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2,4-Dichlorophenol removal by purified horseradish peroxidase enzyme and crude extract from horseradish immobilized to nano spray dried ethyl cellulose particles

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ABSTRACT

Horseradish peroxidase (HRP) is a promising catalyst in the enzymatic process of phenolic compounds removal from wastewaters. Enzyme immobilization provides important benefits in a biotechnological process. Fine particles with a high surface-to-volume ratio composed of ethyl cellulose (EC) polymer were generated by Nano Spray Dryer B-90 as supports for HRP. Carrier particles were prepared by spray caps with hole size of 7 μm, 5.5 μm and 4 μm. Purified HRP and crude extract from horseradish were covalently bound to the carrier using a carbodiimide cross-linker. The attached HRP content, the effect of pH on the activity and the storage stability were investigated. 2,4-dichlorophenol, an extremely persistent chlorinated phenol was removed by the immobilized enzyme, and the effect of main process parameters such as H_2O_2 and 2,4-dichlorophenol substrate concentrations were studied. After immobilization both the purified HRP and the horseradish extract performed better in the pH range of 4–10 and could preserve the activity substantially longer than the free enzyme. The immobilized enzyme was found to be outstandingly efficient (in optimal case close to 100%) in the elimination of 2,4-dichlorophenol, which was also the consequence of the high adsorbing capacity of the fine particles. The reuse study proved the operational stability of HRP attached to EC even after 10 consecutive cycles.

1. Introduction

Most of the phenolic compounds that are present in the waste streams of a wide variety of industrial operations are toxic and some are carcinogens. They get into the food chain and generate important environmental problems. Their removal involves the use of microorganisms, adsorption by active carbon, or chemical oxidation. However, these methods frequently have disadvantages, such as low efficiency, high cost or the generation of products which are even more toxic than the original ones. An alternative for treating wastewaters containing phenolic compounds is enzymatic treatment by peroxidase. In the presence of the peroxidase catalyst, phenols are oxidized to generate the corresponding radicals; the radicals spontaneously react to rapidly form insoluble polymeric phenolic aggregates [1].

An enzymatic process with horseradish peroxidase (HRP) for the removal of phenols from wastewaters was first described by Klibanov et al. [2]. It has already been shown that crude HRP is as effective as purified HRP in catalyzing phenol removal; the significant inactivation of HRP during the process results in the use of a large quantity of HRP to ensure efficient phenol removal [3]. However, the high-efficiency elimination of chlorinated phenols from wastewater is still a challenge.

Horseradish peroxidase is a heme-containing enzyme belonging to the class III of plant peroxidases. It has Fe(III) protoporphyrin IX as the prosthetic group, which plays an important role in its catalytic mechanism. Horseradish peroxidase possesses significant applications in life sciences, including bioassays, DNA-probes, biosensors, bioremediation of phenol and some of its derivatives

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[4]. However, its industrial application is greatly limited by its low thermostability and low reactivity in organic media. HRP is also prone to suicide inactivation by the H_2O_2 substrate in the applications such as diagnostics and biosensors as well as in wastewater treatment [5]. The relatively short lifetime of enzymes and their instability in harsh environment limit their applications. Thus, plenty of trials have been done on enzyme stabilization; such as entrapment of enzyme molecules in sol–gel, polymer matrixes and nanoporous materials, attachment of enzymes onto highly hydrophilic surfaces, e.g. chitosan, dextran and polyethylene glycol, and separation of enzyme layers from sample solutions using polymeric membranes and ion-exchange polyion membranes [4].

The main objective of enzyme immobilization is to maximize the advantages of enzyme catalysis [6]. An important aspect of this is the possibility of reaction interruption by removing the immobilised enzyme, controlling these systems over product formation, which is not possible, when the enzyme is dissolved in the reaction mixture [7].

Conventional spray drying is not suitable for the production of submicron-sized particles, because they are too small to be collected by the cyclone (cutoff diameter ~1–2 μm) of the spray dryer [8]. Recently, a new lab-scale equipment has become available (Büchi Nano Spray Dryer B-90) which is capable of capturing even submicron particles by an electrostatic collector. In addition, a piezoelectrically driven vibrating mesh atomiser is employed, which allows the production of finer droplets (median diameter 1–7 μm, size range 0.5–15 μm, depending on the mesh aperture size) with narrow span membranes [8]. Ethyl cellulose (EC) is an ecofriendly polymer which, according to our knowledge, has not been used as a support for HRP so far.

Fine microparticles with an additionally porous structure should allow even faster and more efficient intraparticle processing as the presence of pores goes along with a higher surface-to-volume ratio, which allows efficient access, especially of macromolecular reactants such as proteins which would encounter serious diffusion limitation to enter densely structured microparticles [9]. Chang and Tang [10] prepared Fe_3O_4 nanoparticles by a co-precipitation method and coated them with a silica layer as carrier for HRP. The activity during the fixing of the enzyme did not decrease substantially, and the immobilized enzyme was significantly more stable against heating and pH variation in comparison with the free HRP. The maximum 2,4-dichlorophenol conversion efficiency was around 80%, and the catalytic performance of the immobilized enzyme was high even after 4 cycles. A composite of graphene oxide and nano Fe_3O_4 as an artificial enzymatic catalyst combined with HRP provided an outstanding synergistic removal of 2,4-dichlorophenol (93%) [11,12].

In our recent work [13] we produced nano spray dried ethyl cellulose and poly(lactic co-glycolic acid) fine particles, respectively, and found the optimal conditions for HRP cross-linking to these supports. In that study purified HRP was immobilized, which due to the high cost of enzyme purification, may make enzymatic processes economically infeasible. In the present paper we compare the 2,4-dichlorophenol converting performance of purified HRP and crude extract from horseradish after attaching to ethyl cellulose supports prepared by nano spray drying using each of the available spray caps. The physical and chemical properties, the working range and storage stability of the immobilized purified as well as crude enzymes were extensively studied; moreover, the removal of persistent 2,4-dichlorophenol was also investigated. The main novelty of the work is the utilization of a new method for the preparation of fine particles of novel composition with high specific surface area for HRP enzyme and horseradish extract immobilization, which complexes were found to be especially effective in the elimination of a persistent chlorinated phenol.

2. Materials and methods

2.1. Materials

Purified HRP (223 U/mg) was purchased in dry solid form from Amresco (Solon, Ohio) and stored at −20 °C until use. One unit of HRP activity was defined as the formation of 1.0 mg purpurogallin from pyrogallol in 20 s at 0.022 M initial pyrogallol concentration and 0.045 M H_2O_2 in 3.0 ml distilled water. Crude extract was gained from horseradish plants and stored after extraction at −20 °C.

Ethyl cellulose (viscosity: 4 mPa s, 5 wt% in 80:20 toluene/ethyl alcohol, 25 °C) was a kind gift from Dow Deutschland Anlagengesellschaft mbH (Germany). Dichloromethane (DCM) was purchased from Scharlab (Hungary). Guaiacol was purchased from Cayman Chemical Company (USA). 30% hydrogen peroxide was obtained from VWR International LLC. *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) (~98%), *N*-hydroxysuccinimide (NHS) (98%) and Folin–Ciocalteu phenol reagent were purchased from Sigma–Aldrich. 2,4-Dichlorophenol was bought from Fluka. Acetonitrile (Scharlau, HPLC grade) was purchased from Scharlab Ltd. (Hungary).

2.2. Extraction of horseradish

Home-grown, 1 year old horseradish plants were harvested in autumn (28th October) and stored in a refrigerator till the extraction, which was achieved after a day. Roots were cleaned with a brush in cold water eliminating soil and other contamination. 400 g horseradish root was ground with a centrifugal juicer device (Moulinex) that allows the extract to pass through a strainer basket while leaving most of the pulp behind. After grinding the crude extract was ultracentrifuged (Sorvall Discovery 90SE) at 32,000 rpm for 50 min to separate 65 ml supernatant from the sediment including the pulp particles. The supernatant containing crude extract was removed and kept in a freezer at −20 °C for further usage. No medium or agent were added to the extract.

2.3. Spray drying with Nano Spray Dryer B-90

Nano Spray Dryer B-90 (BÜCHI Labortechnik AG, Flawil, Switzerland) utilizes piezotechnology to produce fine particles. The piezodriven spray nozzle generates ultra-fine droplets with a narrow size distribution, which are successively dried. The formed solid particles are electrostatically charged and collected at the surface of the cylindrical collecting electrode by electrical field. The main advantage of the Nano Spray Dryer is the novel electrostatic particle collector for the highest yields of fine particles. Two different setups can be established depending on the solvent type. The long version of the drying chamber is needed for aqueous solutions due to the time of evaporation, while the short version of the device is used for organic solvents. The device was operated in closed-mode configuration with the short version of the drying chamber.

Ethyl cellulose was dissolved in DCM to form 1% (w/v) solution for spray drying using spray caps with 7 μm, 5.5 μm and 4 μm hole sizes, which were vibrated at 60 kHz ultrasonic frequency. Nitrogen was used as drying gas; the flow rate was set to 100 L/min. The relative spray rate was 0.2 l/h (100%). During spray drying over 40 °C, EC precipitation was experienced on the spray head due to the relatively high temperature that developed in the glass chamber; therefore, spray head temperature was kept under 40 °C by cooling the EC solution with an ice bath to avoid precipitation and yield loss.