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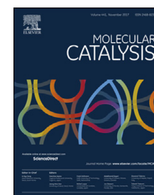
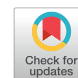


Chitosan hydrogels: A new and simple matrix for lipase catalysed biosyntheses



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ABSTRACT

Lipase from *Mucor miehei* was immobilized on chitosan hydrogels without using any activation agent. These low-cost and easy to prepare lipase-containing hydrogels could be considered as novel solid-phase biocatalysts for use in organic media. When they were applied as biocatalysts towards the model esterification reaction of propyl laurate synthesis they showed an optimum operation temperature at 30 °C and maintained a high catalytic activity in various non-polar organic solvents. The higher initial esterification rates were obtained when biocatalysis took place in branched hydrocarbons where the initial rates recorded were twice the ones observed for straight hydrocarbons, being 0.22 mM/min in isooctane and 0.11 mM/min in hexane, respectively. The novel catalyst presented excellent reusability, with only 10% loss of activity after 12 uses, which is even higher than the one obtained by lipase-containing chitosan beads prepared with a cross-linker. Moreover, enzyme superactivity was evident for several runs.

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1. Introduction

The use of immobilized enzymes appears to be a green, sustainable procedure that has much to offer: mild reaction conditions, usually no by-products, high regio-, chemo- and stereo- selectivity, shorter synthetic routes, less waste and generally environmentally and economically improved high quality results. Enzyme immobilization onto solid supports leads to increased thermal and operational stability and recoverability. These issues have been so far pointed out by several authors [1–4]. Among the enzymes studied, lipases are of particular interest as they find a broad range of industrial applications due to the variety of the reactions they catalyse. They are used in multiple industrial applications in food and flavour making, pharmaceuticals, detergents, cosmetics and perfumery [5], usually by catalysing synthetic instead of hydrolytic reactions.

The type of support as well as the method of immobilization influences the activity and operational stability of immobilized lipases. Various supports have been used so far for lipase immobilization, including inorganic materials (e.g. silica, celite, ceramics, zeolites) [6], synthetic polymers (e.g. amberlite, polyvinyl chloride, polyurethane) [7] or natural polymers (e.g. pectin, starch, cellulose, gelatin, agar, carrageenan, chitin, chitosan) [8]. Among these, chitosan is a polysaccharide obtained easily by alkaline hydrolysis of chitin, a widely abundant natural polymer that can be derived from the exoskeletons of crustaceans, the cuticles of insects and the cell walls of most fungi. Chitosan is biocompatible, non-toxic, able to absorb liquids and able to form films and beads. Therefore, it has been extensively studied in many fields for various applications. These include pharmaceutics as drug carrier, wound dressing material and tissue engineering [9,10], manufacturing materials such as optical and electronic devices [11] or batteries [12], as well as the field of heterogeneous organocatalysis [13]. However, only limited studies refer to chitosan as a matrix for enzyme immobilization [14–16]. Furthermore, these chitosan-based biocatalysts are used in aqueous environment for the catalysis of hydrolytic reactions and not for the synthesis of esters.

Protocols for enzyme immobilization on chitosan matrices often demand an initial surface modification or activation step. In most cases, carbodiimide is used as a coupling agent to activate the hydroxyl groups of chitosan [14,15] and glutaraldehyde to cross-link the lipase to the amino groups [14]. In other cases, sodium tripolyphosphate solution [17] or epichlorohydrin [16] is used followed by amino acid coupling.

In this work, a simpler method is used for lipase immobilization on chitosan hydrogel that does not involve any activation agent. Aqueous solution of the enzyme is mixed with chitosan solution to

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produce an enzyme-containing hydrogel. The produced biocatalyst is then used in organic solvent, to catalyze the model esterification reaction of propyl laurate synthesis. Since the appropriate choice of the immobilization process influences the operational costs of industrial processes, the suitability of the proposed biocatalyst is tested towards various parameters, such as temperature, storage stability and reusability. The aim of this work is to suggest an immobilization process that truly meets the requirements of sustainable chemistry in terms of cost and energy consumption reduction, giving good results as regards recovery and reuse of the catalyst, a parameter that is mandatory for the process to be economically viable at industrial scale.

2. Materials and methods

2.1. Materials

Mucor miehei (*M. miehei*) lipase was supplied by Fluka, Basel, Switzerland and had a specific activity of 1.19 U/mg of protein (1 U corresponds to the amount of enzyme which liberates 1 μmol butyric acid per min at pH 8.0 and 40 °C using tributyrine as substrate). Peroxidase from horseradish (HRP, Type XII) with a specific activity of 382 units/mg solid (using pyrogallol) and 2,2'-Azino-bis(3-ehtylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich, Germany. Chitosan (viscosity 200–600 mPa.s, 0.5% in 0.5% Acetic acid, 20 °C; Deacetylation value: 80%) was purchased from TCI, Belgium. Hydrogen peroxide 30% was purchased from Merck, Darmstadt, Germany. All other materials were at least reagent grade. Millipore Milli-Q water was used for the preparation of gels and buffer solution.

2.2. Preparation of hydrogels

Chitosan hydrogels were prepared by adding acidic water (pH = 3) at 30 °C to the appropriate amount of chitosan. The mixture was stirred until the whole amount of water was absorbed by chitosan. Then the appropriate amount of Tris/HCl buffer, pH = 7.5 containing lipase was added. In a typical experiment, 0.25 g of chitosan is mixed with 0.25 mL of water, followed by the addition of 15 μL of buffer solution containing 0.15 mg of lipase and stirred at room temperature. Each hydrogel was washed three times with 5 mL of isooctane to remove any enzyme molecules that may not be effectively encapsulated to the gel network.

2.3. Lipase-catalyzed reactions

The esterification reactions took place under batch conditions. 0.5 g of hydrogel (containing 0.15 mg of lipase) was placed in a flask with 5 mL of the appropriate organic solvent containing lauric acid and 1-propanol (100 mM/1:1). The flasks were preserved at room temperature without stirring.

At fixed time intervals samples of 10 μL each were withdrawn before less than 10% of the substrate was consumed and analysed by GC. Each reaction was measured in duplicate and conversions were calculated by using calibration curves of butyl laurate ester in the presence of dodecane as an external standard.

For the gas chromatography (GC) analysis a HP5 column (30 m x 0.25 mm i.d. x 0.32 μm film thickness) mounted on a Hewlett-Packard (HP) Model GC-6890C was used. Injector as well as detector temperature was 270 °C and oven temperature was held constant at 200 °C.

2.4. Horseradish peroxidase reaction

For the oxidation reaction, 0.2 g of hydrogel containing 0.17 μg of *Horseradish peroxidase* (HRP) was placed in a cuvette with 1 mL acetate buffer (0.05 M, pH 4.5) containing 0.6 mM ABTS and 1.2 mM H_2O_2. The reaction was followed spectrophotometrically by measuring the one-electron oxidation of ABTS into the corresponding radical-cation $ABTS^{\bullet+}$ at 414 nm. UV–vis spectra were recorded with a Cary 1E Varian spectrophotometer.

3. Results and discussion

3.1. Optimization of chitosan-based hydrogels

In the present work, the ability of a natural polymer, i.e. chitosan, to form hydrogels containing lipase as well as the catalytic effectiveness of these lipase-containing hydrogels towards the synthesis of fatty acid esters, were investigated.

In order to obtain chitosan-based gels, several methods have been proposed to induce chemical or physical cross-linking [10]. In this study, a different approach was tested where no activation agent was used. A simpler method was chosen according to which chitosan was mixed with water followed by addition of aqueous enzyme solution. The conditions under which this chitosan-water mixture should take place had to be determined.

Chitosan is not a common polymer in nature. It is manufactured by the parent, natural molecule chitin by alkaline de-acetylation. The product of the de-acetylation, i.e. chitosan, is a linear polysaccharide of β-(1-4) linked 2-acetamide-2-deoxy-D-glucose (the neutral A-unit) and 2-amino-2-deoxy-D-glucose (the positively charged D-unit). The chain length of the polymer as well as the fraction of A-units (F_A) vary with F_A-value raging from *ca.* 0.7 (70% acetylated) to 0 (0% acetylated). In this study, the chitosan used had an F_A value of 0.2, being 80% de-acetylated, therefore since it is enriched in D-units it acts as a polyelectrolyte. Since the isoelectric point of chitosan is close to 6.3, the amine groups of D-units are positively charged at pH-values below 6.3, influencing its solubility in acidic medium and allowing thus, ionic induced cross-linking. This was experimentally confirmed by forming chitosan hydrogels using water with various pH-values. It was found that gels prepared with acidic water at pH-value 3 were rigid and maintained their integrity in various solvents, while gels prepared with water at higher pH values could not retain their structure when an external solvent was added. Following this procedure of mixing chitosan with acidic water, it was found that chitosan forms gels with a mass fraction of polymer in gels from 0.13 to 0.50. Hydrogels formed at these concentration ranges of polymer were stable in contact with non-polar as well as polar organic solvents at 25 °C and maintained their structural integrity for several days.

The prepared hydrogel was then used as a matrix for enzyme immobilization. Two enzymes, namely *Horseradish peroxidase* (HRP) and *M. miehei* lipase, were encapsulated in chitosan hydrogels and were both able to maintain their catalytic activity towards model reactions. More specifically, HRP was tested towards the oxidation of ABTS by H_2O_2 while *M. miehei* was tested towards the esterification of 1-propanol with lauric acid. Fig. 1 shows a typical reaction profile for both procedures.

The specific activity of the immobilized lipase was 27 μM/min/mg of enzyme for propyl laurate synthesis. The reaction yield was 60% after 24 h of reaction. Similar results were obtained for the synthesis of α-monolaurin by lauric acid and glycidol using *C. rugosa* lipase immobilized on porous chitosan-silica hybrid microspheres [18] where a conversion of 82% was achieved after 50 h of reaction. Similarely, the synthesis of butyl butyrate catalyzed by *C. rugosa* lipase showed an activity of 27.9 μmol/min when immobilized on chitosan flakes [19].

A wide variety of enzyme classes have been reported to keep their catalytic activity when immobilized on chitosan matrix, including oxidases, dehydrogenases, amylases, phosphatases and

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