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Improvement of the Lipase Immobilization Procedure on Monodispersed Fe_3O_4 Magnetic Nanoparticles

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Here we report on the immobilization of lipase (E.C 3.1.1.1) from *Thermomyces lanuginosa* on monodispersed Fe_3O_4 nanoparticles. The nanoparticles were prepared by a bottom-up chemical strategy based on low temperature thermal decomposition of an iron precursor in organic solvent in the presence of surfactants. It offers many advantages, such as experimental easiness, potential low-cost fabrication, very high nanoparticles size control and reproducibility. The nanoparticles were treated with (3-aminopropyl)triethoxysilane (APTES) and then activated via the glutaraldehyde method to couple with lipase by a covalent bond. High recovered activity (77%) was obtained, a new washing procedure was developed to double the immobilization efficiency.

1. Introduction

Magnetic iron oxide nanoparticles have been used for food related applications, for enzyme immobilization, protein purification, and food analysis (Cao et al., 2012). Since a critical concern is their safety, superparamagnetic Fe_3O_4 nanoparticles are very good candidate, because they do not retain residual magnetism, are not toxic, and have good biocompatibility.
Enzymes (carbohydrases, proteases, lipases, lysozymes and oxidoreductases), are widely used in food industry due to their excellent catalytic activity. However, maintaining their structural stability during any biochemical reaction is highly challenging, since free enzymes usually have poor stability towards pH, heat or other denaturating agents and are difficult to recover and reuse. For large commercialization of these bioderived catalysts, their reuse becomes mandatory, failing which they would no longer be economic. Enzyme immobilization is the most usual method to improve enzyme features (Bolivar et al., 2006). Consequently, immobilized enzymes with functional efficiency and enhanced reproducibility are used as alternatives in spite of their expensiveness.
Among the organic and inorganic supports to be used for enzymes immobilization, magnetic nanoparticles are widely investigated because they: (i) allow simple, quick and low-cost collection of enzymes from a complex mixture just with an external magnetic field; (ii) have high enzyme loading capability, due to their large specific surface area; and (iii) suffer lower diffusion limitation in solution.
Several methods are used for immobilization and various factors influence the performance of immobilized enzymes (Zhang et al., 2013). Enzymes immobilization on magnetic nanoparticles can be obtained by both bonding and physical adsorption. In the case of physical adsorption, enzyme can easily cut off from the nanoparticles surface. Maintaining the structural and functional properties of enzymes during immobilization is one of the major roles played by a cross-linking agent. One of such agent is glutaraldehyde, typically used as bifunctional cross-linker, because it is soluble in aqueous solvents and can form stable inter- and intra-subunit covalent bonds. As regards lipases, they have been commonly used in the synthesis of enantio-enriched monomers and macromers, for polymerization reaction, (Gross et al., 2001), for biodiesel production (Xie and Ma 2009, Jegannathan et al., 2010).
In the present work, magnetic Fe_3O_4 nanoparticles were used as support for immobilization. The nanoparticles were prepared for the first time for this application by a bottom-up chemical strategy (Altavilla et al. 2009,

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Altavilla et al. 2011, Sarno et al. 2015) offering many advantages, such as experimental easiness, potential low-cost fabrication, elevated results reproducibility, very high monodisperse nanoparticles size control and thus optimized surface and homogeneous magnetic behaviour. It is based on low temperature thermal decomposition of an iron precursor in organic solvent and in the presence of surfactants. The nanoparticles were treated with (3-aminopropyl)triethoxysilane (APTES) and then activated via the glutaraldehyde method to couple with lipase from *Thermomyces lanuginosa* by a covalent bond. This lipase is the most used thermophilic lipase, due to its wide commercial availability.
High recovered activity (77%) has been obtained. Particular attention has been devoted to one of the crucial aspects related to the immobilization process: the search for appropriate washing conditions allowing to improve the immobilization efficiency.

2. Experimental

2.1 Nanoparticles synthesis and characterization

The nanoparticles synthesis was carried out using standard airless procedures and commercially available reagents. Absolute ethanol, hexane, and dichloromethane (99%) were used as received. Benzyl ether (99%), 1,2-hexadecanediol (97%), oleic acid (OA) (90%), oleylamine (OAM) (>70%), iron(III) acetylacetonate ($Fe(acac)_3$), were purchased from Aldrich Chemical Co.
20 mL of solvent (benzyl ether), 2 mmol of precursor ($Fe(acac)_3$), 10 mmol of 1,2-hexadecanediol, selected for its high reducing ability, 6 mmol of oleic acid and 6 mmol of oleylamine as surfactants, were mixed and magnetically stirred under nitrogen flow. The mixture was heated to 200 °C for 120 min and then, under a blanket of nitrogen, heated to reflux (285 °C) for 60 min. The black-brown mixture was brought to room temperature by removing the heat source and then washed by centrifugation (7500 rpm, 30 min) employing: (i) ethanol, and (ii) an equal volume mixture of hexane and ethanol.
The characterization of nanoparticles was obtained by different techniques. Transmission electron microscopy (TEM) images were acquired using a FEI Tecnai electron microscope operated at 200 KV with a LaB6 filament as source of electrons. XRD measurements were performed with a Bruker D8 X-ray diffractometer using CuKα radiation.
The KBr technique was applied for performing the FT-IR spectra of the samples by using Vertex 70 apparatus (Bruker Corporation). Spectra were recorded in the scanning range from 4000 to 400 cm^{-1}.
Thermogravimetric analysis (TG-DTG) at 10 K/min heating rate in flowing air was performed with a SDTQ 600 Analyzer (TA Instruments).

2.2 Lipase Immobilization

Under ambient conditions, a hexane dispersion of hydrophobic Fe_3O_4 nanoparticles (about 100 mg in 1 mL) was added to a suspension of tetramethylammonium 11-aminoundecanoate (ADATMAS) (about 100 mg in 10 mL) in dichloromethane. The mixture was shaken for about 20 min, during which time the particles precipitated. The suspension was settled and the precipitate was washed several times with dichloromethane, then separated using a magnet to remove excess surfactants before drying under nitrogen. 100 mg of Fe_3O_4 nanoparticles were dispersed in 1.94 mL of ethanol by sonication and then 60 µL of (3-aminopropyl)triethoxysilane (APTES) was added to this solution. The reaction mixture was sonicated and then shaken overnight at room temperature. The supernatant was removed by magnetic separation and the precipitates were washed with distilled water and ethanol. Thereafter, 4 mL of glutaraldehyde (G) solution (10%) were added to the precipitates of APTES-coated magnetic nanoparticles, and the reaction kept at room temperature for 2 h. After 2 h, the glutaraldehyde-activated particles were separated by magnetic decantation and subsequently washed with distilled water.
4 mL of buffer solution (0.1 M phosphate buffer, pH 7.0) containing 10 mg of lipase from *Thermomyces lanuginosa* (Sigma Aldrich) were mixed with the above activated nanoparticles. The mixture was then shaken at room temperature for 2 h. After completion of the reaction, the unbounded enzyme was removed under a magnetic field, and the precipitate was recovered and washed carefully with phosphate buffer (0.1 M phosphate buffer, pH 7.0) for several times and then directly used for the enzyme activity measurements.

2.3 Lipase immobilization efficiency

The amount of lipase protein in supernatant was determined by the Bradford method (Bradford, 1976) using BSA as a protein standard for the calibration curve. The immobilization efficiency of lipase onto the magnetic support was determined from the following equation $q = (C_i\text{-}C_f)V_1 \diagup C_iV_2$, where q is the immobilization efficiency (%), C_i and C_f are the concentrations of the initial soluble lipase, and the final lipase concentrations (mg mL-1) in the supernatant after immobilization, respectively , and V_1 and V_2 are the solution volume (mL). All data in this formula are averages of duplicate experiments.

2.4 Lipase assay

The enzymatic activities of immobilized and free lipase were tested with olive oil emulsion containing 3% (w/v) PVA. A certain amount of the free or immobilized lipase was added to 4 mL of the emulsion and 5 mL of the phosphate buffer (0.025 M, pH 7.0). The hydrolysis reaction was carried out at 40 °C for 15 min. The quantity of fatty acid liberated was measured by titration with 0.1 M KOH solution. One unit (1 U) of activity was defined as the amount of lipase that liberates 1 *μ*mol of oleic acid per minute under the assay conditions.
As a reference test, titration with 0.1 M KOH solution was also performed for the as prepared nanoparticles after the washing step.

3. Results and discussion

Monodispersed nanoparticles (6 nm) of Fe_3O_4 have been obtained successfully, as confirmed by TEM analysis. Figure 1 shows TEM images of the Fe_3O_4 nanoparticles at increasing magnification.

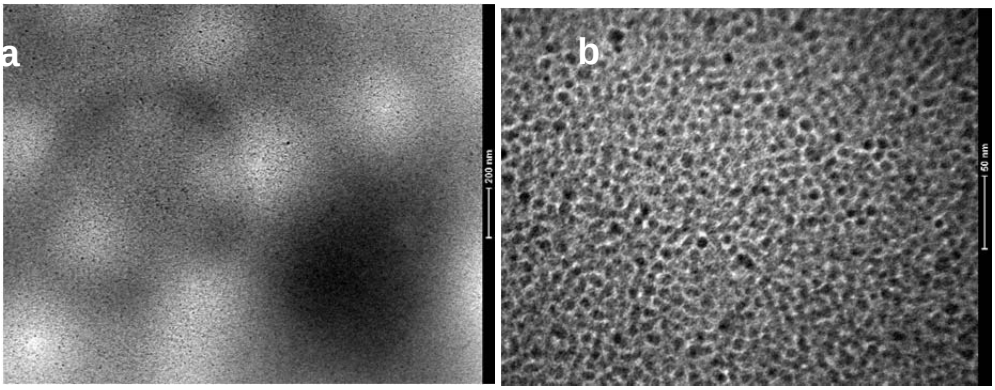

Figure 1: TEM images of Fe_3O_4 nanoparticles at different magnification

The XRD diffraction analysis of synthesized nanoparticles confirms that these are magnetite crystals (Figure 2); the mean crystal size, as determined by Debye-Scherrer equation, is 6 nm (Sarno et al., 2015).

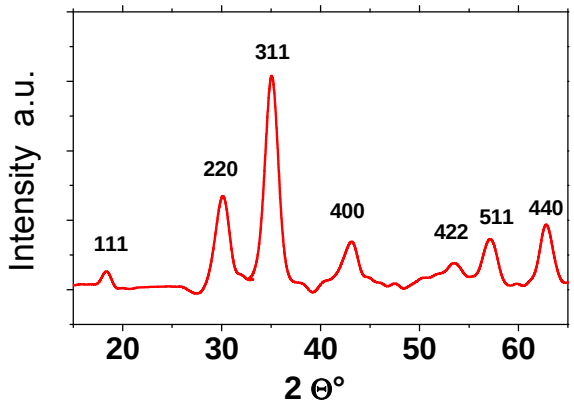

Figure 2: XRD pattern of synthesized Fe_3O_4 nanoparticles

To investigate whether the surface of the nanoparticles was capped with oleic acid and oleylamine, and the functionalization at the end of the different steps, FT-IR spectra were acquired (Figure 3 a and b) on the washed nanoparticles in ethanol alone and at the end of each step of functionalization. The Fe_3O_4 typical peak, due to $\nu 1$ (Fe-O) and $\nu 2$ (Fe-O) bonds at 635 and 590 cm^{-1} (Klokkenburg et al., 2006), respectively, are observed. Oleic acid shows a strong absorption peak of carbonyl stretch band around 1710 cm^{-1}. The band at 1285 cm^{-1} is due to C-O stretch and the bands at 1462 and 937 cm^{-1} are the in-plane and out-plane bands of O-H (Zhang et al., 2010). The strong band at 2855 and 2923 cm^{-1} belongs to methylene and methyl symmetric stretching vibration, respectively (Zhang et al., 2010).

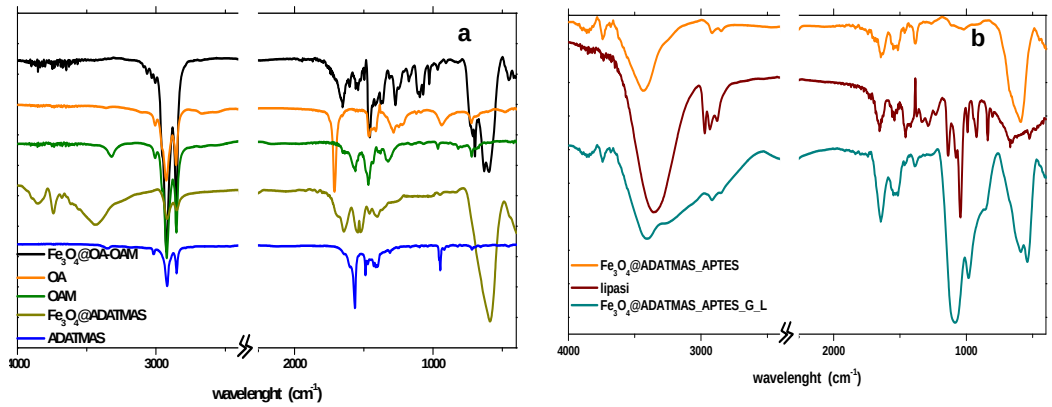

Figure 3: FT-IR spectra in the range of wavenumber 4000-400 cm^{-1} of a) pure surfactants, i.e. oleic acid (OA) and oleylamine (OA), pure tetramethylammonium 11-aminoundecanoate (ADATMAS), nanoparticles synthesized in the presence of surfactants (Fe_3O_4@OA-OAM), after the treatment with ADATMAS (Fe_3O_4@ ADATMAS) b) nanoparticles after the treatment with APTES, (Fe_3O_4@ ADATMAS_APTES), nanoparticles after the treatment with glutaraldehyde and lipase (Fe_3O_4@ ADATMAS_APTES_G_L) and free lipase (lipase).

It is worth noting that the C=O stretch band of carboxyl group, which was present at 1710 cm^{-1} in the spectrum of pure oleic acid, was absent in the curve of the spectrum of the coated nanoparticles. Two new bands at 1541 and 1649 cm^{-1} appear in the Fe_3O_4@OA-OAM spectrum, characteristic of the asymmetric ν_{as}(COO-) and the symmetric ν_s(COO-) stretch, instead. This result can be explained assuming that the bonding of the carboxylic acids on the surface of the nanoparticles was a combination of molecular bonded symmetrically and molecules bonded at an angle to the surface (Zhang et al., 2006). The spectrum of oleylamine shows the characteristic peaks of the oleic group in the 2750-3000 cm^{-1} region, the v(C=C) stretch mode at 1647 cm^{-1}, and the peak at 1468 cm^{-1} due to the (C-H) bending mode. In addition, there are characteristic signals of the amine group: the peak at 3319 cm^{-1} due to the v (N-H) stretching mode of the primary amine, the peak at 1560 cm^{-1} due to the $-NH_2$ scissoring mode, and the peak due to the $-NH_2$ bending mode at 968 cm^{-1} (Altavilla et al., 2011), as well as a C-H (C >7) flexural vibration in the range of 720 cm^{-1}. In the IR spectra of Fe_3O_4@OA-OAM nanohybrid, the presence of the oleylamine as a capping agent on the surface is confirmed by the peak characteristics of the oleic group in the 2750-3000 cm^{-1} region, the v (C=C) stretch mode at 1647 cm^{-1}, and the peak at 1468 cm^{-1} due to the (C-H) bending mode. It can be noted an obvious C-H flexural vibration, indicating that oleylamine was chelated on the surface of the NPs. Although the presence of the amino group is indicated by the $-NH_2$ weak bending mode at 968 cm^{-1} (Salavati-Niasari et al., 2008) the signals of the free primary amine group at 3319 cm^{-1} due to the v (N-H) stretching mode seem to be absent in the spectrum of the nanohybrid, while the peak at 1560 cm^{-1} , due to the $-NH_2$ scissoring mode, results reduced. These phenomena were observed also for other kinds of nanoparticles successfully capped by oleylamine (Huirache-Acuña et al., 2009).

The characteristic bands of pure ADATMAS at 1566, 1487 cm^{-1} are due to COO- group vibrations (Sun et al., 2004), while the peaks appearing at around 1395 and 950 cm^{-1} are associated with the stretching vibration modes of the C–O and CH_2 groups (Zhang at al., 2010).

In Figure 3a the IR spectrum of hydrophilic nanoparticles is also reported (Fe_3O_4@ ADATMAS). The adsorptions around 1560 and 1466 cm^{-1} from the hydrophilic nanoparticles match with the ones from ADATMAS, indicating the presence of free $-COO^-$ group in the sample (Sun et al., 2004). On the other hand, the bands at 1640 and 1539 cm^{-1} suggest the formation of carboxyl-complex between the surfactant and the nanoparticles surface. Probably ADATMAS replaced in a different way the oleic acid and oleylamine molecules on the nanoparticles surface.

In Figure 3b the FT-IR spectra of pure lipase and Fe_3O_4 nanoparticles before and after lipase immobilization step are reported, confirming the binding of lipase to magnetite nanoparticles (peaks around 1000 cm^{-1}, for C=N bond formation after lipase immobilization).

The evaluation of the amount of immobilized enzyme, as determined by the Bradford method (Bradford, 1976), indicates that in our experimental conditions 23 % of lipase immobilized on the nanoparticles surface.

After the immobilization steps of enzyme on nanoparticles, they have been used for enzymatic assay. The results of assay showed that the immobilized enzyme has an activity of 77 % with respect to the free enzyme in the same conditions.

Aiming at increasing the amount of immobilized enzyme on the nanoparticles, and having identified in the organic chains ligand exchange the critical aspect, several attempts have been performed by changing the amount and type (only OA and OAM being used) of surfactants and the washing procedure. From thermogravimetric analysis characterization we found that the washing step after synthesis is crucial to improve the enzyme immobilization efficiency.

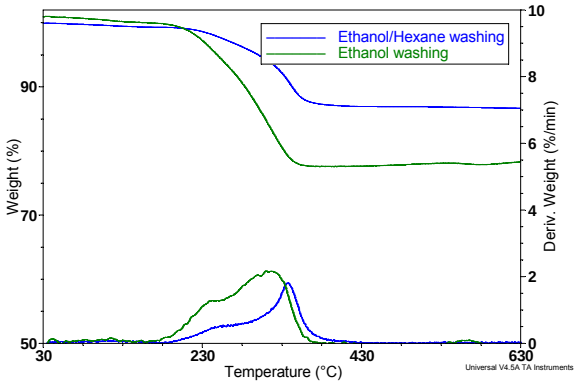

Figure 4: Thermogravimetric analysis of: Fe_3O_4 nanoparticles washed with ethanol alone and Fe_3O_4 nanoparticles washed with an equal volumetric ratio mixture of ethanol and hexane.

The use of surfactants during the synthesis of nanoparticles, that results in a hydrophobic coating around them, is of fundamental importance to avoid the coalescence of seeds and to obtain monodispersed nanometric particles. Moreover, it is obvious that high enzyme immobilization yields need the removal of the excess organic chains, to avoid efficiency reduction during the subsequent steps of functionalization.
On the other hand, it is less obvious that increased immobilization efficiency can be obtained partially removing the organic chains linked to the nanoparticles. Typical washing procedures (Zhang at al., 2006) were performed by using ethanol (see the green profiles in Figure 4). We have developed a washing procedure in a mixture of equal volume of hexane and ethanol (see the blue profiles in Figure 4). In particular, Figure 4 shows the thermogravimetric profiles of two Fe_3O_4 nanoparticles samples washed by the two different routes. The weight losses are due to OA and OAM release, while the oxidized nanoparticles constitute the residue, indeed between room temperature and 600°C the phase transition from Fe_3O_4 to Fe_2O_3 is also to take into account. In particular, the thermal conversion in air flow occurred in a weight loss step, with a total 22 wt.% and 12 wt.% releases, for the nanoparticles after ethanol and ethanol/hexane washing, respectively, indicating that our washing approach permits to remove larger amount of organic chains . Considering a weight ratio of 1/1 for OA and OAM molecules binding directly on the NPs surface and that in a closed packed layer of OA and OAM each molecules occupies an area of 0.21 nm^2 and 0.36 nm^2 respectively, we can calculate the organic molecules weight loss equal to 21.2 wt.%, in agreement with the thermogravimetric results. Thus the more effective washing in the ethanol/hexane mixture partially uncoated the nanoparticles surface.
The amount of immobilized enzyme, as determined by the Bradford method, for the nanoparticles washed in the mixture of ethanol and hexane results higher than that obtained on the nanoparticles washed in ethanol alone, consisting in immobilization efficiency of 52 %. This is probably due to a more easy anchoring of ADATMAS molecules on the nanoparticles surface (FT-IR analysis not shown here indicates an increased intensity of the adsorptions around 1560 and 1466 cm^{-1} due to free $-COO^-$ group of ADATMAS), resulting in an higher amount of immobilized lipase.
It is worth noting, that the complete organic chains removal results in a strong nanoparticles tendency to coalescence.

Conclusions

Fe_3O_4 nanoparticles were obtained by a simple, convenient and reproducible procedure leading to monodispersed nanoparticles of 6 nm diameter, as confirmed by TEM and XRD diffraction analyses. The magnetic nanoparticles are covered by organic chains of oleic acid and oleylamine used as surfactants during the syntheses. An efficient surface modification of nanoparticles is crucial for enzyme immobilization.

The nanoparticles washing step to remove the excess surfactants is fundamental to obtain high enzyme immobilization yields on magnetic nanoparticles. In our case, a new washing procedure was developed to double the immobilization efficiency and to obtain high recovered activity (77%).
This type of immobilization is highly favourable since biological catalysts are often very expensive and the recovery possibility from the reaction vessel by a simple magnetic separation is very interesting.

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