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Covalent immobilization of organophosphorus hydrolase enzyme on chemically modified cellulose microfibers: Statistical optimization and characterization

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ABSTRACT

Organophosphorus hydrolase (OPH) from *Flavobacterium* ATCC 27551 was covalently immobilized on epoxy modified plant cellulose powder. The Taguchi method was applied to optimize the conditions of cellulose activation and binding of the OPH to the carrier surface. The chemical modification of cellulose by covalent coupling with 1, 4-butanediol diglycidyl ether was demonstrated using the FTIR technique. TEM analysis proved good linkage of the OPH over the support surface. At the identified optimum condition of affecting parameters, the activity yield of immobilized OPH on the modified cellulose was found to be 68.32%. The kinetic parameters, K_m and V_{max} values, were calculated and it was determined that the catalytic efficiency of the immobilized OPH was about 4.85-fold lower than that of free enzyme. The storage, thermal, and pH stabilities of the immobilized OPH were improved compared with free counterpart. The results revealed that after incubation for 24 h at 55 °C, the soluble and immobilized OPH retained 8% and 35% of their initial activities, respectively. Furthermore, the immobilized OPH showed a 59% residual activity when used ten times repeatedly. Therefore, plant cellulose as a low-cost carrier has shown excellent properties for enzyme immobilization to be used as biocatalytic material in large scale applications.

1. Introduction

Organophosphorus compounds, are among the most toxic substances known. These compounds are widely used as insecticides, pesticides, and chemical warfare agents [1]. Their extreme toxicity and widespread use of them which pose important hazards to the human and cause great contamination of soil, sediments, and groundwater, has increased public concerns. By considering the recent developments in biotechnology, the use of microorganisms for biodegradation of organophosphorus would appear to be very attractive [2]. Organophosphorus hydrolase (OPH) (EC 3.1.8.1), a biological catalyst, has been shown to effectively hydrolyse a wide range of organophosphorus compounds. But, low specification and thermostability are among the factors decreasing significantly the optimal application of this enzyme [3].

Immobilization of the enzyme will enhance biocatalyst performances by significant improvement in characteristics like stability at extreme pHs and elevated temperatures, more tolerance to organic solvents, convenient recovery, and higher reusability of enzyme. Heterogeneous catalysis could be feasible by using immobilized enzymes. So, the termination of the reaction could be possible by physical isolation of the immobilized biocatalyst from the solution [4,5]. Different techniques such as attachment by covalent binding or adsorption to a support, physical entrapment in polymeric networks, and encapsulation have been employed for enzyme immobilization [6,7]. In general, the covalent methods have various advantages such as biocatalyst leakage prevention even under harsh conditions, easy application of enzyme in different types of bioreactors and stabilization of the biocatalyst tertiary structure due to multipoint tight bonding of the enzyme to the solid surfaces which can rigidify the structure of immobilized enzymes. Furthermore, the slight biocatalyst deactivation in covalent immobilization can be diminished by precise optimization of the process conditions in order to achieve the maximum immobilization yield [8,9].

For biocatalyst immobilization, natural polymers as carriers can represent some advantages such as excellent properties (inert, nontoxic, biodegradable, and biocompatible), easy modification with a variety of functional groups, and absence of impurities coming from chemical reactions [10,11]. Cellulose is the most abundant natural polymer which could be plant-based or synthesized by algae, tunicates,

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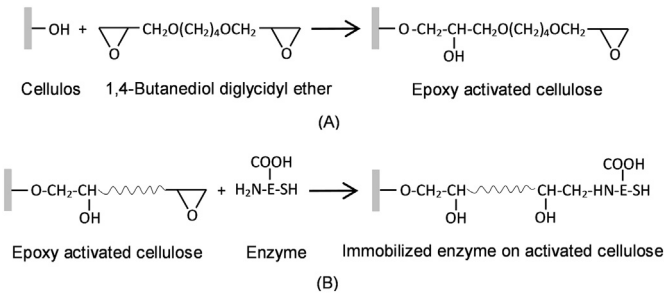
and some bacterias. This material is the cheapest carrier, which offers attractive characteristics like hydrophilicity, renewability, and low contamination risk to environment [12–14]. The genetic engineering approach has been used for immobilization of OPH onto the cellulose. Richins et al. [15] investigated the purification and immobilization of bifunctional fusion proteins consisting of OPH moieties linked to a *Clostridium*-derived cellulose-binding domain (CBD) onto various cellulose matrices in a one-step method. The immobilized CBD-OPH fusion protein was considered as a cost-effective material for the simultaneous adsorption and degradation of stored or spilled organophosphate wastes. As well as, Mansee et al. [16] reported a single step method for purification and immobilization of OPH onto the cellulose matrices by generating a fusion between OPH and a CBD that attaches selectively to the support. The produced bioconjugates were employed in a column bioreactor for detoxification of paraoxon and coumaphos in contaminated wastewaters.

Cellulose has a high chemical reactivity due to the existence of three hydroxyl groups in every monomeric unit (glucose) of its chemical structure. These groups are potential target functional groups to make covalent bonds with amino acids of enzymes for immobilization [12–14]. Since the hydroxyl groups on the cellulose surface can be directly interacted weakly with biocatalyst, the functionalization steps using chemical coupling agents for surface modification of support are required to improve binding efficiency. So, it has been verified that by introducing suitable functional groups onto cellulose surface to prepare an efficient support, strong and stable multipoint covalent attachments can be performed between matrix and amino acid residues of enzyme which are most often involved in covalent binding. Chemical modification of cellulose can be achieved by introducing amino group onto matrix to react with carboxyl group of amino acids (aspartic acid or glutamic acid) or introducing aldehyde, carboxyl or epoxy group which are capable to interact covalently with enzyme molecule through N-terminus (amine group of lysine) [12].

Numerous methods were applied for activation of the hydroxyl groups of cellulose previously by using chemical coupling agents like cyanogen bromide, cyanuric chloride, bisoxiranes, and epichlorhydrine [14,17]. Cellulose-based matrices treated under gentle condition with bisoxiranes such as 1, 4- or 1, 3-butanediol diglycidyl ether, as di-epoxy groups containing compounds, can be used for immobilization of biocatalysts via covalent bond formation between the free reactive epoxy groups created on the support surface and the enzyme [8]. The epoxide functional group is an extremely stable and reactive group employed in many substitution reactions as an electrophile [18]. This group can be attached with a high density to the matrix and the matrices activated by epoxy groups can bind to variety of macro biomolecules. The epoxide groups in modified cellulose with 1, 4-butanediol diglycidyl ether (BTDE) react with nucleophile groups on the enzyme surfaces by a ring-opening process to make a chemically stable bond and this reaction is hardly affected by steric hindrances. So, the terminal epoxide groups would allow multipoint covalent attachment between the biocatalyst and matrix with a very low enzyme leakage which stabilizes the three-dimensional structure of enzyme [19–21]. Also, the biocatalysts attached to the matrix through the long hydrophilic spacer arms like BTDE, exhibit the higher level of biological activity in comparison to direct linkage due to greater enzyme's degree of the mobility provided by this procedure [22]. The epoxide functional groups on the surface of activated cellulose would be capable to make covalent bonds with OPH via available nucleophile groups of its amino acids like amino, thiol, and hydroxyl groups (–NH_2, –SH, and –OH). Epoxy-treated cellulose binds to these different groups, depending on the pH of the binding reaction medium. Therefore, the reaction can happen with primary amine, sulfhydryl, or hydroxyl groups to create stable secondary amine, thioether, or ether bonds, respectively [21,23]. The N—C, S—C, and O—C bonds formed between the enzyme and activated matrix are extremely strong and highly stable [24].

In the current research, OPH from *Flavobacterium* ATCC 27551 was covalently immobilized on epoxy modified plant cellulose powder and the immobilized biocatalyst was used for the degradation of organophosphates. The enzyme immobilization process was demonstrated in the Scheme 1.



Scheme 1. Immobilization of enzyme through the epoxy method. (A) Surface reaction of the cellulose matrix with BTDE. (B) Covalent coupling of enzyme onto the surface of activated cellulose.

The Taguchi technique was applied in present work as one of the widely accepted tools for robust experimental design that recommended a representative set of all possible combinations of parameters and their levels assigned for immobilization process. This method was used to analyse the parameters affecting the preparation of cellulose-OPH system and the results were investigated for obtaining the optimal condition of immobilization process and finding out the significance of each factor to improve the effectiveness of optimization technique. Furthermore, the maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) of Biosensor for direct determination of organophosphate nerve agents OPH were calculated. Then, improvement in thermal, pH, and operational stabilities of the immobilized enzyme were determined and compared with those of free enzyme. All the studies were conducted at the identified optimum conditions and the experiments were performed in triplicate to calculate the standard deviations. The results have proved the advantages of using the enzyme in its supported form.

2. Experimental

2.1. Materials

Flavobacterium ATCC 27551 was gained from Microbial Type Culture Collection (MTCC, Chandigarh, India) and kept in Wakimoto medium on slant [7]. Extraction and purification of OPH enzyme from *Flavobacterium* ATCC 27551 was done by chromatography techniques and the resulting enzyme with the minimum purity of 90% was obtained. Plant cellulose powder (length of fibers: 0.02–0.15 mm) was obtained from Fluka Chemie GmbH (Steinheim, Germany), ethyl parathion (analytical grade) as substrate in determining enzyme activity from Sigma-Aldrich (St. Louis, MO, USA) and p-nitrophenol (PNP) from Aldrich. For the activated reagent, 1, 4-butanediol diglycidyl ether (BTDE) ($C_{10}H_{18}O_4$) was purchased from Sigma Chemical Co., sodium hydroxide was obtained from Merck Co. Ltd. Germany. The other chemicals were of analytical grade.

2.2. Preparation of activated cellulose powder by epoxy method

The plant cellulose powder (0.1 g) was suspended in 0.45 mL of 1.0 M NaOH in a 1.5-mL microfuge tube at room temperature. The cellulose microfibrils cannot be dissolved in NaOH aqueous solution at this condition [25]. So, after treatment of support with this solution, the native cellulose powder retains its fibrillar structure, but the degree of disorder would be increased. However, the NaOH aqueous solution is a swelling agent that often used to enhance the chemical and physical properties of cellulose. The influences of this alkaline solution on the structure, morphology, accessibility, and reactivity of cellulose are well