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Removal of neurotoxic ethyl parathion pesticide by two-stage chemical/enzymatic treatment system using Fenton's reagent and organophosphorous hydrolase

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Abstract−Organophosphate compounds, which are essential ingredients of pesticide, plasticizer, air fuel, and chemical warfare agents, are serious neurotoxic hazardous materials. Therefore, diverse physical-chemical treatments have been attempted to degrade organophosphate compounds. In the present work, we propose a two-stage chemical and enzymatic treatment system. As a first stage, pretreatment of oxidation and coagulation using Fenton's reagent utilizing iron and hydrogen peroxide was employed. Preferentially, 1 mM ethyl parathion (EP) pesticide was largely (~80%) removed by Fenton's reagent reaction within 15 min. To remove residual EP, enzymatic treatment with organophosphorous hydrolase (OPH) from recombinant *Escherichia coli* was employed as a second stage. We successfully demonstrated that the proposed two-stage hybrid treatment process removed 1 mM environmental toxic EP efficiently (~98%) within 30 min.

Key words: Organophosphate Compound, Ethyl Parathion Pesticide, Two-stage Treatment, Fenton's Reagent, Organophosphorous Hydrolase

INTRODUCTION

Organophosphate compounds have been used as a pesticide, plasticizer, air fuel ingredients, and chemical warfare agent. Among these compounds, pesticides are widely used world-wide to control agricultural and household pests [1]. The excessive and continuous use of organophosphate pesticides (OPs) has resulted in contamination of ground and surface waters leading to major environmental concerns. Especially, parathion pesticide, which belongs to organophosphate compounds, was registered by the Environmental Protection Agency [1] as priority pollutant. The farmers and agricultural workers who were directly exposed to parathion showed more symptoms of respiratory diseases such as asthma and wheezing [2]. Particularly, it was known that the exposition of parathion induces the accumulation of acetylcholine (Ach) in the body, disturbing the function of muscular responses and vital organs [3]. It was also reported that OPs are seriously toxic to fish and aquatic invertebrates [4].

To degrade OPs, diverse physical-chemical treatments such as granular activated carbon [5], wet air oxidation [6], photo-Fenton [6,7], UV/O_3 [8], UV/H_2O_2 [9,10], and electro-Fenton [4,11] have been attempted. Among those treatments, advanced oxidation processes have been widely used [7,11] due to the merits of rapid reaction rates and removals of non-degradable compounds [12,13]. However, the advanced oxidation processes using ozone generator and UV lamp have disadvantages, including relatively high treatment cost from consuming high energy [8,12]. The biological OPs treatment using microorganism (pure culture or mixed activated sludge) might be economical compared to the physical-chemical treatment, but it was reported that toxic OPs and generated intermediate compounds inactivated microorganisms and subsequently, decreased degradability [6,12]. In addition, long times (optimal retention time is about 15 days) are generally required for biological treatment of pesticide wastewater under anaerobic condition [14]. Organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* or *Flavobacterium* sp. is a homodimeric organophosphotriesterase that degrades a broad spectrum of neurotoxic OPs [15,16]. The use of OPH in bioremediation is of great interest due to its high turnover rate [17-21]. The OPH-expressing microorganism itself was also attempted to treat OPs as whole cell catalyst, but mass transport problems were not effectively solved yet [17,19,20]. In addition, immobilizations of OPH by physical adsorption or covalent bonding often result in significant reduction of operation activity and stability due to sensitive change in pH or temperature [3].

Therefore, development of new treatment technique for OPs is needed to overcome these problems. In the present work, we, for the first time, employed two-stage chemical/enzymatic treatment system (Fig. 1) for efficient removal of environmental toxic OPs, especially, ethyl parathion (EP) as a target compound. At the first

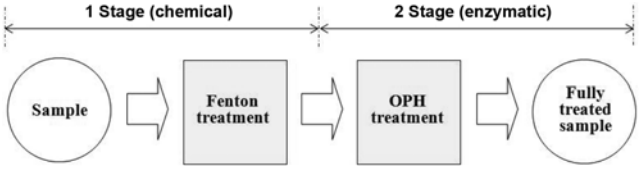

Fig. 1. Schematic diagram of the proposed two-stage chemical/biological treatment system for EP pesticide.

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step, oxidation and coagulation using chemical Fenton's reagent that utilize iron and hydrogen peroxide was employed as a pretreatment process. Then, enzymatic OPH treatment was used to remove residual EP.

MATERIALS AND METHODS

1. Fenton's Reagent Reaction

Sample containing parathion (Supelco, USA) in 300 mL beaker with adjusted pH to 1.5-5.5 using 1 N H_2SO_4 and 1 N NaOH, and Fenton's reagent ($FeSO_4 \cdot 7H_2O$ (Samchun, Korea) and H_2O_2 (Junsei, Japan)) were added into the jar-tester for Fenton oxidation (100 mL final reaction volume). The reaction sample was vigorously stirred under constant mixing (200 rpm) with a mechanical stirrer at room temperature. Then, a flocculation reaction was performed with constant slow mixing under 45 rpm for 10 min. Finally, a precipitation reaction was performed by addition of 0.5 mL of 0.5% cationic polymer flocculant (EC-220; Etec, Korea). The supernatant was analyzed to measure residual parathion concentration after filtering with 0.45 μm membrane filter (MFS, Japan) and used for subsequent biological OPH treatment.

2. OPH Preparation and Reaction

Escherichia coli strain BL21 (DE3) (F^- *ompT hsd*$S_B(r_B^- m_B^-)$ *gal dcm*) (Novagen, USA) was used as a host for recombinant OPH expression. Recombinant plasmid pTOH that contains *oph* gene fused with hexa-histidine affinity tag under T7 promoter was used (Fig. 2) [18]. Recombinant plasmid bearing strain was cultured in M9 minimal medium (12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 mg/L $CaCl_2$, 1 mM $MgSO_4$) containing 0.5% (w/v) glucose and 50 μg/mL of ampicillin at the final concentration. Cells were cultured in 250 mL Erlenmeyer flasks at 250 rpm and 37 °C. When cultures reached a cell density (optical density at 600 nm) of 1.2, 0.1 mM isopropyl-β-D-thiogalactopytanoside (IPTG; Sigma, USA) and 0.1 mM $CoCl_2 \cdot 6H_2O$ (Sigma) were added to induce recombinant OPH expression. The cells were harvested and disrupted by using a sonic dismembrator (Branson, USA). After centrifugation, the supernatant was directly used as crude OPH enzyme to degrade OPs. After OPH reaction at room temperature, the supernatant was analyzed to measure residual EP concentration after filtering with 0.45 μm membrane filter.

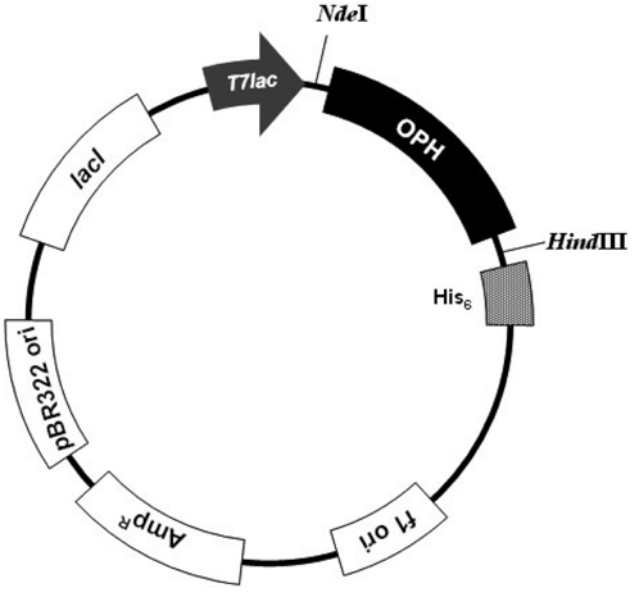

Fig. 2. Gene maps of recombinant plasmid pTOH. Abbreviations: T7lac, T7 and *lac* hybrid promoter; OPH, organophosphorus hydrolase gene; His_6, hexa-histidine affinity tag; lacI, *lac* repressor gene; Amp^R, ampicillin resistance gene; pBR322 ori, replication origin from pBR322 plasmid; f1 ori, replication origin from bacteriophage f1.

3. Analytical Assays

Optical density (OD) was measured with a UV/VIS spectrophotometer (Shimadzu, Japan) at 600 nm. Dry cell weight was calculated from OD by conversion factor 0.414. The EP concentration was determined by measuring the absorbance at 290 nm by high-performance liquid chromatography (HPLC; Waters, USA) with a UV detector and C18 column (4.6 mm×150 mm) at ambient temperature. The mixture of 60% acetonitrile in 40% water was used as a mobile phase with a flow rate of 1 mL/min. Whole cell OPH activity was measured by following the increase in absorbancy of p-nitrophenol from the hydrolysis of substrate (1 mM paraoxon (Supelco)) at 400 nm [22]. 100 μL supernatant sample was added to an assay mixture containing 400 μL 100 mM CHES [2-(N-cyclohexylamino) ethane-sulfonic acid] buffer, pH 9.0, 100 μL 10 mM paraoxon, and 400 μL distilled water. One unit of OPH activity was defined as mmoles paraoxon hydrolyzed per min.

RESULTS AND DISCUSSION

1. Removal of EP using the First Fenton's Reagent Treatment

As a first chemical treatment stage for EP, we used the Fenton method. In this stage, hydrogen peroxide dosage is an important factor, that is, organophosphate compounds will not be sufficiently oxidized under low added concentration of hydrogen peroxide. On the contrary, when hydrogen peroxide concentration is high, organic compounds can be formed by the remaining hydrogen peroxide after completion of Fenton's reagent reaction. Therefore, optimization for added concentration of hydrogen peroxide is required.

We first investigated optimal hydrogen peroxide concentration for efficient removal of 1 mM EP using Fenton's reagent treatment (Fig. 3(a)). At lower molar ratios (0.5 : 1 & 1 : 1) of iron and hydrogen peroxide, dependencies of EP removal efficiency on hydrogen peroxide concentration were not observed. However, from high ratio (2 : 1 & 4 : 1) environments, EP removal efficiencies were increased according to hydrogen peroxide concentration. Especially, 4 : 1 ratio showed the best EP removal efficiency for all treated hydrogen peroxide concentrations. At the 4 : 1 Fe^{2+}/H_2O_2 ratio, addition of both 2.7 and 3.6 mM H_2O_2 showed similar maximum EP removal efficiencies of 78.9 and 79.3%, respectively, while lower H_2O_2 concentrations (0.9 and 1.8 mM) showed low removal efficiencies (28.0 and 60.2%, respectively). Therefore, we found that addition of hydrogen peroxide with higher 2.7 mM is not necessary for efficient EP removal in Fenton's reagent process.

Next, we investigated the effect of initial pH on removal of EP pesticide. Generally, OH radical production rate is limited under high pH ranges in Fenton's reagent reaction. In addition, it was known that Fe^{2+} ions were precipitated as a hydroxide form in high pH environment and, subsequently, their catalytic activities were reduced

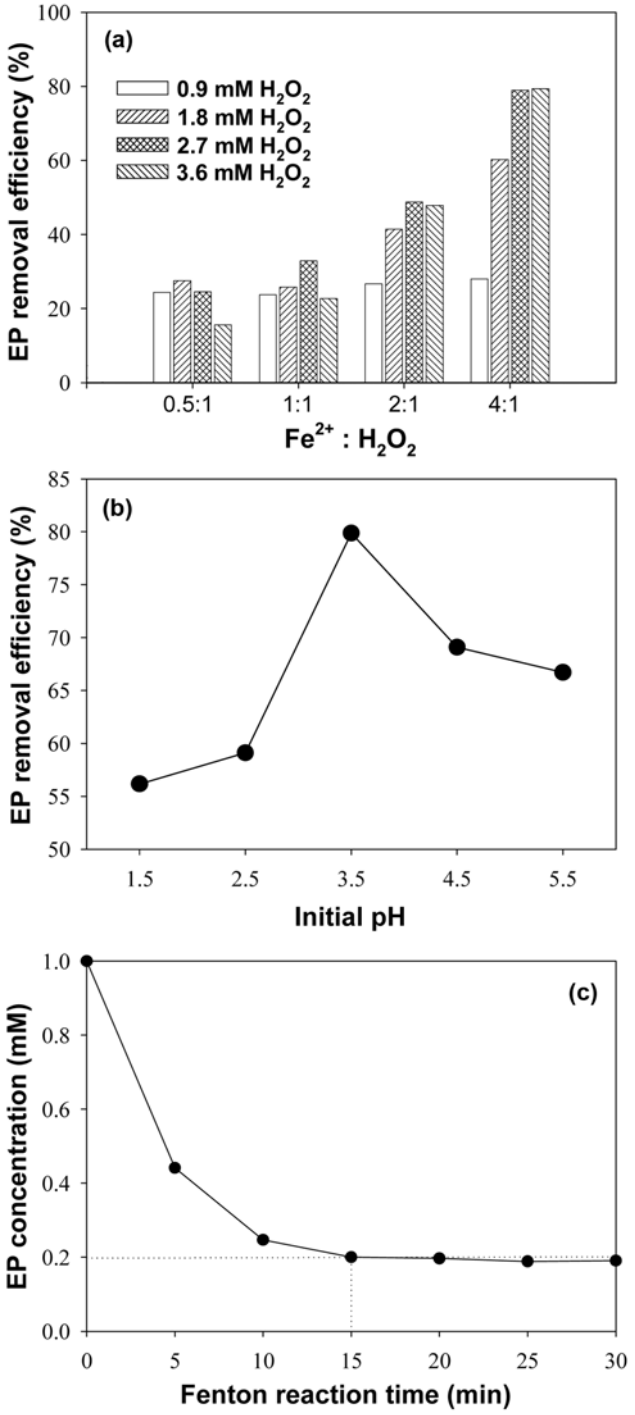

Fig. 3. (a) Effect of Fe^{2+} and H_2O_2 dosage on EP removal efficiency in Fenton process. 1 mM EP sample with initial pH 3.0 was treated for 15 min. (b) Effect of initial pH on EP removal efficiency in Fenton process. 1 mM EP sample with initial pH 3.0 was treated by 2.7 mM H_2O_2 with 4 : 1 Fe^{2+}/H_2O_2 mol ratio for 15 min. (c) Time profile of EP removal in Fenton process. 1 mM EP sample with initial pH 3.5 was treated by 2.7 mM H_2O_2 with 4 : 1 Fe^{2+}/H_2O_2 mol ratio for 15 min.

[23]. Under below pH 2.5, catalytic reactions are also decreased from preventing direct reduction of Fe^{3+} to Fe^{2+} ions [12]. It was also reported that pH value changes in catalytic reaction confer direct effects on equilibrium of Fe^{2+} and Fe^{3+} ions and oxidation ability of Fenton's reagent [12]. We found that maximum (80%) EP removal efficiency was achieved at initial pH 3.5 in this Fenton's reagent reaction condition (Fig. 3(b)). Therefore, we decided on the optimal conditions for Fenton's reagent process to degrade 1 mM EP: 2.7 mM hydrogen peroxide, 4 : 1 Fe^{2+}/H_2O_2 molar ratio, and initial pH 3.5.

Under the determined optimal Fenton's treatment conditions, we investigated the time profile of EP removal (Fig. 3(c)). As soon as Fenton reagents were added, EP was quickly degraded; remaining EP was below 50% of initial concentration within 5 min and about 20% after 15 min. However, after 15 min Fenton treatment, no additional EP removal was observed. Therefore, 15 min was thte optimal reaction time for 1 mM EP removal in this Fenton's reagent process. This first stage Fenton's reagent process can be a pretreatment step for the next enzymatic process.

2. Removal of EP using the Second Enzymatic OPH Treatment

We found that a small portion of the remaining EP was not efficiently removed by just the Fenton chemical process (Fig. 3(c)). Therefore, to remove residual toxic EP (~0.2 mM) from initial 1 mM, we used enzymatic treatment as a second stage process. Isolation of pure enzyme is quite difficult and requires multi-step process, and thus uses in biodegradation or bioremediation of pure enzymes are practically limited [3]. We used two types of cost-effective biocatalysts in this enzymatic parathion removal process: cytoplasmic OPH-expressing whole cell biocatalyst and disrupted cell debris-containing free OPH. In the case of whole cell enzyme (~86 unit/L whole cell OPH activity from 1.05 g/L dry cell mass), the EP removal efficiency was about 25% after 14 min treatment (Fig. 4(a)). This low removal efficiency was from a mass transport problem by cell membrane because OPH enzymes are located in the cytoplasm (intercellular space) and parathion molecules should be transported across the membrane [17,19,20]. Next, we tested parathion removal using cell lysate fraction as an enzyme source after cell disruption. We found that higher parathion removal (90% after 14 min) was achieved using cell-lysed crude OPH enzymes (~1,360 unit/L total OPH activity) due to no problem of mass transport limitation (Fig. 4(a)). Therefore, the use of this crude enzyme type from simple cell disruption without separation and purification might be adequate for environmental applications including biodegradation of toxic chemicals.

3. Removal of EP using Two Stage Chemical/Enzymatic Hybrid Process

Based on the preliminary conditions for each chemical and enzymatic process, we performed a combined two-stage hybrid process for removal of 1 mM EP. As shown in Fig. 4(b), ~80% of EP was degraded in the first chemical Fenton stage after 15 min and finally, ~98% of EP was removed after the second enzymatic OPH stage for the next 14 min. Therefore, we confirmed that 1 mM EP can be efficiently removed by a hybrid system combining Fenton's regent and OPH.

Currently widely used physical treatment techniques such as activated carbon filtration and membrane technology are not regarded as practical solutions for high concentration of pesticide wastewater that is released from agricultural and industrial activities due to

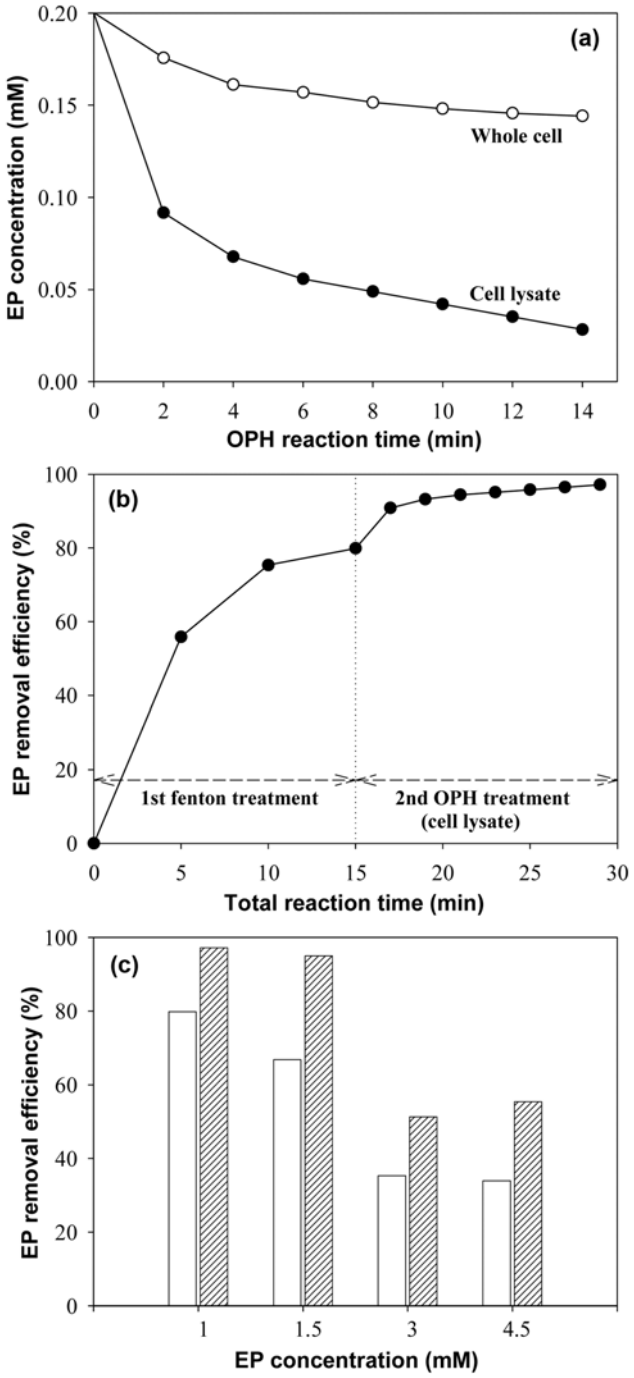

Fig. 4. (a) Time profile of EP removal in OPH process. 0.2 mM sample was treated by 1.05 g/L OPH-expressing whole cells with 86 units/L activity or cell lysate containing OPH with 1,360 unit/L activity. (b) Time profile of EP removal in the two stage Fenton/OPH process. 1 mM EP sample with initial pH 3.5 was treated by 2.7 mM H_2O_2 with 4 : 1 Fe^{2+}/H_2O_2 mol ratio for 15 min in the first Fenton stage and treated by cell lysate containing OPH with 1,360 unit/L activity for next 14 min in the second OPH stage. (c) Effect of EP concentration on EP removal efficiency in the two-stage Fenton/OPH process. EP sample with several concentrations (1-4.5 mM) with initial pH 3.5 was treated by 2.7 mM H_2O_2 with 4 : 1 Fe^{2+}/H_2O_2 mol ratio for 15 min in the first Fenton stage and treated by cell lysate containing OPH with 1,360 unit/L activity for next 14 min in the second OPH stage.

reduction of treatment efficiency [6]. Therefore, a efficient removal technique is required for high concentrations of organophosphate compounds. We investigated the removal of high concentration (1.5, 3, 4.5 mM) of EP using our proposed hybrid treatment system. As results, EP removal efficiencies were 67, 35, and 34% after the first Fenton chemical process with 2.7 mM hydrogen peroxide and 95, 51, and 55% after the second enzymatic process with 1,360 unit/L OPH (Fig. 4(c)). Note that the treatment conditions for high EP concentration were based on the optimal conditions for 1 mM EP. Thus, for application of this proposed hybrid treatment process into diverse concentration ranges of target organophosphate compounds, more detailed studies on the correlation among concentrations of organophosphate compound, H_2O_2, and OPH enzyme will be necessary.

CONCLUSIONS

We demonstrated that a two-stage hybrid treatment process comprised of the first Fenton's reagent chemical stage as a pretreatment step and the second OPH enzymatic stage for residuals can be successfully employed for efficient removal of environmental neurotoxic organophosphate compounds such as EP pesticide without extra pretreatment using UV or ozone. The proposed two-stage hybrid process removed 1 mM EP efficiently (~98%) within 30 min. Based on this feasibility research, further studies on establishment of operation conditions to enhance oxidation reaction of Fenton's reagent and enhancement of OPH production yield for efficient biodegradation of remaining EP will be carried out.

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