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Synthesis of functionalized polyethylenimine-grafted mesoporous silica spheres and the effect of side arms on lipase immobilization and application

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ABSTRACT

In the present study, silicate mesoporous materials (MCM-41), MCM-41-grafted polyethylenimine (MCM-41@PEI), and succinated PEI containing amine, amide, and acid groups were successfully synthesized and characterized by Fourier transform infrared spectroscopy, thermogravimetric analysis, X-ray diffraction, scanning electron microscopy, transmission electron microscopy, Brunauer–Emmett–Teller analysis, and X-ray photoelectron spectroscopy. *Thermomyces lanuginosa* lipase (TLL) was then immobilized onto MCM-41 and polymer-grafted MCM-41 by physical adsorption. Besides, for enzyme immobilization via covalent bonding, glutaraldehyde (GLU), and hexamethylene diisocyanate (HMDI) were used as the bridges for binding the enzyme to supports. The best result was obtained with the immobilized lipase on MCM-41@PEI-GLU. In the study of the enzyme reusability, it was shown that about 83% of the initial activity could be retained after 12 cycles of uses. The immobilized lipase on the selected support was also applied for the synthesis of ethyl valerate. Following 24 h incubation in *n*-hexane and solvent free media, the esterification percentages were 79% and 67%, respectively.

1. Introduction

Remarkable advantages of enzymes including regio-, enantio- and stereoselectivity, as well as specificity and activity under mild conditions made biocatalysts the preferable alternative to the classic chemical modifications especially in pharmaceutical chemistry, food modification, and energy production [1–3]. Lipases (triacyl glycerol acyl hydrolases, EC 3.1.1.3) are one of the most common applied hydrolase enzymes used in wide range of organic reactions such as hydrolysis, ammoniolysis, alcoholysis, Michael-type addition, esterification, and inter-esterification either in organic or neoteric solvents such as ionic liquids, supercritical fluids, and eutectic solvents [4–6]. The lipase originated from *Thermomyces laguginosus* (TLL) is a thermostable and basophilic biocatalyst commercially available in both soluble and immobilized form [5,7]. This single chain protein (269 amino acids with molecular weight of 31.7 kDa) has been widely used from biodiesel production to fine chemicals especially in enantio- and regioselective or specific processes. Like other well-known lipases, the TLL has "lid" domain (a polypeptide chain near the active site) which help lipase to change its conformation from "close" to "open" (more active) form. These drastic changes, known as "interfacial activation", in its structure during catalysis might negatively affect the activity of TLL [7,8]. In fact, it has been shown that immobilization of TLL on hydrophobic supports allows the "open" form of TLL to be more stable. In addition, improvement of regio- and enantio selectivity of lipases after immobilization on suitable supports has been reported by many investigations [9,10].

The literature has featured a number of suggested enzyme immobilization techniques, including enzyme entrapment in solid porous polymers, enzyme encapsulation in semi permeable organic and/or inorganic membranes, physical adsorption, and covalent bonding using a cross-linker among which, the multipoint covalent attachment of enzymes with host materials and physical

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adsorption of biocatalyst on the surface of suitable supports has received considerable attention during last decades [11–13]. Each immobilization method bears assorted advantages and disadvantages, making a comparison of the methodologies difficult. The leakage of protein into the reaction medium due to the weak interaction between the biocatalyst and the applied scaffold is the main problem in enzyme immobilization using physical adsorption methods. On the other hand, support binding via strong covalent attachment prevents the enzyme leaching from the surface. However, this can also be a major drawback, if the enzyme is irreversibly deactivated [14–17]. Enzyme immobilization on chemically inert supports enables the multiple use of biocatalysts, thereby decreasing unit cost and improving physical, chemical, and mechanical stability, as well as enzyme lifetime [18]. Of the different proposed supports for immobilization, inorganic supports are appropriate for enzyme confinement given their chemical and mechanical stability against the influence of microbial agents and organic solvents [19]. MCM-41 (belonging to the family of M41S silicate mesoporous materials) is one of the most popular scaffolds applied for enzyme immobilization especially by physical adsorption due to (i) large pore size which allow loading of the bulky enzymes into the pore and as a result protecting the protein from denaturation, (ii) the presence of silanol groups on the surface of MCM-41 can facilitate immobilization of enzymes via hydrogen bonding, and (iii) mechanical stability which protects immobilized biocatalyst against abrasion and breaking [12,14]. Moreover, introducing of suitable polymers on the surface of ordered mesoporous materials and chemical modification of the desired scaffold provides more appropriate support for immobilization of enzymes [14].

There are many reports on the protective effect of polyethyleneimine (PEI), a water soluble cationic polymer with large number of primary amino groups, on the activity of enzymes such as lipases especially in organic media [10,15]. PEI grafting onto inert, inorganic supports and then cross-linking with bifunctional agents is an extensively used approach for immobilization of various enzymes, such as lipase [20], β-galactosidase [21], and tyrosinase [22]. Glutaraldehyde (containing active aldehyde group), succinic anhydride (a dicarboxylic anhydride), and diisocyanate derivatives have been commonly used as versatile cross-linkers in the enzymes immobilization procedures among which glutaraldehyde introduced as agent of choice due to its low cost and development of efficient immobilized biocatalyst [1,14,15].

In this study, covalently modified MCM-41 with a biologically benign polymer (PEI) was synthesized and subsequently modified with succinic anhydride (SUC) to obtain an adsorption capacity for lipase higher than that of unmodified analogs. Lipase was then immobilized onto PEI and succinated PEI grafted MCM-41by adsorption method and then compared with an enzyme covalently immobilized onto MCM-41@PEI. For the covalent immobilization, glutaraldehyde (GLU) or hexamethylene diisocyanate (HMDI) was used as cross-linking agent to study the effects of immobilization methods on enzyme stability. In addition, immobilized lipase was used for the synthesis of ethyl valerate (green apple flavor) and methyl valerate in free solvent and *n*-hexane media.

2. Materials and methods

2.1. Chemicals and the enzyme

Hyperbranched polyethyleneimine (PEI, MW = 60,000), *p*-nitrophenyl butyrate (*p*-NPB), and [3-(2,3-epoxypropoxy)propyl] trimethoxysilane (EPO, 98% purity) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cetyl trimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), aqueous ammonia solution (28 wt%), glutaraldehyde (GLU), succinic anhydride (SUC), hexamethylene diisocyanate (HMDI), valeric acid and ninhydrin were purchased from Merck (Darmstadt, Germany). Lipase derived from *Thermomyces lanuginosa* (TLL) was kindly donated by Novozymes (Bagsvard, Denmark). TLL was purified before being used in the immobilization experiments by adsorbing on octyl-sepharose beads under continuous stirring in 10 mM sodium phosphate (pH 7). The enzyme preparation was then vacuum filtered by a sintered glass funnel and washed three times with the same buffer. TLL was then desorbed from the used hydrophobic supports by suspending the immobilized lipase in 25 mM sodium phosphate at pH 7 (1/10 w/v) containing 0.6% v/v of CTAB during 1 h at 25 °C [23].

2.2. Preparation of mesoporous supports

MCM-41 was prepared by hydrothermal treatment [24]. Briefly, CTAB (13.0 g) was dissolved in deionized water (167.0 mL). Then, aqueous ammonia (163.7 mL) and absolute ethanol (131.5 mL) were added to the surfactant solution and stirred for 15 min. Subsequently, TEOS (24.8 mL) was added to the mixture. After 2 h of vigorous stirring at 1500 rpm and subsequent filtration, the white precipitate was washed with deionized water and dried at 60 °C for 24 h. The prepared MCM-41 was successfully calcinated under oxygen atmosphere by heat treatment at a rate of 1 °C per min up to 600 °C. This temperature was maintained for 6 h.

PEI was grafted onto the prepared MCM-41 as follows. EPO (1.0 mmol) was added to PEI solution (3.0 mmol) in dry toluene (150 mL). The resultant mixture was allowed to react at 80 °C for 24 h. The calcined MCM-41 (2.5 g) and ethanol (25 mL) were then added to the mixture and stirred at 80 °C for 24 h. The produced MCM-41@PEI was separated by filtration and washed several times with deionized water and ethanol. It was subsequently soxhleted with ethanol for 24 h to remove physically bonded compounds, filtered, and dried at 40 °C for several days [25]. Finally, to introduce amide and acid groups on the surface of MCM-41@PEI, SUC (10 mmol) was added to a stirred solution of dry ethanol (50 mL) containing MCM-41@PEI (1 g). The resultant mixture was allowed to reflux for 24 h. The produced MCM-41@PEI-SUC was filtered and washed several times with methanol and ethanol. After Soxhlet extraction by hot ethanol, MCM-41@PEI-SUC was dried under vacuum for 24 h at 70 °C.

2.3. Immobilization of lipase

The purified TLL solution (1 mL, equal to 5 U), which was selected through a primary experiments in the range of 0.5–25 U/mL lipase activity, was used in both lipase immobilization procedures (physical adsorption or covalent attachment immobilization). After immobilization, the resultant supports were filtered through a 0.45-μm PTFE (Teflon) membrane filter and then dried under vacuum overnight at 4 °C. The immobilization yield (IY) was calculated by measuring protein concentration of the obtained samples before (C_{t0}) and after (C_{tt}) immobilization processes, based on the equation of IY (%) = $[C_{t0} - C_{tt}]/C_{t0} \times 100$; while immobilization efficiency (IE) was calculated according to the relationship of $A_i/A_f \times 100$; where A_i is the activity of the immobilized lipase, and A_f is the activity of lipase added in the initial immobilization solution.

2.3.1. Adsorption onto the PEI- and succinated PEI-grafted MCM-41

PEI- and succinated PEI-grafted MCM-41 (10 mg) were suspended in 10 mL phosphate buffer (0.1 M, pH 7) containing lipase (1 mL, equal to 5 U). The mixture was slowly incubated at 25 °C for