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Zeolites as supports for enzymatic hydrolysis reactions. Comparative study of several zeolites

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Abstract

Fusarium solani pisi recombinant cutinase was immobilised by adsorption on different zeolites and its activity towards the hydrolysis reaction of tricaprylin was measured. It was observed that the enzyme immobilised over most of the zeolites used (NaA, NaX, NaY, NaUSY and modified forms) was active for this reaction, although the specific activity depended strongly on the structure and composition of the zeolite support. This preliminary study of the use of zeolites as supports for enzymes was also extended by the analysis of the influence of the water content and of the thermal stability of one of the preparations. It can be concluded that the prospective use of zeolites as supports for enzymes in reactions involving organic media is very promising.

Keywords: Zeolites; Lipase; Hydrolysis

1. Introduction

The use of immobilised enzymes can increase the productivity of the biocatalytic processes [1,2], by concentrating the catalyst on the reactional media, as well as improving its catalytic and stability properties. The immobilisation process also allows for the enzyme reuse, and in some cases, is responsible for the activation of the active sites themselves. The continuing search for better supports is, therefore, of extreme importance when trying to improve the use of enzymatic preparation in industrial applications.

Lipases have been successfully used in the hydrolysis of triglycerides, for the production of free fatty acids. They are effective biocatalysts when acting at an interface between an insoluble substrate (triglycerides) and an aqueous phase wherein the lipases are soluble, which renders these enzymes quite suitable for the applications in non-conventional media [3]. These reactions can be carried out in reversed micellar media, where the water necessary for the reaction is stored inside reversed micelles [4], while the enzyme carries out its activity in the lipidic interface separating the organic phase from the aqueous phase. When used on a support matrix, the activity and quantity of the water retained by the immobilisation support are very important factors for the hydrolytic enzyme activity in non-aqueous solvents.

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Taking into account the characteristics that have been mentioned above, it is envisaged that it will be worthwhile to test zeolites as lipase immobilisation supports. In fact, zeolites are known to be capable of storing a large amount of water in their intracrystalline void space, and one could easily envisage the use of the external surface of the zeolitic material as the interface between the aqueous phase, contained within the zeolite's framework, and the organic medium where the substrate lies, in much the same way as one uses a reversed micellar medium, only with the distinct advantage of the use of a solid support, thus facilitating enzyme recovery and reuse.

The well-known general features of zeolites arise from their negatively charged aluminosilicate crystalline structures. They consist of a three-dimensional arrangement of SiO_4 and AlO_4^- tetrahedra linked to each other by a shared oxygen atom [5]. The resulting framework leads to the generation of an intracrystalline porous network with pore apertures of molecular dimensions and to the presence of compensating cations within the framework itself. These features have been responsible for the extensive application of these materials in catalysis, separation processes and ion-exchange [6,7], among other fields. The possibility of creating and regulating acid–base, hydrophobic–hydrophilic and selective adsorption properties within a wide range of values constitutes another important factor in the widespread use that zeolites have known. The properties of a particular zeolite will depend mainly on its structure, and, usually, they can be easily modified by a whole range of thermal and chemical treatments of varied complexity, namely by cation exchange or dealumination.

Another important aspect is the possibility of synthesising zeolitic structures that range from hydrophobic to highly hydrophilic, making them suitable both for processes involving aqueous and for those using organic media. The zeolites presenting hydrophilic character, exhibit a remarkable water affinity; with the void spaces within the zeolites framework being able to contain up to 30% of their weight in water, with a strong adsorption interaction.

Very little attention has been focused on this type of zeolite applications, although the use of a widely available solid such as Y zeolite, could bring huge benefits in terms of enzyme immobilisation. Lie and Molin [8] immobilised a lipase in two zeolite samples with very different Si/Al ratios and tested its activity towards the hydrolysis and esterification reactions in a triolein/oleic acid water insoluble system; it was verified that the high Si/Al ratio zeolite preparation (low framework charge – high hydrophobicity) was not efficient for the hydrolysis reaction but could mediate esterification, while the other zeolite immobilisation (low Si/Al ratio – high hydrophilicity) produced the reverse results. The adsorption properties of a commercial 13X zeolite for the immobilisation of an acid phosphatase have also been evaluated by Alfani et al. [9].

In the present study, a *Fusarium solani pisi* recombinant cutinase displaying lipolytic activity was immobilised by adsorption on different zeolites, all presenting hydrophilic character. The immobilised enzyme was tested on the hydrolysis of tricaprylin in organic medium. As this reaction can also proceed through acid catalysis, zeolite samples with a varying degree of acidity were also prepared. The framework composition, expressed by the Si/Al ratio, was also changed.

Other relevant parameters that can influence the catalytic activity of the enzyme, such as the amount of water and the stability of the immobilised enzyme, were also analysed.

2. Experimental

2.1. Preparation and characterisation of zeolite supports

The sodium forms of zeolites A and X, NaA and NaX, were obtained from BDH chemicals.

The sodium form of Y zeolite, NaY (LZY-52), and the ammonium forms of the ultrastabilised Y zeolite, NH_4USY (LZY-82), and of a dealuminated Y zeolite, NH_4DY (LZY-210), were obtained from Union Carbide.

NaUSY and NaDY samples were prepared by ion exchanging NH_4USY and NH_4DY, respectively, with 2 M sodium nitrate solutions; three consecutive ion exchanges were performed at room temperature, and two more at 100°C in order to achieve a more extensive ammonium removal. After each exchange the sample was analysed by thermogravimetry, recording the weight loss corresponding to the residual ammonium.

The strongly acidic form of the ultrastabilised Y zeolite, HUSY, was prepared by calcination at 500°C, under a flow of dry air, of the corresponding ammonium form, NH_4USY.

A series of NaPrY samples was obtained from the NaY sample by exchanging the Na^+ cations with the praseodymium trivalent cations Pr^{3+} in different extensions. This preparation was carried out by ion exchange with different concentrations of $Pr(NO_3)_3$ solutions, followed by calcination at 500°C. The detailed experimental execution, as well as the final composition of the samples, are described elsewhere [10].

The samples were characterised by X-ray diffraction in a model P Philips diffractometer equipped with a CuK α anticathode.

The thermogravimetric analysis were performed in a model TA2 Mettler thermobalance equipped with differential thermal analysis.

The dimensions of the crystallites of the samples were determined by scanning electron microscopy (SEM). The composition and crystallite size of the zeolite supports used in this work are presented in Table 1.

Table 1
Composition and crystallite dimensions of the zeolite supports

Support	Framework Si/Al	Compensating cations	Crystallite size (μm)
NaA	1.0	Na^+	1–2
NaX	1.5	Na^+	1–2
NaY	2.4	Na^+	0.5–1
NaUSY	4.5	Na^+	≈ 1
NaDY	6.0	Na^+	≈ 1
NH_4USY	4.5	NH_4^+	≈ 1
NH_4DY	6.0	NH_4^+	≈ 1
NaPrY1	2.4	0.76 Na^+ + 0.08 Pr^{3+}	0.5–1
NaPrY2	2.4	0.52 Na^+ + 0.16 Pr^{3+}	0.5–1
NaPrY3	2.4	0.34 Na^+ + 0.22 Pr^{3+}	0.5–1
HUSY	4.5	H^+	≈ 1

2.2. Enzyme preparation and characterisation

Cutinase is an extracellular enzyme from the fungus *Fusarium solani pisi* that displays lipolytic activity. Its gene was cloned in *Escherichia coli* and was a gift from Corvas International (Ghent, Belgium). Cutinase production and purification were done according to previously published methods [11]. The enzyme purity (600 U/mg) was controlled by electrophoresis and isoelectric focusing. A single band was observed, corresponding to a molecular weight of 22000 D and an isoelectric point of 7.9 was obtained.

2.3. Enzyme immobilisation

Before the immobilisation all the zeolite samples were dried at 200°C. Cutinase was immobilised by deposition. Enzyme solutions with concentrations ranging from 2 to 20 mg/ml were prepared in 50 mM sodium phosphate buffer (pH 8.0). After some preliminary catalytic tests, the enzyme concentration chosen and used further on in this work was 10 mg/ml. A volume of 0.250 ml of this solution was added to 100 mg of zeolite (2.5 ml/g of the support); after vortex mixing during 1 min the preparation was vacuum dried for at least 24 h.

2.4. Water determinations

Water content of the immobilised cutinase was determined using a Mettler DL 18 Karl Fischer titrator [12]; similar values for the water content were obtained through thermogravimetric analysis.

2.5. Activity assay

The enzymatic activity was determined by the hydrolysis of tricaprylin in organic medium (2,2,4-trimethylpentane), the formation of caprylic acid was followed by the Lowry and Tinsley method [13].

The enzyme preparations (100 mg) were hydrated, by the addition of a controlled amount of water, followed by the addition of 10 ml of a solution of tricaprylin (100 mM) in organic solvent to start the reaction. The reactions were carried out in an orbital stirrer at 400 rpm, pH 8.0 and 30°C. The ratio of water/isooctane was 2.7/97.3 (v/v).

Blank tests were performed for this reactional system, without the presence of the enzyme; no substrate transformation was registered.

The same activity assay was also performed with the pure enzyme, without immobilisation; no measurable enzymatic activity was observed.

2.6. Protein determination

After the activity assay, the organic reaction mixture is removed, the preparation is washed with the organic solvent and dried. The immobilised enzyme was then determined by a modified Folin assay [14].

3. Results and discussion

3.1. Cutinase / support ratio

The effect of the cutinase/support ratio in the activity properties and coupling yield for the immobilisation on NaY zeolite, determined after reaction, can be observed in Table 2. The fraction of cutinase that remains immobilised after reaction is fairly constant and does not present any clear dependence of the enzyme/support ratio for the range values that was studied. This indicates that we are always operating with a non-saturated surface. In fact, taking an average value of 0.75 μm for the size of the spherical crystallites of NaY sample, with density of 1.3 [15], and considering an estimated roughness factor of only 1.5, we will have an external specific surface area of 9.2 m^2/g. From the coupling yields indicated, and considering that each cutinase molecule will occupy an area of 30×30 $Å^2$ (taking into account the known molecular dimensions of cutinase), we will obtain a maximum surface coverage significantly lower than saturation (78% for the case of cutinase/zeolite = 50 mg/g).

Table 2
Effect of cutinase/support ratio on the coupling yield for the immobilisation on NaY zeolite, and respective catalytic activity for the transformation of tricaprylin

Cutinase/zeolite (mg/g)	Activity (U/g sup)	Specific activity (U/mg enzyme)	Coupling yield (%)
5	28.7	9.4	61
10	47.8	6.9	69
20	67.4	6.1	55
25	91.7	5.4	69
30	85.2	4.2	67
40	91.6	3.3	69
50	97.5	3.3	59

The global activities increased with the enzyme concentration up to 25 mg/g sup., after which there was no clear tendency. The above mentioned value was chosen for the further preparations with all the other zeolite samples.

3.2. Immobilised cutinase properties

Zeolite supported enzymes showed a wide range of activity values, depending on the type of zeolite that was used. The effect of the support on the properties of immobilised cutinase can be seen in Table 3.

We can observe that the coupling yields are usually rather high, indicating that the enzyme has a very good affinity towards the zeolite's surface. Although there is not a very large difference between the coupling yields for the various solid samples, the lowest values are obtained for the ammonium forms, while the highest ones are seen on the most acidic solids. The specific activity, however, changes widely

Table 3
Coupling yield for the immobilisation of the enzyme, and respective catalytic activity for the transformation of tricaprylin for different zeolite supports

Support	Framework Si/Al	Activity (U/g sup)	Specific activity (U/mg enzyme)	Coupling yield (%)
NaA	1	13.5	0.74	73
NaX	1.5	20.3	1.08	75
NaY	2.4	91.7	5.41	69
NaUSY	4.5	39.5	2.14	74
NaDY	6.0	77.0	4.03	76
NH_4USY		9.3	0.57	66
NH_4DY		0	0	52
NaPrY1		12.8	0.66	77
NaPrY2		11.1	0.5	79
NaPrY3		12.4	0.60	82
HUSY		4.7	0.19	87

with the zeolite preparation, going from virtually nil to values as high as 5 U/mg of enzyme. Both these factors indicate that zeolites can be *effectively* used in this reaction, but a closer inspection of the way the supports properties influence the enzymatic activity is needed, so that we can understand how the zeolitic material should be designed in order to achieve a maximum specific activity.

The set of zeolites that was used in this study allows us to extract some comparative information regarding several important parameters, and we will look into each of them separately.

3.2.1. Effect of support acidity

The set of zeolites that was used covers a large range of acidity. From the rather basic forms of NaX and NaY, to the highly acidic HUSY zeolite. In the series of PrNaY samples, the progressive substitution of sodium cations by praseodymium can generate moderate acidity. In fact it is well known that a multivalent cation, such as Pr^{3+}, can hydrolyse water molecules producing some acid centres, as shown below [16]:

$$Pr^{3+} + H_2O \rightarrow Pr(OH)^{2+} + H^+$$

Increasing the exchange level leads to an increase in the number of acid sites; their acid force is also improved, particularly for the highest praseodymium contents. This increase in the acid strength is mainly a result of the inductive effect, which should increase the polarisation of the framework–H^+ [10] bond, conferring a higher charge to the hydrogen atom. For the samples in analysis, the exchange levels were not sufficient for the production of strong acidity, as was seen, in a previous study, by their paraffin cracking ability [10]. The protonic forms of the zeolites, namely that of HUSY present a very strong acidity.

It is remarkable the negative effect that the support acidity produces on the immobilised enzyme activity. Even a quite weak acidity, like the one presented by the PrNaY series, significantly reduces the enzyme's catalytic ability. The strong acid sample, HUSY, causes a very drastic reduction in activity.

It is likely that the support acidity may induce some damage to the enzyme, through ion exchange with the buffer enzyme solution cations or by direct interaction of the acid sites in the external crystallite surface in the adsorption process. The coupling yields are always higher in the cases where significant acidity is present, as in the PrNaY series or HUSY, suggesting that there are stronger interactions with the enzyme.

The net balance, however, is clearly negative and we can conclude that acidity must be as low as possible if one is to use the zeolite support in this reaction.

3.2.2. Zeolite framework composition

There are several aspects concerning zeolite composition. The first aspect is related to the zeolite's structure and framework composition (mainly the Si/Al ratio). To analyse this we will focus now on the sodium forms of the zeolites. Several samples of non-acidic zeolites with different framework compositions were then tested as enzyme supports. The results for these zeolites corresponds to the first group of results in Table 3.

The main difference between them can be expressed by the framework Si/Al ratio, since the only significant difference in the structure of the zeolites in question is zeolite A.

Zeolite A presents an intracrystalline porous network with pore apertures of 3–4 Å, while all the other samples have pore apertures of 7–8 Å. Nevertheless, neither the enzyme nor the substrate molecules can penetrate this internal porosity and, thus, the adsorption of the enzyme is bound to occur only on the external surface of the crystallite. Both X and Y zeolites have very similar structures.

The immobilisation coupling yield was not significantly affected by the differences in the crystallite size of the samples (see values given in Tables 1 and 3).

Since the aluminium framework content will be the most determining factor for the framework charge, and its distribution, it is an important parameter in determining the adsorption properties, and several other zeolite features. Considering that the enzyme activity presented by a given preparation will possibly depend on the enzyme–support adsorption interactions, as well as of the substrate partition between the organic solvent and the support surface, it is not surprising that the results for the hydrolysis activity are dependent of the framework composition.

For NaA, NaX and NaY preparations, the enzyme's hydrolytic activity increases as the Si/Al ratio increases. This may be due to a slight decrease in the hydrophilic character of the surface, which can possibly increase the affinity of the substrate to the support.

Further increasing the Si/Al ratio (NaUSY and NaDY samples) implies a decrease in the activity, despite the decrease in the hydrophilic character. The enzyme–support interactions will be eventually affected by the reduction of the framework charges and/or by the change in the adsorption properties of the solid.

In the case of the NaUSY preparation, its 'anomalous' lower activity value when observing the dependence on the framework Si/Al ratio in the series presented in Table 2, might be the consequence of the very particular kind of treatment to which the zeolite has been subjected. In fact, the LZY-82 sample used for the preparation of the NaUSY support is prepared from a Y zeolite sample with lower Si/Al ratio by a hydrothermal dealumination procedure that produces the deposition of the extracted aluminium within the porous structure, as cationic species [17] as well as a significant amount of meso-pores. The presence of these Lewis acid species, such as the extra-framework aluminium, is known to influence the charge distribution on the framework through the inductive effect, thus creating a much different environment from the other Y zeolites that we have studied. In addition, the existence of meso-pores will probably have a large effect on the adsorption properties of this material towards large molecules, such as the enzyme molecule, and it is even possible that some of the enzyme molecules could be entrapped within these pores. So, the result obtained for the NaUSY preparation is probably influenced by these factors, which are not present in the other cases.

Another interesting aspect in relation to the zeolite's composition is the influence of the cation. We have already discussed the presence of acidity, created by the total or partial substitution of the sodium cations by protons or by praseodymium trivalent cations. In both these cases it is possible that the main influence that the presence of these cations induces in the supported enzyme's activity is due to the acidity that is generated. A different behaviour is observed if the sodium cations are substituted by ammonium ions. These should only generate a negligible acidity. However, the net result is a simultaneous decrease of the coupling yield of the enzyme and of the specific activity. In fact, from all the supports that have been tested in this work, the ammonium forms present the lowest values both for the coupling yield and for the specific activity; in the case of the ammonium form of the dealuminated Y zeolite the activity was too small to be measured.

This behaviour of ammonium forms contrasts markedly with the protonic forms, since in the latter case the decrease in activity is not accompanied by a decrease in the coupling yield. This indicates that the main influence in the case of ammonium forms of the zeolites is not acidity.

From all these observations we can conclude that although the interaction of the enzyme with the zeolite probably occurs mainly at the outer surface of the crystallites this interaction is extremely sensitive to the parameters that govern the bulk composition and structure of the zeolite. The nature of these interactions, however, will need further investigation. From all the preparations tested so far, the sodium forms of X and Y zeolites seem to be the most promising.

3.2.3. *Effect of the water / organic solvent ratio*

Since the water content constitutes another important factor in the observed hydrolytic activity, the preparations NaX, NaY, NaUSY and NaDY were tested for different values of the water/organic solvent ratio. The results presented in Fig. 1 show a general increase of activity with increasing values of this parameter, with the exception of NaUSY for high water contents. If one considers the allure of the curves for the less active preparations (those using NaUSY and NaDY), it is conceivable that the activity versus water content goes through a maximum; however, the results obtained so far

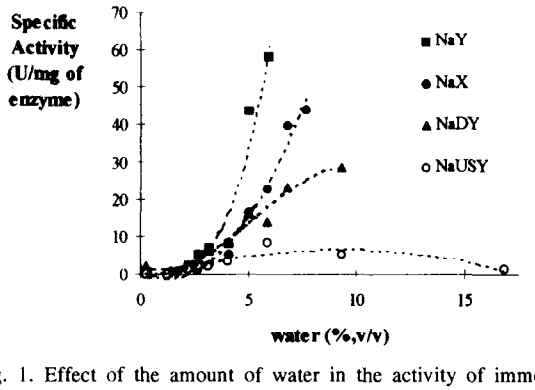

Fig. 1. Effect of the amount of water in the activity of immobilised cutinase.

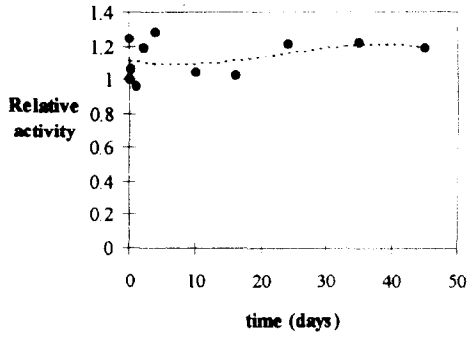

Fig. 2. Thermal stability of immobilised cutinase, stored at 30°C.

can not ascertain this trend and whether or not if the maximum, should it exist, would depend on the zeolite itself. From all the preparations tested, NaY is the one that presents the best effectiveness for this reaction.

Another interesting, and important, observation that can be made is that in the case of the more active preparations the increase of the water content above 5% (v/v) leads to the formation of emulsions. This reinforces the apparent similarity between zeolites and the reversed micellar medium where lipases are known to be active. It should also be noted that the presence of high amounts of water, although favouring the hydrolytic activity, also induce some enzyme desorption, which could present a problem with the operation of continuous reactors.

3.2.4. *Thermal stability*

Another important issue in the use of any catalyst, is its stability. The catalytic preparations should remain stable for sufficiently long periods of time, namely for a successful practical application, although it is known that the activity of supported enzymes do decrease their intrinsic activity with storage time.

The most interesting support preparation, that of the enzyme adsorbed on NaY, was then stored for long periods of time, at 30°C, and its hydrolytic activity was evaluated periodically as a function of the storing time. From the obser-

vation of the results obtained, presented in Fig. 2, it is remarkable the stability of the hydrolytic activity, which is quite better than that normally presented by other supports [18].

4. Conclusions

Zeolites can be used as an efficient means of immobilising cutinase, although its efficiency strongly depends on the characteristics of the zeolitic material. It was observed that even a weak acidity is enough to prevent any significant activity on the enzyme for the hydrolysis reaction studied. The non acidic sodium forms were shown to lead to very good values for the hydrolytic activity.

Although the interaction of the enzyme with the support is probably only at the external surface, the framework composition is also an important factor influencing the immobilisation performances. The best results were obtained with the NaY preparation, their activity values being close to the best ones obtained with other supports commonly used, in the same experimental conditions. Further study should be devoted to investigating the influence of framework and extra-framework composition on the coupling yield and activity of the immobilised enzyme. However, considering that NaY, the one that has shown the best properties for this application, is also one of the most widely used zeolites, shows good prospects indeed for an economical use of this support.

Considering also that the enzyme shows an extremely good stability with this support, the application of zeolite supports in this type of biocatalytic processes seems very promising. We are developing a deeper comparative study between the performances of cutinase immobilised on NaY and on other common supports.

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