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SHORT COMMUNICATION

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THE IMMOBILIZATION OF LIPASE ON SEPABEADS: COUPLING, CHARACTERIZATION AND APPLICATION IN GERANYL BUTYRATE SYNTHESIS IN A LOW AQUEOUS SYSTEM

Lipase from Candida rugosa immobilized on Sepabeads EC-EP was shown to catalyze the esterification of geraniol with butyric acid in a predominantly organic system. The immobilization procedure was adjusted to optimize the enzyme activity and the immobilized enzyme was then used for a geranyl butyrate synthesis as a study model. The immobilized enzyme showed favorable performances in an aqueous system and increased the stability in the presence of organic solvents. The response surface methodology and a 5-level-5--factor central composite rotatable design were performed to identify the factors that influence the ester production and to verify whether any changes should be made in their settings to improve this reaction. The initial water content, the reaction temperature, the enzyme concentration, acid/alcohol molar ratio and time of addition of molecular sieves were the variables investigated. The production of the ester was optimized and an ester production response equation was obtained, making it possible to predict ester yields from the known values of the five main factors. The temperature during the esterification reaction was identified as the factor having the greatest impact on the ester yield.

Key words: Candida rugosa lipase; covalent immobilization; Sepabeads; geranyl butyrate synthesis; nonaqueous system.

Geraniol esters, and geranyl butyrate among them, have strong and pleasant odors and are used extensively as flavor ingredients in the fragrance, food and pharmaceutical industry [1]. One route of the production of geranyl butyrate is the esterification of geraniol with butyric acid, which increases the value of geraniol severalfold. Being applied as catalysts, lipases have certain advantages over chemical ones, including mild reaction conditions, a high catalytic activity, high specificity, and economic viability [2,3]. To fully exploit the technical and economical advantages of lipases, it is recommended to use them in an immobilized state to reduce cost and the poor stability of the soluble form. The immobilization also facilitates the development of continuous bioprocesses, enhances lipase properties such as stability and activity in non--aqueous media, and provides more flexibility with the enzyme/substrate contact by using various reactor configurations [4].

In particular, a multipoint covalent attachment to solid supports is stable and has generally been favored in the case of several enzymes [5]. The formation of the rigid enzyme-support linkage provides both kinetic and thermodynamic stabilization of the three-dimensional structure of the enzyme protecting it against denaturing agents that promote unfolding processes. Among the reactive groups, epoxy groups seem to be very convenient [6]. Immobilization protocols carefully designed to stabilize several enzymes by a multipoint covalent attachment to epoxy-activated supports appear to give the impressive result concerning the enzyme stabilization factor [7]. However, all these strategies have been performed mainly in order to improve the enzyme stability in aqueous systems; not much attention has been paid to the influence of the immobilization techniques on catalytic properties of biocatalysts in non-aqueous systems.

A design of an efficient lipase immobilized system possessing high loading capacity and activity retention, as well as improved stability for the esters

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synthesis in non-conventional media, still has many unresolved issues. Small changes in variables such as nature and characteristics of the immobilization support or water activity can have a significant impact on lipase activity and selectivity. Therefore, a careful design of the reaction medium and the immobilization support is required to obtain a higher synthetic yield of the product in an organic solvent system.

The Sepabeads support material of interest in this work is a synthetic polymethacrylic polymer having good chemical, mechanical and other properties such as low swelling tendency in common solvents, a high flow rate in column procedures, an excellent performance in stirred batch reactors, *etc*. The presence of epoxyde groups in the Sepabeads provides a binding site for enzymes, which makes its use as a matrix for immobilizing several enzymes very attractive [8,9]. However, there are relatively few reports on the use of Sepabeads immobilized enzyme in non-aqueous media with the aim of esters synthesis. For this application, the high water content in the Sepebeads, as well as multi-point attachments between the matrix and the enzyme, could provide a stabilizing effect against the denaturing influence of the organic solvent. Moreover, Sepabeads unlike most other hydrophilic supports were found to be stable in a range of organic solvents.

The aim of this work was to improve the performance of lipase from *Candida rugosa* for catalysis in a low aqueous system by immobilizing it covalently on an inexpensive and industrially applicable carrier such as Sepabeads EC-EP. The synthesis of geranyl butyrate in isooctane was chosen as a model reaction in a low aqueous system. The optimization of the ester synthesis was performed by the application of the factorial design and the response surface methodology. The initial water content, the reaction temperature, the enzyme concentration, the initial acid/alcohol molar ratio and the time of the addition of molecular sieves were the variables investigated.

EXPERIMENTAL

Materials

Candida rugosa lipase (CRL), geraniol and an olive oil emulsion were purchased from Sigma Chemical Co. (St. Louis, MO). Sepabeads EC-EP support was kindly donated by Resindion S.R.L. (Mitsubishi Chem. Co., Italy). All other chemicals used were reagent grade.

The lipase immobilization

The method for lipase immobilization on Sepabeads EC-EP involves a direct enzyme binding on the polymer *via* epoxy groups. The preparation of the enzyme solution and the immobilization procedure was previously described [8]. The samples of the enzyme solution before and after the immobilization, together with the washing solutions, were taken for the protein content and the enzyme activity assay, as previously described [10].

The enzyme hydrolytic activity assay (a test enzyme assay)

Hydrolytic activities of free and immobilized CRL were assayed by the standard olive oil emulsion method and expressed as international units (IU) [10]. 1 IU is defined as the amount of the enzyme required to produce 1 μmol of free fatty acid per min at 37 °C and pH 7.7.

Catalytic properties of the biocatalysts in a non-aqueous medium

Esterification reactions took place with the immobilized CRL in isooctane in screw-capped 100-cm^3 flasks with a working volume of 10 ml. Geraniol and butyric acids were added at different molar ratios followed by different amounts of water and immobilized lipase, according to the experimental design. The reaction mixture was incubated at different temperatures on a shaker at 150 rpm and for 48 h. Reactions were evaluated using a gas chromatography as previously described [11]. The reported percentage ester yield was defined as the amount of the ester produced to the initial substrate in defect. The percentage of esterification determined by both GC analysis and titration were found to be in good agreement.

The influence of the several factors on the ester synthesis was verified using a 5-level-5-factor central composite design requiring 32 experiments (16 factorial points, 10 axial points and 6 central points) (data not shown). The actual and coded settings of each of the five experimental factors are given in Table 1. The data obtained were fitted to a second-order polynomial equation:

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} X_i + \sum_{i=1}^{5} \beta_{kii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} X_i X_j \quad (1)$$

where Y is the response (ester yield in mol %), β_{k0}, β_{ki}, β_{kii} and β_{kij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables. The coefficients of the response function and their statistical significance were evaluated by the method of least squares by using the Matlab software. Response surfaces and contour plots were obtained using the fitted model, by keeping independent variables at a constant value while changing the other two variables.

Table 1. Coded and actual values of variables for a design of the experiment

Variables	Coded levels of variables				
	-2	-1	0	1	2
Water content, X_1 (%, w/v)	1	2	3	4	5
Temperature, X_2 / °C	25	35	45	55	65
Enzyme concentration, X_3 / (g dm^{-3}	2.0	2.5	3.0	3.5	4.0
Substrate molar ratio, X_4	1:2	1:1	3:2	2:1	5:2
Time of addition of molecular sieves, X_5 / h	0	8	16	24	32

RESULTS AND DISCUSSION

The lipase immobilization and characterization

In the case of supports with a high density of epoxide groups such as Sepabeads, the enzyme immobilization is supposed to occur *via* the multipoint covalent attachment, enabling high enzyme stabilization (Figure 1).

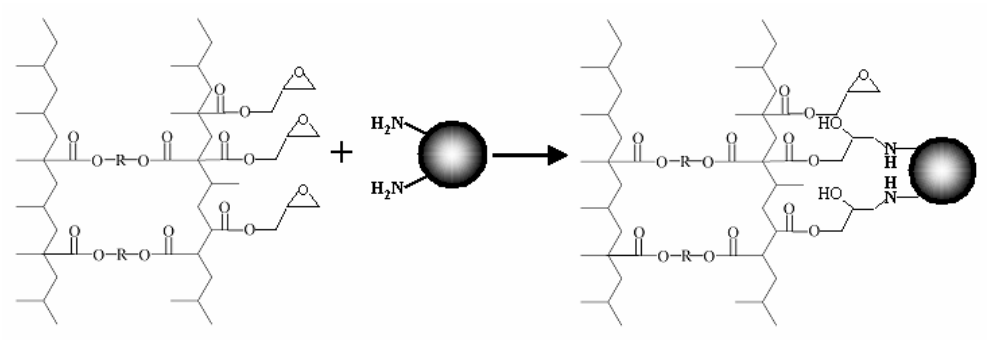

Figure 1. The immobilization of lipase on Sepabeads EC-EP carrier via epoxide groups.

For high efficiency and the activity recovery, the covalent attachment of the enzyme to a support depends not only on the method of the attachment but also on the amount of the enzyme offered. Thus, our initial experiments were carried out to determine the effects of enzyme loadings on the immobilization efficiency (data not shown). The hydrolytic activity of the immobilized lipase appears to increase from 9.6 to 23.2 IU/mg of dry support as more lipase was loaded onto the support (8.18-45.5 mg/g). However, the maximum activity yield of 95.3 % was achieved at the lowest enzyme loading, possibly due to a diffusion limitation. Some other properties of the immobilized CRL were: pH optimum 7; temperature optimum 37 °C; biocatalyst half-life determined from thermal stability assays at 50 and 75 °C were 5.41 and 1.64 h, respectively). The study of deactivation kinetics in various organic solvents at 35 °C revealed that the immobilization method also produces an appreciable stabilization of the biocatalyst in a nonaqueous system, as expected for an enzyme immobilized by the multipoint covalent attachment. The highest stabilization factor was obtained in both *n*-hexane (log$P$ = 3.5) and isooctane (log$P$ = = 4.5), although no obvious trend in the stabilization factor as a function of solvent properties such as log$P$ was observed. The half life of the biocatalyst was determined from these data as being 80 and 100 h, respectively.

Geranyl butyrate synthesis

So far, the use of the immobilized CRL on the epoxy-support has been applied in our laboratory for hydrolytic enzyme reactions [10]. Now, we were also interested in testing the performance of the immobilized lipase for synthetic reactions in the non-aqueous system. The synthesis of geranyl butyrate was chosen as the case study, for being an already known ester of current interest. RSM and 5-level-5-factor central composite rotatable design (CCRD) were used to optimize and understand the relationship between the important reaction parameters. RSM is an optimization technique that determines the optimum process condition by testing several variables at a time, uses special experimental designs to cut the number of required determinations. In addition, this technique allowed us to quantify the individual effect of each factor and to investigate their possible interactions.

The ester synthesis appears to show a wide variation in yield (12.5-94.3 %). Among the various treatments, the greatest molar conversion (94.3 %) was achieved in run No. 20 (water content of 3 %, 25 °C, enzyme concentration of 3 g/dm^3, substrate molar ratio 3:2, time of addition of sieves: 16 h), while the smallest conversion (only 12.5 %) was achieved in run No. 3 (water content of 4 %, 55 °C, enzyme concentration of 2.5 g/dm^3, substrate molar ratio 2:1, 8 h). The data showing the experimental yield of ester for 32 experiments of the statistical design are not presented.

Both *t*-test and *p*-value statistical parameters were used to confirm the significance of the factor studied. It seems that the most relevant variable for the ester synthesis was the temperature with an estimated effect of -29.5, suggesting a significant negative influence on the ester yield. This is in agreement with thermal stability data for this lipase in a non-aqueous medium [12]. Its statistical meaning was significant and negative on the response variable at 95 % probability level ($p = 0.0427$). All quadratic terms were also significant ($p < 0.05$). The response equation obtained was as follows:

$$\begin{aligned} Y = {} & 16.8 - 0.630X_1 - 29.5X_2 + 1.94X_3 - 2.01X_4 - \\ & - 2.96X_5 + 1.99X_1X_2 + 1.65X_1X_3 - 0.496X_1X_4 - \\ & - 2.18X_1X_5 + 1.48X_2X_3 - 3.28X_2X_4 + 0.609X_2X_5 + \\ & + 0.220X_3X_4 - 0.358X_3X_5 - 0.772X_4X_5 + \\ & + 1.80X_1^2 + 12.6X_2^2 + 4.60X_3^2 + 5.28X_4^2 + 6.78X_5^2 \end{aligned} \quad (2)$$

The shape of the three-dimensional surface-representing the yield of ester versus the enzyme concentration and the temperature is shown in Figure 2a. Geranyl butyrate synthesis was found to be strongly dependent on the reaction temperature. For example, as the temperature increased from 25 to 65 °C, the ester yield decreased from ≈100 to 8.2 %, for the enzyme concentration of 3 g/dm^3. This negative effect of the temperature on the yield was probably due to thermal inactivation of the enzyme. The temperature also showed a positive interactive effect with the enzyme concentration, indicating that higher enzyme concentrations could compensate for the lower reaction rates at higher temperatures. Similar behavior was observed using CRL immobilized on macroporous styrene-divinylbenzene resin to catalyze butyl butyrate at different temperatures (37-50 °C) since a decrease in the yield by 39 % was recorded when the temperature changed from 37 to 50 °C [13].

Figure 2b shows the response surface plot for the predicted values of the ester yield as a function of the initial substrate molar ratio at different temperatures for an enzyme concentration of 3 g/dm^3 and an incubation period of 48 h. Substrate molar ratio seems to have a negative effect on the ester yield, possibly due to an inhibitory effect of butyric acid. The ester production is represented by a concave surface described by a second order polynomial with a minimum at an acid/alcohol molar ratio of 1.6 for the tempe-

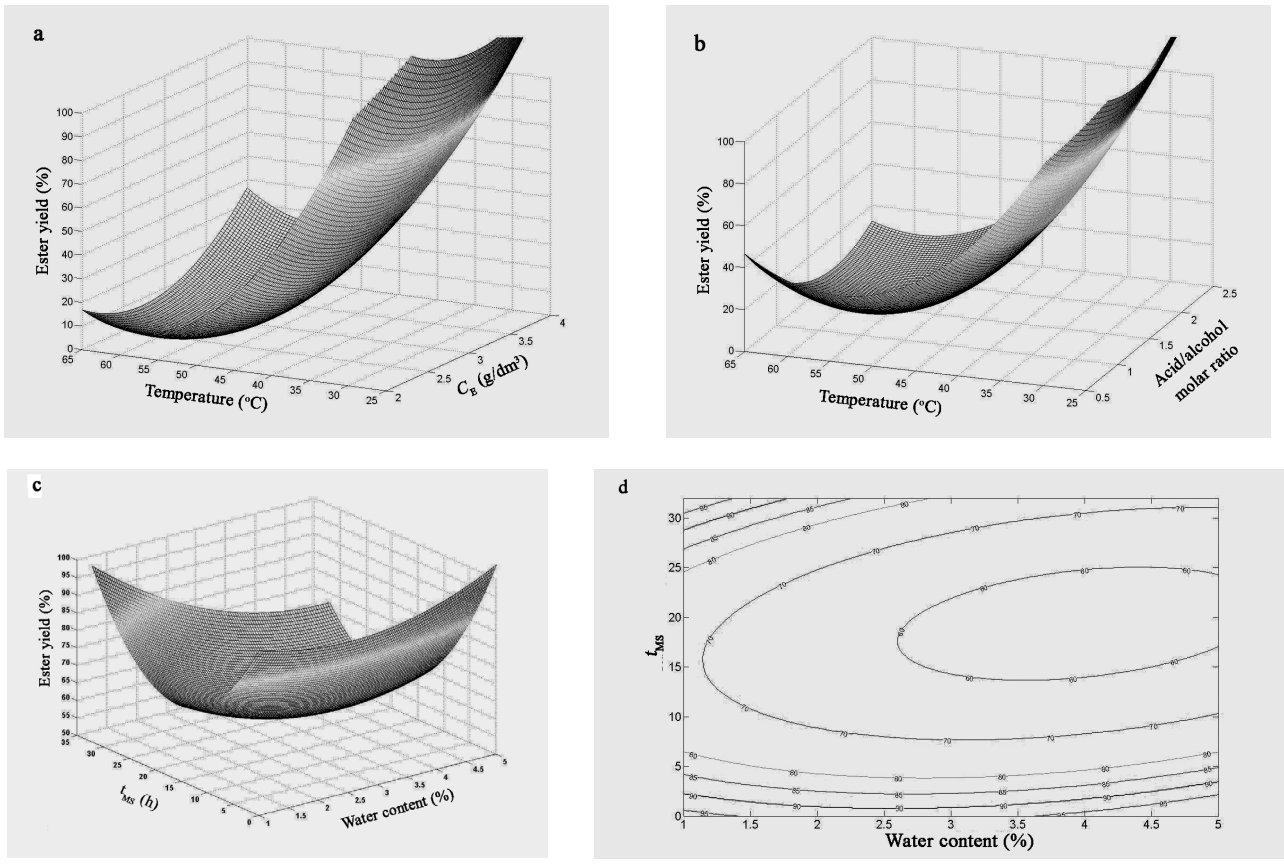

Figure 2. Response surface plots for the ester yield as a function of the enzyme concentration (C_E) and the temperature (a) acid/alcohol molar ratio and the temperature (b); response surface (c) and contour plots (d) for the ester yield as a function of the water content and time of the addition of molecular sieves (t_{MS}).

rature equal to 53 °C. However, a higher concentration of free acid in the reaction system could also be beneficial to the incorporation of acid from the view of the reaction equilibrium [11,13]. In this experimental setup, the substrate molar ratio had negative influence on the ester yield indicating the importance of using an optimal acid/alcohol molar ratio for maximum conversion to the ester. The addition of molecular sieves during the reaction could be a useful strategy for the control of the water content in the system and seems to have a positive effect on the ester yield. A negative interaction between the initial water content and time of the addition of molecular sieves was also observed, indicating the importance of working at low levels of both factors. Overall, these results reveal the importance of a proper selection of the reaction conditions for maximum conversion to the ester.

CONCLUSION

This work provides preliminary results on the use of Sepabeads EC-EP as a support matrix for immobilizing lipase and on the application of the resulting preparation to geranyl butyrate synthesis as a study model. The immobilization results are favorable compared with those reported in the literature in terms of both the activity yield and the stability in the presence of organic solvents. The influence of several important reaction parameters is carefully analyzed by means of an experimental design. The production of the ester was optimized and the ester production response equation was obtained, making it possible to predict ester yields from known values of the five main factors.

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