Bioresource Technology 259 (2018) 268–275

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

4-Chlorophenol biodegradation facilitator composed of recombinant multi-biocatalysts immobilized onto montmorillonite

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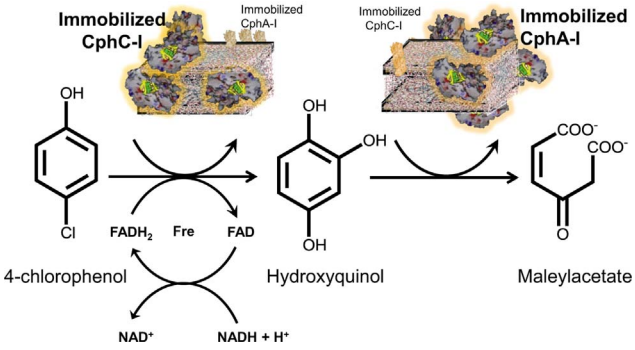
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Biodegradation facilitator
4-Chlorophenol
Recombinant technology
Oxygenase
Immobilization

ABSTRACT

A biodegradation facilitator which catalyzes the initial steps of 4-chlorophenol (4-CP) oxidation was prepared by immobilizing multiple enzymes (monooxygenase, CphC-I and dioxygenase, CphA-I) onto a natural inorganic support. The enzymes were obtained via overexpression and purification after cloning the corresponding genes (*cphC-I* and *cphA-I*) from *Arthrobacter chlorophenolicus* A6. Then, the recombinant CphC-I was immobilized onto fulvic acid-activated montmorillonite. The immobilization yield was 60%, and the high enzyme activity (82.6%) was retained after immobilization. Kinetic analysis indicated that the Michaelis-Menten model parameters for the immobilized CphC-I were similar to those for the free enzyme. The enzyme stability was markedly enhanced after immobilization. The immobilized enzyme exhibited a high level of activity even after repetitive use (84.7%) and powdering (65.8%). 4-CP was sequentially oxidized by a multiple enzyme complex, comprising the immobilized CphC-I and CphA-I, via the hydroquinone pathway: oxidative transformation of 4-CP to hydroxyquinol followed by ring fission of hydroxyquinol.

1. Introduction

Chlorophenol is a representative aromatic hydrocarbon contaminant and can be easily accumulated in the natural media, such as water bodies, soils, and sediments. Due to its high aqueous solubility, volatility, and the resulting diffusible properties, it has been of a great

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https://doi.org/10.1016/j.biortech.2018.03.066
Received 30 January 2018; Received in revised form 11 March 2018; Accepted 12 March 2018
Available online 16 March 2018
0960-8524/

concern in the ecosystem. It has been reported that chlorophenols are present in surface water bodies at the level of 0.005–20 μg/L, which may exhibit chronic effects to human beings (Ge et al., 2017). The lethal concentration 50 of chlorophenol is in the range of 3–4 mg/L (ATSDR, 1999). In particular, 4-chlorophenol (4-CP) is highly toxic and exposure of living organisms to high concentrations of 4-CP induces mutations, anomalies, muscle weakness, tremors, and coma (Farah et al. 2004). Extensive studies have been conducted on 4-CP-degrading microorganisms and their metabolic capability for the biodegradation of 4-CP (Solyanikova et al., 2004). *Pseudomonas knackmussii* B-13, *Ralstonia pickettii* LD1, *Rhodococcus opacus* 1G, *Alcaligenes* sp. A7-2, *Alcaligenes xylosoxidans* JH1, *Arthrobacter ureafaciens* CPR706, *Arthrobacter chlorophenolicus* A6, and *Herbaspirillum chlorophenolicum* CPW301 are known to degrade 4-CP (Arora and Bae, 2014). *A. chlorophenolicus A6* is an aerobic bacterium that is able to degrade a wide range of toxic phenolic compounds and is known to completely degrade 4-CP (Unell et al., 2008). Usually, the best-optimized biodegradation scheme cannot be easily accomplished because the biological degradation efficiency of organic pollutants is greatly influenced by the physicochemical characteristics of target contaminants, characteristics in the contaminated media (waters and soils), and growth conditions of the degrading microorganisms (Margesin et al., 1999).

Therefore, the biochemical degradation technique has received increasing attention since it is based on contaminant-degrading enzymes, rather than contaminant-degrading microorganisms, that can specifically catalyze the reactions for the removal of target organic contaminants (Alcalde et al., 2006). Enzymatic decomposition is a process in which enzymes obtained from contaminant-degrading microbes catalyze the redox reactions that transform target contaminants, such as metabolic substrates, and convert them into non-toxic substances (Caldeira et al., 1999). The treatment period for the enzymatic process is anticipated to be much shorter than that for the microbial process since the enzymatic transformation is usually completed in a minute time-scale. It is highly substrate-specific; therefore, the enzymatic reaction can be designated as contaminant-specific (Alcalde et al., 2006). It has been known that *A. chlorophenolicus* A6 possesses several oxidative enzymes, such as putative monooxygenases, CphC-I and CphC-II; putative flavin reductase, CphB; and dioxygenase, CphA-I, and these enzymes are involved in the initial 4-CP decomposition (Nordin et al., 2005). According to Kwon et al. (2014), the dioxygenase, CphA-I, that catalyzes the ring fission of hydroxyquinol by utilizing it as a primary substrate, without a coenzyme, such as NAD(P)H, exhibited a broad substrate specificity to 4-chlorocatechol, catechol, as well as 3-methylcatechol, which are intermediates produced during 4-CP biodegradation by *A. chlorophenolicus* A6. CphC-I and CphB are the main components of two-component flavin-diffusible monooxygenases (TC-FDM, Perry and Zylstra, 2007). Kang et al. (2017) showed that 4-CP oxidation can be effectively catalyzed by the constitutive enzymes of TC-FDM, which were cloned and expressed using the corresponding genes of *A. chlorophenolicus* A6 and *Escherichia coli*.

However, it should be noted that enzyme inactivation is the biggest concern with regard to environmental applications due to protein denaturation resulting from sudden changes in the surrounding environment, such as pH and temperature, chemical and hydrodynamic shocks, active site poisoning or blockage, or reduction in substrate-enzyme contact as a result of mass transfer limitations (Moore et al., 2004). The nano-scale size of enzymes also limits their field applications due to requirement of special handing (Nazari et al., 2007). To overcome these problems, immobilization of enzymes on organic and inorganic solid supports has been employed in various industrial and environmental processes to improve the enzyme activity, longevity, and stability (Wojcieszyńska et al., 2012). The benefits of enzyme immobilization are stability against abrupt changes in environmental factors, extended lifetime, increased reusability, customizability, and easy handling (Subramanian et al., 1999), whereas the drawbacks associated with enzyme immobilization, such as activity loss of the immobilized enzyme, deterioration of enzyme-substrate binding site, and deformation of the 3,4-dimensional structure of the enzyme, have been reported. The advantages of enzyme immobilization could be maximized by increasing the enzyme activity (Mateo et al., 2007). Various inorganic substances, such as glass beads, chitosan beads, magnetic beads, agarose resins, carbon nano-tubes, and inorganic natural minerals, have been employed as supports for enzyme immobilization (Bayramoglu and Arica, 2008; Bayramoglu et al., 2013; Monier et al., 2010; Suma et al., 2016). In particular, smectite clay has been introduced as an eco-friendly support to allow stable immobilization of various enzymes (Kim et al., 2012; Lee et al., 2013; Sanjay and Sugunan, 2006).

A combination of several enzymes, such as oxidative and reductive enzymes, is required for catalyzing the biochemical reactions for degrading most of the organic compounds. The oxidative enzymes are particularly important since they play a critical role in initiation of recalcitrant hydrocarbon contaminant biodegradation. For instance, halogenated aromatic hydrocarbons are decomposed by stepwise oxidations, which are catalyzed by various oxidoreductases. These initial steps require relatively high activation energy, and therefore, these processes are rate-limiting in biodegradation. Thus, the overall biodegradation process can be facilitated effectively by catalyzing these initial biodegradation steps using key oxidoreductases. However, assembly or immobilization of multiple enzymes to decompose the target substances sequentially has not been reported to date. Therefore, in this study, we aimed to construct a biocatalyst set by immobilizing multiple oxidative enzymes onto a natural inorganic support for catalyzing the initial steps of enzymatic degradation of target contaminant. Monooxygenase and dioxygenase that catalyze the initial oxidation steps of 4-CP, the target contaminant in this study, were produced using a recombinant technique and were immobilized onto montmorillonite. The degradation activity and kinetic characteristics of the enzymes were examined and their stability to the change of the environmental factors and applicability as a biocatalyst were evaluated. These results may form a basis for the development of a biodegradation facilitator targeting oxidative catalysis of initial biodegradation steps using immobilized oxidoreductases and an enzymatic decomposition technique not only for 4-CP, but also for other phenolic hydrocarbons.

2. Methods

2.1. Materials

A. chlorophenolicus A6 and *E. coli* K-12 were obtained from the American Type Culture Collection (ATCC No. 700700). *A. chlorophenolicus* A6 was enriched in the minimal medium (GM medium; ATCC medium 2236; 2.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.5 g NH_4NO_3, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.023 g $CaCl_2{\cdot}2H_2O$, 2 mg $FeCl_3{\cdot}6H_2O$, and 5.0 g yeast extract/L Milli-Q water) supplemented with 0.15 g/L 4-CP as the sole carbon source. The pET-24a(+) vector for gene insertion was purchased from Novagen (Darmstadt, Germany). Competent cells for transformation, *E. coli* BL21 (DE3) and *E. coli* BL21-Codon Plus (DE3)-RIL (Codon+), were purchased from Yeastern (Taipei, Taiwan) and Agilent Technologies (Santa Clara, CA, USA), respectively. The expression inducer, isopropyl β-D-thiogalactopyranoside (IPTG), was purchased from Bioshop (Burlington, Canada). Nickel (II)-nitrilotriacetic acid (Ni-NTA) resin (Ni-NTA His·Bind®Resin) was purchased from Novagen. Blue gel loading dye (6×) was purchased from New England BioLabs (Ipswich, MA, USA). The PageBlue™ Protein Staining Solution was purchased from Thermo Scientific (Rockford, IL, USA). The Quick Start™ Bovine Serum Albumin Standards and the Quick Start™ Bradford dye reagent used in the Bradford assay were purchased from Bio-Rad (Hercules, CA, USA). Luria-Bertani (LB) medium for growing *E. coli* K-12 and competent cells were prepared as described previously (Kim et al., 2012). Montmorillonite and humic acid were purchased from Sigma-Aldrich (St. Louis, Mo. USA). Fulvic acid (FA)

Table 1
Primer design.

Vector	Insert		
	Primer name	Oligo	Amplicon size (bp)
pET-24a	CphC-I-*Nde*I-F	cccaagcttatgaggacaggaaaagaatacctgg	1608
	CphC-I-*Hind*III-R	ccgatatcgggccggcgtggttaccgcag	
	Fre-*Nde*I-F	aaggagatatacatatgacaaccttaagctgtaaag	702
	Fre-*Xho*I-R	ggtggtggtgctcgaggataaatgcaaacgcatcgc	
	CphA-I-*Nde*I-F	gggggcatatgacgacccgtcaagtagcccca	240
	CphA-I-*Bam*HI-R	gggggggatcctcacttcagatcaggattagg	

was isolated as described in a previous study (Kim et al., 2012). All other chemicals used in this study, including 4-CP (analytical grade, purity > 98%), were purchased from Sigma-Aldrich.

2.2. Cloning, overexpression, and purification of enzymes.

Target genes were amplified from the genomic DNA of *A. chlorophenolicus* A6 and *E. coli* K-12 using PCR with forward and reverse primers (Table 1). The amplified PCR products were digested with corresponding endonucleases and cloned into a pET-24a(+) vector that contained *hexa*-histidine (His) affinity tag. *cphC-I* and *cphA-I* were transformed into BL21(DE3) strain and *fre* was transformed into Codon + strain. The competent cells harboring the cloned vector were incubated in LB media supplemented with 50 μg/mL kanamycin (20 μg/mL chloramphenicol additionally supplemented in the case of Codon + strain) and incubated at 37 °C on a rotary shaker at 210 rpm. The bacteria were grown until OD_{600} of the growth medium reached 0.5–0.6., and 0.05 mM IPTG was added to induce the target enzyme. Then, the bacteria were incubated at 15 °C for 24 h by stirring at 200 × *g*. The harvested cells were centrifuged at 6000 × *g* for 30 min at 4 °C, and the supernatant was then discarded. The cells were re-suspended using lysis buffer (0.3 M NaCl, 20% (v/v) glycerol, 0.024% (v/v) 2-mercaptoethanol, 25 mM Tris-HCl, and pH 7.5) and sonicated 12 times at 20 kHz for 40 s each. The bacterial debris were removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The His-tagged recombinant enzyme was purified by passing the supernatant through a column packed with Ni-NTA resin. The resin in the column was washed with 1.5 V (1 V = 1/2 column volume) of wash I (0.5 M NaCl, 15 mM imidazole, 20% (v/v) glycerol, 25 mM Tris-HCl, and pH 7.5) and wash II (0.5 M NaCl, 40 mM imidazole, 20% (v/v) glycerol, 25 mM Tris-HCl, and pH 7.5) buffers sequentially. Finally, the bound protein was eluted with 2.5 V of elution buffer (0.3 M NaCl, 0.3 M imidazole, 20% (v/v) glycerol, 25 mM Tris, and pH 7.5). Eluates from the column were concentrated by overnight dialysis at 4 °C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to identify the production of soluble form of enzymes.

2.3. Enzyme immobilization

Montmorillonite surfaces were activated by adsorbing FA as an enzyme binder for its immobilization as described by Kim et al. (2012). The Freundlich (Eq. (1) and Langmuir (Eq. (2) isotherm models were employed to characterize the adsorption of FA onto montmorillonite. The model parameters were graphically determined as:

$$q_e = K_F C_e^{1/n} \quad (1)$$

where $1/n$ is the sorption intensity (dimensionless) and K_F is the Freundlich constant ($mg^{1-1/n}L^{1/n}kg^{-1}$), and

$$q_e = \frac{Q_{\max} K C_e}{1 + K C_e} \quad (2)$$

where Q_{max} is the maximum sorption capacity (mg/g) and K is the Langmuir constant (L/mg). The surface-activated montmorillonite (~1 g) was added to 5 mL phosphate buffered saline (PBS; 137 mM of NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and pH 7.4) solution containing a pre-determined amount of enzyme. The mixture was stirred on a table-top shaker operating at 20 S/min at 25 °C for 2 h under dark conditions. The mixture was then centrifuged at 3000 × *g* for 20 min. The immobilized enzyme was separated from the supernatant, and rinsed 3 times with PBS solution. The amount of enzyme in the washing solutions was measured to determine the amount of enzyme immobilized onto the supports by calculating the difference.

2.4. Enzyme activity assay and kinetic analysis

Enzyme activity assays of free and immobilized CphC-I were carried out by analyzing their capacity to catalyze the substrate utilization. A pre-determined amount of free or immobilized enzymes along with each substrate and H_2O_2 as an oxygen source at a molar ratio of 1:2 were added to a 1 mL reaction mixture consisting of 200 U Fre, 10 μM FAD, 200 μM NADH, in a PBS solution. The mixture was thoroughly mixed by vortexing at 25 °C for 1 min, and then, the precipitate was separated by centrifugation at 11,000 × *g* for 20 min at 4 °C using a microcentrifuge (Eppendorf, Hamburg, Germany). The supernatant was filtered through 0.45 μm glassfiber filters (GF/D, Whatman, Maidstone, UK). Then, the concentration of substrates remaining in the supernatant was measured to determine the substrate removal rate. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 mmol substrate in 1 min. The relative activity was calculated as:

$$\text{Relative activity (\%)} = (\text{Activity/Maximum activity}) \times 100 \quad (3)$$

A kinetic study was performed by measuring the initial rate of substrate removal at different initial substrate concentrations in the range of 0.1–1.0 mM. The Michaelis–Menten model was employed to describe the kinetic behavior:

$$v = \frac{v_{\max} C_S}{K_M + C_S} \quad (4)$$

where v is the rate of substrate utilization, v_{max} is the maximum rate of substrate utilization, K_M is the half-substrate constant indicating enzyme-substrate affinity, and C_S is the initial substrate concentration. These kinetic parameters were determined by non-linear regression using SigmaPlot (Systat Software Inc., San Jose, USA).

2.5. Enzyme stability, powdering, and reusability test

The effects of pH, temperature, and ionic strength on CphC-I activity were examined by conducting the experiments for the removal of 4-CP with free and immobilized enzymes under various conditions. The pH value was pre-adjusted in the range of 3–11 at 25 °C. The reactions were also conducted at various temperatures in the range of 5–55 °C at pH 7. The ionic strength was varied in the range of 0–1 M by adding NaCl at pH 7 and 25 °C. The immobilized enzyme was freeze-dried for 24 h. The decomposition of 4-CP was carried out using the powdered enzyme particles as described previously. In addition, the reusability of immobilized enzyme was assessed by collecting it at the end of each experiment, separating it by centrifugation at 11,000 × *g* for 5 min at 4 °C using a microcentrifuge, rinsing with PBS solution, and then repeating the degradation study with a fresh solution of each substrate and H_2O_2.

2.6. 4-CP degradation by multiple enzyme complex

Sequential degradation of 4-CP was carried out by CphC-I and

CphA-I. In case 1, both the oxidative enzymes were immobilized onto montmorillonite together at 1:1 ratio (based on enzyme activity, U) and used as a single multiple enzyme complex. In case 2, each enzyme was immobilized onto montmorillonite separately, and then they were added to the solution at 1:1 ratio together as duplicate multiple enzyme complex. The decomposition reactions were carried out as described previously. After completion of reactions, the substances remained in the solution were analyzed by high performance liquid chromatography (HPLC).

2.7. Analytical procedures

Total organic carbon (TOC) concentrations in the aqueous samples were determined using a TOC analyzer (Sievers 5310C, GE Analytical Instruments, Boulder, CO, USA) with potassium hydrogen phthalate as an external standard. Protein concentration was determined by measuring the absorbance at 595 nm using a UV–visible spectrophotometer (Optizen, Mecasys, Seoul, Korea). The purity and size of the enzymes were estimated by SDS-PAGE. The concentrations of substrates were analyzed with a reverse-phase HPLC system (Agilent LC 1200 series, Agilent, Palo Alto, CA, USA) equipped with a ZORBAX Eclipse XDB C-18 column (125 × 3.2 mm, Agilent) and a mobile phase composed of distilled water and acetonitrile (3:7, v/v) at 1 mL/min flow rate. Detection was carried out with a diode array detector at 280 nm or a fluorescence detector (1200 FLD, Agilent) at 270 nm excitation and 310, 315, 320, and 330 nm emission wavelengths.

3. Results and discussion

3.1. Activation of montmorillonite surfaces with fulvic acid

Montmorillonite KSF and K-10 were screened as enzyme immobilizing supports, and montmorillonite K-10 was selected because of its larger specific surface area (Kim, 2012; Sanjay and Sugunan, 2006). Surface activation of clay mineral, which serves as an effective binding agent, has been known to facilitate the crosslinking of enzyme and inorganic support (between amine and carboxylic groups; Kim et al., 2012). In this study, FA was selected due to its high content of oxygenated functional groups. It was adsorbed onto montmorillonite and the adsorption capacity of FA onto montmorillonite was evaluated using Freundlich and Langmuir models (Table 2 and Fig. 1). The Freundlich model parameter, $1/n$, was determined to be 0.49, which indicates a favorable association between FA and montmorillonite. The adsorption capacity constant, K_F, was 52.7, which is known to increase with the increasing adsorbing capacity of adsorbents (Balcke et al., 2002). The maximum adsorption capacity of FA onto montmorillonite, determined by the Langmuir model, was 2057.7 mg-TOC/g-clay, which is similar to the value reported previously by Kim et al. (2012). These results indicated that FA was effectively adsorbed onto montmorillonite. The amount of FA leached from montmorillonite after adsorption was negligible (data not presented), which also indicated the stable binding of FA with montmorillonite. Various functional groups contained in FA can stimulate favorable interactions, such as cation-anion and complex exchanges; ionic, hydrogen, and van der Waals bonds; hydrophobic effect; and covalent cross-coupling, which result in a strong association between soil organic matter and inorganic geo-materials (Aimin et al., 2008).

Table 2
Adsorption isotherm parameters.

Freundlich model parameter	$1/n$	K_F	r^2
	0.49	52.7	0.83
Langmuir model parameter	Q_{max}	K	r^2
	2057.7	0.0025	0.86

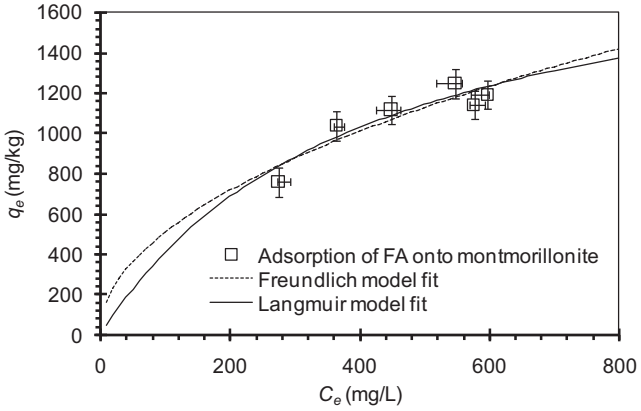

Fig. 1. Adsorption isotherms of FA on soil along with Freundlich and Langmuir model fits. Error bars denote standard deviations (n = 3).

3.2. Cloning, overexpression, and purification of enzyme

According to Kang et al. (2017), CphB cannot be expressed in a soluble form. Thus, Fre from *E. coli* was used as a substitute flavin reductase, in this study. Fre is known to reduce various flavins, such as riboflavin, flavin mononucleotide, and FAD, using NAD(P)H (Fontecave et al., 1994; Xun and Sandvik, 2000). Fre can reduce $FADH_2$ and electrons can be readily transferred to autoxidized oxygen, which can produce superoxide radicals (Coves and Fontecave, 1993). Kang et al. (2017) demonstrated that the initial 4-CP degradation step can be catalyzed by the $FADH_2$ reduced by Fre and CphC-I. *cphC-I*, *fre*, and *cphA-I* were cloned using pET-24a(+) vector. The expression inducer, IPTG, was used to inhibit the *lac* promoter of the vector and to induce high expression of the target genes through binding of T7 promoter and terminator. The vector map showing the replicated *cphC-I*, *fre*, and *cphA-I* are presented in Fig. 2. All the corresponding enzymes, CphC-I, Fre, and CphA-I, were highly expressed in the soluble form (Fig. 3), and therefore, they were effectively purified using Ni^{2+}-NTA resin. The amount of the purified enzymes, CphC-I, Fre, and CphA-I, was as high as 21,972, 1,658, and 23,432 μg/mL, respectively.

3.3. Enzyme immobilization and activity assay

It has been reported that CphA-I can be effectively immobilized onto the humic acid-activated montmorillonite (~63% of the immobilization yield; Lee et al., 2013). Thus, immobilization of CphC-I onto the FA-activated montmorillonite was examined in this study. The yield of CphC-I immobilization reached approximately 60%, which was almost equivalent to that of CphA-I reported previously (Table 3). According to previous studies, when glass and magnetic polymeric beads were used as enzyme support, 37–40% and 67% of enzyme immobilization, respectively, were achieved (Bayramoglu and Arica, 2008; Gomez et al., 2006; Lai and Lin, 2005). On the contrary, FA-activated kaolinite, vermiculite, and montmorillonite exhibited immobilization yields of 36%, 56%, and 66% for horseradish peroxidase, respectively (Kim et al., 2012). Montmorillonite has a 2:1 dioctahedral smectite structure and has inner pores consisting of nano-scale planar layers, which can swell in response to water-wetting. Thus, montmorillonite exhibits very high sorptive capacity for FA as well as excellent electron exchange capability. These characteristics are highly advantageous in accommodating enzymes. Lai and Lin (2005) found that enzyme activity decreased substantially after immobilization owing to restriction of chemical steric hindrance or mass transfer limitation given by undesirable association of enzyme with inorganic support. However, as explained by Kim et al. (2012), the FA-activated clay can not only exhibit a high immobilization yield, but also provide a stable binding between enzyme and support, which is given by various reactive functional groups that

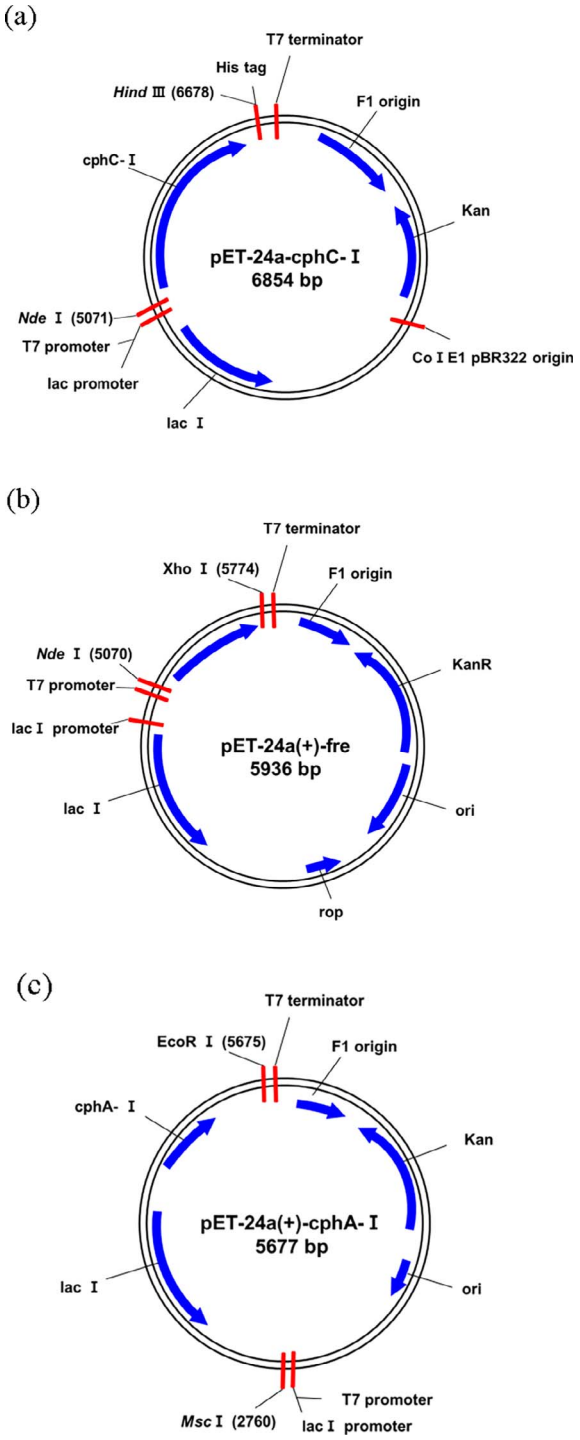

Fig. 2. Recombinant vectors for: (a) *cphC-I*, (b) *fre*, and (c) *cphA-I*.

can induce various chemical bonds, including covalent, ionic, and hydrogen bonds, van der Waals interactions, hydrophobic effects, and complexation. As a result, the activity of CphC-I was retained at a high level of about 83% after immobilization.

3.4. Kinetics of 4-CP removal

4-CP degradation rate was measured using free and immobilized CphC-I, and the results were used to determine the Michaelis-Menten model parameters (Table 4). It was recently found that CphC-I exhibits substrate specificity to 4-CP and catalyzed 4-CP oxidation to its end metabolite, hydroxyquinol (Kang et al., 2017). Thus, the kinetics of

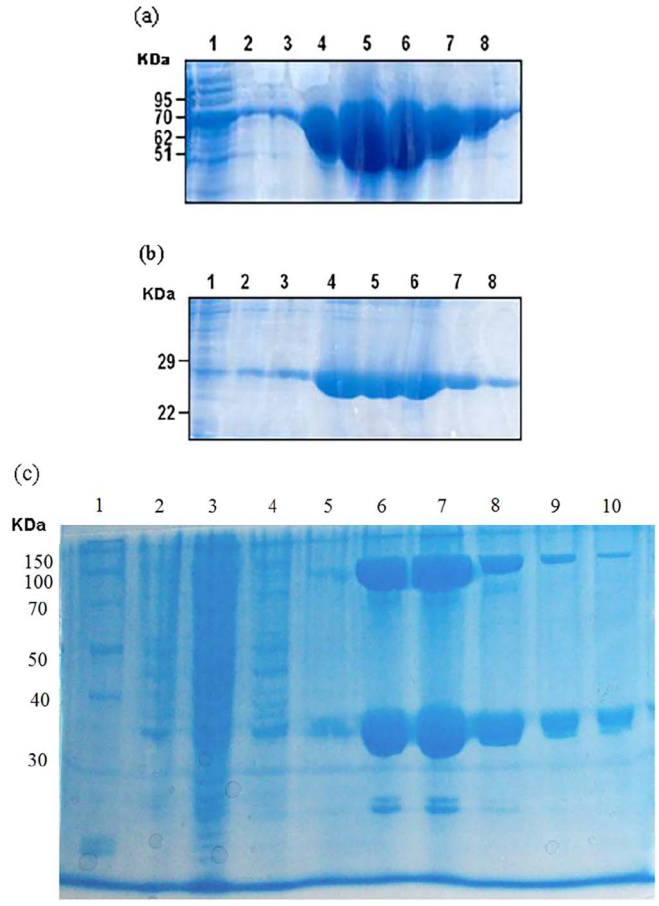

Fig. 3. SDS-PAGE results for the expressed enzymes: (a) CphC-I, (b) Fre, and (c) CphA-I. In legend, 1 corresponds to the protein ladder, 2 to wash I, 3 to wash II, 4–8 to eluates for CphC-I and Fre, and 1 corresponds to the protein ladder, 2 to cell lysate, 3 to supernatant, 4 to wash I, 5 to wash II, 6–10 eluates for CphA-I.

Table 3
Enzyme immobilization yield.

Carrier clay	4-Chlorophenol
Immobilization yield (%)[a]	60 ± 2.54[b]
Amount of enzyme immobilized on clay (mg-protein/g-clay)	0.857 ± 0.013
Activity of immobilized complex enzymes (U/mg-protein)	63.22 ± 0.011
Retained activity of immobilized enzyme (%)[c]	82.6 ± 3.21
Activity of immobilized complex enzymes per clay (U/g-clay)	54.1 ± 0.024

[a] Determined by difference between dose amount and remaining amount of CphC-I after immobilization.
[b] Standard deviation (n = 3).
[c] Relative to the activity of free enzyme.

Table 4
The Michaelis-Menten parameters for free and immobilized CphC-I and CphA-I.

		v_{max} (mM/min)	K_M (mM)	Catalytic Efficiency (k_{cat}/K_M, $min^{-1}\,mM^{-1}$)
4-chlorophenol (CphC-I)	Free enzyme	0.21	0.19	0.04
	Immobilized enzyme	0.18	0.13	0.05
Hydroxyquinol (CphA-I)	Free enzyme	4.61	3.27	1.41
	Immobilized enzyme	2.05	1.83	1.11

CphC-I conversion to hydroxyquinol was also determined in this study. The v_{max} value of the immobilized enzyme for 4-CP was 0.18 mM/min, which is slightly lower than that of the free enzyme (0.21 mM/min). The K_M value for 4-CP was slightly decreased after immobilization, indicating that the affinity of CphC-I for 4-CP was enhanced. These results were consistent with those of the previous studies that employed a similar enzyme support (i.e., humic acid-activated smectite; Kim et al., 2012; Lee et al., 2013) and reported that the loss of enzyme activity specific to the primary substrate was almost negligible or was enhanced after immobilization. They attributed this phenomenon to the denaturation of enzyme and/or the strong association of enzyme with inorganic support, which possibly occurred during enzyme immobilization. It should be noted that the v_{max} value of the free enzyme for 4-CP was approximately 20 times lower than that for hydroxyquinol reported previously (Cho et al., 2017; Lee et al., 2013). This discrepancy in the v_{max} values for 4-CP and hydroxyquinol could be explained by the fact that a greater activation energy is required for the initial step, rather than the later step, of biodegradation, since the chemical stability decreases as the biodegradation proceeds. The overall kinetic efficiency was evaluated using the catalytic efficiency (k_{cat}/K_M), known as the enzyme specific constant. Several previous studies have reported that k_{cat}/K_M is usually decreased when enzyme is immobilized (Cang-Rong and Pastorin, 2009; Zhang et al., 2013). However, the k_{cat}/K_M values for the free and immobilized enzymes for 4-CP were almost similar, and those for hydroxyquinol decreased only marginally after immobilization. This data indicated that the loss of enzyme activity during immobilization was almost negligible in this study.

3.5. Enzyme stability

To examine the stability of the free and immobilized CphC-I against the abrupt changes in environmental factors, such as pH, temperature, and ionic strength, their effects on the enzyme activity were assessed (Fig. 4 (a)–(c)). The optimum pH value for the maximum activity of both the free and immobilized CpHC-I for 4-CP was 9, which is higher than that for CphA-I or analogous dioxygenase (Fernandez-Lafuente et al., 2000; Guzik et al., 2011; Lee et al., 2013; Suma et al., 2016; Wang et al., 2006; Wojcieszyńska et al., 2012). The immobilized CphC-I exhibited higher activity in acidic conditions as compared to that exhibited by the free CphC-I; at pH 11, both the enzymes drastically lost its activity. This may have been attributed to the protective effect caused by the strong association between enzyme and support and the resulting space constraints, which can restrict denaturation or unfolding of enzymes in response to drastic pH changes (Bayramoglu and Arica, 2008; Mateo et al., 2007). Similarly, Sanjay and Sugunan (2006) suggested that strong interactions, including covalent cross-linking, can provide intramolecular forces that prevent conformational changes in enzymes. In addition, the Donnan effect, which may facilitate the variation in the proton distribution around the immobilized enzyme, has been noted as one of the factors providing high stability against the pH changes (Lai and Lin, 2005). Both free and immobilized CphC-I showed the maximum 4-CP degradation activity at 25 °C. In the case of free CphC-I, the relative activity started to decrease with an increase in temperature and eventually dropped to 40% at 55 °C. On the contrary, the immobilized CphC-I showed almost constant relative activity in the temperature range tested. It is well known that high temperature induces structural changes in enzymes, resulting in its denaturation and permanent loss of activity (Boyer, 2005). However, a notable improvement in thermal stability of the immobilized enzyme was reported in a large number of previous studies (Wojcieszyńska et al., 2012). Sanjay and Sugunan (2006) reported that the stable binding of enzyme onto the surface of clay support (e.g., covalent cross-linking) can minimize protein unfolding, which results in irreversible enzyme denaturation. The activity of free CphC-I increased with ionic strength up to 400 mM and then gradually decreased. According to Huang et al. (2005), when the amount of ions in the solution increases, ion exchange becomes active and the oxidation–reduction reaction by the enzyme can be promoted, perhaps due to the enhancement in electron transfer. However, excessive amount of ions is known to provide undesirable effect on enzyme activity (Kim et al., 2012). The immobilized CphC-I exhibited high and constant relative activity over the ionic strength range tested. This was attributable to the large cation exchange capacity of montmorillonite, which may have provided a buffering effect against the high salt concentration (Sanjay and Sugunan, 2006). Moreover, the mass transfer of ions (e.g., diffusion or ionic exchange) to the bulk solution may have been hindered by the microenvironment formed during the enzyme immobilization as suggested by Monier et al. (2010).

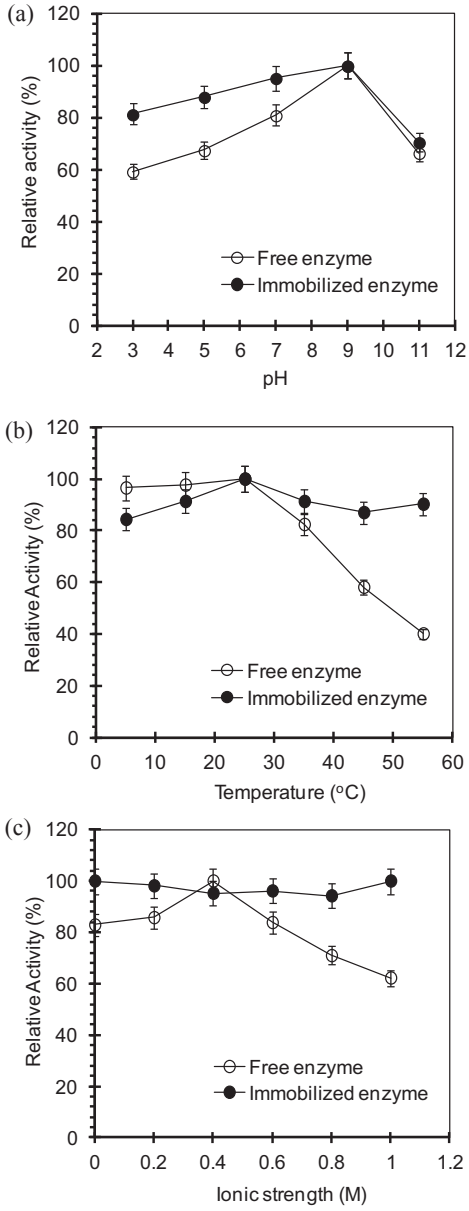

Fig. 4. Effects of (a) pH, (b) temperature, and (c) ionic strength on CphC-I activity. Error bars denote standard deviations (n = 3).

3.6. Powdering and reusability of enzyme

To test the feasibility as a catalyst, the immobilized CphC-I was powdered by freeze-drying and its activity was evaluated. The capacity of the powdered enzyme retained for catalysis of 4-CP oxidation

reached to 66% as compared to that of the original immobilized CphC-I. As discussed earlier, the microenvironment formed by strong association between enzyme and support during immobilization may have contributed greatly to the protection of the enzyme structure against freeze-thawing. Additionally, the immobilized CphC-I was repeatedly used to examine the reusability. After the first use, the activity decreased to 83.5%. However, the activity was maintained at almost a similar level throughout the 7 times of consecutive use, and the average activity level in the 7-times additional re-use was 84.7%. The loss of activity that occurred in the repetitive use is attributable either to enzyme inactivation due to radical attack and active site poisoning, or to limited mass transfer of substrate to the binding sites of enzyme (Moore et al., 2004). Notably, the level of activity retained from our reusability test was markedly higher than those reported in previous studies (50% and 65.8% of residual activities after 5 and 6 times of re-use, reported by Alemzadeh and Nejati et al. (2009) and Monier et al. (2010), respectively). Consistent with this study, Suma et al. (2016) reported that the activity of immobilized CphA-I decreased to about 40% of the original activity after 7 times of repetitive use. These results indicated that the enzyme immobilization employed in this study provides a highly useful advantage for the industrial production and commercialization of biocatalyst by improving the easiness in handling and application in the field.

3.7. 4-CP degradation by immobilized multi-enzyme complex

To examine the consecutive oxidative degradation of 4-CP by multiple enzyme complex, CphC-I and CphA-I were used together. It has been confirmed that 4-CP is initially oxidized to hydroxyquinol through the formation of intermediates, such as benzoquinone and hydroxyquinone, by CphC-I, and then hydroxyquinol is finally decomposed into maleylacetate by ring-fission catalyzed by CphA-I (Cho et al., 2017). In particular, halogenated aromatic hydrocarbons are resistant to microbial attack due to the stability of ring structure, which is strengthened by halogenated functional groups (Unell et al., 2008). The oxidation of such chlorinated aromatic hydrocarbons is initiated by addition of hydroxyl group, which is catalyzed usually by monooxygenase, and further decomposition, such as ring fission, which is catalyzed by dioxygenase (Nordin et al., 2005). Therefore, in this study, 2 ways of the multiple enzyme complex are: (i) case 1: a single enzyme complex consisting of CphC-I and CphA-I immobilized together onto montmorillonite, and (ii) case 2: dual enzyme complex consisting of CphC-I and CphA-I immobilized separately onto montmorillonite and used as a mixture. 4-CP decomposition and its intermediates were confirmed by HPLC analysis (data not presented). 4-CP was completely decomposed perhaps to maleylacetate by the multi-enzyme complex in the cases 1 and 2. As reported by numerous previous studies (Cho et al., 2017; Kang et al., 2017; Nordin et al., 2005), the hydroquinone pathway for the initial 4-CP degradation can be catalyzed by TC-FDM composed of monooxygenase, CphC-I, and flavin reductase, CphB. Kang et al. (2017) assembled the TC-FDM using recombinant CphC-I and Fre, and used it for the initial oxidation of 4-CP. It has been recently confirmed that the CphC-I of TC-FDM catalyzes 4-CP degradation through the hydroquinone pathway (Cho et al., 2017), resulting in formation of final product, hydroxyquinol: 4-CP → benzoquinone → hydroquinone → hydroxyquinol. It was also demonstrated that hydroxyquinol is the primary substrate of CphA-I, dioxygenase which does not require a co-enzyme (Kwon et al., 2014), and the sequential oxidative decomposition of 4-CP can be catalyzed by CphC-I and CphA-I (Cho et al., 2017). In our HPLC analysis, the formation of any intermediate products, such as benzoquinone, hydroquinone, and hydroxyquinol, was not detected, which indicated that the decomposition through the hydroquinone pathway and ring fission to maleylacetate was readily catalyzed by CphC-I and CphA-I. The immobilized enzyme can form separate heterogeneous layers on the surface of the solid enzyme support, and steric hindrance, which occurs on the immobilized enzyme, can also limit its activity (Kim et al., 2012; Suma et al., 2016). Due to these factors, the activity of the immobilized enzyme is generally lower than that of the free enzyme. When two or more enzymes are immobilized together, the enzymes can be randomly located on the support due to interactions between enzymes and support (Jia et al., 2014). This also attributed to the reduced the degradation capacity in the case 1. Overall, approximately only 20% and 32% of 4-CP in the case 1 and 2, respectively, was degraded by both enzymes. This means that CphC-I was not sufficiently provided to catalyze the complete decomposition of the given amount of 4-CP, while CphA-I activity was enough. Additionally, of note, 4-CP removal efficiency was somewhat higher in the case 2 than in the case 1. This may have been attributed to the competitive binding of several enzymes onto the support, which may have blocked the substrate binding sites of enzymes in the case 1. It was also plausible that higher quantity of enzymes immobilized together onto a support can limit the effective mass transfer of substrates in the microenvironment around the immobilized enzymes in the case 1 to a higher degree than that in the case 2. More studies, thus, are required to optimize the way of enzyme immobilization and application scheme. It should be also noted that the product obtained after ring fission, such as maleylacetate, is neither toxic nor hazardous; it should be easily accessible to almost any bacteria, and it should be degraded easily and fast in nature.

4. Conclusions

A biodegradation facilitator composed of multiple oxygenases was developed to catalyze the initial 4-CP biodegradation steps. The recombinant monooxygenase CphC-I and dioxygenase CphA-I were obtained from *A. chlorophenolicus* A6. CphC-I was effectively immobilized onto FA-activated montmorillonite, and a high level of enzyme activity was preserved after immobilization. The Michaelis-Menten parameters of the immobilized CphC-I were similar to those of the free enzyme. The immobilized CphC-I was highly stable against the abrupt changes in environmental factors, and the immobilized CphC-I exhibited a relatively high activity after repetitive use and powdering. 4-CP can be sequentially oxidized by the multiple enzyme complex (CphC-I and CphA-I).

Acknowledgment

This research was supported by the Faculty Research Fund of Konkuk University 2017.

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