPH	BAA85881.1	*Arthrobacter* sp.	415	44	Ohshiro et al., 1999
OPAA	AAB05590.1	*Alteromonas* sp.	517	60	Cheng et al., 1996
OPAB	AAA25371.1	*Mycobacterium* sp.	409	43	Mulbry, 1992
OPDA	AAK85308.1	*Agrobacterium radiobacte* P230	384	41	Horne et al., 2003
OPDB	AAT67170.1	*Burkholderia* sp. FDS-1	324	34	Zhang et al., 2006
MPDB	AAY18224.1	*Burkholderia cepacia*	331	35	Ekkhunnatham et al., 2012
OPHC2	CAE53631.1	*Pseudomonas pseudoalcaligenes*	324	35	Chu et al., 2006
MPH	AAP06948.1	*Pseudomonas* sp. WBC-3	331	35	Wei et al., 2009
MPD	AAk14390.1	Plesiomonas sp. M6	331	35	Fu et al., 2004

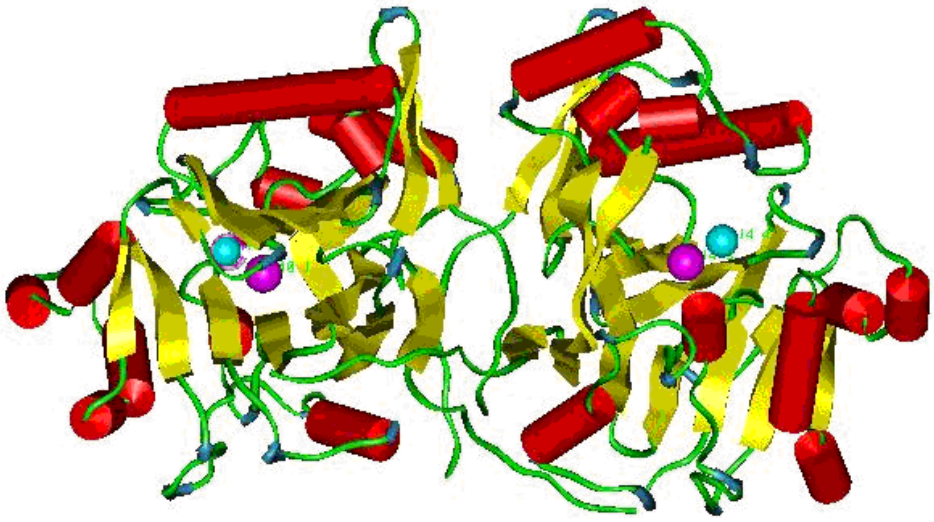

Figure 2. Representation of the OPH dimmer. A dimmer consist of two monomers. The mental binding active central consist of two metal ions (shown as pink and green spheres) (Zheng et al., 2008).

Vilanova, 2002; Kang et al., 2012), which had been isolated from both *Flavobacterium* sp. ATCC 27551 (Mulbry and Karns, 1989) and *Pseudomonas diminuta* MG (Phillips et al., 1990). Nowadays these unique enzymes have been isolated, purified and characterized, and has been demonstrated that these enzymes differ in high sensitivity to chemicals, substrate specificity, molecular mass and optimum reaction conditions (Table 2). Most of them were isolated from bacteria with little reports on organophosphorus compounds biotrans-formation by fungi. Liu et al. (2004) purified a novel fungal enzyme capable of hydrolyzing methyl parathion, parathion, paraoxon and coumaphos, and it degrades organophosphorus pesticides containing both P-O bond and P-S linkage effectively, which is the first broad-spectrum organophosphorus compound hydrolase iso-lated and purified from fungi.

THREE-DIMENSIONAL STRUCTURET AND MUTATIONAL STUDIES OF OPH

X-ray crystallographic studies and NMR spectroscopy analysis (Benning et al., 2001; Vanhooke et al.,1996) have verified that OPH is a dimeric metalloenzyme that contains two equivalents of zinc per monomer, which can be described as αβ/βα two-fold like metallo-hydrolase former (Figure 2). Two internal β-sheets are flanked each side by three α-helices, a β-lactamase-like domain forms in the subunit domain, which includes the binuclear metal centre and several metals (Co^{2+},Cd^{2+},Ni^{2+},Mn^{2+},or Fe^{2+}) can substitute for the native zinc with varying affects on rates of hydrolysis (Zheng et al.,2008; Omburo et al.,1992).

Different metal-associated form of the enzyme have demonstrated the varying hydrolytic capabilities for each of the OP neurotoxins, and the activity of OPH (Co^{2+}) is consistently higher than that of OPH (Zn^{2+}) by 5- to 20-folds (di Sioudi et al., 1999).

To increase the enzyme activity or enhance their broad-spectrum property, a variety of techniques have been used to evolve OPH enzyme. New active sites can be introduced into naturally occurring enzymes by the chemical modification of specific amino acid residues with the use of appropriately designed coenzyme analogs (Kaiser and Lawrence, 1984). Small-scale mutations such as point mutations, insertions and deletions were usually selected and operated (Grimsley et al., 2005). di Sioudi et al. (1999). have used the site-directed mutations of histidines near the native OPH active site and gained three OPH variants with a 4- to 30-fold increase in substrate specificity and enhanced rates of hydrolysis for demeton-S (P-S bond) and NPPMP (P-O bond), analogs for the chemical warfare agents VX and soman, respectively. Another successful variant (H254R) in which the histidine at position 254 of OPH was changed to an arginine showed a 4-fold increase in the hydrolysis of demeton-S (VX analog) and a 14-fold decrease with paraoxon (Grimsley et al.,2005). Substitution of the histidine at position 254 of OPH enzyme could also affect the kinetic properties of substrates except paraoxon. The histidine at position 254 (His-254) is currently believed to assist the reaction by the shuttling of the proton from Asp-301 to the bulk solvent and away from the active site (Aubert et al.,2004). The *opd* gene was shuffled in search for enhanced variants for methyl parathion hydrolysis and the mutation H257Y was found in all isolates that displayed higher activities (Cho et al., 2002). These researches clearly demonstrate that the active site of OPH can be manipulated for optimum decontamination and decomposition of the OPs and the OPH enzyme can be modified and direct evolution.

POTENTIAL APPLICATIONS

The traditional approaches (landfilling, recycling, pyrolysis and incineration) for the detoxification of organophosphorus compounds are harmful and possess serious environmental consequences. Therefore, utilizing functional strains or enzymes for the biodegradation and decontamination of organophosphorus agents has received considerable attention and it offers a promising strategy for economical and safe detoxification of OPs (Sogorb and Vilanova, 2002).

Bioremediation

Bioremediation is the use of microorganism metabolism to remove OPs pollutants, which is an eco-friendly, cost effective, highly efficient approach and can be considered as a superior, promising alternative to physical and chemical methods (Vermelho et al., 2012; Nawaz et al., 2011; Niu et al., 2011). The technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation is the process whereby OPs pollutants are biodegraded under natural conditions to either carbon dioxide and water or an attenuated transformation product. It is a low-cost, environment-friendly and sustainable approach for the cleanup of polluted sites, whereas the cost of *ex situ* bioremediation approaches can be high, relative to *in situ* methods (Ely et al., 2008). OPs in soil and water can be biodegraded by functional microorganisms and it is the primary mechanism of OPs breakdown, mineralization and detoxification in contamination soils (Surekha et al., 2008).

Mohan et al. (2004) applied the soil bioslurry-sequencial batch reactors (SS-SBRs) for treating chlropyrifos contaminated soil under anoxic-aerobic-anoxic condition. Rapid degradation of the substrate in soil matrix was observed in slurry phase system compared to the *in situ* decontaminate systems (it showed efficient performance in SBR that enforced short-term unsteady state conditions coupled with periodic exposure of the microorganisms to defined process conditions).

Pesticide-degrading epiphytic bacterium could also become a new way for *in situ* phyllosphere bioremediation where the hostile niche is unsuitable for other pesticide-degrading bacteria isolated from soil and water (Ning et al., 2012).

Immobilization

The immobilization of desired enzyme on suitable materials as carriers is currently gaining much attention in various fields of bioremediation. The enzyme immobilization has been recognized as a potential candidate for extending the process of organophosphorus compounds detoxification. In early research in immobilizing OPH, a partially purified enzyme extract was covalently attached to porous glass and silica beads and were used in a continuous flow reactor to treat pesticide manufacturing wastewater. These studies indicate that the immobilized enzyme accomplished 95% hydrolysis of up to 250 ppm parathion with no loss of activity after 70 days of continuous flow experiments (Munnecke, 1979). Pure OPH enzyme was first immobilized in trityl agarose in a fixed bed reactor. The immobilized enzyme was shown to behave chemically and kinetically similar to the free enzyme when paraoxon was utilized as a substrate. Several organophosphorus pesticides, methyl parathion, ethyl parathion, diazinon, and coumaphos were also hydrolyzed by the immobilized phosphotriesterase (Caldwell and Raushel, 1991). The insecticides paraoxon and parathion were successfully hydrolyzed by phosphotriesterase (PTE) that was immobilized on a trityl

agarose matrix. The immobilization resulted in a relative increase in the stability of PTE and the maximum efficiency of the system was about 40% relative to the free enzyme (Wang et al., 2002; Ghanem and Raushel, 2005).

Construction of genetically engineering bacteria

With recent advances in biomolecular engineering, the bioremediation of organophosphorus compounds using genetically modified microorganisms has become a rapidly growing and promising fields of research for environmental protection. Two main biomolecular approaches, rational design and directed evolution, have been developed to engineer enhanced microorganisms and enzymes for the biodegradation of Ops (Ang et al., 2005). Lan et al. (2006) used a coexpression vector for the purpose of developing bacteria that can detoxify different pesticides. The vector pETDuet was designed for coexpression of two target genes simultaneously. The organophosphorus hydrolase gene (opd) from *Flavobacterium* sp. and carboxylesterase B1 gene (b1) from *Culex pipiens* were cloned in the coexpression vector. A genetically engineering bacterium was capable of producing both enzymes for degradation of organophosphorus, carbamate and pyrethroid pesticides. Zhang et al. (2004) constructed the recombinant insecticide-resistant mosquito carboxylesterase B1 to detoxify organophosphorous compounds. A combination of carboxylesterase B1 with the uncharged oxime diacetylmonoxime was used and demonstrated that the recombinant insecticide-resistant mosquito carboxylesterase with oxime is an effective approach for detoxification of organophosphorous compounds (Zhang et al., 2004). Another genetically engineered *Escherichia coli* cell expressing both organophosphorus hydrolase (OPH) and a cellulose-binding domain (CBD) on the cell surface was constructed, enabling the simultaneous hydrolysis of organophosphorus nerve agents and immobilization via specific adsorption to cellulose. The immobilized cells degraded paraoxon rapidly at an initial rate of 0.65 mM/min/g of cells (dry weight) and retained almost 100% efficiency over a period of 45 days (Wang et al., 2002).

The specificity of native OPH has been further enhanced by alterations to the metal content of the enzyme as well as mutations to specific active site residues, which productively enhanced the ability of the enzyme to degrade specific OP compounds. di Sioudi et al. (1999) utilized a alternative expression systems to facilitate the native OPH protein aimed at the elucidation of cellular processing and secretion, and the optimization of purification protocols. OPH was chemically synthesized with a codon bias toward *E. coli* and followed by its cloning and heterologous over-expression in *E. coli* under induction with 0.1 M IPTG. The expressed enzyme was solubilised by mild treatment with incubation at 4°C to precipitate SDS. Results indicate that recombinant OPH was able to detoxify parathion and methyl parathion ranging between 10 to 80% and 3.6 to 45% in enzyme reaction cycles after immobilization on Ca-alginate and agar-agar, respectively (Kapoor and Rajagopa, 2011). Khodi et al. (2012) used the recombinant *E. coli* display- ing organophosphorus hydrolase (OPH) to overcome the diffusion barrier limitation of organophosphorus pesticides, and the ability of recombinant *E. coli* that express significant activity to utilize diazinon as the sole source of energy without growth inhibition (Islam et al., 2010).

CONCLUSIONS

Organophosphorus compounds are most widely used in agriculture for crop production and other fields. The fate of organophosphorus compounds in the environment is associated with both abiotic and biotic processes, including volatization, photooxidation, chemical oxidation, bioaccumulation, and microbial transformation. Microorganisms are the predominant entities in the biosphere with an exceptional ability to degrade various OPs for their growth. Many functional microorganisms capable of degrading a special or several kinds of OPs have been isolated and characterized from different niches. They have evolved various novel degradation enzyme and pathway(s) for their metabolism. Organophosphorus hydrolase (OPH) is a typical enzyme with the ability of hydrolyzing a wide variety of organophosphorus pesticides and chemical warfare agents. The organophosphorus pesticide hydrolase, including OPD, MPD, MPH, etc, belong to organophosphorus hydrolases family.

Organophosphorus hydrolase holds an intense interest and close attention should be paid by researcher for its higher enzyme activity and broad spectrum for the detoxification of OPs. The gene encoding OPH protein have been cloned and characterized from different functional strains, and was highly efficiently expressed not only in prokaryotic expression vector (Islam et al., 2010; Khodi et al., 2012) but also in eukaryocyte expression vector (Fukuda et al., 2010; Chu et al., 2006).

The specificity of native OPH has been further enhanced by alterations to the metal content of the enzyme as well as mutations to specific active site residues, which productively enhanced the ability of the enzyme to degrade specific OP compounds. To increase the enzyme activity or enhance their broad-spectrum property, native activated OPH was used to modify activated sites by the chemical modification of specific amino acid residues with the use of appropriately designed coenzyme analogs.

Functional strains and OPH enzyme have been applied extensively in the bioremediation of OPs pollutants. Bioremediation technologies can be generally classified as *in situ* or *ex situ*. In recent years, the immobilization of

OPH enzyme on suitable materials as carriers is currently gaining much attention in various fields of bioremediation. Along with the development of the biochemistry and the molecular biology, and the other modem biological technology, such as genetic engineering, enzyme engineering, fermentation engineering and so on, many works have been carried out to change biology characteristic of OPH, and to construct a versatile genetically engineering bacteria, which have creative and promising future in OPs detoxification.

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