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Enzymatic degradation of aromatic hydrocarbon intermediates using a recombinant dioxygenase immobilized onto surfactant-activated carbon nanotube

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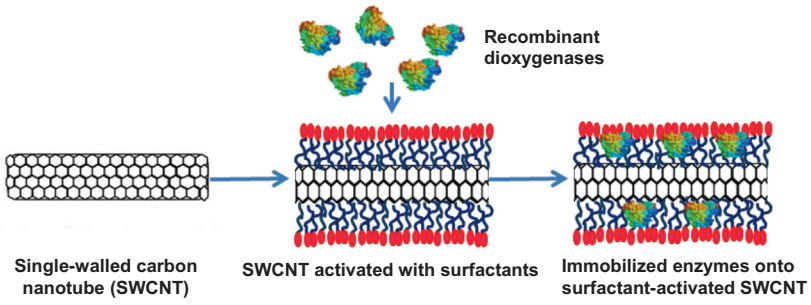
HIGHLIGHTS

- Various aromatic hydrocarbon intermediates can be decomposed by enzymes effectively.
- SWCNTs activated with nonionic surfactants can serve as an effective enzyme support.
- Loss of enzyme activity was minimal during enzyme immobilization.
- Enzyme immobilization improved the enzyme stability significantly.

GRAPHICAL ABSTRACT



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ABSTRACT

This study examined the enzymatic decomposition of aromatic hydrocarbon intermediates (catechol, 4-chlorocatechol, and 3-methylcatechol) using a dioxygenase immobilized onto single-walled carbon nanotube (SWCNT). The surfaces of SWCNTs were activated with surfactants. The dioxygenase was obtained by recombinant technique: the corresponding gene was cloned from *Arthrobacter chlorophenolicus* A6, and the enzyme was overexpressed and purified subsequently. The enzyme immobilization yield was 62%, and the high level of enzyme activity was preserved (60–79%) after enzyme immobilization. Kinetic analyses showed that the substrate utilization rates and the catalytic efficiencies of the immobilized enzyme for all substrates (target aromatic hydrocarbon intermediates) tested were similar to those of the free enzyme, indicating that the loss of enzyme activity was minimal during enzyme immobilization. The immobilized enzyme was more stable than the free enzyme against abrupt changes in pH, temperature, and ionic strength. Moreover, it retained high enzyme activity even after repetitive use.

1. Introduction

Microbial biotreatment of organic hydrocarbon contaminants has the limitations associated with slow growth of degrading microbes and difficulty in control and maintenance of the optimal microbial environment (Vidali, 2001). Hence, enzymatic decomposition of organic compounds has been suggested as an alternative approach on account of its rapid and highly specific reactions with target contaminant substrates (Kim et al., 2012). However, direct application of enzymes in the environmental treatment process has not been very successful due to loss of enzyme activity and low stability as a result of enzyme inactivation or protein denaturing, abrupt changes in the environment, active-site poisoning or blockage, and reduction in the enzyme–substrate association (Alemzadeh and Nejati, 2009; Schnell and Hanson, 2007).

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Enzymes have been immobilized on various inorganic supports since activity, preservability, and stability of enzymes can be enhanced after immobilization (Bickerstaff, 1997; Wojcieszyńska et al., 2012). In particular, enzyme immobilization on nano-materials has received increasing interests in the areas of biocatalyst, biofuel cell fabrication, and biosensor processes (Broering et al., 2006; Katz et al., 2001; Kim et al., 2006a). Nano-materials have excellent physico-chemical properties as the carrier for enzyme immobilization such as large surface area/volume ratio, low mass transfer constraints, and high mobility (Kim et al., 2006b; Pavlidis et al., 2012). Carbon nanotube (CNT) which is nano-scale tabular allotropes of carbon provides the unique characteristics useful for enzyme immobilization owing to its high surface area, excellent thermal and mechanical properties, and low electrical percolation thresholds (Asuri et al., 2006; Pedrosa et al., 2010). Single-walled CNT (SWCNT) consists of a single sheet of graphite and it is characterized by high surface area, small size, good dispersivity in the aqueous solution, and low mass transfer resistance (Wang et al., 2010). Asuri et al. (2006) demonstrated that enzymes can be effectively immobilized to SWCNTs by covalent bonds. The covalent binding between enzymes and SWCNTs can enhance the enzyme stability and the accessibility and selectivity of enzyme to substrates (Jiang et al., 2004). Although the covalent binding of enzyme with SWCNTs provides stable association, this technique may disrupt the electrochemical and mechanical properties of the enzyme support and structure, which can eventually lead to enzyme inactivation. In the meantime, it has been reported that non-covalent immobilization of enzymes onto SWCNTs is much less likely to cause such detrimental changes in the enzyme properties (Karajanagi et al., 2004). Thus, physical adsorption techniques have been commonly employed for non-covalent enzyme immobilization onto CNTs (Cang-Rong and Pastorin, 2009).

CNTs exhibit a strong hydrophobic character and tend to aggregate and deposit in the polar solvents. Therefore, the homogeneous dispersion of CNTs in the aqueous phase is necessary for their applications as nanocomposites, nano-films, nanotransistors, and catalyst supports (Chen et al., 2009; Das et al., 2009; Liao et al., 2009; Satishkumar et al., 2008; Wu et al., 2004). Surfactants can enhance the stabilization of CNTs in water since they can improve the dispersibility of CNTs in the aqueous phase (Han et al., 2008; Hilding et al., 2003; Islam et al., 2003; Vaisman et al., 2006). It has been reported that surfactants are adsorbed effectively on the CNT surfaces and they induce electrostatic and/or steric repulsions, which can counterbalance van der Waals attractions between CNT particles (Han et al., 2008; Hilding et al., 2003). The dispersion and aggregation of CNTs are regulated by thermodynamic equilibrium, which is given by the balance between repulsive and attractive forces (Hilding et al., 2003).

The objective of this study was to assess the feasibility of surfactant-activated SWCNTs as an enzyme carrier for the oxidative enzyme which catalyzes the ring cleavage of various aromatic hydrocarbon intermediates. The dioxygenase was obtained via recombinant technique using *Arthrobacter chlorophenolicus* A6 which is known to degrade 4-chlorophenol completely. This enzyme was immobilized onto the SWCNTs of which surfaces were activated with surfactants. The dispersion of SWCNTs in the presence of surfactants, the enzyme immobilization yield, the retained enzyme activity after enzyme immobilization, and the kinetic characteristics were assessed correspondingly. The stability of the enzyme in free and immobilized forms was also examined for practical applications.

2. Methods

2.1. Material

The recombinant dioxygenase (hydroxyquinol 1,2-dioxygenase) was prepared as described by Kwon et al. (2014). SWCNTs (1–2 nm in diameter and 10 μm in length) were purchased from Carbon Nano-Material Technology (Gyeongbuk, Korea). The target organic substrates, catechol (analytical grade, purity 99%), 4-chlorocatechol (purity 97%), 3-methylcatechol (purity 98%), were obtained from Sigma–Aldrich (St. Louis, USA). Nonionic and anionic surfactants (Triton X-100, Tween 80, and sodium dodecyl sulfate (SDS)) were selected as CNT dispersing agents, and they were purchased from Sigma–Aldrich. All other chemicals used in this study were obtained from Sigma–Aldrich as well.

2.2. Surface activation of SWCNT with surfactant

SWCNTs were dispersed in each of surfactant solutions and the optimal SWCNT–surfactant mass ratio was determined as follows. First, the SWCNT concentration was varied in the range of 15–50 mg/mL at a constant surfactant concentration (1%, v/v). The SWCNT concentration for the maximum dispersivity in the 1% surfactant solution was determined for each surfactant. In the second set of experiments, the surfactant concentration was varied in the range of 1.1–1.9% (v/v) at a constant SWCNT concentration for the maximum dispersivity as determined previously. The samples were sonicated for 2 h in a sonication bath operated at 50 Hz. It has been demonstrated that the UV–Vis absorption is not responsive to the aggregates or bundles of CNTs but to the dispersed CNTs (Yu et al., 2007). Thus, the dispersion of SWCNTs can be analyzed using UV–vis absorption spectroscopy. The dispersed SWCNT concentrations were measured by UV–vis spectroscopy at 500 nm (Ikeda et al., 2006; Sinani et al., 2005) based on the specific extinction coefficient (ε_{500} = 28.6 cm^2/mg), which can be used for determining the SWCNTs present in either dissolved or dispersed form (Ikeda et al., 2006). The amount of SWCNTs dispersed in the solution can be calculated as:

$$\% \text{ dispersivity} = (c_1/c) \times 100 \quad (1)$$

where c_1 is the concentration of SWCNTs dispersed in the solution and c is the concentration of SWCNTs initially present in the solution. This parameter was used as the measure of dispersion of SWCNTs in the solution.

2.3. Enzyme immobilization

SWCNTs activated with Triton X-100 were separated by centrifugation at 11,000*g* for 20 min at 4 °C using a microcentrifuge (Centrifuge 5424, Eppendorf, Hamburg, Germany), and the solution phase was replaced with a 50-mM phosphate buffer solution (pH 7). Then, it was sonicated for 2 h in a sonication bath operated at 50 Hz. This washing procedure was repeated 4 times. The activated SWCNTs were then added to the same phosphate buffer solution containing enzymes and the mixture was stirred for 2 h at 4 °C on a rotating shaker operated at 500 rpm. Then, the solution was centrifuged at 11,000*g* for 5 min at 4 °C using a microcentrifuge and the supernatant was removed. The immobilized enzyme-SWCNT composite was washed with fresh phosphate buffer solutions (50-mM and pH 7) to remove unbound enzymes until no enzyme was leached. The amount of enzyme in the washing solutions was measured by its protein concentration to determine the amount of immobilized enzymes by difference.

2.4. Enzyme activity assay and kinetic analysis

The enzyme activity was determined in terms of its capacity to catalyze the oxidation of catechol, 4-chlorocatechol, and 3-methylcatechol. Free and immobilized enzymes were added to a solution containing each substrate and H_2O_2 as an oxygen source at a molar ratio of 1:2. The mixture was thoroughly mixed by vortexing at 25 °C for 1 min, and then, the precipitate was separated