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Biotransformation with whole microbial systems in a continuous flow reactor: resolution of (*RS*)-flurbiprofen using *Aspergillus oryzae* by direct esterification with ethanol in organic solvent

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ABSTRACT

Cell-bound lipases of dry mycelium of *Aspergillus oryzae* were used in organic solvent for the resolution of racemic flurbiprofen by direct esterification with ethanol in a flow-chemistry reactor. Under flow conditions a significant reduction of the reaction time and an increase of the enantioselectivity were achieved compared to the batch mode. Moreover, the process was implemented by adding an in-line purification step integrated with the racemization of the unreacted flurbiprofen directly into a polymer-supported resin.

Introduction

Carboxylesterases (mostly lipases) have been used in monophasic organic solution under controlled values of water activity (a_w) for catalyzing ester formation; the strategies more frequently employed for shifting the equilibrium toward ester formation imply the use of interesterification or *trans*esterification, because direct esterification is often hampered by water formation, which negatively influences the equilibrium.[1] The use of cell-bound enzymes of different microorganisms has proved an effective method for direct esterification of different alcohols and carboxylic acids in organic solvent.[2] Dry whole mycelia of filamentous eumycetes can be directly used as biocatalyst, showing few advantages, such as: high stability in organic solvents, high resistance to the inactivation due to free carboxylic acids (including acetic acid), and high molar conversions enabled by favorable partition of water.[3] Previous observations suggested that the mycelium contributes marginally to the water take-up: rather than sequestrating water inside the cell wall, it seems that the mycelium provides a micro-environment where the water produced during the esterification is promptly removed.[4] Furthermore, mycelial microorganisms can be employed without immobilization, because their morphological structure allows for easy filtration and re-utilization; this last feature favors simple set-up of continuous bioreactors.

Direct esterification of racemic alcohols or carboxylic acids with dry mycelia of strains of *Aspergillus oryzae* and *Rhizopus oryzae* often resulted in an efficient kinetic resolution.[5] For example, *A. oryzae* has been used in pure organic solvent for the resolution of (*RS*)-flurbiprofen,[6] displaying good enantioselectivity toward (*R*)-flurbiprofen and furnishing results competitive with the data obtained using commercial enzymes.[7] However, the biotransformations showed limited productivity and space time yield due to substrate inhibition effects and low solubility of flurbiprofen in the solvents where the best results, in terms of activity and enantioselectivity, were observed (e.g. aliphatic hydrocarbons).[6] Kinetic resolution of racemic flurbiprofen is attractive because (*S*)-flurbiprofen is a nonsteroidal anti-inflammatory drug, whereas its *R*-enantiomer shows anticancer effects in vivo and in vitro.[8] Moreover, as recently reviewed by Kourist and co-workers,[9] despite the considerable number of biocatalytic routes developed so far for the production of (*S*)-profens, there is still room for improvement with the perspective of more efficient and sustainable processes.

Flow reactors can dramatically improve the performances of lipase-catalyzed reactions.[10] Recently, the kinetic resolution of flurbiprofen has been performed in a continuous flow reactor using an

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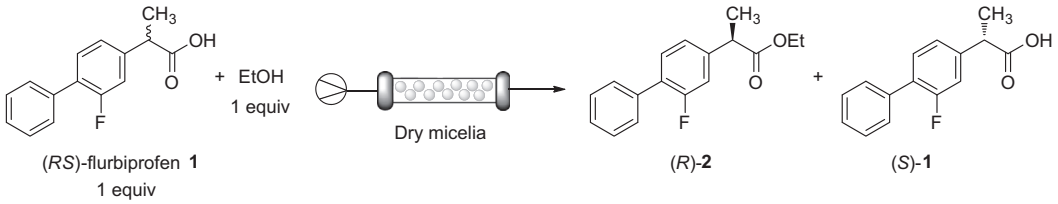


Scheme 1. Esterification of (*RS*)-flurbiprofen **1** with EtOH using mycelium of *Aspergillus oryzae* MIM in a continuous flow reactor.

Table 1
Screening of different flow rates in the enantioselective esterification of (*RS*)-flurbiprofen **1** with EtOH using *Aspergillus oryzae* MIM in a continuous flow reactor

Entry	Time (min)	Flow rate (μl/min)	Molar conversion[a] (%)	ee_s[b] (%)	ee_p[b] (%)	*E*[a]	*r*[c] (μmol/min g)
Batch	1440	—	20	22	86	16	0.14
1	15	46	58	90	66	18	1.48
2	8	85	45	66	82	19	2.12
3	6.5	105	28	34	88	21	1.63
4	5	137	19	20	88	19	1.44

Reaction conditions: 10 mM solution of (*RS*)-flurbiprofen in *n*-heptane, 1 equiv of EtOH, 180 mg of lyophilized mycelium of *Aspergillus oryzae* MIM, *T* = 50 °C.
[a] Calculated according to Ref. 17.
[b] Determined by chiral HPLC.[14]
[c] Calculated according to Ref. 18.

immobilized lipase B from *Candida antarctica* (Novozym 435®) allowing a significant reduction of the reaction time compared to the classical batch method.[11]

In the present work, we have studied the use of dry mycelium of *A. oryzae*[12] in an organic solvent for the resolution of racemic flurbiprofen in a continuous flow reactor, combining the advantages of an easy to produce (and use) biocatalyst with a process-intensification technology. The process was implemented by adding an in-line purification step integrated with the racemization of the unreacted flurbiprofen directly into a polymer-supported resin.

Results and discussion

The esterification reaction catalyzed by dry mycelia of *A. oryzae* was firstly performed in a batch mode using stoichiometric amounts of racemic flurbiprofen and ethanol in *n*-heptane at 50 °C.[13] The progress and stereobias of the reaction was monitored by chiral HPLC,[14] which showed that after 8 h the enantiomeric excess of (*S*)-flurbiprofen **1** was 22%, the enantiomeric excess of the product (*R*)-flurbiprofen ethyl ester **2** was 86%, and the conversion was 20%.

Flow experiments were initially focused on reaching a degree of conversion similar to the one obtained in the batch process.[15] To this aim, a 10 mM solution of (*RS*)-flurbiprofen **1** in *n*-heptane containing 1 equiv of EtOH was made to flow through a glass column loaded with dry mycelium of *A. oryzae* MIM (180 mg, Scheme 1). Temperature was kept constant at 50 °C, while flow rate, which sets the residence time, was varied and the best results are reported in (Table 1).[16]

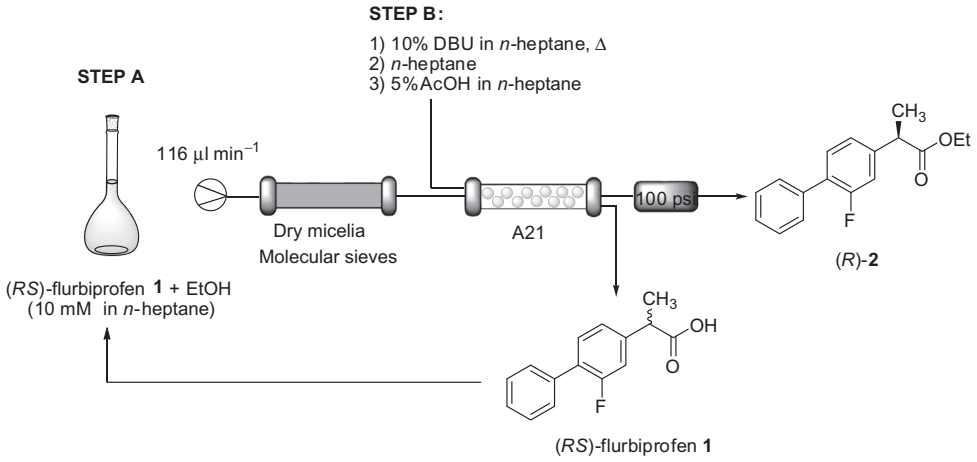
The data reported in Table 1 indicate that the use of a flow reactor dramatically reduced the reaction time and slightly increased the enantioselectivity. In fact, a 19% conversion was reached in only 5 min of residence time with 88% enantiomeric excess (ee) of the product (Table 1, entry 4). On the other hand, a residence time of 15 min (entry 1) resulted in a marked increase of the conversion (58%) allowing, in this case, the obtainment of (*S*)-flurbiprofen with a good enantiomeric excess (90% ee). Therefore, by simply modulating the flow rate, optically enriched substrate [(*S*)-flurbiprofen, entry 1] or product [(*R*)-flurbiprofen ethyl ester, entries 3 and 4] could be obtained.

The specific reaction rate (*r*) of the batch reaction was 0.14 μmol/min g, whereas in the flow process, using the conditions reported in Table 1, entry 4, which provided a similar degree of conversion of the batch reaction, the specific reaction rate was 1.44 μmol/min g, that means an increase of the productivity of about 10 times. It must be noted that the resolution is obtained through direct esterification with formation of water, which can affect the equilibrium of the reaction and, consequently, the overall stereoselectivity. Therefore, experiments in the presence of molecular sieves were carried out. For this purpose, we prepared a mixed bed column filled with an equal weight of lyophilized mycelium of *A. oryzae* MIM and molecular sieves. Keeping constant the temper-

Table 2
Screening of different temperatures in the enantioselective esterification of (*RS*)-flurbiprofen with EtOH using *Aspergillus oryzae* MIM in the presence of molecular sieves in a continuous flow reactor

Entry	*T* (°C)	Molar conversion[a] (%)	ee_s[b] (%)	ee_p[b] (%)	*E*[a]	*r*[c] (μmol/min g)
1	50	38	54	88	26	2.45
2	30	30	38	88	22	1.93
3	40	32	42	88	23	2.06
4	60	41	62	90	35	2.64
5	70	42	60	84	21	2.70

Reaction conditions: 10 mM solution of (*RS*)-flurbiprofen in *n*-heptane, 1 equiv of EtOH, 180 mg of lyophilized mycelium of *Aspergillus oryzae* MIM, and 180 mg of molecular sieves (powder, 4 Å). Residence time: 6.5 min; flow rate 116 μL/min.
[a] Calculated according to Ref. 17.
[b] Determined by chiral HPLC.[14]
[c] Calculated according to Ref. 18.



Scheme 2. Schematic representation of the overall process.

ature and the residence time at 6.5 min, it was possible to observe an increase of the molar conversion from 28% (Table 1, entry 3) to 38% (Table 2, entry 1). Different temperatures were then tested (Table 2, entries 2–5). A slight increase of both the molar conversion and ee of the product was achieved working at 60 °C (Table 2, entry 4). No significant differences in the conversion or in the ee of the product were observed by increasing the equivalents of EtOH.

The addition of molecular sieves generally increased the specific reaction rates and enantioselectivity; under the best conditions (Table 2, entry 4), the specific rate was 19 times higher than the one registered in the batch mode and the enantiomeric ratio (*E*) was increased from 16 to 35.

Moreover, the stability of the lyophilized mycelia was evaluated. The thermal stability was already demonstrated by us in a previous work,[3a] whereas the mycelia stability over continuous work was checked by performing the resolution under the conditions reported in Table 2 entry 4, and running the reactor for 3 h. Samples were collected and analyzed every hour and the matching of the results obtained over the time was verified both in terms of conversion and enantiomeric purity of substrate and product.

As previously described,[11] the overall process can be implemented by adding an in-line purification step of the exiting solution, consisting in a catch and release protocol, which allows the easy separation and recovery of both (*S*)-flurbiprofen and (*R*)-flurbiprofen ethyl ester. Following the reported procedure, the unreacted carboxylic acid was trapped by flowing the exiting solution into a column containing the polymer-supported base Amberlyst A21 (Scheme 2). To further improve the process, the racemization of the trapped (*S*)-flurbiprofen **1** was included. The complete racemization was achieved by filling the column with a 10% solution of DBU in *n*-heptane (prepared through dilution of the DBU charged in one injection loop with the *n*-heptane flow stream) and, after stopping the flow stream, heating the column at 115 °C for 1 h (lower concentrations of DBU did not allow the complete racemization of the trapped acid). The column was then washed with *n*-heptane for 10 min at a total flow rate of 300 μL/min, and subsequently, flurbiprofen was released with a 5% solution of AcOH in *n*-heptane and analyzed by chiral HPLC.

Conclusion

Dry mycelia of *A. oryzae* can be effectively used for enantioselective esterification of racemic flurbiprofen in organic solvent in a flow reactor. As previously observed in the kinetic resolution of flurbiprofen using the commercially available immobilized lipase B from *Candida antarctica*,[11] the use of a continuous flow reactor allows a significant reduction of the reaction time compared to the classical batch method and dramatically improved the productivity of the batch biotransformation, with beneficial effects also on the enantioselectivity. The procedure here reported appears to be notably simple because dry mycelium can be directly used in the continuous reactor without immobilization. To the best of our knowledge, this is the first application of microbial whole cells as an enantioselective biocatalyst in a continuous flow reactor.

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12. *Aspergillus oryzae* MIM was maintained on malt extract (8 g/L, agar 15 g/L, pH 5.5), cultivated in 500 mL-Erlenmeyer flasks containing 100 mL medium and incubated for 48 h at 28 °C on a reciprocal shaker (100 rpm). Liquid media contained a basic medium (yeast extract 1 g/L, $(NH_4)_2SO_4$ 5 g/L, K_2HPO_4 1 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, pH 5.8) supplemented with Tween 80 (0.5%). Suspensions of spores (1.6×104) were used as inocula. Mycelia grown for 48 h in

submerged cultures were harvested by filtration at 4 °C and resuspended in deionized water, homogenized for 5 min (Silverson L2R, Silverson Machines, Waterside, UK), frozen at −20 °C, and finally lyophilized (Alfa Criosec, Milan, Italy) at plate temperature of 25 °C.

13. *Resolution of (RS)-flurbiprofen in batch:* The dry mycelium of *Aspergillus oryzae* MIM (30 mg/mL) was suspended in 6.0 mL of *n*-heptane. Equimolar concentrations (10 mM) of racemic flurbiprofen (14.6 mg, 0.06 mmol) and ethanol (2.88 mg, 0.06 mmol) were added to the suspension and the reaction mixture was maintained in a thermostated bath at 50 °C under constant magnetic stirring for 8 h. The biotransformation medium was then centrifuged and mycelium removed; the organic phase was treated with a NaOH aqueous solution (4 mL, 0.1 N) and the organic extract washed with water (4 mL), dried over Na_2SO_4, and evaporated to give flurbiprofen ethyl ester (3.3 mg, 20% conversion, ee_p = 86%); the alkaline solution was brought to pH 1 with HCl (0.5 N) and extracted twice with EtOAc and the resulting organic phase was dried over Na_2SO_4 and evaporated to give unreacted flurbiprofen (11.7 mg, ee_s = 22%). The reaction was followed by HPLC. Withdrawals of 100 μL were taken from the reaction mixture, filtered, evaporated, re-dissolved in acetonitrile (100 μL) and analyzed by chiral HPLC.
14. HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975 (wavelength: 254 nm). Column: Lux Amylose-2, 4.60 mm i.d. × 150 mm, Phenomenex. Eluent: acetonitrile/water/formic acid (1:1:0.2, v/v/v). Flow: 1.0 mL/min. (*R*)-flurbiprofen 1: retention time 4.3 min; (*S*)-flurbiprofen 1: retention time 5.7 min; (*R*)-flurbiprofen ethyl ester 2: retention time 14.4 min; (*S*)-flurbiprofen ethyl ester 2: retention time 15.9 min.
15. The continuous flow biotransformations were performed using a R2+/R4 flow reactor commercially available from Vapourtec equipped with Omnifit glass column (6.6 mm i.d. × 100 mm length).
16. *Resolution of (RS)-flurbiprofen in flow:* Racemic flurbiprofen 1 (14.6 mg, 0.06 mmol) and EtOH (1 equiv) were dissolved in *n*-heptane (6 mL) and the solution was flowed through a glass column (6.6 mm i.d. × 100 mm length) filled with lyophilized cells of *Aspergillus oryzae* MIM (180 mg). When molecular sieves were used (Table 2), a mixed bed column was prepared using 180 mg of lyophilized cells of *Aspergillus oryzae* MIM and 180 mg of molecular sieves (powder, 4 Å). A 100 psi back-pressure regulator was applied to the system. Temperature and flow rate were varied and their values are reported in Table 1 and Table 2 After collecting a total volume of 8 mL, the reaction outcome was analyzed by chiral HPLC. To this aim, a sample of 200 μL was withdrawn, evaporated, and re-dissolved in acetonitrile (150 μL).

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