Ethyl Oleate Synthesis Using *Candida rugosa* Lipase in a Solvent-Free System. Role of Hydrophobic Interactions

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The solvent-free esterification reaction of a commercial oleic acid and ethanol was selected as the test reaction for *Candida rugosa* lipase immobilized on polypropylene (PP) at 318 K (initial molar ratio 1:1). Adding of water from 0 to 30 wt. % (in gram per gram of fatty acid × 100) and the pretreatment of *Candida rugosa* lipase with polyethylenglycol (PEG), octane, and acetone increases the conversion to ethyl esters. The role of hydrophobic interactions of the lipase with PP and PEG was studied using molecular mechanics (MM2) for calculation of steric energies and the parametrized model (PM3) for calculation of enthalpy changes upon interaction. The nonpolar lateral groups of amino acids interact strongly with PP, whereas polar groups interact more strongly with PEG. Both interactions stabilize the open, active conformation of the lipase from *Candida rugosa*. Activities ranged from 5×10^{-5} to 2.0×10^{-4} mol ethyl oleate/h/mg enzyme, depending on reaction conditions. Steric energy changes vary between +30 and −10 kcal/mol, whereas the enthalpy changes ranged from +10 to −10 kcal/mol.

1. Introduction

Lipases catalyze hydrolysis but also several reactions such as esterification, transesterification, and aminolysis in organic solvents or in solvent-free systems (SFS). The reversal of the original hydrolytic activity is obtained by using lipases in organic solvents at low water activity (a_w)[1] or in solvent-free media only composed by substrates.[2]

In the SFS, supporting the enzyme guaranties the reuse, especially if water is present in the reaction media to activate the enzyme. The support permits an easy recovery, reuse, greater stability of the enzyme, and the possibility of continuous operation. The temperature and pH of the immobilization media, the support election (hydrophobic or hydrophilic) and the kind of the selected enzyme are crucial to obtain an active and stable supported enzymatic catalyst.[3]

Recent reports about immobilization of *Candida rugosa* and *Rhizomucor meihei* lipases on polymer supports have been published.[4,5] The adsorption on a hydrophobic support activates the enzyme, leading to a more active enzymatic catalyst than the nonsupported lipase. After activation, Guisán et al.[4] further incubated the enzyme with polyethylenimine (PEI) in a low ionic strength reaction media. The supported enzyme is proposed to be covered by a layer of a hydrophilic polymer and protected from inactivation in organic solvents by adsorption, not reaction or covalent bonding.[6] Leakage of the lipase toward an apolar and hydrophobic reaction media is an improbable event.[4] Thermodynamic and kinetic studies have been published using solvent-free systems.[7,8] Computer modeling has been applied to substrate binding to lipases from *Rhizomucor miehei*, *Humicola lanuginosa*, and *Candida rugosa*.[9] Free-energy methods have been criticized by these authors as not straightforward. Computational methods have been used to study the extensive conformation change of *Rhizomucor meihei* lipase upon activation[10] in a vacuum. A hydrophobic environment is not able to make local or extensive polar interactions with the protein. Moreover, several approaches have been used to overcome convergence criteria. Fluctuations in calculations of the overall structures of lipases are common also when a hydrophobic environment is considered. Reported works on hydrophobic/hydrophilic modification of CRL are scarce, and they mainly involve chemical modifications of the lipase through covalent coupling, using glycoside or PEG.[8]

This manuscript presents results of pretreatments with modifiers (before contact with the SFS reaction media) of PP immobilized lipase from *Candida rugosa* (CR). The modifiers were a hydrophilic polymer such as polyethyleneglycol (PEG) and different solvents (octane, acetone). When PEG was used, the catalyst is noted as PEG/CR/PP (CRI). The effect of the initial water amount in the range 0−30 wt % (g H_2O/gFA*100) was analyzed to test the importance of the reaction media polarity and character. In fact, we studied the transition from a SFS (0% water) to a microemulsion (5−10% water) and finally a biphasic/microemulsion system (20−30% water). Different pretreatments before reaction for Novozyme 435 and Lipozyme IM 60 were carried out in order to test the sensibility of these supported enzymes to further activation. One of these pretreatments was carried out with an imprint molecule such as 9-decen-1-ol. Besides, the possibility of further interfacial activation was studied for these commercial lipases using a hydrocarbon/buffer interface. The aim was to study how CR/PP (a catalyst with a nonporous support PP of low area, with lipase adsorbed

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on the external surface) is affected by these pretreatment vs commercial lipases, supported on macroporous exchange resins (with BET areas near 100−200 m^2/g and pore radius of near 180−270 Å, with lipases adsorbed on pore walls). No PEG adsorption was tested in commercial lipases because these preparations include 10−12% water and supports are hydrophilic whereas PP is hydrophobic. The possibility of reuse of CRI was considered. The affinity between lateral amino acid groups different than H with PP/PEG was studied using MM2 and PM3 methods. This study emphasizes the local character of the interaction.

To the best of the author's knowledge, this is the first report dealing with the use of PEG/CR/PP in the esterification of oleic acid performed in a SFS using *Candida rugosa* lipase and with initial water content as high as 20% (g/gFA × 100). Although there are several reports on the effect of PEG on CR activity and the use of PP supported lipase, there are no reports on solvent free esterification of oleic acid using PP supported CR. Hydrophobic interactions of residues in the lipase (mainly Ala, Gly, Leu, Ile and residues containing hydrocarbon chains/rings such as Ser or Pro) with PP are proposed to play a role in the adsorption/activation of *Candida rugosa* lipase.

2. Materials and Methods

2.1. Materials. Lipozyme IM 60 (a lipase from *Rhizomucor miehei* immobilized on a macroporous anion-exchange resin) and Novozyme 435 (a lipase from *Candida antartica* immobilized on a macroporous acrylic resin) were kindly donated from Novo-Nordisk. *Candida rugosa* was donated by Amano USA Inc. as Lipase AY "Amano" (30.000 U/g), and no further purification was carried out on the lipases. Lipases provided by Amano Inc. (from *Candida rugosa*, *Pseudomonas fluorescens* and *Pseudomonas cepacia*) have been tested elsewhere and their purity is enough to present comparative results on the effect of supporting on the activity. The huge amount of published data on commercial lipases support this prompt [see references]. Sigma and Dorwill provided standards and other chemicals. Commercial oleic acid was used, with 79% oleic acid, 11.7% palmitoleic acid, 5.7% palmitic acid, 1.9% miristoleic acid, and 1.6% miristic acid, checked by chromatographic analysis (all in weight). Grinded Molecular Sieves 13 X from Grace Davison were used. The buffer is a monophosphate-monobasic buffer (KH_2PO_4−NaOH).

2.2. Definitions. The initial molar ratio N was defined as initial mol of alcohol/initial mol of fatty acids (FA); the enzyme content in the reaction media E was defined as mass of immobilized enzyme/mass of fatty acids (initial g of immobilized enzyme/g of FA). The initial water content, W, was defined as initial mass of water/initial mass of fatty acids (initial g of water/g of FA). Low contents of water such as 1% (g/gFA × 100) reach water activities (a_w) near 1 when SFS are analyzed.[8] Therefore, we used concentration of water as W. It has no sense to present data in terms of water activity, because, for the water percentages used here, a_w is near 1 in all cases. We found a relation with the percentage of water, not the activity as usual in enzymatic reactions using organic solvents. Water activity will play a crucial role in the system with the organic compound as the solvent. Elsewhere, calculations were performed and the optimum is near $a_w = 1.6$, using UNIFAC as the calculation method for this solvent-free system (an extremely nonideal system).[11] CRS is the nonsupported *Candida rugosa* lipase, whereas CRI is equivalent to PEG/CR/PP.

2.3. Experimental. The reactions were carried out in vials of 10 mL volume operating batchwise, using 4.5 g of oleic acid and 0.75 g of absolute alcohol (ethanol). The magnetic stirrer speed was set at 1000 rpm to minimize mass transfer limitations. The reaction temperature (T) was set at 318 K, $N = 1$, E was varied from 0.7 up to near 8 wt % (g/gFA × 100; from near 30 up to 350 mg supported lipase), and W was varied from 0 to 30 wt % (g/gFA × 100). The substrates were introduced into the vials where they were heated to the desired temperature. When the reaction temperature was reached, the catalyst was added and the reaction started.

2.4. Analytical Procedures. Samples (near 0.2 g) were withdrawn from the reaction mixture periodically and analyzed titrimetrically by duplicate or even triplicate for the residual acid content using a basic solution of potassium hydroxide of concentration (near 0.05 N) standardized using carefully dried potassium-monoacid biphthalate. The percentage of conversion of fatty acids was calculated comparing the obtained value of fatty acid consumed with that at the beginning of the reaction. The amount of immobilized lipase on PP was checked with a kit provided by Wiener Lab., Proti 2 (Rosario, R. Argentina). The reaction is based on the reaction of the peptidic bonds of proteins with cupric ion in an alkaline medium. The violet resulting complex absorbs at 540 nm.

2.5. Enzyme Activities. Enzyme activities of the lipases were calculated as follows:

$$\text{enzymatic activity}\left(\frac{\text{mol of fatty acids}}{h^*\text{mgenz}}\right) \cong \frac{(N_{FA})^0 * (\text{Xac})_t}{(t)w_0} \quad (1)$$

where $(N_{FA})^0$ = initial mol of fatty acids (mol of oleic acid); $(\text{Xac})_t$ = conversion of fatty acids at time t; t = time (hours); w_0 = initial immobilized enzyme content (mg). Catalyst is the support plus the adsorbed enzyme.

2.6. Immobilization Procedure on Polypropylene Powder. 100−200 mg of nonporous polypropylene of near 25 m^2/g (M_w = 28000 g/mol, obtained using metallocenes in our laboratory) were contacted in 10 mL of buffer at pH 7 with 40−80 mg of lipase using 950 rpm at room temperature by 24 h. The buffer is a monophosphate-monobasic buffer (KH_2PO_4−NaOH). The solid was recovered by filtration and washed with distilled water (efficiency near 50% enzyme loading: 250 mg/g catalyst). 50 mg of poly(ethylene glycol) (PEG) was added to 140−280 mg of supported enzyme and stirred for 1 h at room temperature in 5 mL pH 7 buffer. The solid was recovered by filtration and finally dried at 313 K (1 h). The remaining unsupported enzyme was checked in the solution by several UV/visible methods.

2.7. Pretreatments. Open literature reported several times that pretreatments with selected hydrocarbon/buffer mixtures, organic solvents, or amphiphiles resulted in improved

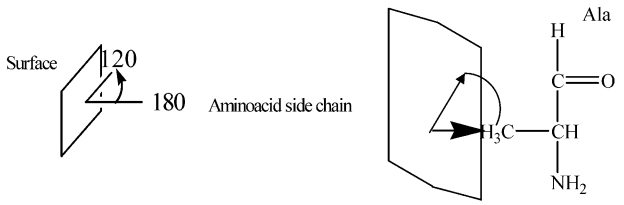
synthesis activity. Considering the nature of the supports, we tried different pretreatments depending on their nature, hydrophobic or hydrophilic.

We selected *n*-tetradecane as the hydrocarbon and 9-decen-1-ol as the amphiphile to test with commercial lipases. 30 mg of novozyme/lipozyme was added to 0.25 mL of *n*-tetradecane in 5 mL of buffer at pH 7 for 1 h at 313 K. When 9-decen-1-ol was used, 35 μL was added to 30 mg of novozyme 435/lipozyme IM 60 in 3 mL of buffer at pH 7 for 24 h at 313 K. A washing step with toluene was done for 24 h at room temperature. Octane (log $P = 4.5$) and acetone (log $P = 0.45$) were selected for a pretreatment with an organic solvent considering their log P.

PP/CR was treated with PEG in a 0.5:1 mass ratio for 1 h at room temperature. PP/CR/PEG was contacted with 5 mL of organic solvent (octane or acetone) for 1 h at room temperature. The solid was recovered by filtration and dried before use until constant weight. Reusing studies were done after a first ethyl ester synthesis using catalysts washed with and without ethanol, after storage at low temperatures. Around 80 mg of catalyst was used to compare different treatments.

2.8. Theoretical Method. The steric energies were calculated using the MM2 (molecular mechanics Allinger program version 2) modified improved version included in Chem 3D Pro 5.0 from Cambridge Soft 1999. Although there are more advanced versions (MM3 and the yet nonavailable commercially MM4), the MM3 version of the Allinger program has demonstrated to have some drawbacks in the calculation of interactions of long/short terms in biopolymers. PM3 (parametrized model 3) included in MOPAC software was used to calculate the ΔH_f (standard enthalpy of formation). Simple models of polypropylene (PP) and PEG structures were employed to analyze the interactions of side chains of different amino acids with local environments in PP or PEG. The PP model includes two chains with a total of 48 C atoms, whereas the PEG model contains six C_2H_4 residues and 7 O. Being the side chain of the amino acid residue available for interaction, what is the trend in enthalpy or steric energy changes considering local environments of PP and PEG? These simple calculations give tools to understand the interaction of the side chain of the amino acid residues of *Candida rugosa* lipase with PP and PEG. No strong electrostatic effects take place in the state of the enzyme, soluble or supported in a solvent-free reaction media, in this case with oleic acid as the solvent. Moreover, the preparation of immobilized lipase is done using buffer pH 7, and therefore, no effects of acid/alkaline media are expected to take place at this step. The amino acid main chain was modeled with a NH_2 group by one side and an aldehyde group by another. This choice was done because in the enzyme there is not a $COOH/NH_2$ group in the residues. With an aldehyde group, nonacidic character is included in the residue, and this fact is important to address the conformation of the lateral chain. The criterion was to select hydrogen to model the chain before and the chain after the main structural groups in the protein structure at the level of the amino acids. The models of amino acid's lateral groups were approached to the simple models of PP and PEG at distances between 3 and 2 Å, the conformation was minimized, and after this, a PM3 calculation was performed. Changes in steric energies are calculated between adsorbent and adsorbate far away from each other and in contact.

Scheme 1



We focused on the interaction of R (side chain amino acid) with different surfaces, in a vacuum. However, different orientations (angle of R with a plane delimited by the surface: from 180° to near 120°) were considered.

Scheme 1 shows the general procedure for the amino acid side chain approach for the case of alanine. The side chain of the amino acid was placed initially at a long distance from PP surface and approached to the surface by 0.2 Å steps. The reported energies are those from the minimum. The final energies practically did not differ from different configurations. The starting configurations do not have this effect because minimization always changed to a similar configuration in steric energy. Considering only the side chain, the possible orientations are reduced. The minimization performed by molecular mechanics gives the possibility to compare conformational changes when adsorbent and adsorbate are located far away from each other and in close contact. With MM2 calculations, the obtained steric energy for a particular conformation has no physical meaning, but the differences between conformers have physical meaning. In case of PP/amino acid, we performed an additional PM3 minimization to compare the effects of electrostatic interactions vs steric interactions.

Semiempirical methods of an electronic nature represent poorly the electronic distribution in different conformers to predict partial charges, especially with polar compounds. For conformationally flexible molecules, if the charge set is changed then, the torsional parameters must also be adjusted to maintain relative conformational energies. However, these calculations are being used in a qualitative basis, and we are not analyzing charges because interactions are analyzed in a vacuum and between side-chain amino acid residues and polypropylene models. The importance of the main chain $(-C{=}O-CHR-NH-)_n$ is neglected to focus on the side chain, and the above-reported calculations focus on the overall amino acid and the interaction with water. The interaction of the enzyme with the interface is much difficult to model.

The final aim is to know if these methods can be used to qualitatively analyze the hydrophillic/hydrophobic interactions of the side chain of amino acids and to relate the results with a coherent explanation of the experimental results. The searching of the energy surface for minimum energy conformations is usually too expensive to do using MO methods, and generally, the results are not in agreement with the experiment. This is why we used PM3 calculation at the minima found by MM2 in this particular case. Besides,

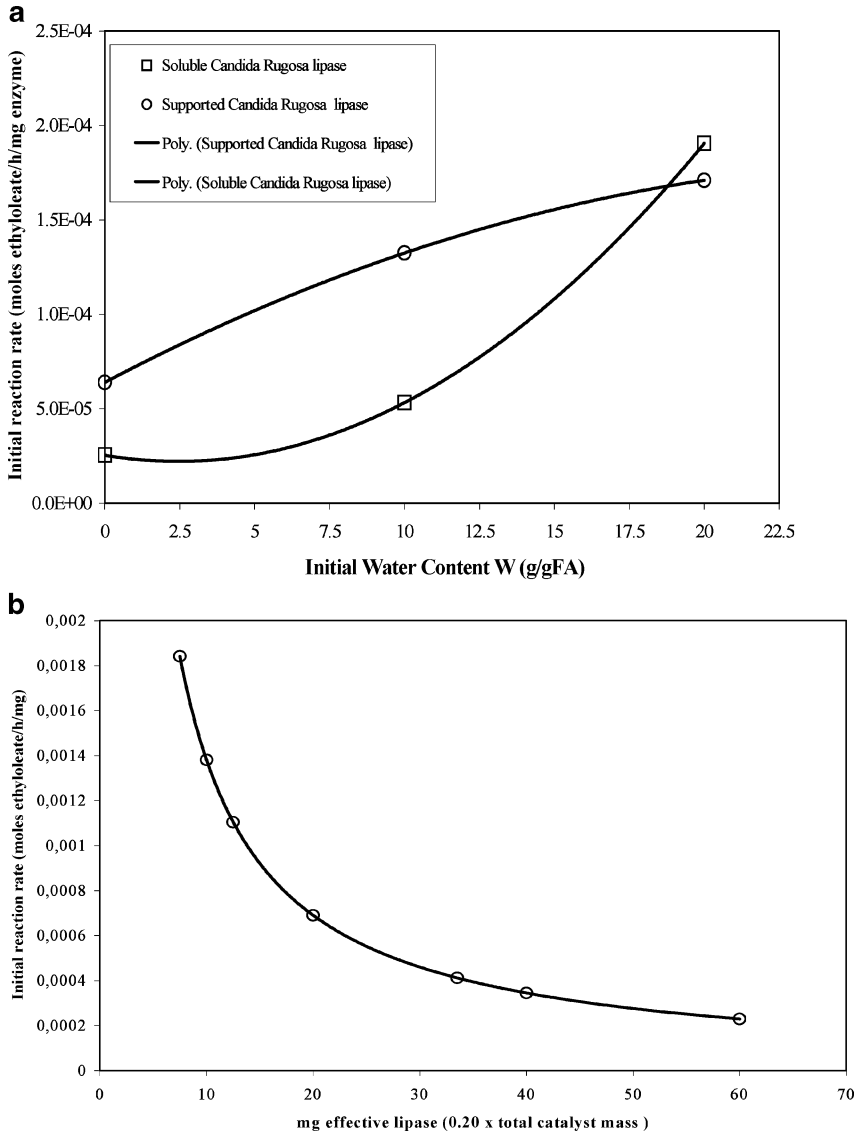


Figure 1. (a) Initial reaction rate of ethyl esters synthesis versus initial water content E for soluble and supported *Candida rugosa* lipase. Reactions conditions: N = 1, T = 45 °C, 30 mg enzyme soluble, 80 mg supported enzyme. (b) Initial reaction rates of ethyl esters synthesis vs enzyme content *E* for supported *Candida rugosa* lipase. Enzyme loading: 25%. Reactions conditions: *N* = 1, *T* = 45 °C, *W* = 20% g/gFA *x* axis in mg of lipase.

we performed a PM3 minimization for the amino acid side chain/PP interactions. Atoms are automatically assigned a formal charge based on the atom type parameter for that atom and its bonding in MM. Since different methods rely on different assumptions about a given molecule, values from different methods should not be compared. The application of QM/MM methods that model the actual reactivity patterns could be realistically formulated. These methods are still approximate. However, they showed that focusing the attention on the reactive region of the enzyme with quantum mechanics calculations, while keeping tracking of the enzyme environment influence through molecular dynamics, is the right way.[12]

ΔSE is the change in the steric energy. A spontaneous process needs a negative ΔSE value. A positive ΔSE value can be correlated with an esterically not favored process. $\Delta(\Delta H_f)$ is the change in the standard enthalpy, positive for endothermic process and negative for exothermic process. A spontaneous process is favored with a negative $\Delta(\Delta H_f)$ value. They are estimates of steric and enthalpic contributions to the total energy (Gibbs energy).

3. Results and Discussion

3.1. *Candida rugosa* Lipase: Free and Immobilized. The enzymatic activity of CRS increased as the water content increased.[22] The enzyme is deactivated with more than 20 wt % water (g/gFA × 100) in the reaction media because probably the interface oleic acid/(water + ethanol) is unavailable to it. Fatty acids are almost insoluble in water, but the unsupported enzyme is soluble. For initial water content lower than 20 wt % (g/gFA × 100), the activity of CRI was higher than for CRS (see Figure 1a). Different amounts of soluble lipase (10 and 30 mg) gave the same results in conversion. We used 10 and 30 mg because these amounts are reported in the open literature as the more convenient. We found that the activity does not increase when the amount of lipase is increased, giving support to aggregation theories in the case of CRL. We selected 30 mg

Table 1. Effect of Pretreatment with PEG and Nonpolar Solvents on the Enzymatic Activity of CRI[a]

	mol fatty acid/h*mgenz*10^5 for CRI	
	reaction time	
pretreatment	after 1 h	after 2 h
no PEG	4.4	3.6
PEG	12.5	7.7
PEG + octane	18.3	18.5
PEG + acetone	31.9	19.9

[a] Reaction conditions: $N = 1$, $T = 45$ °C, $W = 20$ wt % (g/gFA × 100). Mass of CRI: 20 mg (80 mg of total catalyst).

Table 2. Effect of the Reuse on the Conversion

	conversion (% of fatty acid consumed)	
	after 1 h	after 2 h
fresh catalyst (total catalyst mass =134 mg)	9.2	13.7
condition 1[a]	4.7	11.6
condition 2[b]	3.3	8.1
condition 3[c]	0.4	1.1

[a] Condition 1: After use, the catalyst was filtered but not washed and later stored at 0−5 °C. [b] Condition 2: The immobilized enzyme was rinsed with 20 mL ethanol after first use, dried and used again 45 days of storage at 0−5 °C. [c] Condition 3: After use, without ethanol washing, with 25 wt % (g/gFA × 100) H_2O. 343 mg supported PP−CR−PEG.

of free lipase and 80 mg of CR/PP (or 20 mg lipase) to compare. Only 25% of the catalyst mass is enzyme, following the analytical procedure (see Materials and Methods).

The results using CRS are different if we use pure oleic acid (99%) instead of commercial oleic acid (79% w/w oleic acid, 21% a mix of C16, C14, C16:1, and C14:1). With CRI, reproducibility is good, whatever the kind of oleic acid. Up to a value of 10 wt % (g/gFA × 100) the conversion obtained for CRI was higher than for CRS but when this 10 wt % (g/gFA × 100) was exceeded, the situation was the opposite. The maximum activity per mg of enzyme is found for a lower amount of effective supported enzyme for CRI (10 mg) than in case of CRS (30 mg).

The key point is the adsorption of the lipase to the interface.[13−15,23] Other authors observed an increase in the reaction rate as the water content increased, using organic solvents, because a biphasic system is created.[16] Dissolution of lipase in the aqueous phase before adding the oleic acid/ethanol inactivated the lipase because it becomes unavailable to the interface. The fact that free lipase is soluble in the water phase does not explain the increase in activity that is related in the open literature to the increase in interfacial area in biphasic systems.[16] Excess water around lipases attacks the polar site of the lipase causing reduction of activity or even deactivation.

3.2. Effect of Pretreatments in CR/PP. When the PEG pretreatment of CR/PP was omitted, the activity of CRI decreased two or three times (see Table 1). *Candida rugosa* lipase is activated when PEG is used as a protector at room temperatures and using 80% oleic acid.[17] The effect of medium-chain fatty acids cannot be ruled out. This means that the increase in activity when the PEG treatment is included is due, not to oleic acid, but to C18:0, C14, and C16 fatty acids present. When PEG plus octane or plus acetone is used, the activity increases. The surrounding of the lipase is crucial to obtain a high activity. PEG would ensure water, and octane would ensure that oleic acid (o better C14 and C16) is present near the lipase. The nonpolar (although) polarizable character of acetone could be associated with the high activity obtained with it: it improves the transfer of oleic acid and shorter acids and polar substrate ethanol in the neighbor of the lipase. However, PEG cannot avoid the ethanol deactivation. Final conversion with octane and acetone is the same. The total enzyme mass varies from 7 (35 mg of total catalyst) to 60 mg (240 mg of total catalyst). Figure 2a shows the enzymatic activity vs catalyst mass for 1 and 2 h of reaction. The enzymatic activities for C*andida rugosa* immobilized, pretreated with PEG, decrease as the

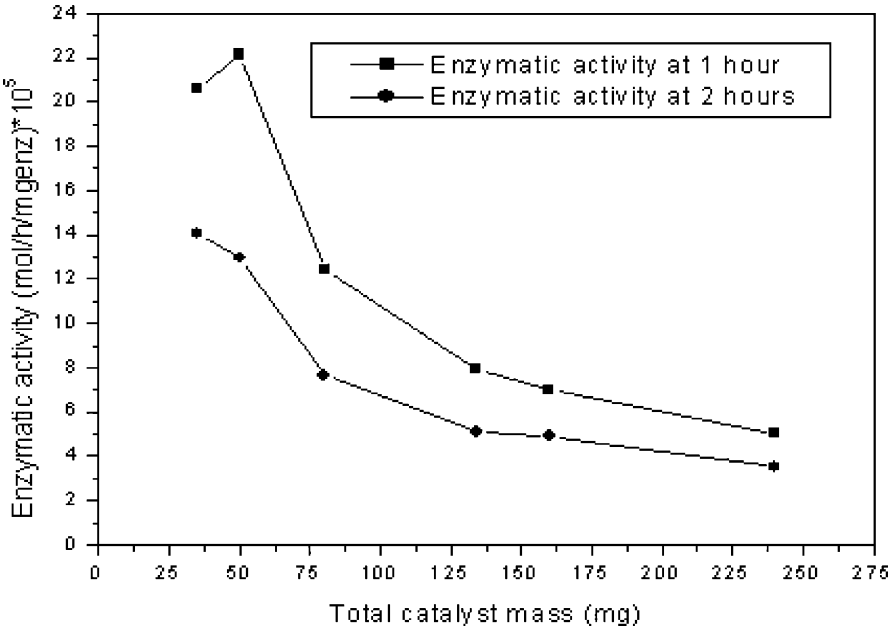


Figure 2. Effect of the total catalyst mass on the enzymatic activity for 1 and 2 h of reaction. Reaction conditions: $N = 1$, $T = 318.15$ K, $W =$ 20 wt % (g/gFA × 100). *x* axis in catalyst mass units.

Table 3. Amino Acid Lateral Group Adsorption on PP Model-Steric Energies and Enthalpy Changes or the Steric MM2 Minimum and for the PM3 Minimization[a]

AMINOAL	SE	SE AM+PP	ΔH_f	ΔH_f PM3	ΔSE	ΔH_f	PM3	$\Delta(\Delta H_f)$	$\Delta(\Delta H_f)$ PM3	H−H AM−PP[b]
Lys	3.90	74.6	−48.1	**−51.2**	−3.19	−278	**−172**	1.22	−8.90	2.52
Arg	8.42	112	123	**109**	28.1	−80.1	**−7.93**	26.4	10.2	1.84
His	13.2	82.5	2.06	**−7.06**	−6.03	−228	**−122**	−0.89	−3.50	2.53
Cys	2.78	73.3	−29.4		−4.78	−259		−0.61		2.25
Met	4.67	76.2	−38.9	**−43.9**	−3.80	−267	**−160**	0.74	−4.40	3.79
Glu	7.04	70.3	−125	**−133**	−12.0	−362	**−249**	−7.62	−2.10	3.11
Asp	−1.23	69.5	−125	**−129**	−2.06	−354	**−247**	−0.41	−6.40	
Ser	0.95	71.6	−78.7	**−82.6**	−4.63	−309	**−199**	−1.69	−4.60	2.04
Thr	2.64	74.7	−84.2	**−88.9**	−3.24	−315	**−205**	−1.80	−4.80	2.40
Asn	−3.19	67.7	−72.2	**−78.9**	−1.40	−304	**−194**	−2.55	−3.60	
Gln	−1.63	81.2	−78.7	**−84.0**	7.56	−292	**−199**	15.5	−3.70	2.20
Gly	−0.68	71.3	−35.1	**−36.8**	−3.25	−265	**−151**	−0.99	−3.11	2.69
Ala	1.49	73.5	−41.6	**−43.7**	−3.28	−272	**−161**	−1.61	−5.90	2.25
Val	4.19	75.5	−49.6	**−51.9**	−3.96	−281	**−172**	−2.52	−8.20	2.38
Leu	6.18	74.7	−53.6	**−59.5**	−6.8	−285	**−186**	−2.53	−14.7	2.79
Ile	6.14	76.1	−53.5	**−56.8**	−5.32	−285	**−176**	−2.07	−7.40	2.83
Phen	−1.25	69.2	−12.2	**−14.7**	4.72	−241	**−134**	0.05	−7.50	2.23
Tyr	−2.47	71.7	−55.9	**−59.8**	−1.11	−281	**−175**	4.41	−4.10	2.48
Trp	7.52	79.2	11.1	**3.68**	−3.59	−221	**−115**	−2.61	−7.10	3.32
Pro	12.2	82.7	−38.5	**−43.2**	−4.77	−268	**−161**	0.12	−6.20	2.33

[a] In bold, enthalpies after PM minimization. Energies in kcal/mol. SE steric energy; ΔH_f standard enthalpy; PP model −log P = 9.9 SE 75.275 kcal/mol; ΔH_f −229.6134 kcal/mol, ΔH_f for PM3 = −111.86 kcal/mol; AMINOAL-AM-Lateral structure of the amino acid modeled $\Delta\Delta H_f = \Delta H_f$ residue adsorbed on PP − (ΔH_f PP + ΔH_f residue). ΔSE = SE (residue adsorbed on PP) (SE PP+ SE residue) [b] Minimum distance H (from lateral residueAM) to H (from PP).

Table 4. Interaction with PEG-Steric Energies and Enthalpy Changes upon Adsorption[a]

AMINOAL residue AM	log P	SE	SE AM+PEG	ΔH_f	(ΔH_f) AM+PEG	ΔSE	$\Delta(\Delta H_f)$	H−H (PEG)
Lys	−1.18	3.90	28.5	−48.1	−333	−3.14	−0.87	2.89 (N)
Arg	−4.70	8.42	16.0	123	−170	−20.1	−9.13	2.13 (O)
His	−2.12	13.2	36.8	2.1	−280	−4.14	1.21	2.47
Cys		2.78		−29.4				
Met	−0.83	4.67	29.3	−38.9	−323	−3.08	−0.63	2.33
Glu	−1.66	7.04	22.2	−125	−416	−12.5	−7.28	2.14
Asp	−1.94	−1.23	21.1	−125	−409	−5.47	−0.06	2.22 (O)
Ser	−2.45	0.95	26.6	−78.6	−364	−2.07	−1.37	2.25
Thr	−1.67	2.65	27.6	−84.2	−390	−2.80	−21.6	3.01
Asn	−2.59	−3.15	15.1	−72.1	−353	−9.51	3.25	2.20 (O)
Gln	−2.31	−1.63	19.8	−78.7	−361	−6.30	1.38	2.24
Gly	−1.21	−0.68	24.5	−35.1	−320	−3.80	−1.19	2.49
Ala	−1.29	1.49	26.4	−41.6	−327	−2.77	−1.71	2.81
Val	−0.63	4.19	29.5	−49.6	−335	−2.43	−1.41	2.41
Leu	−0.17	6.18	29.5	−53.6	−340	4.36	−3.20	2.29
Ile	−0.17	6.14	30.7	−53.5	−339	−3.14	−1.57	2.54
Phen	0.19	−1.24	24.5	−12.2	−296	4.52	0.21	2.41
Tyr	−0.20	−2.47	21.9	−55.9	−339	3.36	0.84	2.82
Trp	0.05	7.52	29.0	11.1	−273	−6.24	−0.52	2.49
Pro	−0.61	12.2	36.2	−38.5	−323	−3.20	−1.15	2.78

[a] Steric energy PEG = 27.72 kcal/mol − 284.17 kcal/mol − log P = −1.57. $\Delta\Delta H_f = \Delta H_f$ residue adsorbed on PEG − (ΔH_f PEG + ΔH_f residue.

total mass catalyst increase but a maximum value of activity appears around 50 mg of the total catalyst mass (or 12.5 mg lipase) for 1 h of reaction (see Figure 2). The conversion increases as the total catalyst mass does, and it seems to reach a constant value for amounts of catalyst higher than 100 mg (or the enzyme content of 20 mg). This fact implies that the agglomeration of the lipase using higher catalyst concentration in this SFS is so important that only 100 mg of catalyst are effective, even when we add 150 or 200 mg to the reaction media. Agglomeration explains also that using 10 or 30 mg of CRS gives the same total conversion.

The catalyst can be reused at least once without problems, having an activity slightly lower than that of fresh catalyst, even after storage. The percentage of recovered activity after 1 reuse was around 90% when no organic solvent was used at the washing step (see Table 2). Washing steps with ethanol decreased the activity. Ethyl ester residues can block the active site for further reaction, and this can be the reason for the apparent activation of the enzyme after the first hour. The use of high amounts of catalyst (condition 3) decreases the activity, as it happens with fresh supported catalyst.

3.3. Theoretical Methods MM2 and PM3. Changes in local conformation because of the interaction with PP/PEG can explain the increase in activity found at our conditions with the CR because of effects in the opening of the lid for *Candida rugosa* lipase. PP and PEG interact in different ways with nonpolar or polar lateral amino acid residues of the lipase, in such a way that their effects are different. These

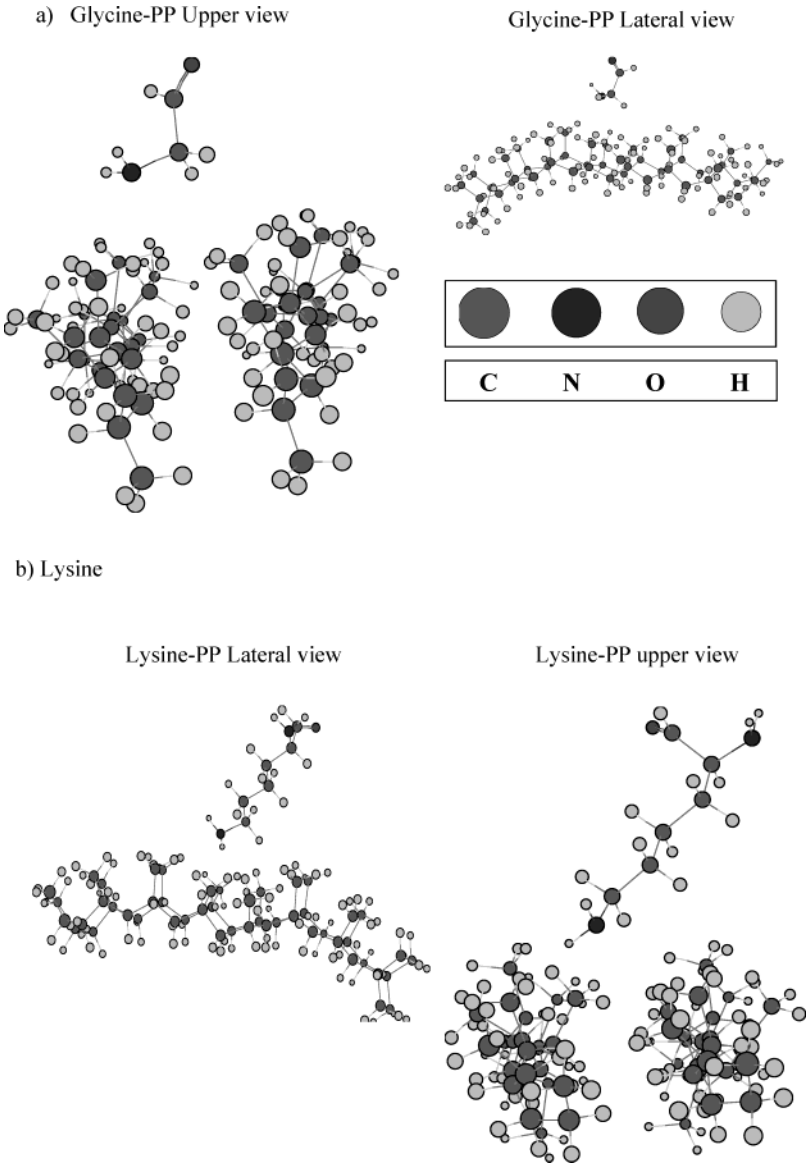


Figure 3. Conformational minima found for lysine side chain group and glycine side chain amino acid residue models. Adsorption on PP.

kinds of interactions in an aqueous media can explain the opening of the lid because of the stabilization of the lateral chain that, due to the hydrophobic effect, would be expected to be located to inside the structure and supports the idea of PP as a solid hydrocarbon, as a part of the interface water /hydrocarbon.[26] This is very difficult because of the sophistication of the preparation system (water, ions from buffer, surfaces, liquid−liquid interfaces, adsorption in case of PEG, that is soluble in water). Moreover, lipase from CR has several identified isomorfs (Lip1, Lip2, and Lip3) from a total of 7 lipases, closely related.[11] Tables 3 and 4 include the minimum H−H distance or (H−O/N in the case of PEG) to show the strong interaction of lateral residues with PP and PEG in some cases, where the distance is lower than the van der Waals ratios. The adsorption is steric and enthalpically favored when ΔSE and ΔH are negative. These results demonstrate that the steric interactions and enthalpy interaction energies of residues side chains with PP are in the range of +4 up to −15 kcal/mol, excluding Arg, which presents the strongest repulsion. It is interesting that the flap of lipase from *Candida rugosa* does not include Arg.

From the trends that arise considering ΔSE, $\Delta(\Delta H_f)$, and PM3 overall minimization, we can conclude the following:

1. The steric energies using MM2 follow different trends than enthalpy energy changes calculated with PM3 for side chain interactions of amino acids with PP.

2. In cases where an hydrocarbonated chain is present in the side chain of the amino acid, the longer this chain, the stronger the attractive interaction with PP, even if the chain is cyclic, as in case of Proline.

3. Considering steric interactions only, there are attractive forces between PP and Glu, and considering the electronics of this system using PM3 minimization, the interaction is repulsive.

It is clear that Arg, Gln, and Phe have steric repulsive interactions with PP, whereas Trp, Val, Leu, Ile, Ala, and Glu have attractive interactions with PP. The H−H distance is longer in this last case. Lys, Met, Arg, Tyr, Phen, and Pro show positive ΔH values for the conformational minimum. The minimization using PM3 shows that the electronic interactions are strong in the case of Lys, Val, and Leu. The length of the hydrocarbon side chain is important to increase

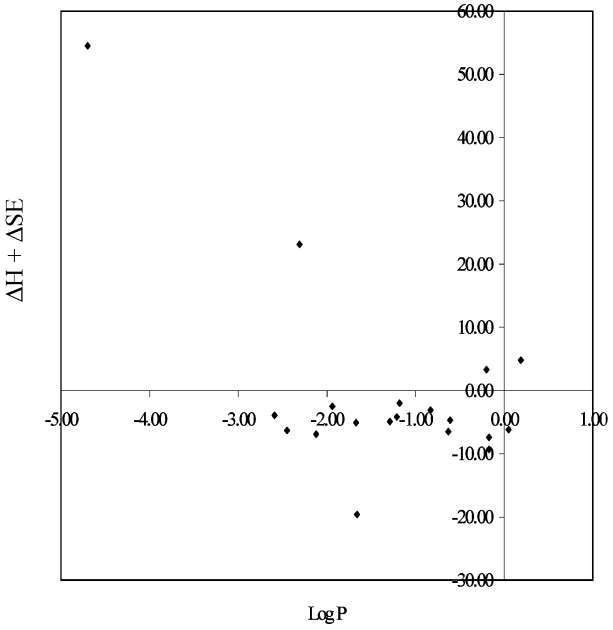


Figure 4. Analysis of energy change (as $\Delta H + \Delta SE$) upon adsorption of amino acid lateral groups on PP vs log *P*.

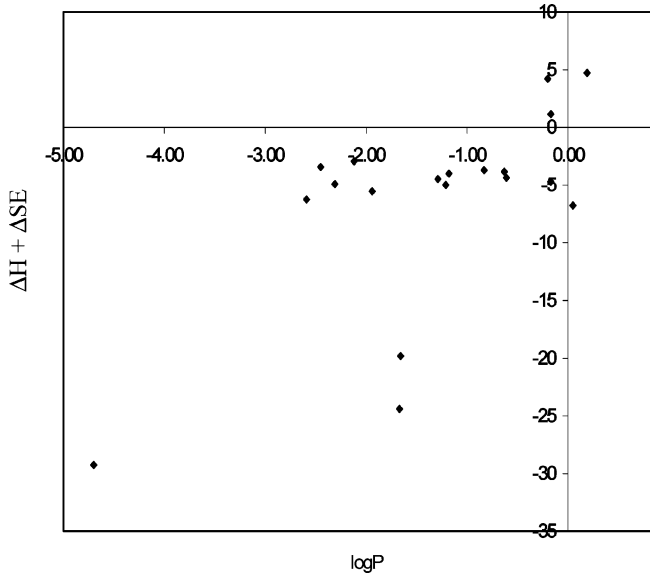


Figure 5. Analysis of energy change ($\Delta H + \Delta SE$) upon adsorption of amino acid lateral groups on PEG versus log *P*.

the value of the adsorption enthalpy (compare Gly with Ala with Val-Leu). Arg always presents a strong repulsive interaction.

Arg, Glu, and Thr show the most negative values of $\Delta(\Delta H_f)$ for the interaction with PEG. His, Asn, and Gln show positive values for $\Delta(\Delta H_f)$, and Phen and Tyr both show positive values. Gly, Ala, Val, Leu, and Ile present negative values for $\Delta(\Delta H_f)$. Because no clear trend could be found considering only the polar−nonpolar character of the side chain group, log *P* (partition coefficient in octanol/water) was used to test the importance of the complete residue in the adsorption.

Lateral and upper views of the same conformation show the placement of the side chain of the amino acid related to the surface's model (see Figure 3). Methyl groups from the surface seem suitable to suffer attractive van der Waals interactions with side chains from amino acids, especially ending with nonpolar H-containing groups. For log *P* values between −2.5 and +0.5, the values for both parameters are mainly negative, with no more than −5 kcal/mol for the interaction energy. Steric/enthalpic effects govern the interaction of side chain groups of amino acids with PP or PEG. The interaction with PP follows the trend found for the ΔH_f + SE of the amino acids versus log *P* (see Figures 4 and 5).

In case of the PEG model, data dispersion is wider and increases with values of log *P* more negative than −1.5. Enthalpy and steric energy changes follow opposite trends (see Table 4). The movement of the flap of CR is associated with a rearrangement of its secondary structure and the cis−trans isomerization of the peptide bond of Pro 92.[18] From results of Grochulski et al.,[18] the more affected residues from closed to open conformation are Glu 66, Thr 68, Lys 85, and Pro 92, that changes from cis to trans. Glu and Thr suffer strong interactions with PP, the same residues that are mainly involved in the opening of the flap of CR. Exposed residues in the flap in the closed and open forms are crucial to understand interfacial activation.[24,25,27]

3.4. Integrated Discussion. The strongest interactions of the PP model are with the nonpolar residues that in the nonactive conformation (native) of lipase are directed to inside the protein core. Interaction with PP would change the preferred location of the side chain nonpolar groups from inside the protein to outside the protein. Movements and changes in the secondary structure are proposed to take place in the water/PP interface, resulting in a final structure at the PP surface, different from aqueous solution. This interaction resembles the interfacial activation. We propose that adsorbed PEG can stabilize the exposed hydrophilic residues of the open CRL through interactions with exposed polar lateral groups and also increase the strength of the H bonds.

Lipase activity in the hydrolysis of esters in aqueous media increases when the lipase was previously treated with nearly anhydrous organic media.[19] Recent kinetic studies have been published.[28] Lateral groups probably interact favorably with the nonpolar solvent octane, increasing the stability of the conformation achieved at the immobilization step.[5] Data published from Sugimura et al. (2000) demonstrated that the surfaces of hydrophilic proteins become more locally hydrophobic due to induced conformational change.[20] In conclusion, PP/pH 7 buffer media resembles the needed interface, improving the opening of the flap by strong interactions of side chain groups of lipase's lid with PP. This proposal has been suggested before.[21] PEG seems to interact with polar lateral groups, stabilizing the open conformation of CR and promoting reaction with medium chain fatty acids. Treatment with octane after PEG/pH7 buffer contact increases the PP effect. The remaining PEG/buffer in the immobilized CR ensures the needed interface to maintain the lid opening.

4. Conclusion

An active catalyst for SF ethyl oleate synthesis was obtained when *Candida rugosa* was immobilized onto polypropylene. At 20 wt % (g/gFA × 100) of initial water

content, the activity was the highest for CRS and CRI. The effect of medium-chain-length fatty acids (C14−C16) present in commercial oleic (20%) cannot be ruled out. The use of PEG as a protector and the pretreatments with solvents such as octane and acetone increased the activity. We could not change the activity of immobilized, commercial enzymes (such as novozyme and lipozyme) using pretreatment. *Candida rugosa* lipase is activated during the immobilization procedure and the supported lipase can be further activated with a proper selected organic solvent treatment. The interesting observation is the effect of pretreatment with PEG and organic solvents of polypropylene adsorbed enzyme in the conversion of this commercial oleic acid (79% oleic acid). Such effects have been reported before, but mainly for lyophilized enzyme powders, and their occurrence with enzymes attached to polypropylene is a useful aid to our understanding of their mechanism. The activating effect can be related to the interaction with nonpolar residues with PP and of polar residues with PEG/water in such a way that the open conformation is stabilized in some way. The catalyst can be used twice without significant changes in conversion. MM2 and PM3 methods are useful to study the interaction of the side chain groups of the CR, or even lipases in general, with different surfaces/compounds and to analyze conformational effects at the local level. Forthcoming reports will present the use of these results in the analysis of different lipases activities.

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