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Candida rugosa Lipase Supported on High Crystallinity Chitosan as Biocatalyst for the Synthesis of 1-Butyl Oleate

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Abstract Lipase from *Candida rugosa* was immobilized onto chitosan using four different protocols. The variation of crystallinity (5.57–92.86%), which was a result of thermal treatments and crosslinking of the chitosan, influenced the protein load (7.46–25.15 mg g^{-1} chitosan) and protein load efficiency (21.67–41.68%) for immobilization assays made with identical lipase solution concentration (1.3 mg of protein/mL). The effects of protein load (10, 30, 50 and 70 mg of lipase), reaction temperature (30, 40, 50, 60, 70 °C) and substrates molar ratio (0.05–0.30 M) have been studied in the butyl oleate synthesis in iso-octane when water activity of the free and immobilized enzymes were fixed around 0.53 ± 0.04. The catalytic activity of the immobilized lipase has also been tested. The Ping–Pong bi–bi mechanism with dead end complex of *n*-butanol was found to fit the initial rate data. The values of the apparent kinetic parameters were determined by graphic and parametric method as: V_{max} = 18.2–19.0 mmol min^{-1} g^{-1}; $K_{M;\ Acid}$ = 0.599–0.640 mol L^{-1}; $K_{M;\ Alcohol}$ = 0.128–0.149 mol L^{-1}; and $K_{i;\ Alcohol}$ = 1.933 mol L^{-1}.

Keywords Chitosan · Immobilized *Candida rugosa* lipase · 1-Butyl oleate · Iso-octane

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1 Introduction

Enzymatic catalysis in nonaqueous solvents has gained considerable interest for the preparation of natural products, pharmaceuticals, fine chemicals and food ingredients [1–3]. Lipases (glycerol esters hydrolases, E.C.3.1.1.3) are the most frequently used enzymes in organic media because they have an inherent affinity for hydrophobic environment [4]. They have been widely used to produce organic chemicals, biosurfactants, oleochemicals, agrochemicals, paper, cosmetics, fine chemicals and pharmaceuticals [5].

Esterification is usually catalyzed by an acid catalyst such as sulfuric acid, p-toluene sulfonic acid or phosphoric acid which are toxic, corrosive, and hard to remove from the reaction medium. An alternative is to use solid acid catalysts at 110–120 °C as reaction temperatures [6]. However, the green synthesis of esters in organic medium catalyzed by using immobilized lipases can overcome problems such as product separation, Hazards in handling of the corrosive acids, high energy consumption and degradation of esters in elevated temperature processing, since the enzymatic synthesis offers mild reaction conditions and often high yields. 1-Butyl oleate is a useful ester used as a diesel additive during winter use, a poly vinyl chloride plasticizer, a water-resisting agent and in hydraulic fluids [7, 8].

Lipase from *Candida rugosa* has been proved to be suitable for butyl oleate synthesis. On screening 25 commercially available lipases in the absence of additional organic solvent the lipase from *C. rugosa* was found to give both high ester yields and superior cost:benefit ratio in direct biocatalytic synthesis of 1-butyl oleate [7]. For the same ester production covalent nylon-immobilized lipase from *C. rugosa* in *n*-hexane allowed nearly complete conversion (>90%) of the substrates [9].

Chitosan, a poly-*N*-acetylglucosamine, is a transformed oligosaccharide obtained by the deacetylation of chitin, an unelastic and nitrogenated polysaccharide, which is found on the walls of the fungi and outer skeleton of arthropods. The concurrent presence of hydroxyl and amino groups in the chitosan molecules, which are excellent functional groups for the anchoring of a large variety of organometallic complexes, makes chitosan a good support for heterogeneous molecular catalysts [10–13]. In order to use the biopolymer as enzyme support it is important to control its crystallinity because this property controls its sorption ability and prevents polymer dissolving [12]. The adjustment of crystallinity could be obtained as a result of crosslinking and the process of manufacture of chitosan supports such as thermal treatment or drying procedure [14].

The present paper focuses on the *C. rugosa* lipase immobilization on chitosan membrane carriers prepared with a freeze-thaw method that allows handling their crystallinities and the effect of this property on protein load and protein load efficiency. Another objective of this work is to determine the proper rate equations and kinetic parameters for the immobilized enzyme in the esterification reaction of 1-butyl oleate in iso-octane.

2 Experimental

2.1 Materials

Chitosan flakes (high molecular weight, degree of deacetylation 76.5%), *C. rugosa* lipase (with a nominal specific lipolytic activity of 1,104 U mg^{-1} solid and containing ~18 protein based on the Biuret protein assay) from Sigma Chemical Co. (St Louis, MO, US). All other organic and inorganic reagents were of analytical grade.

2.2 Support Production

According to previous work [15] crystallinity of chitosan membranes is affected by the use of freezing and thawing cycles as drying method. To modify the crystallinity of chitosan supports three different protocols, made by triplicate, were tested as follows.

Procedure 1. 1.5 g of chitosan powder was diluted in 50 mL of 1% v/v acetic acid solution. After the powder was completely suspended, the dispersion was sonicated until the disappearance of trapped air bubbles, and poured on petri dishes (10 cm, i.d.). The films were cured using 30 mL of NaOH/Ethanol gelling solution during 2 h. Preparation of dispersions and curing was conducted at 20 °C. Subsequent to casting the samples were brought into the freezer at −20 °C for 22 h. After cryogelling, samples were thawed (3 h) at 4 °C; the liquid phase was withdrawn to take apart the wet gelled matrices or supports. These supports were immersed successively for 30 min into 30 mL of ethyl–ether and 30 mL of acetone and rinsed with abundant distilled water, until pH 7 was reached. Gelled matrices were frozen at −45 °C during 30 min, thawed and exposed to the air for 3 h at 4 °C; this freezing–thawing (F/T) procedure was repeated once more time.

Procedures 2 and 3. These protocols were performed following the same procedure 1 except in the number and conditions of F/T cycles (see Table 1).

Procedure 4. 1.5 g of chitosan powder was diluted in 50 mL of 1% v/v acetic acid solution. After the powder was completely dissolved, the dispersion was treated with glutaraldehyde until cross-linker content was 0.0003 M. Subsequent to that the sample was treated by the same procedure 3 described above.

The resulting films were cut into 2 cm × 2 cm sections. To complete drying all the resulting chitosan film samples were stored for a week at 4 °C before packing in high barrier plastic bags.

2.3 X-Ray Diffraction Analysis (XRD)

Dried supports X-ray diffraction measurements were recorded by Bruker AXS D8 Advance diffractometer, using Cu,Kα monochromatic radiation ($\lambda = 1.5406$ Å) at 40 kV and 30 mA Scanning diffraction angle (2θ) range was set at 5–30° with a step angle 0.020° and step time 2 s. Crystallinity index (*%I*) was determined by a modified method of proposed by Focher et al. [16]

$$\%I = \left(\frac{I_c - I_a}{I_c}\right) \times 100 \quad (1)$$

where, according to Ogawa et al. [17], maximum intensity I_c, at $2\theta = 21°$ of the (200) lattice diffraction and the amorphous region diffraction, I_a, at $2\theta = 16°$.

Table 1 Support production protocols

Procedure/sample	Cryogelling	Thawing	Freeezing/thawing	F/T cycles
1	−20 °C, 22 h	4 °C, 3 h	−45 °C, 30 min/4 °C, 3 h	2
2	−20 °C, 22 h	4 °C, 3 h	−45 °C, 30 min/4 °C, 3 h	3
3	−20 °C, 22 h	4 °C, 3 h	−45 °C, 30 min/4 °C, 3 h	6
4[a]	−20 °C, 22 h	4 °C, 3 h	−45 °C, 30 min/4 °C, 3 h	6

[a] Crosslinked with glutaraldehyde

2.4 Immobilization of Lipase on Chitosan Supports

About 50 mL of lipase solution (1–3 mg protein/mL) in phosphate buffer (pH 7.2) was pre-incubated at 35 °C for 2 h. After that 1 g of chitosan films was submerged into the enzyme solution for 20 h at 20 °C under reciprocal agitation at 60 strokes/min (Thermo Fisher Scientific shaker bath model 2870, Marieta, OH, USA). Subsequent to immobilization, films were taken out, washed two times with phosphate buffer solution and stored at 4 °C until their water activity (25 °C) reached 0.53 ± 0.04. In this moment they were ready for the assay of immobilized lipase activity. The remaining lipase solution and washing buffer solutions were stored for protein determinations. The protein load of the enzyme on the membrane was defined as the amount of protein (mg) per gram of the chitosan membrane. The protein loading efficiency was defined as the ratio of the amount of protein coupled on the chitosan membrane to the amount of the protein added into the immobilization solution.

2.5 Water Activity of the Immobilized Lipase

The a_w of the lipase dried film samples were determined at using a Novasina Thermoconstanter TH-200 water activity machine (Novasina, Switzerland) with controlled temperature from 0 to 50 °C, accuracy: ±0.01 a_w, ±0.1 °C. The water activity machine was calibrated at 25 °C prior to making each set of measurements by using standard saturated solutions of LiCl, $MgC1_2$, K_2CO_3, NaCl and $BaCl_2$ [18, 19].

2.6 Protein Loading Assay

The amount of lipase adsorbed onto the chitosan membranes was determined as the total protein quantity from the difference between the amount of protein in solution before and after adsorption and in washing solutions by the Biuret method [20]. Absorbance measurements were done in wavelength 550 nm, using a Perkin–Elmer Lambda 20 spectrophotometer. In a typical experiment, 2.0 mL of a protein or washing solution was mixed with the Biuret reagent and the protein concentration was determined spectrophotometrically using a calibration curve of bovine seroalbumin.

2.7 Esterification Reaction

The esterification of 1-butanol and oleic acid in iso-octane catalyzed with the free and immobilized enzymes was investigated. The main aspects considered were: the contribution of temperature in the conversion without catalyst, optimum reaction temperature, inhibition effects of substrates concentration, control of water activity of the catalytic system, the effect of both substrate concentrations on the initial rate of ester synthesis and finally, the kinetic model and mechanism of esterification.

Substrate concentrations ranges between 0.05 and 0.3 mol L^{-1} in 75 mL flasks under agitation (120 rpm), varying the temperature within 30 and 70 °C, the enzyme concentration between 10 and 70 mg of protein on free enzyme and 10–30 mg of protein on chitosan supports.

Specific activity of free and immobilized lipase were expressed as initial rate per gram protein in free enzyme or immobilized onto membranes. Initial rates were estimated from the slope of plots of the concentrations of oleic acid at conversions of <10% versus time and reported as mmol min^{-1} g^{-1} of protein in catalyst.

All of the experiments were carried out in triplicate. The amount of lipase in the film supports was determined from the protein loading data. Controls without enzyme were also checked at equimolar concentration of substrates (0.1 M) and different temperatures (30, 40, 50, 60 and 70 °C) to study contribution of temperature in the conversion of the reaction.

At fixed intervals, 1 mL aliquots of the reaction mixture were withdrawn, heated to 90 °C and mixed with 4 mL of anhydrous ethanol as a quenching agent. The residual acid content was assayed by titration against 0.01 M KOH in anhydrous ethanol using phenolphthalein as an indicator. The volume in the reaction was maintained at 75 mL by adding iso-octane.

Oleic acid conversion (%) was also determined using reversed phase high performance liquid chromatography (HPLC) as modified from [21] using a Hitachi-Elite LaChrom (Tokyo, Japan), L-2130 pump module, LiChroCART RP18 4 nm × 250 mm column with 5-μm silica particles, UV detector, EZChrom Elite software and HP 7540 professional computer. Samples (ca. 50 μL) were heated to evaporate iso-octane and then diluted with tetrahydrofuran to about 0.5% (w/v) and passed through a 0.5 μm Nylon filter. The phase mobile was acetonitrile/tetrahydrofurane (73/27 v:v) at 1.0 mL min^{-1}, 20 °C, 4.5 min. Detection by UV was carried out at 265 nm. Initial rates and yields determined by HPLC analysis and titrimetry were found to be in good agreement.

2.8 Effect of the Amount of Lipase on the Synthesis of Butyl Oleate

To estimate the effect of lipase content on the time courses of the synthesis and the disappearance of the fatty acid in the esterification, the reaction was carried out at 40 °C in iso-octane using variable amounts of free *C. rugosa* lipase (10, 30, 50 and 70 mg of protein on reaction mix) with an initial equimolar mixture of oleic acid and butyl alcohol

(0.1 M) in a 75 mL flask under agitation (150 rpm). The free lipase was pre-equilibrated to $a_w = 0.53$, by exposing the enzyme powder to an humidity controlled container with a $Mg(NO_3)_2$ saturated salt solution at 25 °C [18]. During a period of 8 h, at a given time 1 mL of a mixture was collected and the free oleic acid was determined. The yield, in percent, was calculated from oleic acid consumed on the basis of the limiting substrate.

2.9 Effect of Temperature on the Lipase Activity

Effect of temperature on the activity of the immobilized lipase preparations was studied in the temperature range of 30–70 °C. In this assay the esterification reaction consisted of *n*-butanol (0.1 M), oleic acid (0.1 M), free or immobilized enzyme (30 mg of protein) and iso-octane (75 mL). The mixtures were incubated at different temperatures (30, 40, 50, 60, 70 °C) for the measurement of activity.

3 Results and Discussions

3.1 Effect of Crystallinity on Protein Load on Chitosan Membranes

Figure 1 shows four X-ray diffractograms of chitosan dried supports. According to the experimental data, support crystallinity increase in the order of $1 < 2 < 3 < 4$. It was evident that the number of cycles of F/T strongly increases the crystallinity of the samples. In view of the fact that chitosan is a cation polyelectrolyte having a free amino group on each D-glucosamine residue, it forms salt with acetic acid. Among the four conformations of chitosan salts that have been found (I-anhydrous, extended, two fold helix, II-hydrated, relaxed, two fold helix, IIa-hydrated, 4/1 helix and III-anhydrous, 5/3 helix) the commonly one accepted for the acetic acid salt is type II, hydrated [17]. In this study, and taking account the X-ray data, apparently, the more cycles of F/T treatment of the salt, the higher acetic acid that was removed from chitosan molecules simultaneously with water, bringing about the formation of more anhydrous crystals of chitosan, and consequently producing membranes with greater crystallinity. In the case of the sample treated with glutaraldehyde (Six F/T cycles) the final crystallinity index is even larger than chitosan sample without crosslinker (Six F/T cycles) (Table 1).

Such variation of crystallinity, which was a result of thermal treatments and crosslinking of the chitosan membrane samples, influenced the surface properties of membrane supports [12], as can be observed in the results showed in Table 2. The protein load and protein load efficiency rose with the increment of crystallinity index for the immobilization assays made with identical lipase

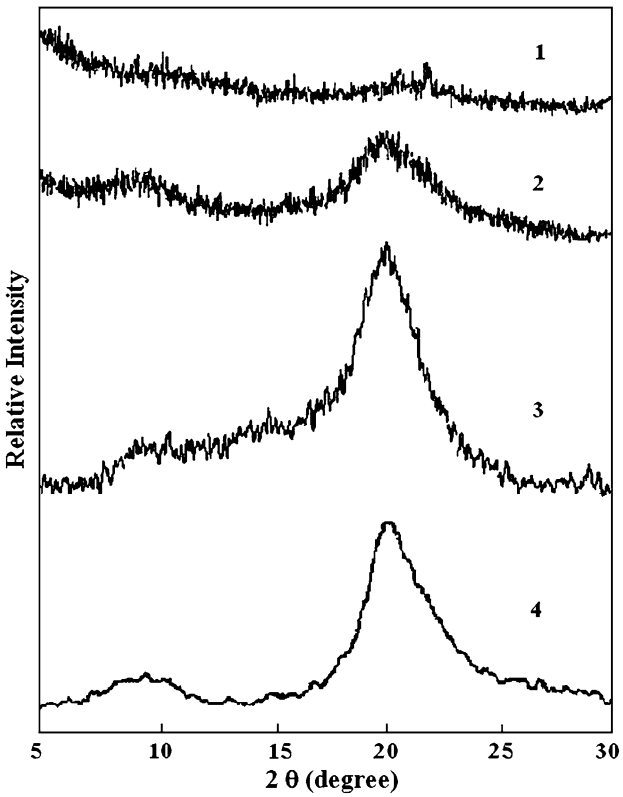

Fig. 1 A comparison of X-ray diffraction diagrams for chitosan supports prepared according to the protocols described in Table 1. *Sample 1*: Uncross-linked chitosan membrane (Two F/T cycles); *sample 2*: uncross-linked chitosan membrane (Three F/T cycles); *sample 3*: uncross-linked chitosan membrane (Six F/T cycles); *sample 4*: glutaraldehyde cross-linked chitosan membrane (Six F/T cycles)

solution concentration (1.3 mg of protein/mL). Glucosamine moieties in chitosan carry free amine groups. Some researchers have suggested that the availability of amine groups are controlled by the crystallinity of the biopolymer [12, 14]. Consequently, as the free amino groups are responsible of the surface charge of the support, which in turn handle the capacity of protein adsorption, the raise in the crystallinity originated by the cryogelling, F/T treatments and mild crosslinking, could explain the superior protein load and efficiency capacity of the chitosan supports obtained. The relationship between the crystallinty of chitosan and its metal adsorption selectivity and capacity is commonly accepted [12]. However, studies on the influence of this property on protein load capacity in enzyme immobilization are not available.

Protein load and protein load efficiency obtained from this study were better than the reported values from three different papers [22–24] dealing with *C. rugosa* lipase immobilization on chitosan. Table 3 presents the differences between these published parameters and the achieved in the present work using the chitosan membrane

Table 2 Effect of crystallinity on protein load

Sample	Crystallinity index (%) (Eq. 1)	Protein load (mg g^{-1} chitosan)	Protein loading efficiency (%)
1	5.75 ± 0.80	7.46 ± 0.28	21.67 ± 1.00
2	48.95 ± 2.62	12.34 ± 0.52	26.55 ± 1.53
3	72.39 ± 3.23	16.00 ± 1.12	31.81 ± 2.22
4	92.86 ± 3.01	25.15 ± 1.82	41.68 ± 3.40

The values are given as the mean ± SD of three experiments

Table 3 Comparison of yields of protein load and protein load efficiency obtained in the present study and similar data of other published works on the *Candida rugosa* lipase immobilization on chitosan supports

CR lipase solution (mg mL^{-1})	Protein load (mg g^{-1} chitosan)	Protein loading efficiency (%)	References
10.00	0.07–0.11	–	[26]
3.00	1.24–6.28	12.10–69.30	[29]
0.10	0.12–0.29	10.80–25.20	[28]
1.10	11.49	18.11	(*)
1.30	16.00	31.81	(*)
1.83	27.34	44.11	(*)
2.37	32.68	56.41	(*)
3.01	34.78	61.34	(*)

(*) Present study, for chitosan support treated with glutaraldehyde (sample 4). Mean values from three replicates

crosslinked with glutaraldehyde (procedure 4, Table 1) as enzyme support.

Therefore, it was decided to follow the procedure 4 for the immobilization of lipase onto chitosan and to study the performance of the immobilized lipase in the butyl oleate synthesis in iso-octane.

3.2 Water Activity of Lipases

The water activity (a_w) of a medium is an important factor in lipase catalyzed ester synthesis [25, 26]. According to the study of Wehtje and Adlercreutz [27] the shape of the profiles of enzyme activity in non aqueous media versus water activity for four different lipases (*Rhizopus arrhizus*, *Pseudomonas* sp., *C. rugosa* and *Lipozyme*) were independent of the reaction used to determine the activity (esterification, two different hydrolysis and transesterification). *C. rugosa* lipase showed an optimum at water activity was around 0.33–0.75 (max. at $a_w = 0.53$) for all four reactions. Identical optimum range and maximum were obtained on dimethyl adipate ester production in hexane via esterification using immobilized and native *C. rugosa* lipase [28]. However, the optimum water activity of lipases is influenced by the concentration of reactants [27, 29]. In the synthesis of ethyl butyrate in hexane by transesterification there was a shift in optimal water activity of *C. rugosa* lipase from lower (0.33) to higher (0.54) with increase in substrate concentration (50, 100–150, 200 mM) [29].

Since preliminary trials showed that lipases at 40 °C and $a_w < 0.4$ show low esterification activities, and taking account that the substrate concentrations, temperature ranges and lipase loads were similar than that used in the cited references, the initial water activity of the free and immobilized enzymes were fixed around 0.53 ± 0.04.

3.3 External Mass Transfer and Internal Diffusional Limitations in Esterification Assays

When solid catalysts are used, there is a possibility of mass transfer effect due to external mass transfer and internal diffusion limitations, which must be considered in the kinetic model. The first aspect can be conveniently investigated by computing the time constants for the reaction (t_r) and diffusion (t_D) which are defined as [30]:

$$t_r = \frac{S_0}{V(S_0)}, \qquad t_D = \frac{D}{k_{SL}^2} \tag{2}$$

where S_0 is the *n*-butanol or oleic acid concentration (M) in the bulk organic phase, $V(S_0)$ is the inicial rate of reaction (Ms^{-1}), D is the diffusivity of substrates in organic phase ($m^2\ s^{-1}$) and k_{SL} is the solid–liquid film mass transfer coefficient (m/s). The liquid phase diffusivity of *n*-butanol in iso-octane (D_A) at 40 °C was calculated by using the Wilke–Chang correlation [31] to be $2.25 \times 10^{-9}\ m^2\ s^{-1}$. Similarly, for oleic acid, D_B, was calculated to be $9.78 \times 10^{-10}\ m^2\ s^{-1}$. The value of solid–liquid mass transfer coefficient k_{SL} was calculated from the Sherwood number for the agitated reaction system, $Sh = k_{SL} \times dp/D = 326$ [32]. The values of liquid side mass transfer coefficients, $k_{SL\text{-}A}$ and $k_{SL\text{-}B}$ were found to be 1.95×10^{-5} and $4.49 \times 10^{-5}\ ms^{-1}$, respectively, taking the equivalent catalyst particle diameter of 0.016 m. The time constants for diffusion (Eq. 2), $t_{D\text{-}A}$ and $t_{D\text{-}B}$ were 2.57 and 1.12 s.

Reaction rates were between 1 and 5 mmol $min^{-1}\ g^{-1}$ of immobilized lipases for 0.05 and 0.25 M (1.67 and 8.35×10^{-5} M/s for 30 mg of lipase present in reaction medium). The time constant for the reaction was estimated in 3,000 s (Eq. 1). Since $t_r \gg t_D$ there was no external mass transfer limitation. To verify this theoretical

calculations experiments at three different stirring speeds (60, 120 and 180 rpm) were carried out. No significant changes in the conversion values obtained were observed, so, external mass transfer limitations can be neglected. The stirring speed was fixed at 120 rpm.

Further evaluation of the intra-particle diffusion limitation was done. The catalyst particle is a film with 0.02 × 0.02 × 0.0005 m dimensions. The time constant for the internal diffusion of oleic acid from the reaction mixture into the lipase on chitosan slab, was calculated from the Fourier number, $Fo_m = D_{eff}\ t/b^2 = 1.96$ [32] using the unsteady state mass transfer equation (effective diffusivity, $D_{eff\text{-}B,}$ of 9.9×10^{-11} m^2 s^{-1} and a bulk concentration of 0.25 M). The time constant for intra-particle diffusion $t_{D\text{-}A}$ was 12.35 s. Again, since $t_r \gg t_D$ there was no internal mass transfer limitation.

Thus, it was concluded that the reaction is seen to be not influenced by mass transfer but controlled by the intrinsic enzyme kinetics.

3.4 Effect of Protein Load on Progress of Esterification Reaction

The reactions were carried out at 40 °C in iso-octane using variable amounts of free lipase (10, 30, 50 and 70 mg of protein) and an initial equimolar mixture of oleic acid and butyl alcohol (0.1 M) in a 75 mL flask under agitation (120 rpm). The hyperbolic curves obtained the first 2 h of reaction indicated that the reaction followed Michaelis–Menten type kinetics. The dynamics of butyl oleate synthesis by *C. rugosa* lipase in iso-octane, revealed that after 120 min of the reaction, the amount of oleic acid (or ester) in reaction mixture presents instability within the initial 8 h of esterification (Fig. 2). It is believed that water produced during the reaction gives rise to a reverse reaction, namely the ester hydrolysis. The same phenomena was reported by Szczesna-Antczak et al. [33] for butyl and propyl oleates, and caprylates synthesis in petroleum ether catalyzed by Mucor circinelloides and Mucor racemosus lipases.

As expected, for the range of amount of lipase studied, the conversion percentage of oleic acid is increased as more lipase was present in the mixture. However, after 8 h the differences between conversion of the esterification with 30, 50 and 70 mg of enzyme were almost leveled off. With identical substrate concentrations in hexane, at 37 °C, the yield of butyl oleate also increased with the amount of immobilized lipase from *Rhizopus oryzae* but remained constant after certain value of protein content [8].

The effect of the catalyst concentration on the specific kinetic rate constant is shown in Fig. 3. Again, initial rates for 30, 50 and 70 mg of enzyme were very similar.

Consequently, subsequent kinetic experiments were carried out with 30 mg of immobilized *C. rugosa* lipase.

3.5 Contribution of Temperature in the Conversion Without Catalyst

Blanks at 30, 40, 50, 60 and 70 °C (reaction conditions: 75 mL of reaction mixture, 1:1 mol ratio of 1-butanol and oleic acid (0.1 M) and agitation speed of 120 rpm) were performed to study contribution of temperature in the conversion of the reaction. After 6 h of progress of esterification the maximum oleic acid conversion reached was 2.67% (40 °C).

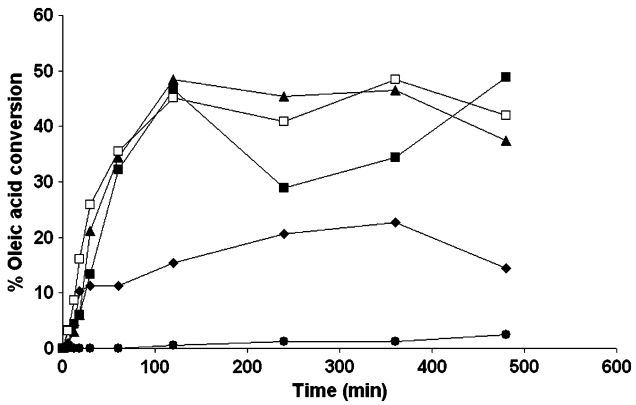

Fig. 2 Effect of protein content on the synthesis of butyl oleate in iso-octane by *Candida rugosa* lipase. Reaction conditions: 75 mL of reaction mixture, 40 °C, 1:1 mol ratio of 1-butanol and oleic acid (0,1 mol L^{-1}), initial aw of 0.53 ± 0.04, agitation speed: 120 rpm. Protein on lipase: ● 0 mg (*blank*); ◆ 10 mg; ■ 30 mg; ▲ 50 mg; □ 70 mg

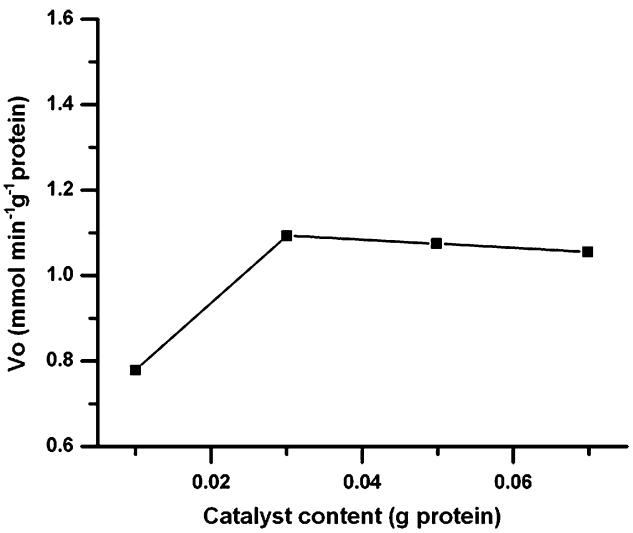

Fig. 3 Effect of catalyst content content on the specific kinetic rate constant for the synthesis of butyl oleate in iso-octane by *Candida rugosa* lipase. Reaction conditions: 75 mL of reaction mixture, 40 °C, 1:1 mol ratio of 1-butanol and oleic acid (0.1 M), initial aw of 0.53 ± 0.04, agitation speed: 120 rpm

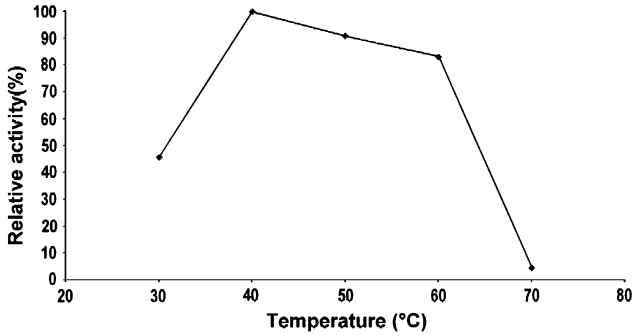

Fig. 4 Percent relative activity of immobilized lipase (30 mg g^{-1} support) on chitosan support treated with glutaraldehyde (sample 4, Table 1) at various temperatures. Reactions were performed in iso-octane with 1:1 mol ratio of 1-butanol and oleic acid (0.1 M) at 40 °C and initial a_w of 0.53 ± 0.04

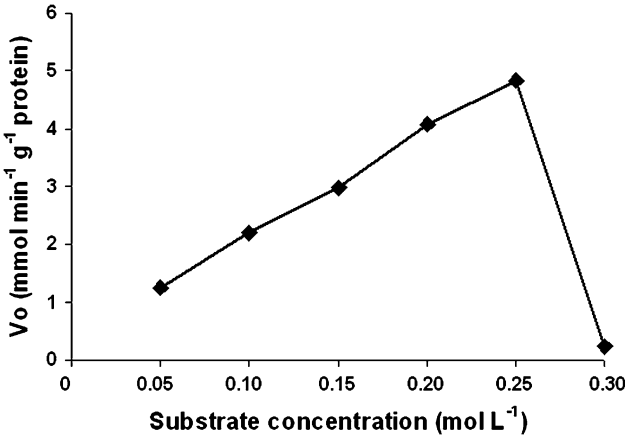

Fig. 5 Effect of substrate concentration on the inicial rate of butyl oleate synthesis in iso-octane. Reaction conditions: Butyl alcohol and oleic acid (0.05–0.3 mol L^{-1}, 1:1 mol ratio); agitation speed: 120 rpm; temperature: 40 °C; incubation period: 21 min

3.6 Optimum Reaction Temperature

The effect of the reaction temperature on the initial velocity is shown in Fig. 4. Since the highest activity was found at 40 °C, the relative activities were determined as percentage yield of activities at different temperature compared to the activity of reaction at 40 °C as

$$\text{Relative activity } (\%) = \frac{\%\text{ activity at different temperature}}{\text{maximum }\%\text{ activity } (40\,^{\circ}\text{C})}$$

The activity was increased from 30 to 40 °C but decreased when temperature was increased from 50 to 70 °C. The optimum temperature for lipase activity was observed at 40 °C. This value is in agreement with the optimal temperatures reported for free and immobilized *C. rugosa* lipase catalyzed esterifications in non aqueous media for the synthesis of butyl oleate in hexane, 40 °C [28], amyl isobutyrate in iso-octane, 45 °C [34] and citronellyl laurate in iso-octane, 37 °C [35].

3.7 Kinetic Studies

For the design of suitable reactors for enzymatic processing, kinetic information on the rate equations and kinetic parameters in system conditions is needed. The effect of both substrate concentrations on the initial rate of ester synthesis was investigated. The reaction conditions were: concentration ranges 0.05–0.3 M for oleic acid and *n*-butanol substrates; 75 mL of reaction mixture in iso-octane, 40 °C; *C. rugosa* lipase on glutaraldehyde treated support, 30 mg; initial a_w of immobilized lipase, 0.53 ± 0.04; and agitation speed: 120 rpm.

When the substrates were in equimolar amounts, the reaction rate (Fig. 5) increased gradually with the increase of substrate concentration, reaching a maximum of about 0.027 mmol min^{-1} g^{-1} with a concentration of 0.24 M. Above this concentration, an inhibition was observed.

This approach suggests that either or both the fatty acid and the alcohol have inhibiting effects on the catalytic mode behavior of the enzyme. To investigate these possible effects individually, initial rates of ester synthesis were carried out maintaining one substrate at constant concentration while varying the concentration of the other reactant. Two sets of experiments were conducted using 30 mg of immobilized *C. rugosa* lipase on glutaraldehyde crosslinked chitosan. The mechanism of enzymatic reactions can be obtained from the Lineweaver–Burk double reciprocal plots (Figs. 6, 7).The double reciprocal plot of the initial reaction rate versus the reciprocal of initial butyl alcohol concentration at five butyl alcohol concentrations is shown in Fig. 6. A set of approximately parallel lines was

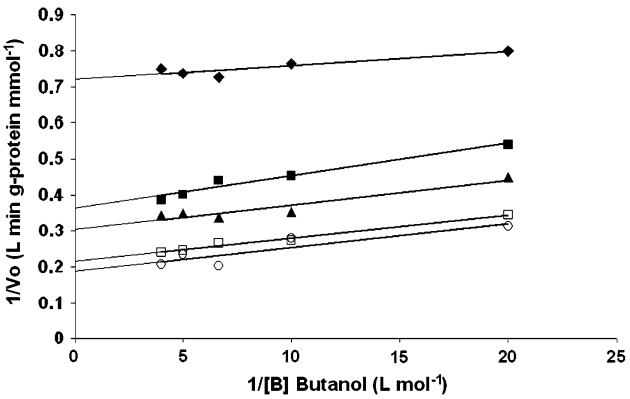

Fig. 6 Double-reciplocal plot of the initial rate and the initial *n*-butanol [*B*] concentration with a fixed oleic acid [*A*] concentration (*M*). Symbols: ◆ [*A*] = 0.05, ■ [*A*] = 0.10, ▲ [*A*] = 0.15, □ [*A*] = 0.20, ○ [*A*] = 0.25

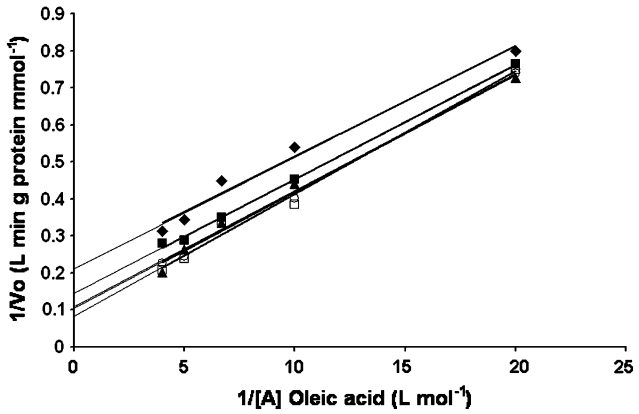

Fig. 7 Double-reciplocal plot of the initial rate and the initial oleic acid [*A*] concentration with a fixed *n*-butanol [*B*]concentration (*M*). Symbols: ◆ [*A*] = 0.05, ■ [*A*] = 0.10, ▲ [*A*] = 0.15, □ [*A*] = 0.20, ○ [*A*] = 0.25

obtained indicating Ping–Pong, Bi–Bi mechanism. By plotting the *y*-axis intercepts of the primary plot presented in Fig. 6 against the reciprocal of oleic acid concentration (Fig. 8), a secondary plot results that has a slope of K_A/V_{max} of 0.0330 min g protein mol L^{-1} mmol and a *y*-axis intercept of $1/V_{max}$ of 0.0551 min g protein $mmol^{-1}$. Repeating this procedure with the double reciprocal plot of the initial reaction rate versus the reciprocal oleic acid concentration, and its respective secondary plot (Fig. 9), the value of K_B/V_{max} was of 0.0082 with the *y*-axis intercept ($1/V_{max}$) of 0.0548. The values for the model parameters that were determined from these graphs were: $K_B = 0.149$ mol L^{-1}, $K_A = 0.599$ mol L^{-1} and $V_{max} =$ 18.198 mmol min^{-1} g^{-1} protein.

The Lineweaver–Burk plot ($1/V_0$ vs. 1/[*A*]) shows that at low fixed *n*-butanol [B] concentrations, the plots appear parallel. As the concentration of *n*-butanol increases, the slope increases and the intercept on $1/V_0$ axis decreases to the limit of $1/V_{max}$ (Fig. 7). Since variations in slope 1/*A* results only from the competitive inhibition by *B*, a replot

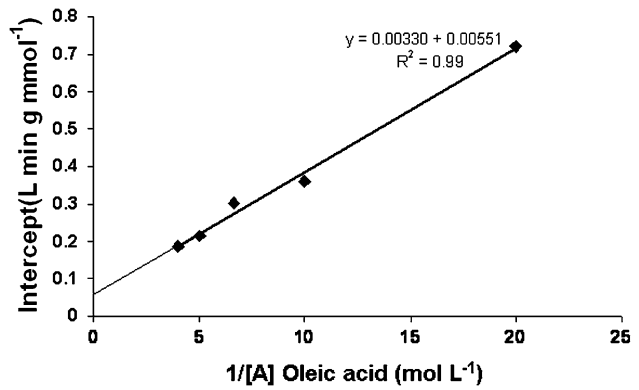

Fig. 8 Secondary plot of *y*-axis intercepts of Fig. 6 versus reciprocal of oleic acid concentration

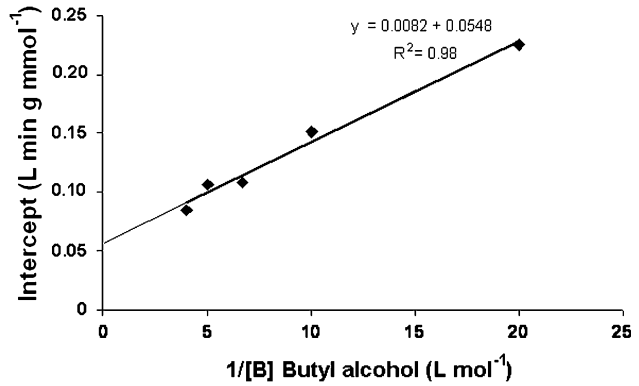

Fig. 9 Secondary plot of *y*-axis intercepts of Fig. 7 versus reciprocal of n-butanol concentration

of slope 1/*A* versus [*B*] is linear and yields K_i directly. This result is typical for competitive susbstrate inhibition in Ping–Pong mechanism with dead end complex [36]. By plotting the variations in slope of the Fig. 7 versus butyl alcohol concentration, the intercept with *x*-axis is—K_i (1,93 mol L^{-1}) as it is shown in Fig. 10.

As in this work, previous studies have shown that the lipase-catalyzed esterification and transesterification in organic media can be described by the Ping–Pong mechanism for two-substrate two-product (bi–bi) kinetic model [9, 37–39]. In this reaction, the first step is the binding of fatty acid to the enzyme to form a non-covalent lipase-oleic acid complex which then is transformed to by a unimolecular isomerization reaction to an acyl–enzyme intermediate and water is released. The second reactant, the *n*-butanol binds to the acyl–enzyme complex to form a modified enzyme–butanol complex which isomerizes unimolecularly to an enzyme oleic acid–butyl ester complex which then yields the butyl–oleate ester and the free enzyme. After the release of the ester, another molecule of oleic acid can bind to the enzyme. Nevertheless, above certain concentration of substrates, competitive inhibition is reported. The lipase may react with *n*-butanol to yield a dead end enzyme *n*-butanol complex or it may react with oleic acid to yield the effective lipase oleic acid complex. However, fatty acid

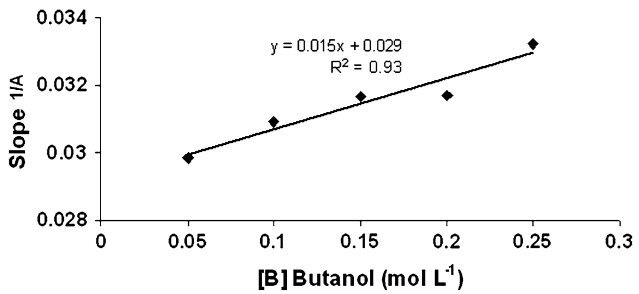

Fig. 10 Plot of the slope 1/[*A*] of Fig. 7 against *n*-butanol concentration

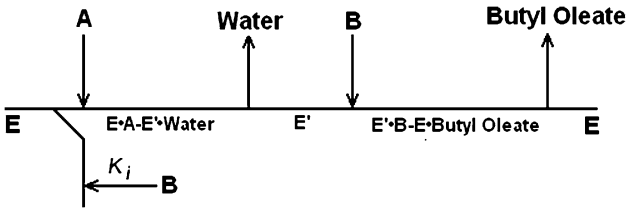

Fig. 11 Scheme of the enzymatic mechanism for butyl oleate synthesis: *E*, Lipase; *A*, Oleic Acid; *B*, *n*-butanol; *E*′, acyl–enzyme intermediate

inhibition effect was reported especially for short chain fatty acids [9, 40]. In the present study, besides alcohol inhibition, it has not observed fatty acid inhibition in the tested range of oleic acid concentration.

The reaction mechanism for the Ping–Pong bi–bi mechanism with dead end complex of *n*-butanol can be illustrated by the Cleland conventional diagram as shown in Fig. 11.

The resultant equation for the Ping–Pong bi–bi reaction with dead end complex of *n*-butanol sequence is expressed by Eq. (1)

$$v_0 = \frac{V_{\max}[A][B]}{\frac{K_A}{K_{iB}}[B]^2 + K_A[B] + K_B[A] + [A][B]} \quad (3)$$

where v_0 is the initial reaction rate, $V_{\max}$ the maximum reaction rate, $[A]$ the oleic acid concentration, $[B]$ the butyl alcohol concentration, K_A and K_B the Michaelis constants of oleic acid and butyl alcohol and K_{iB} is the butyl alcohol inhibition constant.

Using the initial rates data set, kinetic parameters of Eq. 1 were also optimized by the least square error estimation using Matlab 7.6.0 software. The resulting values were very close to the parameters found with the graphical method and are shown in Table 4.

The value of K_M for *n*-butanol ($K_B = 0.149$ mol L^{-1}) is smaller of K_M value for oleic acid ($K_A = 0.599$ mol L^{-1}), implying that the lipase in its immobilized form has higher affinity for the alcohol substrate. This could be partially explained by a slower diffusion rate of the long-chain oleic acid into the support. In the butyl oleate synthesis in hexane catalized by immobilized *Rhizopus oryzae* lipase, butanol inhibition was observed above 0.1 M alcohol concentration [8]. In the current study it can be observed, from the relatively large value of inhibition constant ($K_{iB} = 1.933$ mol L^{-1}) that alcohol inhibition occurred above 0.25 M butanol (Fig. 4) and is much less significant bellow this concentration. At superior concentration levels the alcohol, being polar in nature, is adsorbed by the immobilization support thereby blocks the entry of oleic acid and/or may be accumulated in the aqueous microenvironment that surrounds the enzyme, reaching a concentration level sufficient to denature the protein and causing the reaction to stop .

Equilibrium of lipase catalized esterification reactions is normally reached after six or more hours. The initial rate method for kinetic studies is intended to focus in the very beginning of the reaction with conversion bellow 10% and short time courses. In these cases the systems are considered to operate under irreversible conditions. Although the complete analysis of the system demanded a study under reversibility, the analysis under irreversible conditions would be much more attractive from an industrial point of view, since the larger conversion reached leads to higher

Table 4 Kinetic parameters of Ping–Pong model of lipase catalized esterifications in non aqueous media

Substrates/solvent/lipase	$V_{\max}$ (mmol min^{-1} g^{-1})	K_A (mol L^{-1})	K_B (mol L^{-1})	K_{iA} (mol L^{-1})	K_{iB} (mol L^{-1})	Reference
Isobutyric acid, amyl alcohol/iso-octane/freeCRL	0.167	0.60	0.750	–	0.020	[31]
Lauric acid, sulcatol/toluene/CRL	0.400	0.439	2.522	–	0.795	[36]
Palmitic acid, menthol/iso-octane/CRL	0.640	1.00	0.011	–	0.013	[38]
Oleic acid, methanol/hexane/CAL	4.9	0.013	0.016	–	0.003	[39]
Oleic acid, ethanol/hexane/RML	1,336.2	1.2	1.16×10^{-8}	–	9.46×10^{7}	[40]
Oleic acid, ethanol/hexane/freePPL	4.0	0.066	0.103	–	0.020	[41]
Oleic acid, ethanol/hexane/MML	23.0	0.45	0.6	–	0.06	[42]
Oleic acid, ethanol/SCCO2/MML	14.0	0.16	1.60	–	0.065	[42]
Oleic acid, butanol/hexane/CRL	3.2	0.380	0.190	0.23	0.780	[9]
Oleic acid, *n*-butanol/iso-octane/CRL	18.2	0.599	0.149	–	1.933	(*)
	19.0	0.640	0.128	–	–	(**)

MML Mucor miehei lipase, *PPL* Porcine pancreatic lipase, *RML* R.Miehei lipase, *CAL* Candida Antarctica lipase

(*) Present work, graphic method

(**) Present work, parametric determination

quality of the final product [41]. This work and the studies referenced in Table 4 considered only irreversible conditions for the esterification reactions.

In reference to the comparison of the kinetic parameters of the biocatalysts prepared in this contribution for the butyl oleate synthesis with other chitosan immobilized lipases reported in literature showed to be very scarce. Only one work dealing with immobilized *C. rugosa* lipase on nylon was found [9]. The authors reported the same mechanism for the esterification with competitive inhibition by both oleic acid and *n*-butanol, using substrate concentrations from 0.1 to 1.0 M, lower reaction temperature, and hexane as non aqueous media. Alternatively, the kinetic constants obtained in the present study are summarized and compared with some published data for *C. rugosa* and other lipase catalized esterifications that follow the same enzymatic mechanism in Table 4.

In different esterifications in non aqueous media the lipase from *C. rugosa* synthesizes more efficiently the short-chain fatty acids than for the long-chain fatty acids [39]. For this lipase, the higher values of *Km* for long-chain fatty acids (Table 4) apparently corroborate this observation.

4 Conclusions

The variation of crystallinity, which was a result of thermal treatments and crosslinking of chitosan, influenced its protein load capacity when the biopolymer used as lipase immobilization support. Among four protocols tested and three different published procedures dealing with *C. rugosa* lipase on chitosan immobilization, the highest crystallinity chitosan support, produced with six freezing and thawing cycles and treated with glutaraldehyde, gave the better yields of protein load and protein load efficiency. The synthetic activity of this biocatalytic system was assayed in a model esterification reaction: the *n*-butanol–oleic acid esterification, which was carried out in iso-octane. The kinetic constants were estimated by using a graphical method and a non-linear regression method. The analysis of the initial rates showed the reaction in organic solvent was well approximated by Ping–Pong bi–bi kinetic model with inhibition by butyl alcohol. No evidence of enzyme inhibition by oleic acid up to 0.25 M. The value of K_M for *n*-butanol ($K_B = 0.128$–0.149 mol L^{-1}) is smaller of K_M value for oleic acid ($K_A = 0.599$–0.640 mol L^{-1}), implying that the lipase in its immobilized form has higher affinity for the alcohol substrate. This could be partially explained by a slower diffusion rate of the long-chain oleic acid into the support.

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