Geranyl Acetate Synthesis in a Packed-Bed Reactor Catalyzed by Novozym in Supercritical Carbon Dioxide and in Supercritical Ethane

ARTICLE *in* INDUSTRIAL & ENGINEERING CHEMISTRY RESEARCH · FEBRUARY 2011

Impact Factor: 2.59 · DOI: 10.1021/ie101489j

9 AUTHORS, INCLUDING:

Ricardo Couto
University of Alberta
14 PUBLICATIONS **176** CITATIONS
SEE PROFILE

Pedro Vidinha
University of São Paulo
32 PUBLICATIONS **294** CITATIONS
SEE PROFILE

Olga Ferreira
Instituto Politécnico de Bragança
22 PUBLICATIONS **189** CITATIONS
SEE PROFILE

José M Loureiro
University of Porto
141 PUBLICATIONS **2,138** CITATIONS
SEE PROFILE

I&EC research
Industrial & Engineering Chemistry Research

ARTICLE

pubs.acs.org/IECR

Geranyl Acetate Synthesis in a Packed-Bed Reactor Catalyzed by Novozym in Supercritical Carbon Dioxide and in Supercritical Ethane

Ricardo Couto,[†] Pedro Vidinha,[†] Célia Peres,[†] Adriano S. Ribeiro,[‡] Olga Ferreira,[§] Manuela V. Oliveira,[‡] Eugénia A. Macedo,[‡] José M. Loureiro,[‡] and Susana Barreiros*[,†]

[†]REQUIMTE/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

[‡]Laboratory of Separation and Reaction Engineering, Departamento de Engenharia Química, Faculdade de Engenharia da Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[§]Laboratory of Separation and Reaction Engineering, Departamento de Tecnologia Química e Biológica, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5301-857 Bragança, Portugal

ABSTRACT: The esterification reaction of geraniol with acetic acid (100 mM/100 mM) catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435) was studied in supercritical carbon dioxide (sc-CO_2) and in sc-ethane in a packed-bed reactor (PBR). In sc-CO_2 it was easy to adjust the water activity (a_w) in the reaction mixture to levels leading to good enzyme performance. Enzyme stability was high and steady-state conversions could be achieved that exceeded the largest conversions measured in batch stirred-tank reactors (BSTRs), which is probably due to the lower a_w levels achieved in the PBR. In sc-ethane, where the solubility of water is lower, high steady-state conversions could be attained only by preventing the accumulation on the enzyme bed of the water produced during reaction. The kinetic parameters for the reaction in sc-CO_2 were determined using previously published data obtained in a BSTR, and a model was developed for the PBR that included those kinetic parameters. This model was able to predict with reasonable accuracy the behavior of the PBR. Slight differences were observed for some operating regions, probably due to the influence of a_w in the activity of the enzyme, which is not included in the model.

1. INTRODUCTION

The extension of biocatalysis to nonaqueous media has contributed to the observed increase in industrial applications of enzymes in the past few years.[1] In nonaqueous media, enzymes can catalyze reactions that involve substrates with poor solubility in water or that are not favored in water and, in addition, can be more stable and exhibit altered and better selectivity.[2] The use of supercritical fluids (sc-fluids; any substance above its critical temperature and pressure) as solvents can be a way to comply with the need for green(er) chemistry, also in biocatalysis.[3−5] Supercritical fluids can be easily eliminated without leaving residues and are thus an environmentally friendlier alternative to more conventional solvents. Another useful characteristic of sc-fluids is their adjustable solvation ability and properties that facilitate mass transfer.

Batch stirred-tank reactors (BSTRs) are probably the reactors most widely used for biocatalysis.[6] However, their volumetric productivity is relatively low. For larger-scale applications, continuous packed-bed reactors (PBRs) are often the preferred option. PBRs provide a practical way to separate the catalyst from the reaction mixture entering and exiting the reactor, are highly efficient, are easy to operate, and have low maintenance requirements. In the case of reactions in sc-fluids, continuous reactors help to reduce operating costs by avoiding the pressurization/depressurization cycles of feeding the reactor/recovering the reaction mixture, allow the adjustment of the properties of the sc-fluid in real time, and are safer to use because the volume of pressurized medium is smaller.[7]

Water activity (a_w) is the most adequate parameter to correlate enzymatic activity in nonaqueous media.[8] a_w can affect enzymatic activity through enzyme dynamics, mass action, or kinetic effects. Providing a_w control is thus very important when using enzymes in nonaqueous media, especially in esterification reactions that produce water. In this case, low enzymatic activity at high a_w is usually attributed to the accumulation of water on the enzyme particles[9] and favorable hydrolysis of the ester substrate. a_w control in esterification reactions is complicated by the fact that the solubility of water in the reaction medium becomes lower as reaction conversion increases and the reaction medium becomes richer in the ester product and thus less polar. This makes it more difficult to predict the gradients for water and the other solutes along a PBR.[10]

One of the best ways to achieve good a_w control in organic media is the circulation of saturated salt solutions able to exchange water with the reaction mixture through a semipermeable membrane, thus ensuring in situ continuous a_w control.[11,12] Pervaporation can also be used to efficiently control a_w continuously.[13] None of the two methods just referred to can be easily applied to sc-fluids. In this case, and also in organic solvents, authors have taken care to set the initial hydration level of the enzyme, e.g., by correlating that parameter with the water content of the solvent

Received: July 12, 2010
Accepted: December 15, 2010
Revised: October 21, 2010
Published: January 18, 2011

through water sorption isotherms.[14−16] Partial elimination of the water produced has led to significant increases in esterification conversion. For example, Omar et al.[17] used a system with two PBRs containing the immobilized enzyme, separated by a column filled with molecular sieves. Whetje et al.[12] also used two PBRs, the second of which was filled with a mixture of the immobilized enzyme and molecular sieves. Mensah and Carta[18] also tested a system of two reactors mounted sequentially. These authors stated that water tended to accumulate more rapidly in the first reactor, especially closer to the reactor entrance, and that the regeneration of the catalyst should be done before it reached its maximum water sorption capacity. Periodically, the first PBR was removed from the circuit for regeneration (washing with a solvent for removal of water sorbed by the catalyst), the second PBR took its place, and a third reactor occupied the second position in the series, typically a PBR containing regenerated catalyst. Trusek-Holownia and Noworyta[19] used a membrane to separate the enzymatic reactor from a column filled with molecular sieves, achieving close to 100% conversion of geraniol into geranyl acetate.

Regarding theoretical work, several reactor models in enzyme catalysis have been proposed (e.g., refs 20−25). Xiu et al.[24] addressed the case of kinetic resolution in a batch reactor and the influence of mass transfer limitations in the enantiomeric ratio; most of the remaining work deals with the case of one-substrate reactions following Michaelis−Menten kinetics in several types of reactors. More recently, Van Roon et al.[25] developed a batch reactor model for a more complex reaction system for the synthesis of cephalexin. Regarding the development/application of such reactor models to enzyme catalysis in supercritical media, Goddard et al.[23] dealt with the case of esterification reactions, assuming a simplified Michaelis−Menten kinetics when one of the substrates is present in excess ($C_i \gg K_{Mi}$).

Geranyl esters can be found in many natural fragrances. The enzymatic synthesis of geranyl esters has attracted a lot of attention.[13,19,26−31] Geranyl acetate is one of the most important members of the group. Most of the biotransformations aimed at producing this ester employ a lipase, in particular *Candida antarctica* lipase B, to catalyze the esterification of geraniol with acetic acid. In the present study we look at how this reaction system performs in a PBR containing immobilized *Candida antarctica* lipase B (Novozym 435), using sc-CO_2 or sc-ethane as solvent. Kinetic parameters were calculated using experimental data previously obtained in a BSTR, in sc-CO_2.[29] The kinetic model developed was then used to predict the behavior of the PBR in sc-CO_2.

2. EXPERIMENTAL SECTION

2.1. Materials. Novozym 435 (*Candida antarctica* lipase B immobilized on a macroporous acrylic resin; average catalyst particle size ca. 0.5 mm), with a reported activity of 7000 PLU (propyl laurate units) g^{-1}, was a gift from Novo Nordisk Bioindustrial, Spain. Geraniol (98% purity) was purchased from Sigma; acetic acid, KCH_3COO, and KNO_3 were from Merck; *n*-decane (99% purity), geranyl acetate (98% purity), and zeolite NaA (in powder form) were from Aldrich; Hydranal Coulomat A and C Karl Fischer reagents were from Riedel de Haën. Geraniol and *n*-decane were dried over molecular sieves (with 3 Å pores, from Merck). Acetic acid was distilled. Ethane, CO_2, and nitrogen were supplied by Air Liquide and guaranteed to have purities of over 99.95 mol % (ethane) and 99.995 mol %.

Table 1. Kinetic Parameters for the Novozym 435 Catalyzed Esterification Reaction of Acetic Acid with Geraniol in sc-CO_2 at 40 °C and 100 bar

parameter	value
V_{max} (mol/g·min)	0.0859
K_{MA} (M)	21.4
K_{MB} (M)	30.1

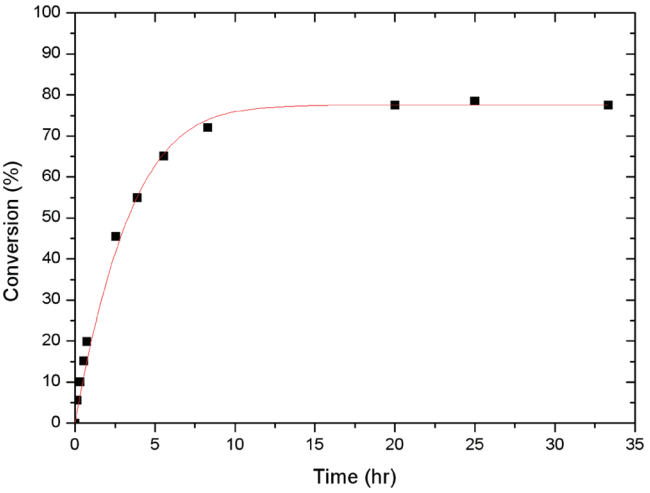

Figure 1. BSTR conversion versus reaction time for an equimolar mixture of both substrates (100 mM) in sc-CO_2. T = 40 °C; P = 100 bar; [Novozym 435] = 2.2 mg cm^{-3}; a_w = 0.25. ■, experimental data;[29] —, model results.

2.2. Apparatus and Technique for PBR Experiments. Experiments were performed using a single PBR or a system of PBR(s) mounted in series. In both cases, the solvent (CO_2 or ethane) passed through a molecular sieve bed, was cooled and pressurized with an HPLC (master) pump before being heated to the supercritical state in a thermostatic bath, and mixed with a freshly prepared mixture of geraniol and acetic acid that was pressurized with a second HPLC (slave) pump. The reaction mixture was then fed to the PBR(s) previously loaded with Novozym, kept in a thermostatic bath. The pressure in the system was controlled with a back-pressure regulator and measured with pressure transducers mounted at several points of the circuit. Solutes were precipitated in cooled traps. CO_2 and ethane were recirculated. HPLC valves allowed the sampling of the reaction mixture that entered and exited the reactor(s). We used tubular reactors built from 3.2 mm internal diameter stainless steel tubing cut to accommodate the amount of enzyme selected (ca. 12 cm reactor length when using 300 mg of enzyme). The enzyme was kept between two pieces of glass wool. Novozym was pre-equilibrated to a_w = 0.23 or a_w = 0.94 through the vapor phase with a saturated salt solution of KCH_3COO or KNO_3, respectively, at 25 °C.[32] a_w values in the reaction mixture were calculated by dividing the water concentration in the mixture by the water concentration in the same mixture at saturation.[33] To increase the water content of the reaction mixture, water was added to the substrate mixture. The water contents of solids and fluids were measured by direct

Karl Fischer titration. *n*-Decane (20 mM) was used as internal standard for gas chromatographic (GC) analysis. The enzyme load in the reactor was replaced only when the enzyme bed became excessively hydrated in the course of experiments with sc-ethane. The reaction studied was the esterification of geraniol with acetic acid (100 mM equimolar mixture; volume fraction ca. 2.5% in the reaction stream) at 100 bar and 35 °C. Flow rates of 0.25, 0.50, and 1.0 mL min^{-1} were used to vary the residence time.

2.3. Analysis. GC analyses were performed with a Trace 2000 Series Unicam gas chromatograph. GC conditions: 50 m × 0.32 mm i.d. DB-Wax capillary column from J&W Scientific; oven temperature program, 50 °C for 5 min, 50−240 °C ramp at 6.5 °C min^{-1}, 240 °C for 3 min; injection temperature, 250 °C; flame ionization detector (FID) temperature, 250 °C; carrier gas, helium (2.3 cm^3 min^{-1}); split ratio, 20:1. The results reported are the average of at least two measurements, except where stated otherwise.

3. MODELING PROCEDURE

3.1. Kinetic Modeling. The ping-pong bibi mechanism is widely used to describe esterification reactions.[34] Based on this mechanism, the reaction rate (R) can be obtained from the following equation:[35]

$$R = \frac{V_f V_r \left([A][B] - \frac{[W][Es]}{K_{eq}} \right)}{V_r K_{MB}[A] + V_r K_{MA}[B] + \frac{V_f K_{MEs}[W]}{K_{eq}} + \frac{V_f K_{MW}[Es]}{K_{eq}} + V_r[A][B] + \frac{V_f K_{MEs}[A][W]}{K_{eq} K_{IA}}} + \frac{V_f[W][Es]}{K_{eq}} + \frac{V_r K_{MA}[W][Es]}{K_{IEs}} \quad (1)$$

where [A] represents, in this case, the concentration of acetic acid, [B] is the concentration of geraniol, [W] is the concentration of water, and [Es] is the concentration of geranyl acetate. K_{Mi} and K_{Ii} represent Michaelis and inhibition kinetic constants, and V_r and V_f are the reverse and forward maximum reaction rates, respectively. K_{eq} is the equilibrium constant.

Although there are nine parameters in the ping-pong bibi reaction rate equation, the influence of some of them can usually be neglected if the enzyme has significantly higher affinity for some of the components or if there are large differences in their concentrations. In the simplest case, when the affinity for the substrates is much higher than the affinity for the products, the following simplified equation can be used instead to describe the reaction rate:

$$R = \frac{V_{max} \left([A][B] - \frac{[W][Es]}{K_{eq}} \right)}{K_{MB}[A] + K_{MA}[B] + [A][B]} \quad (2)$$

where V_{max} is the forward maximum reaction rate, which was represented by V_f in eq 1.

Additional terms may appear to account for inhibition by any of the components. In the case of esterification reactions, competitive inhibition by the alcohol is often encountered.[34] High concentrations of acetic acid have also been reported to cause enzyme inhibition when Novozym 435 is used.[27,29,36] In previous work,[29] we reported that acetic acid had an inhibitory effect at concentrations higher than 100 mM. Inhibition by geraniol was only verified above 200 mM, when the acetic acid concentration was 50 mM.

3.2. Reactor Modeling. As mentioned previously, among the usually stated advantages of supercritical fluids as solvents for reaction processes are the reduction of mass transport resistances that arise from the high gaslike diffusivities. Thus the reactor models developed—BSTR and PBR—are based on the assumptions that the reactors are isothermal and that the mass transfer resistances (both external and internal) are negligible.

On the basis of these assumptions, the material balance for substrate i in a BSTR yields

$$\frac{dC_i}{dt} = -[\text{Novozym}]R \quad (3)$$

where C_i represents the concentration of substrate i, t is the time variable, and [Novozym] is the amount of catalyst particles within the reactor.

For the PBR, we also considered that the flow inside the reactor can be described as plug flow. In laboratory packed-bed reactors axial dispersion is seldom negligible,[37] which would go against the above assumption of plug flow; however, given the ratio between the length of the reactors used and the size of the catalyst particles (approximately 250 for the smallest reactor), axial dispersion should not be relevant.[37]

On the basis of the assumptions referred to above, the steady-state material balance, in a PBR, for substrate i yields

$$\frac{dC_i}{dz} = -R\frac{\rho_B}{u_S} \quad (4)$$

where z represents the position along the reactor, ρ_B is the bulk density, and u_S is the superficial velocity.

Equations 3 and 4 were solved using the LSODE code.[38,39]

4. RESULTS AND DISCUSSION

4.1. Kinetic Parameters in sc-CO_2. The kinetic parameters were estimated using the experimental data previously obtained in a BSTR, using sc-CO_2 as solvent, at 40 °C and 100 bar.[29] A Levenberg−Marquardt algorithm[40,41] was used in the optimization procedure implemented for the estimation.

Given the concentration range used (10−100 mM initial concentration of acetic acid, 50−300 mM initial concentration of geraniol, and an initial water activity of 0.25), it should not be necessary to account for the inhibitory effects referred to in section 3.1. Therefore, eq 2 was used as the kinetic model.

In order to minimize the number of parameters to be fitted simultaneously, initial rate values from experiments with excess acetic acid were used to estimate V_{max} and K_{MB}. Similarly, using initial rate data from experiments with excess geraniol, K_{MA} (and V_{max}, again) were obtained. K_{eq} was determined through the equilibrium conversion: 3.68 at 40 °C. The parameters obtained this way were then used as initial guesses for the complete optimization using all the available experimental data. The kinetic parameters obtained in the optimization are shown in Table 1. The sum of the square residues (SSQ) was 2.7×10^{-2} (SSQ/N_{points} = 6.0×10^{-4}).

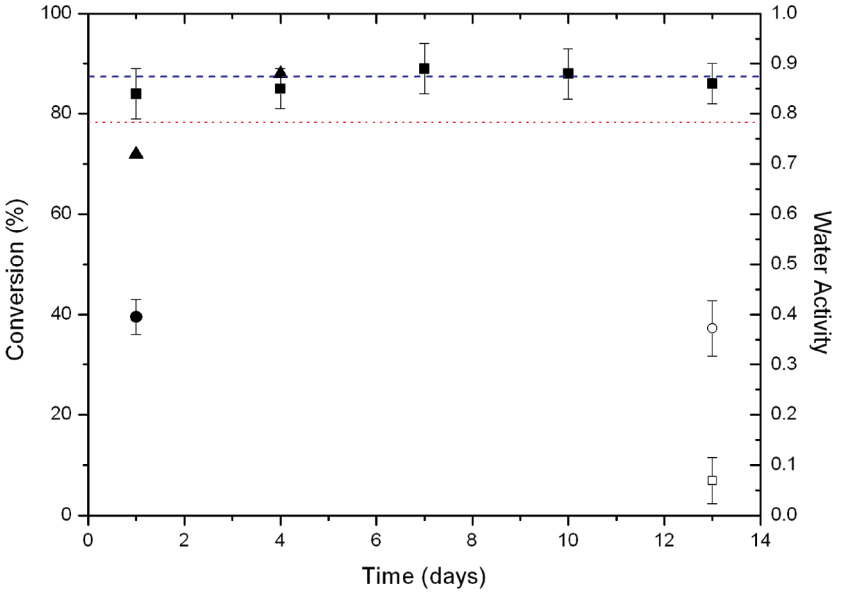

Figure 2. PBR steady-state conversion of geraniol to geranyl acetate in sc-CO_2 in two series of consecutive assays of ca. 8 h each, performed with the same enzyme load pre-equilibrated at a_w = 0.23 (■) or in 1-day runs in which water was added to the substrates before mixing with CO_2 (●), and a_w of the reaction mixture exiting the reactor (□ and ○, respectively). Also shown is reaction conversion at the end of assays performed with enzyme pre-equilibrated at a_w = 0.94 (▲). Amount of Novozym = 900 mg; [geraniol] = [acetic acid] = 100 mM; flow rate = 0.25 mL min^{-1}; T = 35 °C; P = 100 bar; Model results of steady-state conversion for a_w = 0.07 (---) and a_w = 0.23 (· · ·).

Given the satisfactory results obtained with the simplified form of the ping-pong bibi rate equation (eq 2), it is not necessary to include any additional terms; however, care should be taken when using these kinetic parameters outside the operating conditions used in the optimization since, as mentioned above, inhibition by any of the substrates may arise.

Figure 1 compares the experimental and simulated results for one of the BSTR runs used in the estimation of the parameter values displayed in Table 1.

The kinetic model, with the parameter values obtained from the BSTR runs, will be included in the PBR model, in order to simulate the experimental runs of operation of the PBR.

4.2. Packed-Bed Reactor in sc-CO_2. Our first experiments in the packed-bed reactor were performed with Novozym 435 pre-equilibrated at a_w = 0.23, a value that led to higher initial rate and reaction conversion in a BSTR.[29] CO_2 was fairly dry, both before and after mixing with the substrates, and thus the reaction mixture had the ability to remove water from the enzyme. Water production in the course of the reaction had an opposite effect that led to an increase of a_w in the reaction mixture exiting the reactor, which nonetheless remained below the a_w value of pre-equilibration of Novozym (Figure 2). Reaction conversion varied little during each run, or from one run to the other, even after long waits between consecutive runs during which the reactor was kept under CO_2 pressure. Pre-equilibration of Novozym at a_w = 0.94 impacted on reaction conversion essentially during the first day of utilization of that enzyme load. As the enzyme bed lost water, the conditions of the system approached those of experiments with Novozym pre-equilibrated at a_w = 0.23 and led to the same reaction steady-state conversion values, showing that the low water content of the CO_2 stream was the main factor in determining the a_w profile of the reaction mixture. On the other hand, when water was added to the substrates and a_w of the reaction mixture was consistently higher, reaction conversion was significantly lower. Also presented in Figure 2 are the steady-state conversions calculated by the PBR model developed assuming a_w values of 0.07 and 0.23 throughout the entire reactor. Note that, for the experiments where Novozym was pre-equilibrated with a_w= 0.23, the measured value of a_w at the bed outlet was 0.07. The model results for a_w = 0.07 are in good agreement with the experimental data and seem to confirm that water removal by CO_2 leads to higher conversions because of its effect on the reaction equilibrium.

An increase in the flow rate of the reaction mixture led to a decrease in the steady-state reaction conversion (Figure 3), which must be due to the related reduction in residence times. However, reaction conversion was still reasonably high at the highest flow rate tested. Whetje et al.[12] obtained similar results for a different esterification reaction catalyzed by Novozym 435 in a PBR, using an organic solvent. The specifications of our HPLC pumps and the fact that the one used for the substrate mixture was a slave pump did not allow experiments with the 100 mM equimolar substrate mixture at flow rates below 0.25 mL min^{-1}. Without this limitation, we should have been able to reach higher conversion values. Nonetheless, and given the similarity of our reaction conversion vs flow rate profile with that reported by Whetje et al., the increase in conversion for flow rates below 0.25 mL min^{-1} should have been small. When working with Novozym 435, Whetje et al. found that the production rate increased as the flow rate of the reaction mixture increased, a situation also encountered in the present study (Figure 3). That is, in the flow rate range tested, their reaction was not kinetically controlled.

As seen from Figures 2 and 3, the use of a large reactor containing 900 mg of Novozym led to a higher reaction conversion than with the small reactor containing 300 mg of Novozym at the same flow rate. However, the observed increase in conversion appeared to be too small for the increase in the amount of enzyme that brought it about, suggesting that reaction equilibrium was attained before the end of the large reactor

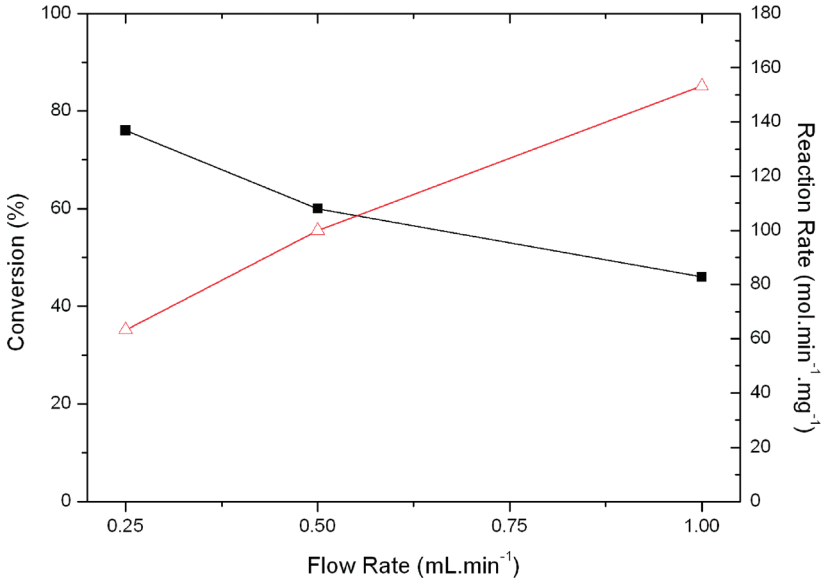

Figure 3. PBR steady-state reaction conversion (■) and esterification rate (Δ) in sc-CO_2, obtained in a reactor containing 300 mg of Novozym pre-equilibrated at a_w = 0.23. Inlet concentrations: [geraniol] = [acetic acid] = 100 mM; T = 35 °C; P = 100 bar.

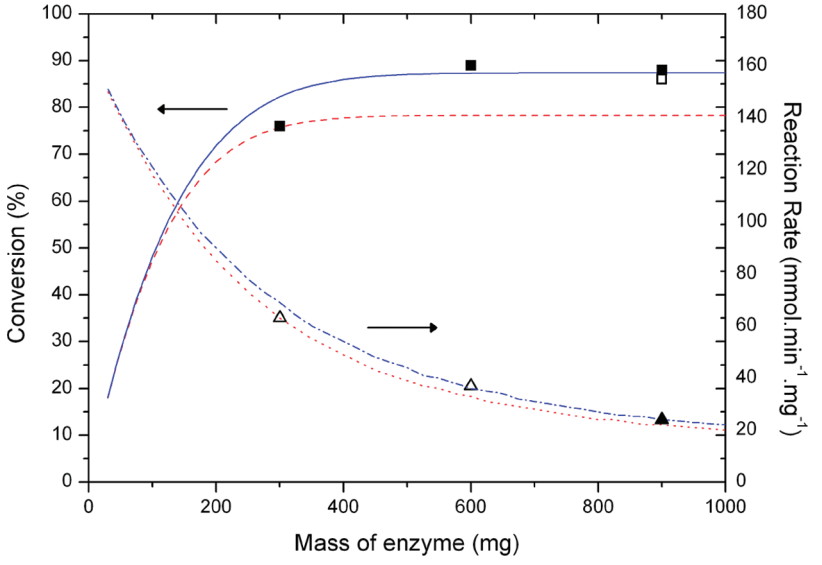

Figure 4. PBR steady-state conversion (■, experimental, 3 × 300 mg reactors in series; □, experimental, 900 mg reactor; —, simulated with a_w = 0.07; ---, simulated with a_w = 0.23) and esterification rate (Δ, experimental, 3 × 300 mg reactors in series; ▲, experimental, 900 mg reactor; — · —, simulated with a_w = 0.07; · · ·, simulated with a_w = 0.23) in sc-CO_2 for a flow-rate of 0.25 mL min^{-1}. Novozym pre-equilibrated at a_w = 0.23. Other reaction conditions set as in Figure 3.

and, consequently, part of the enzyme in the reactor was no longer necessary for reaching the measured reaction conversion. According to the model simulations, around 450 mg of enzyme would be necessary to reach equilibrium at a flow rate of 0.25 mL min^{-1} and an a_w of 0.07. To further study the process and test the model, experiments were performed with small reactors containing 300 mg of Novozym 435 mounted in series. The experimental results obtained are presented in Figures 4−6, along with the model simulations. While an arrangement of two reactors brought about an increase in reaction conversion, as expected, no further benefit ensued when a third reactor was added. The reaction rates for two and three reactors were calculated from the conversion values measured at the end of the last reactor and the total amount of enzyme. By doing this we were assuming identical behavior of single reactors and their arrangements in series. This seems a reasonable assumption, as evidenced by the similarity of the results obtained with the large reactor containing 900 mg of enzyme and the 3 × 300 mg enzyme reactor arrangement (Figures 4 and 5). A comparison of the experimental results obtained for a single reactor and for two reactors in series suggested an optimum amount of ca. 400 mg of Novozym 435, which is in agreement with the value obtained through simulation. We cut and tested a reactor holding that amount of catalyst, and obtained reaction rates that fell reasonably in line with the other sets of results (Figure 5). In agreement with a lack of kinetic control at the highest flow rate tested in the present study, the

dx.doi.org/10.1021/ie101489j |*Ind. Eng. Chem. Res.* 2011, 50, 1938–1946

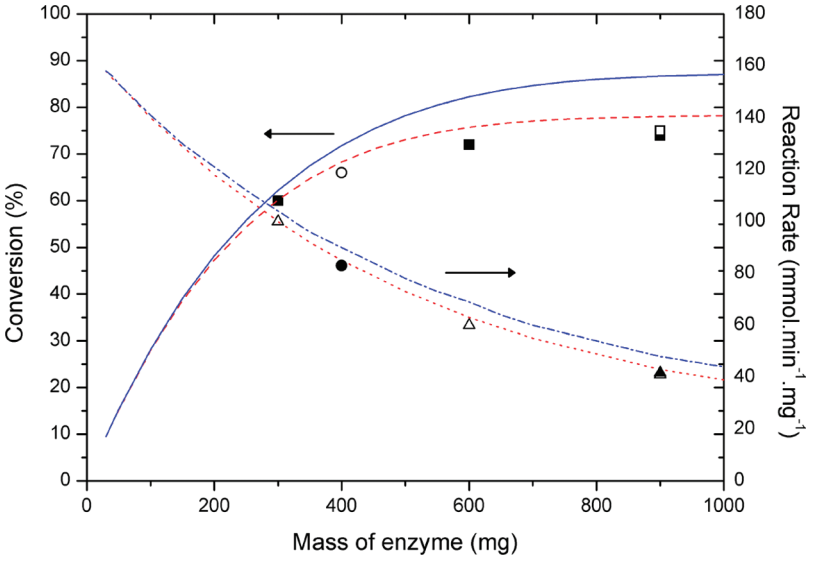

Figure 5. PBR steady-state conversion (■, experimental, 3 × 300 mg reactors in series; ○, experimental, 400 mg reactor; □, experimental, 900 mg reactor; —, simulated with a_w = 0.07; ---, simulated with a_w = 0.23) and esterification rate (Δ, experimental, 3 × 300 mg reactors in series; ●, experimental, 400 mg reactor; ▲, experimental, 900 mg reactor; — · —, simulated with a_w = 0.07; · · ·, simulated with a_w = 0.23) in sc-CO_2 for a flow rate of 0.50 mL min^{-1}. Novozym pre-equilibrated at a_w = 0.23. Other reaction conditions set as in Figure 3.

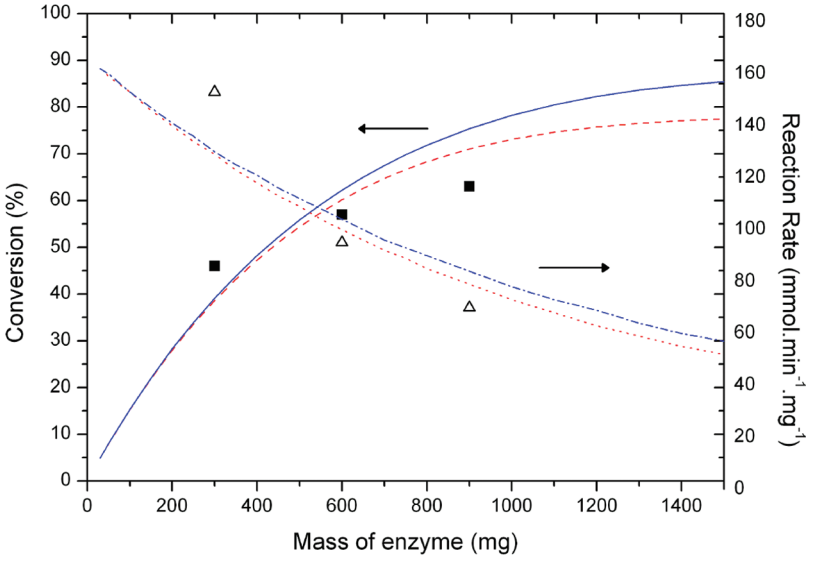

Figure 6. PBR steady-state conversion (■, experimental; —, simulated with a_w = 0.07; ---, simulated with a_w = 0.23) and esterification rate (Δ, experimental; — · —, simulated with a_w = 0.07; · · ·, simulated with a_w = 0.23) in sc-CO_2 for a flow rate of 1.0 mL min^{-1}. Novozym pre-equilibrated at a_w = 0.23. Other reaction conditions set as in Figure 3.

maximum reaction rate recorded was lower than the initial reaction rate measured in the BSTR;[29] the conversions obtained in all experiments with the packed-bed reactors were above 40%, which is outside the linear portion of the reaction profiles obtained in BSTRs.

To analyze the model predictions, it is useful to plot the experimental results as a function of the space time (Figure 7). For long residence times (i.e., long enough to reach equilibrium), the experimental results are consistent with the simulations considering an a_w of 0.07, whereas for lower residence times, the conversions tend to be lower than predicted even considering an a_w of 0.23. In all experiments, CO_2 exiting the reactor(s) presented a low value of a_w if compared with the initial value of pre-equilibration.

As mentioned previously, a_w can influence enzyme catalysis in several ways. This usually leads to the existence of an optimum water activity. In our previous work[29] an optimum a_w of around 0.25 was reported. When a_w is lower than this optimum value, the esterification rate at the early stages of the reaction will drop; nevertheless, a lower a_w will lead, in this case, to higher conversion values. The effect of a_w in the equilibrium conversion is accounted for in the rate equation that was used, and the model successfully predicts the steady-state conversion values that are close to equilibrium; however, the other effects are not considered. In the early stages of the reaction, the reaction rates predicted by the kinetic model will be overestimated, which explains the lower conversions obtained experimentally for intermediate values of

dx.doi.org/10.1021/ie101489j |*Ind. Eng. Chem. Res.* 2011, 50, 1938–1946

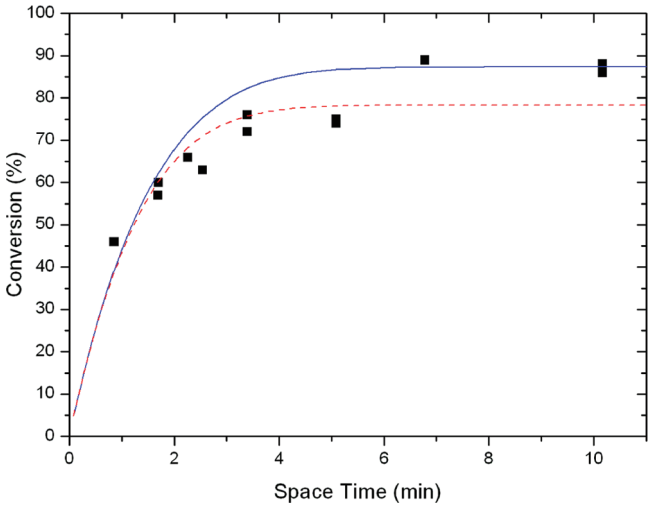

Figure 7. PBR steady-state conversion vs space time (■, experimental; —, simulated with a_w = 0.07; ---, simulated with a_w = 0.23). Novozym pre-equilibrated at a_w = 0.23. Other reaction conditions set as in Figure 2.

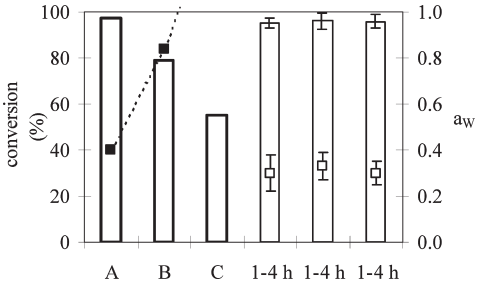

Figure 8. PBR steady-state conversion of geraniol to geranyl acetate in sc-ethane at the end of three consecutive assays (A−C) of ca. 6 h each performed with the same enzyme load (heavy bars) and steady-state reaction conversion in three consecutive assays of ca. 4 h with the same enzyme load where washing with sc-CO_2 at a flow rate of 5−10 mL min^{-1} for ca. 30 min was provided between assays (light bars). Also shown is a_w of the reaction mixture exiting the reactor (■ and □, respectively). Novozym (900 mg) was pre-equilibrated at a_w = 0.23; [geraniol] = [acetic acid] = 100 mM; flow rate = 0.25 mL min^{-1}; T = 35 °C; P = 100 bar.

the residence time. For shorter residence times, the deviations between the experimental and predicted conversions are smaller. The experiments performed in BSTRs[29] showed that the differences in the reaction rates obtained with different water activities tend to be higher for intermediate reaction times than for the initial period, which is in agreement with the trends presented in Figure 7.

Since water is being produced, a_w probably changes along the reactor. Additionally, water is not produced at a constant rate; unless the reactor is in kinetic control, the rate of water production is higher at the beginning of the reaction. The polarity of the medium also changes as the reaction proceeds, due to the transformation of acetic acid and geraniol in geranyl acetate. This leads to a decrease in the polarity of the medium that contributes to a gradual increase in a_w. Accounting for all these effects would be a complex task, but it would most likely improve the model predictions.

4.3. Packed Bed Reactor in sc-Ethane. Running the PBR with sc-ethane required a different approach. Ethane is less polar and is able to dissolve ca. 5 times less water than CO_2 at an identical temperature and pressure. Figure 8 depicts a sequence of assays performed with the same enzyme load (A−C). Reaction conversion remained high during the first assay but had already dropped on the second, as a_w increased, a situation that became worse during the third assay. On the basis of previous results, the enzyme bed was washed between assays with sc-CO_2 for ca. 1 h at the same flow rate as used with sc-ethane, and kept under a CO_2 atmosphere overnight, in an attempt to prevent the accumulation of water on the enzyme. However, and as shown in Figure 8, this approach was not successful. The enzyme taken from the reactor was visibly highly hydrated. The fact that the calculation of a_w as described in the Experimental Section sometimes led to values higher than 1, which are absurd, suggests that portions of water were sometimes released from the enzyme bed and carried away as an emulsion. That is, the water sorption capacity of the enzyme bed was exceeded and a third (water) phase was formed. This situation can restrict access of intervening species to the enzyme and can behave as an interface for enzyme denaturation.[42] Many authors working with PBRs have referred to irreversible enzyme inactivation caused by enzyme hydration above a certain level.[10,14,15] For example, on comparing *n*-hexane and sc-CO_2 for a given esterification reaction, Marty et al.[15] pointed out that although on a first approach *n*-hexane seemed to be a better solvent than sc-CO_2, the capacity of *n*-hexane to eliminate the water produced during reaction was too low to prevent its accumulation on the enzyme bed in a continuous process, leading to a decrease in reaction conversion and irreversible deactivation of the enzyme.

Mixing the enzyme with a salt hydrate pair or with molecular sieves, dry or pre-equilibrated to a given a_w, led to some improvement, but these approaches were not easy to optimize. We thus returned to the idea of using the higher water solubility of (dry) sc-CO_2 to prevent excessive hydration of the enzyme bed in experiments with sc-ethane. The success of the concept had been illustrated by Colombié et al.[43] (intermittent air stripping of the water produced), Mensah and Carta[18] (washing with isoamyl alcohol), and Sánchez et al.[44] (washing with fresh solvent and drying overnight in a silica gel drier). The difference now was the flow rate at which sc-CO_2 was passed through the enzyme bed, which was considerably higher than that used for regular experiments. Figure 8 shows results obtained when the reactor was run in cycles comprising a ca. 4 h run with sc-ethane followed by washing with an abundant flow of sc-CO_2 for ca. 30 min. This mode of operation prevented the accumulation of water at high levels on the enzyme bed and the need to replace the enzyme load, and afforded high reaction conversion. It could be adapted to the type of setup described by Mensah and Carta[18] of two reactors mounted sequentially, allowing for continuous reaction/enzyme regeneration.

4.4. sc-CO_2/sc-Ethane: Comparison. The equilibrium conversions that we obtained in sc-CO_2 and sc-ethane in BSTRs at a_w = 0.25 were ca. 73 and 98%, respectively.[29] These values must reflect differences in the solvation of the intervening species in the two solvents. Solvents in which the solubility of water is smaller favor esterification.[45] For example, Whetje et al.[12] obtained equilibrium conversions of 95 and 64% for a given esterification reaction in *n*-hexane and in a ketone, respectively. In the present work, we obtained maximum conversion values of ca. 96 and 86% in sc-ethane and sc-CO_2, respectively. In the case of sc-ethane, a slightly lower value might be due to the fact that

equilibrium was most likely not attained at the lowest flow rate tested, given that a_w was similar in the PBR and in the BSTR. In the BSTR, we obtained an equilibrium conversion of ca. 98% in *n*-hexane. For the same reaction system, Bartling et al.[13] give values of 94.0% at 30 °C and 93.2% at 40 °C, without a_w control, and thus measured at higher a_w (for a concentration of water at equilibrium of nearly 0.1 M). The maximum conversion value obtained in the PBR with sc-CO_2, which was higher than the equilibrium conversion measured in the BSTR, must be due to the lower a_w at which the PBR was run.

5. CONCLUSIONS

The PBR could be operated for many consecutive runs with sc-CO_2 at constant a_w without any loss of enzyme performance. The lower a_w values at which the reactor was run brought about a significant improvement in reaction conversion relative to the BSTR. The reactor model presented, which includes the kinetic parameters obtained, describes reasonably well the behavior of the PBR using sc-CO_2 as solvent; improvements in the model predictions are expected by accounting for other effects of a_w on the reaction rate. In sc-ethane, a solvent with lower water solubility, it was difficult to take advantage of the higher equilibrium constant. There was a tendency for water to accumulate on the enzyme bed, and this led to a marked decrease in reaction conversion. It was possible to overcome this difficulty by running the PBR in cycles of shorter reaction periods alternating with washing with sc-CO_2 at higher flow rates than normally used for reaction. This type of strategy is successfully applied to organic media. In the present case, however, experimental procedures are more demanding.

AUTHOR INFORMATION

Corresponding Author

*E-mail: sfb@dq.fct.unl.pt. Tel.: 351 21 2947885. Fax: 351 21 2941999.

ACKNOWLEDGMENT

This work was supported by Fundação para a Ciência e Tecnologia (FCT, Portugal) through grants SFRH/BD/13084/2003 (A.S.R.) and SFRH/BPD/41546/2007 (P.V.), FEDER, and by the European Commission in the framework of the Marie Curie Research Training Network "Green Chemistry in Supercritical Fluids: Phase Behaviour, Kinetics and Scale-up" (EC Contract No. MRTN-CT-2004-504005). We thank Novo Nordisk Bioindustrial, Spain, for the gift of Novozym 435.

REFERENCES

(1) Liese, A.; Seelbach, K.; Buchholz, A., Haberland, J. Processes. In *Industrial Biotransformations*, 2nd ed.; Liese, A., Seelbach, K., Wandrey, C., Eds.; Wiley-VCH: Weinheim, Germany, 2006; pp 147−513.

(2) Klibanov, A. M. Improving Enzymes by Using them in Organic Solvents. *Nature* **2001**, *409*, 241–246.

(3) Garcia, S.; Lourenço, N. M. T.; Lousa, D.; Sequeira, A. F.; Mimoso, P.; Cabral, J. M. S.; Afonso, C. M. A.; Barreiros, S. A Comparative Study of Biocatalysis in Non-Conventional Solvents: Ionic Liquids, Supercritical Fluids and Organic Media. *Green Chem.* **2004**, *6*, 466–470.

(4) Cantone, S.; Hanefeld, U.; Basso, A. Biocatalysis in Non-Conventional Media—Ionic Liquids, Supercritical Fluids and the Gas Phase. *Green Chem.* **2007**, *9*, 954–971.

(5) Sheldon, R. A. E Factors, Green Chemistry and Catalysis: An Odyssey. *Chem. Commun.* **2008**, 3352–3365.

(6) Buchholz, K.; Kasche, V.; Bornscheuer, U. T. *Biocatalysts and Enzyme Technology*; Wiley-VCH: Weinheim, Germany, 2005.

(7) Hyde, J. R.; Licence, P.; Carter, D.; Poliakoff, M. Continuous Catalytic Reactions in Supercritical Fluids. *Appl. Catal., A* **2001**, *222*, 119–131.

(8) Halling, P. J. Thermodynamic Predictions for Biocatalysis in Nonconventional Media: Theory, Tests, and Recommendations for Experimental Design and Analysis. *Enzyme Microb. Technol.* **1994**, *16*, 178–206.

(9) Vázquez Lima, F.; Pyle, D. L.; Asenjo, J. A. Factors Affecting the Esterification of Lauric Acid Using an Immobilized Biocatalyst: Enzyme Characterization and Studies in a Well-Mixed Reactor. *Biotechnol. Bioeng.* **1995**, *46*, 69–97.

(10) Mensah, P.; Gainer, J. L.; Carta, G. Adsorptive Control of Water in Esterification with Immobilized Enzymes. II. Fixed-bed Reactor Behavior. *Biotechnol. Bioeng.* **1998**, *60*, 445–453.

(11) Whetje, E.; Svensson, I.; Adlercreutz, P.; Mattiasson, B. Continuous Control of Water Activity During Biocatalysis in Organic Media. *Biotechnol. Tech.* **1993**, *7*, 873–878.

(12) Wehtje, E.; Kaur, J.; Adlercreutz, P.; Chand, S.; Mattiasson, B. Water Activity Control in Enzymatic Esterification Processes. *Enzyme Microb. Technol.* **1997**, *21*, 502–510.

(13) Bartling, K.; Thompson, J. U. S.; Pfromm, P. H.; Czermak, P.; Rezac, M. E. Lipase-Catalyzed Synthesis of Geranyl Acetate in n-Hexane with Membrane-Mediated Water Removal. *Biotechnol. Bioeng.* **2001**, *75*, 676–681.

(14) Dumond, T.; Barth, D.; Perrut, M. Continuous Synthesis of Ethyl Myristate by Enzymatic Reaction in Supercritical Carbon Dioxide. *J. Supercrit. Fluids* **1993**, *6*, 85–89.

(15) Marty, A.; Dossat, V.; Condoret, J. S. Continuous Operation of Lipase-Catalyzed Reactions in Nonaqueous Solvents: Influence of the Production of Hydrophilic Compounds. *Biotechnol. Bioeng.* **1997**, *56*, 232–237.

(16) Mensah, P.; Gainer, J. L.; Carta, G. Adsorptive Control of Water in Esterification with Immobilized Enzymes. I. Batch Reactor Behavior. *Biotechnol. Bioeng.* **1998**, *60*, 435–444.

(17) Omar, I. C.; Nishio, N.; Nagai, S. The Role of Water on the Equilibrium of Esterification by Immobilized Lipase Packed-Bed Column Reactor. *Biotechnol. Lett.* **1988**, *11*, 799–804.

(18) Mensah, P.; Carta, G. Adsorptive Control of Water in Esterification with Immobilized Enzymes. Continuous Operation in a Periodic Counter-Current Reactor. *Biotechnol. Bioeng.* **1999**, *66*, 137–146.

(19) Trusek-Holownia, A.; Noworyta, A. An Integrated Process: Ester Synthesis in an Enzymatic Membrane Reactor and Water Sorption. *J. Biotechnol.* **2007**, *130*, 47–56.

(20) Engasser, J. M.; Hisland, P. Diffusional Effects on the Heterogeneous Kinetics of Two-Substrate Enzymic Reactions. *J. Theor. Biol.* **1979**, *77*, 427–440.

(21) Bódalo, A.; Gómez, J. L.; Gómez, E.; Bastida, J.; Máximo, M. F. Fluidized Bed Reactors Operating with Immobilized Enzyme Systems: Design Model and its Experimental Verification. *Enzyme Microb. Technol.* **1995**, *17*, 915–922.

(22) Abu-Reesh, I. M. Predicting the Performance of Immobilized Enzyme Reactors Using Reversible Michaelis−Menten Kinetics. *Bioprocess Eng.* **1997**, *17*, 131–137.

(23) Goddard, E.; Bosley, J.; Al-Duri, B. Lipase-Catalysed Esterification of Oleic Acid and Ethanol in a Continuous Packed Bed Reactor, Using Supercritical CO_2 as Solvent: Approximation of System Kinetics. *J. Chem. Technol. Biotechnol.* **2000**, *75*, 715–721.

(24) Xiu, G.-H.; Jiang, L.; Li, P. Mass-Transfer Limitations for Immobilized Enzyme-Catalyzed Kinetic Resolution of Racemate in a Batch Reactor. *Ind. Eng. Chem. Res.* **2000**, *39*, 4054–4062.

(25) Van Roon, J. L.; Arntz, M. M.; Kallenberg, A. I.; Paasman, M. A.; Tramper, J.; Schroen, C. J.; Beeftink, H. H. A Multicomponent Reaction-Diffusion Model of a Heterogeneously Distributed Immobilized Enzyme. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 263–278.

(26) Yahya, A. R. M.; Anderson, W. A.; Moo-Young, M. Ester Synthesis in Lipase-Catalyzed Reactions. *Enzyme Microb. Technol.* **1998**, *23*, 438–450.
(27) Ikeda, Y.; Kurokawa, Y. Synthesis of Geranyl Acetate by Lipase Entrap-Immobilized in Cellulose Acetate-TiO_2 Gel Fiber. *J. Am. Oil Chem. Soc.* **2001**, *78*, 1099–1103.
(28) Abbas, H.; Comeau, L. Aroma Synthesis by Immobilized Lipase from *Mucor sp*. *Enzyme Microb. Technol.* **2003**, *32*, 589–595.
(29) Peres, C.; Gomes da Silva, M. D. R.; Barreiros, S. Water Activity Effects on Geranyl Acetate Synthesis Catalyzed by Novozym in Supercritical Ethane and in Supercritical Carbon Dioxide. *J. Agric. Food Chem.* **2003**, *51*, 1884–1888.
(30) Larios, A.; García, H. S.; Oliart, R. M.; Valerio-Alfaro, G. Synthesis of Flavor and Fragrance Esters Using *Candida antarctica* Lipase. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 373–376.
(31) Barahona, D.; Pfromm, P. H.; Rezac, M. E. Effect of Water Activity on the Lipase Catalysed Esterification of Geraniol in Ionic Liquid [bmim]PF_6. *Biotechnol. Bioeng.* **2005**, *93*, 318–324.
(32) Greenspan, L. Humidity Fixed Points of Binary Saturated Aqueous Solutions. *J. Res. Natl. Bur. Stand. (U.S.A.)* **1977**, *81A*, 89–96.
(33) Fontes, N.; Partridge, J.; Halling, P. J.; Barreiros, S. Zeolite Molecular Sieves Have Dramatic Acid-Base Effects on Enzymes in Nonaqueous Media. *Biotechnol. Bioeng.* **2002**, *77*, 296–305.
(34) Gandhi, N. N.; Patil, N. S.; Sawant, S. B.; Joshi, J. B. Lipase-Catalyzed Esterification. *Catal. Rev.—Sci. Eng.* **2000**, *42*, 439–480.
(35) Segel, I. H. *Enzyme Kinetics*; Wiley-Interscience: New York, 1993.
(36) Claon, P. A.; Akoh, C. C. Enzymatic Synthesis of Geranyl Acetate in *n*-Hexane with *Candida antarctica* Lipase. *J. Am. Oil Chem. Soc.* **1994**, *71*, 575–578.
(37) Froment, G. F.; Bischoff, K. B. *Chemical Reactor Analysis and Design*, 2nd ed.; John Wiley and Sons: New York, 1990.
(38) Hindmarsh, A. C. ODEPACK, a Systematized Collection of ODE Solvers. In *Scientific Computing*; Stepleman, R. S., Ed.; North-Holland: Amsterdam, 1983, pp 55−64.
(39) Petzold, L. R. Automatic Selection of Methods for Solving Stiff and Nonstiff Systems of Ordinary Differential Equations. *SIAM J. Sci. Stat. Comput.* **1983**, *4*, 136–148.
(40) Levenberg, K. A Method for the Solution of Certain Problems in Least Squares. *Q. Appl. Math.* **1944**, *2*, 164–168.
(41) Marquardt, D. W. An Algorithm for Least-Squares Estimation of Nonlinear Parameters. *SIAM J. Appl. Math.* **1963**, *11*, 431–441.
(42) Parker, M. C.; Moore, B. D.; Blacker, A. J. Measuring Enzyme Hydration in Nonpolar Organic Solvents Using NMR. *Biotechnol. Bioeng.* **1995**, *46*, 452–458.
(43) Colombié, S.; Tweddell, R. J.; Condoret, J. S.; Marty, A. Water Activity Control: A Way to Improve the Efficiency of Continuous Lipase Esterification. *Biotechnol. Bioeng.* **1998**, *60*, 362–368.
(44) Sanchéz, A.; Valero, F.; Lafuente, X.; Solà, C. Highly Enantioselective Esterification of Racemic Ibuprofen in a Packed Bed Reactor Using Immobilized *Rhizomucor miehei* Lipase. *Enzyme Microb. Technol.* **2000**, *27*, 157–166.
(45) Valivety, R. H.; Johnston, G. A.; Suckling, C. J.; Halling, P. J. Solvent Effects on Biocatalysis in Organic Systems: Equilibrium Position and Rates of Lipase Esterification. *Biotechnol. Bioeng.* **1991**, *38*, 1137–1143.