Progress in protein engineering of Organophosphorus hydrolase (OPH)

Qiamin Gu, Chuanxin Zhao, Gaoyun Chen, Xiuli Qi and Min Liu*

Institute of NBC Defense, Beijing, 102205, China

*Corresponding author’s e-mail: lm@nbcdef.cn

Abstract. The biodegradation of organophosphorus pesticides is characterized by high efficiency, mild reaction conditions, no stimulation, environmental friendliness and no secondary pollution, however, the hydrolysis activity, expression level and thermal stability of wild-type enzymes restrict the practical application of biodegradation seriously. Using protein engineering methods, researchers have made many achievements in improving the properties of enzymes.

1. Introduction

Organophosphates (OPs) play an important role in the development of human industry and agriculture, but their pollution to the environment can not be ignored. The general formula of action is shown in Fig. 1. Residues in the environment are highly toxic to mammals and other target animals such as invertebrates[1]. Its toxic mechanism is mainly through the inhibition of Acetylcholinesterase (AChE) activity to affect the body's physiological response. OPs covalently modify the active site of the serine residues to form a relatively stable phosphorylated Acetylcholinesterase, leading to the loss of AChE activity and the ability to break down the neurotransmitter Acetylcholine (ACh), the accumulation of acetylcholine in the body can lead to physiological disorders of nerve conduction, nerve hyperactivity and muscle contraction symptoms, such as restlessness, salivation, unconsciousness, convulsion and respiratory failure. Eventually it led to death. [2-3]. In addition, the OPS toxicity also includes by the mitochondria-mediated nutrition metabolism disorder, induced oxidative stress and apoptosis process, presents the chronic poisoning syndrome [4].

Traditional physical and chemical methods for degradation of OPs are expensive, environmentally unfriendly and difficult to be applied in a wide range. Biodegradation has the advantages of economy, high efficiency, broad spectrum and environmental friendliness, so it is a hot spot in the Research of degradation of OPs.

![Figure 1. General structure formula and main degradation pathway of Ops.](image-url)
2. OP Hydrolases

Hydrolases are widely used in the biodegradation of OPs. Phosphoric triester hydrolase (PTH, EC 3.1.8) is a common broad-spectrum OP hydrolase, which is widely distributed in environmental microorganisms, animals and plants [5]. Most of these enzymes belong to the superfamily of amidohydrolases, which are lactonases with good stability[6]. PTH can be divided into two subclasses: one is phosphotriesterase (PTE, EC 3.1.8.1). The hydrolytic substrates tend to parathion and other organophosphate compounds containing P-O bond, including Organophosphorus hydrolase (OPH) and Methyl parathion hydrolase (MPH). The other is Diisopropyl-fluorophosphatase (DFPase, EC 3.1.8.2). The hydrolytic substrates tend to contain P-F or P-CN bond organophosphate monoester compounds, including Diisopropyl Fluorophosphatase (DFPase) and Organophosphorus acid anhydrolase (OPAA) and Human serum paraoxonase (OPN 1).

Organophosphorus hydrolase (OPH, EC. 3.1.8.1) was originally isolated and purified from Flavobacterium sp. ATCC 27551 and Pseudomonas diminuta MG. The coding gene was opd. Molecular biological evidence showed that although the plasmids of the two strains were significantly different, the coding sequences of opd gene contained in the two strains were identical[7]. OPH enzyme family is the product of the molecular evolution of lactonases. In the process of evolution, the specificity of the enzyme gradually declined, and finally developed into an OP hydrolase which can hydrolyze a variety of OPs. OPH can hydrolyze P-O, P-CN, P-F and P-S bonds, and the most suitable substrates are parathion, parathion and other OPs containing two ethyl groups.

Methyl parathion hydrolase (MPH, EC. 3.1.8.1) was isolated and purified from soil bacterium Pseudomonas sp. WBC-3[8], which was composed of 331 amino acid residues, and had no sequence homology with the known OPH containing PTE domain. The range of MPD hydrolysis substrate is relatively narrow, which tends to methyl paraoxon, methyl parathion and other OPs containing two methyl groups. Methyl parathion can be used as the sole carbon or phosphorus source for the growth of MPH, and it can be completely hydrolyzed to p-nitrophenol and dimethyl phosphoric acid.

Diisopropyl Fluorophosphatase (DFPase, EC 3.1.8.2) is widely distributed in mammals and the ganglion and hepatopancreas of squid. It is the first enzyme with the activity of hydrolyzing OPs[9]. In 1946, Abraham Mazur found an enzyme in the plasma and tissues of rabbits and human beings, and named DFPase. DFPase mainly acts on P-F bond and P-CN bond, which can hydrolyze DFP and organophosphorus nerve agents such as GB and GD, but has no hydrolysis ability to VX and paraoxon.

Organophosphorous acid anhydrolase (OPAA, EC. 3.1.8.2) was isolated from Alteromonas sp. Strain JD6.5 for the first time [10]. OPAA consists of 517 amino acids with a molecular weight of 58 kDa. It is a typical proline dipeptidase and has partial phosphotriesterase activity. This enzyme has similar hydrolysis activity, catalytic mechanism and enantiomeric selection mechanism with OPH and MPH [11], but their gene coding sequences are not similar. OPAA showed high activity in the hydrolysis of P-F bond, which could efficiently hydrolyze DFP, GB and GF, but had low ability to hydrolyze P-O and P-CN bond, and could not hydrolyze P-S bond.

In addition to the above hydrolases, there are many kinds of OP hydrolases, such as OPHC2, PON, HOCA, ADPase, OPDA and so on. Different hydrolases exhibit different enzymatic properties and substrate ranges. Under specific needs, different hydrolases can be selected or combined to achieve the best biodegradation effect.

3. The protein engineering of OPH

Driven by the achievements of structural biology, the protein engineering of OP hydrolase has developed rapidly. Due to the different sources, structures and mechanisms of enzymes, the enzymatic properties, catalytic mechanism and substrate range of each hydrolase are quite different. Among them, OPH has a good application prospect because of its clear structure, definite coding, good broad spectrum and mature hydrolysis mechanism. It is an ideal protein engineering object and the most deeply understood OP hydrolase.
3.1. Crystal structure and hydrolysis mechanism of OPH

The crystal structure of OPH is a homodimer protein with 336 amino acid residues and a molecular weight of 36 kDa[5]. The high-level structure of OPH is regular TIM barrel folding, that is, eight α-helices surround eight β-sheets, each β-sheets and α-helices are wound into a ring at the end of the barrel, and the active center is located at the C-terminal of the β-sheets of TIM barrel, which conforms to the structural characteristics of amide hydrolase superfamily proteins [12]. Its active site contains a pair of binuclear metal centers, and the natural hydrolase is generally Zn2+. Bivalent metal ions CO2+, Cd2+ or Ni2+ can replace Zn2+ and maintain all enzyme activity [13]. There are three hydrophobic pockets in the active center of the hydrolase, which correspond to the three substituents of the substrate molecule, which affect the release rate of the leaving group and determine the specificity of the enzyme. Large pocket (H254, H257 and L271) and small pocket (M317, G60, 1106, I303 and S308) recognize hydrophobicity of substrate side chain and chirality of central phosphorus atom; pocket (W131,F132, F306 and Y309) control the release of leaving groups [14].

The hydrolysis mechanism of OPH is as follows: the metal center activates water molecules into bridging hydroxyl radicals, which coordinate with D301 and attack the central phosphorus atom of the substrate. The first leaving group is released after the single bond of phosphorus and oxygen in the substrate is broken (p-nitrophenol is released as the first product in the case of paraoxon). Protons are transferred to h254 via D301 and are far away from the active site, releasing the second product. The active cavity returns to its original state and enters the next round of catalysis [15-16].

3.2. Progress in protein engineering of OPH

Protein engineering is the main way to improve enzyme function, including rational design strategy and irrational design strategy. Rational design is based on the analysis of enzyme protein crystal structure combined with computer-aided design, using modification, mutation and other technologies to change the enzyme hydrolysis activity, substrate range and stability; irrational design uses directed evolution technology to carry out extensive and targeted random mutation of enzyme protein, and then screen functional mutants according to needs. The two strategies complement each other and promote the research, development and production of enzymes.

The hydrolytic activity, expression level and thermal stability of wild-type OPH restrict the practical application of the enzyme. In order to solve this problem, many researchers have devoted themselves to improving the catalytic efficiency of the enzyme by changing the special amino acid, broadening the substrate spectrum of the enzyme, increasing the expression of the enzyme and strengthening the stability of the enzyme, so as to make the enzyme evolve towards the direction of pre design.

3.2.1. Point mutation of OPH gene

The three hydrophobic pockets of the enzyme activity center, which affect the release rate of the leaving group and determine the specificity, are the focus of the researchers.

Shim[17] used site directed mutagenesis to construct mutant m317a, which had a relatively higher ability to catalyze the hydrolysis of phosphodiester bond. The rate of hydrolysis of phosphodiester catalyzed by mutant M317A was 200 times faster than that of wild-type OPH. Because Met317 is located in the "protein-s-binding" pocket of the active center of the hydrolase. By changing to Ala, the attack of ammonium cation in the active site on the negative charge of oxygen atom of phosphodiester is considered to be the reason for the increase of hydrolysis rate. The substrate binding site pocket is mainly composed of hydrophobic residues, which can easily accommodate a variety of nonpolar organic phosphates. This explains the relatively wide substrate specificity of the enzyme to some extent.

Discoudi B[18] carried out two point mutations in H254R / H257L of OPH, which increased the substrate specificity (kcat / km) of Demeton S (an analogue for VX, containing P-S bond) by 2-30 times, and the specificity for NPPPMP (the analogue for soman) by 18 times. H254 and H257 are located near the active site of the enzyme, which catalyze the reaction between the active site and the
substrate. At the same time, these two sites are located in the large hydrophobic pocket of hydrolase. The mutation of arginine and leucine can expand the hydrophobic pocket diameter, better combine with the substrate with larger molecular weight, and improve the hydrolysis rate.

Sriram gopal[19] constructed eight mutants based on the crystal structure of OPH and its similarity with AChE. L136Y increased the ability of OPH to hydrolyze nerve agent VX by 33%. L136y is a mutant based on the crystal structure of OPH, covering the interaction region between s2dpe leaving group of VX and OPH, in order to create a favorable space environment for the departure group. The enhanced activity of 1136y to VX can be explained by the increased hydrogen bonding potential between VX and mutant enzymes compared with natural enzymes. This binding can be used to stabilize the substrate at the active site and accelerate the separation of hydrolysates after hydrolysis. The other seven mutants reduced the relative hydrolysis rate of VX by 55% - 76%.

Cho[20] screened the OPH variant B3561 (A14T, L17P, A80V, V116I, K185R, A203T, I274N, P342S) by directed evolution technology, which increased the hydrolysis rate of chlorpyrifos by 725 times and other insecticides by 12 to 39 times.

Studies by D. A. Schofield showed that[21], OPH mutants, such as G60V, A80V, I106V, F132D, I274N, K185R, H257W, F306V and S308L, could improve the hydrolysis efficiency of P-S pond OPs or agent VX. Compared with the wild-type lysates, the specific activities of demeton-S methyl and malathion were increased by 177 and 1800 times, respectively, and the specific constants of the purified mutant protein for demeton-S methyl and malathion were increased by 25 times.

Young Su Jeong[22] showed that the double mutant L271A / Y309A showed a 150 fold higher catalytic efficiency for VX than the wild type. Studies showed that expanding the hydrophobic pocket of the active site was conducive to the entry of the substrate into the active site, and also conducive to the hydrolysis of the substrate, and the substrate concentration required for the half maximum rate (Km) also increased. This result strongly supports the current mainstream view that the method of enlarging the substrate binding site entrance can improve the catalytic efficiency of class V agents. Their research also showed that Y309A mutation also led to a significantly higher soluble expression level of the hydrolase than that of the wild type, overcoming the problem that the low soluble expression of OPH restricted the practical application of the hydrolase.

The 211 site was identified by Liu XY as the key residue of the new OPH activity[23]. Compared with the wild-type OPH, the activity of OPH I211A was significantly increased, and the activity of lactonase was decreased, but the activity of esterase was similar. The single mutant OPH (OPH from Acinetobacter sp.) I211A showed significant activity to methyl parathion and ethyl parathion, but the wild-type aboph was not detected. The activity of OPH I211A to fenitrothion was 0.54 μmol·min⁻¹·mg⁻¹, which was 11 times higher than that of wild-type OPH. It is speculated that the substitution of I211A reduces the size of the side chain, thus providing enough space for the movement of the flexible ring.

Farnoosh[24] selected appropriate amino acid pairs and introduced disulfide bond to improve the thermal stability of protein through the design software disulpide. Data analysis showed that the half lives of A204C / T234C and T128C / E153C mutants were extended to 4 and 24 minutes, respectively. For T128C / E124C mutants, thermal stability and catalytic efficiency (kcat) were also increased.

3.2.2. Modifying the recombinant enzyme to improve the enzymatic properties of OPH

Studies by Fei[25] showed that the modification of protein after translation has a great influence on the hydrolytic activity, and the properties of the enzyme can be effectively improved by post-translational modification of the enzyme. Yu[26] improved the stability and heat resistance of the hydrolytic through glycosylation. Shen[27] improved the enzyme activity by glycosylation. The existence of metal ions is necessary for enzyme activity, which can be improved by replacing the metal ions in the recombinant enzyme. Chu[28] added different divalent metal ions in the protein translation process to replace the metal ions in the natural OPH structure, so as to improve the enzyme activity.
3.2.3. Secretory expression system of OPH

In order to achieve the needs of foreign gene protein expression, researchers have constructed a variety of expression systems as a molecular biological technology to express foreign gene protein. The common expression systems include: E.coli Expression System, plant expression system, insect expression system and mammalian expression system. Different expression systems have their own advantages, disadvantages and limitations. In order to achieve the expression of exogenous proteins, we should consider the expression amount, production cost, production cycle and other factors to select the most appropriate expression mode.

At present, Escherichia coli is the most commonly used host for the expression of OPH. Its system is mature, the genetic background is clear, the reproduction is fast, the cost is relatively low, the scope of application is wide, and the expression products can be obtained in a short time. However, E.coli usually generates foreign proteins in the form of inclusion bodies. The proteins in the inclusion bodies are non-folded aggregates with poor biological activity and high difficulty in isolation and purification [29-30], which is not the most suitable system for the expression of organophosphorus hydrolase. Srinivasan[31] cloned the opd gene into the expression system of Ralstonia eutropha, indicating that the ability to express active enzyme protein was positively correlated with the copy number of opd gene. When the recombinant strain with two copies of opd gene was used for high-density fermentation, the enzyme protein production could reach 4.3g/L, which was 30 times of that in E.coli. Chu[32] expressed ophc2 in P. pastoris GS115, which effectively increased the secretion of the enzyme protein, but there was no study on the expression of OPH in Pichia pastoris. Acharya[33] expressed OPH in Bacillus subtilis, and studied the related properties and characteristics.

3.2.4. Cell surface display of OPH

It is also an effective method to improve the expression and degradation efficiency of organophosphorus hydrolase by displaying on the cell surface. Richins[34] used the LPP-OmpA fusion system to "display" OPH on the cell membrane of E.coli, so that the cells can cells show the activity of degrading enzymes in intact condition; compared with the cells with the same expression level that locate the enzyme in the cytoplasm, the degradation efficiency of parathion is increased by seven times, and there is no diffusion restriction, but the expression of OPH on the surface causes serious growth inhibition And the enzyme was not stable enough. Shimazu[35] used the ice nucleation protein (IanV) anchoring system of Pseudomonas syringae Ina5 to display the phosphate degrading enzyme OPH, and the constitutive expression of OPH on the surface of InaV anchor did not lead to cell lysis or growth inhibition. Suspension culture also showed good stability and maintained almost 100% activity for 3 weeks. Takeshi Fukuda[36] used Flo1p anchor system to display organic phosphorus hydrolase OPH on yeast surface. The enzyme activity of the recombinant strain reached 2000 units / (mg dry cell). Yang C[37] constructed a fusion vector (INPNC-OPH-GFP) with organophosphate hydrolase, green fluorescent protein and ice nucleation protein, and displayed it on the surface of P. putida JS444. It was found that the recombinant protein displayed on the surface of the cells did not inhibit the growth of the cells or affect the cell viability. The recombinant strain could completely degrade parathion (100 mg / kg) in 15 days. These studies indicate that immobilization of hydrolases on the surface of cells to improve the degradation efficiency has a good application prospect.

4. Prospects

The protein engineering of OPH improves the performance of the hydrolase from many aspects, which greatly improves the hydrolysis rate of organophosphate compounds, widens the range of hydrolysis substrates, enhances the thermal stability and improves the secretion expression level. With the gradual deepening of scientific research, it has become a reality to obtain hydrolases with excellent performance and high practical value. Many high activity degrading bacteria have been isolated, most of the work is still in the stage of laboratory research. There are still many problems to be solved and many problems need to be further discussed. However, it can be expected that with the discovery of
new degrading microorganisms, degrading enzymes and the development of bioengineering technology, biodegradation technology will play an increasingly important role in OPs degradation.

4.1. Deep degradation of OPs
At present, biodegradation can only degrade pollutants into intermediate products with less toxicity, such as p-nitrophenol from paraoxon. And in practice, the treatment of OPs pollution is more complex, especially the pesticide residues in farmland are usually combined with a variety of pesticides. So it is urgent to find more complete degradation bacteria, build more effective degradation bacteria and broaden the substrate of degradation bacteria. According to the different needs of degradation, several microorganisms with high degradation ability for different compounds and symbiotic relationship were used to breed high-efficiency mixed strains. The synergistic effect between microorganisms is used to improve the degradation efficiency, so that the degradation products can provide nutrients such as nitrogen, phosphorus, carbon source for the growth of microorganisms, and finally realize the complete degradation of OPs.

4.2. Integration of hydrolase genes
The hydrolytic bacteria screened from nature have different degradation characteristics, broken bond sites and optimal substrates. At present, the research of protein engineering is mainly based on wild enzymes for gene transformation. Therefore, it is possible to integrate different hydrolytic enzyme plasmids into one strain by genetic methods, integrate different enzyme protein coding sequences into one plasmid, or integrate multiple hydrolase active sites into the basic protein skeleton to construct a "super strain" with high degradation ability, so as to broaden the degradation spectrum and enhance the degradation ability.

4.3. Research and development of hydrolase application products
At present, the research on organophosphorus degrading microorganisms is mainly focused on the laboratory research on the function of hydrolase protein. However, in the actual industrial production and application of hydrolases, it is necessary to consider the selection of living strain or enzyme preparation and other application methods.

4.4. Safety considerations of hydrolases
The construction of genetically engineered bacteria can effectively solve the problems that are difficult to be solved by other technologies, and brings great economic and environmental benefits. At the same time, more and more attention has been paid to the biosecurity problem. The widespread use of disease-resistance genes has led to the spread of disease-resistance genes in the environment, which may have serious consequences. Therefore, it is necessary to further monitor the biosafety of transgenic bacteria in order to avoid secondary pollution to the environment.

References
[1] Karamimohajeri S, Abdollahi M. (2013) Mitochondrial dysfunction and organophosphorus compounds. Toxicology & Applied Pharmacology, 270(1):39-44.
[2] Abdollahi M, Ranjbar A, Shadnia S, et al. (2004) Pesticides and oxidative stress: a review. Med Sci Monit, 10(6):RA141.
[3] Karami-Mohajeri S, Abdollahi M. (2011) Toxic influence of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: a systematic review. Human & Experimental Toxicology, 30(9):1119-40.
[4] Abdollahi M, Karami-Mohajeri S. (2012) A comprehensive review on experimental and clinical findings in intermediate syndrome caused by organophosphate poisoning. Toxicology & Applied Pharmacology, 258(3):309-314.
[5] Bigley A N, Rauschel F M. (2013) Catalytic mechanisms for phosphotriesterases. Biochimica Et Biophysica Acta Proteins & Proteomics, 1834(1):443-453.
[6] Elias M, Dupuy, Merone L, et al. (2008) Structural Basis for Natural Lactonase and Promiscuous Phosphotriesterase Activities. Journal of Molecular Biology, 379(5):1017-1028.
[7] Iyer R, Iken B, Damania A. (2013) A comparison of organophosphate degradation genes and bioremediation applications. Environmental Microbiology Reports, 5(6):787-798.
[8] Yan-Jie Dong, Mark Bartlam, Lei Sun, Ya-Feng Zhou, Zhi-Ping Zhang, Cheng-Gang Zhang, Zihe Rao, Xian-En Zhang. (2005) Crystal Structure of Methyl Parathion Hydrolase from Pseudomonas sp. WBC-3. Journal of Molecular Biology, 353(3).
[9] Mazur A. (1946) An Enzyme In Animal Tissues Capable of Hydrolyzing the Phosphorus-Fluorine Bond of Alkyl Fluorophosphates. Journal of Biological Chemistry, 164:271.
[10] Theriot C M, Grunden A M. (2011) Hydrolysis of organophosphorus compounds by microbial enzymes. Applied Microbiology and Biotechnology, 89(1):35-43.
[11] Nand K. Vyas, Alexei Nickitenko, Vipin K. Rastogi, Saumil S. Shah and Florante A. Quiocio. (2010) Structural Insights into the Dual Activities of the Nerve Agent Degrading Organophosphate Anhydrolase/Prolidase. Biochemistry, 49(3):p.547-559.
[12] Xia M, Zhou C, Ma X, et al. (2017) Assembly of the active center of organophosphorus hydrolase in metal–organic frameworks via rational combination of functional ligands. Chemical Communications, 53(82):11302.
[13] Theriot C M, Grunden A M. (2011) Hydrolysis of organophosphorus compounds by microbial enzymes. Applied Microbiology and Biotechnology, 89(1):35-43.
[14] Bigley A N, Raushel F M. (2013) Catalytic mechanisms for phosphotriesterases. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1834(1):443-453.
[15] Aubert S D, Li Y, Raushel F M. (2004) Mechanism for the hydrolysis of organophosphates by the bacterial phosphotriesterase. Biochemistry, 43(19):5707.
[16] Frank M, Raushel. (2002) Bacterial detoxification of organophosphate nerve agents. Current Opinion in Microbiology, 5(3):288-295.
[17] Shim H, Hong S B, Raushel F M. (1998) Hydrolysis of phosphodiesterases through transformation of the bacterial phosphotriesterase. Journal of Biological Chemistry, 273(28):17445.
[18] Disioudi B, Grimsley J K, Lai K, et al. (1999) Modification of near active site residues in organophosphorus hydrolase reduces metal stoichiometry and alters substrate specificity. Biochemistry, 38(10):2866.
[19] Gopal S, Rastogi V, Ashman W, et al. (2000) Mutagenesis of Organophosphorus Hydrolase to Enhance Hydrolysis of the Nerve Agent VX. Biochem Biophys Res Commun, 279(2):516-519.
[20] Mee-Hie Cho C, Mulchandani A, Chen W. (2004) Altering the Substrate Specificity of Organophosphorus Hydrolase for Enhanced Hydrolysis of Chlorpyrifos. Applied and Environmental Microbiology, 70(8):4681-4685.
[21] Schofield D A, Dinovo A A. (2010) Generation of a mutagenized organophosphorus hydrolase for the biodegradation of the organophosphate pesticides malathion and demeton-S. Journal of Applied Microbiology, 109(2):0-0.
[22] Jeong Y S, Choi J M, Kyong H H, et al. (2014) Rational design of organophosphorus hydrolase with high catalytic efficiency for detoxifying a V-type nerve agent. Biochemical and Biophysical Research Communications, 449(3):263-267.
[23] Jie Chen; Xiao-Jing Luo; Qi Chen; Jiang Pan; Jiahai Zhou; Jian-He Xu. (2015) Marked enhancement of Acinetobacter sp. organophosphorus hydrolase activity by a single residue substitution Ile211Ala. Biocatalysts and Bioprocessing, Vol.2(No.1).
[24] Farnoosh G, Khajeh K, Latifi A M, et al. (2016) Engineering and introduction of de novo disulphide bridges in organophosphorus hydrolase enzyme for thermostability improvement. Journal of Biosciences, 41(4):1-12.
[25] Fei W, Wang X, Yu X, et al. (2015) Correction: High-Level Expression of Endo-[beta]-N-Acetylgalactosaminidase H from Streptomyces plicatus in Pichia pastoris and Its Application for the Deglycosylation of Glycoproteins. Plos One, 10(3):e0120458.
[26] Yu X, Chao Z, Xing Z, et al. (2015) High-level expression and characterization of carboxypeptidase Y from Saccharomyces cerevisiae in Pichia pastoris GS115. Biotechnology Letters, 37(1):161-167.

[27] Shen W, Shu M, Ma L, et al. (2016) High level expression of organophosphorus hydrolase in Pichia pastoris by multicopy ophcM assembly. Protein Expression & Purification, 119:110-116.

[28] Chu X Y, Wu N F, Deng M J, et al. (2006) Expression of organophosphorus hydrolase OPHC2 in Pichia pastoris: Purification and characterization. Protein Expression & Purification, 49(1):0-14.

[29] Sreekrishna K, Nelles L, Potenz R, et al. (1989) High-level expression, purification, and characterization of recombinant human tumor necrosis factor synthesized in the methylotrophic yeast Pichia pastoris. Biochemistry, 28(9):4117-25.

[30] Shu M, Shen W, Wang X, et al. (2015) Expression, Activation and Characterization of Porcine Trypsin in Pichia pastoris GS115. Protein Expression and Purification:S1046592815300024.

[31] Srinivasan S, Barnard G C, Gerngross T U. (2003) Production of recombinant proteins using multiple-copy gene integration in high-cell-density fermentations of Ralstonia eutropha. Biotechnology and Bioengineering.

[32] Chu X Y, Wu N F, Deng M J, et al. (2006) Expression of organophosphorus hydrolase OPHC2 in Pichia pastoris: Purification and characterization. Protein Expression & Purification, 49(1):0-14.

[33] Acharya K P, Shilpkar P, Shah M C, et al. (2015) Biodegradation of Insecticide Monocrotophos by Bacillus subtilis KPA-1, Isolated from Agriculture Soils. Appl Biochem Biotechnol, 175(4):1789-804.

[34] Richins R D, Kaneva I, Mulchandani A, et al. (1997) Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase. Nature Biotechnology, 15(10):984-987.

[35] Shimazu M, Mulchandani A, Chen W. (2010) Cell Surface Display of Organophosphorus Hydrolase Using Ice Nucleation Protein. Biotechnology Progress, 17(1).

[36] Fukuda T, Tsuchiyama K, Makishima H, et al. (2010) Improvement in organophosphorus hydrolase activity of cell surface-engineered yeast strain using Flo1p anchor system. Biotechnology Letters, 32(5):655-659.

[37] Yang C, Liu R, Yuan Y, et al. (2013) Construction of a Green Fluorescent Protein (GFP)-Marked Multifunctional Pesticide-Degrading Bacterium for Simultaneous Degradation of Organophosphates and γ-Hexachlorocyclohexane. J Agric Food Chem, 61(6):1328-1334.