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Preparation and modification of chitosan particles for *Rhizomucor miehei* lipase immobilization

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ABSTRACT

Chitosan particles, suitable as immobilization support, were prepared by precipitation and modified by reductive amination in order to graft linear aliphatic chains of 12 carbon atoms to their native amine groups. Their physical characterization was performed by different techniques: differential scanning calorimetry (DSC), X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), air–water contact angle analysis, among others. Lipases from *Rhizomucor miehei* (RM) were immobilized by adsorption at low ionic strength onto different modified chitosan microspheres. Their ability to catalyze the acidolysis reaction between sunflower oil and palmitic and stearic free fatty acids was evaluated in a solvent medium. Effects of modification conditions on the particles hydrophobic character, lipase adsorption and acidolysis activity were investigated. Modified particles were bigger and more hydrophobic than unmodified ones. The most active biocatalyst achieved a change in the composition of palmitic and stearic acid from a value of 9.6% in the original oil to 49.1% in the final structured lipids, representing an almost 3-fold enzyme hyperactivation. This high conversion was maintained during 7 reuse cycles (168 total hours). The results demonstrated that the chitosan modification was effective in order to adsorb and hyperactivate RM lipases.

1. Introduction

Lipases have been widely accepted as biocatalysts for the modification of oils and fats [1,2]. A particular use is the obtaining of structured lipids, which are tailor-made lipids with desired characteristics, like certain physical or chemical properties, and/or nutritional benefits [3]. Among others, they can be synthesized by acidolysis reactions catalyzed by lipases, where it is possible to incorporate a desired acyl group onto a specific position of the triacylglycerol through the hydrolysis and reesterification reaction steps. These reactions can be performed at low temperatures in a solvent free medium or an organic solvent medium, and in continuous or batch systems [4–6]. In view of the current high cost of lipases, the possibility of reusing the enzyme is an attractive feature of biocatalysis. The immobilization allows the lipase to be physically separated from the reaction mixture, and readily reused. Furthermore, it provides some benefits for industrial application such as pH or heat stability, easy recovery, reduction of inhibition by the medium or products, and especially, it makes possible to operate in continuous mode [5,7,8]. However, the high cost and the easy attrition of the commercially available immobilized lipases restrict its use in large scale processes. Therefore, the objective of this work is the preparation of a biological catalyst, suitable to produce structured lipids using a support with two desirable characteristics: low cost and high resistance to attrition.

It has been shown that lipases are able to adsorb on hydrophobic supports at very low ionic strengths, involving the hydrophobic surface that surrounds the catalytic site. This makes possible to stabilize the open conformation of lipases and promotes their hyperactivation after their immobilization [9–12]. Hydrophobic supports mimic the hydrophobic surface of the substrates, and the lipase becomes adsorbed on them via interfacial activation. Thus, lipases have been immobilized, hyperactivated, and in some cases even purified by immobilization on a wide variety of hydrophobic supports or coated supports with hydrophobic groups [13]. Moreover, by changing the nature support and its hydrophobicity, it has been possible to control the adsorption strength, activity, and even selectivity of the lipase [14,15]. The adsorption of the lipase on these supports can often be very strong, making it possible to use the biocatalysts with hydrophobic substrates having small–medium size even in the presence of moderate concentrations of organic solvents. However, their full desorption could be achieved by permitting to recover and reuse the support after the enzyme inactivation. Furthermore, this immobilization may produce biocatalysts that are very stable under different experimental conditions, making this technique especially suitable for lipase immobilization [12,13,15].

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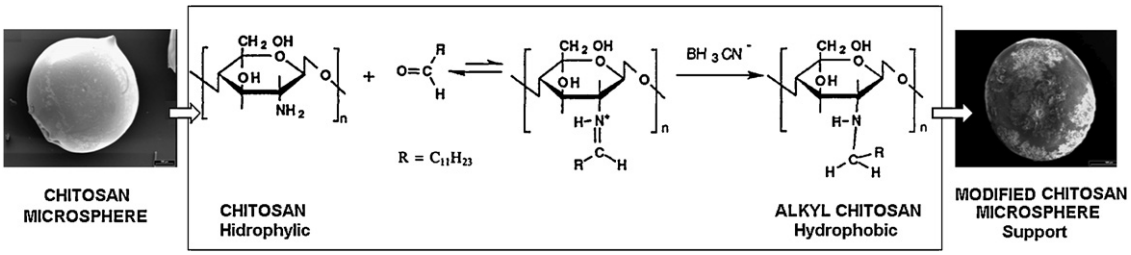

Fig. 1. Scheme of the reductive amination reaction with SEM micrographs of unmodified and modified chitosan particles (scale bar 200 μm at 66× magnification and scale bar 500 μm at 54× magnification, respectively).

Any material that is to be considered as enzyme support must accomplish some requirements, especially if it is thought to be used in a packed bed reactor: high affinity for proteins, availability of reactive functional groups, mechanical stability, rigidity, and high loading capacity [16]. Nowadays, chitosan is becoming a very interesting raw material because of its actual and potential applications due to its chemical versatility, natural abundance, and ecological compatibility. It is also well known for its high mechanical strength, non-toxicity, water insoluble properties, and being chemically modified easily. These properties have allowed the use of chitosan to immobilize enzymes [17,18]. Even though this material is completely suitable for adsorbing lipases, it is necessary to modify its hydrophilic nature. By applying reductive amination reaction, using aldehydes, different length alkyl chains can be grafted on the chitosan backbone to change its hydrophilic character to a more hydrophobic one [19]. Up to now, this technique has been only used on chitosan powder or flakes and chitosan solutions [17,20]. So, one innovative aspect of the present contribution is the application of this technique on chitosan particles previously prepared by precipitation. Different characterization methods were employed in order to corroborate the modification and study the characteristics of the obtained supports.

Additionally, the ability of these particles as support for immobilization of lipases from *Rhizomucor miehei* (RM) was analyzed, evaluating not only the protein content in the obtained biocatalysts but also their catalytic activity in acidolysis reaction using long chain substrates. This last feature is of great importance, since the immobilization process can cause a reduction in the activity compared to free enzymes activity [9,12,21], especially when long chain substrates are used. This contribution focused on obtaining strong biocatalysts with good catalytic activity to the acidolysis reactions so, all of them were performed in batch systems as part of a preliminary study, for future work in a continuous system. The selected reaction was acidolysis between sunflower oil and palmitic and stearic free fatty acids catalyzed by RM lipases, converting vegetable liquid oil into semi-solid fats without the formation of the undesired *trans* fatty acids. Semi-solid fats can also be obtained by hydrogenation of liquid oils, but this process usually generates *trans* fatty acids, which are unhealthy for their consumers.

2. Materials and methods

2.1. Materials

Chitosan was generously donated by Laboratorio de Investigaciones Básicas y Aplicadas en Quitina of Universidad Nacional del Sur, with a degree of 80.2% of deacetylation and 9.3% of moisture, having the original chitin been isolated from shrimp shells (*Artemesia longinaris* source). Refined sunflower oil was purchased from a local grocery store and it was used as received (approximate composition of predominant fatty acids as fatty acid methyl esters (FAME): 56.6% C18:2 (linoleic acid), 31.0% C18:1 (oleic acid), 6.3% C16:0 (palmitic acid), 3.2% C18:0 (stearic acid); peroxide value (PV): 1.94 mequiv/kg). *R. miehei* lipase (>20,000 U/g, Novozymes) from *Aspergillus oryzae* conditioned in the form of liquid, dodecyl aldehyde (92%, Aldrich), sodium cyanoborohydride $NaCNBH_3$ (>95%, Fluka), palmitic-stearic acids blend with purity grade 49–54% and 40–51%, respectively (SPFA) (Fluka) were purchased from Sigma–Aldrich (Germany). Fatty acid methyl esters (FAME) standards were purchased from Supelco (Bellefonte, USA). All the other reagents, gases and solvents were of analytical or chromatographic grade.

2.2. Chitosan characterization

The chitosan mean viscosimetric molecular weight (M) was calculated through the Mark–Houwink equation, which relates the intrinsic viscosity (η) and M through out two parameters, K and a [22]. Viscosity measurements were performed using an Ubbelohde viscometer size 1 (Cannon Instrument Co.) at 25 °C and solutions of chitosan with 0.25 M AcH/0.25 M AcNa buffer solution as solvent. Solution concentrations were lower than 1% (w/v). All measurements were made following the specifications fixed by the D 2857-95 (2001) ASTM standard [23]. Parameters of the Mark–Houwink equation were obtained from Kasaai et al. [24] to used conditions, being $K = 1.57 \times 10^{-2}$ and $a = 0.79$ to express η in $cm^3\ g^{-1}$ and M in Da (valid parameters for molecular weight values between 35 and 2220 kDa).

2.3. Preparation and modification of chitosan particles

Chitosan microspheres were prepared by using 3% (w/v) solution of chitosan powder in 2% acetic acid. This concentration was selected because it achieves particles with a good mechanical resistance and regular shape [25]. Chitosan solution was added dropwise to 3:1 (v/v) mixture of 1 N sodium hydroxide and methanol using hypodermic needles of 1.5 mm diameter. Obtained microspheres were filtered and washed with distilled water.

A reductive amination procedure widely discussed in literature to modified chitosan powder [19,22,26] was applied to synthesize alkylated chitosan particles. This reaction was performed in two stages as suggested by Abdel-Magid [27] with some modifications (Fig. 1). Initially, chitosan microspheres previously prepared were suspended in ethanol (10 mL/g initial dried chitosan powder). Then, an amount of dodecyl aldehyde determined by the monomolar ratio NH_2/aldehyde (AA ratio) was dissolved in ethanol (28.0 mL/g aldehyde to the stoichiometric ratio) and added to this medium under very intensive stirring. Different contact times (amination reaction time, ART), 0.5–2 h, were used to obtain different modification degrees. Finally, the reduction was performed adding an excess of $NaCNBH_3$ (3 mol $NaCNBH_3$/monomole initial dried chitosan powder). The mixture was stirred for 1 h and the alkylated chitosan microspheres were separated, washed with ethanol in order to remove reaction waste, washed with distilled water and then dried at 40 °C until constant weight. Different aspects of that procedure were analyzed in order to obtain the suitable support

to immobilize lipases: ART, AA ratio and their interaction. From this procedure, following modified supports were obtained: Q (AA ratio: 1:0, ART:0), QM0.5–1 (AA ratio: 1:0.5, ART:1 h), QM0.5–1.5 (AA ratio: 1:0.5, ART:1.5 h), QM0.5–2 (AA ratio: 1:0.5, ART:2 h), QM1–0.5 (AA ratio: 1:1, ART:0.5 h), QM1–1 (AA ratio: 1:1, ART:1 h), QM1–1.5 (AA ratio: 1:1, ART:1.5 h), and QM1–2 (AA ratio: 1:1, ART:2 h).

2.4. Chitosan microspheres characterization

Several characterization techniques were used to corroborate the chemical modification of the microspheres and the degree of hydrophobicity achieved in each case due to the variations in its preparation.

2.4.1. Particle size analysis

Digital optical micrographs of microspheres were taken with a Karl Zeiss optical microscope of polymerization (Phomi III Pol). Images of 30 particles per case (according to obtained particles described in Section 2.3) were processed with ImageJ software, measuring 4 diameters per particle, and then the mean diameter of the microspheres was calculated as

$$D_{\text{mean}} = \frac{\sum_{i=1}^{30}\sum_{j=1}^{4} D_{ij}/4}{30} \quad (1)$$

2.4.2. Air–water contact angle analysis

The particle contact angle measurement was made using the method developed by Forward et al. [28]. Digital pictures of the water–particle–air interface were taken under an optical microscope (Phomi III Pol). To that end, microscope slides were constructed in polycarbonate and the spacers were performed in glass with a thickness of 1 mm. The ImageJ image processing software was used to obtain the contact angle.

2.4.3. Differential scanning calorimetry (DSC)

Thermal properties of microspheres were measured on a DSC Pyris I (Perkin Elmer) system. Nitrogen was used as purge gas with a flow rate of 20 cm^3/min. Samples of 10 mg were encapsulated in aluminum pans, holding for 1.0 min at 30 °C and heated from 30 to 350 °C with a rate of 10 °C/min.

2.4.4. X-ray diffraction (XRD)

X-ray diffraction patterns of the microspheres were recorded on a computer-based Rigaku X-ray diffractometer D-max III-C, with a detector operating at a 35 kV and 15 mA, using Cu Kα radiation. The scanning scope of 2θ was from 3° to 50° at room temperature. The crystallinity index (CrI) was determined following the method applied by Focher et al. [29], using the equation:

$$\text{CrI} = \frac{I_{110} - I_{\text{am}}}{I_{110}} \times 100 \quad (2)$$

where I_{110} is the maximum intensity at $2\theta = 20°$ of the lattice diffraction and I_{am} is the intensity amorphous diffraction at $2\theta = 16°$.

2.4.5. Scanning electron microscopy (SEM)

The morphology of chitosan microspheres and modified chitosan microspheres were studied using a JEOL 35CF microscope, operated at 15 kV, equipped with a secondary electron detector and energy dispersive X-ray microanalysis (EDX). Samples were dried under vacuum and coated with gold before observation.

2.4.6. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of chitosan microspheres and modified chitosan microspheres were recorded on Nicolet FTIR 520 spectrometer. Samples were prepared as KBr pellets and were scanned against a blank KBr pellet background at wavenumber range of 4000–400 cm^{-1}.

2.4.7. Solubility test

The solubility of modified chitosan microspheres was evaluated at a concentration of 6 mg/ml using different solvents: H_2O/HCl solution at pH 3.0, dichloromethane and chloroform, having been shaken for 72 h at room temperature, with short intermediate heatings.

2.5. Lipase immobilization

Immobilization on chitosan microspheres were carried out at specific conditions following the procedure described by Esteban et al. [30] with modifications: 10 g of liquid lipase was dissolved in 20 mL of phosphate buffer (pH 6.0, 20 mM) and 1 g of support, chitosan microspheres or modified chitosan microspheres, was added. The suspension was stirred at room temperature for 24 h. Immobilized lipase was recovered by filtration, washed three times with phosphate buffer (pH 6.0, 20 mM) at 0 °C, dried under vacuum at 35 °C until constant weight and stored at 5 °C until use. With this procedure, 8 biocatalysts were obtained (according to particles described in Section 2.3).

2.6. Protein assay

Protein concentration in the immobilization solution was determined by a colorimetric method (Proti2, Wiener Lab, Argentina) based on the absorbance observation at 540 nm by UV–vis spectroscopy when copper ions in an alkaline solution (EDTA/Cu in NaOH) binds to protein. Absorbance intensity is proportional to protein concentration. So a calibration curve obtained with albumin standard solution was used. Adsorbed protein on particles was estimated by differences between initial and final enzyme solution concentration.

2.7. Enzymatic reaction

Enzymatic acidolysis reactions were carried out between refined sunflower oil and a mixture of free palmitic and stearic acids, at specific conditions [31]. In general, substrates (1:6 molar ratio, oil:free fatty acids) and hexane (3 mL/g substrate) were mixed and heated (60 °C). The reaction began when immobilized or free enzyme (33.0 mg protein/g substrate) was added. Reactions were performed in a screw-capped test tube in a water bath with temperature controller and magnetic agitation at 250 rpm. After 24 h, reactions were stopped removing enzymes by filtering (when immobilized enzyme was used) or adding acetone to the reaction mixture prior to the filtering process (when free enzymes were used). All reactions were performed in duplicate and mean values are reported.

2.8. Reuse stability

The reuse stability study was performed with all biocatalysts. After the sample was withdrawn for analysis, 5 mL hexane was added to the biocatalyst and it was vortexed to be washed (3 times). A new reaction was performed by transferring the enzyme to a fresh mixture of substrates and hexane. Reaction conditions were the same as they were described above.

2.9. Acidolysis products analysis

Samples were analyzed according to Carrín and Crapiste [31]. Acidolysis reaction products were purified by alkaline deacidifi-

cation and recovered structured lipids were transesterified by cold methanolic KOH (AOCS Official Method Ce2-66) [32] to obtain their corresponding FAME. FAME were analyzed by gas chromatography and identified by comparing their retention times with authentic standards.

2.10. Statistical analysis

Statistical differences between average values were evaluated with the *t*-test. A confidence value of 95% was used. Results are shown as average value of two results (when no other value was reported) ± standard deviation. When the variation coefficient between two replicates was higher than 10%, a third independent replicate was made to disregard the wrong value. Variables and their interaction effects were analyzed using ANOVA statistical analysis, by Design-Expert 8.0.2 software.

3. Results and discussion

3.1. Chitosan characterization

The mean viscosimetric molecular weight of chitosan was calculated through the Mark–Houwink equation. The values of intrinsic viscosity, η, and viscosity average molecular weight, M, obtained for assayed experimental conditions were 553.26 $cm^3\,g^{-1}$ and 569.8 kDa, respectively. This result was consistent with the one previously reported by Kasaai [33] for chitosan samples with similar degree of deacetylation.

3.2. Chitosan microspheres characterization

3.2.1. Particle size analysis

The mean diameter (±standard deviation) of the prepared microspheres were as follows: Q, 1260 ± 100 μm; QM0.5–1, 1525 ± 116 μm; QM0.5–1.5, 1534 ± 106 μm; QM0.5–2, 1537 ± 93 μm; QM1–0.5, 1659 ± 103 μm; QM1–1, 1665 ± 135 μm; QM1–1.5, 1675 ± 104 μm; and QM1–2, 1703 ± 108 μm. Chitosan microspheres turned out to be the smallest particles, with a mean size of 1260 μm. Significant differences with regard to the size of the modified microspheres ($p < 0.05$) were found. As it can be observed, obtained microspheres using half of the required stoichiometric aldehyde amount during reductive amination reaction (QM0.5 series) seem to follow a trend where mean diameter increases as reaction time does. However, significant differences were not found. A similar behavior was shown for the obtained microspheres using a stoichiometric quantity of aldehyde (QM1 series), but differences were not found, either. QM1 series showed higher particle mean diameters than the QM0.5 one ($p = 0.0593$). So, it could be said that a size increment was obtained by the modification performed on the microspheres, probably due to the superficial incorporation of long carbonated chains to the polysaccharide original structure.

3.2.2. Air–water contact angle analysis

It is well known that the hydrophobicity/hydrophilicity of films can be easily analyzed by air–water contact angle measurements [34]. Similarly, in recent times, methods have been developed for characterizing the wettability of particulate materials by the contact angle described in the liquid–particle–air interface. These methods are broadly used for a large range of particles sizes, shapes and compositions [28]. Contact angle values for obtained particles are shown in Fig. 2. The contact angle for chitosan microspheres was 87° ± 0.6. This result is consistent with literature reports for the contact angle of water on chitosan films (89° ± 6) [34], validating our results. Besides, all modified microspheres presented higher contact angles than the unmodified material showing that their hydrophobic character had been increased. Microspheres of the QM0.5 series seem to follow a linear trend, where the contact angle increases with higher aldehyde reaction times (ART). A similar behavior is shown for the QM1 series microspheres, but with higher values of contact angles (as expected for a higher surface modification). The effect of both parameters, ART and AA ratio ($p < 0.0001$) and their interaction ($p = 0.0063$) resulted statistically significant for the modification of the contact angle. In other words, the stoichiometric series (QM1) showed a higher hydrophobic character than the half-stoichiometric series (QM0.5) and the hydrophobic character increased with the time of reductive amination reaction in both cases. This technique highlighted the hydrophobic character of the material surface, independently of the modification degree inside of microsphere. Therefore, this analysis was useful in order to know the kind of surface which *R. miehei* lipases have more affinity for adsorption to.

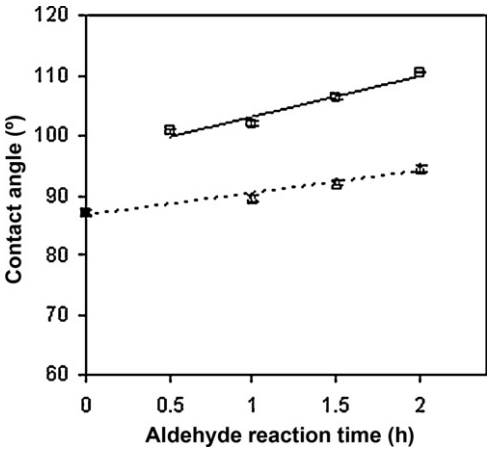

Fig. 2. Air–water contact angle of chitosan microspheres: Q (■), QM0.5 series: (△) and QM1 series: (□). For nomenclature description see Section 2.3.

3.2.3. Differential scanning calorimetry

DSC thermograms of the chitosan microspheres and modified chitosan microspheres are shown in Fig. 3. No obvious glass transition was observed for any type of microspheres, but thermal decomposition peaks were detected. Chitosan microspheres, Q, showed a decomposition peak at 294 °C and an endothermic peak at 260 °C, probably due to crystalline structure melting. The exothermic peaks of QM1–0.5, QM1–1, QM1–1.5 and QM1–2 at 289, 282, 277 and 273 °C, respectively, and the exothermic peaks of QM0.5–1, QM0.5–1.5, and QM0.5–2 at 282, 278, and 273 °C, respectively, corresponded to their thermal decomposition. It could be observed that the higher the amination reaction time was, the smaller the decomposition temperature was, independently of the aldehyde ratio. Thus, from the thermal stability point of view, the structural modification of chitosan particles seemed to be controlled by reaction time but not by aldehyde ratio (in the range studied). Compared to chitosan microspheres, the modified microspheres exhibited decreasing decomposition temperatures with increasing degree of modification. This indicates that thermal stability could be decreased by the introduction of the alkyl groups. Introduction of substituents into polysaccharide structures could disrupt the crystalline structure of chitosan, especially through the loss of the hydrogen bonding [35,36]. Small peaks appearing beyond decomposition point are likely due to further degradation reactions as observed by Zong et al. [35] who suggested that this behavior is due to the decomposition, causing disintegration into tiny fragments.

These results corroborate indirectly the modification of the particles towards a hydrophobic character. They also confirm that the obtained supports would be thermally stable in the temperature

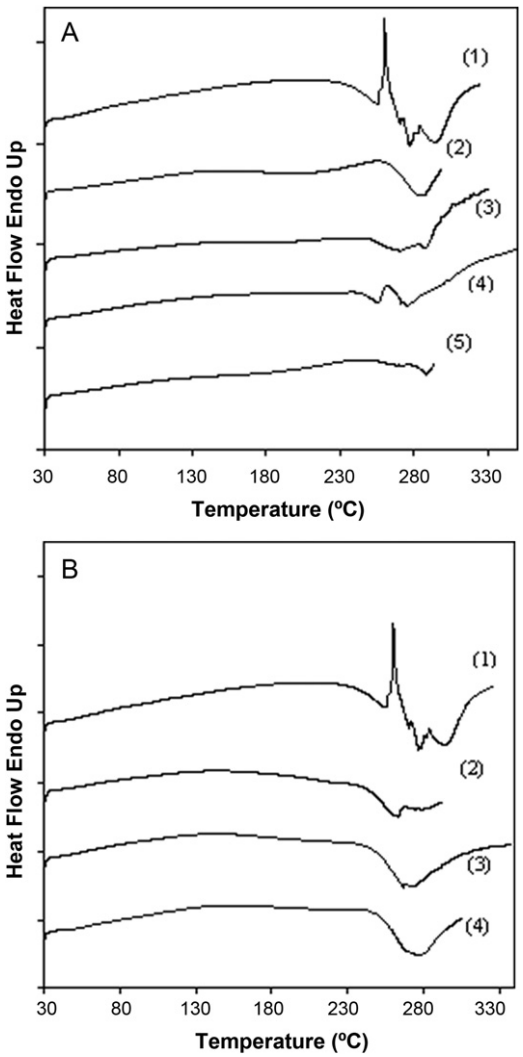

Fig. 3. DSC thermogram of (A): (1) chitosan microspheres, Q, and modified chitosan microspheres, (2) QM1–0.5, (3) QM1–1, (4) QM1–1.5, and (5) QM1–2; (B), (1) chitosan microspheres, Q, and modified chitosan microspheres: (2) QM0.5–1, (3) QM0.5–1.5, and (4) QM0.5–2.

range generally used during enzymatic acidolysis reactions (lower than 100 °C) [4] and would let future users know the limit temperature to which modified supports could be exposed.

3.2.4. X-ray diffraction

In order to observe modifications on the crystallinity of chitosan microspheres induced by the modification processes, X-ray diffraction of Q and QM1–1 were performed. The diffractograms of both samples showed some structural resemblance (Fig. 4). They exhibited characteristic crystalline peaks of chitosan powder at $2\theta = 9.96^\circ$ and 20.16° [37]. However, based on the calculation of CrI, the modified material presented lower crystallinity than unmodified chitosan, 55% vs. 70%, respectively. It means that the reductive amination reaction applied to chitosan particles, a process happening from particle surface, was responsible for this change in crystallinity (being according to the results obtained about decomposition temperature presented in Section 3.2.3).

3.2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out to examine the morphology of chitosan microspheres and modified chitosan microspheres. Only the QM1–1 image is presented for comparison. Micrographs showed that microspheres, modified or not, exhibited

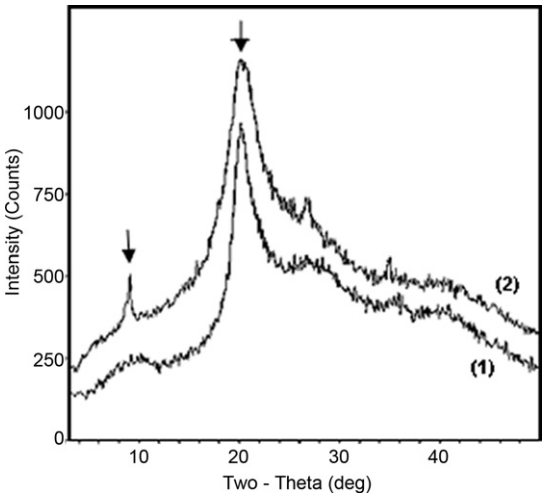

Fig. 4. XRD patterns of (1) Q and (2) QM1–1.

good sphericity (Fig. 1). The unmodified chitosan microsphere surface was smooth (Fig. 5A) while the modified chitosan microsphere surface, QM1–1, was rough, exhibiting a similar appearance to filaments (Fig. 5B). This image confirmed the presence of long chains at the particle surface.

With regard to the microanalysis technique, it has been used to the structural characterization of chitosan and its derivatives. It would allow to determine the degree of substitution of the macromolecular chain and the presence of mono or disubstituted units, estimating the carbon (C) and nitrogen (N) weight content [20].

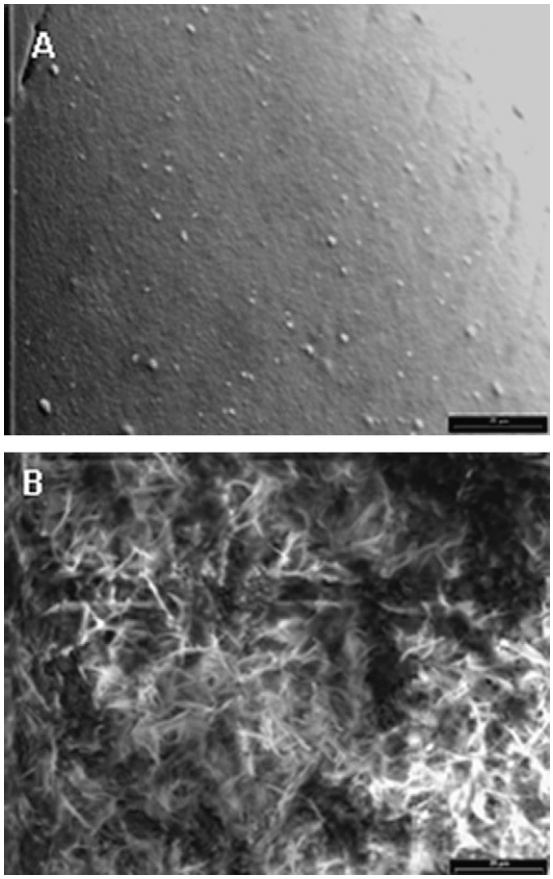

Fig. 5. Micrographs of (A) chitosan microsphere and (B) modified chitosan microsphere, at 1000× magnification (scale bar 20 μm).

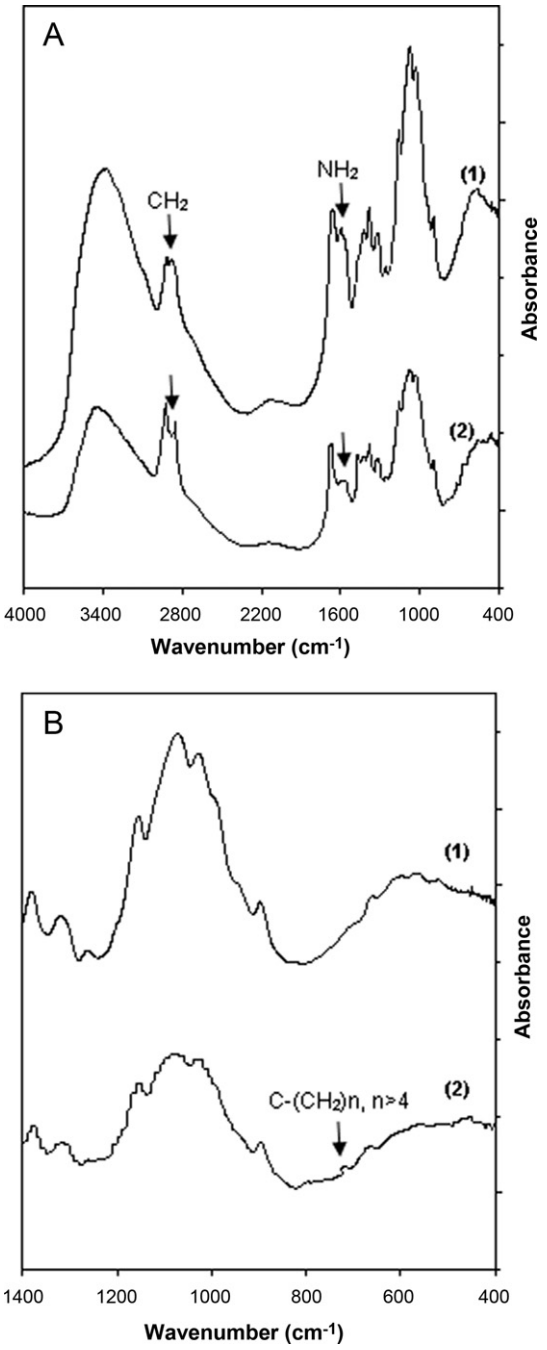

Fig. 6. (A) FTIR spectra of (1) chitosan microspheres and (2) modified chitosan microspheres, QM1–1 series. (B) Amplified scale of (A).

This technique could be very useful in order to characterize the supports, but in this work the bands of C and N obtained from DRX spectra were overlapping. Consequently, no calculations could be done.

3.2.6. Fourier transform infrared spectroscopy

The FTIR spectra of chitosan microspheres and QM1–1 modified chitosan microspheres (Fig. 6A), although similar to each other, showed differences in the absorption intensities. Chitosan characteristic peaks in FTIR spectrum were in agreement with the ones reported in other works [36,38]. As expected, compared with chitosan microspheres, a higher intensity peak at 2924 and 2853 cm^{-1} assigned to the asymmetric and symmetric mode of CH_2, respectively and a weakening of the peak at 1599 attributed to amine NH_2

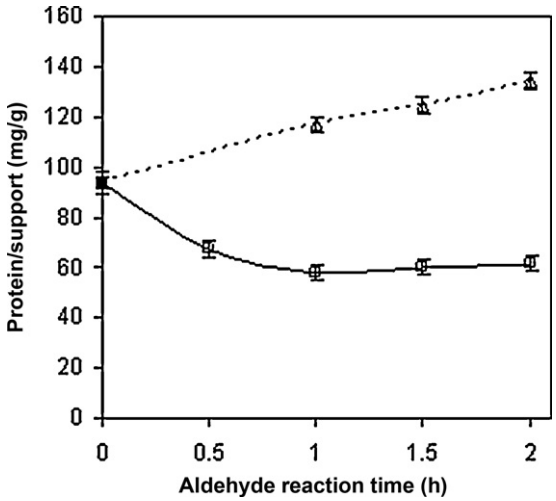

Fig. 7. Protein content adsorbed onto prepared biocatalysts, identified in terms of the conditions used to obtain the supports: Q (■), QM0.5 series (△), and QM1 series (□).

could be observed in modified chitosan microspheres, characterizing the imposed modification by addition of alkylated chains to NH_2 groups. Amplifying the scale (Fig. 6B), it is possible to distinguish a new peak at 720 cm^{-1} assigned to C-$(CH_2)_n$ with $n > 4$, due to the grafted carbon chain in the molecular structure of chitosan.

3.2.7. Solubility test

All modified chitosan microspheres were not soluble in acidic media as well as not in the tested organic solvents. So, the substitution degree in the modified chitosan microspheres could not be determined using the H NMR technique as reported by Rinaudo et al. [26]. This would suggest that the microspheres prepared in this work would be substituted to a greater degree than N-dodecyl chitosan obtained in the mentioned research. Problems in quantifying the degree of substitution using H NMR due to the material insolubility were also detected by Sajomsang et al. [38] when D_2O/CF_3COOD is used to dissolve N-octyl chitosan. On the other hand, modified particles insolubility showed these supports would be suitable to be used in reactions where organic solvents are present.

3.3. Protein content

Measurements of the protein content in the commercial preparation of lipase and in the developed biocatalysts were made with two purposes. First, it was used to examine the effect of support modification on the relationship between its hydrophobic character and the lipases adsorption. On the other hand, with this information available it was possible to compare biocatalysts activity with free enzymes activity, adding the same amount of protein in the acidolysis reaction media. Results of proteins adsorbed on each biocatalyst are shown in Fig. 7. Supports of the QM0.5 series (1:0.5 NH_2/aldehyde ratio) showed that, with a major modification of the material (obtained with increased amination reaction times), more proteins were adsorbed. This behavior seems to follow a positive linear trend. Otherwise, supports of the QM1 series (1:1 NH_2/aldehyde ratio) incorporated less amount of proteins than the unmodified material (Q), independently of the amination reaction time. It is worth mentioning that QM1 supports resulted more hydrophobic than QM0.5 ones (Section 3.2.2). The results suggest that support hydrophobicity and the morphology of support surface, obtained by different concentration of aldehyde chains grafted on it, may define the adsorption of lipases

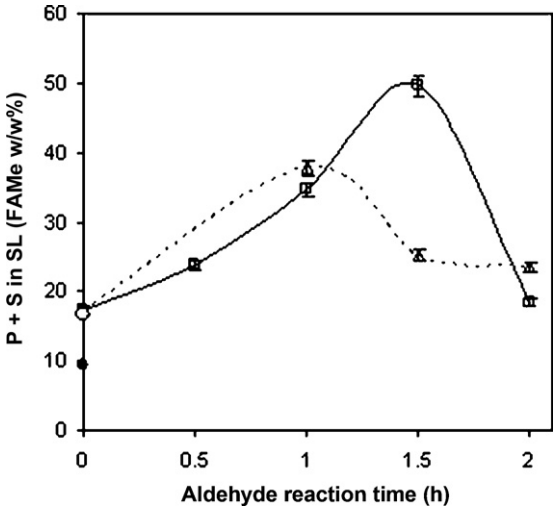

Fig. 8. Palmitic and stearic acid composition (P+S) in the structured lipids (SL) obtained by acidolysis with each biocatalyst, identified in terms of the conditions used to obtain the supports: Q (■), QM0.5 series (△), and QM1 series (□). Acidolysis reaction with free lipase RM: (○). Original sunflower oil: (●). Reactions conditions were described in Section 2.7.

on a support. Nieto et al. [11] carried out the immobilization of *R. miehei* lipases by adsorption on commercial hydrophobic supports (butyl-Sepabeads, octyl-agarose, and octadecyl-Sepabeads), observing that the protein load decreased when the material was extremely hydrophobic. This is consistent with the results found in this work. An explanation of this finding is presented in next section. On the other hand, Branco et al. [39] studied the immobilization of lipases from *Pyrococcus furiosus* on butyl-Sepabeads and octadecyl-Sepabeads and observed that the more the support hydrophobicity increases, the more lipase is adsorbed on it. The results suggest that adsorption is not only determined by support characteristics but also by lipase features (size of the lid, hydrophobic residues number, etc.).

3.4. Enzymatic reaction

The catalytic behavior of the biocatalysts and the free enzyme was evaluated in the acidolysis reaction. Palmitic and stearic acids composition (P + S) in the obtained structured lipids (SL), products after 24 h of reaction, are reported in Fig. 8. It can be observed, almost all the obtained biocatalysts using modified chitosan microspheres as support, QM0.5 and QM1 series, were more active than those obtained from unmodified chitosan microspheres, Q (zero aldehyde reaction time). Thus, in both used ratios of NH_2/aldehyde, adsorbed proteins showed higher activity to perform the acidolysis reaction as amination reaction time increased, until reaching a condition from which the activity fall to the "zero time" biocatalyst (unmodified chitosan particles) level. The NH_2/aldehyde ratio, the amination reaction time, and their interaction presented significant effects over immobilized lipase activity ($p = 0.0351$, 0.0002, and 0.0009, respectively). QM1–1.5 was the biocatalyst that achieved the highest modification on sunflower oil, reaching a change in the composition of palmitic and stearic acids from a value of 9.6% in the original oil to 49.1% in the final structured lipid. It is worth mentioning that QM1–1.5 was one of the supports with less adsorbed protein (Fig. 7). However, according to the contact angle analysis, it turned out to be one of the most hydrophobic supports. During stoichiometric ratio, it seems that higher superficial modification, related to more hydrophobic character, makes lipases be adsorbed in a more open conformation. At first, this seemed to be a more active conformation but then, when the support modification rose above a certain degree, the immobilized lipase conformation would be too open and/or with the active site closer to the support being less active or presenting steric hindrance to the substrate access [40]. This open or expanded immobilized lipase would involve more aldehyde chains than in the case of 1:0.5 ratio (a closer conformation). So, the capacity of the 1:1 series support to adsorb proteins could be restricted because of this fact justifying the lower protein load reached with these supports (Fig. 7). The increment of activity due to immobilized lipases in a strongly hydrophobic support has been reported in numerous works [11,12]. Other research works reported that greater hyperactivation occurred when a lower amount of protein was adsorbed on the support. To explain this, the authors suggest a microenvironment was formed, involving the support surface, the active site of the enzyme and the substrate, which favored the catalytic efficiency of the enzyme [39,41].

A similar incorporation of saturated fatty acids in the reaction products was reached with free RM lipases and with biocatalysts obtained from unmodified supports (Fig. 8, reaction time: 0 h). However, the most active biocatalyst (QM1–1.5) performed an increment of almost a 3-fold factor in its activity compared to free lipases. These results showed that the immobilized lipase achieved a hyperactivation condition in some modified supports, but not as high as those reported in other works [9,11,12]. This may be caused by the differences in the analyzed reactions and the type of the used substrates. For instance, Palomo et al. [12] showed that immobilized *M. miehei* on octadecyl-Sepabeads was 20 times more active than the soluble enzyme hydrolyzing p-nitrophenyl propionate. However, the same enzyme derivative reached a 5-fold increment compared to soluble enzyme when it was used in ethyl butyrate hydrolysis.

On the other hand, both free and bound lipases exhibited similar preference towards stearic acid incorporation instead of palmitic one. Using the same amount of proteins, palmitic and stearic acid incorporations were obtained as follows: 60.0% and 104.1%, respectively, with free RM lipase and 351.3% and 536.4%, respectively, with QM1–1.5.

This suggests that the obtained supports together with the used immobilization process did not change the characteristics of RM lipases [42].

Otherwise, the imposed condition on the biocatalysts to be highly resistant to attrition was corroborated after each acidolysis reaction by visual inspection. All the biocatalysts remained unaffected in the studied conditions.

The comparison of the performance of prepared biocatalysts in this contribution with immobilized lipases on chitosan microspheres reported in previous works could not be carried out because production of structured lipids with the latter was not found.

Published information showed that other lipases have been immobilized on unmodified chitosan microspheres and generally used in hydrolysis reaction. For instance, immobilized *Candida rugosa* lipase was assayed in hydrolysis of p-nitrophenyl palmitate [43]. Mainly, other enzymes have been immobilized, for example β-galactosidase in order to be used in lactose hydrolysis [25,44]. Also, β-glucosidase, acid phosphatase, and α-amilase, among others, have been used in their specific reactions [45]. This shows that this work is innovative not only from the point of view of the modification performed on chitosan to obtain a more adequate support but also by the application that gives the immobilized biocatalyst.

3.5. Reuse stability

An operational stability study was performed with all the biocatalysts. Stability was moderately kept for repeated 3 uses when obtained biocatalysts from modified chitosan particles (all QM series) were tested (data not shown). However, the activity of bio-

catalyst from unmodified chitosan (Q) decreased with reuses in accordance with findings reported by Tang et al. [46]. The stability for the best obtained biocatalyst, QM1–1.5, was analyzed over 10 repeated uses whereby activity was maintained during 7 consecutive uses of 24 h each ($p > 0.05$). These results show that, during the first 7 cycles of using, adsorbed lipases on modified chitosan microspheres were not inactivated by acidolysis reaction conditions, neither released to the reaction medium nor desorbed in the intermediate washings. Moreover, after 10 consecutive cycles of using, more than 70% of initial activity was retained being yet in a higher level than free lipase activity. This would reveal a strong adsorption of lipases to hydrophobic support, perhaps, due in part to the specific physical interaction between lipase and support, which was improved by the organic phase where acidolysis reaction is performed.

4. Conclusions

Modified chitosan microspheres were successfully prepared by precipitation and reductive amination in order to be used as supports for the immobilization of lipases. Chemical modification was corroborated by different materials characterization techniques. Results revealed that, for the ranges under study, aldehyde concentration in the reaction media and the contact time with it had influence in the hydrophobicity of the obtained particles. A size increment was obtained by the modification performed on the microspheres, probably due to the superficial incorporation of long carbonated chains to the polysaccharide original structure. All obtained particles showed thermal stability until at least 250 °C. RM lipases were effectively immobilized by adsorption on modified chitosan microspheres, showing major activity in more hydrophobic materials. However, this behavior was no longer observed with supports of higher hydrophobicity. The prepared biocatalyst QM1–1.5, obtained from one of the most modified supports, achieved the highest modification on sunflower oil during the acidolysis reaction, reaching a change in the composition of palmitic and stearic acids from a value of 9.6% in the original oil to 49.1% in the final structured lipids. This high conversion, 3-fold higher than the one obtained with free lipase, was maintained during 7 practiced reuses of 24 h each, during which attrition problems were not detected. These results indicate that this biocatalyst could be suitable for being used in batch systems with high speed agitation. Likewise, biocatalyst performance in continuous systems, for instance, as catalyst of a packed bed reactor, will be a subject for future study.

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