Catalysis Today 157 (2010) 315–320

Contents lists available at ScienceDirect

Catalysis Today

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

Development of a continuous steroid biotransformation process and product extraction within microchannel system

P. Žnidaršič-Plazl *, I. Plazl

University of Ljubljana, Faculty of Chemistry and Chemical Technology, Aškerčeva 5, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Available online 18 February 2010

Keywords:
Progesterone
Rhizopus nigricans
Hydroxylation
Optimization
Microchannel

ABSTRACT

Results of an integrated approach for the improvement of progesterone 11α-hydroxylation by *Rhizopus nigricans*, which is one of the key steps in the production of corticosteroid drugs and hormones, are presented. Several issues have been addressed in order to increase process productivity, including biocatalyst immobilization, improvement in the solubility of the lipophilic substrate, setting-up of continuous reaction process and easing downstream processing. After preliminary studies on the effects on *Rhizopus nigricans* morphology, fungal pellets of a desired size and structure could be obtained. The mycelia grown in spherical agglomerates further enabled its continuous use in the process of biotransformation. The problem of low steroid solubility was addressed by the addition of organic solvents or β-cyclodextrin. In order to develop an integrated biotransformation process, a continuous two-liquid phase extraction within microchannel device has been set up, where very efficient steroid extraction was achieved within a few seconds.

1. Introduction

Since historical discovery of anti-inflammatory activity of cortisone in 1949, which together with the findings related to the structure of hormones of the adrenal cortex has provided the basis for the Nobel Prize for medicine in 1950 awarded to E.C. Kendall, T. Reichstein and Ph.S. Hench [1], steroid hormones and their derivatives have been used for a wide range of therapeutic purposes. Beside the established utilization as immunosuppressive, anti-inflammatory, antirheumatic, progestational, diuretic, sedative, anabolic and contraceptive agents, recent applications of steroid compounds include the treatment of some forms of cancer, osteoporosis, HIV infections etc. [2]. Nowadays, steroids represent one of the largest sectors in the pharmaceutical industry with world markets in the region of US$ 10 billion and the production exceeding 1,000 000 tons per year [3,4].

Regarding very limited amounts of their natural sources, a great demand for steroid compounds with biological activity has spurred an intense effort to find cost-effective routes and economical industrial processes for their synthesis. The initial chemical transformation of deoxycholic acid from bile acids to corticosteroids developed for laboratory synthesis at Merck in 1940s required 31 steps, resulted in very low yield and was not economically relevant. Slightly better was another chemical route developed in early 1950s at Syntex, which based on transformation of naturally abundant phytosterol diosgenin. A major chemical problem of both synthetic pathways was the introduction of oxygen into C11 of the steroid ring, which was shown to be mandatory for the biological activity of cortisone [5]. Therefore, a real breakthrough in process development was a patent from 1952 covering 11α-hydroxylation of progesterone (PR) by aerated cultures of the Mucorales fungi from *Rhizopus* species [6], which drastically reduced the number of the required chemical steps in the process and cheapen cortisone on the market by several folds. Several microorganisms that catalyze steroid hydroxylations, hydrogenations, dehydrogenations and the splitting of carbon–carbon bonds were identified already in 1950s and 1960s and several microbial steroid transformations have been introduced into industrial processes, among which hydroxylations at different positions are of highest commercial significance [3]. Half a century later, significant progress in this area continues to be made, mainly in the isolation of new microorganisms capable of performing the required steroid transformation and genetic engineering of currently used strains. Among the most impressive achievements is the total biosynthesis of steroids from simple carbon source accomplished by genetically modified yeast [7].

Abbreviations: β-CD, β-cyclodextrin; DMF, *N,N*-dimethylformamide; DMSO, Dimethyl sulfoxide; HP, 11α-hydroxyprogesterone (11α-hydroxy-4-pregnene-3,20-dione); PR, Progesterone (4-pregnene-3,20-dione).

* Corresponding author.
E-mail address: polona.znidarsic@fkkt.uni-lj.si (P. Žnidaršič-Plazl).

0920-5861/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.cattod.2010.01.042

Moreover, rational design of fermentation media based on by-products or wastes from industry and even on steroid precursors of microbial or plant tissue origins contributes to improved productivity of steroid biotransformations [2]. An engineering approach towards the increased productivity of this processes include biocatalyst immobilization, improvement in the solubility of lipophilic substrates, setting-up of continuous reaction processes and easing downstream processing [2]. All of these issues were addressed in the present work, which summarizes the extensive research towards the integrated process of PR 11α-hydroxylation with filamentous fungus *R. nigricans*.

The preferential use of whole cells over enzymes for this biotransformation is mainly resulting from necessity of co-factor regeneration and problems associated with the isolation of enzyme complex and its *in vitro* regeneration [8,9]. Namely, PR 11α-hydroxylase consists of a microsomal membrane-bound cytochrome P450 and NADPH-cytochrome P450 reductase [8]. While classically immobilized *R. nigricans* cells failed to maintain hydroxylase activity after a few repeated batch processes [10], the use of mycelial pellets as a naturally immobilized biomass was proposed [11]. In order to obtain pellets of a desired size and structure, a part of our research has focused on the morphology of *R. nigricans* during submerged cultivation, which is known to depend on several environmental conditions including the size and type of inoculum, agitation, medium composition, temperature and pH and has to be examined closely for each particular strain [12,13].

The low solubility of steroid compounds in aqueous media has been found as a limiting factor for process productivity [14]. In order to overcome this problem, different organic solvents and β-cyclodextrin (β-CD), which is known to form inclusion complexes with many kinds of organic molecules [15], were added in aqueous transformation media.

Furthermore, a long-term PR biotransformation with a continuous use of *R. nigricans* biomass in the form of pellets and with nutrient supply, where PR was added stepwise at concentrations above aqueous saturation was compared with the previously performed continuous process employing pellets, where the aqueous solution of PR in the presence of β-CD was continuously supplied in a laboratory-scale bioreactor [16].

For the isolation of steroid biotransformation products from the aqueous phase, simple variations of several primary solvent extraction procedures conventionally take place [17]. A continuous extraction of steroids in a microchannel was proposed in our recent work [18], which could replace the generally applied batch separation process. Thereby an integrated system of PR 11α-hydroxylation by *R. nigricans* in the form of pellets could be developed.

2. Material and methods

2.1. Microorganism and growth conditions

The submerged growth of *Rhizopus nigricans* ATCC 6227b, harvested from malt agar slants after 14 days of incubation at 26 °C, was performed in growth medium based on glucose, soya flour, yeast extract, NaCl and K_2HPO_4 as described previously [16]. Erlenmeyer flasks with 100 mL of medium were inoculated with spores, inoculum concentrations varying from 2.5 10^2 to 2.5 10^6 spores/L. Submerged growth of *R. nigricans* was performed on a thermostated rotary shaker at 100 or 225 rpm and at 23 °C. After 48 h, pellets were harvested and photographed and further analyzed for pellet average size as previously described [19].

2.2. Biotransformation in shake-flasks

Pellets from the above-described submerged cultures were filtered from the growth medium and 5 g (wet weight) of mycelia was resuspended in Erlenmeyer flasks containing 100 mL of phosphate buffer with 50 mg/L of deoxycorticosterone (Sigma, St. Louis, USA) as previously described [11]. After induction period of 2.5 h at 28 °C and 100 rpm on a rotary shaker with orbital diameter of 25 mm, mycelia was transferred into 100 mL of phosphate buffer with 10 mg/L or 50 mg/L PR (Merck, Darmstadt, Germany), previously dissolved in 0.3 mL of *N,N*-dimethyl-formamide (DMF, Merck Schuchardt OHG, Hohenbrunn, Germany); in phosphate buffer with 250 mg/L of β-CD (Roquette Frères, Lestrem, France) and 50 mg/L PR; or in 95 mL phosphate buffer, where PR was added dissolved in 5 mL of dimethyl sulfoxide (DMSO, Kemika, Zagreb, Croatia) or DMF to give final PR concentration of 50 mg/L. At the beginning of process, 0.2 g/L of cycloheximide (Sigma, St. Louis, USA) was added in order to inhibit further enzyme synthesis and cultures were incubated at 180 rpm and 28 °C on a rotary shaker. Samples without biomass were taken from the flasks and further analyzed for steroid concentration using HPLC as described previously [11]. All chemicals used were of analytical grade. All the experiments were performed in at least triplicates with standard deviations below 5%.

2.3. Biotransformation in a laboratory-scale bioreactor

For the process with the continuous use of *R. nigricans* biomass and with stepwise PR addition, 60 g (wet weight) of the induced pellets of average diameter of 6.97 ± 1.08 mm was aseptically transferred from shake flasks into a sterilized stirred-tank bioreactor (Bia d.o.o., Ljubljana, Slovenia) with one propeller and geometry described previously [19], containing 1.6 L of transformation buffer and 0.1 L of growth medium described above. PR was added dissolved in DMF (100 mg/mL), initially to give final concentration of 260 mg/L and later on in several additions in various time intervals to give an average feed of 25 mg/h of PR. Furthermore, stepwise aseptic addition of concentrated growth medium (40 g/L glucose, 24 g/L soya flour, 22.8 g/L yeast extract, 16 g/L NaCl and 7.92 g/L K_2HPO_4 in water, pH 5.5) with an average flow rate of 3.5 mL/h was assured. The bioprocess was performed at 28 °C and at 150 rpm for the first 46 h, while afterwards agitation was increased to 210 rpm. A constant air supply was ensured at aeration rate of 1.0 vvm. The working volume was kept constant with deviation up to 0.1 L by means of aseptical sampling. Steroids in samples were analyzed by HPLC as previously described [11].

Another continuous process employing 22.5 g (wet weight) of *R. nigricans* pellets of average diameter of 3.64 ± 0.4 mm with continuous PR supply of 27 mg/h in the presence of β-CD was performed in a laboratory scale bioreactor with 2 L of working volume and with 2 Rushton turbines without the additional supply of growth medium as previously described [11].

2.4. Extraction within microchannel system

The extraction of PR was studied in a microscale device as described previously [18].

2.5. Modelling

2.5.1. Progesterone 11α-hydroxylation process

The rate of 11α-hydroxylation of dissolved PR by the pelleted growth form of *R. nigricans* could be described by Michaelis-Menten kinetic equation [16]

$$r = \frac{V_m S_L}{K_M + S_L} \quad (1)$$

where r is the rate of substrate consumption (mg/L min), V_m is the maximum rate of 11α-hydroxylation (mg/L min), S_L is the concentration of substrate in solution (mg/L) and K_M is Michaelis constant (mg/L).

When PR was used in concentrations above its saturation in aqueous media (11.5 mg/L), the overall biotransformation rate was considered to depend also on the rate of substrate dissolution and the mass balance for a batch process could be described as [16]

$$\frac{dS_L}{dt} = K_1 S_S(S^* - S_L) - r \quad (2)$$

where K_1 is a kinetic rate constant of dissolution (L/mg min), S_S is the concentration of solid substrate (mg/L) and S^* is the solubility of the substrate (mg/L). Eq. (2) includes the convective transport with a linear dependence between solid substrate concentration and effective area for mass transfer [16,20]. The model assumes that diffusion resistance of the liquid–solid interface is negligible and that mass transfer through the mycelium does not affect hydroxylation rate [20].

When water-miscible organic solvents were added to the aqueous media, a second order deactivation of hydroxylase enzyme system was considered [21]:

$$r_i = \frac{V_m S_L}{K_M + S_L}\left(\frac{1}{1 + k_d t}\right) \quad (3)$$

where k_d is a deactivation rate constant (min^{-1}).

Simple mathematical model of a continuous process of PR 11α-hydroxylation in a stirred tank bioreactor was developed to describe the process, where β-CD was used to enhance steroid solubility in aqueous transformation media [22]

$$\frac{dS_L}{dt} = \frac{S_F - S_L}{\tau} - r, \quad (4)$$

$$\frac{dP}{dt} = -\frac{P}{\tau} + \beta r \quad (5)$$

where S_F is the concentration of substrate in feeding solution (mg/L) and τ is residence time (min) and β is the ratio between molecular masses of HP and PR.

2.6. Product extraction within microchannel system

In order to analyze and optimize the continuous ethyl acetate extraction of 11α-hydroxyprogesterone (HP) in microstructured devices, a 3D mathematical model with convection and diffusion terms was developed considering the velocity profile for laminar flow of two parallel phases in a microchannel at steady-state conditions. The transfer of HP across the water–organic interphase was described by the diffusion and based on the assumption of equilibrium relation between the concentration of steroid in water and ethyl acetate at the interface, while the continuity of flux on both sides of the interface was considered as a boundary condition at the ethyl acetate-side interface [18].

3. Results and discussion

3.1. Immobilization of biocatalyst

According to our previous findings on *R. nigricans* morphology [11,23], very low inoculum concentrations were used in order to obtain pelleted morphological form of this filamentous fungus. This growth form may be considered as naturally immobilized biomass, where there is no additional mass transfer resistance to the surface of agglomerates as it is in the case of encapsulation or entrapment of cells. A slower rate of 11α-hydroxylation of PR by alginate-immobilized *Aspergillus ochraceus* mycelium compared to free cells was also found to be a result of poorer access of the PR through the hydrophilic gel matrix [24]. Furthermore, there is no need for extra chemicals, time and equipment for immobilization, so the rationalization of this step by using natural aggregation of the mycelium is obvious.

As seen in Fig. 1, we were able to obtain pellets of a relatively uniform size and structure. Lower inoculum concentration resulted in larger pellets and *vice versa*. Final pellet size was significantly influenced also by the agitation intensity. While at 100 rpm final pellet diameter varied from 3.4 ± 0.8 to 32.5 ± 3.2 mm, a much more vigorous shaking of 225 rpm resulted in pellets with diameters from 1.6 ± 0.3 to 13.9 ± 1.6 mm. The inverse proportional relationship between energy input and final pellet diameter was established also when *R. nigricans* was grown in a stirred-tank bioreactor [19]. The differences in sizes were accompanied also with huge variations in pellet structure – while pellets grown at 100 rpm were very fluffy and with "hairy" surface, the others obtained at 225 rpm were very compact and smooth because they were exposed to much higher shear forces. Furthermore, higher number of pellets was developed from the same inoculum size at vigorous agitation. This was a consequence of the fragmentation of pieces of hyphae from outer pellet surface, resulting in hyphal fragments, which might serve as new centers for biomass growth [13].

Since membrane-associated hydroxylase complex in *R. nigricans* is inducible and the induction was shown to be greatly

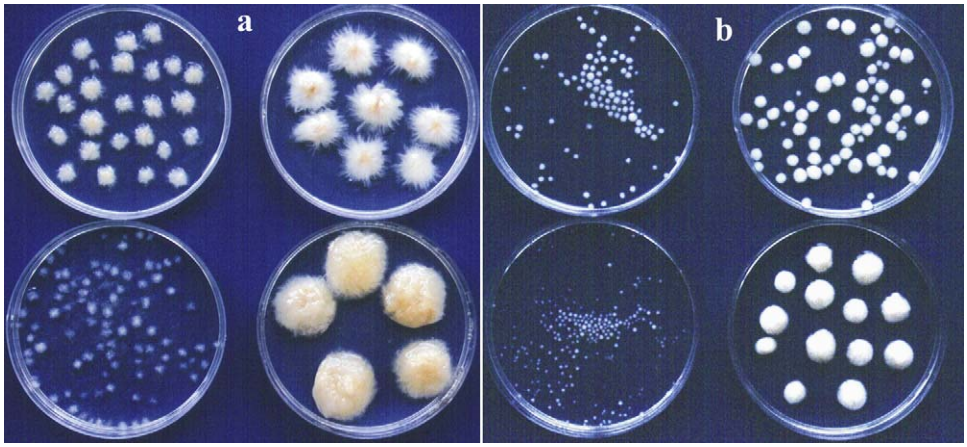

Fig. 1. *R. nigricans* pellets, obtained in shake-flasks at various inoculum concentrations and shaking frequency of (a) 100 rpm and (b) 225 rpm. The same position of Petri dish in both figures represents pellets from cultures with identical inoculum, which was the highest in the cultures at the left bottom and decreased clockwise.

Table 1
The comparison of maximum rate of PR 11α-hydroxylation normalized on biomass dry weight with pellets of different size and structure at PR concentration below saturation. Kinetic constants were determined on the basis of the best fit of model using Eq. (1) with experimental data.

Pellet diam. (mm)	Pellet structure	V_m (mg/$g_{dry\ weight}$ h)
7.22 ± 0.9	Smooth	6.15
9.85 ± 1.3	Fluffy	9.78

dependent on the physiological state of the mycelium [11,25], the morphology of *R. nigricans* was supposed to influence the inducement and consequently the rate of PR 11α-hydroxylation. Moreover, intraparticle diffusion into a spherical pellet depends on its porosity and density, which again differ among agglomerates of different structures [26]. For this purpose, mycelial pellets of various sizes and structures were induced at the same conditions and further tested for biotransformation. The increase in specific hydroxylation rate correlated with lower diameters and thus larger specific surface area of agglomerates, harvested from the intensive growth phase of batch cultivation, which could be contributed to higher enzyme concentration per biomass [11,22].

Furthermore, pellets with fluffier surface expressed an increased biotransformation capability compared to smooth agglomerates, which again shows a relationship with larger outer surface exposed to aqueous media with steroids (Table 1). Since transport limitations restrain inducing molecules to reach pellet interior, the inducement of hydroxylation system predominantly takes place in outer layers of pellets, which also contain more RNA and thereby have higher capability for protein synthesis compared to older hyphae in inner layers of mycelial agglomerates, where the limitation of nutrients causes cell lysis [22,26]. However, smaller and fluffier pellets were less favorable for the use in repeated batch [11] or continuous process [22], which indicated that compromises between optimal inducibility of mycelium and stability of biocatalyst are necessary.

3.2. Solving the problem of low steroid solubility

In order to solve the main problem of steroid biotransformations, which is the low water solubility of these organic compounds resulting in non-bioavailability of substrate, different organic solvents or solubilizing agent were added. The influence of the replacement of a part of water with DMSO or DMF on PR 11α-hydroxylation kinetics was evaluated. Results were compared with biotransformations performed in the presence of β-CD or in aqueous transformation media with PR concentration below or above saturation (Fig. 2). From the time course of PR biotransformation with *R. nigricans* pellets at initial PR concentration of 10 mg/L, which is below its water solubility, kinetic parameters V_m = 1.037 mg/L min and K_M = 8.09 mg/L were evaluated according to the proposed enzyme kinetic model (Eq. (1)) [16]. When PR was transformed in the same media at concentrations above its saturation, the slow transport of substrate between the solid and aqueous phase lowered the global reaction rate, which was confirmed by the agreement of experimental data and model containing dissolution term (Eq. (2)) with previously defined dissolution rate constant K_1 = 0.005 L/mg min [17].

As evident from Fig. 2, the addition of 5 vol % of DMSO improved the biotransformation rate, while the same amount of DMF in aqueous media had detrimental effect on investigated reaction. In both cases, experimental data were in good agreement with the model based on the second order enzyme deactivation (Eq. (3)) and the obtained deactivation rate constant were 0.012 and 0.09 min^{-1} for DMSO and DMF, respectively. Higher deactivation constant for DMF correlates with findings on much lower maximum membrane concentration of DMSO compared to DMF in *R. nigricans*, which

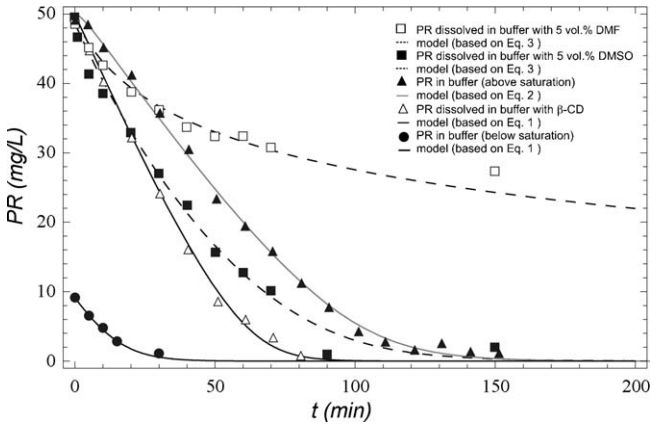

Fig. 2. Progesterone biotransformation with *R. nigricans* pellets obtained at identical growth conditions in aqueous transformation media with or without the presence of different solvents or solubilizing agent.

was together with high log P value exposed as the most important physicochemical property for the retention of biocatalyst activity in organic solvents [27]. The toxic effect of organic solvent on microbial cells could arise from both direct contact of cells with the solvent fraction and from the interactions of the cells with the solvent fraction dissolved in the aqueous phase [28]. Furthermore, recent studies suggested that organic solvents have a potential to behave as competitive inhibitors of P450 s [29]. It was also shown that the alginate immobilization of *Aspergillus ochraceus* did not protect the cells sufficiently for prolonged PR hydroxylations in different aqueous-organic solvent systems, as the cells ceased hydroxylation within the first hour of reaction [24].

On the other hand, the best results of biotransformation were obtained using β-CD in amounts previously shown to give water-soluble complex with steroid substrate at used concentrations [16]. As evident from Fig. 2, β-CD addition resulted in nearly two-fold faster process and the experimental values corresponded very well to the model with previously defined parameters for biotransformation without solid substrate present (Eq. (1)).

3.3. Biotransformations with the continuous use of biomass

The growth of *R. nigricans* in a pelleted morphological form enabled the continuous use of biomass in the biotransformation process. Initial experiments were performed in aqueous media with initial PR concentrations above its water saturation. The results of a laboratory-scale process performed in a stirred tank bioreactor with stepwise addition of PR giving the average feed of 25 mg/h, while maintaining constant working volume by sampling are presented in Fig. 3a. As evident, steady state conditions were reached after approximately 60 h of process with considerable amount of unreacted PR. Despite stepwise nutrient supply, which was found to be beneficial for PR biotransformation with *R. nigricans* pellets [30] due to the need for preservation of general metabolism required for NADPH regeneration, as well as for maintaining microbial slow growth, the biomass concentration after 98 h of bioprocess was 10% lower as at the beginning, while average pellet diameter changed from 6.97 ± 1.08 to 5.20 ± 0.99 mm, indicating partial autolysis of the mycelia, as well as pellet fragmentation.

On the other hand, very efficient steroid conversion with almost complete PR conversion was set up with smaller pellets, using β-CD in order to dissolve PR in the initial transformation media in laboratory-scale reactor, as well as in feeding solution (Fig. 3b). The process was also successfully described by the model without dissolution term (Eq. (4)) with previously defined kinetic parameters for the biotransformation [22].

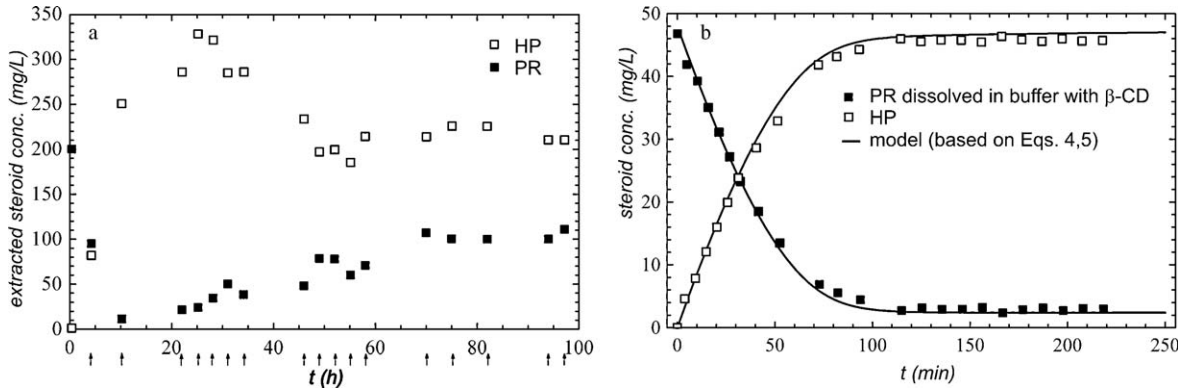

Fig. 3. Time-courses of biotransformation processes with the continuous use of *R. nigricans* pellets with (a) stepwise addition of PR above saturation at indicated time intervals and with nutrients supply and (b) the continuous feed of PR in aqueous solution with β-CD (Fig. 3b adapted with permission from reference [22]).

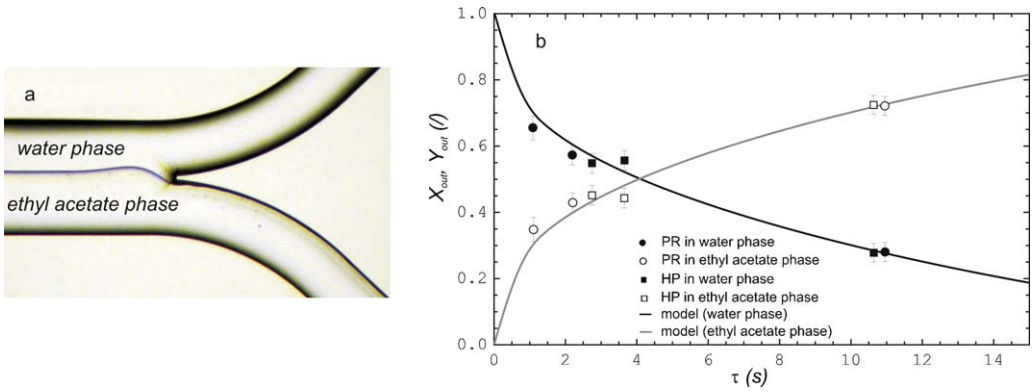

Fig. 4. (a) The efficient phase separation at stable laminar flow with the aqueous-organic interface formed in the middle of the microchannel at the exit of the microchannel device. (b) The experimental values and mathematical model simulations of average dimensionless concentrations of PR and HP in both phases at the outlet of the microchannel at different flow velocities. (Adapted with permission from reference [18]).

3.4. Steroid extraction in a microchannel system

Further process development included steroid extraction in a continuously operated microchannel system. Microreaction technology has impressively demonstrated the advantages for chemical processes as a new concept in chemical engineering, mainly due to the very large surface-to-volume ratio, enhanced heat and mass transfer efficiency, product quality and safety, as well as opening the possibilities for new process technologies and new product syntheses [31]. The experiments performed in custom-made glass microchannels of 220 μm width, 50 μm height and 332 mm length revealed that very efficient transfer of PR and HP from water into ethyl acetate phase was possible within a few seconds and that adjustment of fluid flow rates enabled phase separation at the y-shaped exit of the microchannel device (Fig. 4) [18]. Moreover, a developed mathematical model agreed very well with experimental data, which enables us to further optimise the extraction in a microchannel device. For increasing the capacity of the system, a numbering-up approach by using parallel microreactors was proposed [18].

4. Conclusions

The determination of conditions of *R. nigricans* submerged growth leading to the formation of pellets further enabled the set up of an efficient continuous operation of PR 11α-hydroxylation. β-CD was found as the best additive to achieve higher concentrations of soluble substrate and also higher biotransformation rates. Furthermore, very efficient continuous steroid extraction was carried out in a pressure driven flow microchannel system. As the end goal of this research, a continuous biotransformation process with integrated removal of product in the organic phase within continuously operated microchannel system, and recycling of β-CD with aqueous phase is envisaged. Thereby an integrated system of PR 11α-hydroxylation by *R. nigricans* in the form of pellets could be developed.

Conflicts of interest

The authors have not declared any conflict of interest.

Acknowledgements

This work was supported through Grant P2-0191, provided by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia. We are especially grateful to Dr. U. Roglič and M. Milosević, B.Sc., for their contribution to this work, and to Dr. M. Lakner for his help in numerical analysis.

References

[1] R.P. Rubin, A brief history of great discoveries in pharmacology: in celebration of the centennial anniversary of the Founding of the American Society of Pharmacology and Experimental Therapeutics, Pharmacol. Rev. 59 (2007) 289–359.
[2] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.M.S. Cabral, Microbial conversion of steroid compounds: recent developments, Enzyme Microb. Technol. 32 (2003) 688–705.
[3] M. Bureik, R. Bernhardt, Steroid Hydroxylation: Microbial Steroid Biotranformations Using Cytochrome P450 Enzymes, in: R.D. Schmid, V.B. Urlacher (Eds.), Modern biooxidation: enzymes, reactions and applications., Wiley-VCH Verlag GmbH & Co, KGaA, Weinheim, 2007, pp. 155–176.
[4] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Industrial biocatalysis today and tomorrow, Nature 409 (2001) 258–268.

[5] K.E. Smith, F. Ahmed, T. Antoniou, Microbial transformations of steroids, Biochem. Soc. Trans. 21 (1993) 1077–1080.
[6] H.C. Murray, D.H. Peterson, Oxygenation of steroids by Mucorales fungi, US. Pat. (1952), 2 602 769 (Upjohn Company, USA).
[7] F.M. Szczebara, C. Chandelier, C. Villeret, A. Masurel, S. Bourot, C. Duport, et al., Total biosynthesis of hydrocortisone from a simple carbon source in yeast, Nat. Biotechnol. 21 (2003) 143–149.
[8] T. Makovec, K. Breskvar, Purification and characterization of NADPH-cytochrome P450 reductase from filamentous fungus *Rhizopus nigricans*, Arch. Biochem. Biophys. 357 (1998) 310–316.
[9] D.F. Broad, S. Pontin, P. Dunnil, Studies for the large scale isolation of membrane-associated progesterone 11α-hydroxylase: enzyme stability in *Rhizopus nigricans* ATCC 6227b cells, Enzyme Microb. Technol. 9 (1986) 546–548.
[10] I.S. Maddox, P. Dunnill, M.D. Lilly, Use of immobilized cells of *Rhizopus nigricans* for the 11α-hydroxylation of progesterone, Biotechnol. Bioeng. 23 (1981) 345–354.
[11] P. Žnidaršič, R. Komel, A. Pavko, Studies of a pelleted growth form of *Rhizopus nigricans* as a biocatalyst for progesterone 11α-hydroxylation, J. Biotechnol. 60 (1998) 207–216.
[12] M. Papagianni, Fungal morphology and metabolite production in submerged mycelial processes, Biotechnol. Adv. 22 (2004) 189–259.
[13] P. Žnidaršič, A. Pavko, The morphology of filamentous fungi in submerged cultivation as a bioprocess parameter, Food Technol. Biotechnol. 39 (2001) 237–252.
[14] B. Angelova, H.-P. Schmauder, Lipophilic compounds in biotechnology - interactions with cells and technological problems, J. Biotechnol. 67 (1999) 13–32.
[15] J. Szejtli, Cyclodextrin Technology, Kluwer Academic Publisher, Dordrecht, 1988.
[16] U. Roglič, P. Žnidaršič-Plazl, I. Plazl, The influence of β-cyclodextrin on the kinetics of progesterone transformation by *Rhizopus nigricans*, Biocatal. Biotransform. 23 (2005) 299–305.
[17] T.L. Miller, Steroid fermentations, in: H.W. Blanch, S. Drew, D.I.C. Wang (Eds.), Comprehensive Biotechnology, Vol.3, Pergamon Press, Oxford, 1985, pp. 297–318.
[18] P. Žnidaršič-Plazl, I. Plazl, Steroid extraction in a microchannel system–mathematical modelling and experiments, Lab Chip 7 (2007) 883–889.
[19] P. Žnidaršič-Plazl, The influence of some engineering variables upon the morphology of *Rhizopus nigricans* in a stirred tank reactor, Chem. Biochem. Eng. Q. 20 (2006) 275–280.
[20] K.-C. Chen, H.-C. Wey, Dissolution – enzyme kinetics of 11β-hydroxylation of cortexolone by *Curvularia lunata*, Enzyme Microb. Technol. 12 (1990) 616–621.
[21] O. Levenspiel, Chemical reaction engineering, 3rd ed, John Wiley & Sons, Ltd, New York, 1999.
[22] U. Roglič, I. Plazl, P. Žnidaršič-Plazl, Batch and continuous transformation of progesterone by *Rhizopus nigricans* pellets in the presence of β-cyclodextrin, Biocatal. Biotransform. 25 (2007) 16–23.
[23] P. Žnidaršič, R. Komel, A. Pavko, Influence of some environmental factors on *Rhizopus nigricans* submerged growth in the form of pellets, World J. Microb. Biot. 16 (2000) 589–593.
[24] E.G. Ceen, J.P.R. Herrmann, P. Dunnill, Solvent damage during immobilised cell catalysis and its avoidance: studies of 11α-hydroxylation of progesterone by *Aspergillus ochraceus*, Appl. Microbiol. Biotechnol. 25 (1987) 491–494.
[25] T. Hudnik-Plevnik, B. Črešnar, Factors affecting the induction of 11α-hydroxylase of progesterone in the filamentous fungus *Rhizopus nigricans*, J. Steroid Biochem. Molec. Biol. 35 (1990) 749–751.
[26] J. Nielsen, M. Carlsen, Fungal pellets, in: R.G. Willaert, G.V. Baron, L. De Backer (Eds.), Immobilised living cell systems: modelling and experimental methods, John Wiley & Sons, Ltd, Chichester, 1996, pp. 273–293.
[27] S.J. Osborne, J. Leaver, M.K. Turner, P. Dunnill, Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane, Enzyme Microb. Technol. 12 (1990) 281–291.
[28] R. Bar, in: C. Laane, J. Tramper, M.D. Lilly (Eds.), Biocatalysis in organic media, Elsevier Science Publishers B.V, Amsterdam, 1987, pp. 147–153.
[29] R. Vappugalla, S.-Y. Chang, H. Zhang, P.H. Marathe, D.A. Rodrigues, Effect of commonly used organic solvents on the kinetics of cytochrome P450 2B6- and 2C8-dependent activity in human liver microsomes, Drug Metab. Dispos. 35 (2007) 1990–1995.
[30] P. Žnidaršič, K. Uršič, A. Pavko, Studies on supporting media for progesterone 11α-hydroxylation with a pelleted growth form of *Rhizopus nigricans*, Acta Chim. Slov. 48 (2001) 533–544.
[31] K. Jähnisch, V. Hessel, H. Löwe, M. Baerns, Chemistry in microstructured reactors, Angew. Chem. Int. Ed. 43 (2004) 406–446.