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Modelling the production of ethyl butyrate catalysed by *Candida rugosa* lipase immobilised in polyurethane foams

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Abstract

Response surface methodology was used to model and optimise the esterification of ethanol with butyric acid in *n*-hexane, catalysed by *Candida rugosa* lipase immobilised in two hydrophilic polyurethane foams (“FHP 2002^{TM}” and “FHP 5000^{TM}”). Experiments were carried out following central composite rotatable designs (CCRD), as a function of the initial water activity of the biocatalyst (a_w), initial butyric acid concentration (A) and ethanol:acid molar ratio (MR) in the organic medium. Ester production increased with increasing a_w of the biocatalysts, probably due to the hydrophilicity of both substrates in contrast with the hydrophobicity of the product, which is released to the bulk medium. Thus, for each biocatalyst ($a_w = 0.98$) another CCRD was performed as a function of A and MR. With both preparations, higher conversions (>95%) were observed for low A values. For the “FHP 2002^{TM}” system, a maximum ester production of 0.23 M is expected, after 18-h reaction, at initial 0.35 M A and 1.51 MR, corresponding to a_w of 0.95 and 0.84 M A and 1.65 M ethanol in lipase microenvironment. With “FHP 5000^{TM}” system, predicted initial conditions of 0.54 M A and 0.75 MR (0.32 M A; 0.75 M ethanol in microenvironment; a_w of 0.95), will lead to the maximum ester production of 0.27 M. These maxima were experimentally confirmed.
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Keywords: Ethyl butyrate; Immobilised enzymes; Lipase; Modelling; Optimisation; Polyurethane foam

1. Introduction

Esters of short-chain carboxylic acids and alcohols are widely used in the food industry as flavouring compounds [1]. Nowadays people are concerned about their health and claim for natural ingredients and foods. However, the sources of natural flavour compounds are frequently not enough to meet the demand. Also, the extraction of flavours from natural sources is expensive and often gives low yields. The search for enzymatic processes, recognized as natural, to produce these molecules has been a challenge for the food industry.

Lipases have been successfully used to catalyse esterification [1–16] and interesterification reactions [4,7,17,18] aimed at the production of flavouring esters for food, pharmaceutical and cosmetics purposes.

However, lipases must be used in immobilised forms, presenting both high catalytic activity and operational stability, in order to lower the costs of the biocatalyst in the process by reusing it in batch cycles or using it in continuous reactors [19].

Among several immobilisation techniques, lipase immobilisation in polyurethane foams, where entrapment is coupled to chemical binding during polymer synthesis, has been reported by several authors [5,20–31]. In previous studies, *Candida rugosa* lipase was immobilised in hydrophilic polyurethane foams and successfully used as a catalyst for the following reactions: esterification of ethanol with butyric acid [5], glycerolysis [23–25,29] and hydrolysis of olive and olive residue oils [22,26,28].

All these reactions were carried out in the presence of an organic phase. The displacement of the reaction equilibrium towards synthesis (esterification or interesterification reactions) or hydrolysis is mainly determined by the amount of water in

Abbreviations: CCRD, central composite rotatable design; RSM, response surface methodology

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Nomenclature

a_w	thermodynamic water activity
A	initial butyric acid concentration (M) in the organic medium
FHP 2002	foamable hydrophilic polyurethane pre-polymer "HYPOL FHP 2002^{TM}" from Dow Chemicals, UK
FHP 5000	foamable hydrophilic polyurethane pre-polymer "Hypol FHP 5000^{TM}" from Dow Chemicals, UK
MR	initial ethanol:butyric acid molar ratio in the organic medium
R^2	coefficient of determination
R^2_{adj}	adjusted coefficient of determination

these systems. In fact, water is essential to enzyme activity, due to its role in the formation of hydrogen bonding and in van der Waals interactions, which are responsible for the maintenance of the native catalytically active conformation of the enzyme.

Rather than the knowledge of the water content of a system, the thermodynamic water activity (a_w), i.e. the ratio between the vapour pressure of the water molecules in that system and that of pure water at the same temperature, is a most useful parameter since it is related with the water availability as reactant. In principle, it is expected that in systems with low water activity values, esterification and interesterification reactions are favoured, while water activity values very close to 1 promote hydrolytic reactions [32].

The aim of the present study is to model and optimise, by response surface methodology (RSM), the production of ethyl butyrate, a pineapple-like flavouring ester, in *n*-hexane catalysed by *C. rugosa* lipase immobilised in biocompatible polyurethane foams with different hydrophilicities. Experiments were carried out as a function of the initial water activity (a_w) of the biocatalyst, initial butyric acid concentration (A) and the molar ratio ethanol:butyric acid (MR) in the organic medium.

When the foams containing the immobilised lipase molecules are added to the reaction medium (substrates in *n*-hexane), the various molecular species will partition between the foams and the organic phase, with the migration of the hydrophilic substrates towards the microenvironment. Thus, the concentration of substrates in the microenvironment of the lipase becomes very different from that of the organic medium [31]. The partitioning of substrates (butyric acid and ethanol), product (ethyl butyrate) and water between the microenvironment of the *C. rugosa* lipase immobilised in these foams and bulk reaction medium was previously investigated [30,31]. From these studies, model equations were established to predict the water activity and the microenvironmental composition for these systems, from the knowledge of the initial bulk composition (prior to the addition of the biocatalyst). Therefore, modelling and optimisation of the production of ethyl butyrate and conversion into ester, as a function of the composition of the lipase microenvironment, were also attempted.

2. Materials

2.1. Enzyme

The lyophilised *C. rugosa* lipase (lipase AY 30) was a generous gift from Amano Enzyme Europe Ltd., UK.

2.2. Immobilisation matrix

The hydrophilic polyurethane pre-polymers ("Hypol FHP 2002^{TM}" and "Hypol FHP 5000^{TM}"), for lipase immobilisation, were kindly donated by Dow Chemical Company Limited, UK. "Hypol FHP 2002^{TM}" is a toluene diisocyanate (TDI) pre-polymer and "Hypol FHP 5000^{TM}" contains diphenylmethane-4,4′-diisocyanate (4,4′-MDI) groups. "Hypol FHP 2002^{TM}" and "Hypol FHP 5000^{TM}" foams have aquaphilicity values [33] of 3.2 and 2.6, respectively [31]. The aquaphilicity of immobilisation supports is a parameter proposed by Reslow et al. [33] as an indicator of the ability of the support to adsorb water from water-saturated diisopropyl ether. It is defined as the quotient (amount of water on the support):(amount of water in the solvent). In fact, aquaphilicity is not a true partition coefficient since it depends on the ratio (dried support: water saturated diisopropyl ether) used for the assay. Reslow et al. [33] proposed the use of a 1:5 (w/w) ratio.

2.3. Reagents

Butyric acid, ethanol, ethyl butyrate, *n*-hexane and 4-methyl 2-penthanol (used as internal standard) were analytical grade and obtained from various commercial sources.

3. Methods

3.1. Preparation of immobilised lipase

Hydrophilic polyurethane foams were prepared by mixing the polyurethane pre-polymer (0.60 g of "Hypol FHP 2002^{TM}" and 0.35 g of "Hypol FHP 5000^{TM}") with the aqueous phosphate buffer solution (0.020 M KH_2PO_4 + 0.027 M Na_2HPO_4; pH 7.0), containing lipase powder (0.35 or 0.30 g for "Hypol FHP 2002^{TM}" and "Hypol FHP 5000^{TM}" foams, respectively), in a ratio of 1:1 (w/w) [28]. During polymerisation, water reacts with free radicals of the pre-polymer and carbon dioxide is released [34]. In spite of the different amounts of pre-polymer used, a final volume of about 1.7 cm^3 was exhibited by each foam preparation. This is explained by the higher porosity of FHP 5000 foam (0.73) when compared with the other counterpart (0.64), as previously estimated by water displacement in a graduated cylinder [5].

The amount of lipase used in each foam corresponds to the maximum load above which severe internal mass transfer limitations are encountered [28,31].

Table 1
Experimental design matrix used (CCRD-1) as a function of initial thermodynamic water activity of the biocatalyst (a_w), the initial butyric acid concentration (A) and the initial ethanol:butyric acid molar ratio in the organic medium (MR)

Experiment number	Initial thermodynamic water activity, a_w	Organic medium		FHP 2002 foam		FHP 5000 foam	
		A (M)	MR	[ESTER] (M)	CONV (%)	[ESTER] (M)	CONV (%)
1	0.350	0.150	0.700	0.022	21.0	0.046	43.5
2	0.350	0.150	2.000	0.021	13.9	0.049	32.6
3	0.350	0.500	0.700	0.023	6.5	0.059	16.7
4	0.350	0.500	2.000	0.020	4.1	0.038	7.5
5	0.750	0.150	0.700	0.045	43.2	0.060	57.1
6	0.750	0.150	2.000	0.036	23.8	0.144	96.3
7	0.750	0.500	0.700	0.112	32.1	0.130	37.1
8	0.750	0.500	2.000	0.062	12.4	0.102	20.4
9	0.214	0.325	1.350	0.023	7.1	0.037	11.5
10	0.886	0.325	1.350	0.162	49.8	0.305	93.9
11	0.550	0.031	1.350	0.019	60.0	0.027	88.7
12	0.550	0.619	1.350	0.025	4.1	0.042	6.8
13	0.550	0.325	0.257	0.030	35.7	0.082	98.0
14	0.550	0.325	2.443	0.021	6.3	0.031	9.5
15	0.550	0.325	1.350	0.022	6.7	0.032	9.8
16	0.550	0.325	1.350	0.022	6.6	0.056	17.2
17	0.550	0.325	1.350	0.023	7.1	0.060	18.5

Experimental results of ethyl butyrate concentration ([ESTER]) and conversion into ethyl butyrate (CONV), after 18 h reaction, at 30 °C, for each experiment with both biocatalysts.

3.2. Preparation of biocatalysts with different initial a_w values

After polymerisation, both FHP 2002 and FHP 5000 preparations have high a_w values (about 0.98 at 30 °C) [30]. To achieve immobilised lipases at different initial a_w values, foams were dried under reduced pressure, for different times, according to the procedure previously described [28]. The foams containing immobilised lipase molecules, prepared in triplicate, were cut into small cuboids (~0.07 cm^3) and immediately placed in a Heraeus D-6450 Hanaw vacuum oven, at 40 °C under 15 or 50 kPa absolute pressure. At different times, foams were taken from the oven and their thermodynamic water activity value was measured at 30 °C with a Rotronic Hygroskop DT humidity sensor (DMS-100H). After drying and a_w measurement, the immobilised biocatalysts were immediately added to the organic reaction medium.

3.3. Esterification experiments

The FHP 5000 or FHP 2002 foams containing immobilised lipase molecules and cut in cuboids (~0.07 cm^3) were immersed in 12 cm^3 *n*-hexane solutions containing butyric acid and ethanol. The initial a_w value of the biocatalyst and concentrations of substrates varied according to the experimental design followed (cf. Section 3.4).

The esterification reaction was carried out at 30 °C in a thermostated-capped cylindrical glass vessel under magnetic stirring at 1400 rpm. After 18 h contact time, samples of 500 μL of organic medium were taken and assayed for ethanol, butyric acid and ethyl butyrate content. These samples were added to equal volumes of 0.4 M 4-methyl-2-penthanol (internal standard) in *n*-hexane, prior to the analysis by gas chromatography, as previously described [31].

3.4. Experimental design

Response surface methodology [35–37] was used to model and optimise the esterification of ethanol with butyric acid, catalysed by *C. rugosa* lipase immobilised in polyurethane foams. In a first set of experiments, the effects of the initial a_w of the biocatalyst (lipase in FHP 2002 foams or in FHP 5000 foams), initial butyric acid concentration (A) and molar ratio ethanol:butyric acid (MR) on ester production were investigated. For each biocatalyst, a total of 17 esterification experiments was carried out following a central composite rotatable design (CCRD-1), where the following five levels were tested for each independent variable: two factorial levels; two star levels; and the centre level [35–37]. The following ranges were considered in this experimental design: a_w varied from 0.21 to 0.89, A from 0.03 to 0.62 M and MR from 0.26 to 2.44 (Table 1). The choice of a wide range for all the variables tested was aimed at identifying real effects of each variable on the esterification reaction and also at limiting the boundaries of the experimental space.

Taking into account the results obtained in this first CCRD, another set of esterification experiments was carried out, for each biocatalyst type, following a second CCRD (CCRD-2) only as a function of A and MR, and using the biocatalyst at its original a_w of about 0.98. Also, five levels were tested for each variable and the experimental design consists of four factorial points, four star-points and three centre points, in a total of 11 experiments per biocatalyst. A varied from 0.08 to 0.57 M and MR from 0.43 to 2.27 (Table 2). In this second set of experiments, the CCRD followed was the same used in previous experiments carried out with the same reaction systems, to evaluate the partitioning of water, butyric acid and ethanol between the microenvironment of the immobilised lipase in polyurethane foams and the organic solvent [30,31].

Table 2
Experimental design matrix used (CCRD-2) as a function of the initial butyric acid concentration (*A*) and the initial ethanol:butyric acid molar ratio in the organic medium (MR)

Experiment number	Organic medium		FHP 2002 foam		FHP 5000 foam	
	A (M)	MR	[ESTER] (M)	CONV (%)	[ESTER] (M)	CONV (%)
1	0.150	0.700	0.093	88.6	0.105	100.0
2	0.150	2.000	0.122	81.2	0.135	90.3
3	0.500	0.700	0.027	7.6	0.311	89.0
4	0.500	2.000	0.142	28.3	0.115	23.1
5	0.078	1.350	0.077	98.8	0.078	100.0
6	0.572	1.350	0.179	31.3	0.196	34.3
7	0.325	0.431	0.129	92.3	0.124	88.6
8	0.325	2.269	0.159	48.8	0.109	33.5
9	0.325	1.350	0.225	69.2	0.245	75.5
10	0.325	1.350	0.198	60.9	0.213	65.5
11	0.325	1.350	0.258	79.4	0.249	76.6

Experimental results of ethyl butyrate concentration ([ESTER]) and conversion into ethyl butyrate (CONV) after 18 h reaction, for each experiment with both biocatalysts at their original water activity of 0.98 at 30 °C.

3.5. Statistical analysis

For every experiment of each CCRD, the ethyl butyrate concentration (ESTER) and the respective conversion values were analysed using the software "Statistica™", version 5, from Statsoft, Tulsa, USA. The conversion into ethyl butyrate (CONV) was defined as the ratio between ethyl butyrate concentration and the initial concentration of the limiting substrate in the organic medium.

Linear and quadratic effects of the independent variables and their linear interactions on ESTER and CONV were calculated. Their significance was evaluated by analysis of variance. Four- and three-dimensional surfaces were fitted to each set of estimated ESTER and CONV values, for the CCRD-1 and CCRD-2 experiments, respectively. These surfaces were described by a first- or second-order polynomial. First- and second-order coefficients of these equations are usually unknown and, therefore, were estimated from the experimental data by using the statistical principle of least squares. The fit of the models was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2_{adj}) [35–38]. The R^2 value provides a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions. However, the R^2 should be used with caution since it will always increase with the inclusion of a new variable to the model. The use of R^2_{adj} is preferred and is related with R^2 by the following equation:

$$R^2_{adj} = 1 - \frac{n-1}{n-p}(1 - R^2) \qquad (1)$$

where n is the number of experiments and p is the number of variables in the model. The R^2_{adj} takes into account the fact that the number of residual degrees of freedom in the polynomial regression changes as the order of the polynomial changes. R^2_{adj} is an unbiased estimate of the coefficient of determination and is always smaller than R^2. In practice, R^2 should be at least 0.75 or greater; values above 0.90 are considered to be very good [36].

3.6. Validation of the esterification models

To investigate the applicability of the models established by RSM to describe ester production, esterification experiments were carried out for each biocatalyst, under the predicted initial optimised conditions. Thus, initial 0.35 M *A* and 1.51 MR were used, for the system containing the immobilised lipase in FHP 2002 foams, and 0.54 M *A* and 0.75 MR for the lipase in FHP 5000 foam system. Along 48 h reaction, samples of 500 μL of organic medium were taken and assayed for substrates and product as previously described (cf. Section 3.3). The obtained experimental results were compared to the theoretical values predicted by the models.

4. Results and discussion

4.1. Preparation of biocatalysts with different initial a_w values

The drying curves for FHP 2002 and FHP 5000 foams containing immobilised lipase molecules, obtained at 40 °C under 15 and 50 kPa, are presented in Fig. 1. Similar profiles were observed for all cases, but when drying was performed at 15 kPa,

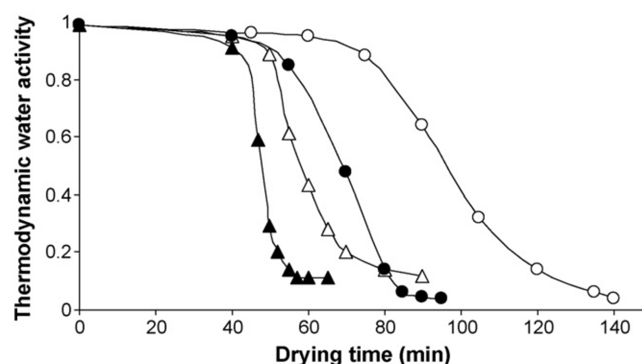

Fig. 1. Drying curves of FHP 2002 (circles) and FHP 5000 (triangles) foams with immobilised lipase molecules, obtained at 40 °C under 15 kPa (filled symbols) and 50 kPa (open symbols) pressures.

Table 3
Effects and respective *p*-levels (values between brackets) of the initial thermodynamic water activity of the biocatalyst (a_w), of the initial butyric acid concentration (A), of the molar ratio ethanol:acid (MR) and the respective interactions ($a_w \times A$), ($a_w \times$ MR) and ($A \times$ MR) on the production of ethyl butyrate (ESTER) and on conversion into ethyl butyrate (CONV) with the biocatalyst immobilised in the FHP 2002 and FHP 5000 foams

Factor	FHP 2002 foam[a]		FHP 5000 foam[a]	
	ESTER (M)	CONV (%)	ESTER (M)	CONV (%)
a_w (linear term)	0.059 (0.00038)	20.189 (0.0080)	0.102 (0.0022)	36.504 (0.026)
a_w (quadratic term)	0.0472 (0.0024)	11.409 (0.102)	0.0814 (0.011)	20.034 (0.203)
A (linear term)	0.0151 (0.147)	−20.625 (0.0072)	0.00783 (0.728)	−41.821 (0.014)
A (quadratic term)	−0.0027 (0.798)	13.960 (0.055)	−0.0154 (0.538)	16.513 (0.285)
MR (linear term)	−0.0114 (0.257)	−14.359 (0.035)	−0.00683 (0.761)	−21.433 (0.141)
MR (quadratic term)	−0.00023 (0.982)	6.125 (0.346)	−0.00008 (0.997)	20.741 (0.188)
$a_w \times A$	0.0233 (0.097)	0.45 (0.952)	0.00648 (0.825)	−11.006 (0.536)
$a_w \times$ MR	−0.0138 (0.294)	−7.400 (0.338)	0.0185 (0.532)	10.629 (0.549)
$A \times$ MR	−0.0108 (0.404)	1.100 (0.883)	−0.0341 (0.266)	−13.569 (0.448)

[a] System.

lower drying times were needed. Thus, by controlling pressure and drying time, different final a_w values can be reached in the foams, as previously reported [28]. In addition, the time required to dry FHP 2002 foams was higher than that needed to dry FHP 5000 foams, under the same experimental conditions. This suggests that water molecules are more strongly bound to FHP 2002 foams than to the other counterpart, which is in agreement with their aquaphilicity values (cf. Section 2.2).

4.2. Modelling ethyl butyrate production

To model the esterification reaction and optimise the initial bulk composition and the thermodynamic water activity of both FHP 2002 and FHP 5000 foams, linear and quadratic effects of a_w, A and MR and the respective linear interactions ($A \times a_w$), (MR $\times a_w$) and ($A \times$ MR), on ethyl butyrate concentration and on the conversion into ester were calculated (Table 3). For a given factor (a_w, A or MR), a positive or negative effect on a response (ESTER or CONV) indicates that an increase in the value of that factor is accompanied by a corresponding increase or reduction in the response, respectively. Therefore, the response CONV significantly decreases at linear level and increases at quadratic level with the factors A and MR (Table 3).

In these first sets of experiments, a low effect of the initial acid concentration alone on ester production is observed. However, the effect of A cannot be ignored since some of its linear interactions are important enough to be neglected.

For every system under study, the initial a_w showed significant positive linear and/or quadratic effects on ESTER and CONV. This indicates that high initial a_w values of the immobilised lipase will promote the esterification reaction. The positive effect of initial a_w of the biocatalyst on ester synthesis is unexpected since, in esterification reactions, water is a product. Therefore, low a_w environmental conditions should promote the shift of the chemical equilibrium towards the synthesis [32]. However, due to the hydrophylicity of the polyurethanes used as immobilisation supports, a competition for the free water remaining from the polymerisation step will occur between the foams, the enzyme molecules and, though in much less extent, the organic solvent [30]. Also, due to partition effects, higher ethanol concentrations in the microenvironment of the enzyme, particularly in the most hydrophilic foams (FHP 2002 foam) will be observed, since ethanol is considerably more soluble in the aqueous phase (i.e. near the enzyme) than in the organic medium [31]. A loss of biocatalyst activity, due to ethanol-induced dehydration of the protein, may also occur. This may be particularly dramatic when the amount of free water is insufficient to compensate for the dehydration, i.e. under low water activity conditions. Thus, a high initial a_w value of the biocatalyst seems to promote an adequate water activity for the esterification reaction, after the immersion of the immobilised lipase preparations in the organic media and when water partition equilibrium is attained. In addition, high a_w values are not detrimental to ester synthesis because both substrates are hydrophilic and the product is hydrophobic. Thus, the ester is released to the bulk organic medium, becoming not accessible to the reverse hydrolytic reaction [31]. In fact, the reaction systems used can be approximated as a reverse emulsion, with *n*-hexane as the continuous phase and water droplets entrapped in the support particles [30].

For each system, the experimental data (ESTER and CONV values) could be fitted to four-dimensional response surfaces, described by second-order polynomial models, as a function of the selected factors with important effects on them (Table 4). These surfaces are presented in two three-dimensional surfaces each (Figs. 2 and 3), as a function of only two factors (a_w versus A; a_w versus MR), keeping the third factor constant, at its central point value (i.e., MR = 1.35 or A = 0.325 M, respectively).

Concerning the results obtained in these first CCRDs, a second set of esterification experiments was carried out, only as a function of A and MR, using the biocatalysts at its initial thermodynamic water activity of 0.98. The migration of free water from the polyurethane foams to the bulk organic medium will occur, leading to high a_w values when partition equilibrium is attained [30].

The production of ethyl butyrate and conversion into ester, after 18 h reaction time, are presented in Table 2. With both foams, the highest conversions (higher than 95%) were observed at low A values.

The conversion into ester significantly decreases with the initial butyric acid concentration and/or the molar ratio ethanol/butyric acid, as indicated by the estimated effects of A,

Table 4
Model equations for the response surfaces fitted to the production of ethyl butyrate (ESTER) and to the conversion into ethyl butyrate (CONV), after 18 h reaction, as a function of the initial thermodynamic water activity (a_w), of the initial butyric acid concentration (A) and the initial molar ratio ethanol:butyric acid (MR), with the biocatalyst immobilised in the FHP 2002 and FHP 5000 foams and respective R^2 and R^2_{adj}

System	Model equations	R^2	R^2_{adj}
FHP 2002 foam	$\text{ESTER} = 0.119 - 0.548a_w + 0.599a_w^2 - 0.0757A + 0.0356\text{MR} + 0.332a_w A - 0.0529a_w \text{MR} - 0.0473A\,\text{MR}$	0.915	0.848
	$\text{CONV} = 72.981 - 67.985a_w + 142.618a_w^2 - 207.08A + 227.926A^2 - 14.964\text{MR} + 7.249\text{MR}^2 - 28.462a_w \text{MR}$	0.857	0.746
FHP 5000 foam	$\text{ESTER} = 0.223 - 0.912a_w + 1.061a_w^2$	0.793	0.764
	$\text{CONV} = 175.989 - 184.21a_w + 250.427a_w^2 - 294.729A + 269.598A^2 - 82.761\text{MR} + 24.546\text{MR}^2$	0.744	0.591

MR and of the linear interaction ($A \times \text{MR}$) (Table 5). In addition, for FHP 5000 preparation, the negative effect of the linear interaction ($A \times \text{MR}$) cannot be neglected, either on the conversion or on ester production.

A decrease of the ester conversion with increasing substrate concentration was also reported by others [6,12,14]. This is probably ascribed to the accumulation of water during the progress of the reaction, which will promote the reverse hydrolysis reaction and/or an inhibitory effect of ethanol [12]. For both immobilised preparations, the production of ethyl butyrate could be fitted to convex surfaces (Fig. 4A and B), described by second-order polynomial models (Table 6) with stationary points (maxima) inside the experimental domain. The conversion into ester could be described by a first- or second-order polynomial equation

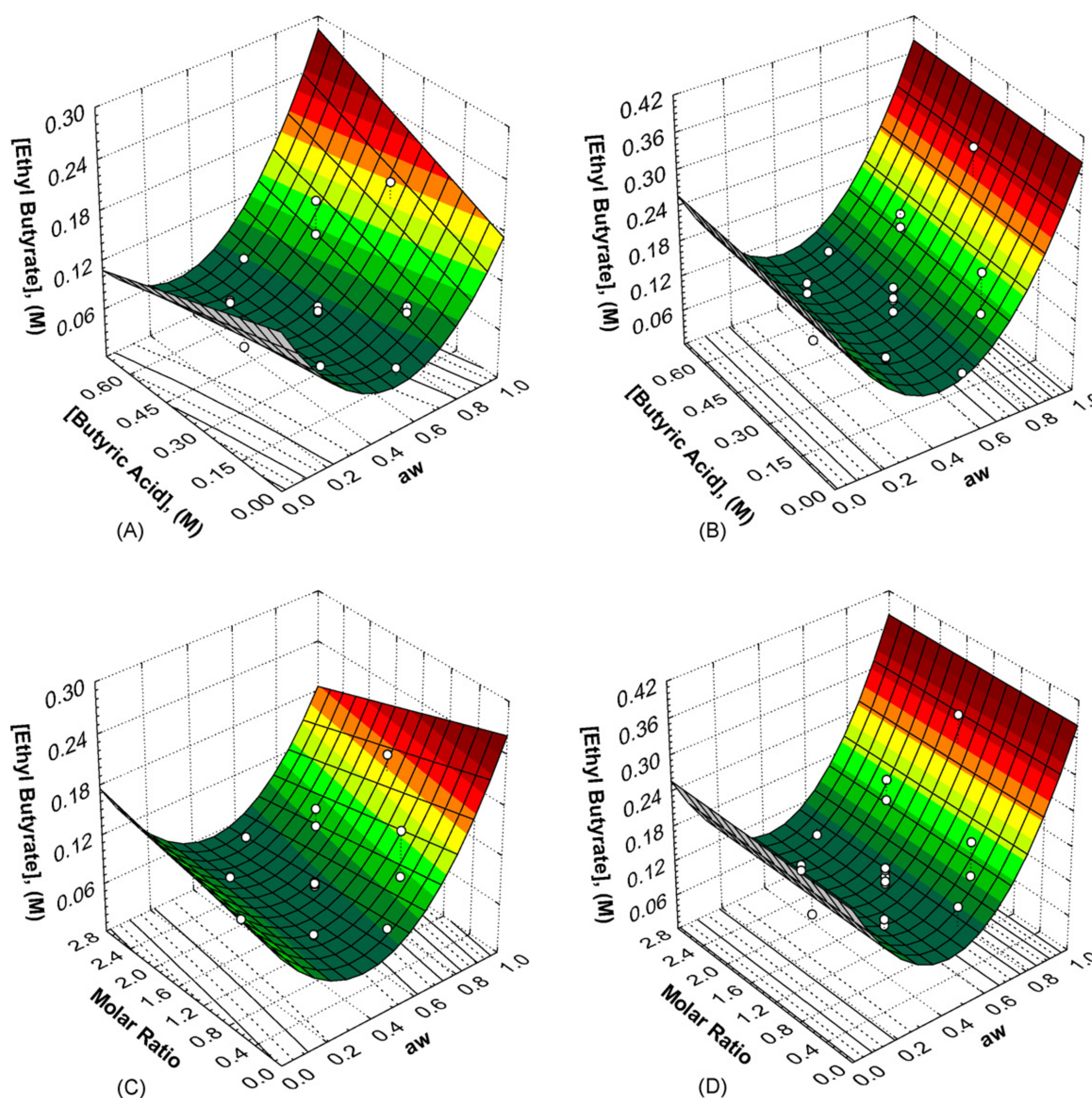

Fig. 2. Response surfaces fitted to the production of ethyl butyrate, as a function of (i) initial a_w of the biocatalyst and initial butyric acid concentration, and of (ii) initial a_w of the biocatalyst and initial molar ratio ethanol/butyric acid, catalysed by *C. rugosa* lipase immobilised in FHP 2002 (A and C) and FHP 5000 (B and D) foams, respectively.

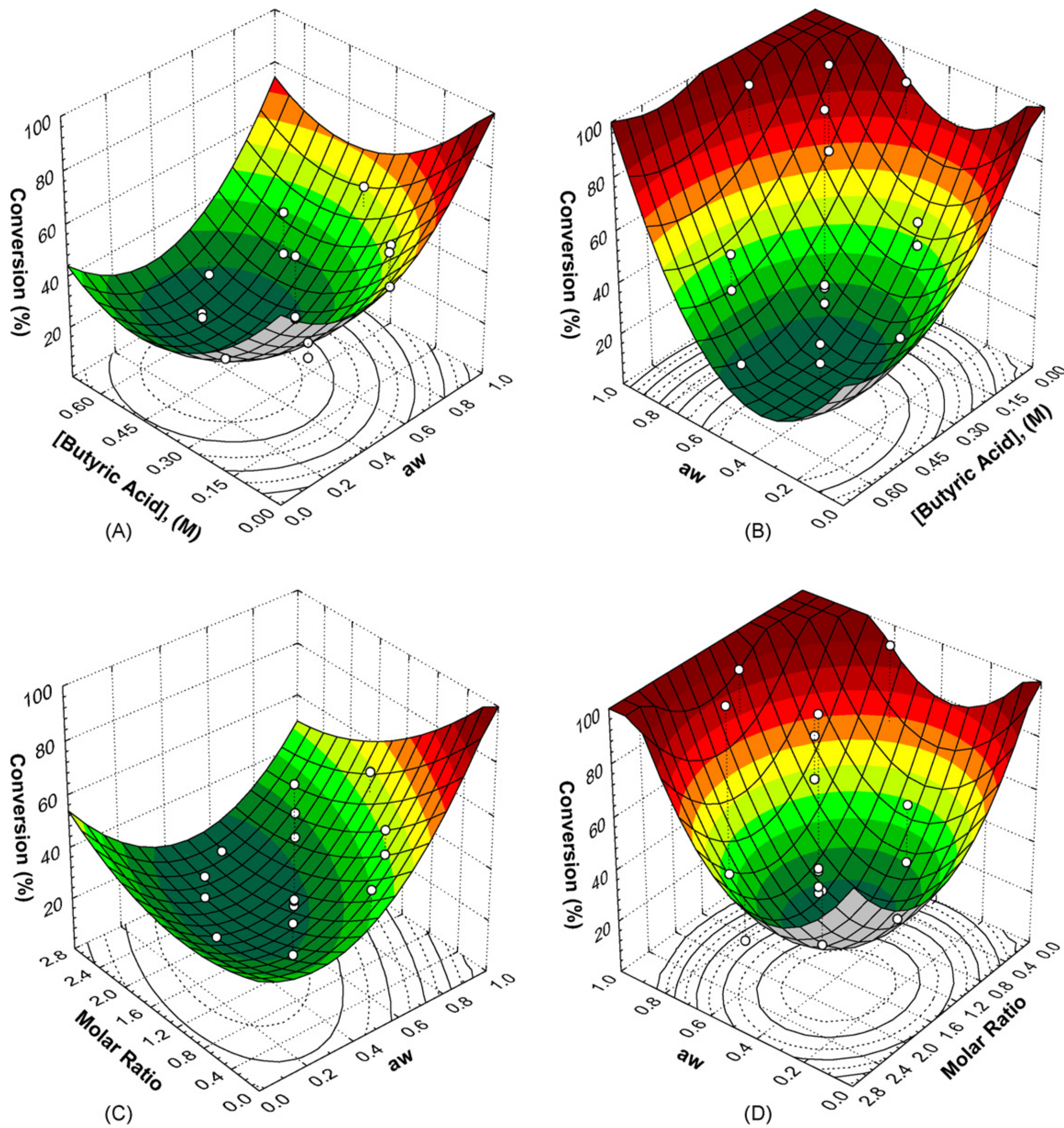

Fig. 3. Response surfaces fitted to the conversion into ethyl butyrate, as a function of (i) initial a_w of the biocatalyst and initial butyric acid concentration, and of (ii) initial a_w of the biocatalyst and initial molar ratio ethanol/butyric acid, catalysed by *C. rugosa* lipase immobilised in FHP 2002 (A and C) and FHP 5000 (B and D) foams, respectively.

Table 5

Effects and respective *p*-levels (values between parentheses) of the initial butyric acid concentration (*A*), of the molar ratio ethanol:acid (MR) and interaction ($A \times$ MR) on the production of ethyl butyrate (ESTER) and on the conversion into ethyl butyrate (CONV) with the biocatalyst immobilised in the FHP 2002 and FHP 5000 foams, at its original a_w (about 0.98)

Factor	FHP 2002 foam[a]		FHP 5000 foam[a]	
	ESTER (M)	CONV (%)	ESTER (M)	CONV (%)
A (linear term)	0.0244 (0.493)	−57.399 (0.0061)	0.0687 (0.086)	−42.853 (0.0009)
A (quadratic term)	−0.1194 (0.029)	−13.051 (0.425)	−0.0668 (0.142)	0.375 (0.961)
MR (linear term)	0.0464 (0.219)	−12.041 (0.383)	−0.0538 (0.155)	−38.360 (0.0015)
MR (quadratic term)	−0.103 (0.047)	−7.438 (0.641)	−0.0924 (0.060)	−5.690 (0.466)
$A \times$ MR	0.0431 (0.397)	14.069 (0.466)	−0.0989 (0.081)	−28.089 (0.022)

[a] System.

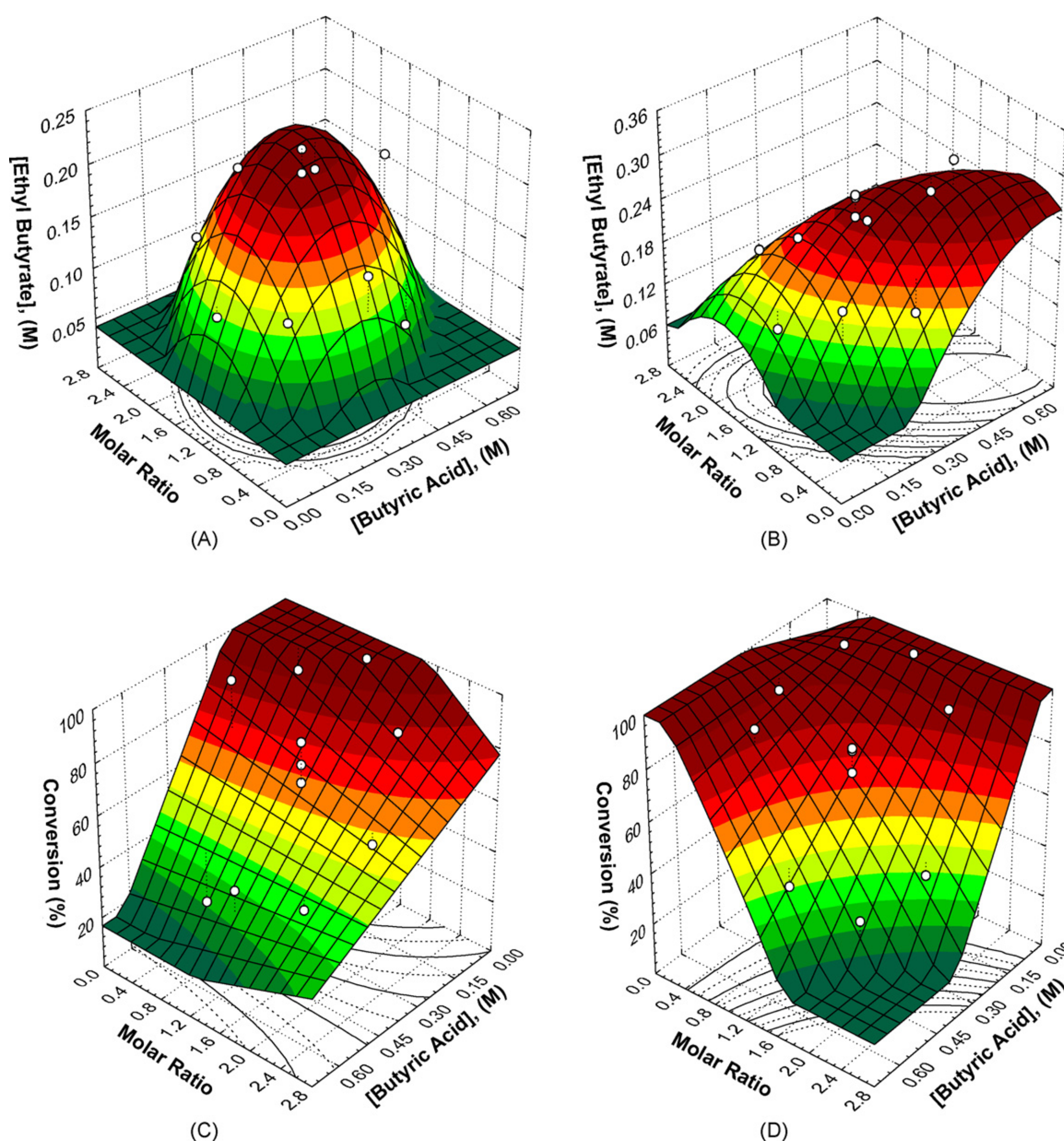

Fig. 4. Response surfaces fitted to the production of ethyl butyrate and to the conversion into ethyl butyrate, catalysed by *C. rugosa* lipase immobilised in FHP 2002 (A and C) and FHP 5000 (B and D) foams, respectively, as a function of initial butyric acid concentration and initial molar ratio ethanol/butyric acid.

(Table 6), when lipase was immobilised in FHP 2002 and FHP 5000 foams, respectively (Fig. 4C and D).

By partial differentiation of the polynomial equations for ESTER (Table 6), a maximum ester production, after 18 h esterification, and the respective initial *A* and MR in the organic medium, needed to attain this maximum, can be predicted (Table 7).

In previous studies, model equations were established to predict the a_w of these systems [30] and also the composition of the microenvironment of these biocatalysts [31], as a function of

Table 6
Model equations for the response surfaces fitted to the production of ethyl butyrate (ESTER) and to the conversion into ethyl butyrate (CONV) after 18 h reaction, as a function of the initial butyric acid concentration (A) and the initial molar ratio ethanol:butyric acid (MR), with the biocatalyst immobilised in the FHP 2002 and FHP 5000 foams, at its original a_w (about 0.98), and respective R^2 and R^2_{adj}

System	Model equations	R^2	R^2_{adj}
FHP 2002 foam	$\text{ESTER} = -0.189 + 1.081A - 1.950A^2 + 0.303\text{MR} - 0.122\text{MR}^2 + 0.190A\,\text{MR}$	0.761	0.521
	$\text{CONV} = 155.347 - 247.483A - 29.361\text{MR} + 61.84A\,\text{MR}$	0.792	0.704
FHP 5000 foam	$\text{ESTER} = -0.278 + 1.493A - 1.092A^2 + 0.395\text{MR} - 0.109\text{MR}^2 - 0.435A\,\text{MR}$	0.793	0.585
	$\text{CONV} = 85.636 + 44.245A + 29.150\text{MR} - 6.863\text{MR}^2 - 123.467A\,\text{MR}$	0.953	0.922

Table 7
Predicted initial medium composition (initial acid concentration [A], molar ratio, MR, and ethanol concentration [EtOH]), and the respective predicted composition in the microenvironment of the biocatalyst ($[A]_{microenv}$; $[EtOH]_{microenv}$), to maximize ester production, [ESTER], the corresponding conversion into ester, CONV, and productivity, after 18 h esterification reaction, and a_w values of the systems

System	Initial [A] (M)	Initial MR	Initial [EtOH] (M)	$[A]_{microenv}$ (M)	$[EtOH]_{microenv}$ (M)	[Ester] (M)	CONV (%)	Productivity (μmol/mL h)	a_w
FHP 2002 foam	0.35	1.51	0.53	0.84	1.65	0.23	66	12.8	0.945
FHP 5000 foam	0.54	0.75	0.41	0.32	0.45	0.27	66	15.0	0.949

the bulk initial A and MR. Using these equations, it is possible to predict the water activity of our systems and the concentration of ethanol and butyric acid in the microenvironment of the biocatalysts, under the predicted optimised conditions and when partition equilibrium is reached. All these predicted values are presented in Table 7.

For the immobilised lipase in FHP 2002 foam, a maximum ester production of 0.23 M (volumetric productivity of 12.8 μmol/mL h), is expected, after 18 h reaction, under optimised initial conditions (0.35 M of A, MR of 1.51). When FHP 5000 foam is used, optimum initial conditions (0.54 M of A, MR of 0.75) will lead to the production of 0.27 M of ethyl butyrate (volumetric productivity of 15.0 μmol/mL h). For both biocatalysts, predicted maxima for ester production are attained under similar a_w conditions (about 0.95) and correspond to 66% conversion. The conversion values are calculated on the basis of the limiting substrate, i.e. butyric acid and ethanol for the FHP 2002 and FHP 5000 system, respectively.

According to the models, with FHP 5000 foam, the maximum predicted ester concentration is expected under much lower concentrations of butyric acid and ethanol in the vicinity of the biocatalyst, than with FHP 2002 foam under optimised conditions (Table 7).

4.3. Validation of esterification models

To investigate the applicability of these models describing ester production, esterification experiments were carried out with each biocatalyst, under the predicted initial optimised conditions. The time-course of the reaction catalysed by the lipase in FHP 2002 and FHP 5000 foams is presented in Figs. 5 and 6, respectively. The obtained values were compared with the theoretical values predicted by the models (Table 7). After 18 h reaction, the amounts of ethyl butyrate produced are similar to the values predicted by the models. This confirms the goodness of fit of the models to the experimental data, validating them. In addition, for both systems, a quasi-equilibrium state is only attained after about 24 h reaction when 0.243 M (69% conversion) and 0.28 M (68% conversion) of ester is produced by the lipase in FHP 2002 and in FHP 5000 foams, respectively.

The better performance of FHP 5000 foam can be ascribed to its lower hydrophilicity than FHP 2000 foam, leading to lower substrate concentrations in the microenvironment, under similar initial conditions. Thus, substrate inhibition seems to be alleviated by the use of FHP 5000 foam for lipase immobilisation. These aspects will be addressed in a forthcoming article.

4.4. Comparison with other studies

The production of ethyl butyrate by direct esterification catalysed by immobilised lipases has been previously described [5,6,12,14]. The majority of these studies used equimolar amounts of ethanol and butyric acid in n-hexane [5,14] or in n-heptane [5,12].

The immobilised *C. rugosa* lipase immobilised in FHP 2002 foam was previously tested as a catalyst for the esterification of 0.3 M ethanol with 0.3 M butyric acid in n-hexane or n-heptane: final ester concentrations of 0.21 and 0.23 M, corresponding to volumetric productivities of 8.58 and 9.63 μmol/mL h, were obtained, respectively [5]. When the same lipase was

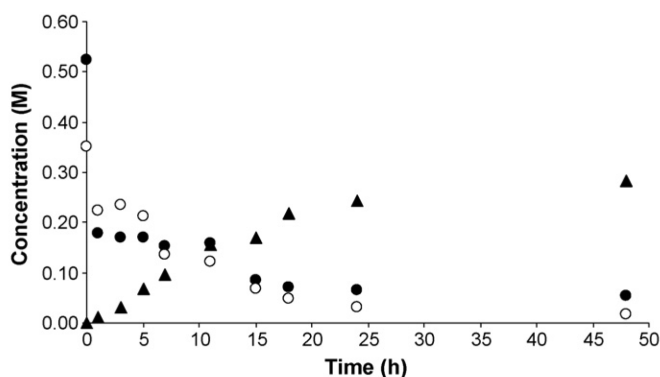

Fig. 5. Time-course of the production of ethyl butyrate (triangles) by the esterification of ethanol (filled circles) with butyric acid (open circles), catalysed by *C. rugosa* lipase immobilised in FHP 2002 foam.

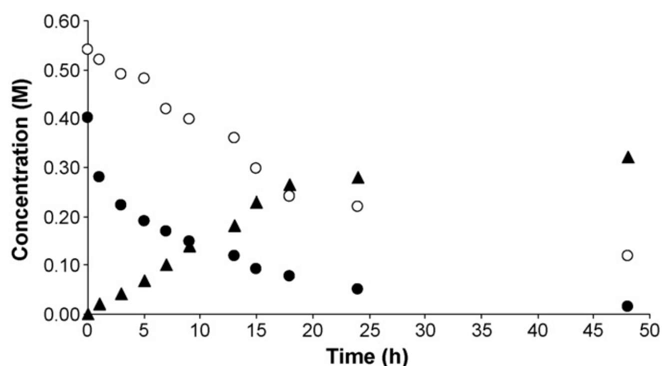

Fig. 6. Time-course of the production of ethyl butyrate (triangles) by the esterification of ethanol (filled circles) with butyric acid (open circles), catalysed by *C. rugosa* lipase immobilised in FHP 5000 foam.

immobilised on silica-gel and used in *n*-hexane containing initial equimolar substrates concentration (0.2 M), 0.19 M ester was obtained after 96 h reaction time (productivity of 1.98 μmol/mL h), [14]. A maximum conversion to ethyl butyrate of 72.9%, corresponding to 0.03 M ester production, was predicted by RSM upon 96 h reaction at 34 °C in *n*-heptane (0.312 μmol/mL h), catalysed by a commercial immobilised preparation of *C. antarctica*, also when initial equimolar substrate concentrations were used [12].

In the presence of ethanol in excess (0.1 M *A* and 0.2 M of ethanol) in *n*-hexane, 98% conversion of acid in ester was achieved, corresponding to a volumetric productivity of 4.1 μmol/mL h, when the lipase from *Rhizomucor miehei* immobilized in celite was used [6].

The ethyl butyrate productivity values obtained in the present study are considerably higher than the values reported by other authors. In fact, in terms of scale-up of these systems and process implementation in continuous bioreactors, the volumetric productivity is a more important parameter to consider than conversion, since in the majority of these systems, high conversions are only attained upon long-term reactions and/or when low substrates concentration is used. These situations are thus not competitive with the chemical route. Clearly, the use of hydrophilic polyurethane foams as lipase immobilisation matrices enables the manipulation of the microenvironment of the biocatalyst, leading to the optimisation of the esterification reactions in the presence of organic solvents.

5. Conclusions

The present study shows that the prediction of optimal initial conditions for ester production using an immobilised biocatalyst may be tackled through the use of response surface models. In addition, it confirms the feasibility of RSM to predict: (i) the microenvironmental composition corresponding to the initial substrate composition that maximize ester production and also (ii) the water activity of these systems after partition equilibrium of water and substrates between bulk medium and microenvironment, by using the models developed in previous studies on the same reaction systems [30,31].

The developed methodology can be easily transposed to other systems containing immobilised biocatalysts, for the modelling and optimisation of the reaction conditions.

References

[1] M. Liaquat, R.K.O. Apenten, Synthesis of low molecular weight flavor esters using plant seedling lipases in organic media, J. Food Sci. 65 (2000) 295–299.

[2] D.W. Armstrong, H. Yamazaki, Natural flavours production: a biotechnological approach, Trends Biotechnol. 4 (1986) 264–268.

[3] B. Gilles, H. Yamazaki, D.W. Armstrong, Production of flavor esters by immobilized lipase, Biotechnol. Lett. 9 (1987) 709–714.

[4] G. Langrand, C. Triantaphylides, J. Baratti, Lipase catalyzed formation of flavour esters, Biotechnol. Lett. 10 (1988) 549–554.

[5] S.F. Dias, L. Vilas-Boas, J.M.S. Cabral, M.M.R. Fonseca, Production of ethyl butyrate by *Candida rugosa* lipase immobilized in polyurethane, Biocatalysis 5 (1991) 21–34.

[6] A. Manjón, J.L. Iborra, A. Arocas, Short-chain flavour ester synthesis by immobilized lipase in organic media, Biotechnol. Lett. 13 (1991) 339–344.

[7] H.F. Castro, P.C. Oliveira, E.B. Pereira, Evalluation of different approaches for lipase catalysed synthesis of citronellyl acetate, Biotechnol. Lett. 19 (1997) 229–232.

[8] A. Thakar, D. Madamwar, Enhanced ethyl butyrate production by surfactant coated lipase immobilized on silica, Process Biochem. 40 (2005) 3263–3266.

[9] D.R. Hamsaveni, S.G. Prapulla, S. Divakar, Response surface methodological approach for the synthesis of isobutyl isobutyrate, Process Biochem. 36 (2001) 1103–1109.

[10] S.H. Krishna, A.P. Sattur, N.G. Karanth, Lipase-catalyzed synthesis of isoamyl isobutyrate—optimisation using a central composite rotatable design, Process Biochem. 37 (2001) 9–16.

[11] G.A. Macedo, G.M. Pastore, M.I. Rodrigues, Optimising the synthesis of isoamyl butyrate using *Rhizopus* sp. lipase with a central composite rotatable design, Process Biochem. 39 (2004) 687–693.

[12] J.M. Rodriguez-Nogales, E. Roura, E. Contreras, Biosynthesis of ethyl butyrate using immobilized lipase: a statistical approach, Process Biochem. 40 (2005) 63–68.

[13] L.L.M.M. Melo, G.M. Pastore, G.A. Macedo, Optimized synthesis of citronellyl flavour esters using free and immobilized lipase from *Rhizopus* sp., Process Biochem. 40 (2005) 3181–3185.

[14] R. Dave, D. Madamwar, Esterification in organic solvents by silica-gel immobilized *Candida rugosa* lipase, in: C. Larroche, A. Pandey, C.-G. Dussap (Eds.), Current Topics on Bioprocesses in Food Industry, Asiatech Publishers Inc., New Deli, India, 2006, pp. 70–80.

[15] J.C. Santos, H.F. Castro, Lipase-catalyzed synthesis of butyl butyrate by direct esterification: optimization by factorial design, in: C. Larroche, A. Pandey, C.-G. Dussap (Eds.), Current Topics on Bioprocesses in Food Industry, Asiatech Publishers Inc., New Deli, India, 2006, pp. 120–127.

[16] M. Karra-Châabouni, H. Ghamgui, S. Bezzine, A. Rekik, Y. Gargouri, Production of flavour esters by immobilized Staphylococcus simulans lipase in a solvent-free system, Process Biochem. 41 (2006) 1692–1698.

[17] W.-D. Chiang, S.-W. Chang, C.-J. Shieh, Studies on the optimised lipase-catalyzed biosynthesis of *cis*-3-hexen-1-yl acetate in *n*-hexane, Process Biochem. 38 (2003) 1193–1199.

[18] G.D. Yadav, A.H. Trivedi, Kinetic modelling of immobilized-lipase catalysed transesterification of *n*-octanol with vinyl acetate in non-aqueous media, Enzyme Microb. Technol. 32 (2003) 783–789.

[19] B.H. Lee, Fundamentals of food biotechnology, VCH Publishers, New York, 1996.

[20] S. Fukui, A. Tanaka, T. Iida, Immobilisation of biocatalysts for bioprocesses in organic solvent media, in: C. Laane, J. Tramper, M.D. Lilly (Eds.), Biocatalysis in Organic Media, Elsevier Science Publishers B.V., Amsterdam, 1987, pp. 21–41.

[21] T. Kawamoto, K. Sonomoto, A. Tanaka, Esterification in organic solvents: selection of hydrolases and effects of reaction conditions, Biocatalysis 1 (1987) 137–145.

[22] A.C. Correia, S. Ferreira-Dias, The effect of impurities of crude olive residue oil on the operational stability of the *Candida rugosa* lipase immobilized in polyurethane foams, in: A. Ballesteros, F.J. Plou, J.L. Iborra, P. Halling (Eds.), Stability and Stabilization of Biocatalysts, Elsevier, 1988, pp. 71–76.

[23] S. Ferreira-Dias, M.M.R. da Fonseca, Enzymatic glycerolysis of olive oil: a reactional system with major analytical problems, Biotechnol. Techniques 7 (1993) 447–452.

[24] S. Ferreira-Dias, M.M.R. da Fonseca, Production of monoglycerides by glycerolysis of olive oil with immobilized lipases: effect of the water activity, Bioprocess Eng. 12 (1995) 327–337.

[25] S. Ferreira-Dias, M.M.R. da Fonseca, Glycerolysis of olive oil: batch operational stability of *Candida rugosa* lipase immobilized in hydrophilic polyurethane foams, Bioprocess Eng. 13 (1995) 311–315.

[26] S. Ferreira-Dias, M.M.R. da Fonseca, The effect of substrate hydrophobicity on the kinetic behaviour of immobilized *Candida rugosa* lipase, Biocatal. Biotransform. 13 (1995) 99–110.

[27] K.E. LeJeune, A.J. Russell, Covalent binding of a nerve agent hydrolyzing enzyme within polyurethane foams, Biotechnol. Bioeng. 51 (1996) 450–457.
[28] S. Ferreira-Dias, A.C. Correia, F.O. Baptista, Activity and batch operational stability of *Candida rugosa* lipase immobilized in different hydrophilic polyurethane foams during hydrolysis in a biphasic medium, Bioprocess Eng. 21 (1999) 517–524.
[29] S. Ferreira-Dias, A.C. Correia, M.M.R. da Fonseca, Response surface modelling of glycerolysis catalysed by *Candida rugosa* lipase immobilized in different polyurethane foams for the production of partial glycerides, J. Mol. Catal. B: Enzym. 21 (2003) 71–80.
[30] P. Pires-Cabral, E. Dubreucq, M.M.R. da Fonseca, S. Ferreira-Dias, Partitioning of water in organic systems with lipase immobilized in polyurethane foams, Biochem. Eng. J. 26 (2005) 29–37.
[31] P. Pires-Cabral, M.M.R. da Fonseca, S. Ferreira-Dias, Modelling the microenvironment of a lipase immobilised in polyurethane foams, Biocatal. Biotransform. 23 (2005) 363–373.
[32] P.J. Halling, Thermodynamic predictions for biocatalysis in non-conventional media—theory, tests and recommendations for experimental design and analysis, Enzyme Microb. Technol. 16 (1994) 178–206.
[33] M. Reslow, P. Adlercreutz, B. Mattiasson, On the importance of the support material for bioorganic synthesis, Eur. J. Biochem. 172 (1988) 573–578.
[34] D.W. Fasset, Cyanides and nitriles, in: Industrial Hygiene and Toxicology, vol. 2, Interscience Publishers, New York, 1963, p. 2032.
[35] M.C. Gacula Jr., J. Singh, Response surface designs and analysis, in: Statistical Methods in Food and Consumer Research, Food Science and Technology, A Series of Monographs, Academic Press, 1984, p. 214.
[36] P.D. Haaland, Experimental Design in Biotechnology, Statistics: Textbooks and Monographs, D.B. Owen (Ed.), Marcel Dekker Inc., New York and Basel, 1989, p. 258.
[37] D.C. Montgomery, Design and Analysis of Experiments, John Wiley and Sons, New York, 1991, p. 649.
[38] S. Weisberg, Applied Linear Regression, John Wiley & Sons, 1985, pp. 217–218.