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Novel chitosan membranes as support for lipases immobilization: Characterization aspects

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ABSTRACT

Membranes of chitosan (QS), chitosan treated with glutaraldehyde (QGA) and chitosan crown ether (QCE) were utilized as carriers for immobilization of *Candida antarctica* and *Candida rugosa* lipases. Membrane supports were characterized by several techniques (Raman spectroscopy, elemental analysis by CHN determination and Energy Dispersive X-ray (EDX), water sorption isotherms, and surface area from nitrogen sorption data). To verify the presence of enzymes, some of these techniques were also used for lipase on chitosan biocatalytic systems. Measurements of protein load from Biuret assays and catalytic activity in esterification in nonaqueous media were also made for the immobilized enzymes. Sorption isotherms at 20, 30, 40 and 50 °C for QS, QGA and QCE supports were fitted to the Guggenheim, Anderson and Böer model. GAB monolayer moisture parameter, Xm, varied between 0.029 and 0.051 for QS, 0.039 and 0.058 for QGA and 0.039–0.075 g of water g^{-1} s.s. for QCE membranes. Elemental analysis and Raman spectra measurements of the lipase, supports and immobilized lipase systems gave evidence of the presence of enzymes on supports. Chitosan supports with internal surface area (m2 g^{-1}) among 3.31 and 1.26 were obtained. Regardless of these low values, acceptable protein load (0.61 to 3.21%) and esterification initial rates were achieved (0.88–2.75 mmol min^{-1} g of $protein^{-1}$).

1. Introduction

Enzymatic catalysis in nonaqueous solvents has gained considerable interest for the preparation of natural products, pharmaceuticals, fine chemicals and food ingredients (Carrea & Riva, 2000; Faber & Franssen, 1993; Margolin, 1993; Ru et al., 2000). Improved thermostability, favored synthesis over hydrolysis, tunable enzyme selectivity via medium engineering, and the simplicity of product recovery owing to the more easily evaporated reaction solvent, are reasons why nonaqueous bioprocessing is so attractive (Lee & Dordick, 2002; Turner & Vulfson, 2000; van Unen, Engbersen, & Reinhoudt, 2001; Vermuë & Tramper, 1995). Lipases (glycerol esters hydrolase, E.C.3.1.1.3) have been widely used to produce organic chemicals, biosurfactants, oleochemicals, agrochemicals, paper, cosmetics, fine chemicals and pharmaceuticals (Sharma, Chisti, & Banerjee, 2001). Lipases can also catalyze ester synthesis reactions in organic solvent systems. Among these esters, the 1-butyl oleate is used to decrease cloud point and pour point of diesel and biodiesel during winter, as poly vinyl chloride plasticizer, water-resisting agent and hydraulic fluid (Ghamgui, Karra-Chaábouni, & Gargouri, 2004; Linko et al., 1998).

Chitosan is an amine polysaccharide obtained from alkaline deacetylation of chitin, an unelastic and nitrogenated polysaccharide, which is found on the walls of the fungi and outer skeleton of arthropodes such as insects, crustaceans and beetles. Amino groups make chitosan one of the few found in nature cationic polyelectrolyte ($pK_a \approx 6.5$). Chitosan is known for its biocompatibility allowing its use in various medical applications (Chandy & Sharma, 1990; Okamoto et al., 1993), the production of value-added food products (Muzzarelli, 1996; Shahidi, Arachchi, & Jeon, 1999) and can be considered as biodegradable (Berger, Reist, Mayer, Felt, & Gurny, 2004).

Chitosan has been used as a matrix for immobilization of lipases (Alsarra, Betigeri, Zhang, Erans, & Neau, 2002; Betigeri & Neau, 2002) and many other enzymes (Krajewska, 2004; Spagna, Barbagallo, Casarini, & Pifferi, 2001). Enzymes bound to sugars, or sugar-based polymers like chitosan are stabilized during

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lyophilization and in nonaqueous environments. This may be due to a reduction of autolysis, that is a multipoint attachment limiting enzyme distortions or microenvironmental effects (Pandey, Benjamin, Soccol, Nigam, & Soccol, 1999; Wang et al., 1992).

The water activity (a_w) of a medium is an important factor in lipase catalyzed synthesis (Graber, Bousquet-Dubouch, Sousa, Lamare, & Legoy, 2003; Malcata, Reyes, Garcia, Hill, & Amundson, 1992). The retention (or release) of water on a solid carrier in which an enzyme is immobilized affects enzyme action in various ways: by influencing enzyme structure via noncovalent bonding and disruption of hydrogen bonds; by facilitating reagent diffusion. The relationship between total moisture content and the water activity of the chitosan support, over a range of values at constant temperature yields a moisture sorption isotherm. This type of isotherms gives information about the water sorption mechanism, interactions between the biopolymer and water, and also helps to establish the final moisture content on dehydration (Rodríguez-Aragón & López-Fidalgo, 2007; Telis, Kohayakawa, V.S., Pedro, & Gabas, 2005).

In previous studies we evaluated the results of the combination of freezing and thawing cycles and the addition of glutaraldehyde and 18-crown-6 ether on superficial chitosan membranes characteristics (Orrego & Valencia, 2009). Particularly we study the modulation of chitosan crystallinity derived by those treatments and the effect of biopolymer crystallinity on protein load. We also studied kinetic aspects for the immobilized *Candida rugosa* lipase in the esterification reaction of 1-butyl oleate in iso-octane (Orrego, Valencia, & Zapata, 2009). The aim of the present work is to compare and expand the results of the above approaches with the characterization by solid state techniques of three different chitosan membranes intended now as supports for two enzymes (*Candida rugosa* lipase and *Candida antarctica* lipase). Measurements of protein load and catalytic activity in butyl oleate synthesis in iso-octane were also made for the immobilized lipase.

2. Materials and methods

2.1. Reagents and materials

Chitosan flakes (high molecular weight 602 kDa, degree of deacetylation 76.5%), *Candida rugosa* lipase (with a nominal specific lipolytic activity of 1104 U mg^{-1} solid and containing ~18.26% protein based on the Biuret protein assay) were obtained from Sigma Chemical Co. (St. Louis, MO, United States), *Candida antarctica* lipase (with a nominal specific lipolytic activity of 910 U mg^{-1} solid and containing ~9.02% protein based on the Biuret protein assay) was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark); *iso*-octane and *n*-butanol were purchased from Panreac (Barcelona, Spain) and oleic acid from Carlo Erba (Milan, Italy). All other organic and inorganic reagents were of analytical grade.

2.2. Support production

2.2.1. Cryogelled chitosan support (QS)

The procedure for support production was described in a previous work (Orrego & Valencia, 2009).

2.2.2. Supports treated with glutaraldehyde (QGA) and with 18-crown-6 ether (QCE)

The procedure for support production was described in a previous work (Orrego & Valencia, 2009).

2.3. Moisture sorption isotherms

Moisture sorption isotherms of QS, QGA and QCE membrane supports were made by static gravimetric method (Rockland, 1960) in a laboratory set up consisted of eight glass hermetic flasks, six of them with different saturated salt solutions in their base (LiCl, $MgC1_2$, K_2CO_3, NaCl, KCl and $BaCl_2$). These salts have a range of relative humidity from 5% to 90% (Greenspan, 1977; Bizot, 1987). In the upper side of each flash was placed ca. 0.2 g of dried membranes obtained after 3 days of dehydration under air at 60 ± 0.7 °C in a forced convection oven PJTECH Thermolab Kryoven (Medellín, Col). The dried membranes enclosed under the humidity controlled environment of the solutions into the sealed flasks were placed in the oven at four different temperatures (20, 30, 40 and 50 °C). The gain of water was measured with an Ohaus Adventurer Balance (Pine Brook, NJ, USA, 65–310 g, precision, ±0.1 mg). When the weight of the sample stayed constant for 3 consecutive days it was considered that membrane moisture equilibrium was accomplished. This equilibrium was reached between 7 and 20 days.

2.4. Surface area measurements

Surface areas assays of QS, QGA and QCE supports were made in a porosity and surface area analyzer ASAP 2020 (Micromeritics Instrument Corporation, Gosford, New South Wales, Australia). As calibration standards were used α-alumin and kaolinite, with 0.52 ± 0.03 $m^2 g^{-1}$ and 15.8 ± 0.09 $m^2 g^{-1}$, of surface area, respectively. Samples were degasified at 100 °C with a heating rate of 10 °C/min for 24 h, under evacuation rate of 10 mmHg/s, until 7 μmHg of stable pressure was reached. After this procedure, sorption isotherms were obtained at 77 K. Adsorbed volume data at standard conditions of temperature and pressure were mathematically transformed according with the BET method (Brunauer, Emmett, & Teller, 1938).

2.5. Immobilization of lipase on chitosan supports

Lipases from *C. rugosa* (E.C.3.1.1.3) and *C. antarctica* were immobilized onto QS, QGA and QCE film supports to obtain the systems showed in Table 1.

During immobilization, the enzymes were dissolved (final protein content in lipase solutions: 1.2 ± 0.1 mg ml^{-1}) and pre-incubated at 35 °C in 50 ml of phosphate buffer (pH 7.2) under gentle stirring for 2 h. After that; 1 g of chitosan film was submerged into the enzyme solution for 20 h at 20 °C under agitation (120 rpm). Subsequent to immobilization, films were taken out, washed thoroughly with deionized water and rinsed with phosphate buffer solution. The resultant immobilized lipase on chitosan films were taken out and stored at 4 °C. During storage the films dehydrated and the water activity of the films were measured periodically at 4 °C in a thermoconstanter NOVASINA analyzer until their water activity were between 0.5 and 0.6. Before that they were packed in high barrier plastic bags. After immobilization the catalytic supports were placed in controlled humidity chambers until the equilibrium water activity (0.53 ± 0.04) was reached.

2.5.1. Protein loading assay

The amount of immobilized enzyme on the membrane was determined by measuring the initial and final concentrations of protein within the enzyme and washing solutions using the Biuret

Table 1
Lipase on chitosan immobilized systems.

Support or membrane	Immobilized system	
	C. rugosa lipase	*C. antarctica* lipase
QS	QSR	QSA
QGA	QGAR	QGAA
QCE	QCER	QCEA

method (Robson, Goll, & Temple, 1968) at a wavelength of 540 nm using a Lambda 20 double beam spectrophotometer from Perkin-Elmer with a 1-cm path length cell and a standard calibration curve of bovine serum albumin (BSA). Protein load of lipase was expressed as the percentage of protein (%) on the chitosan membrane.

2.6. Measurement of immobilized lipase activity

Lipase activity was measured using esterification reaction in nonaqueous media. The enzymatic reaction consisted of 1-butanol (0.1 M), oleic acid (0.1 M), 1 g of immobilized enzyme (0.61–3.21% of lipase on 1 g of immobilized support) and iso-octane (75 ml). The reaction mixtures were agitated in a shaking water bath at 40 °C and 120 rpm. The immobilized enzyme was incubated for 20 min. in the mixture of *iso*-octane and oleic acid, and then, 1-butanol was added to start the reaction.

At fixed intervals of every 3 min, 1 ml samples of the mixture were collected from the reaction medium and analyzed for residual oleic acid. The decrease of fatty acid was estimated by titrating the reaction mixture against 0.01 M KOH in anhydrous ethanol using phenolphthalein as indicator. These measurements were made after inactivation of the enzyme and solvent evaporation by heating. Initial rates were estimated from the slope of plots of the concentrations of oleic acid at conversions of less than 10% versus time and reported as mmol min^{-1} g^{-1} of protein in catalyst. The initial rate measurement was carried out in triplicate. The amount of lipase in the film supports was determined from the protein loading data.

2.7. Raman spectroscopy

Specimens of samples described in Table 1 were examined using a LabRAM HR micro-Raman spectrophotometer (Horiba Jobin Yvon, France) in the 200–1800 cm^{-1} region. The 473 nm solid state laser with 12 mW of power was focused over a membrane circle area by using a Raman microprobe with a 50× eyepiece. The scattered light, dispersed by the spectrophotometer, was detected by a CCD chamber with a spectra resolution at 3 cm^{-1}. The system was calibrated using Si spectra at 520 cm^{-1} before and after measurement. All Raman spectra were acquired at room temperature (20 °C).

2.8. Elemental analysis

Elemental analysis using CHN analyzers has been used for estimation of the nitrogen, hydrogen and carbon contents as well as N/C ratio in chitin and chitosan characterization (Julkapli & Akil, 2008; Yen, Yang, & Mau, 2008). This technique was also used for total quantification of amino groups in enzyme immobilization on chitosan (Yi et al., 2007). Energy Dispersive X-ray spectroscopy measurements are useful for confirm chitosan surface coatings, chitosan modification or the presence of some molecules inside chitosan fibers (Ismail et al., 2007; Xi, Liu, Wu, & Lin, 2008). Kjeldahl technique allows to measure nitrogen in organic compounds, proteins and food. It has been used for nitrogen determination in chitin and chitosan (Chandumpai, Singhpibulporn, Faroongsarng, & Sornprasita, 2004) and for the quantification of chitosan in fibers and composites (Hou, Liu, Liu, Duan, & Bai, 2008; Liua, Nishi, Tokurab, & Sakairi, 2001).

Elemental analysis of supports and lipase-membrane systems was conducted using the combined results of three techniques: CHN determination by high temperature combustion using a LECO CHN-600 equipment (precision: C ± 0.3%; H_2 ± 1.5%; N_2 ± 3%); nitrogen analysis by Kjeldahl method (Precision: ±0.4%) and Energy Dispersive X-ray in six micro-zones of each sample by means of an ESEM/EDX XL30 TMP Philips, 20 KV accelerator voltage. Complimentary determination of moisture content was made for each sample in a vacuum oven at 70 °C for 24 h, under pressure <6.7 kPa (AOAC, 1990).

3. Results and discussion

3.1. Characterization of chitosan supports

3.1.1. Sorption isotherms

The obtained data were adjusted to Guggenheim, Anderson and de Böer model (Telis et al., 2005; Van den Berg, 1981):

$$\frac{X_e}{X_m} = \frac{Cka_w}{(1 - ka_w)(1 - ka_w + Cka_w)} \quad (1)$$

where X_e is the equilibrium moisture after water adsorption (mass of water/mass of dry solid), X_m the monolayer moisture; C is the Guggenheim constant; k, a multilayer molecule factor and a_w the water activity. C is related to the chemical potential difference between adsorption upper layers and the monolayer. Factor k ranges between 0.5 and 1; for proteins k varies from 0.78 to 0.85, whilst for electrolytes and polyelectrolytes is approximately 0.92 (Rahman, 1995; Timmermann, 2003; Timmermann, Chirife, & Iglesias, 2001).

Moisture sorption isotherms of chitosan membranes were intended to find model (GAB) parameters with physical meaning with X_m being a monolayer moisture value and C and K relating to interaction energies between water and membranes, and between the multiple layers of water, respectively. It is also known that the shape and position of the isotherm are influenced by these parameters, the sample composition and its physical structure (crystalline or amorphous). It was considered of interest to verify the outcome of the thermal treatments and the addition of glutaradehyde and 18-crown-6 ether on these parameters and their possible effects on the protein load and catalytic activity.

Table 2 presents the GAB constants of QS, QGA and QCE film supports sorption isotherms obtained by nonlinear regression analysis using software ORIGIN® v.7.0383.

The parameter X_m is a critical moisture content below of which water is not enough to allow reagent diffusion and, consequently, chemical and enzymatic reactions in food materials are very slow (Rahman, 1995). The monolayer moisture X_m varied between 0.029 and 0.051 for QS, 0.039 and 0.058 for QGA, and 0.039 and 0.075 g of water g^{-1} (dry basis) for QCE membranes.

In the present work, since chitosan was used as lipase immobilization support for use in nonaqueous media catalysis, these values were the lower practical limit of moisture in the membrane. Supports with water content above X_m will assure enough water availability for the lipase attached to chitosan to exert its catalytic function in organic media. It is interesting to note that, at 40 °C (the temperature used to measure catalytic activity of immobilized lipases), the corresponding water activity minimum for X_m moisture contents in GAB fitted curves were 0.49, 0.51 and 0.55 for QS, QGA and QCE chitosan supports. Assays of QSR, QGAR, QCER, QSA, QGAA and QCEA catalysis of butyl oleate synthesis in *iso*-octane below those water activity values did not show catalytic activity. These results agree with the study of Wehtje and Adlercreutz (1997). The shape of the profiles of lipase activity versus water activity for four different lipases (*Rhizopus arrhizus*, *Pseudomonas* sp., *Candida rugosa* and *Lipozyme*) were independent of the reaction used to determine the activity. The optimum water activity range was 0.33–0.75 (maximum at $a_w = 0.53$). Similar results were obtained for the methyl adipate production in hexane using *Candida rugosa* lipase (Rahman et al., 2008) and the ethyl butirate synthesis (Chowdary & Prapulla, 2002).

Table 2
GAB isotherm parameters of three chitosan supports.

Sample	GAB parameter	20 °C	30 °C	40 °C	50 °C
QS	C	2.0597	1.9755	1.9184	1.2518
	k	0.9318	0.9983	0.8515	0.8721
	X_m	0.0496	0.0292	0.0512	0.0427
	r^2	0.9928	0.9974	0.9982	0.9916
QGA	C	1.4808	1.3249	1.2381	0.8383
	k	0.9118	0.9511	0.9281	0.8712
	X_m	0.0528	0.0407	0.0391	0.0577
	r^2	0.9977	0.9946	0.9942	0.9980
QCE	C	1.7995	1.7824	1.1164	1.1088
	k	0.9309	0.8794	0.8823	0.9755
	X_m	0.0504	0.0536	0.0751	0.0389
	r^2	0.9959	0.9959	0.9985	0.9993

The listed determination coefficient, r^2, was found to be satisfactory.

Sorption isotherms at 20, 30, 40 and 50 °C for QS, QGA and QCE supports are shown in Fig. 1a, b and c, respectively.

3.2. Surface area measurements

Table 3 shows the average surface areas obtained from two analyses of chitosan samples. Dried chitosan surface areas are strongly influenced by the dehydration procedure. In these data the internal surface area; QCE membrane was lower than that of QS membrane because the applications of crown ethers are based on their capability for selective complexation with cations. Protonated amino groups of chitosan were partially complexed with 18-crown-6 ether in the QCE membrane. This lower availability of amino groups in crown ether treated chitosan could partially explain the diminished internal surface area and protein load capacity of QCE membrane.

Chitosan beads produced by convective drying have surface areas below 0.5 $m^2\ g^{-1}$ (Rege, Garmise, & Block, 2003; Roberts & Taylor, 1989). Spray drying of chitosan allows slightly higher values (0.74–3.01 $m^2\ g^{-1}$) to be achieved (Rege et al., 2003). Vacuum drying and freeze drying permit 10–150 $m^2\ g^{-1}$ to be reached (Juang, Wu, & Tseng, 2002; Rorrer, Hsien, & Way, 1993). Highest areas are obtained using supercritical drying (up to 200 $m^2\ g^{-1}$) (Rorrer et al., 1993; Valentin, Bonelli, Garrone, Di Renzo, & Quignard, 2007). The measured surface areas obtained in the present work were among the typical values for convective and spray drying chitosan materials.

It is commonly accepted that immobilization efficiency is established by the support surface area. However, this concept is questionable for lipases because of the amphoteric character of these enzymes. In this case the affinity between supports and enzyme is more important for the efficient immobilization of enzyme (Al-Duri, Robinson, McNerlan, & Bailie, 1995; Chiou & Wu, 2004). This appears to be the case in this study in which, despite low support surface area, proper protein load for acceptable esterification initial rates were achieved as can be observed in Section 3.5.

3.3. Analysis of Raman spectra

Table 4 summarizes relevant IR and Raman spectra information related to chitosan and lipase immobilization.

Comparison of this data with the Raman spectra obtained for different supports and lipases immobilized (Fig. 2) allows proposing some explanations about the effect of physical–chemical treatments used for the production of chitosan supports. Evidence of lipase immobilization is also confirmed from this information.

Raman spectra of chitosan and chitosan treated supports are typified by three intense wide bands: 800–940 cm^{-1}, 970–1188 cm^{-1} and 1240–1440 cm^{-1}. In the first band it can be observed two peaks, at 900 cm^{-1} and 912 cm^{-1} attributed to NH_2 wagging (Valentin et al., 2007). The second intense band encloses three minor bands with wavenumbers at 1055 cm^{-1}, 1118 cm^{-1} and 1155 cm^{-1} assigned to the C–O, C–O–C, C–C and C–N of the biopolymer. That is a characteristic of its saccharide structure

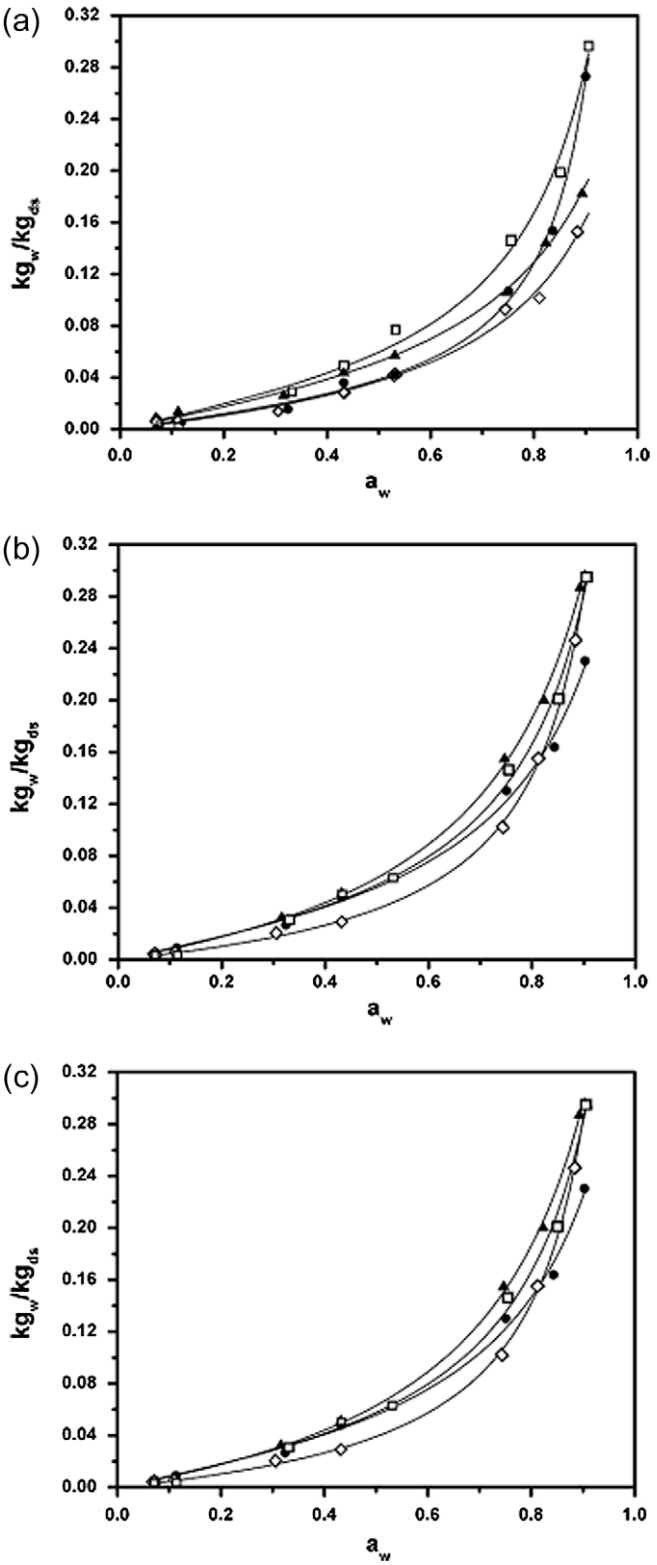


Fig. 1. Adsorption isotherms. Symbols: □ 20 °C; ● 30 °C; ▲ 40 °C; ◇ 50 °C. (a) QS, (b) QGA and (c) QCE.

Table 3
Internal surface area of chitosan membranes.

Sample	Internal surface area ($m^2\ g^{-1}$)
QS	0.40
QGA	1.26
QCE	0.31

(Boonsongrit, Mueller, & Mitrevej, 2008; Kanti, Srigowri, Madhuri, Smitha, & Sridhar, 2004). The third of these intense bands has also three peaks: 1280 cm^{-1}, 1340 cm^{-1} and 1380 cm^{-1} due to different vibrational modes of CH, CH_2 and CH_3 groups (Kanti et al., 2004; Synytsya, Blafková, Copíková, Spevácek, & Uher, 2008; Valentin et al., 2007). For chitosan the wavenumber 1380 cm^{-1} has been associated to CH_3 in amide group (Pawlac & Mucha, 2003). All of the spectra show typical band of amide group (C=O–NHR at 1665 cm^{-1}). The similarities of the spectra in Fig. 2(a) show that the glutaraldehyde and 18-crown-6 ether treated chitosan membranes, QGA and QCE, retained the essential characteristics of the original structure of chitosan (QS film). The interaction between chitosan and glutaraldehyde, that would be evidenced by the presence of free aldehyde group band (1720 cm^{-1}) or by the imine bond peak (1626–1655 cm^{-1}), was not observed from the QS and QGA spectra comparison in Fig. 2(a). However, the minor intensity of the amino-ammonium peak (1530 cm^{-1}) of the QGA with respect of the QS spectra is a confirmation of weak crosslinking owing to the low glutaraldehyde concentration (0.0003 M) in the initial chitosan dispersion.

Crown ethers are well-known for their unique capacity to form complexes with cations and neutral molecules (Terekhova, Kulikov, Kumeev, Nikiforov, & Al'per, 2005). The new bands at 1560–1700 cm^{-1} of chitosan treated with 18-crown-6 ether (QCE) are attributed to the membrane – ether poly-ionic complex formed due to chitosan cationic polyelectrolyte nature (Kanti et al., 2004).

Raman spectra assessment of the lipase and immobilized lipase systems gave confirmation of the presence of enzymes on supports. While in Raman spectra of *Candida antarctica* lipase were evident the amide I and amide II bands at 1638 and 1549 cm^{-1}, respectively (Fig. 2(c) and (d)), in the *Candida rugosa* lipase spectra (Fig. 2(b)) these bands were overlapped into a wide band (1500–1670 cm^{-1}), centered at 1570 cm^{-1}. After *Candida rugosa* lipase immobilization on QS support (QSR spectra in Fig. 2(b), it was marked the increase of the intensity of the peak at 1380 cm^{-1}, assigned to CH_3 in amide group and the amide II band (1550 cm^{-1}). Similar observations can be made from the comparison of immobilized *Candida antarctica* lipase (QGAA) and its support (QGA) spectra (Fig. 2(c)). In the *Candida antarctica* immobilized systems amide I band was also found at 1638 cm^{-1} in QGAA and at 1670 cm^{-1} in QSA.

3.4. Elemental analysis

3.4.1. EDX measurement

For the immobilized lipase QGAR the presence of eight elements was confirmed from X-ray EDX signals (Fig. 3).

Sodium, potassium and phosphorus were derived from the buffer salt solution used for lipase immobilization; calcium and magnesium were attributed to the original reagent lipase support.

Six sets of similar data shown in Fig. 3 were recorded for each sample. The dispersion on elemental analysis information obtained from this technique was taken as evidence of heterogeneity of the materials.

3.4.2. Nitrogen by Kjeldahl

In Table 5 are shown the results of nitrogen content (dry basis), adjusted according to moisture determinations for membrane supports, lipases and immobilized lipases.

3.4.2.1. Elemental analysis using CHN analyzer. Table 5 shows hydrogen and carbon content (dry base corrected) for supports and immobilized lipases. The nitrogen results were not shown because of high dispersion of data. Table 5 combines nitrogen data from Table 5 and carbon and hydrogen content from Table 5. There is also included in this table the corresponding elemental content derived from theoretical calculations of the chitosan powder reagent (Chitosan, DDA 76.5%). Besides polymeric chains of chitin and chitosan, commercial purified chitosan has other complex carbohydrates but it must be free of protein because of alkaline treatment implicated in its production (Yen et al., 2008).

From the comparison of the two left columns of Table 5 it can be concluded that impurities in chitosan reagent (Q) are mainly carbohydrates. In the last row of the same table is shown the variation of N/C ratio for the samples. For supports and lipase-support systems, lower ratios were observed for QS and QGA. The required addition of high carbon content chemicals (acetic acid and glutaraldehyde) for the production of those supports explains the reduction of the N/C proportion. Conversely, the N/C ratio showed an increase for immobilized lipases (QGAR and QGAA). This result clearly shows that lipases are present on the biocatalytic systems.

3.5. Protein load and catalytic activity

Protein load and initial rate measurements in oleic acid and 1-butanol catalyzed esterification in *iso*-octane are shown in Table

Table 4
Main observed Raman and IR wavenumbers (cm^{-1}) and vibrational assignments for chitosan and lipase.

Mode	Wavenumber (cm^{-1})	References
Free aldehydic group	1720	Monteiro and Airoldi (1999)
Amide I band (C=O stretching peptide linkages)	1650–1660	Kanti et al. (2004), Lin, Yan, Hu, and Ju (2004), Mei, Miller, Gao, and Gross (2003), Nunthanid et al. (2004), Qi, Xu, Jiang, Hu, and Zou (2004)
Imine (C=N)	1626–1655	Monteiro and Airoldi (1999), Vasconcelos (2007)
Amide II band	1540–1598	Kanti et al. (2004), Lin et al. (2004), Mei et al. (2003), Osman and Arof (2003), Paulino, Simionato, García, and Nozaki (2006), Qi et al. (2004), Vachoud, Zydowicz, and Domard (1997), Valentin et al. (2007)
Ammonium (NH_3^+) and amino ($-NH_2$) groups	1514–1540	Boonsongrit et al. (2008), Osman and Arof (2003)
CH bending, CH_2 wagging, CH_2 twisting	1376–1460	Kanti et al. (2004), Synytsya et al. (2008), Valentin et al. (2007)
CH_3 bending	1300–1383	Gümüşderelioğlu and Agi (2004), Synytsya et al. (2008)
Glycosidic linkage (–C–O–C–)	1150–1040	Pawlac and Mucha (2003)
C–O, C–N, CC stretching	1100–1000	Gümüşderelioğlu and Agi (2004), Valentin et al. (2007)
C–O stretching pyranose ring	1040–1070	Kanti et al. (2004), Liu and Bai (2005), Vachoud et al. (1997)
NH_2 wagging	898–950	Valentin et al. (2007)

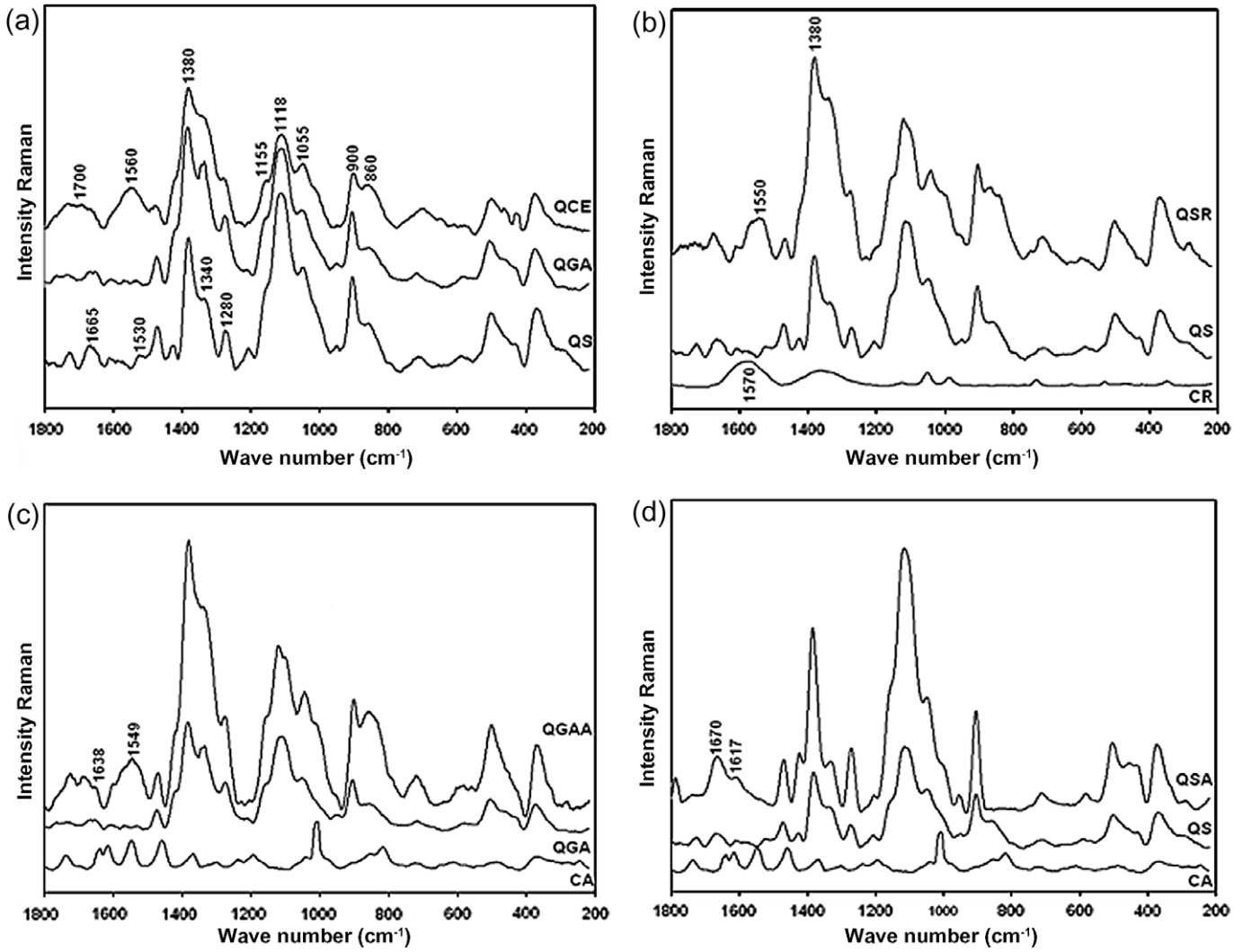
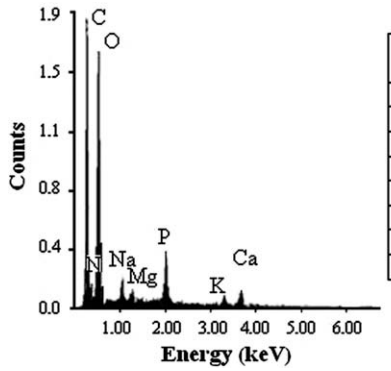


Fig. 2. Raman spectra of supports and immobilized lipases. (a) Chitosan (QS), chitosan treated with glutaraldehyde (QGA), chitosan treated with 18-crown-6 ether (QCE). (b) *Candida rugosa* lipase (CR), QS and *C.R.* lipase immobillized on QS (QSR). (c) *Candida antarctica* (CA), QGA and *C.A.* lipase immobilized on QGA (QGAA). (d) *Candida antarctica* lipase (CA), QS and *C.A.* lipase immobilized on QS (QSA).



Element	Content (%)
C	47.46
N	4.71
O	41.51
Na	2.08
Mg	0.84
P	2.04
K	0.48
Ca	0.88

Fig. 3. EDX analysis of a sample of lipase from *Candida rugosa* immobilized on chitosan-treated with glutaraldehyde (QGAR).

6. The amounts of immobilized lipases on supports were superior or similar to the values reported in comparable studies (Hung, Giridhar, Chiou, & Wu, 2003; Orrego & Valencia, 2009; Zaidi et al., 2002). Chitosan membrane crosslinked with glutaraldehyde appears to be the more suitable biocatalytic system for this reaction (Orrego et al., 2009), because crystallinity of QGA membrane was higher than QS and QCE membranes. The increment of crystallinity, which was a result of freezing and thawing treatments and glutaraldehyde and crown ether treatments increased the protein load capacity because the availability of amine groups are controlled by the crystallinity of the biopolymer. The relationship between the crystallinity of chitosan and its metal adsorption selectivity and capacity is commonly accepted. For protein adsorption similar relationship was also demonstrated in our previous work (Orrego & Valencia, 2009).

In Table 7, we made a comparison between activities of *Candida rugosa* lipase immobilized on chitosan membranes used on this work and the same information published for the same lipase on similar supports for use as catalysts in nonaqueous media.

4. Conclusions

In this paper, the properties of hydrophilic chitosan network membranes obtained by cryogelling and six F/T cycles (QS) and biopolymer membranes produced by the same procedure but treated with glutaraldehyde (QGA) and 18-crown-6 ether (QCE) are studied. Sorption water isotherms data obtained by static

Table 5
Carbon, hydrogen and nitrogen content of three supports, two lipase-support systems and lipases.

Element	Q[a]	Q	QS	QGA	QGAR	QGAA	CR	CA
C	45.44	52.15	48.86	53.27	48.39	58.3	24.66	76.10
H	6.71	7.80	6.91	6.66	7.90	5.86	3.25	8.33
N	8.19	7.80	7.37	6.73	7.16	9.04	18.26	9.07
N/C	0.179	0.150	0.151	0.126	0.148	0.155	0.757	0.119

[a] Theoretical calculation for 76.5% DDA chitosan reagent.

Table 6
Protein load and catalytic activity of chitosan supports. Reaction conditions: 75 ml of reaction mixture, 40 °C, 1:1 mol ratio of 1-butanol and oleic acid (0.1 mol L^{-1}), initial a_w of 0.53 ± 0.04, agitation speed: 120 rpm, 1 g of immobilized lipase.

Catalytic system	Protein in immobilized lipase (mg of protein/g of membrane)	Esterification initial rate (mM/min g protein)	Reference
QSR	16	0.88	This study
QGAR	30	2.21	This study
QCER	6.1	1.78	This study
QSA	10.5	1.21	This study
QGAA	19.3	2.75	This study
QCEA	32.1	2.39	This study
CRL on Nylon	14.7	0.45	Zaidi et al. (2002)[a]

[a] Oleic acid and butanol in *n*-hexane, 25 °C, 100 rpm, 0.1 M substrate concentration.

Table 7
Catalytic system activities for *Candida rugosa* lipase on chitosan in nonaqueous media.

Reference	Support	Activity (mM min^{-1} g $protein^{-1}$)
This work[a]	QSR	0.88
This work[a]	QGAR	2.21
This work[a]	QCER	1.78
Magnin, Dumitriu, Magny, and Chornet (2001)[b]	Chitosan/xanthan	0.0008
Chen and Lin (2003)[c]	Chitosan beads	0.0033–0.0167

[a] This work: 40 °C, *n*-butanol 0.1 M and oleic acid 0.1 M/*iso*-octane.
[b] Magnin et al.: 37 °C, olive oil hydrolysis/*iso*-octane.
[c] Chen and Lin: 35 °C, ethanol 0.25 M and butyric acid 0.3 M/*n*-hexane.

gravimetric method at 20, 30, 40 and 50 °C of QS, QGA and QCE followed the Guggenheim, Anderson and de Boer (Telis et al., 2005) model. According to the surface area determinations all membranes were macroporous. Regardless of the low values of surface internal area, protein load and activity in nonaqueous media indicate that chitosan membranes had a high *Candida rugosa* and *Candida antarctica* lipase affinity because they showed acceptable protein load (0.61–3.21%) and esterification initial rates (0.88–2.75 mmol min^{-1} g of $protein^{-1}$).

The experimental results from the application of Raman spectroscopy and elemental analysis demonstrated the presence of immobilized lipase on the supports. From Raman spectroscopy, when the spectra for supports and the immobilized lipase system were compared, an increasing on the signal from the carbonyl group in the amide I band was observed. The presence of amide II band and an increment on the CH_3 signal of the amide group (1380 cm^{-1}) was also observed in all immobilized lipase supports.

The presence of lipases on the supports was verified with elemental analysis according to an increase in the nitrogen content (Kjeldahl analysis) as well as an increase on the C/N ratio (combustion and Kjeldahl analysis). This presence was also confirmed by mass balance of immobilization lipase solutions by the Biuret method.

In the Raman spectra, neither imine bond (C=N) nor a possible crosslinking of chitosan glutaraldehyde in immobilized lipase systems was possible to be detected, probably due to the overlapping of this band (1626–1655 cm^{-1}) with the amide I band (1650–1660 cm^{-1}). Despite the relative decrease of the peak corresponding to ammonium group (1530 cm^{-1}) no conclusion can be drawn about the formation of covalent bonds between lipases and supports during immobilization with the membrane treated with glutaraldehyde.

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