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Journal of Molecular Catalysis B: Enzymatic 27 (2004) 19–27
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Application of factorial design to the study of an alcoholysis transformation promoted by cutinase immobilized on NaY zeolite and Accurel PA6

F.N. Serralha, J.M. Lopes*, F. Lemos, F. Ramôa Ribeiro, D.M.F. Prazeres, M.R. Aires-Barros, J.M.S. Cabral

Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

Received 2 February 2003; received in revised form 28 July 2003; accepted 2 September 2003

Abstract

Fusarium solani pisi recombinant cutinase was immobilized on sodium form of zeolite Y (NaY) and polyamide Accurel PA6 and used to catalyze the alcoholysis reaction of butyl acetate with hexanol, in isooctane. The influence of some of the relevant parameters to the enzyme alcoholysis activity, such as temperature, buffer molarity and pH of the enzyme solution, hexanol and butyl acetate concentrations, were studied by means of a factorial design plan. By knowing the system response to the experimental design, the effects of each factor and its interactions were determined. In the case of the preparations with NaY zeolite, the main effects are due to temperature and buffer molarity, while pH and hexanol concentration have little effect on activity. In case of the cutinase adsorbed on Accurel PA6 the main effects are due to temperature, buffer molarity and pH of the enzyme solution.

Keywords: Zeolites; Cutinase; Immobilization; Alcoholysis; Factorial design

1. Introduction

Enzymatic catalysis in non-conventional media has been receiving increased attention [1–4]. Zeolites have been used as supports for enzymatic reactions carried out in organic medium [5–10].

Previous studies described the use of a recombinant cutinase from *Fusarium solani pisi* immobilized on several zeolites to the promotion of the alcoholysis reaction of butyl acetate with hexanol [6,11,12], in isooctane. One of the most active preparations was obtained with the immobilization of the enzyme on the sodium form of zeolite Y (NaY). The water content of the catalytic system is of paramount importance in the enzymatic activity of the preparation and its influence on the observed activity, for the alcoholysis transformation, was previously studied [6]; an optimum value was observed, which depends on the particular support used, but that always corresponds to approximately the same water activity ($a_w \approx 0.97$); this hydration level was used in the following work. In this study, the influence of some relevant reaction parameters on the alcoholysis transformation, promoted by the immobilized cutinase on NaY zeolite and on the commonly used polyamide Accurel PA6, was analyzed through the factorial design methodology. This methodology has been recently used in system optimization [13–17]. The major advantage of studying the influence of several parameters by means of factorial design methodology is to distinguish possible interactions among factors, which would not be possible by classical experimental methods. Moreover, factorial design requires fewer experiments and allows the study of each variable for different conditions of the others [18].

The study of reaction parameters influence on alcoholysis activity was planned as a 2^{5-1} fractional factorial design [18] accommodating five variables, each one at two levels (−1/+1), where 16 runs were performed. The design was further expanded to a central composite design (CCD) [19] by introducing the extreme levels −2/+2. Consequently, it is possible to investigate up to a quadratic polynomial relationship.

* Corresponding author. Tel.: +351-21-841-9286; fax: +351-21-841-9062.
E-mail address: jmlopes@popsrv.ist.utl.pt (J.M. Lopes).

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doi:10.1016/j.molcatb.2003.09.005

At the medium point (0), independent replicates were run to estimate the standard deviation. The variation between them reflects the variability of all design.

After the runs, the response obtained (in this case the specific activity displayed in the alcoholysis reaction) is submitted to the algorithm of Yates to calculate the effects of each factor (parameter) and the important interactions [18].

The response is also used to calculate the coefficients of a second order polynomial equation. The obtained Eq. (1) shows the dependence of specific activity on all effects and interactions:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{15}x_1x_5 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{25}x_2x_5 + b_{34}x_3x_4 + b_{35}x_3x_5 + b_{45}x_4x_5 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{55}x_5^2 \quad (1)$$

where y is the theoretical response (the value predicted by the model), x_i the factors (i from 1 to 5 represents the factors) and b_i the coefficients determined by matrix calculation. By applying this equation, two variables can be represented while keeping the other ones at a constant value, to obtain the response surface.

2. Materials and methods

2.1. Enzyme preparation

F. solani pisi cutinase was produced by a *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 original procedure of Lauwereys et al. [20]. 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. Immobilization procedure

The immobilization was accomplished, by adsorption/deposition on the supports: the enzyme solutions were prepared in sodium phosphate buffer and added to the supports (25 mg of cutinase/g of support); the preparations were then vacuum dried after vortex mixing [6].

2.3. Alcoholysis reaction

The enzyme immobilizations were equilibrated in closed vessels with salt solutions at well-defined water activity ($a_w = 0.97$) at 30 °C, during 3 days [21] and used to catalyze the alcoholysis of butyl acetate with hexanol, in isooctane.

The reactions were carried out in a batch stirred reactor (BSTR), at 30 °C, placed on an orbital stirrer operating at 400 rpm. Hexyl acetate formation and butyl acetate consumption were followed by UV at 220 nm, using a HPLC system with a C18 reverse-phase column with isocratic elution and using a mixture of 60% acetonitrile and 40% water. Initial reaction rates were determined by linear regression. The values of the specific enzymatic activities are expressed as enzyme units (U)/mg of enzyme, where one enzyme unit corresponds to 1 μmol of product formed per minute.

Some blank tests were performed for this reaction system: when no enzyme was present no substrate transformation was detected; when free enzyme was used in isooctane, in the absence of the support, the observed enzyme activity was negligible.

2.4. Protein determination

The amount of the immobilized remaining on the support, before and after the reaction and washing of the preparation with the organic solvent, was determined by a modified Folin assay [22], using BSA as reference protein. Similar values, before and after the reaction, were obtained, indicating that no significant desorption of the enzyme during the reaction process occurs. Typical amounts of immobilized enzyme are, approximately, 15 mg enzyme/g of support.

2.5. Parameters under study

The reaction parameters, whose influence on the enzymatic activity was studied, are temperature of the reaction, buffer molarity, pH of the enzyme solution used in the immobilization procedure, hexanol concentration and butyl acetate concentration. The corresponding values, at each level, are presented in Table 1.

2.6. Supports

NaY was obtained from Union Carbide. The reference support, Accurel PA6 was obtained from AKZO (EP 700). Further details concerning the composition, crystallite dimensions and porosity of these supports can be found elsewhere [6].

Table 1
Parameters studied on the planned design

Factor	Level				
	−2	−1	0	+1	+2
1- Temperature (°C)	20	27.5	35	42.5	50
2- Buffer molarity (mM)	20	65	110	155	200
3- pH of the enzyme solution	7.2	8.1	9	9.9	10.8
4- Hexanol concentration (mM)	100	250	400	550	700
5- Butyl acetate concentration (mM)	100	450	800	1150	1500

Some conditions were maintained constant: concentration of enzyme solution [E] = 0.25 mg/ml; 25 mg cutinase/g of support; water activity a_w = 0.97.

Table 2
Specific activities (U/mg of enzyme) of levels −2/+2 (expansion of design)

Reaction	Temperature (°C)	Buffer molarity (mM)	pH	[Hexanol] (mM)	[Butyl acetate] (mM)	Specific activity of NaY (U/mg)	Specific activity of PA6 (U/mg)
1	20	110	9	400	800	4.0	12.6
2	50	110	9	400	800	9.2	23.7
3	35	20	9	400	800	6.8	7.2
4	35	200	9	400	800	8.5	13.0
5	35	110	7.2	400	800	10.7	19.0
6	35	110	10.8	400	800	7.5	14.3
7	35	110	9	100	800	3.0	12.2
8	35	110	9	700	800	10.8	19.9
9	35	110	9	400	100	1.6	9.6
10	35	110	9	400	1500	15.0	23.4

Table 3
Specific activities (U/mg of enzyme) of central point (replicates)

Central point	Specific activity of NaY (U/mg)	Specific activity of PA6 (U/mg)
1	15.4	5.1
2	17.1	6.1
3	12.6	7.0
4	13.8	7.5
5	13.8	8.9
6	17.7	8.1
7	15.2	5.5
8	16.5	6.5
9	18.9	
10	16.7	
11	12.6	

3. Results and discussion

The specific activity for each experimental condition is presented in Tables 2–4. The responses obtained were submitted to Yates' algorithm in order to determine the effect of each factor and the important interactions.

This algorithm performs simple calculations with the experimental activities obtained for different values of the parameters whose influence on the activity is under study. Taking the adequate experiments, the result of the calculation may express the effect of the variation of each parameter on the enzymatic activity; higher effect means stronger influence of the corresponding factor changes on the activity. In a similar way, it is possible to obtain the interactions between factors, which will express the dependence of the influence of a given parameter for different values of other factors.

The effects of the variables and their interactions, for the design with NaY zeolite preparations and with Accurel PA6 preparations, are shown in Tables 5 and 6, respectively. The most important factors and interactions are marked in bold.

The main effects are due to temperature, buffer molarity and butyl acetate concentration. In case of NaY zeolite preparations, the main interactions are the pairs temperature/butyl acetate concentration, buffer molarity/hexanol concentration and hexanol concentration/butyl acetate concentration. In case of Accurel PA6 preparations, the main interactions are the pairs temperature/butyl acetate

Table 4
Specific activities (U/mg of enzyme) of levels −1/+1

Reaction	Temperature (°C)	Buffer molarity (mM)	pH	[Hexanol] (mM)	[Butyl acetate] (mM)	Specific activity of NaY (U/mg)	Specific activity of PA6 (U/mg)
1	27.5	65	8.1	250	1150	17.4	8.0
2	42.5	65	8.1	250	450	14.1	6.3
3	27.5	155	8.1	250	450	8.5	9.6
4	42.5	155	8.1	250	1150	23.4	10.3
5	27.5	65	9.9	250	450	5.5	4.2
6	42.5	65	9.9	250	1150	18.4	6.3
7	27.5	155	9.9	250	1150	15.8	5.8
8	42.5	155	9.9	250	450	14.8	6.1
9	27.5	65	8.1	550	450	7.0	5.2
10	42.5	65	8.1	550	1150	14.7	12.5
11	27.5	155	8.1	550	1150	16.3	11.4
12	42.5	155	8.1	550	450	25.1	8.0
13	27.5	65	9.9	550	1150	10.2	6.9
14	42.5	65	9.9	550	450	15.6	4.6
15	27.5	155	9.9	550	450	10.2	3.3
16	42.5	155	9.9	550	1150	23.5	15.2

Table 5
Main effects and interactions analysis of factorial design with NaY zeolite preparations

Factor	Effect (U/mg)	Factor	Interaction (U/mg)
Mean	**15.0**	1–2	2.54
1	**49.6**	1–3	0.09
2	**16.9**	1–4	1.92
3	2.2	1–5	**4.70**
4	0.29	2–3	0.45
5	**21.9**	2–4	**6.1**
		2–5	0.05
		3–4	0.39
		3–5	0.29
		4–5	**9.1**

Table 6
Main effects and interactions analysis of factorial design with Accurel PA6 preparations

Factor	Effect (U/mg)	Factor	Interaction (U/mg)
Mean	**7.7**	1–2	0.69
1	**8.4**	1–3	3.19
2	**9.2**	1–4	**5.46**
3	**13.4**	1–5	**3.32**
4	4.2	2–3	0.07
5	**31.8**	2–4	0.12
		2–5	0.21
		3–4	0.83
		3–5	0.31
		4–5	**16.2**

concentration, temperature/hexanol concentration and hexanol concentration/butyl acetate concentration.

The values of the model coefficients, determined by fitting Eq. (1) to the experimental enzymatic activities, are shown in Table 7.

3.1. Effect of buffer molarity and temperature

In the range of values that was studied (20–50 °C), the response surfaces (Fig. 1) show, in both cases, that an increase in temperature clearly enhances the specific activity. Usually the activity is improved by raising the temperature

Table 7
Values of polynomial coefficients determined by adjustment

Coefficient	NaY	PA6
b_0 (U/mg)	35.9	1.26
b_1 (U/(mg °C))	-6.52×10^{-1}	-1.02
b_2 (U/(mg mM))	1.35×10^{-1}	-6.16×10^{-2}
b_3 (U/mg)	-6.84	-17.8
b_4 (U/(mg mM))	-2.80×10^{-2}	-5.71×10^{-2}
b_5 (U/(mg mM))	2.43×10^{-2}	-2.16×10^{-2}
b_{11} (U/mg °C^2))	1.01×10^{-2}	-1.90×10^{-3}
b_{22} (U/(mg mM^2))	-6.34×10^{-4}	8.37×10^{-5}
b_{33} (U/mg)	2.37×10^{-1}	6.70×10^{-1}
b_{44} (U/(mg mM^2))	9.16×10^{-6}	-9.14×10^{-8}
b_{55} (U/mg mM^2))	1.19×10^{-6}	2.76×10^{-6}
b_{12} (U/(mg °C mM))	2.47×10^{-3}	7.93×10^{-4}
b_{13} (U/(mg °C))	2.37×10^{-2}	8.52×10^{-2}
b_{14} (U/(mg °C mM))	6.42×10^{-4}	6.69×10^{-4}
b_{15} (U/(mg °C mM))	-4.31×10^{-4}	2.23×10^{-4}
b_{23} (U/(mg mM))	-8.64×10^{-3}	2.12×10^{-3}
b_{24} (U/(mg mM^2))	7.35×10^{-5}	1.65×10^{-5}
b_{25} (U/(mg mM^2))	7.05×10^{-6}	9.32×10^{-6}
b_{34} (U/(mg mM))	2.42×10^{-3}	2.17×10^{-3}
b_{35} (U/(mg mM))	8.98×10^{-4}	5.68×10^{-4}
b_{45} (U/(mg mM^2))	-3.00×10^{-5}	2.47×10^{-5}

up to a certain limit dependent on the structural stability of the enzyme.

When cutinase was microencapsulated in AOT micelles, and used to catalyze the same alcoholysis reaction, a similar positive effect was observed in the activity, resulting from a temperature increase up to 50 °C [13]. The studies performed with cutinase immobilized on NaY zeolite and Accurel PA6 to catalyze tricaprylin hydrolysis showed an optimum at 30 °C [5].

The activation energy was determined from the results obtained at different temperatures, with constant values for the other factors (medium point), through the application of the Arrhenius' law, and assuming that in the experimental conditions the specific activity is proportional to the rate constant. The values obtained are 5.07 and 5.09 kcal/mol, respectively, for the NaY zeolite preparations and Accurel PA6 preparations.

These values are very similar, what reveals the weak influence of the support in the enzymatic activity increase due

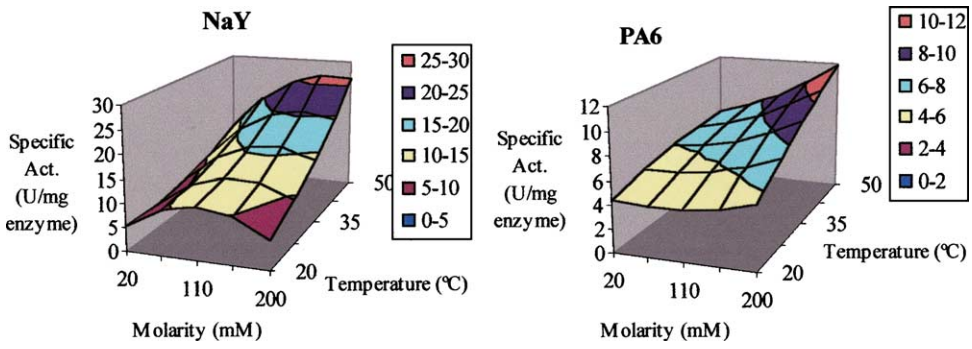

Fig. 1. Effect of buffer molarity and temperature on alcoholysis reaction using pH 9, [hexanol] = 400 mM and [butyl acetate] = 800 mM.

to the temperature effect, at least in the range of experimental conditions that was studied. These values are also close to other values that have been determined for immobilized enzymes on supports [23] and microencapsulated enzymes in reversed micelles [24].

The buffer molarity of the enzyme solution produces a strong influence on specific activity in both cases. The studies with NaY zeolite preparations show an optimum between 110 and 150 mM, while the studies with Accurel PA6 preparations show that an increase in buffer molarity proportionally enhances the specific activity, in the range of studied values.

The preparations with NaY zeolite and PA6 contain different amounts of water, corresponding to the situation of similar water activity imposed in all the immobilization experiments, and shall present different properties of the water phase. This is a consequence of the different nature of the support surface, namely the much more hydrophilic character in the case of the zeolite. Different water phase features and adsorption properties of the support surface possibly induce different conformational states in the enzyme, thus affecting the resulting activity. In fact, the cutinase fluorescence emission spectra were previously used to detect different conformational enzyme states in a series of preparations, obtained as in the present study, using different zeolites and other reference supports [11]. Significant conformational changes were observed, with important consequences on the enzymatic activities: native (for NaA zeolite, NaY zeolite and alumina) or partially denaturated (PA6, NaZSM-5 (Si/Al = 19) zeolite) conformational states have been observed for the active preparations; a strong denaturation was observed in other cases (NaDY zeolite, NaZSM-5 (Si/Al = 40) zeolite and silica).

Changes in the salt concentrations of the aqueous buffer solution, probably induce different conformational alterations in the enzyme adsorbed on the NaY zeolite or PA6. The increase of alcoholysis activity with increasing buffer molarity was also observed in studies with microencapsulated cutinase [13], where the effect of salts in the water pool of AOT reversed micelles is of great importance and include an influence on reversed micelles radius and, consequently, on enzyme conformation.

The interaction between temperature and buffer molarity is not significant, as predicted by Yates' algorithm.

3.2. Effect of pH and temperature

Despite the fact that the alcoholysis reaction occurs in the non-aqueous phase, the pH value of the enzyme solution used in the preparation also influences the enzymatic activity; the pH values for the most interesting activities depend on the exposed residues of the protein near the active site.

As shown in Fig. 2, the pH of the enzyme solution influences the specific activity of the NaY zeolite preparations to a much lesser extent than the strong influence it has in the case of Accurel PA6 preparations, as already predicted by the Yates' algorithm. In this latter case, an increase of pH results in a decrease of specific activity, especially for low temperatures.

In both cases the best pH values are from 7.2 to 8, in the range of values under study, which are very close to the isoelectric point of cutinase, which is 7.9.

The minor effect for the NaY zeolite preparations can be explained by the fact that the zeolite presents a high framework charge, compensated by the easily exchanged counter-ions located in the internal porous network, and therefore acts as a proton buffer. A similar result was obtained with cutinase immobilized on zeolites used to catalyze tricaprylin hydrolysis [5].

In the case of cutinase microencapsulation in reversed AOT micelles, an important effect of pH was observed, and the best activity values, in the range under study, were also obtained for pH 7–8, at the lowest water levels, and for pH 11 at the highest water levels [13].

3.3. Effect of hexanol concentration and temperature

Hexanol concentration can play two roles concerning the effect on the cutinase activity: it has an obvious positive effect because hexanol is a substrate; nevertheless, it can also present an inhibitory effect, as previously reported [25], due to the formation of a dead-end complex. As evidenced in the proposed reaction mechanism for the alcoholysis of butyl acetate with hexanol [26], this complex is formed from

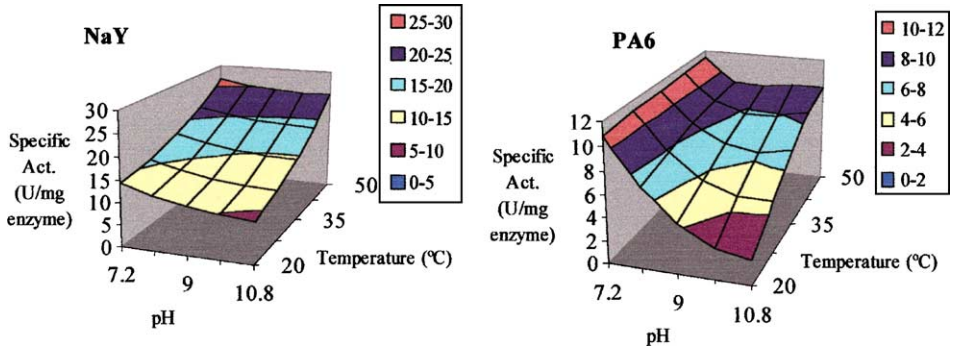

Fig. 2. Effect of pH and temperature on alcoholysis reaction using 110 mM carbonate buffer, [hexanol] = 400 mM and [butyl acetate] = 800 mM.

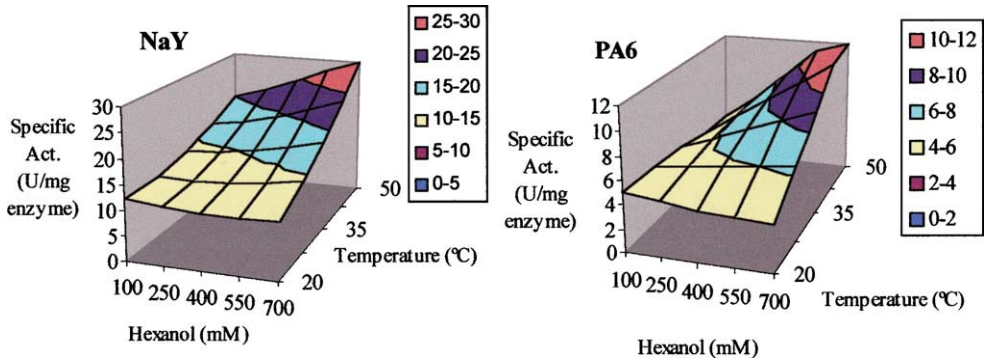

Fig. 3. Effect of hexanol concentration and temperature on alcoholysis reaction using 110 mM carbonate buffer, pH 9 and [butyl acetate] = 800 mM.

the reaction between hexanol and the enzyme, and is unable to participate in the reaction.

At low temperatures the increase of hexanol concentration does not have much influence on specific activity, while at high temperatures an increase of hexanol concentration enhances the specific activity (Fig. 3). This fact is more significant for Accurel PA6 preparations. The observed enhancement of the hexanol concentration effect, for higher temperatures, can be related to a possible inhibition of the dead-end complex formation.

The cutinase in AOT reversed micelles system clearly showed an optimum value at the hexanol concentration of 490 mM.

3.4. Effect of butyl acetate concentration and temperature

In both cases the butyl acetate concentration has a major influence on specific activity (Fig. 4). This fact can be explained by the reaction mechanism: in its first step the catalytic serine of cutinase acts as a nucleophile to the carbonyl carbon of butyl acetate, forming a stable tetrahedral intermediate. Therefore, increasing the ester concentration near the enzyme active site enhances the reaction [26]. This result had also been observed in the cutinase AOT reversed micelles system [13].

The increase of specific activity with increasing temperature is more pronounced for lower butyl acetate concentrations, when NaY zeolite preparations are used, and for higher butyl acetate concentrations, in the case of Accurel PA6 preparations (Fig. 4).

3.5. Effect of pH and buffer molarity of the enzyme solution

The best immobilization conditions for NaY zeolite preparations, in the range of values under study, are: buffer molarity between 110 and 155 mM and pH from 7.2 to 8.1 (Fig. 5), as previously observed. However, this interaction is not very significant, because the best values for one effect are independent of the other, inside the range covered in this study for these parameters.

For Accurel PA6 preparations, the increase of buffer molarity and pH of the enzyme solution, in the range of studied values, respectively, enhances an increase and decrease on enzyme activity, as previously showed. This interaction is also not very significant.

3.6. Effect of pH of the enzyme solution and hexanol concentration

For both support preparations, increasing hexanol concentration induces an increase of the specific activity, more

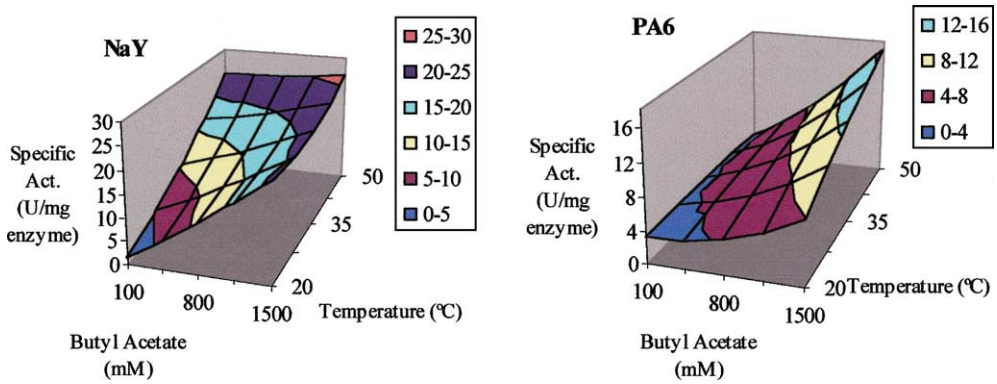

Fig. 4. Effect of butyl acetate and temperature on alcoholysis reaction using 110 mM carbonate buffer, pH 9 and [hexanol] = 400 mM.

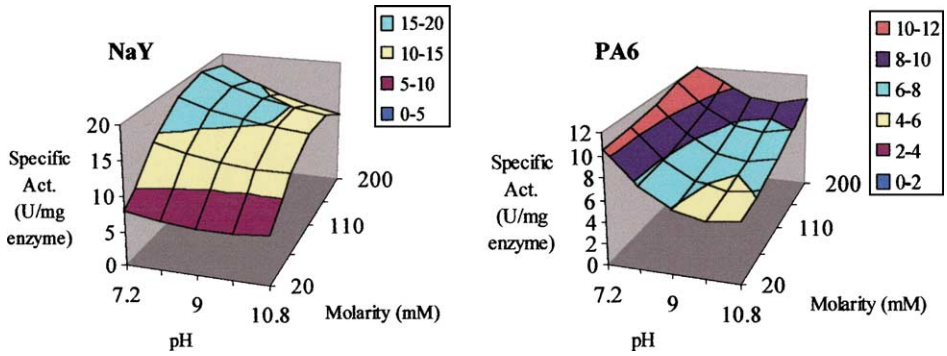

Fig. 5. Effect of pH and buffer molarity on alcoholysis reaction using [hexanol] = 400 mM and [butyl acetate] = 800 mM at 35 °C.

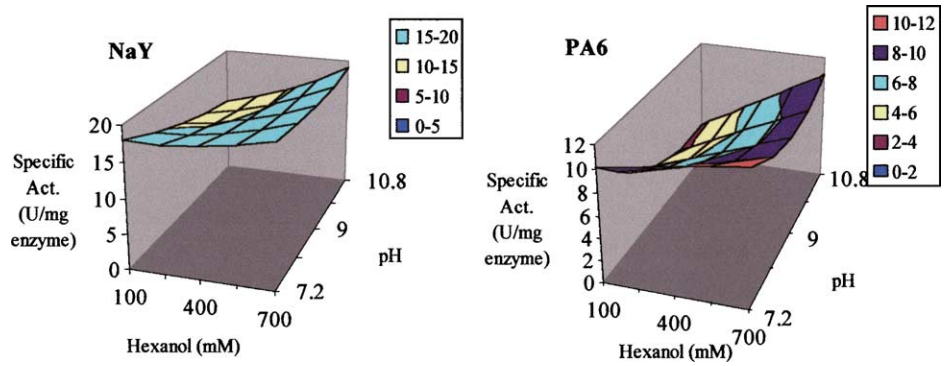

Fig. 6. Effect of pH and hexanol concentration on alcoholysis reaction using 110 mM carbonate and [butyl acetate] = 800 mM at 35 °C.

significant for higher pH values, and stronger in the case of Accurel PA6 preparations (Fig. 6). This stronger interaction in the case of PA6 shall be a consequence of the already mentioned action of the zeolite as a proton buffer; the real value of pH in the aqueous phase near the active site of the enzyme adsorbed on the zeolite NaY should then not be changed as extensively as in the case of PA6 preparation.

3.7. Effect of pH of the enzyme solution and butyl acetate concentration

The increase of specific activity with increasing butyl acetate concentration takes place in the entire range of pH values that were studied, for both supports (Fig. 7). This increase is more important for higher pH values, especially in the case of the PA6 preparation, in a similar behavior to that observed in the study of pH and hexanol concentration. Moreover, specific activity slightly decreases with increasing pH value, in the entire range of butyl acetate concentrations, for both supports.

3.8. Effect of hexanol and butyl acetate concentrations

As predicted by Yates' algorithm, Fig. 8 shows a strong interaction between the concentrations of both substrates. In the case of the preparation with NaY, the induced

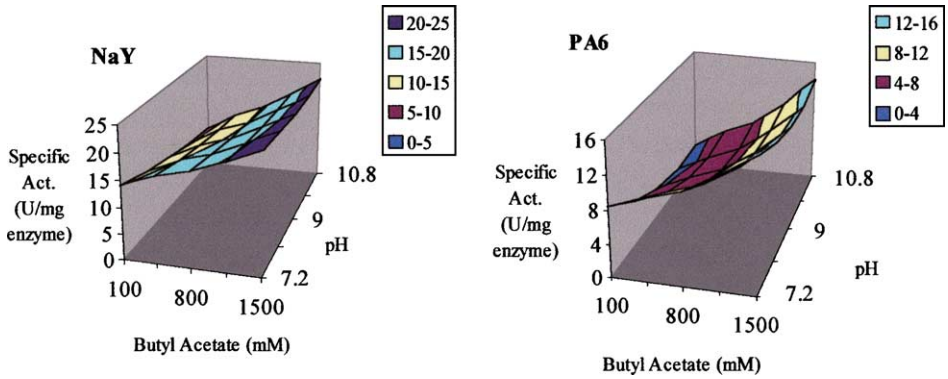

Fig. 7. Effect of pH and butyl acetate concentration on alcoholysis reaction using 110 mM carbonate and [hexanol] = 400 mM at 35 °C.

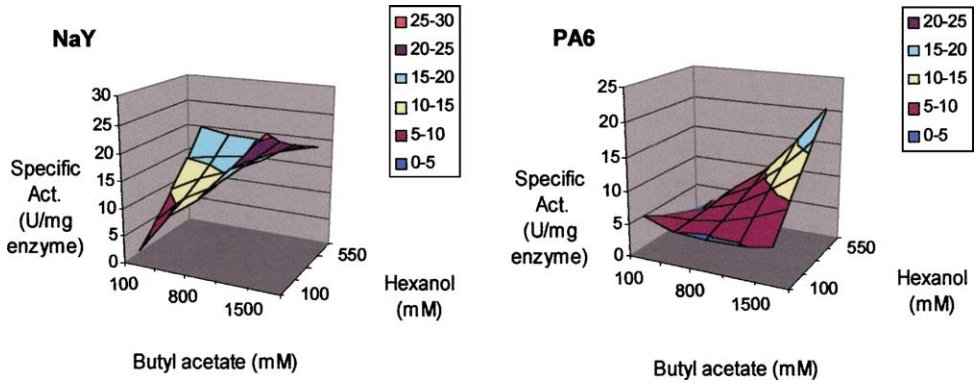

Fig. 8. Effect of hexanol and butyl acetate concentrations on alcoholysis reaction using 110 mM carbonate and pH 9 at 35 °C.

enhancement of the butyl acetate concentration in the enzymatic activity, decreases as the hexanol concentration increases; this might be a consequence of the hexanol inhibitory effect. The opposite behavior is observed for the PA6 immobilization. Taking into account the low hydrophilic character of PA6, and being the enzyme adsorbed on the particle internal walls of its porous network and not only on the external surface, as in the NaY support, the hexanol partition between the vicinity of the adsorbed enzyme active sites and the bulk organic phase shall be less favorable in PA6 relative to NaY. For the lowest hexanol concentrations the reaction should then be strongly limited.

3.9. Statistical analysis of the model

The independent replicates at the medium point were performed on different days, along the period of time corresponding to the complete set of experiments, so that the estimated standard deviation reflects the variability of all design.

The values of standard deviation obtained for NaY zeolite and Accurel PA6 designs were 2.1 and 1.3 U/mg, respectively. The lower variability associated with the PA6 preparations is a consequence of the lower sensibility of their enzymatic activities relative to the water amount; slight differences in the water amount present in the final immobilization samples induce more significant changes on the activity of the NaY zeolite preparations [6].

The correlation parameter is 0.936 for NaY zeolite preparations and 0.945 for Accurel PA6 preparations, thus expressing a good agreement between the experimental results and the results given by the model. The quality of this agreement, and also the observed variability, reflect the particular sensitivity of this reaction system to the water content of the preparations; correlation values closer to one and lower variabilities should be obtained in other situations.

4. Conclusions

Information about several parameters that influence the reaction and their interactions can be obtained by the factorial methodology, requiring a limited number of experiments, when compared with classical methods.

In the case of NaY zeolite preparations, the main conclusions on the factors that influence the observed activity, and on their interactions are:

- the temperature increase, in the range 20–50 °C, enhances the specific activity of the supported cutinase;
- the maximum enzymatic activity occurs for values of buffer molarity between 110 and 150 mM;
- the largest values for the enzymatic activity were observed for values of pH of the enzyme solution between 7.2 and 8, although the pH value has little influence on activity;
- the hexanol concentration does not significantly influence the specific activity.

For Accurel PA6 preparations, the main conclusions on the factors that influence the observed activity, and on their interactions are:

- the temperature increase enhances the specific activity;
- the enzymatic activity increases with increasing buffer molarity;
- the maximum enzymatic activity was observed for values of pH of the enzyme solution between 7.2 and 8, and decreases rapidly for higher pH values;
- the increase of enzymatic activity with increasing hexanol concentration is more relevant at higher temperatures.

Acknowledgements

F.N. Serralha acknowledges a Ph.D. grant (BD/l277/95) on Project Praxis 2/2.1/BIO/34/94, Junta National de Investigação Científica e Tecnológica (JNICT). Financial support by JNICT through Project PBIC/C/QUI/2378/95 is also gratefully acknowledged.

References

[1] J.S. Dordick, Enzyme Microb. Technol. 11 (1989) 194.
[2] P. Adlercreutz, Biotechnol. Genet. Eng. Rev. 12 (1994) 231.

[3] A. Tanaka, T. Kawamoto, in: R.F. Taylor (Ed.), Protein Immobilization, Marcel Dekker, New York, 1991, p. 183.
[4] E. Wehtje, P. Adlercreutz, Biotechnol. Bioeng. 55 (1997) 798.
[5] A.P.V. Gonçalves, J.M. Lopes, F. Lemos, F. Ramôa Ribeiro, D.M.F. Prazeres, J.M.S. Cabral, M.R. Aires-Barros, J. Mol. Catal. B: Enzym. 1 (1996) 53.
[6] F.N. Serralha, J.M. Lopes, F. Lemos, D.M.F. Prazeres, M.R. Aires-Barros, J.M.S. Cabral, F. Ramôa Ribeiro, J. Mol. Catal. B: Enzym. 4 (1998) 303.
[7] E. Lie, G. Molin, J. Chem. Technol. Biotechnol. 50 (1991) 549.
[8] G.-W. Xing, X.-W. Li, G.-L. Tian, Y.-H. Ye, Tetrahedron 56 (2000) 3517.
[9] Z. Knezevic, L. Mojovic, B. Adnadjevic, Enzyme Microb. Technol. 22 (1998) 275.
[10] A.P.V. Gonçalves, J.M. Lopes, F. Lemos, F. Ramôa Ribeiro, D.M.F. Prazeres, J.M.S. Cabral, M.R. Aires-Barros, Enzyme Microb. Technol. 20 (1997) 93.
[11] F.N. Serralha, J.M. Lopes, L.F. Vieira Ferreira, F. Lemos, D.M.F. Prazeres, M.R. Aires-Barros, J.M.S. Cabral, F. Ramôa Ribeiro, Catal. Lett. 73 (1) (2001) 63.
[12] F.N. Serralha, J.M. Lopes, F. Lemos, D.M.F. Prazeres, M.R. Aires-Barros, J.M.S. Cabral, F. Ramôa Ribeiro, Enzyme Microb. Technol. 31 (2002) 29.
[13] C.M.L. Carvalho, M.L.M. Serralheiro, J.M.S. Cabral, M.R. Aires-Barros, Enzyme Microb. Technol. 21 (1997) 117.
[14] E. Uosukainen, M. Lämsä, Y.Y. Linko, P. Linko, M. Leisola, Enzyme Microb. Technol. 25 (1999) 236.
[15] D.G. Hayes, E. Gulari, Biocatalysis 11 (1994) 223.
[16] M.L.M. Serralheiro, J.M.S. Cabral, in: J. Tramper, M.H. Vermue, H.H. Beeftink, U. Von Stockar (Eds.), Biocatalysis in Non-Conventional Media, Elsevier, Amsterdam, 1992, p. 725.
[17] C. Pizarro, M.A. Fernández-Torroba, C. Benito, J.M. González-Sáiz, Biotechnol. Bioeng. 53 (1997) 497.
[18] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experiments, Wiley, New York, 1985.
[19] T.B. Barker, Quality by Experimental Design, Marcel Dekker, New York, 1985.
[20] M. Lauwereys, P. De Geus, J. De Meutter, P. Stanssens G. Matthyssens, in: L. Alberghina, R.D. Schmid, R. Verger (Eds.), Lipases: Structure, Mechanism and Genetic Engineering, VCH, New York, 1990, p. 243.
[21] L. Greenspan, J. Res. Natl. Bureau Stand. A: Phys. Chem. 81A (1977) 89.
[22] O.H. Lowry, N.J. Rosenbrough, L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[23] A.P.V. Gonçalves, J.M.S. Cabral, M.R. Aires-Barros, Appl. Biochem. Biotechnol. 60 (1996) 217.
[24] D.M.F. Prazeres, F.A.P. Garcia, J.M.S. Cabral, J. Chem. Technol. Biotechnol. 53 (1992) 159.
[25] M.J. Sebastião, J.M.S. Cabral, M.R. Aires-Barros, Biotechnol. Bioeng. 42 (1993) 326.
[26] F.N. Serralha, J.M. Lopes, F. Lemos, D.M.F. Prazeres, M.R. Aires-Barros, J.M.S. Cabral, F. Ramôa Ribeiro, J. Mol. Catal. B: Enzym. 11 (2001) 713.