Chemical Engineering Journal 160 (2010) 708–714

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

On the feasibility of *in situ* steroid biotransformation and product recovery in microchannels

M.P.C. Marques[a,*], P. Fernandes[a], J.M.S. Cabral[a], P. Žnidaršič-Plazl[b], I. Plazl[b]

[a] *IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*
[b] *University of Ljubljana, Faculty of Chemistry and Chemical Technology, Aškerčeva 5, SI-1000 Ljubljana, Slovenia*

ARTICLE INFO

Article history:
Received 9 November 2009
Received in revised form 11 March 2010
Accepted 23 March 2010

Keywords:
Steroids
Oxidation
Microreactor
Reaction-diffusion dynamics
Modeling
Cholesterol oxidase

ABSTRACT

Microchannel reactor technologies are gaining widespread use in a large range of areas, which comprise biotechnology and chemistry. The small volumes involved and the favorable mass and heat transfer inherent to these devices make them particularly useful for the screening of biocatalysts and rapid characterization of bioconversion systems.

In the present work, the enzymatic oxidation of cholesterol to 4-cholesten-3-one performed within microchannels by cholesterol oxidase, was studied in a two-phase system, comprising an organic phase as substrate and product pool and an aqueous phase with dissolved enzyme. A mathematical model based on mass balances for cholesterol, 4-cholesten-3-one and dissolved oxygen concentrations, comprising double-substrate Michaelis–Menten kinetics and the velocity profile of two immiscible fluids, was developed in order to describe and predict the process of cholesterol oxidation. The numerical procedure of solving the non-linear 3D model was based on an implicit finite-difference method improved by non-equidistant differences.

In a Y-shape microreactor geometry, roughly up to 70% conversion of cholesterol was achieved at residence times below 1 min. The suitable adjustment of the ratio of the fluid flow rates was performed by taking into account the viscosity of the fluids involved. This allowed for phase separation to be reestablished at the Y-shaped exit from the microreactor and thereby enabled *in situ* product separation from the aqueous phase containing the enzyme.

1. Introduction

Microchannel reactor technologies have found application in research, development and production processes in several areas, including (bio)chemistry and biotechnology, having already been introduced in industrial-scale applications [1–5].

In contrast with conventional reactors, microchannel reactors offer the option for achieving a more precise control of the process, higher efficiency and safety allied to better selectivity, improved yield and flexible production [5,6].

These reactors can be assembled by microfabrication techniques or by the modification of microcapillaries, using reaction apparatus with small dimensions, typically in the range of micrometers (μm) with volumetric capacity in the range of microliters (μl) [7]. These systems take advantage of micro- or nano-fluidics, that require low volumes of reactant solutions, offering high-performance efficiency and repeatability [8], thus making microchannel reactors an extremely efficient tool for the rapid screening of biocatalysts.

The flow patterns in microfluidic systems are mostly laminar, as opposite to macro-scale systems, a feature that favors the strict control of reaction conditions and time [9].

In addition, microchannel reaction systems provide large surface to volume ratio, which gives the microreactors superior performance w.r.t. heat and mass transfer compared to conventional reactors, e.g. in extractions and multiphase (bio)catalytic reactions [10].

Given their flexibility, microchannel reactors allow furthermore for faster transfer from the development to the production stage, reducing associated cost of scale translation, materials and energy, and manpower.

In the particular case of enzymatic microchannel reactors, using either dissolved or immobilized enzymes, these were originally developed mostly in order to improve the routine work in biochemical analyses of proteomic and genetic material [8,11]. The use of microreactors has however expanded to other areas, including production processes, as demonstrated by the increased number of

* Corresponding author. Tel.: +351 218 419 138.
E-mail address: mpc.marques@ist.utl.pt (M.P.C. Marques).

1385-8947/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.cej.2010.03.056

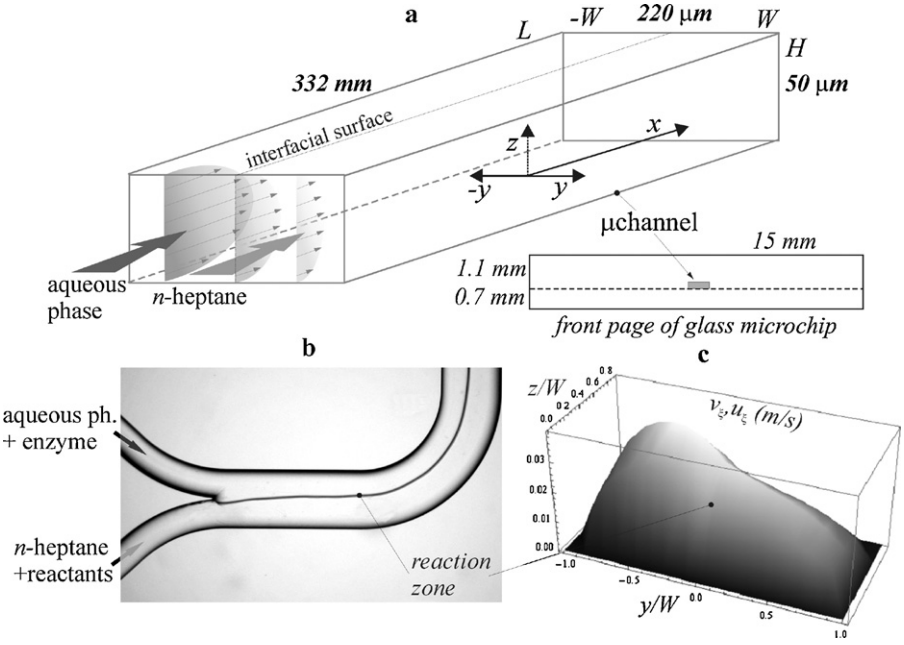

Fig. 1. (A) Scheme of microchannel reactor ($2W$ = 220 μm, H = 100 μm and L = 332 μm). Parallel aqueous–organic two-phase flow (B) and velocity profile (C) at the inlet of the microchannels at combined flow rate of 14 μl min^{-1} (4.4 μl min^{-1} aqueous phase and 9.6 μl min^{-1} organic phase).

patents and articles on this matter [12,13]. Microchannel enzymatic reactors have been mainly used for kinetic studies and imaging of biotransformations [11].

Within the scope of biotransformation, the use of enzymes and microbial cells has been investigated aiming at the reduction of cholesterol level in foods, the production of precursors in the manufacturing of pharmaceutical steroids from cheap sterols and for clinical assays of serum cholesterol. Microbial cholesterol oxidases have received much attention in recent years, mainly due to their large use in medical practice for determination of free and bound cholesterol [14].

Cholesterol oxidase (3β-hydroxysteroid oxidase, EC 1.1.3.6) is a bifunctional enzyme catalyzing the oxidation of Δ^5-3β-hydroxysteroids to the corresponding Δ^5-3-ketosteroid, alongside with the isomerization of this compound to the Δ^4-3-ketosteroid, with reduction of oxygen to hydrogen peroxide [15]. Given its broad substrate specificity, cholesterol oxidase can oxidize any 3β-hydroxysteroid to its corresponding ketones, which are important precursors of chemically synthesized hormones. Alongside with the bioconversion of cholesterol to cholestenone, other relevant examples include the production ergosta-4,7,22-triene-3-one, progesterone from pregnenolone, androstenedione from dehydroepiandrosterone or testosterone from androstenediol. Besides their application in preparative steroid transformation, cholesterol oxidases have also been used for the optical resolution of allylic alcohols [16,17].

The aim of this study is to perform and characterize the enzymatic oxidation of cholesterol in microchannels, using an aqueous–organic two-phase system flow with defined interface. Pioneering work has already been performed by Maruyama et al. [18] for the dehalogenation of p-chlorophenol catalyzed by laccase in a water–isooctane system while Žnidaršič-Plazl and Plazl [19] synthesized isoamyl acetate using lipase in a water–hexane system.

Within this system, the organic phase (*n*-heptane) acts as a pool for the poorly water-soluble sterol and steroid, whereas the aqueous phase (phosphate buffer) stores the enzyme. This allows for an integrated bioconversion system, combining cholesterol oxidation and *in situ* product recovery in microchannel reactors. To the author's knowledge, the two-phase microfluidic approach for the present bioconversion system has not been reported before.

2. Theoretical background

2.1. Velocity profile in a microchannel

Laminar flow characterizes microreactor flow conditions [19–22]. In order to simulate cholesterol oxidation in a microchannel reactor, the velocity profile was first set up. As shown in Fig. 1A, the main microchannel was fed by two-phase inflows: an aqueous phase containing cholesterol oxidase and an organic phase containing cholesterol where the concentration of oxygen is variable.

Since both inflow phases had different viscosities, flow rates had to be adjusted so that the two phases occupied the same fraction of the channel. The assumption of the parabolic velocity profile, developed only in the smallest z-dimension, and therefore the uniform velocity profile in the y-direction was not possible due to the small width/height ratio of the microchannel used in our experiments, which was of 2.2:1 [19,22] (Fig. 1A).

Therefore, the compressibility and gravitational force were neglected. Considering these presumptions, dimensionless continuity and momentum equations for a fully developed Poiseuille-type flow were solved [19,20].

2.2. Reaction-diffusion dynamics of cholesterol conversion to cholestenone in a microchannel

For the description and prediction of microreactor performance, a 3D model was developed considering convection in the flow direction (x) and diffusion in 3D grid directions. Dimensionless partial differential equations for steady-state conditions in the single pass microchannel reactor system with the associated boundary conditions are as follows:

For cholesterol in organic phase (c_{CH}):

$$v_\xi(\psi,\omega)\frac{\partial c_{CH}}{\partial \xi} = \frac{D_{CH}}{W}\left(\frac{\partial^2 c_{CH}}{\partial \xi^2} + \frac{\partial^2 c_{CH}}{\partial \psi^2} + \frac{\partial^2 c_{CH}}{\partial \omega^2}\right) \quad (1)$$

with the associated boundary conditions:

$$\begin{aligned} &c_{CH}\left(0,\psi,\omega\right)=c_{CH,i}, && \frac{\partial c_{CH}((L/W),\psi,\omega)}{\partial\xi}=0; && -1\le\psi\le 0, 0\le\omega\le\frac{H}{W}\\ &\frac{\partial c_{CH}\left(\xi,-1,\omega\right)}{\partial\psi}=0, && \frac{\partial c_{CH}\left(\xi,0,\omega\right)}{\partial\psi}=-\frac{v_r\cdot W^2\,\Delta\psi}{D_{CH}}; && 0<\xi<\frac{L}{W}, 0\le\omega\le\frac{H}{W}\\ &\frac{\partial c_{CH}\left(\xi,\psi,0\right)}{\partial\omega}=\frac{\partial c_{CH}(\xi,\psi,(H/W))}{\partial\omega}=0; && && 0<\xi<\frac{L}{W}, -1\le\psi\le 0 \end{aligned} \quad (2)$$

For oxygen in organic phase (c_{O_2}):

$$v_\xi(\psi,\omega)\frac{\partial c_{O_2}}{\partial\xi}=\frac{D_{O_2}}{W}\left(\frac{\partial^2 c_{O_2}}{\partial\xi^2}+\frac{\partial^2 c_{O_2}}{\partial\psi^2}+\frac{\partial^2 c_{O_2}}{\partial\omega^2}\right) \quad (3)$$

with the associated boundary conditions:

$$\begin{aligned} &c_{O_2}\left(0,\psi,\omega\right)=c_{O_2,i}, && \frac{\partial c_{O_2}((L/W),\psi,\omega)}{\partial\xi}=0; && -1\le\psi\le 0, 0\le\omega\le\frac{H}{W}\\ &\frac{\partial c_{O_2}\left(\xi,-1,\omega\right)}{\partial\psi}=0, && \frac{\partial c_{O_2}\left(\xi,0,\omega\right)}{\partial\psi}=-\frac{v_r\cdot W^2\,\Delta\psi}{D_{O_2}}; && 0<\xi<\frac{L}{W}, 0\le\omega\le\frac{H}{W}\\ &\frac{\partial c_{O_2}\left(\xi,\psi,0\right)}{\partial\omega}=\frac{\partial c_{O_2}(\xi,\psi,(H/W))}{\partial\omega}=0; && && 0<\xi<\frac{L}{W}, -1\le\psi\le 0 \end{aligned} \quad (4)$$

In Eqs. (1)–(4) c_{CH} and c_{O_2} represent the concentrations of cholesterol and dissolved oxygen, respectively (mM), and D_{CH} and D_{O_2} represent the diffusion coefficients for cholesterol and oxygen in *n*-heptane, respectively ($cm^2\,s^{-1}$), c_i denotes inlet concentrations, v_ξ is the *x*-directional velocity of water ($m\,s^{-1}$).

For the investigated reaction in a water/*n*-heptane two-phase system, the Michaelis–Menten kinetic expression was used:

$$v_r=\frac{v_{max}\cdot c_E\cdot c_{CH}}{k_m^{CH}+c_{CH}}\frac{c_{O_2}}{k_m^{O_2}+c_{O_2}} \quad (5)$$

where v_{max} is the maximal reaction rate ($mM\,min^{-1}$), while k_m^{CH} and $k_m^{O_2}$ are the Michaelis constants for cholesterol and oxygen respectively (mM).

2.3. Estimation of diffusion coefficients

For the estimation of the molecular diffusion coefficients of cholesterol, 4-cholesten-3-one and oxygen in *n*-heptane, the Reid empirical correlation was used [24]:

$$D=\frac{7.4\times 10^{-8}(\sqrt{\varphi.M_B})T}{\eta_B V_A^{0.6}} \quad (6)$$

where D is the diffusion coefficient ($cm^2\,s^{-1}$), M_B is the molecular weight of the solvents, T is the temperature (K), η_B is the viscosity of the solvent (cP), V_A is the molar volume of the solute ($cm^3\,mol^{-1}$) and φ is the association factor of the solvent.

2.4. Numerical analysis

Finite differences on the 3D Cartesian grid were used to replace the partial derivatives in the presented model equations. Static equidistant finite differences were transformed to non-equidistant finite differences according to Žnidaršič-Plazl and Plazl [19,20].

3. Materials and methods

3.1. Materials

Cholesterol oxidase was purchased from BBI Enzyme Ltd. (Gwent, UK). 4-Cholesten-3-one was from Acros (Geel, Belgium), and cholesterol and progesterone were obtained from Sigma (St Louis, MO, USA). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade, and purchased from various suppliers.

3.2. Methods

Cholesterol biotransformation was carried out in glass microchannel reactors with Y-shaped inflow and outflow channels. The main channel had the following dimensions: 220 μm width, 100 μm height and 332 mm length (Micronit Microfluidics B.V., Enschede, The Netherlands). The reactor was fixed in stainless steel housing coupled with two syringe pumps (PHD4400 Syringe Pump Series, Harvard Apparatus, Holliston, USA) for solution supply.

Oxygen-saturated, half saturated and unsaturated cholesterol solutions in *n*-heptane were alternatively fed from one inflow, and an air saturated cholesterol oxidase solution in 50 mM phosphate buffer (pH 7) was fed from another inflow of the Y-shaped microchannel reactor.

Different concentrations of cholesterol were used at the inlet: $c_{i,CH}$ = 0.17, 0.5 or 1 mM, while enzyme concentration at the inlet was kept constant $c_{i,CHO}$ = 0.1 mg ml^{-1}. The organic and aqueous phases were supplied at constant flow rates between 5 and 72 μl min^{-1} for buffer and between 10 and 154 μl ml^{-1} for *n*-heptane, and collected at the two outlets, where samples for further analysis were taken.

Experiments were performed at 30 °C, using a thermostat-controlled water-bath. Blank experiments were performed under the same conditions but without enzyme. All experiments were carried out at least in triplicates. The samples from the microchannel reactor were diluted in 0.1 mol l^{-1} HCl in order to stop the enzymatic reaction prior to the HPLC analysis.

Batch reactions were performed in 250 ml reactors filled with 50 ml with 1:1 two-phase system composed of 50 mM phosphate buffer and *n*-heptane containing 0.1 mg ml^{-1} of cholesterol oxidase. Temperature was set at 30 °C and agitation was provided by magnetic stirring (700 rpm). Continuous aeration with oxygen was provided to assess the influence of oxygen on the reaction rate. Air flow rate was set to obtain the following oxygen concentrations in the medium: 0.24, 0.65 and 1.00 mmol l^{-1}.

3.3. Analytics

3.3.1. Oxygen concentration

An oxymeter (WTW pH/Oxi 340, electrode WTW Cellox 325, Weinheim, Germany) was used to measure dissolved oxygen concentration in the reaction media.

3.3.2. Steroid analysis

HPLC analysis (Lichrospher Si-60 column, 5 μm particle size, Merck, Germany) with 1 ml min^{-1} isocratic elution was performed for the quantification of the concentration of the products, with UV

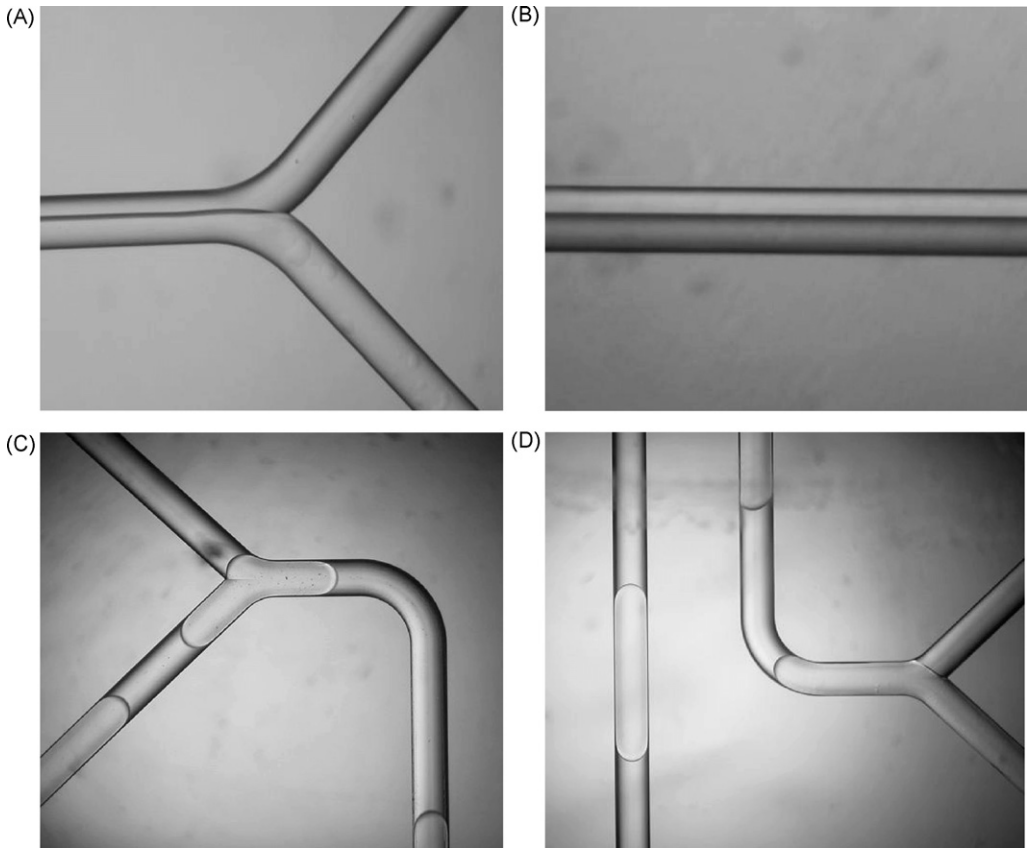

Fig. 2. (A and B) Microscopic observation of a flow pattern of colored buffer and *n*-heptane entering through the separate inflow channels (total flow rate through the main microchannel 14 μl min^{-1}). (C and D) Slug flow formation in the microchannels at equal inlet flow rates of both aqueous and organic phase (total flow rate through the main microchannel 14 μl min^{-1}).

detection at 254 nm. The mobile phase was composed of *n*-heptane and ethanol (90:10, v/v).

Conversion yields were calculated on the basis of product formation since no product, besides 4-cholesten-3-one was formed.

4. Results and discussion

4.1. Fluid flow in a microchannel

Previously, it was demonstrated that relatively small differences in the viscosity can have significant effects on the relative distribution of two fluid streams within the microchannel devices [19]. In order to have laminar flow parallel to the sidewalls of the channels, the flow of the different inlets were set as the difference of the ratios of the fluid viscosities, $Faq = (\mu_{org}/\mu_{aq})F_{org}$ (Fig 1B and C).

The results of the 3D numerical simulation of the pattern of fluid flow in the microchannel with the position of the interface area in the middle of the channel showed that under steady-state conditions a fully developed profile is established at the beginning of the microchannel. A fully developed velocity profile was therefore considered in further simulations of concentration profiles along the whole length of the microchannel.

In order to validate the assumptions, phosphate buffer with a colored dye and *n*-heptane were fed through separate inflows. Microscopic observations (Fig. 2A and B) confirmed the laminar flow parallel to the sidewalls of the channels for the flow rates used in the present studies (flow rates of buffer system ranging from 4.4 to 72 μl min^{-1} and of *n*-heptane system from 9.6 to 153.6 μl min^{-1}). Moreover, the position of the interface was exactly in the middle of the channel, which again confirmed the assumptions for the model. For different flow rates ratios (e.g. equal flow rates between aqueous and organic phase), Taylor flow was observed (Fig. 2C and D).

Despite improving mass transfer from the liquid to the wall due to the recirculation flow in the liquid slugs (enhanced radial mass transfer and reduced axial mass transfer), two-phase flow was preferred as it gave a defined interfacial area and phase separation at the Y-shaped microchannel end. Nonetheless, previous work has shown the applicability of a combined Taylor flow with fine dispersion for the synthesis of isoamyl acetate catalyzed by lipase CALB L in 1-butyl-3-methylpyridinium dicyanamide/*n*-heptane two-phase system [23].

Enzymes tend to form clusters above a critical concentration [25,26], however in a literature survey, no critical concentration for aggregation of cholesterol oxidase was found. Parallel studies were performed in order to assess the enzyme concentration leading to cluster formation and consequentially to clogging of the microchannels.

Clogging resulted in the formation of Taylor flow as well as in the increased back pressure leading to lower substrate conversion yields. Clogging occurred for enzyme concentrations above 0.1 g l^{-1}, with consequent accumulation of protein, mainly at the Y-shape end of the microchannels, as well as in the channel turns (Fig. 3).

4.2. Estimation of model parameters

Estimated diffusion coefficients of cholesterol, 4-cholesten-3-one and oxygen in *n*-heptane at 30 °C were of 1.77×10^{-9}, 1.78×10^{-9} and 1.49×10^{-9} m^2 s^{-1}, respectively. Kinetic parameters of cholesterol oxidation in two-phase system were previously

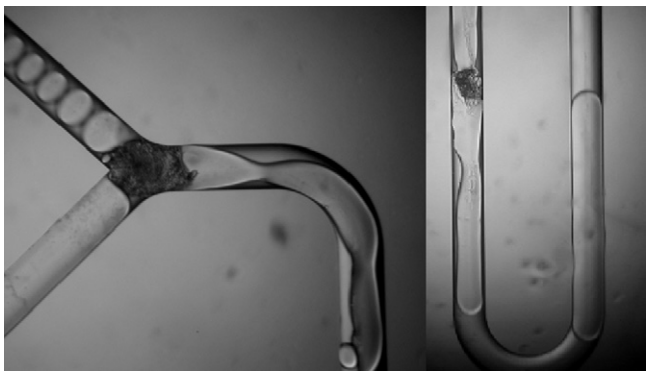

Fig. 3. Clogging of the microchannels due to possible protein denaturation at enzyme concentration of 0.2 g l^{-1} and 30 °C.

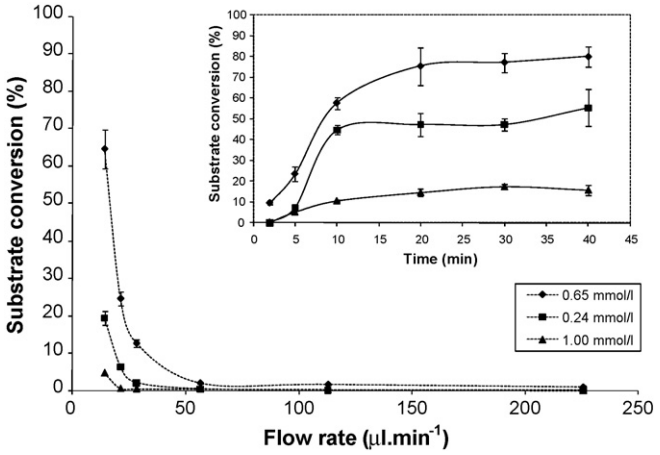

Fig. 4. Conversion yields in batch reactor (insert) and in microchannel reactor at several concentrations of dissolved oxygen. Concentration of substrate of 0.1 mM, enzyme of 0.1 g l^{-1} performed at 30 °C.

estimated in a batch reactor and used for mathematical modeling of enzyme reaction within the microchannel: ν_{max} = 0.05 mM min^{-1}, k_m^{CH} = 0.242 mM and $k_m^{O_2}$ = 0.3 mM.

4.3. Enzyme reaction

Oxidation of cholesterol was both performed in batch reactors as well as at steady-state conditions in a microchannel reactor, investigating the effect of different inlet oxygen concentrations and different combined fluid flow rates (Fig. 4). Both in the microchannel as well as in the batch reactor a clear influence of oxygen was observed on substrate conversion yields. In the batch reactor an increase of the substrate conversion yield of roughly 25% was observed when increasing the oxygen concentration from 0.24 to 0.65 mM, reaching 77% substrate conversion at 0.65 mM.

Nonetheless, higher concentrations of oxygen, such as 1 mM, apparently led to enzyme deactivation, since conversion yields lowered to 10%. Similar effect was observed in the microchannel reactor at combined flow rate of 14 μl min^{-1}. With intermediate concentration of oxygen, 0.65 mM, conversion yields reached approximately 70%. At 0.24 mM of oxygen, a 20% conversion of cholesterol was observed, whereas for 1 mM of oxygen, only 4% of cholesterol was converted.

Similar studies were carried out in the same microchannel system with L-DOPA, where the influence of oxygen on the biotransformation. Tišma et al. [22] observed that an increase of dissolved oxygen concentration of 50% led to an increase in conversion yields of L-DOPA from roughly 75 to 90%.

The results in Fig. 5 show that the use of microchannel reactor is advantageous when compared with traditional batch reactor with continuous aeration, since for achieving the same conversion yields a 20-fold decrease in time was possible, reducing from 20 min of incubation to only 1 min residence time, to achieve similar conversion yields of cholesterol (70% conversion of substrate).

In previous work, the enzymatic (lipase) synthesis of isoamyl acetate was performed both in two-phase system with defined interface [19] and in Taylor flow system [23], in a microchannel reactor, and compared with classical stirred reactors (working volume of 10 ml). In both cases, a significant reduction in residence/operation time for similar conversion yields was observed. Thus, in a two-phase system with defined interface [19], a 35% conversion was achieved after roughly 1 min residence time, as compared with 20% after 2.5 h in a traditional reactor. On the other hand, Pohar et al. [23] showed that a roughly 3-fold increase in the reaction rate of isoamyl acetate synthesis was achieved in a microchannel reactor operated with Taylor flow, as compared to a perfectly mixed batch reactor. Such behavior was ascribed to the specific flow pattern that provided highly efficient mixing inside the microchannels, and to the high interfacial area for the reaction, along with simultaneous extraction and product removal.

A conversion yield of 66% of cholesterol was reached with the lowest inlet concentration of 0.17 mM in oxygen-half saturated medium (0.65 mM) and at the longest residence time of 62 s (total fluid flow rate = 14 μl min^{-1}). At higher flow rates, and thereby shorter residence times, lower conversions were obtained in the chosen microchannel reactor configuration. For residence times under 15 s, conversion yields dropped below 5% (Fig. 4).

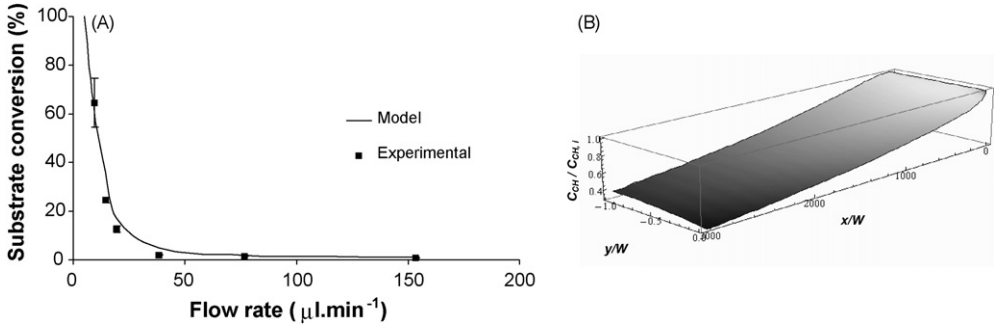

Fig. 5. (A) Correlation of the mathematical simulation to the experimental results for enzyme concentration of 0.1 g l^{-1}, cholesterol of 0.17 mM and oxygen of 0.65 mM. (B) Cholesterol concentration profiles within organic phase along the total length of the microchannel at 14 μl min^{-1} (enzyme concentration of 0.1 g l^{-1}, inlet cholesterol concentration of 0.17 mM and inlet oxygen concentration of 0.65 mM).

At the highest fluid flow rate tested (225 μl min^{-1}, σ = 1.1 s), residual conversion yields, below 1%, were observed within the microchannel for all tested reaction conditions. At higher substrate concentrations, as expected, biotransformation yields lowered. Nonetheless, this can be overcome if the length of the microchannel is increased (data not shown).

Mathematical model simulations were performed in order to predict the microchannel reactor performance. As shown in Fig. 5, the experimental conversions of cholesterol performed in a microchannel reactor are in good agreement with the model predictions for all applied flow rates.

4.4. Concentration profiles within the microchannel

Concentration profiles of a particular component within the main microchannel were obtained by a numerical solution of the non-linear system of partial differential equations, based on the previously described velocity profile and double-substrate Michaelis–Menten kinetic model. The results for cholesterol conversion along the microchannel reactor, with inlet concentration of 0.17 mM, in oxygen-half saturated *n*-heptane phase at the lowest tested total flow rates (14 μl min^{-1}) are presented in Fig. 5B.

The cholesterol concentration profiles in the organic phase were calculated based on the following assumptions:

(a) No substrate or product diffused to the aqueous phase due to the low solubility of sterols and steroids in aqueous phase, usually below 1 μM and 0.1 mM respectively [27], and to the high partition coefficient towards the organic phase (above 100).
(b) The reaction occurred at the interface, so no alteration in enzyme concentration was observed along the reactor and aqueous phase.
(c) The aqueous phase was fully saturated with oxygen and additionally the oxygen content was higher in the organic phase. During oxygen consumption there was no significant depletion from the aqueous phase, since equilibrium was established between organic and aqueous phase. Due to the high content of oxygen in the organic phase, no significant decrease was observed.

A decrease in cholesterol concentration due to the enzymatic reaction along the microchannel is evident from Fig. 5B. For prolonged residence times there is a steady decrease in cholesterol concentration along the microchannel and at the interface. This pattern is clearly noticeable at higher flow rates (data not shown) where substrate decrease is observable only in the surroundings of the interface and near the end of the microchannel. The same reasoning could be used also for the poor results of cholesterol oxidation at higher inlet concentration and at shorter residence times.

Further studies are envisaged to increase conversion yields either by: (i) the use of longer and/or consecutively bounded microreactors; (ii) employing Taylor flow; (iii) the use of micromixers; (iv) a combination of the several approaches.

If industrial application of the experimental setup is desired, the throughput required can be achieved by numbering-up the microchannel reactors. In such case strategies allowing for the sustained use of the biocatalyst through several consecutive batch cycles or in continuous operation are required, in order to insure the economical viability of the process. Immobilization of the enzyme on the surface of the channels or recycle of the free enzyme can be considered. Additionally, enzyme deactivation must be taken into account. This approach is expected to allow for a significant cost reduction when biocatalysts and equipments (reactors and associated equipments). Nonetheless, economical studies are necessary to validate our assumptions.

5. Conclusions

Cholesterol oxidation was performed in a microchannel reactor by free cholesterol oxidase in organic-aqueous two-phase flow. A conversion yield of 67% was reached for initial concentrations of 0.17 mM of cholesterol and 0.65 mM of oxygen, and for a residence time of 62 s. For higher inlet concentration of cholesterol, the oxidation in the same microreactor was less efficient at the same operational conditions, suggesting the use of a longer microchannel for overcoming this limitation. Furthermore this productivity of the reaction system was enhanced in the microreactor when compared with the traditional batch system. Results showed that *in situ* formation and recovery of product is feasible in microchannel reactors, therefore allowing for a reduction of cost and equipment associated with downstream processing, a feature of paramount relevance particularly when industrial applications are foreseen.

Based on the developed model simulations, which were in good agreement with experimental data, further microchannel reactor design and process optimization is feasible.

Acknowledgements

M.P.C. Marques and P. Fernandes acknowledge Fundação para a Ciência e Tecnologia (Portugal) for financial support in the form of PhD grant SFRH/BD/24433/2005 and program Ciência 2007, respectively. This work was supported through Grant P2-0191, and M.P.C. Marques was partly supported through CMEPIUS Grant 2008/2009 - 5976, both provided by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia.

References

[1] P. Watts, S.J. Haswell, The application of micro reactors for organic synthesis, Chem. Soc. Rev. 34 (2005) 235–246.
[2] T. Chovan, A. Guttman, Microfabricated devices in biotechnology and biochemical processing, Trends Biotechnol. 20 (2002) 116–122.
[3] C.J. Cullen, R.C. Wootton, A.J. de Mello, Microfluidic systems for high-throughput and combinatorial chemistry, Curr. Opin. Drug Discov. Dev. 7 (2004) 798–806.
[4] K.-I. Sotowa, K. Takagi, S. Sugiyama, Fluid flow behavior and the rate of an enzyme reaction in deep microchannel reactor under high-throughput condition, Chem. Eng. J. 135 (2008) S30–S36.
[5] G. Kolb, V. Hessel, Micro-structured reactors for gas phase reactions, Chem. Eng. J. 98 (2004) 1–38.
[6] L. Kiwi-Minsker, A. Renken, Microstructured reactors for catalytic reactions, Catal. Today 110 (2005) 2–14.
[7] S.V. Gokhale, R.K. Tayal, V.K. Jayaraman, B.D. Kulkarni, Microchannel reactors: applications and use in process development, Int. J. Chem. React. Eng. 3 (2005) R2.
[8] M. Miyazaki, H. Maeda, Microchannel enzyme reactors and their application for processing, Trends Biotechnol. 24 (2006) 463–470.
[9] N.-T. Nguyen, Z. Wu, Micromixers—a review, J. Micromech. Microeng. 15 (2005) R1–R16.
[10] A.M. Thayer, Harnessing microreactions, Chem. Eng. News 83 (2005) 43–52.
[11] P.L. Urban, D.M. Goodall, N.C. Bruce, Enzymatic microreactors in chemical analysis and kinetic studies, Biotechnol. Adv. 24 (2006) 42–57.
[12] Z. Zhang, G. Perozziello, P. Boccazzi, A.J. Sinskey, O. Geschke, K.F. Jensen, Microbioreactors for bioprocess development, JALA 12 (2007) 143–151.
[13] V. Hessel, C. Knobloch, H. Löwe, Review on patents in microreactor and micro process engineering, Recent Pat. Chem. Eng. 1 (2008) 1–16.
[14] J. MacLachlan, A.T.L. Wotherspoon, R.O. Ansell, C.J.W. Brooks, Cholesterol oxidase: sources, physical properties and analytical applications, J. Steroid Biochem. Mol. Biol. 72 (2000) 169–195.
[15] G.E. Turfitt, The microbiological degradation of steroids, part IV, Biochem. J. 42 (1948) 376–383.
[16] J.F. Aparicio, J.F. Martín, Microbial cholesterol oxidases: bioconversion enzymes or signal proteins? Mol. Biosyst. 4 (2008) 804–809.
[17] A. Gupte, R. Nagarqjan, A. Kilara, Enzymatic oxidation of cholesterol in reverse micelles, Ind. Eng. Chem. Res. 34 (1996) 2910–2922.
[18] T. Maruyama, J.-I. Uchida, T. Ohkawa, T. Futami, K. Katayama, K.-I. Nishizawa, K.-I. Sotowa, F. Kubota, N. Kamiya, M. Goto, Enzymatic degradation of p-

chlorophenol in a two-phase flow microchannel system, Lab Chip 23 (2003) 308–312.
[19] P. Žnidaršič-Plazl, I. Plazl, Modelling and experimental studies on lipase-catalyzed isoamyl acetate synthesis in a microreactor, Process Biochem. 44 (2009) 1115–1121.
[20] P. Žnidaršič-Plazl, I. Plazl, Steroid extraction in a microchannel system—mathematical modelling and experiments, Lab Chip 7 (2007) 883–889.
[21] A. Pohar, I. Plazl, Laminar to turbulent transition and heat transfer in a microreactor: mathematical modeling and experiments, Ind. Eng. Chem. Res. 47 (2008) 7447–7455.
[22] M. Tišma, B. Zelić, Đ. Vasić-Rački, P. Žnidaršič-Plazl, I. Plazl, Modelling of laccase-catalyzed l-DOPA oxidation in a microreactor, Chem. Eng. J. 149 (2009) 383–388.
[23] A. Pohar, I. Plazl, P. Žnidaršič-Plazl, Lipase-catalyzed synthesis of isoamyl acetate in an ionic liquid/n-heptane two-phase system at the microreactor scale, Lab. Chip. 9 (2009) 3385–3390.
[24] R.C. Reid, J.M. Prausnitz, B.E. Poling, The Properties of Gases and Liquids, fourth ed., McGraw-Hill, New York, 1987.
[25] H. Bohr, A. Kühle, A.H. Sørensen, J. Bohr, Hierarchical organization in aggregates of protein molecules, Z. Phys. 40 (1979) 513–515.
[26] F. van Rantwijk, F. Secundo, R.A. Sheldon, Structure and activity of *Candida antarctica* lipase B in ionic liquids, Green Chem. 8 (2006) 282–286.
[27] R. Goetschel, R. Bar, Formation of mixed crystals in microbial conversion of sterols and steroids, Enzyme Microb. Technol. 14 (1992) 462–469.