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Zeolites as supports for an enzymatic alcoholysis reaction

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Abstract

A recombinant cutinase from *Fusarium solani pisi* was immobilized by adsorption on several zeolites and its activity towards the alcoholysis reaction of butyl acetate with hexanol, in organic media (isooctane), was measured as a function of the water content and water activity. The effects of the zeolite framework composition (including cation nature) and acidity were studied. The results were compared with other commonly used supports: polyamide Accurel-PA6, silica and alumina. Both the nature of the cation and the silica:alumina (Si:Al) ratio of the framework revealed to be important parameters. The most promising results were obtained for supports with little acidity and with lower Si:Al ratio. This last observation is in accordance with the results obtained with silica and alumina.

Keywords: Zeolites; Enzymatic catalysis; Cutinase; Alcoholysis; Enzyme immobilization

1. Introduction

Supports have a very significant influence on the activity of supported enzymes. In the case of reactions carried out in organic media, the water content of the support is also of paramount importance [1]. Water soluble lipases are a very important example; in organic media they have to be adsorbed on a solid support in order to have significant catalytic activity [2,3]. Deposition by adsorption on a support is one of the simplest methods of physical immobilization of enzymes. Parameters such as particle size, surface area, pore diameter, mechanical strength, microbial resistance, thermal stability, chemical durability, hydrophobic/hydrophilic character, ease of regeneration, loading capacity and cost, will influence the behaviour of the catalyst and its practical and technical applications [4]. In the particular cases of hydrolysis, esterification and transesterification, the water content of the support is another important parameter [3].

Zeolites are crystalline silico-aluminates with a very precise micro-porous structure and present some well-known general features responsible for their extensive applications in catalysis, adsorption and ion exchange [5]. These properties are related to the possibility of generating and regulating the acid–base and hydrophobic–hydrophilic character, and strong selective adsorption affinities. The properties of the zeolite depend mainly on the particular zeolite structure, framework composition, cation nature and pore structure, and can be easily

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modified. The water affinity and adsorption capacity of zeolites can be manipulated over a broad range [5]. Thus, the zeolite external crystallite surface, presenting interesting properties for the adsorption of enzymes, can be used as an interface between an aqueous phase, within the zeolite, and an organic medium containing the substrates.

Lipase immobilized on zeolites has already been tested in the hydrolysis and esterification reactions in a triolein/oleic acid water insoluble system [6]; it was verified that the results were quite dependent on the zeolite framework composition (Si:Al ratio). Immobilization of other enzymes on zeolite supports has also been described [7–10], as well as the use of these solids for applications in other areas of biotechnology [11–17].

In the present study, a *Fusarium solani pisi* recombinant cutinase was immobilized by adsorption/deposition on different supports (several zeolites and other supports that were used for comparison). Cutinase is a lipolytic enzyme that catalyses the hydrolysis of the water-insoluble biopolyester cutin that covers the surface of plants [18]. It has been used in an immobilized form [19] or encapsulated in reversed micelles [20–23] to carry out esterification and transesterification reactions, as well as ester synthesis [24]; reactions which have a great importance in the food [25,26], chemical [27] and pharmaceutical [28] industries, among others.

In a previous study, zeolites have been successfully used as supports for cutinase on the hydrolysis reaction of tricaprylin [29,30]. The immobilizations prepared in this work were used to catalyze the butyl acetate alcoholysis with hexanol in isooctane, a reaction that can be promoted if appropriate water activity levels are reached. The catalytic tests were performed for a broad range of water contents and water activities. Several series of related zeolite samples were used to study a possible correlation between zeolite properties and the catalytic properties of the enzyme. The effect of the nature of the cation contained within the zeolitic structure was analyzed using lithium, sodium, potassium, caesium and calcium A zeolite. The effect of the Si:Al ratio was studied on faujasites and ZSM-5 in their sodium forms. As the reaction can also proceed through acid catalysis, a ZSM-5 sample with Brönsted acidity was tested. The results obtained with the zeolites were also compared with other commonly used supports, namely polyamide Accurel-PA6, silica and alumina.

2. Experimental

2.1. Enzyme

F. solani pisi cutinase was produced by an *Escherichia coli* WK-6, a kind gift from Corvas International (Ghent, Belgium). The fermentation, extraction and purification were carried out following a protocol developed in our laboratory from the procedure of Lauwereys et al. [31]. The enzyme purity was controlled by electrophoresis and isoelectric focusing. A single band was observed, corresponding to a molecular weight of 22,000 Da and an isoelectric point of 7.9 was obtained.

2.2. Preparation and characterization of the supports

The sodium form of Y zeolite, NaY, was obtained from Union Carbide. The sodium forms of A and X Zeolites, NaA and NaX, and zeolite CaA correspond to commercial designations 4A, 13X and 5A, obtained from Aldrich. Zeolites KA, CsA and LiA were prepared by ion exchanging zeolite NaA, using 2M nitrate solutions of the corresponding cation. The ion exchange procedure, for each zeolite preparation, consisted in five consecutive ion exchanges, the first at room temperature and the others at approximately 100°C with reflux. Each one was performed for 5 h with stirring. The zeolite $HNaZSM5_1$ was obtained by thermal treatment

at 550°C of zeolite $ZSM5_1$ supplied by UOP Molecular Sieves, and its sodium form, $NaZSM5_1$, was obtained by ion exchanging the $HNaZSM5_1$ with a 2 M sodium nitrate solution. The zeolite $NaZSM5_2$ was obtained from $ZSM5_2$ (supplied by PQ Zeolites) by a similar treatment to that of zeolite $NaZSM5_1$. The zeolites NaDY and NaM were obtained by thermal treatment at 550°C of zeolites DY (dealuminated Y zeolite) and Mordenite, respectively, followed by ion exchanging with 2 M sodium nitrate solutions. The zeolites DY and M were supplied by PQ Zeolites.

The silica gel tested was from BDH, with particle sizes between 200 and 350 μm and average pore dimensions of 375 Å. Alumina was from Condea (Pural SB, alpha-alumina monohydrate), with particle sizes distribution up to 90 μm; and Accurel-PA6 was obtained from AKZO (EP 700), with particle sizes < 1000 μm and pore dimensions between 0.05 and 0.3 μm.

Zeolites were characterized by X-ray diffraction in a model P Philips diffractometer equipped with a CuKα anticathode, in order to check their crystallinity level. The dimensions of their crystallites were evaluated by scanning electron microscopy (SEM). The composition, crystallite size and pore apertures [32] of the zeolites tested in this work are presented in Table 1. The elemental chemical analysis showed that all the sodium forms and the cation exchanged samples presented levels higher than 90% for the main cation (relative to the total cationic positions), with the exception of the calcium (62%) and caesium (40%) samples.

Table 1
Composition, crystallite dimensions and pore apertures of the zeolites [32]

Zeolite	Si:Al ratio	Crystallite size (μm)	Pore apertures (Å)
NaY	2.7	0.5–1	7.4
NaDY	20	0.5–1	7.4
NaX	1.5	2	7.4
NaA	1	1–2	3.8–4
CaA	1	1–2.5	4.3–4.5
KA	1	1–2	3–3.3
LiA	1	1–2	3.8–4
CsA	1	1–2	–
$NaZSM5_1$	19	0.5–1.5	5.3×5.6
$HNaZSM5_1$	19	0.5–1.5	5.3×5.6
$NaZSM5_2$	40	0.5–1	5.3×5.6
NaM	18	0.2–0.5	5.9×7.1

2.3. Enzyme immobilization

Cutinase was immobilized by deposition. Enzyme solutions prepared in 50 mM sodium phosphate buffer (pH 8.0) were added to the solid (25 mg of cutinase/g of support), according to the procedure previously developed [29]. The volume of cutinase solution added to the supports was 4.0 ml/g for polyamide Accurel PA6 and 2.5 ml/g for the other supports. After vortex mixing for 1 min, the preparations were vacuum dried. The final water content was controlled by the duration of the vacuum drying procedure.

2.4. Protein and water determinations

The amount of enzyme immobilized on the support, just before the reaction and after reaction and washing of the preparation with the organic solvent, was determined by a modified Folin assay [33]. The water content of the preparations was determined using a Mettler DL 18 Karl Fischer titrator [34].

2.5. Activity assay

The immobilized enzyme was used to catalyze the alcoholysis reaction of butyl acetate (500 mM) with hexanol (400 mM) in organic media (isooctane), resulting in the formation of hexyl acetate and butanol. The reaction was initiated by the addition of butyl acetate, and was carried out in a batch reactor, on an orbital stirrer at 400 rpm and 30°C.

The activity was determined following the formation of the hexyl acetate, by UV absorption at a wavelength of 220 nm, using a HPLC system with a C18 reverse-phase column with

isocratic elution using a mixture of 60% acetonitrile and 40% water.

Blank tests were also performed for this reaction system. When no enzyme was present, no substrate transformation was registered. When free enzyme was used (in the absence of the support), the observed enzyme activity was less than 1% of the highest value obtained with the immobilized enzyme preparations.

2.6. Water activity determination

Water activity determinations of the preparations with different amounts of water were done at 30°C in a Rotonic HygrosKop DT equipped with a humidity sensor of lithium chloride (DMS-100H). This determination was limited to water activity values up to ≈ 0.95. To obtain samples with water activities higher than 0.95, the samples were equilibrated with salt solutions presenting known water activities [35], and then the water amounts were obtained.

3. Results and discussion

The influence of the water content on the initial reaction rate is similar for all the samples inspected. As the water content is increased, the activity goes through a sharp maximum and finally levels-off for higher water contents, as it can be seen in Figs. 1, 3, 5 and 6. This allure corresponds to the competition between two different factors: a certain amount of water is necessary to hydrate the enzyme and allow it to function properly, so that the activity increases as the amount of water increases, for low water contents; as more water is made available, the hydrolytic transformation of the ester begins to compete with the reaction under study, resulting in a decrease of the alcoholysis reaction rate. Therefore, the optimal water content for the alcoholysis reaction in the organic medium should be a compromise between the required hydration of the enzyme and a low hydrolytic activity.

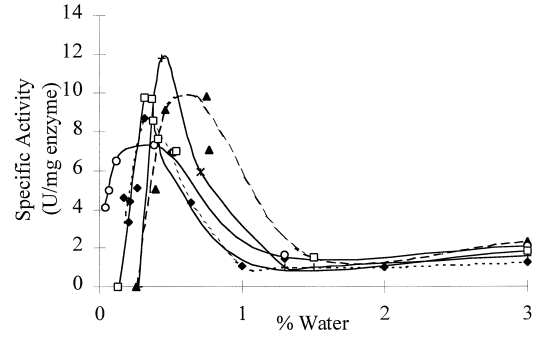

Fig. 1. Effect of the water amount (volume of water/volume of reactional media) on the specific activity of immobilized cutinase on zeolites NaA (♦), KA (o), CaA (▲), LiA (x) and CsA (□).

It is also apparent from the experimental data that, although the general behaviour is similar for all the supports, the values of activity obtained for a given water content value are very much dependent on the support itself. As it was previously said, the ability of the support to adsorb water is of paramount importance in these applications, as clearly demonstrated in the study of Reslow et al. [36].

The amount of cutinase immobilized on the supports in this work presents similar values before and after the reaction, so that we can infer that no significant desorption of the enzyme during the reaction process occurs. The observed amounts of enzyme immobilized on the supports ranged from 12–18 (mg enzyme)/(g support) for the zeolite preparations, without any noticeable systematic variation, while a range from 10.5–17 (mg enzyme)/(g support) was observed for the other supports. A slight tendency for the amount of enzyme immobilized to decrease when the water content increases is observed.

3.1. Effect of the cation within the zeolite

Zeolites being crystalline silico-aluminates, where the aluminium is in a tetrahedral arrangement, have within their structure one cation per each aluminium atom in the framework. These cations are easily exchangeable and affect very drastically the properties of the zeolites in their

various applications. In the present case, the effect of the nature of the cation was studied using zeolite A exchanged with different cations: Li, Na, K, Cs and Ca. This zeolite, the most aluminium rich, presents the highest number of cations per unit mass. The data for the initial enzymatic activities are presented in Fig. 1, as a function of the preparation water content (expressed as the volume of water/volume of reaction media).

The range of specific activities that were observed was not much different from one sample to the other, probably because all the preparations were done using the same zeolite framework. Nevertheless, the optimal activity value was obtained for different amounts of water in the reaction; this water amount increased in the following order: KA ≈ CsA ≈ NaA < LiA < CaA. This indicates that the higher the charge density of the cation, the higher the water content for optimal activity will be.

Additional information can be obtained by looking at the water activities for the preparations of this set of zeolites, as a function of the water amount (Fig. 2). The amount of water needed to reach the same water activity increases with the increase of the acidity of the cation. As it can be seen in the data presented in Table 2, the optimal enzymatic activities were obtained when the water activity approached its maximum value; from this situation on, increasing the water content will only result in decreasing the activity.

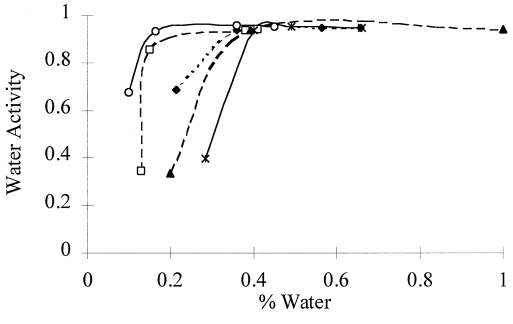

Fig. 2. Water activity of the preparations of immobilized cutinase on zeolites NaA (♦), KA (o), CaA (▲), LiA (x) and CsA (□).

Table 2
Enzymatic activities, water amounts and water activities for preparations using zeolite A with different compensating cations

Zeolite	Enzyme activity (U/mg enzyme)	Water amount (%)	Water activity
CaA	0	0.25	0.48
	9.1	**0.46**	**0.95**
	9.9	**0.75**	**0.95**
	7.1	0.77	≈ 1
	1.52	1.5	≈ 1
LiA	0	0.28	0.40
	11.8	**0.44**	**0.97**
	5.9	0.71	≈ 1
	0.99	1.3	≈ 1
	1.0	1.5	≈ 1
NaA	3.3	0.21	0.68
	5.1	0.27	0.77
	8.7	**0.32**	**0.90**
	6.9	0.51	0.96
	4.3	0.64	≈ 1
	1.1	1.0	≈ 1
	1.4	1.03	≈ 1
KA	6.0	0.07	0.68
	6.5	0.12	0.70
	7.3	**0.38**	**0.96**
	7.0	0.52	0.97
	1.7	1.3	≈ 1
CsA	0	0.13	0.35
	9.8	**0.32**	**0.96**
	9.7	0.37	0.97
	8.6	0.38	≈ 1
	7.6	0.41	≈ 1
	7.0	0.54	≈ 1
	1.5	1.5	≈ 1

This reinforces the idea that the ability of the support to supply water to the enzyme is critical. Thus, the nature of the cation in the zeolite structure, which will affect the water binding to the support, will influence the amount of water that must be present in the reaction media for a high activity to be obtained. Zeolites containing cations with a higher charge density will bind water more tightly and, thus, will produce smaller water activities for the same water contents, which is in general accordance with the results presented, since charge density increases in the following order Cs < K < Na < Li < Ca. Adding water beyond the necessary water activity level will result in increasing the competing hydrolysis reaction and, thus, decreasing the alcoholysis reaction rate.

These results are similar to the results obtained by Adlercreutz [3], in his study of the water effect on the alcoholysis activity of chymotrypsin on different supports. This author concluded that most support materials did not greatly affect the shape of the activity vs. water activity profile, though the absolute activities did vary. In this case, the highest rate was observed with celite as support and a water activity of 0.94.

3.2. Zeolite framework composition

One of the most important parameters in the zeolite framework composition is its Si:Al ratio. In fact, the aluminium content of the crystalline network will determine the global framework charge, as well as the amount of cations in the structure, and will drastically influence the hydrophobic/hydrophilic and acid/base character of the structure.

Several sodium forms of zeolites, with different framework compositions, were tested. The results obtained with the preparations using zeolites NaX, NaY, NaDY and NaM are shown in Fig. 3A. The results for the zeolites $NaZSM5_1$ and $NaZSM5_2$ are presented in Fig. 3B. Zeolites NaX, NaY and NaDY have a related structure (faujasite), but different Si:Al ratios (see Table 1). Zeolite NaM has a Si:Al ratio similar to that of NaDY, but a different structure. The $NaZSM5_1$ and $NaZSM5_2$ samples correspond to two samples of zeolites with a ZSM-5 structure but different Si:Al ratios, as can be seen in Table 1.

Both figures show that zeolites with a high Si:Al ratio present lower enzymatic activities. High silica zeolites carry less framework charge and are commonly referred to as hydrophobic, presenting also the lowest Lewis basic character; the opposite holds true for high alumina zeolites that are labelled hydrophilic and present the highest Lewis basic character. Hence, by increasing the Si:Al ratio of a given zeolite framework, the hydrophilic and the basic Lewis character decrease, and so does the activity. It is

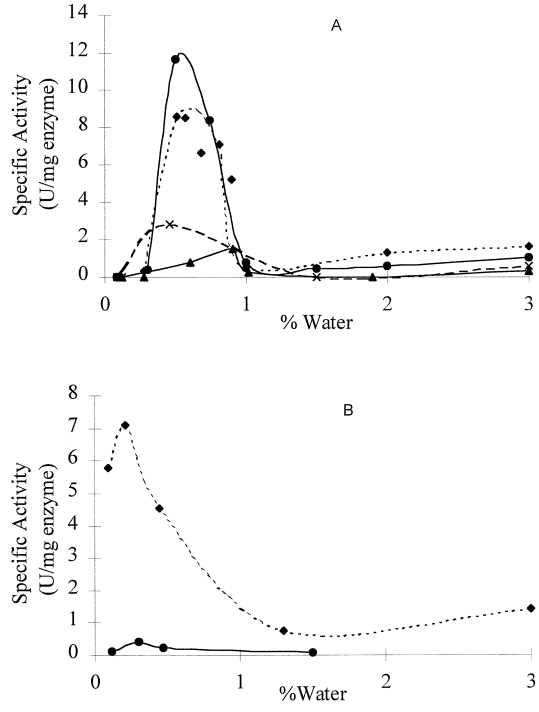

Fig. 3. Effect of the water amount on the specific activity of immobilized cutinase on zeolites NaY (●), NaX (♦), NaDY (▲) and NaM (x) (A), and $NaZSM5_1$ (♦) and $NaZSM5_2$ (●) (B).

not clear, however, if the basicity is the main factor, since zeolite NaX, a more basic zeolite, has a smaller maximum activity than NaY. NaA (see Fig. 1) which has the lowest Si:Al ratio has a behaviour similar to NaX. All these results seem to indicate that there is an optimum Si:Al ratio, which corresponds roughly to the composition of NaY.

Water activity, however, seems not to be relevant in this context. In fact, all faujasitic structures revealed similar water activity curves as a function of the water amount in the reaction media, despite the wide difference in specific enzymatic activities. (Fig. 4)

3.3. Effect of acidity

The study of the effect of zeolite Brönsted acidity was carried out with the comparison of the results for the samples of $NaZSM5_1$ and $NaHZSM5_1$, the latter presenting a strong acid-

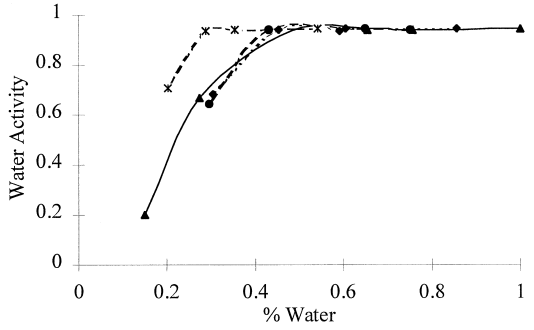

Fig. 4. Water activity of the preparations of immobilized cutinase on zeolites NaY (●), NaX (♦), NaDY (▲) and NaM (x).

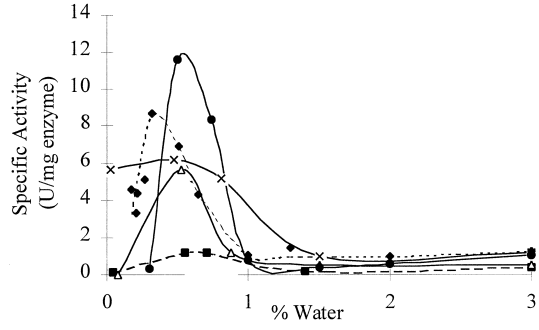

Fig. 6. Effect of the water amount on the specific activity of immobilized cutinase on zeolites NaY (●) and NaA (♦) and other supports silica (■), alumina (△) and Accurel-PA6 (x).

ity. The negative effect observed in the immobilized enzyme activity (see Fig. 5) is remarkable. The acidity of the support may induce some damage to the enzyme, either by ion exchange with the buffer enzyme solution cations or by direct interaction of the acid sites in the external surface. This negative effect of acidity was already observed in a previous work concerning hydrolysis reaction [29].

3.4. Comparison with other supports

Supported enzymes showed a wide range of activity values, depending on the type of support used. Besides the various series of zeolitic materials, other supports that are commonly used for immobilization, such as silica, alumina and a polyamide support (Accurel-PA6) were also tested. The results can be seen in Fig. 6, where they are compared with the results obtained with NaY and NaA. The activities observed for the preparations with these zeolites presented maximum values that were higher than those for the other three supports.

The average amount of immobilized enzyme was also slightly higher in the zeolite preparations. Therefore, if the activity values were expressed per unit mass of the support, the differences would be slightly higher, despite the fact that cutinase adsorption occurs only at the external surface of the zeolite crystal while it also occurs inside the macroporosity of the other supports.

From the water activity vs. water amount representations in Fig. 7, it is clear that the water activity reaches values close to unity much earlier in silica and PA6, while alumina has a behaviour which is closer to those of the NaA and NaY zeolites; this, however, cannot explain

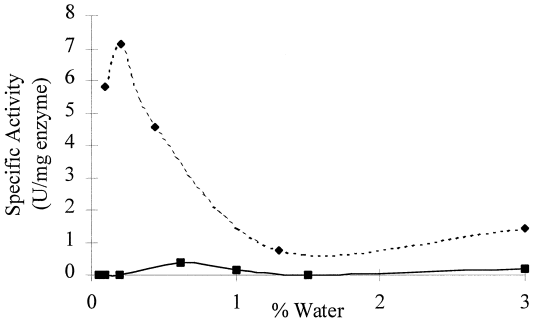

Fig. 5. Effect of the water amount on the specific activity of immobilized cutinase on zeolites $NaZSM5_1$ (♦) and $NaHZSM5_1$ (■).

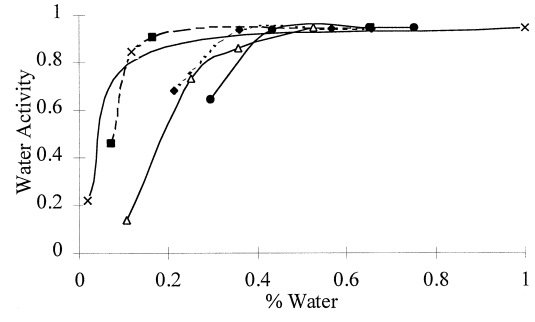

Fig. 7. Water activity of the preparations of immobilized cutinase on zeolites NaY (●) and NaA (♦) and other supports silica (■), alumina (△) and Accurel-PA6 (x).

the relative catalytic activities that are observed. It is also interesting to note that the enzymatic activity with silica is considerably lower than that for all other supports in this comparison, and comparable to those that are observed for the zeolites with high silicon content. The activity obtained with alumina is also lower than that of the zeolites with higher aluminium content, a fact which is consistent with the idea that an optimum Si:Al ratio exists for this reaction.

4. Conclusions

Cutinase can be efficiently adsorbed on zeolites. The corresponding immobilizations are effective in the promotion of the alcoholysis reaction in isooctane, although dependent on the zeolite characteristics.

The structure and composition of the zeolite have a marked influence on the catalytic activity that is observed and there seems to be an optimum Si:Al ratio for the structures, which corresponds roughly to the composition of NaY zeolite. This is indeed fortunate, since this is one of the most widely available and cheapest zeolites.

Considering the highly heterogeneous surface of the zeolite samples, with multiple adsorption sites (framework oxygen, silanol groups and some compensating cations) and the chemical structure of the enzyme, it is easily envisaged that a strong interaction could exist between the enzyme and the surface. Assuming that the enzyme activity presented by a given immobilization depends on the enzyme–support adsorption interactions, as well as on the substrate partition between the organic solvent and the support surface, it is quite understandable that the results are dependent on the framework composition. The enzyme–support interactions are probably affected by the amount of framework charges either directly or indirectly due to the change in the adsorption properties of the solid.

As already observed for other systems used in transesterification reactions in organic media, the enzymatic activity dependence of the water content of the zeolite immobilizations shows a maximum value for an optimal water content. This should correspond to a compromise between the required minimum hydration of the enzyme and low hydrolytic activity.

In this context, water interactions with the support also seem to be critical on the catalytic performance of the preparations, although water activity seems to be insufficient to assess this effect. Although some correlation was observed between the amount of water necessary to attain near-unity water activity and the amount of water for optimal activity, in the case of the LiA, NaA, KA, CsA and CaA series, this could not be observed for the influence of other parameters related to the zeolite composition. It is thus envisaged that we should look at other forms of quantifying the water–support interaction.

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