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Immobilization of oxidative enzymes onto Cu-activated zeolite to catalyze 4-chlorophenol decomposition

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Abstract. In this study, a biocatalyst composite was prepared by immobilizing oxidoreductases onto Cu-activated zeolite to facilitate biochemical decomposition of 4-chlorophenol (4-CP). 4-CP monooxygenase (CphC-I) was cloned from a 4-CP degrading bacterium, Pseudarthrobacter chlorophenolicus A6, and then overexpressed and purified. Type X zeolite was synthesized from non-magnetic coal fly ash using acetic acid treatment, and its surfaces were coated with copper ions via impregnation (Cu-zeolite). Then, the recombinant oxidative and reductive enzymes were immobilized onto Cu-zeolite. The enzymes were effectively immobilized onto the Cu-zeolite (79% of immobilization yield). The retained catalytic activity of CphC-I after immobilization was 0.3423 U/g-Cu-zeolite, which was 63.3% of the value of free enzymes. The results of this study suggest that copper can be used as an effective enzyme immobilization binder because it provides favorable metal-histidine binding between the enzyme and Cu-zeolite.

Keywords: biocatalyst; 4-chlorophenol; enzyme immobilization; Cu-zeolite; coal fly ash

1. Introduction

Chlorophenols are phenolic compounds to which chlorines are substituted, and phenol is an aromatic compound derived from benzene (ATSDR 1999). These chemical substances are extensively applied in industries, such as in the manufacturing processes for antiseptics, disinfectants, herbicides, and pesticides. Chlorophenols including monochlorophenols, polychlorophenols, chloronitrophenols, chloroaminophenols, and chloromethylphenols are highly toxic to living organisms because of their carcinogenic, mutagenic, and cytotoxic properties (Arora and Bae 2014). They mostly enter the environment in the form of mono- and dichlorophenols when used as pesticides and herbicides. Exposure to a large amount of chlorophenols can damage the environment and human bodies. In particular, considerable attention has been given to 4-chlorophenol (4-CP), one of the chlorophenols, because of its highly toxic effects on the surrounding environment.

Arthrobacter chlorophenolicus A6 is a gram-positive bacterium known to completely degrade 4-CP (Westerberg *et al*. 2000). It can also decompose most phenolic compounds including 4-nitrophenol and 4-bromophenol (Westerberg *et al*. 2000). The 4-CP is sequentially oxidized, and then, the aromatic ring is cleaved to maleylacetate by *A. chlorophenolicus* A6. These oxidative decomposition processes are catalyzed by 4-CP monooxygenases, CphC-I and CphC-II, and hydroxyquinol 1,2-dioxygenase, CphA-I (Nordin *et al*. 2005). *A. chlorophenolicus* A6, originally classified as a member of the *Arthrobacter* genus, was reclassified into the genus *Pseudarthrobacter* (Hans-Jürgen Busse 2016). The initial steps of the 4-CP biodegradation pathway by *P. chlorophenolicus* A6 are known to be promoted by two-component flavin-diffusible monooxygenase (TC-FDM) including CphC-I (Cho *et al*. 2017). CphC-I exhibits substrate specificity for 4-CP, benzoquinone (BQN), and hydroquinone (HQN) (Cho *et al*. 2017). In addition, it was confirmed that TC-FDM catalyzes the oxidation of 4-CP and the sequential conversion of 4-CP to BQN, HQN, and finally hydroxyquinol (HQL, Cho *et al*. 2017).

Enzymes are biological catalysts and are highly effective and specific for biochemical conversion of organic compounds (Baumer *et al*. 2018, Qayyum *et al*. 2009). Enzymes have numerous advantages over microorganisms in terms of degradation activity and kinetics for organic contaminants (Ye *et al*. 2019). They lower the activation energy, thus increasing the reaction kinetics, and the reaction becomes more economic because it generates less waste (Sheldon and van Pelt 2013). Horseradish peroxidase (HRP) and laccase have been extensively investigated because they can catalyze a number of organic compounds. HRP is one of the most important enzymes obtained from plant sources (Lopes *et al*. 2014, Veitch 2004). HRP is useful in broad pH and temperature ranges in the presence of various compounds usually found in municipal wastewaters (Melo *et al*. 2015).

However, enzymatic reactions require strict reaction conditions such as ambient temperature, atmospheric

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pressure, and natural pH. Otherwise, enzymes are subject to inactivation, which has been noted as a critical drawback. Enzyme immobilization has been introduced as a means of preserving the selectivity, stability, and kinetics of enzymes because physical and chemical properties of enzyme carriers provide a protection effect (Basso and Serban 2019). Enzyme immobilization can be classified into two types: physical and chemical immobilization. For example, adsorption and physical entrapment are major physical immobilization methods and crosslinking and covalent binding are representative examples of chemical immobilization methods. According to the study of Kwean *et al*. (2018), multiple enzymes (monooxygenase, CphC-I and dioxygenase, and CphA-I) can be immobilized onto fulvic acid-activated montmorillonite. The immobilization yield was 60%, and the high enzyme activity (82.6%) was retained 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 oxidative transformation of 4-CP to HQL followed by ring fission of HQL.

The objective of this study was to immobilize enzymes onto an inorganic support, zeolite, which was obtained from coal ash as a recycled material. Copper was chosen as an activator to bind enzymes onto the surfaces of an inorganic enzyme support. 4-CP monooxygenase was cloned from the corresponding genes of *P. chlorophenolicus* A6 and then immobilized onto zeolite synthesized from coal fly ash coated with copper ions. Then, the immobilization efficiency and enzyme activities were examined. This was done by first immobilizing those enzymes onto support materials such as zeolite and then introducing the enzyme to a system containing the pollutant.

2. Materials and methods

2.1 Materials

P. chlorophenolicus A6 and *Escherichia coli* K-12 were obtained from the American Type Culture Collection (ATCC No. 700700) and the Korean Collection for Type Cultures (KCTC No. 1116), respectively. Competent *E. coli* cell (BL21 (DE3)) was purchased from Yeastern Biotech (Taipei, Taiwan). Isopropyl-β-D-thiogalactopyranoside (IPTG), an expression inducer, and growth media were purchased from Bioshop (Burlington, Canada). Antibiotic chloramphenicol solution, protein size reagent GangNam-BLUE™ Prestained Protein Ladder, 1 M Tris-HCl (pH 6.8), 1 M Tris-HCl (pH 7.5), 1.5 M Tris-HCl (pH 8.8), and Acylamide-BisSol (40%) were purchased from iNtRON Biotechnology (Sungnam, Korea). Blue gel loading dye (6×) was purchased from New England BioLabs (Ipswich, MA, U.S.A.). 3.5K MWCO SnakeSkin™ Dialysis Tubing for PageBlue™ protein staining solution and enzyme dialysis solution were purchased from Thermo Scientific (Rockford, IL, U.S.A.). To determine the Bradford assay, Quick Start™ Bovine Serum Albumin Standards and Quick Start™ Bradford 1× dye reagent were purchased from Bio-Rad (Hercules, CA, U.S.A.). Reagents for buffers used for the purification of enzymes such as 99.5% glycerine, sodium chloride, and anhydrous ethyl alcohol were purchased from Daejung (Siheung, Korea). Nickel(II)-nitrilotriacetic acid (Ni^{2+}-NTA), antibiotics kanamycin and ampicillin, and reagents 4-CP, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), flavin adenine dinucleotide disodium salt hydrate, and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetic acid was purchased from Duksan Chemicals (Ansan, Korea), and NaOH beads were purchased from Showa Chemical Industry (Tokyo, Japan). Coal fly ash used for the synthesis of zeolite was obtained from the Korea Western Power Co., Ltd. (Taean, Korea).

2.2 Cloning, overexpression, and purification of enzymes

The procedure for cloning and overexpression of monooxygenase and flavin reductase was adopted from Kwean *et al*. (2018) and slightly modified in this study. After the overexpression was completed, the supernatant was removed, and the cells were transferred to 20-mL lysis buffer solution (0.3 M NaCl, 20% glycerol, 0.024% 2-mercaptoethanol, 25 mM Tris-HCl, and pH 7.5). After 30 min, the samples were sonicated by repetition of 40 s on and 20 s off for 12 min to rupture the cells. The supernatant was separated by centrifugation at 9,000×*g* for 30 min at 4 °C. Soluble hexahistidine-tagged enzymes were purified using Ni^{2+}-NTA resin at 4 °C. One half of the column was washed once with ethanol, twice with Milli-Q water, and twice with lysis buffer. The enzyme supernatant was injected into the column at a flow rate of 1 mL/min. Then, 20 mL of wash I buffer (0.5 M NaCl, 15 mM imidazole, 20% (*v/v*) glycerol, 25 mM Tris-HCl, and pH 7.5) was injected, followed by 20 mL of wash II buffer (0.5 M NaCl, 40 mM imidazole, 20 % (*v/v*) glycerol, 25 mM Tris-HCl, and pH 7.5). Then, the elution buffer (0.3 M NaCl, 0.3 M imidazole, 20% (*v/v*) glycerol, 25 mM Tris, and pH 7.5) was passed through five times to recover the purified enzyme. The size of the soluble enzyme was confirmed before and after purification using sodium dodecyl sulfate polyacrylamide gel electrophoresis and quantitated by Bradford protein assay. Imidazole, a component of the elution buffer, was used to separate the enzyme from Ni^{2+}-NTA. It was removed by dialysis with 3.5K MWCO SnakeSkin™ Dialysis Tubing (Sigma-Aldrich) 3 times.

2.3 Preparation of enzyme support

Synthetic zeolite-XF12 was prepared using coal fly ash as described by Lim *et al*. (2019). Then, the surfaces of zeolite were activated with copper ions (Cu^{2+}) to facilitate the binding between enzymes and zeolite as follows (Cu-zeolite). The experimental process is demonstrated in the Fig. 1. The zeolite was sonicated with Milli-Q water for 5 min. The sample was mixed with $CuCl_2$ solution in a conical flask and stirred with a magnetic stirrer for 2 h. Then, the solution was dried in an oven at 105 °C overnight.

The dried powder was calcined in a ceramic jar at 350 °C for 2 h. The calcined powder was washed with Milli-Q water three times and ethanol three times. The washed powder was oven-dried again overnight and then vacuum-dried overnight.

2.4 Enzyme immobilization

The purified enzymes were mixed with Cu-zeolite contained in a 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 on a shaking incubator for 4 h. Then, the samples were centrifuged to separate the immobilized enzyme at 9,000×*g* for 30 min at 4 °C, and the supernatant was discarded. The samples were washed with PBS solution and centrifuged. Then, the samples were quantified in a Bradford assay to determine the enzyme concentration. The immobilized enzyme was examined using Eq. (1):

$$\text{Immobilization yield (\%)} = \frac{Amount\ of\ immobilized\ enzyme}{Amount\ of\ total\ enzyme\ dosage} \times 10 \quad (1)$$

Two adsorption models were employed to examine the enzyme immobilization: Langmuir and Freundlich adsorption models. These are the most commonly used models to describe the adsorbent-absorbate relation. Langmuir-type adsorption was considered to be a monolayer process (Chung *et al*. 2015). The maximum adsorption capacity per unit adsorbent mass was determined along with the Langmuir constant showing solute affinity to the adsorbent. The Langmuir model is described by Eq. (2):

$$Q_e = \frac{Q_{max} b C_e}{1 + bC_e} \quad (2)$$

The Freundlich adsorption isotherm is a model that exhibits the empirical relation between the concentrations of a solute on the surface of an absorbent to the concentration of the solute in the liquid with which it is in contact. In other words, Freundlich-type adsorption is a multi-layer sorption process in which the amount of adsorbed solute per unit adsorbent mass increases gradually (Chung *et al*. 2015). The Freundlich model is described by Eq. (3):

$$q_e = \frac{x}{m} = K_F \cdot C_e^{\frac{1}{n}} \quad (3)$$

where Q_e is the solute concentration in the solid phase at equilibrium (μg/mg), C_e is the solute concentration in the liquid phase at equilibrium (μg/mL), Q_{max} is the maximum sorption capacity (μg/mg), and b is the Langmuir constant (L/mg). x is the mass of adsorbate, m is the mass of adsorbent, K_F is the constant of the Freundlich constant ($mg^{1-1/n}L^{1/n}kg^{-1}$), and $1/n$ is the sorption intensity (dimensionless).

2.5 Enzyme activity assay

4-CP decomposition experiments were conducted as follows. The reaction was initiated by adding 1 mL of NADH solution. The reaction solution was rigorously mixed by a vortex at 25 °C for 1 min. Acetic acid was added to stop the reaction. Then, the samples were centrifuged at 9,000×*g* for 30 min at 4 °C. The supernatant was collected and filtered with a glass microfiber filter. One unit (U) of enzyme-specific activity was defined as the amount of enzyme required to catalyze the conversion of 1 mmol of substrate in 1 min at 25 °C. The relative activity was obtained using Eq. (4):

$$\text{Relative activity (\%)} = \frac{Activity}{Maximum\ activity} \times 100 \quad (4)$$

2.6 Analytical procedures

High performance liquid chromatography (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with an Eclipse XDB C-18 column (4.6 mm × 150 mm, 5 μm) was used to determine the concentrations of 4-CP. The mobile phase solvent was composed of acetonitrile, and water was employed at a flow rate of 1 mL/min. 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 nm emission wavelengths. Bradford protein assay was conducted to measure the quantity of proteins produced. Bovine serum albumin was used as a standard, with a linear range of 125–1,000 μg/mL. Protein samples (20 μL) were mixed with 1 mL dye reagent (1×) in a 1.5 mL centrifuge tube. The absorbance of each sample was measured at 595 nm using a UV-VIS spectrophotometer (Optizen, Mecasys, Daejeon, Korea). The surfaces of the enzyme supports were analyzed by a field emission scanning electron microscope (FE-SEM, SIGMA VP, Carl Zeiss, Munich, Germany) equipped with an energy dispersive X-ray spectrometer (EDS, Carl Zeiss).

3. Results and discussion

3.1 Cloning, overexpression, and purification of enzymes

Cho *et al*. (2017) demonstrated that the initial 4-CP degradation pathway can be promoted by CphC-I and Fre. The corresponding genes, *cphC-I* and *fre*, obtained from *P. chlorophenolicus* A6 and *E. coli* K-12, respectively, were also cloned using the pET-24a(+) vector in this study. The expression inducer, IPTG, was used to inhibit the lac promoter of the vector as well as to induce high expression of the target genes through binding of the T7 promoter and terminator. The optimum condition of production of soluble enzymes obtained from Kang *et al*. (2017) was employed as well. CphC-I and Fre were effectively expressed in a soluble form and were then purified using Ni^{2+}-NTA resin in a high quantity. The results of expression and purification are presented in Table 1. The amounts of the purified enzymes, CphC-I and Fre, were as high as 2,949 and 990 μg/mL, respectively.

3.2 Preparation of enzyme support

Zeolite-XF12, which showed the largest specific surface area (486.75 m^2/g) among the X type zeolites synthesized

Table 1 Concentration of CphC-I and Fre after purification

Protein	Eluate	Concentration (μg/mL)[a]	Concentration (μM)[b]
CphC- I	1	2761	45.12
	2	2949	48.18
	3	2916	47.65
	4	2729	44.60
	5	1884	30.79
Fre	1	394	15.14
	2	544	20.91
	3	990	38.06
	4	398	15.30
	5	147	5.66

[a] Concentration was determined using Bradford protein assay.

[b] Molar concentration was calculated with protein size in Da (= 1 atomic mass unit)

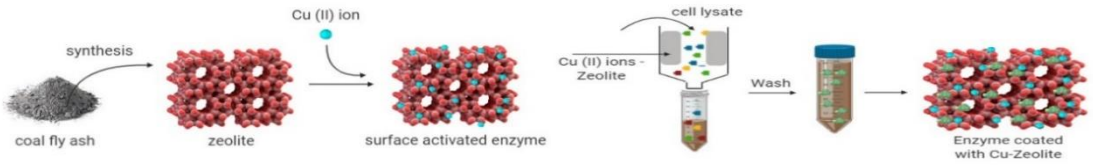

Fig. 1 Schematic diagram of enzyme immobilization using Cu-zeolite synthesized by coal fly ash

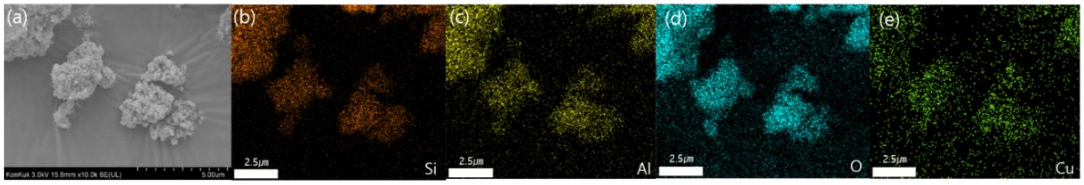

Fig. 2 SEM images of Cu-zeolite (a) and electron mapping of Si (b), Al (c), O (d), and Cu (e) on the Cu-zeolite

from coal ash according to Lim *et al*. (2018), was used in this study. FE-SEM confirmed that Cu-zeolite (Fig. 2(a)) was mainly composed of Si (b), Al (c), and O (d), which are major constituents of typical zeolite. In addition, the zeolite was coated with copper ions homogeneously on its surface (e).

3.3 Enzyme immobilization

Several prior studies have reported that CphC-I can be effectively immobilized onto natural mineral material (clay) which was activated with soil organic matter (Kim *et al*. 2012; Lee *et al*. 2013; Kwean *et al*. 2018). Likewise, enzyme immobilization onto synthetic zeolite activated with copper ions was examined in this study to catalyze the biodegradation of 4-CP. The adsorption capacity of enzyme, CphC-I, on Cu-zeolite was evaluated using Langmuir and Freundlich models (Fig. 3). The adsorption demonstrated a typical model of favorable adsorption characteristics and a strong non-linear adsorption pattern: high adsorption capacity in the low sorbate concentration range, followed by leveling-off of sorbent phase concentration in the high sorbate concentration range. The maximum adsorption capacity of the Cu-zeolite for enzyme was confirmed by the Langmuir model parameters: 45.67 μg/mg for Q_{max}. At an enzyme dosage of 180 μg/mL, the yield of enzyme immobilization onto the enzyme support was up to 79%. The high Q_{max} value indicates that the enzymes were effectively bound onto the surface of Cu-zeolite. The Freundlich model parameter, $1/n$, was determined to be 0.34, which indicates a favorable association between the enzyme and Cu-zeolite. The adsorption capacity constant, K_F, was 4.94, which is known to increase with the increasing adsorbing capacity of adsorbents (Balcke *et al*. 2002). These results indicate that the enzyme was effectively adsorbed/immobilized onto the Cu-zeolite because of the favorable and strong crosslinking between copper ions and histidine proteins, which was expressed by histidine tags of cloning vector. In addition, the synthetic zeolite showed high cation exchange capacity and high specific surface area (Querol *et al*. 2002; Lim *et al*. 2019). The surface of zeolite was activated with copper ions, which is subject to the strong coordinating bond between metal ions and the amine groups of enzyme proteins. These characteristics are highly advantageous in creating an environment to immobilize enzymes effectively. The Langmuir adsorption

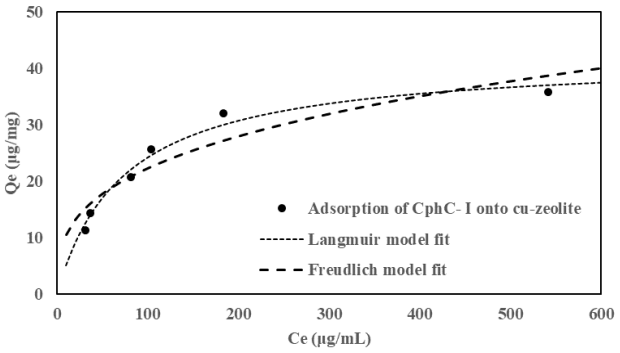

Fig. 3 Langmuir and Freundlich model fits for CphC-I adsorption onto Cu-zeolite

Table 2 Enzyme immobilization results

Immobilization yield (%)	30.26
Amount of enzyme immobilized on Cu-zeolite (mg-protein/ g-Cu-zeolite)	19.65
Activity of immobilized enzyme (U/mg-protein)[a]	17.42
Activity retained of immobilized enzyme (%)	63.31
Activity of immobilized enzyme per Cu-zeolite (U/mg-Cu-zeolite)	34.23

[a] mmol/min·mg·protein

model that assumes the same activity for monolayer adsorption was found to fit the experimental data better than the Freundlich adsorption model (r^2 = 0.96 and 0.89 for the Langmuir and the Freundlich models, respectively).

3.4 Enzyme activity assay

The activity analysis of 4-CP decomposition by immobilized enzyme is presented in Table 2. The yield of CphC-I immobilization reached 30%, which is slightly lower than the values reported by the previous studies (Lee *et al*. 2013; Kwean *et al*. 2018). The surface of zeolite was activated with copper ions, which is subject to the strong coordinating bond between metal ions and the amine group of enzyme proteins. These characteristics are highly advantageous in accommodating enzymes. The specific activity of the enzymes immobilized on the Cu-zeolite was evaluated by utilizing 4-CP as a primary substrate, and the results are presented in Table 2. The activity of the immobilized enzyme for decomposition of 4-CP was comparable to the value reported previously (Kwean *et al*. 2018). The retained activity of the immobilized enzymes was as high as 63%, which suggests that Cu-zeolite provides a stable protection effect for the enzyme. These results indicate that Cu-activation and its crosslinking with histidine protein are effective and useful for enzyme immobilization. In addition, enzyme immobilization with Cu-zeolite is effective to retain the specific activity of enzyme after immobilization. Notably, enzyme activity decreased after immobilization because of the restriction of chemical steric hindrance or mass transfer limitation by the undesirable association of enzymes with inorganic supports (Lai and Lin 2005).

4. Conclusions

In this study, zeolite synthesized from coal fly ash was activated with copper ions, and the Cu-zeolite was used as an enzyme support for immobilizing 4-CP oxidative enzyme, CphC-I, to catalyze the decomposition of 4-CP. The corresponding genes were cloned from *P. chlorophenolicus* A6, and CphC-I was overexpressed and purified. The zeolite was synthesized from coal fly ash, and copper ions were coated onto its surfaces by impregnation. Then, CphC-I was immobilized onto Cu-zeolite. The immobilization yield was 79%, and the enzyme immobilization was examined by the Langmuir adsorption model, which indicated favorable binding between enzymes and the support material. The specific activity of the immobilized enzyme was 17.42 U/mg-protein. Sixty-three percent of activity of the immobilized enzyme was retained with a value of 34.23 U/g-Cu-Zeolite. It can be concluded that copper ions provide favorable and strong metal-histidine binding between enzyme and enzyme support. The results of this study are expected to provide a new enzyme immobilization method for the biochemical decomposition of hazardous and recalcitrant organic contaminants.

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