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Interfacial activation and bioimprinting of *Candida rugosa* lipase immobilized on polypropylene: effect on the enzymatic activity in solvent-free ethyl oleate synthesis

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Abstract

Lipase from *Candida rugosa* adsorbed on polypropylene powder (CR/PP) was subjected to activation pre-treatments in order to enhance its activity in solvent-free ethyl oleate synthesis. The lipase activation achieved upon adsorption onto a hydrophobic support like PP was further enhanced through oil–water interfacial activation and bioimprinting of the immobilized catalyst. Several aliphatic hydrocarbons/buffer pH 7 mixtures were used in the pre-activation of CR/PP with specific activity increments of up to 29%. Molecular bioimprinting was also performed, with specific activity enhancement of near 70% with respect to non-treated CR/PP. The effect of several fatty acids used as templates and the water present in the reaction medium was studied. The oil–water activation and bioimprinting treatments that led to the best activities were assayed at the immobilization step. Instead of pre-treating CR/PP adsorbed in buffer medium, interfacial activation with octane/buffer and bioimprinting with a mix of fatty acids were carried out *in the immobilization vial*. The best results were found for CR/PP immobilized in 5/95 octane/buffer (v/v, %) medium. In that way, a biocatalyst with enhanced specific activity is obtained right from the immobilization vial with no need of further activation steps prior to reaction.
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Keywords: Adsorbed lipase; Interfacial activation; Bioimprinting

1. Introduction

To perform lipase-mediated synthesis in solvent-free media has a number of advantages. In absence of solvent, substrate concentration can be maximized, solvent cost and solvent separation are avoided, and downstream processing is reduced. Different strategies have been adopted in order to improve enzyme activity/stability in non-aqueous media: immobilization on supports of varied nature, surface modification with amphiphiles, enzyme crystal cross-linking, introduction of new bonds by protein engineering, change of solvent nature, incorporation into reverse micelles, use of surfactant coated enzymes and molecular bioimprinting [1].

Lipases are known to contain an amino acid lid covering their active site, which opens in presence of an oil–water interface. This phenomenon, called interfacial activation, has been found for *Candida rugosa* lipase (CRL) upon its adsorption onto hydrophobic supports [2], and especially onto polypropylene powder [3]. The immobilized lipase is fixed in an “open conformation” and enhanced enzymatic activity is achieved. In this work, further lipase activation is obtained through activating pre-treatments involving liquid–liquid interfaces. Maruyama et al. have reported that the closing of the lipase lid is caused by water, and the opening is caused by an oil–water interface in oil–water two-phase system. Both, closing and opening, cannot take place in organic solvents, where the lids of the lipase molecules will be closed because of the absence of an interface. If the lids can be kept open in advance, lipase is expected to have a high activity in organic solvents [4]. In this contribution, several hydrocarbon–buffer

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mixtures have been used as pre-treatment for lipase from *C. rugosa* immobilized on polypropylene powder (CR/PP) which was later used in ethyl oleate synthesis.

Improvement of enzymatic activity and stability in non-aqueous media has also been achieved by bioimprinting [5,6]. Bioimprinting consists of loading the enzyme's active site with a substrate analogue in an aqueous solution. A complex similar to an enzyme–substrate complex is formed and small conformational changes are supposed to occur—the induced fit. Then the ligand is washed away, but the enzyme is unable to adopt its former conformation due to its rigid structure which results from strong electrostatic interactions in the media possessing low dielectric constants [5]. González-Navarro and Braco found that the benefits of molecular bioimprinting strategy seem valid, not only for organic solutions but also for solvent-free mixtures of substrates [6].

In this contribution, both interfacial activation through oil–water interfaces and bioimprinting are tested in the pre-treatment of a biocatalyst already activated upon adsorption onto a hydrophobic interface. In particular for CRL adsorbed on polypropylene, in the author's knowledge, *this is the first time* that additional activation is obtained through liquid–liquid interface-pre-treatments and/or simple bioimprinting procedures. The water presence in reaction media (something apparently prohibited for bioimprinted molecules) has also been addressed.

2. Experimental

2.1. Materials

Low-molecular weight polypropylene powder (30,000 g/mol; BET area: 23 m^2/g) was obtained by polymerization using metallocenes. *C. rugosa* AY lipase (EC 3.1.1.3) (64,000 g/mol) was kindly donated by Amano Enzyme Inc. Oleic acid (99%) was purchased from J.T. Baker. Commercial fatty acid mixture (C_{14}: 01.6%, C_{14}: 11.9%, C_{16}: 05.7%, C_{16}: 111.7%, C_{18}: 179.1%—wt.%) was purchased from Quimicar-Olavarría, Argentina). Absolute ethanol (99%) and sulphuric ether (99%) were both purchased from Dorwil. Buffer solution of pH 7 (di-sodium hydrogenophosphate) and potassium hydroxide were both from Merck. Laboratory Ceblaco provided octane used in pre-activation experiments. Isooctane (2,2,4-trimethylpentane) was purchased from U.V.E., *n*-tedradecane (99%) from Sigma and polyethylenglycol (PEG; $M_w = 35{,}000$ g/mol) from Fluka.

2.2. Immobilization procedure

Four hundred milligrams of *C. rugosa* lipase was added to 50 ml of buffer solution of pH 7, and subjected to strong stirring during 30 min in order to solubilize lipase. A filtering step was performed to retain carbohydrates and other insoluble compounds. One gram of ethanol pre-treated PP was added to lipase solution and immobilization began. Samples from supernatant solution were periodically withdrawn, filtrated with special filters for small particle's powder, and diluted up to 5 ml with buffer for UV/vis analysis of lipase content. At the end of the immobilization period, the catalyst (CR/PP) was washed with distilled water, separated from solution and dried to constant weight.

2.3. Characterization of adsorption/desorption of water for free and immobilized *C. rugosa* lipase

In the open literature, there are several reports on water adsorption isotherms performed on free and immobilized lipases. In these works, organic solvents are used to equilibrate the water activities selected (a_w, defined as the water vapor pressure in equilibrium with the solution divided by the vapor pressure of pure water evaluated at the same temperature [7]), and construct an adsorption isotherm [8–10]. In this work, we propose a different way to study the behavior of free and immobilized lipase: successive adsorption/desorption of water, for increasing values of a_w of the gas phase. A Cahn Electrobalance 1000 with a device containing the salt solutions at the desired a_w was used to give the desired relative humidities or water activities of the gas phase. Successive water adsorption/desorption cycles were carried out to analyze the changes in free and immobilized lipase (10 mg of sample in all the cases) upon contact with water gas at different a_w (0.33, 0.44, 0.58, 0.75, 0.84, 0.94). Blank (without sample) and samples were equilibrated until no change in weight was found (each point required one full-day work).

2.4. Interfacial activation of CR/PP with aliphatic hydrocarbon/buffer pH 7 mixtures

2.4.1. Pre-treatment procedure

Fifty milligrams of immobilized lipase (CR/PP) was typically contacted with 5 ml of octane/buffer of pH 7 mixture for 30 min. The two-phase mixture was magnetically stirred at 45 °C (a temperature equal to reaction temperature) and 1000 rpm. The oil–water interfacially activated lipase was obtained by filtration of the mixture and dried at 45 °C for 1 h. When pure octane was used as pre-treatment medium (two experiments performed), the periods of contact were 10 and 60 min.

2.4.2. Nature of the pre-treatment mixtures

Increasing amounts of octane were used for the activation mixtures. The volume of the mixtures was always of 5 ml, with percentages of octane ranging from 2.5 to 100%. Total volume was completed with buffer of pH 7 solution. In the octane/buffer relationship that produced the catalyst with highest esterification activity, *n*-tetradecane and isooctane were tested. In the best condition, the addition of 25 mg of polyethylenglycol to pre-treatment mixture was also assayed (0.5:1, w/w, PEG:CR/PP). Pre-treatment of CR/PP with oleic acid and oleic acid/buffer pH 7 in the amounts used for reac-

tion during 30 min was also performed (approximately 80/20, v/v, oleic acid/buffer).

2.5. Bioimprinting

2.5.1. Bioimprinting procedure

Fifty milligrams of CR/PP was incubated for 20 min in a stirred mixture of very low amounts of oleic acid, ethanol (0.2 ml) and buffer of pH 7 (1.5 ml), kept at room temperature and 1000 rpm. The masses of oleic acid used (30–145 mg) ensured a molar ratio of 37.8 and 7.4 (mol ethanol/mol oleic acid), respectively. After incubation, the solid was recovered by filtration, washed with octane to remove the imprinted molecule and dried at 50 °C for 1 h to remove the solvent.

2.5.2. Effect of the acid used in the bioimprinting

Three different templates were used to bioimprint the catalyst: pure oleic acid (99%), undecenoic acid (99%) and a commercial mixture of fatty acids (C_{14}–C_{18}) with the major component being oleic acid (please see Section 2.1).

2.6. The reaction: esterification of oleic acid

The biocatalysts subjected to the described pre-treatments were tested in the direct esterification of ethanol (0.5 g) and oleic acid (3 g), with 0–0.6 g of buffer of pH 7 typically added. Mixtures were kept in 10 ml vials at 45 °C with magnetic stirring at about 350 rpm. They look like microemulsions where only one phase is observed. Characterization of the reaction medium is currently under study. Reaction began when either the non-activated or activated biocatalyst was added to the reactants. After 2 h, the reaction was stopped and the ester content was quantified by calculating the residual fatty acid in the reaction mixture using KOH titration methods. Samples were withdrawn using a needle inserted through the stopper of the reactor-vial. Sampling and weighting procedures were carefully designed to avoid mistakes and to assure reproducibility. They were dissolved in ethanol–sulphuric ether 50/50 (v/v, %) mixture (5 ml/5 ml), and titrated with KOH solution in ethanol, using phenolphthalein as the end-point indicator. It must be pointed out that activity measurements at 2 h of reaction do not belong to equilibrium conversions but to the maximum activity achievable in the present system with the current biocatalyst [3].

2.7. Activating treatments performed at the immobilization step

CRL was adsorbed on PP in different coupling media. In the same conditions detailed in Section 2.2, the original coupling medium (50 ml of buffer of pH 7) was changed for a medium consisting of 2.5 ml of octane and 47.5 ml of buffer (to resemble the best conditions found for oil–water interfacial activation) or in a mixture of 40 ml of buffer, 5.3 ml of ethanol and 800 mg of fatty acids mixture (to resemble the best conditions found by us for bioimprinting CR/PP prior to reaction). The activated biocatalysts were recovered by filtration, washed with distilled water and dried to constant weight.

An immobilization batch using octane as coupling medium was also performed. In this case, CR and PP were contacted with 15 ml of octane for only 2 h at room temperature and 350 rpm. The recovered solid was washed with distilled water and dried to constant weight.

3. Results and discussion

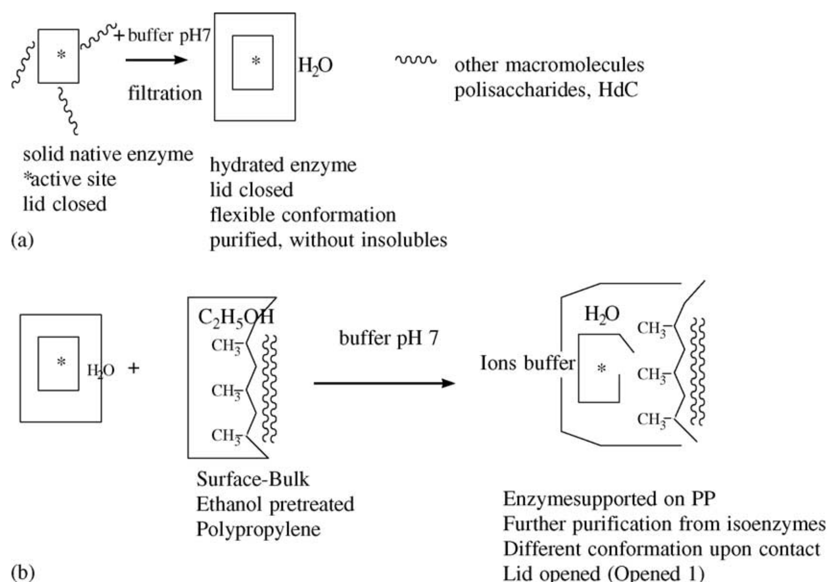
3.1. Activity of non-pre-treated CR and CR/PP

Soluble and PP-immobilized CR's specific activities were determined for the reaction described in Section 2.6. The specific enzymatic activity of the non-activated free lipase in ethyl oleate synthesis was determined to be 0.04583 (specific activity is defined as mmol of oleic acid converted/(h mg) of catalyst, in this case free lipase). Immobilized lipase, containing about 250 mg of lipase/g of catalyst, showed a specific enzymatic activity of 0.01136 mmol/(h mg) CR/PP (activity developed in 2 h at 45 °C using 50 mg of immobilized biocatalyst), or 0.04543 mmol/(h mg) CR present in CR/PP. From here and thereafter, when specific activity is mentioned, it is always in units of mmol/(h mg) of catalyst. Scheme 1 shows the changes suffered by free lipase upon filtration (a) and immobilization on PP in a pH 7 buffered-medium (b).

3.2. Characterization of free and immobilized lipase by adsorption/desorption of water

Fig. 1a and b shows, for free and supported lipase, respectively, the amount of water adsorbed from gas phase for each selected humidity (or a_w) always starting from clean sample. That is, each time the equilibration at one specific a_w was achieved, instead of going on adsorbing water, the sample was forced to desorb the humidity previously adsorbed from gas phase. Then, for the next a_w chosen, the value of adsorbed water presented belongs to an initial state in which no water is present in the sample.

The figures presented show two main important differences: the first one is the amount of water adsorbed in each case (please note the scales of the graphics). While the free lipase shows a maximum of 0.09 (adsorbed mass/dry sample mass), the one presented by the immobilized lipase is one order of magnitude lower. This finding was expectable, since the lipase present in 10 mg of CR/PP is much less (about 2–2.5 mg) than the one present in 10 mg of free lipase. Furthermore, the free lipase sample had not been previously dissolved and filtrated (as CRL used for CR/PP was), so other macromolecules, polysaccharides, etc. present in the commercial soluble lipase could also account for the higher water adsorption determined. Besides the magnitude of water adsorption, the other important difference between free and supported catalysts is the form of the adsorption curves ob-



Scheme 1.

tained. While only one peak in the curve of water uptake versus a_{w} or relative humidity is found for free lipase, two peaks are found for immobilized one. It seems that after the highest uptake of water (and its desorption), changes induced upon water adsorption greatly reduced its capacity to adsorb water at higher a_{w}. In the case of CR/PP (immobilized in a buffer medium), its behavior appears to be much more stable, with a more constant and reversible water uptake for higher a_{w}.

This experiment gave much more information than a traditional adsorption isotherm, demonstrating that lipase is supported on PP in a conformation different from free lipase, and that it is able to adsorb/desorb water in a more reversible way than free lipase does. These observations confirm that lipase is adsorbed on PP in a different conformation that the one presented by free lipase, a fact we had previously proposed in another manuscript using FTIR results [3,11].

3.3. Interfacial activation of CR/PP with aliphatic hydrocarbon/buffer pH 7 mixtures

Several hydrocarbon/buffer pH 7 mixtures were used in the pre-treatment of CR/PP. As a guide for the selection of hydrocarbons to use in the pre-treatment of the catalyst, their performance *as solvents of* lipase-catalyzed reactions was considered. Logarithm P ($\log P$) is a parameter widely used to account for the characteristics of the organic phase. It has been reported that if the solvent used has a $\log P > 3.5$, then the catalyst is protected from the organic media, since the solvent is not able to distort the water layer around it that is essential for maintaining the tertiary structure of the protein and thus, its activity [12]. In accordance with that observation valid for hydrocarbons *used as solvents*, *pre-activation of CR/PP* was performed using hydrocarbons with $\log P$ above 3.5: *n*-octane ($\log P$: 4.5), isooctane (4.51) and *n*-tetradecane (7.6).

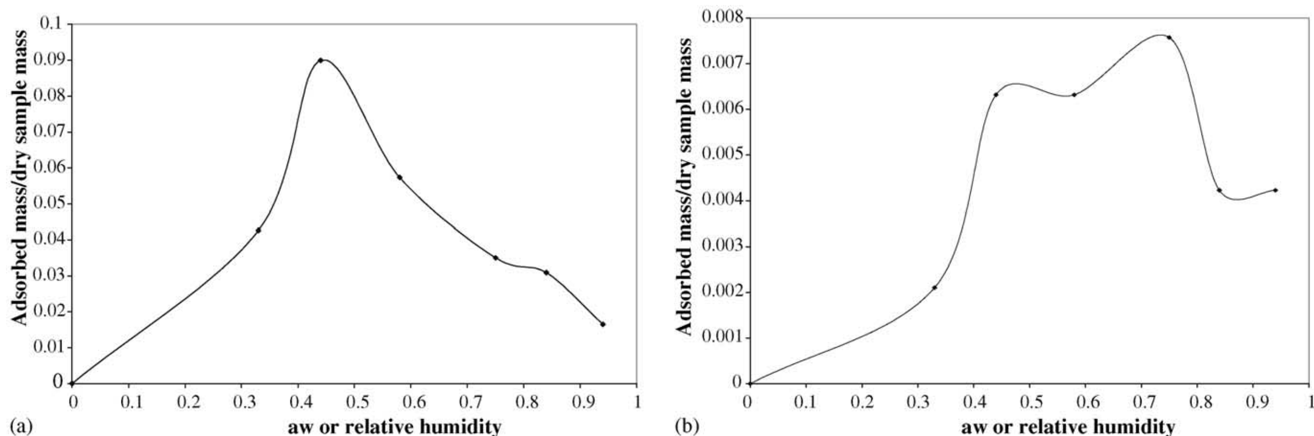


Fig. 1. Water uptake from gas phase in adsorption/desorption cycles: (a) free CR lipase and (b) CR/PP.

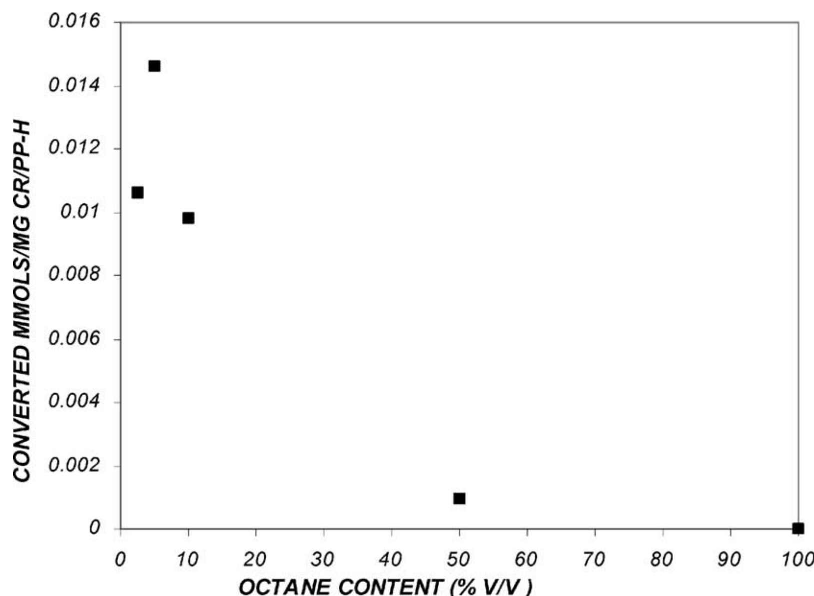


Fig. 2. Conversion to ethyl oleate catalyzed by 50 mg of pre-treated CR/PP. Effect of the amount of octane in the octane/buffer of pH 7 mixture of pre-treatment. Reaction conditions detailed in text (specific enzymatic activity of untreated CR/PP = 0.01136 mmol/(h mg) CR/PP).

3.3.1. Effect of the hydrocarbon/buffer pH 7 (v/v) ratio

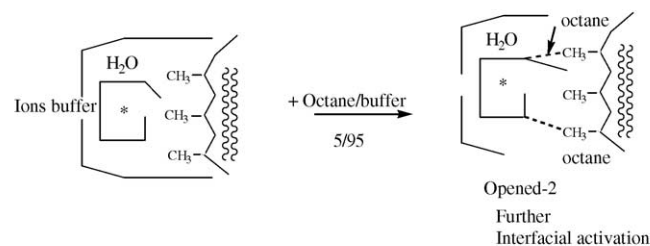
The activity of the pre-treated biocatalyst showed to be strongly dependent on the octane/buffer (v/v) relationship used. Fig. 2 clearly demonstrates that while 5/95 octane/buffer mixture led to an increase in specific enzymatic activity of nearly 30% was found—0.01480 per mg CR/PP or 0.05972 per mg CR in CR/PP—(experiment performed twice), pure octane pre-treatment completely deactivated the lipase (please see Table 1).

When using pure octane as pre-activating medium, the two experiments carried out (10 and 60 min of contact with the hydrocarbon) led to a complete inactivation of the biocatalyst. In anhydrous octane (even if its log *P* is high), the immobilized enzyme is completely surrounded by a hydrophobic solvent possibly leading to a dramatic irreversible inactivation and the subsequent absence of conversion in the esterification reaction of oleic acid [13].

Fig. 2 shows that when 2.5% of octane in volume was used, enzymatic activity showed to be lower than 5/95 pre-treated CR/PP. This suggests that the concentration of lipase in this experiment required a certain interfacial area to be activated, with a 5% oil phase providing sufficient interfacial area for successful lipase activation. Maruyama et al. also found a minimum amount of oil-phase needed for interesterification activity enhancement [4].

3.3.2. Effect of the hydrocarbon used

In the best ratio octane/buffer found (5/95, v/v), *n*-tetradecane and isooctane were tested. Octane was replaced with those two hydrocarbons in order to investigate if the use of hydrocarbons of longer or branched carbon chain had any influence in the activity of the pre-treated CR/PP. It was found that the activating effect of the oil–water interface did not vary with the length or ramification of the acid chain. No significant differences in activity were found with those hydrocarbons, all having a high log *P*. This behavior found for hydrocarbons used in pre-activating mixtures agrees with what Yahya et al. reported for their use as solvents. With solvents with high log *P* values, there is no general trend of increasing enzyme activity with increasing solvent log *P* [13]. However, the behavior found with pre-treated CR/PP differs from the one found by Maruyama et al. in the lipase-catalyzed interesterification of tripalmitin with stearic acid in *n*-hexane [4]. In their work, activity was significantly dependent on the hydrocarbon used for activation, with activity increasing with the carbon number of the aliphatic hydrocarbon up to *n*-tetradecane. They found that when hydrocarbons of higher number of carbons were used, the activity shifted to decrease gradually.



Scheme 2.

It had been previously reported that pH 7 was the optimum buffer pH to use in pre-activating mixtures [4], so no other buffer solutions were assayed. Scheme 2 shows the proposed changes upon pre-treatment with 5/95 octane/buffer.

Table 1
Effect of several pre-treatments and immobilization media on oleic acid conversion and CR/PP specific activity

Treatment of CR/PP	Description	Conversion of oleic acid (%)	Specific activity	
			mmol/(h mg)$_{cat}$	mmol/(h mg)$_{lip}$
None free CR (10 mg)	–	9	0.04583	0.04583
None	–	10	0.01136	0.04544
5 Octane/95 buffer pH 7	Pre-treatment, 30 min, 45 °C, 1000 rpm	14	0.01480	0.05972
Octane	Pre-treatment, 30 min, 45 °C, 1000 rpm	0	0	0
5 Octane/95 buffer pH 7 + PEG	Pre-treatment, 30 min, 45 °C, 1000 rpm, weight ratio PEG/CR/PP = 0.5	8	0.00846	0.03384
Oleic acid/oleic acid + buffer pH 7	Pre-treatment, 10 and 60 min, 45 °C, 1000 rpm	0	0	0
Bioimprinting C_{18}	*N* (ethanol/fatty acid) = 37.8, 20 min, 0.2 ml ethanol, 1.5 ml buffer	11	0.01285	0.05139
Bioimprinting mix C_{18}, C_{16}, C_{14}	*N* (ethanol/fatty acid mix) = 33.4, 20 min, 0.2 ml ethanol, 1.5 ml buffer	18	0.01912	0.07647
Bioimprinting C_{11}	*N* (ethanol/fatty acid) = 29.1, 20 min, 0.2 ml ethanol, 1.5 ml buffer	8	0.00853	0.03411
At the immobilization step	Immobilization in octane/buffer 5/95 (v/v, %)	14	0.01402	0.05609
At the immobilization step	Immobilization in octane	6	0.00618	0.02472
At the immobilization step	Immobilization in "bioimprinting medium" (conditions similar to bioimprinting with mix of fatty acids)	11	0.011508	0.04604

Reaction and pre-treatment conditions described in Section 2.

3.3.3. Hydrocarbon/buffer + PEG

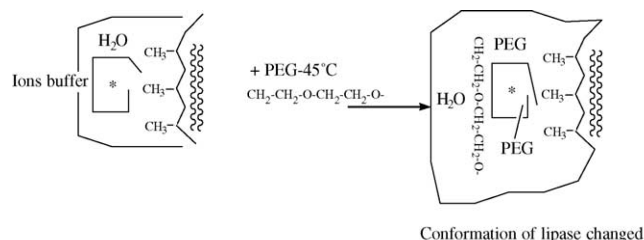
In the best condition of pre-treatment, found at 5/95 octane/buffer (v/v), 25 mg of PEG was added to the pre-activating mixture. The combination of interfacial adsorption of *C. rugosa* lipases on hydrophobic support surfaces plus additional hydrophilization of the rest of lipase with another random coil polycationic polymer (polyethyleneimine, PEI) had given excellent activity and stability properties in anhydrous organic media [14]. However, the specific activity found in the esterification reaction using the octane/buffer/PEG pre-treated biocatalyst (0.00846 per mg CR/PP) was more than 40% lower than the one achieved with 5/95 (v/v) octane/buffer pre-treated CR/PP (0.01480) and even lower than the specific enzymatic activity developed when no pre-activation experiments were performed on CR/PP (0.01136). The use of PEI or PEG is supposed to provide the immobilized lipases with a highly hydrophilic environment that preserves the essential layer of water molecules surrounding each immobilized lipase. However, in this case, the pre-judicial effect of the adding of PEG might be explained in terms of agglomeration of the catalyst. Being the catalyst more hydrophilic due to the contact with PEG, clumping of lipase is favored, leading to aggregate formation and inhomogeneous enzyme distribution in the reaction media. The enzyme molecules on the outer surface of such particles are exposed to high substrate concentrations (especially high when solvent-free media are used), but mass transport into a particle of clumped enzyme can severely limit the concentration of substrate inside the particle, reducing global enzymatic activity [3].

On the other hand, enhanced activity had been found in previous experiments using CR/PP/PEG in similar conditions using as substrate a mix of fatty acids (see Section 2.2) instead of pure oleic acid [15]. The good results achieved in that opportunity (CR/PP/PEG enhanced activity did not appear to be decreased by clumping), let us think not only of agglomeration but also of a PEG-induced change in lipase conformation that reduces its reactivity to C_{18}.

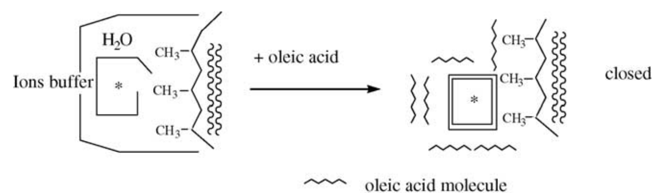
Scheme 3 shows a proposal of the PEG pre-treated lipase with a changed conformation upon contact with PEG.

3.3.4. Pre-treatment of CR/PP with oleic acid, oleic acid + buffer

Mingarro et al. reported a rational activation strategy to provide lipases with a surfactant–water interface, resulting in substantial enhancement of esterification activity in anhydrous solvents [16]. The oleic acid being a surfactant, CR/PP was pre-treated with oleic acid during 10 min at 45 °C and 1000 rpm. Neither the pre-treatment with pure oleic acid nor the pre-activation with oleic acid/buffer did show any conversion of substrates to ethyl oleate. Probably, the pre-contact of lipase with pure oleic acid (log P = 6.2) or with oleic acid/buffer mixture did not allow the alcohol to reach the active site, closed the lid, or both, and esterification did not proceed (see Scheme 4 that shows a proposal of the interaction of CR/PP with oleic acid).



Scheme 3.



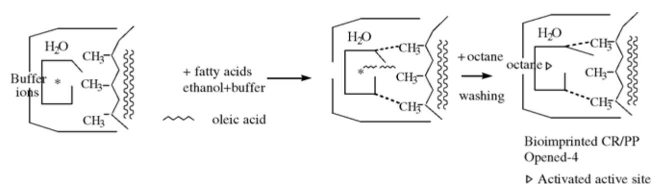
Scheme 4.

3.4. Bioimprinting of CR/PP

The strategy in molecular bioimprinting is to generate a ligand-induced beneficial conformational change in the enzyme in aqueous solution, and later use it in non-aqueous media where the enzyme is supposed to preserve the imprinted conformation. Lipases from *C. rugosa* seem to be successfully activated to a different extent by imprinting with different amphiphiles [1].

3.4.1. Effect of the acid used as template

The templates used for the imprinting of CR/PP were fatty acids in the range of C_{11}–C_{18}. Fatty acids have been previously used as imprint molecules showing to be useful for enhancement of the enzymatic activity in organic solvents [5]. Since the catalyst would be used in the esterification of oleic acid and ethanol, the first fatty acid chosen to be used as template was pure oleic acid. When ethanol/fatty acid molar ratio (N) was equal to 7.4, no enhancement in activity was found. A higher N of 37.8 led to a 13% enhancement in lipase activity with respect to non-pre-treated CR/PP (0.01136 per mg CR/PP or 0.04544 per mg CR present in CR/PP). However, this increase in enzymatic activity was not significant compared to the one found when a commercial mixture of fatty acids was used as template (please see Section 2.1 for details). CR/PP was imprinted with the fatty acids mixture with a molar ratio similar to the highest tested for pure oleic acid, and significant enhancement in enzymatic activity was achieved. The specific enzymatic activity achieved with the bioimprinted catalyst (0.01912 per mg CR/PP or 0.07647 per mg CR present in CR/PP) was 68% higher than the one achieved with untreated CR/PP, and almost 50% higher than the one achieved using pure oleic acid in the same alcohol/fatty acid ratio. Conversion of oleic acid increased from 10% for CR/PP to 18% in this case. It seems that the shorter fatty acids present in the commercial mixture accounted for



Scheme 5.

the bioimprinting of CR/PP. Since the main requirements for an imprint molecule are its solubility in the aqueous phase and resemblance to the natural substrate of the enzyme, the lower solubility of oleic acid in water might have made it a poor imprint molecule. Solubility in water in g/l at 20 °C for C_{14}:0 and C_{14}:1 is near 0.02 g/l, C_{16}:0 and C_{16}:1 near 0.007 g/l, whereas for C_{18}:1, solubility in water is near 0.003 g/l (see Scheme 5 representing the opened conformation of lipase bioimprinted with the mix of fatty acids).

In organic media, the dependence of the extent of imprinting-based activation on the carbon chain length of the fatty acids used as templates has shown an optimum at C_{10} acid [5], with a solubility of 0.15 g/l water. However, for our solvent-free system, bioimprinting with undecenoic acid led to an enzymatic activity of 0.00853 per mg CR/PP or 0.03412 per mg CR present in CR/PP, 25% lower than non-pre-treated CR/PP, and more than 55% lower than the one achieved with the bioimprinted CR/PP with the commercial fatty acid mixture.

Table 1 resumes all the data discussed above.

3.4.2. Effect of the amount of aqueous media in the reaction catalyzed by the bioimprinted biocatalyst

Activity enhancement due to bioimprinting is said to be restricted to anhydrous or microaqueous organic solvents, since the “memory” is lost in aqueous systems, unless additional stabilization measures are used [5]. It has been shown that, as a result of protein conformational relaxation facilitated by the ‘flexibilizing’ effect of water, the imprinted lipase ‘memory’ is gradually lost as the water content of the enzyme sample or the reaction medium is increased [6]. In the present work, when reaction was performed in absence of an aqueous medium, the activity increase upon bioimprinting of CR/PP was of 2.6 times. By the way, when 20% of buffer was used in the reaction mixture, the activity of the biocatalyst upon bioimprinting was increased 1.7 times. Although in a lower percentage, the activity of the bioimprinted biocatalyst was still enhanced in a medium containing as much as 20% of aqueous medium. Possibly, in a viscous media like this, water molecules were not able to strongly interact with the hydrophilic enzyme and introduce the flexibility needed for the molecule to revert to its native conformation eliminating the activation effect of bioimprinting. Besides, the possibility of reverse micelles formation and unavailability of lipase (that works at the interface) of an important fraction of water is certain.

3.5. Pre-treatment or bioimprinting at the immobilization step

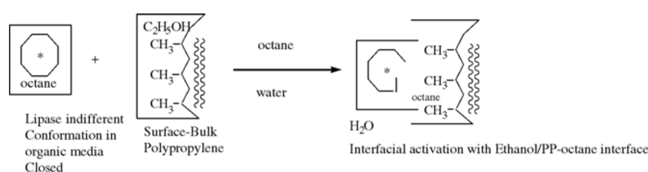
3.5.1. Immobilization + 5/95 octane/buffer

We studied the effect of 5/95 octane/buffer as immobilization media. In this case, the buffer medium usually used for the production of CR/PP was changed to an emulsion of buffer with 5% of octane. The produced biocatalyst ($CR/PP_{oct5\%}$) was tested in the typical ethyl oleate synthesis with no additional pre-activation pre-treatment. The specific activity developed (0.01402) was near 25% higher than the one achieved with untreated CR/PP. Thus, in this case, it appears that besides the support hydrophobicity, the use of a hydrocarbon/water interface with a solvent of low polarity was able to create around the enzyme a specific microenvironment that enhanced its activity. Similar results have been described by several researchers, indicating a new trend in the use of organic apolar solvent as a coupling medium for lipase immobilization on different support types (see reference [7] and references therein).

Furthermore, from a comparison of the specific activities obtained developed by $CR/PP_{oct5\%}$ and the ones achieved with octane/buffer 5/95 (v/v) pre-treated CR/PP, the values are surprisingly similar (0.01402 versus 0.01480). It appears that similar activation is achieved by pre-treating CR immobilized on PP in a buffered medium or by using $CR/PP_{oct5\%}$ with no additional pre-activation step. The importance of this fact is that, if the enhancement in activity due to the octane/buffer interface can be achieved at the immobilization step, catalyst handling and extra time devoted to pre-activation steps prior to each reaction would be avoided.

3.5.2. Immobilization + 100/0 octane/buffer (immobilization in octane media)

It has been reported that the adsorption of *C. rugosa* lipase using heptane as coupling media gave an immobilized catalyst far more active than using sodium phosphate buffer solution as immobilization medium [17]. Immobilization of CR/PP in a medium of octane was assayed (see conditions in Section 2.7). However, if the enhancement in enzymatic activity reported in reference [17] was to happen with CR/PP immobilized in pure octane, no correspondence with the 5/95 case would be found. That is, CR/PP immobilized in buffer solution and pre-treated with 5/95 octane/buffer (v/v) and CR/PP immobilized in a medium of 5/95 octane/buffer (v/v) led to the same esterification activity. So, if the same was to happen with pure octane, the octane coupling medium was supposed to completely deactivate $CR/PP_{100\%oct}$. In fact, the



Scheme 6.

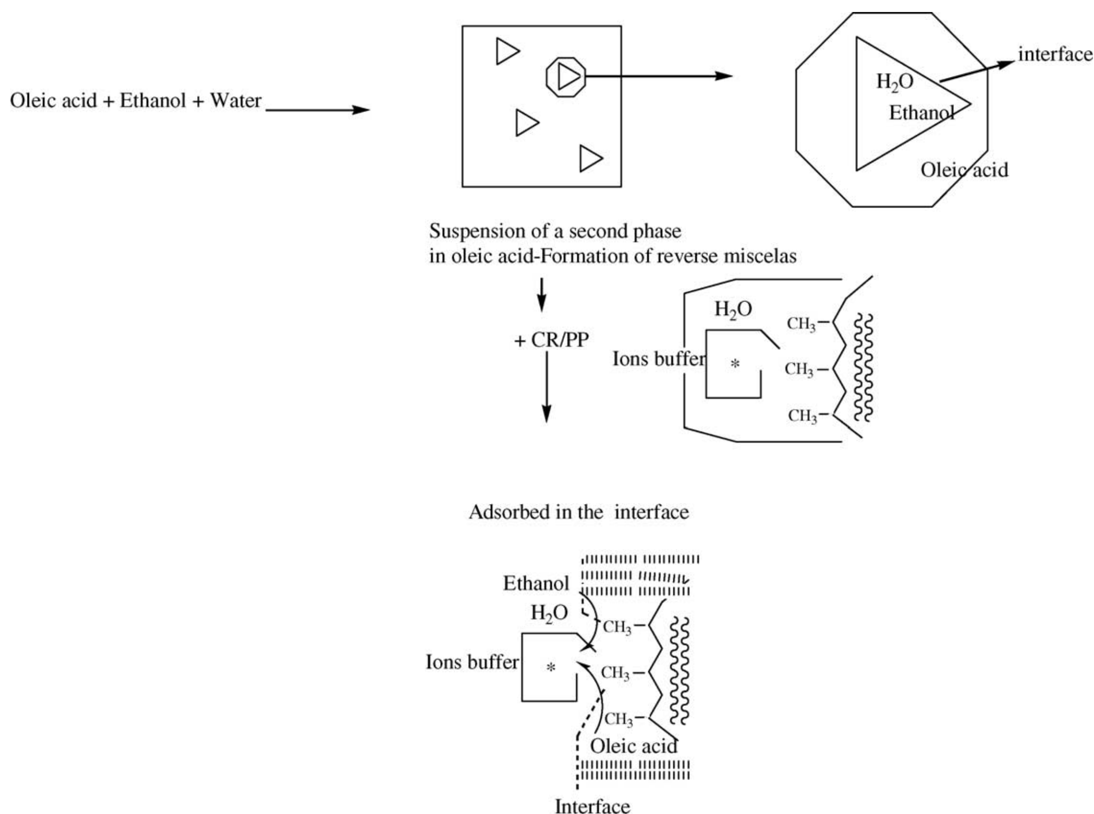
lipase adsorbed in a medium of octane, *washed with distilled water before use*, led to a specific synthetic activity of 0.00618 (reaction conditions were the ones described in Section 2.6). This value is definitely better than the one obtained with CR/PP pre-treated with octane (in that case, no activity was detected), but nearly one-half of the specific activity developed by non-pre-treated CR/PP (and nearly 60% lower than the one achieved with octane/buffer 5/95 pre-treatment or with $CR/PP_{oct5\%}$). Apparently, the deleterious effect of pure octane on CRL can be partially reversed if contacted with water before reaction.

According to our data, this may be explained in terms of the conformation of the enzyme molecule which changes according to the medium polarity. Whereas in buffer solution, the enzyme tends to fold so that more hydrophilic amino acids become exposed to the coupling solution, in a medium of low polarity, the enzyme should attain a different conformation with the more hydrophobic amino acids at the surface [17]. Because of changes in equilibrium between open and closed forms, it is probable that in octane as immobilization media, the conformation at surface of PP is not "as open as" in the case of the adsorption in buffer pH 7 or in buffer pH 7/octane in 95/5 (v/v) medium. With these results in mind, a trend in the "grade of opening of CR" or the "quality of the opening" at the surface of PP can be identified: buffer pH 7/octane (interface) > buffer pH 7 > octane.

In the case of the water/octane mix, the order of immobilization/contact with octane/buffer did not affect the final result in the CR conformation at the PP surface, but in the case of pure octane, it showed to be crucial. It seems that in octane-pre-treated lipases, the lids are closed and unavailable to the interface at the reaction media: the supported lipases are surrounded by octane molecules where water and ethanol are poorly soluble and therefore, no reaction is found. Although when octane is the immobilization media, the same phenomenon should take place; the in situ formation of an octane/water interface at the washing step seems to be enough to partially revert deactivation. Scheme 6 shows the proposed local structure and surroundings of CR/PP immobilized in octane.

3.5.3. Immobilization + bioimprinting

An additional immobilization experiment was carried out in order to find out if the combination of bioimprinting with adsorption onto PP was a powerful technique to obtain an even more active and stable enzyme preparations. In the work of Fishman and Cogan, the combination of imprinting with immobilization of CRL onto celite or cellulose showed to be a synergistic process that led to further enhancement of the activity of lipases in organic solvents [5]. However, for CR lipase immobilized on PP in a "bioimprinting medium" (CR/PP_{BIOIMP}), the enzymatic activity found (0.011508) was



Scheme 7.

Table 2
Lid structure, composition of the surroundings of the biocatalyst and substrate with lowest solubility in them, detailed for different pre-treatments and immobilization procedures described in the text

Treatment of CR/PP	Description	Surround immobilization	Interfacial Activation	Initial structure lipase at the reaction media		
				Lid	Surrounding	Substrates with lowest solubility in the surrounding
Free CR (10 mg)	–	–	–	Opened in situ	Reaction media interface	–
None	–	Buffer pH 7	I = yes	Opened 1 (Scheme 1)	Water buffer pH 7	Oleic acid
5 Octane/95 buffer pH 7	Pre-treatment, 30 min, 45 °C, 1000 rpm	Buffer pH 7	I = yes, T = YES	Opened 2 (Scheme 2)	Octane/buffer distributed 5/95	None
Octane	Pre-treatment, 10/60 min, 45 °C, 1000 rpm	Buffer pH 7	I = yes, T = no	Closed	Octane + remaining buffer	Water/ethanol
5 Octane/95 buffer pH 7 + PEG	Pre-treatment, 30 min, 45 °C, 1000 rpm, weight ratio PEG/CAT = 0.5	Buffer pH 7	I = yes, T = yes	Opened 2 + PEG (Scheme 3)	Octane/buffer distributed + PEG on polar side chains	Oleic acid
Oleic acid/oleic acid + buffer pH 7	Pre-treatment, 10 min, 45 °C, 1000 rpm	Buffer pH 7	I = yes, T = no	Closed (Scheme 4)	Oleic acid, oleic acid + water	Water/ethanol
Bioimprinting C_{18}	*N* (ethanol/fatty acid) = 37.820 min, 0.2 ml ethanol, 1.5 ml buffer	Buffer pH 7	I = yes, T = yes, active site not locked	Opened 3 (Scheme 5)	Water + ethanol + remaining octane	Oleic acid
Bioimprinting mix C_{18}, C_{16}, C_{14}	*N* (ethanol/fatty acid) = 37.820 min, 0.2 ml ethanol, 1.5 ml buffer	Buffer pH 7	I = yes, T = yes, active site locked	Opened 4 (Scheme 5)	Water + ethanol + remaining octane	Oleic acid
Bioimprinting C_{11}	*N* (ethanol/fatty acid) = 37.820 min, 0.2 ml ethanol, 1.5 ml buffer	Buffer pH 7	I = yes, T = yes, active site not locked	Opened 5 (Scheme 5)	Water + ethanol + remaining octane	Oleic acid
At the immobilization step	Immobilization in octane/buffer 5/95 (v/v, %)	Octane/buffer–water (washing)	Yes	Opened 2	Octane/buffer distributed	Oleic acid
At the immobilization step	Octane 100%, the immobilization media	Octane–water (washing)	I = no, after washing = yes	Partly closed (Scheme 6)	Octane–water	Ethanol
At the immobilization step	Immobilization in a “bioimprinting medium”, (conditions similar to the ones described for bioimprinting with mix C_{18}, C_{16}, C_{14})	Mix $C_{18} + C_{16} + C_{14}$ + water + ethanol–water (washing)	I = yes, active site not locked	Opened 6	Water + ethanol	Oleic acid

Opened 1—lid opened by contact with PP surface at the PP/buffer pH 7 interface; opened 2—lid opened by contact with PP surface at the PP/buffer pH 7 interface plus contact with interface octane/buffer-reinforced effect; opened 3—lid opened by contact with PP surface at the PP/buffer pH 7 interface. Active site partially reacted with oleic acid and ethanol available. Formation of acyl enzyme at least in some of the active sites; opened 4—lid opened by contact with PP surface at the PP/buffer pH 7 interface. Distribution of C_{18}–C_{14} following their solubility. Stronger effect of shorter fatty acids (20%, w/w) than oleic acid (80%, w/w); opened 5—lid opened by contact with PP at the PP/buffer pH 7 interface. Negative effect of C_{11} on the active site structure or on the availability of C_{18} for *Candida rugosa* lipase; opened 6—interfacial activation + bioimprinted combined. Lipase supported in a no imprinted structure and activity similar to no treatment.

Note: All catalysts subjected to bioimprinting *as pre-treatment* were washed with octane prior to their use in reaction.; I = immobilization step; T = treatment step.

40% lower than the one obtained when immobilization in buffer medium and bioimprinting with fatty acids mixture were consecutive steps. The last three rows of Table 1 present the results obtained when "activating" treatments were performed at the immobilization step.

3.6. Surrounding of the pre-treated catalyst and its influence on activity

Camacho Paez et al. [18] reported that conflicting results published on the influence of water on the rate of the lipase catalyzed reactions are due to a physical phenomenon: the proximity of the enzyme's active sites to the interface and therefore, its access to the substrates. In this work, the reaction medium is composed of oleic acid (our 'solvent'), ethanol, water and the free or immobilized lipase, *with a particular surrounding and structure depending on the immobilization medium and the pre-treatment or washing solution used before reaction*. Scheme 7 shows the reaction medium faced by pre-treated (or not) CR/PP.

Each pre-treatment/washing changes the local surrounding of the lipase and also the lipase conformation. Moreover, in different lipase microenvironments, substrates have different solubilities and therefore, their opportunity to reach the active site is also conditioned. Table 2 summarizes the local surroundings of lipases added to reaction medium according to the pre-treatment that they have been subjected to. A comparison of the items of the table demonstrates that no activity is found when lipase presents an irreversible closed form that reaction medium is not able to revert. In particular, when ethanol/water have solubility problems in the lipase surrounding (due to its non-polar nature), the catalyst does not work in the proper manner. The fact that the use of octane as coupling medium produces an active catalyst if washed with water, whereas as pre-treatment deactivates completely the catalyst, demonstrates that even low amounts of water near the enzyme are enough to assure the availability of the active site to ethanol. In reference to water, it has shown to be crucial not only for polar substrate solubilization but also for achieving an adequate interfacial area [3]. The proper amount of water needed in reactions carried out in SFS deserves careful analysis. In this SFS, for example, we strongly believe that from the amount of water added to reaction medium (20%), a high fraction is unavailable to the free or PP-immobilized lipase that works at the interface. The possibility of a mix from microemulsion to microemulsion plus biphasic system in this kind of SFS when water is added will be the topic of a forthcoming paper.

4. Conclusion

CR/PP was subjected to several pre-treatments in order to enhance its catalytic activity in the synthesis of ethyl oleate. Among the pre-treatments that involved the formation of an oil/water interface, the mixture containing 5% of hydrocarbon and 95% of buffer of pH 7 showed to be the best one. Using this ratio, a specific activity increase of 29% was found when compared with the one developed by untreated CR/PP. The experiments using octane, isooctane and tetradecane in the same ratio demonstrated that in this system, the length or branching of the hydrocarbon used as oil phase did not affect the activation achieved.

In the bioimprinting experiments performed, not only the solubility but also the length of the hydrocarbon chain and the position of the double bond of the fatty acid used as template did show it to be very important (undecenoic acid with the highest solubility of all the fatty acids tested and one terminal double bond did not show to be useful for bioimprinting purposes). It seems that a mixture of fatty acids of different lengths, saturated and no saturated (C_{14}: 01.6%, C_{14}: 11.9%, C_{16}: 05.7%, C_{16}: 111.7%), performs better than pure oleic acid, leading to an increase of more than 68% in specific activity compared with untreated CR/PP. In reference to the water content of the reaction medium, in this SFS, the use of 20% buffer pH 7 did not revert the bioimprinting achieved with the mix of fatty acids. Although the enhancement of activity due to bioimprinting is higher in absence of water, it seems that the high viscosity of a solvent-free reaction medium avoids conformational relaxation and makes it possible for the catalyst to retain its new structure, even in a reaction medium with a relatively high amount of water.

Whereas bioimprinting at the immobilization step did not significantly increase synthetic activity, immobilization using a 5/95 octane/buffer pH 7 emulsion as coupling medium resulted in a biocatalyst with enhanced specific activity. Yet more important is the fact that the specific activity of the obtained catalyst was similar to the one achieved with pre-treated octane/buffer 5/95 (v/v) CR/PP (immobilized in a buffer medium), but with the benefit of an "activated catalyst" taken straight from the bottle. When pure octane was used as coupling medium, although the produced catalyst was active in SFS, the activity developed showed to be lower than the one achieved in the octane/buffer emulsion and even lower than the obtained with CR/PP immobilized in an aqueous medium.

The experiments performed pointed out the importance of the surroundings of the enzyme that remain after immobilization or pre-treatment steps, and the solubility of substrates in those surroundings.

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