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Continuous steroid biotransformations in microchannel reactors

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The use of microchannel reactor based technologies within the scope of bioprocesses as process intensification and production platforms is gaining momentum. Such trend can be ascribed a particular set of characteristics of microchannel reactors, namely the enhanced mass and heat transfer, combined with easier handling and smaller volumes required, as compared to traditional reactors.
In the present work, a continuous production process of 4-cholesten-3-one by the enzymatic oxidation of cholesterol without the formation of any by-product was assessed. The production was carried out within Y-shaped microchannel reactors in an aqueous–organic two-phase system. Substrate was delivered from the organic phase to aqueous phase containing cholesterol oxidase and the product formed partitions back to the organic phase. The aqueous phase was then forced through a plug-flow reactor, containing immobilized catalase. This step aimed at the reduction of hydrogen peroxide formed as a by-product during cholesterol oxidation, to avoid cholesterol oxidase deactivation due to said by-product. This setup was compared with traditional reactors and modes of operation. The results showed that microchannel reactor geometry outperformed traditional stirred tank and plug-flow reactors reaching similar conversion yields at reduced residence time. Coupling the plug-flow reactor containing catalase enabled aqueous phase reuse with maintenance of 30% catalytic activity of cholesterol oxidase while eliminating hydrogen peroxide. A final production of 36 M of cholestenone was reached after 300 hours of operation.

Introduction

Miniaturized devices are gaining widespread use in biocatalysis because this approach contributes to the rationalization of process development with significant reduction in manpower, in the quantity of reagents required and in waste production, concomitantly contributing for a significant cost reduction [1]. These microreactors have been shown to outperform conventional, large-scale vessels operating in batch mode, given the favorable mass and heat transfer characteristics due to large area to volume ratio, and the possibility to operate in continuous mode [2–4]. Recent reviews have compiled current applications of microreactors in biotransformation processes, as well as set trends for future applications [1,5,6].

Regarding the fluid phase composition, enzymatic microreactors can operate in single or multiphase environment. In the latter, two or more phases are separately added to the reactor, typically in cocurrent mode [1]. The single phase encompasses merely the use of, for example, a single aqueous phase, where both substrates and enzymes are fed separately to the microreactor and the reaction takes place over the entire length of the microchannel [7]. Multiphase systems are preferred when the conversion of scarcely water-soluble substrates is envisaged or when the enzymatic resolution of

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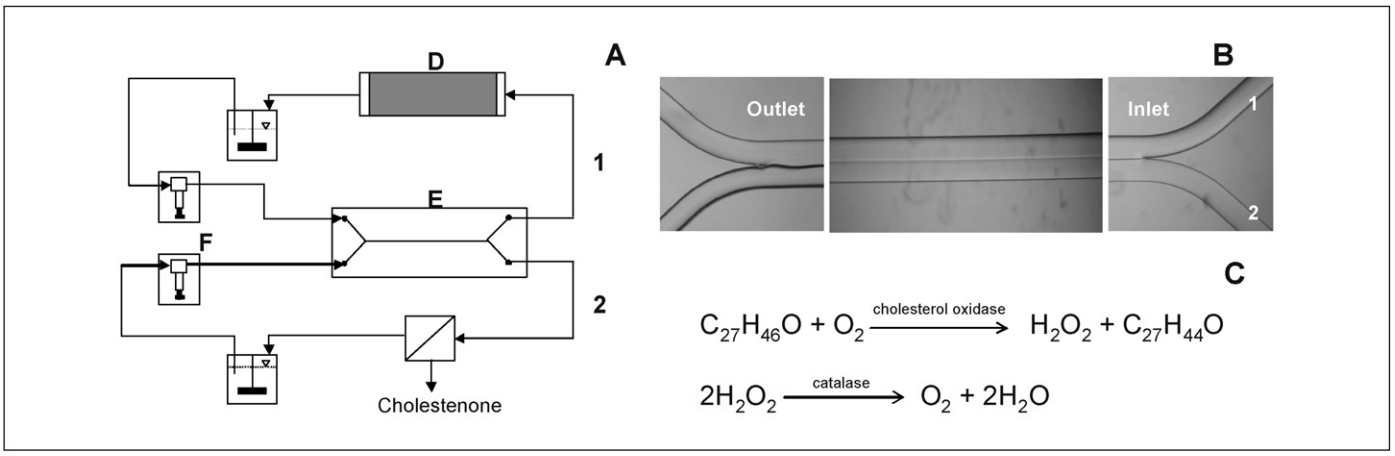

FIGURE 1

(a) Experimental setup. **(d)** Microchannel reactor, **(e)** plug-flow reactor and **(f)** syringe pumps, (i) aqueous phase and (ii) organic phase. **(b)** parallel aqueous–organic two-phase flow obtained along the entire length of the microchannel reactor at a combined flow rate of 14 μL min^{-1}. (1) Aqueous phase (harboring the enzyme) and (2) organic phase (harboring substrates). **(c)** Stoichiometry of the biocatalytic reactions.

chiral compounds is targeted [8–11]. In multiphase systems, proper fluid hydrodynamic must be chosen taking into account both biotransformation yields and downstream operations. If increased volumetric productivity or improved enzyme stability is sought, the use of immobilized enzymes can also be considered. The techniques regularly applied in larger-scale immobilization can also be used in microreactors, with particular focus on adsorption or covalent binding methods, use of cross-linked enzyme aggregates and monolith-type formulations [1].

Within the scope of cholesterol biotransformation, enzyme formulations have been used within the food and pharmaceutical areas. Quantification of blood cholesterol is another well-known application of cholesterol oxidase formulations [12]. Cholesterol oxidase is a bifunctional enzyme that oxidizes Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, while reducing oxygen to hydrogen peroxide [13]. Given such catalytic nature cholesterol oxidase has been advantageously used for the preparative production of intermediate steroids as well as in the optical resolution of allylic alcohols [14,15].

The aim of this study was to set up a continuous microproduction platform based on the bio-oxidation of cholesterol performed in microchannel reactors in a closed system setup (Fig. 1a). This system will account for enzyme reuse as well as for the elimination of the by-product, hydrogen peroxide, that can hinder the industrial application of this specific reaction because it can deactivate cholesterol oxidase [16].

The suitability of carrying out enzymatic cholesterol oxidation in an aqueous–organic two-phase system with defined interface has been previously established [11]. Within this system, the organic phase will act as a reservoir for substrates and products (oxygen, sterol and steroid), whereas the aqueous phase harbors the enzyme. To remove the H_2O_2 formed, a second enzymatic reaction was coupled, using a packed bed reactor (PBR) filled with immobilized catalase.

To the authors' knowledge, this is the first report of such an integrated approach for the continuous bioproduction in microchannel concerning this specific reaction, despite previous reports on chemical synthesis [17] and microextraction after larger-scale production of steroids [18].

Materials and methods

Materials

Cholesterol oxidase and catalase were provided by BBI Enzyme Ltd. (Gwent, UK) and Sigma (St Louis, MO, USA), respectively. Polyethylene glycol (PEG) 600 was from Fluka (Deisenhofen, Germany) and LentiKat® liquid, a PVA-based material was from GeniaLab (Braunschweig, Germany). 4-Cholesten-3-one was purchased from Acros (Geel, Belgium) and progesterone and cholesterol from Sigma (St Louis, MO, USA). All other chemicals used during this work were of analytical or HPLC grade, purchased from assorted suppliers.

Methods

Enzyme immobilization

Catalase and cholesterol oxidase were entrapped in PVA according to the protocol established by Fernandes *et al.* [19]. Briefly, the LentiKat® liquid was heated to about 90°C until complete melting, and then cooled afterwards to 40°C. A volume of 0.4 mL of 1 g L^{-1} enzyme suspension, in 50 mM phosphate buffer pH 7, was added to 8 mL of the LentiKat® liquid and the whole thoroughly mixed under magnetic stirring. The solution formed was extruded into PEG 600 where capsules were formed. The capsules were harvested after a three-hour period, washed with 50 mM phosphate buffer pH 7 and either immediately used or stored at 4°C until use.

Biotransformation runs

Microchannel reactors

Cholesterol biotransformation was carried out in glass microchannel reactors with Y-shaped inflow and outflow channels according to Marques *et al.* [11]. The microchannel reactors (W = 220 μm, H = 100 μm and L = 332 mm) were purchased from Micronit Microfluidics B.V. (Enschede, The Netherlands).

Oxygen-saturated cholesterol solutions in *n*-heptane (μ = 0.3696 Pa s at 30°C) were fed to one inflow and an air saturated 50 mM phosphate buffer pH 7 containing cholesterol oxidase (μ = 0.7978 Pa.s at 30°C) was fed to the other inflow of the Y-shaped microchannel reactor. The concentration of cholesterol at the inlet was 1 g L^{-1} while enzyme concentration was 0.1 mg mL^{-1}. The aqueous phase was supplied at constant flow rate between