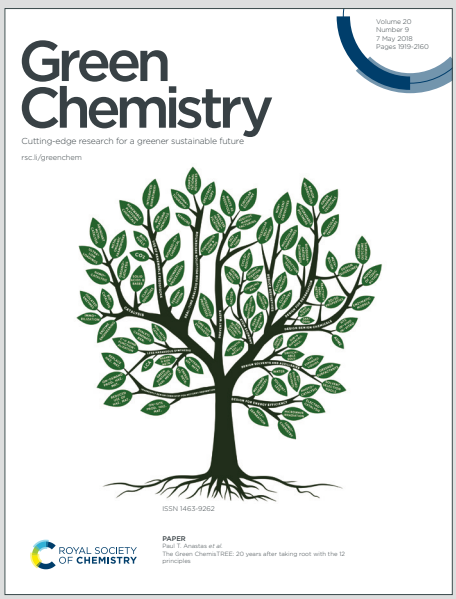
View Article Online
View Journal

Green Chemistry

Cutting-edge research for a greener sustainable future

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: F. Annunziata, A. Guaglio, P. Conti, L. Tamborini and R. Gandolfi, *Green Chem.*, 2021, DOI: 10.1039/D1GC03786B.



View Article Online
DOI: 10.1039/D1GC03786B

ARTICLE

Continuous-flow stereoselective reduction of prochiral ketones in a whole cell bioreactor with natural deep eutectic solvents

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Francesca Annunziata,[a] Alessandra Guaglio,[a] Paola Conti,[a] Lucia Tamborini*[a] and Raffaella Gandolfi[a]

Immobilized whole cells of *Rhodotorula rubra* MIM147 were used in a packed bed flow reactor for the enantioselective reduction of β-ketonitriles and for the efficient production of a key building block for the synthesis of the antidepressant drug duloxetine. A choline chloride-glucose natural deep eutectic solvent (NADES) was employed with a dual function, as a co-solvent and as a source of glucose, fundamental for the cofactor regeneration. To develop a fully automated protocol, an in-line purification procedure was also developed. Firstly, an in-line liquid-liquid extraction of the desired β-hydroxynitriles was performed with a flow stream of ethyl acetate, then the unreacted ketone was trapped by a polymer-supported benzylamine. The optimized protocol allowed the obtainment of (*S*)-β-hydroxynitriles in 80 minutes with >95% conversion and excellent e.e. (96-99%).

Introduction

Biocatalyzed redox reactions have received increased attention in the last decades, due to the mild operational conditions and the high enzyme selectivity.[1] Alcohol dehydrogenases (ADHs, EC.1.1.1.x) represent a sustainable alternative to non-enzymatic methods for the production of optically active alcohols from prochiral ketones.[2-4] However, this class of biocatalysts relies on expensive cofactors [e.g., NAD(P)+] that require an efficient recycling system usually obtained by coupling a second enzyme (e.g., glucose dehydrogenase and formate dehydrogenase) to achieve an economic and feasible redox transformation. As an alternative, whole-cells offer some unique advantages over the use of crude, purified, or immobilized enzyme preparations. First, the presence of native metabolic pathways, as well as endogenous cofactors, can make redox processes self-sufficient. Moreover, lower costs are required for whole-cell catalyst production in comparison with isolated enzymes, which are obtained after, at least in part, cost-intensive purification steps.[5-7] To further reduce the environmental impact of the processes, the use of natural deep eutectic solvents (NADESs) as green co-solvents in enzymatic transformations has recently gained attention.[8-12] NADESs are defined as a mixture of two or more natural and degradable compounds, generally plant primary metabolites (i.e., alcohols, amines, amino acids, carboxylic acids, sugar or choline derivatives), that, if combined in specific molar ratios, interact via intermolecular forces, often strong hydrogen bonds, and produce an eutectic mixture with the melting point much lower than that of either of individual components.[13] Noteworthy, compared to ionic liquids (ILs) and traditional metal-based Deep Eutectic Solvents (DESs), NADES systems are generally considered to be less toxic and more environmentally friendly.[14,15]

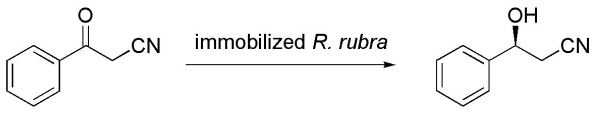
In this context, we focused on the development of bioreductions for the synthesis of enantiopure alcohols, using an immobilized whole cell biocatalyst and an aqueous-NADES mixture in a continuous flow environment.[16-18] In particular, we selected *Rhodotorula rubra,* which was reported to be a valid biocatalyst for the enantioselective reduction of different substituted β-ketonitriles with good conversion and excellent enantiomeric excess,[19] to obtain β-hydroxynitriles, which are important building blocks for the synthesis of different drugs such as fluoxetine, duloxetine, ezetimibe, atorvastatin, atomoxetine, and selective inhibitors of the serotonin and norepinephrine reuptake.[20] Although it is known that *Rhodotorula* species can cause localized infections or fungemia in patients with severe underlying illnesses and compromized host defense,[21,22] they are the object of increasing interest, due to their huge biotechnological potential and several examples of their use for food pharma applications have been reported.[23,24]

Results and discussion

R. rubra MIM147 whole cells were prepared following a slightly modified protocol previously reported by us.[19] In view of developing a continuous flow approach, the cells were immobilized. The whole cell immobilization should keep the cells in a viable state, allowing the stabilization of their catalytic efficiency and enabling their repeated use. Among the several methods,[25] one of the most used techniques is based on the formation of stable porous gels based on ionotropic gelation of water soluble polyelectrolytes, such as alginate, carrageenan, chitosan, with oppositely charged ions.[26,27] Therefore, we first immobilized *R. rubra* MIM147 whole cells in alginate beads, a form of immobilization which already proved to be suitable for

[a] Department of Pharmaceutical Sciences (DISFARM), University of Milan, Via Mangiagalli 25, 20133 Milan, Italy. E-mail: lucia.tamborini@unimi.it
†Electronic supplementary information (ESI) available. See DOI:

a continuous flow application thanks to the good stability over the time and after subsequent cycles of biotransformations.[28-30] 3-Oxo-3-phenylpropanenitrile **1a** (Scheme 1) was first used as model substrate to compare the activity and stability of the free and immobilized biocatalyst in a batch mode.



3-oxo-3-phenylpropanenitrile, **1a** (*S*)-**2a**

Scheme 1. Reduction of model compound **1a** using immobilized *R. rubra* MIM147.

To this aim, 12 mg/mL of free cells (dry weight) corresponding to 50 OD_{530nm} were suspended in a physiological solution containing glucose (50 mg/mL) and 2 mg/mL of substrate, previously solubilized in DMSO (10% v/v). *R. rubra* MIM147 was immobilized in sodium alginate from brown algae (2% w/v) and used in a batch biotransformation. After 24 h, the biocatalyst was recovered and reused under the same conditions. Already after the first cycle of biotransformation, the molar conversion obtained with the free cells resulted to be much lower than the one of the immobilized cells (68% *vs* 88%, respectively). After 3 cycles of repeated batch reactions, the conversion with the free cells was less than 10%, whereas the immobilized cells still gave about 55% of molar conversion, indicating that the immobilization matrix increases the stability of the biocatalyst (Figure 1).

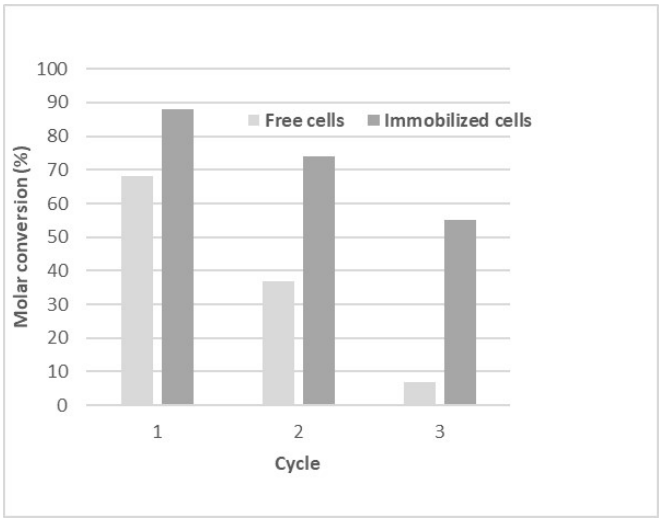


Figure 1. Molar conversions (after 24 h) obtained with 12 mg/mL of free cells in 2 mL of physiological solution and 12 mg/mL of immobilized cells in 2 mL of 2% w/v alginate from brown algae. Cells were grown in YPD liquid medium for 72 h at 28 °C.

To select the most resistant immobilization matrix for a continuous flow application and further increase the activity and stability of the biocatalyst, other matrices with different types of sodium alginate were tested: i) low density (LD) alginate from *Macrocystis pyrifera*; ii) low density (LD) alginate from brown algae; iii) medium density (MD) alginate from brown algae. The biotransformations were carried out under the same conditions as reported above immobilizing the cells in 2% of alginate. The molar conversions were similar to those obtained with the matrix prepared with 2% alginate from brown algae (i.e., 28% after 3 h of biotransformation), with the exception of those prepared with alginate from *Macrocystis pyrifera,* which gave a slightly better performance (i.e., 35% after 3 h of biotransformation) and thus was selected for our studies. To improve the resistance of the beads, we increased the concentration of the alginate from 2% w/v to 6% w/v.

We then immobilized in the selected matrix the cells of *R. rubra* MIM147 grown for 48 h, 72 h and 96 h in YPD liquid medium. Those grown for 48 h gave the best molar conversion (i.e., 58% after 3 h of biotransformation) and were thus selected for the next batch and continuous flow reactions.

A Multisimplex approach was exploited for optimization, considering substrate concentration, buffer pH, and immobilized cells concentration as control parameters;[31] conversion, determined by HPLC, was the response variable. Finally, batch biotransformations were carried out using 26 $mg/mL_{alginate}$ of cells at 30 °C in 10 mL vial using 1 mg/mL substrate dissolved in Tris-HCl buffer (0.1 M, pH 7.0, 2 mL) and DMSO (10% v/v) with 2 mL of alginate beads. Glucose (50 mg/mL) was added to the mixture for cofactor recycling. Optimized batch conditions gave (*S*)-**2a** with 93% molar conversion and ee = 99% after 3 h. Notably, as previously reported, no by-product formation was observed.[19,32]

Immobilized *R. rubra* MIM147 cells were re-used in repeated batch reduction of compound **1a**, in order to evaluate the operational stability. To this aim, the beads were filtered from the reaction mixture, washed with deionized water and re-used under the same conditions every 24 h. The molar conversion was evaluated after 3 h of biotransformation. The immobilized biocatalyst resulted to be very stable, being used for 14 reaction cycles, even if a decrease of the reaction rate was observed particularly from the 9th cycle, where conversions after 3 h are below 80% (Figure 2). However, it must be noted that full conversion was again achieved after 24 h of biotransformation. In order to avoid the use of DMSO as co-solvent and to increase the sustainability of the reaction, we decided to evaluate the use of a NADES as co-solvent.[33,34] In particular, we selected a choline chloride : glucose (ChCl : Glc, 1.5 : 1 mol/mol) NADES. ChCl is a popular choice as hydrogen bond acceptor because it is a low cost, non-toxic, biocompatible, and biodegradable reagent which could be produced from natural sources through a very high economy process. On the other hand, glucose serve both as a hydrogen bond donor and for cofactor regeneration.[9,35-38] The molar ratio between choline chloride and glucose was selected in order to have a significant amount of glucose for cofactor recycling without the disadvantage of using a viscous and dense NADES at higher glucose concentrations, which would make it difficult to use in a flow environment. The NADES was prepared through a straightforward waste-free process by simply mixing solid ChCl and Glc at 75 °C for 3 h to form a viscous liquid that was used in the batch biotransformations (10% v/v).

View Article Online
DOI: 10.1039/D1GC03786B

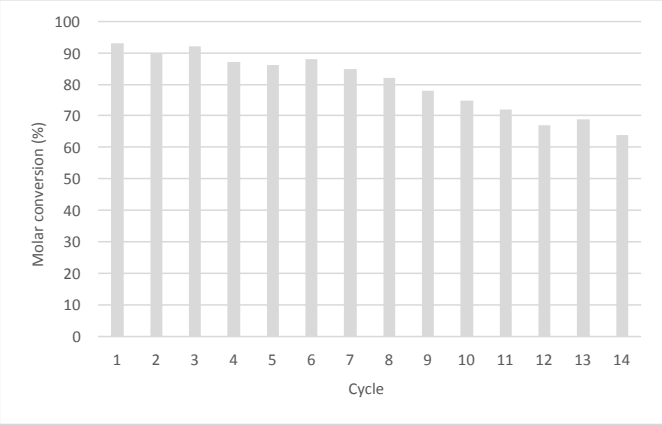


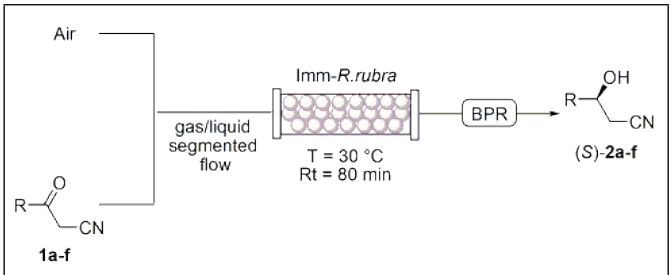
Figure 2. Molar conversions after 3 h of biotransformation in repeated batch with immobilized cells using DMSO as co-solvent.

The use of this less toxic co-solvent, instead of DMSO, also speeds up the biotransformation, enabling a molar conversion of 99% after 3 h. To evaluate the operational stability in the presence of NADES, we set up a repeated batch process (14 cycles), and again we found that the immobilized cells were very stable. For each batch cycle, the substrate solution was replaced with a fresh one, guaranteeing optimal pH conditions (pH = 7) and the presence of an adequate amount of glucose for cofactor regeneration.

Moved by these promising results, we tested the bioreduction in a continuous flow environment. Freshly prepared alginate beads were packed into a glass column and washed with a Tris-HCl buffer flow stream. However, a constant increase of the pressure was observed during the washing, leading to a partial disintegration of the beads at the bottom and at the top of the reactor, close to the frits. This issue was easily overcome inserting glass beads at the two ends of the column. Considering the preliminary optimization performed in batch, we decided to work in Tris-HCl buffer at 30 °C and we evaluated the effects on the conversion of residence time, pressurization and oxygen supply, using again β-ketonitrile **1a** as substrate. Oxygen, which, in the batch biotransformations, is in the head-space of the vials, takes part in the regeneration of cofactors involved in the biotransformation, therefore an efficient strategy to guarantee a suitable oxygen supply in the aqueous phase was necessary. First, a solution of compound **1a** (1 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.0) and ChCl : Glc NADES (10% v/v) was flowed through the packed bed reactor. The residence time was set at 60 min and we evaluated if the oxygen dissolved in the buffer by bubbling it in the stock solution was enough to guarantee good results. Under these conditions, the biotransformation only reached 40% of conversion. This poor result was ascribed to lack of available oxygen in the flow stream. So, a segmented gas-liquid flow was applied:[39-41] the gas phase (air, delivered at a constant flow rate through a peristaltic pump) and the liquid phase merged in a T-junction, thus generating air-liquid segments in the flow stream entering the packed bed reactor. The system was pressurized at 20 psi. In this way, the conversion reached 80% in 60 min of residence time. To achieve full conversion, we further increased the residence time to 80 min. We then evaluated the stability of the system under continuous work, but, unfortunately, the pressure of the system started increasing after 18 h of continuous work, leading to a blockage for overpressure after 24 h (set P_{max} = 8 bar). When the immobilized cells were removed from the column, we noticed that a partial breakup of the beads occurred. For this reason, we decided to evaluate a different matrix for immobilization. In particular, we consider a mixture polyvinyl alcohol (PVA)/alginate a good option because PVA, which is a non-toxic, and cheap synthetic polymer with strong mechanical properties, makes the beads more deformable,[42,43] suitable to better tolerate the pressure inside the reactor. We prepared the matrix for immobilization using 12% w/v and 15% w/v PVA and 1% w/v alginate. The molar conversions obtained after 3 h of batch biotransformation with NADES as co-solvent were comparable, in the range of 51 54%. Since the matrix prepared with 15% w/v PVA was too viscous and made the immobilization difficult, we chose the one prepared with 12% w/v PVA, which allowed the obtainment of stable reusable beads with better thermal, mechanical and chemical stability.[44-46] In order to obtain higher molar conversions, the cell concentration was set at 30 mg/mL, obtaining a molar conversion of 96% in 3 h of batch biotransformation using substrate **1a**. This immobilization protocol gave quantitative yield in terms of cell loading into the beads: in fact, after the immobilization, no activity was found in the supernatant. Moreover, to determine the activity recovery (AR (%) = activity immobilized cells/activity free cells), the activity of immobilized and free cells under the same batch conditions was evaluated. After 60 min, the obtained AR was 53%. Before moving to flow, the operational stability was tested by reusing the cells every 24 h: up to the 10th cycle of biotransformation, a molar conversion between 96% and 99% was obtained after 3 h, then a 21% reduction in biocatalyst performance was observed at the 14th cycle.

The PVA/alginate beads (12 mL) were packed into a glass column and a solution of the substrate, prepared as described above, was passed through it. Full conversion of substrate **1a** was obtained again after 80 min of residence time. No increase of the pressure was observed. Specific productivity (*SP*) under flow condition was about 1.6 times the one obtained in batch (SP_f = 0.94 μmol/min*g vs SP_b = 0.60 μmol/min*g). Different substrates (**1b-1f**) were then tested, obtaining in all the cases conversions > 95% and excellent e.e. (Table 1).

View Article Online DOI: 10.1039/D1GC03786B



Substrate	R	m.c. (%)[a]	e.e. (%)[b]	SP_f (µmol/min*g)[c]
1a	Ph	100	99	0.94
1b	*p*-CH_3Ph	100	97	0.86
1c	*p*-CF_3Ph	96	96	0.62
1d	*p*-OCH_3Ph	100	99	0.78
1e	*p*-FPh	97	96	0.82
1f	thiophen-2-yl	95	99	0.86

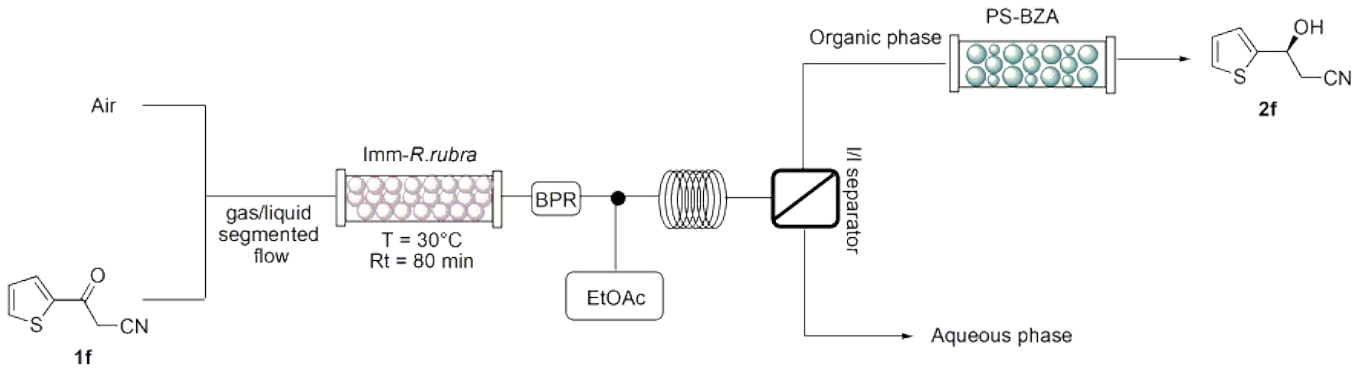
Table 1. Molar conversion (m.c.) and enantiomeric excess obtained under optimized conditions. [a]Molar conversion determined by HPLC analysis (**1a**) or by ^{1}H NMR.[47] [b]E.e. determined by chiral HPLC. [c]Specific productivity (*SP*) for flow biotransformation (SP_f) was calculated from the concentration of the formed product ([P] expressed as µmol/mL), the flow rate of the liquid phase (f expressed as mL/min), and the mass of the biocatalyst expressed as weight of the cells employed (m expressed as g), according to the following equation: SP_f = [P]·f/m (µmol/min*g).[48] For details, see materials and methods.

In the case of 3-oxo-3-(thiophen-2-yl)propanenitrile **1f**, whose reduction product is the valuable precursor of the antidepressant drug duloxetine, the system was left running for 120 h. The biocatalyst showed excellent performance; in fact, samples were collected every 12 h and a constant conversion (95%) was observed. After that time, the bioreactor was washed with Tris-HCl buffer, the system was stopped, the cells were recovered from the column and used for a batch biotransformation, obtaining again 97% conversion of substrate **1a** after 3 h of biotransformation. Under optimized flow conditions, turnover number was about 2.8 fold the batch one (1.07 $g_{product}/g_{cells}$ vs 0.38 $g_{product}/g_{cells}$) and the flow bioreactor productivity (PBR = 12 mL) was 78 $mg_{product}/day$. Finally, with the aim of developing a fully automated procedure for the synthesis of compound **2f**, an in-line purification procedure has been developed (Scheme 2). In fact, continuous integration for in situ product removal can significantly contribute to process intensification, together with miniaturization, in line process analytics, and cascade reactions.[41,49,50] First, an in-line liquid/liquid extraction of the desired product was performed adding a flow stream of EtOAc followed by a separation with a Zaiput l/l separator. The organic stream containing the unreacted substrate and the desired product was further purified by flowing it through a column packed with polymer supported benzylamine (PS-BZA), able to trap the ketone **1f** by forming the corresponding imine, allowing the recovery of the pure final product **2f** after simple solvent evaporation.

Experimental

Materials and methods.

Reagents and solvents were obtained from commercial suppliers and were used without further purification. For cell immobilization, the following alginic acids, purchased from Merck, were used: alginic acid sodium salt from brown algae suitable for immobilization of microorganisms, alginic acid sodium salt from brown algae low and medium viscosity and alginic acid sodium from *Macrocystis pyrifera* (low viscosity). Continuous flow biotransformations were performed using a R2$^+$/R4 or a Series E Vapourtec flow reactor equipped with an Omnifit® glass column (15 mm i.d. × 150 mm length). The temperature sensor sits on the wall of the reactors. Pressure was controlled by using a 20 psi back-pressure regulator. For the in-line extraction, an additional HPLC pump (ThalesNano) was used. In-line liquid/liquid separations were performed using a Zaiput separator. MS analyses were performed by using a Thermo Finnigan (MA, USA) LCQ Advantage system MS spectrometer with an electronspray ionization source and an 'Ion Trap' mass analyser. The MS spectra were obtained by direct infusion of a sample solution in MeOH under ionization, ESI positive**.** Biocatalyzed reactions were monitored by HPLC analysis with a Merck-Hitachi L-7100 instrument equipped with



Scheme 2. Representation of the final reactor configuration for the biocatalyzed enantioselective reduction of 3-oxo-3-(thiophen-2-yl)propanenitrile **1f** and in-line purification. Substrate solution: 1 mg/mL in Tris-HCl buffer (0.1 M, pH 7.0) and 10% (v/v) ChCl:Glc (1.5:1) NADES. Back pressure regulator (BPR): 20 psi.

Detector UV6000LP and reverse phase column (Waters' Spherisorb ODS2) or chiral column (OJ-H Chiralcel). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian Mercury 300 MHz. Chemical shifts (in ppm) were referred to the residual hydrogen/carbon solvent peaks. FTIR spectra were collected by using a Perkin Elmer (MA, USA) FTIR Spectrometer 'Spectrum One' in a spectral region between 4000 and 450 cm^{-1} and analyzed by transmittance technique with 32 scansions and 4 cm^{-1} resolution. Turnover number (TTN) is defined as the amount of the product per amount of the biocatalyst over the biocatalyst lifetime.[49]

***Rhodotorula rubra* MIM147: culture conditions.** *Rhodotorula rubra* MIM147 was routinely maintained on YPD medium slants (18 g/L agar, 10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, pH = 5.6). The strain, grown on YPD medium slants for 72 h at 28 °C, was inoculated in a 100 mL Erlenmeyer flask containing 20 mL of the same liquid medium (OD_{530nm} = 2) and incubated on a reciprocal shaker (150 rpm) for 48 h at 28 °C. Two more passages in liquid medium were made over the next few days, leaving the microorganism to grow for 48 h each time. Then, a pre-inoculation was carried out, leaving the microorganism to grow for 24 h and then inoculating it into 50 mL of liquid medium in a 500 mL Erlenmeyer flask ($OD_{530nm=}$ 5). The microorganism was incubated on a reciprocal shaker (150 rpm) for 48 h at 28 °C before testing.

Cell growth evaluation. Cell growth was evaluated by UV/Vis analysis, using optical density as a parameter, and by evaluation of cell's dry weight. Different dilutions of the test sample (1:10, 1:100 and 1:500) were analyzed using a spectrophotometer at 530 nm (UV/Vis Jasco V-530). For the evaluation of dry weight, 50 mL of broth culture were centrifuged, the pellet was washed three times with tap water and re-suspended in deionized water. The cell suspension was placed in an oven for 12 h at 104 °C.

Preparation of alginate – PVA/alginate matrix. Alginate matrix was obtained by mixing low density sodium alginate from *Microcystis pyriferia* (6% w/v) in deionized water previously heated at 60 °C. For the preparation of PVA/alginate matrix, PVA (12% w/v) was dissolved in deionized water previously heated at 80 °C under magnetic stirring. Sodium alginate (1% w/v) was then added. The immobilization solution was then cooled to room temperature.

Immobilization procedure. Cells obtained by centrifugation (3500 rpm for 5 min) of the culture broth (50 mL) were washed two times with tap water and then re-suspended in a certain volume of immobilization matrix to obtain a desired concentration cells. The mixture was dropped through a 0.7 mm diameter syringe needle into a cross-linking solution containing $CaCl_2$ (2% w/v) under stirring to make alginate beads (30 min) or containing H_3BO_3 (5% w/v) and $CaCl_2$ (2% w/v) under stirred conditions to make PVA/alginate beads (50 min). After washing with deionized water, the PVA/alginate beads were placed in a 0.5 M Na_2SO_4 solution for about 30 min. At the end, the beads were filtered and washed with deionized water before being used (average beads diameter: 2.0-2.2 mm). To rule out a possible negative effect on cell activity of H_3BO_3, a biotransformation with free cells in the immobilization solution containing H_3BO_3 5% (w/v) and $CaCl_2$ 2% (w/v) was carried out, obtaining a molar conversion of 93% after 3 h.

Preparation of choline chloride : glucose NADES. A mixture of choline chloride : glucose (molar ratio: 1.5 : 1) was placed in a round bottle flask and heated at 75 °C under magnetic stirring till a clear liquid was formed (3 h). The obtained viscous liquid was used in the batch and flow biotransformations.

General procedure for the batch/repeated batch biotransformation using free cells. Fresh cells from sub-merged cultures were centrifuged (3500 rpm for 5 min) and washed with tap water before using. The biotransformation was carried out in 10 mL screw-capped test tubes re-suspending *R. rubra* cells in physiological solution (9 g/L NaCl) at twice the concentration of the sub-merged culture (12 mg/mL). Glucose (50 mg/mL) and the substrate (2 mg/mL) dissolved in DMSO (10% v/v) were added to 2 mL of the suspension. The reaction mixture was magnetically stirred at 28 °C. In repeated batch biotransformations, the suspension was centrifuged and the cells washed twice with water. The product was extracted with ethyl acetate (3 times), dried with Na_2SO_4 and the solvent was removed under vacuum. In order to determine the molar conversion of the product, samples were analyzed using HPLC Water 600E by Waters Coorporation equipped with Waters' Spherisorb ODS2 reverse phase column, eluent: water:MeCN:TFA= 60:40:0.1, flow rate = 1.0 mL/min and λ=210 nm; room temperature; rt: **1a** = 8.0 min, **2a** = 5.0 min.

General procedure for the batch/repeated batch biotransformation using immobilized cells. A solution of the substrate (1 mg/mL) was prepared in a mixture of Tris-HCl (0.1 M, pH = 7) and DMSO (10% v/v) and glucose (50 mg/mL) was added. As an alternative, DMSO and glucose were replaced with the ChCl:Glc NADES (10% v/v). 2 mL of immobilized cells beads were placed in a vial and then 2 mL of the starting solution containing the substrate was added. The mixture was kept under gentle magnetic stirring at 30 °C. After 1 h, 2 h, 3 h and 24 h, 100 µL samples were withdrawn from the reaction mixture, and extracted with ethyl acetate (100 µL). The molar conversion was determined by HPLC. At the end of each cycle, the beads were filtered from the reaction mixture, washed with deionized water and re-used under the same conditions. The molar conversion was determined by HPLC.

General procedure for the flow reduction of β-ketonitriles using immobilized whole cells of *R. rubra*.

PVA/alginate beads (12 mL) were packed into a glass column (i.d. 15 mm). A stock solution of the β-ketonitrile (1 mg/mL of substrate in Tris-HCl pH 7 0.1 M, 10% v/v NADES) was prepared. The mixture was sonicated (5 min) to completely dissolve the substrate. The solution was pumped using an HPLC pump at 50 µL/min, mixed with an air inlet delivered using a peristaltic pump at 100 µL/min, and flowed through the packed bed reactor maintained at 30 °C. A 20 psi back-pressure regulator (BPR) was applied to the system. After the residence time (80 min), a sample of the exiting flow stream was collected and analyzed. Molar conversion was determined by reverse phase HPLC analysis (**1a**) or by ^{1}H NMR[47] and e.e. by chiral HPLC analysis. Chiral HPLC condition: eluent hexane: 2-propanol = 90:10, flow=1 mL/min, λ= 216 nm; room temperature. Compounds: **2a** rt (*S*) = 24.6 min, (*R*) = 30.9 min; **2b** rt (*S*) = 20.2 min, (*R*) = 22.9 min; **2c** rt (*S*) = 15.3 min, (*R*) = 17.8 min; **2d** rt (*S*) = 40.3 min, (*R*) = 42.2 min; **2e** rt (*S*) = 20.4 min, (*R*) = 24.8 min; **2f** rt (*S*) = 28.9 min, (*R*) = 33.0 min.

Synthesis of (*S*)-3-hydroxy-3-(thiophen-2-yl)propanenitrile (*S*)-2f and in-line purification.

A solution of 3-oxo-3-(thiophen-2-yl)propanenitrile **1f** was prepared (1 mg/mL in Tris-HCl pH 7 0.1 M, 10% v/v NADES, total volume: 400 mL) and flowed through the packed bed reactor (12 mL, T = 30 °C) as described in the general procedure (flow rate of the substrate solution: 50 µL/min, air inlet: 100 µL/min, BPR = 20 psi). The exiting flow stream was extracted in-line by adding an inlet of EtOAc pumped with an external HPLC pump at 50 µL/min. The outlet flow stream was directed to a liquid/liquid separator and the organic was directed into a column packed with polymer supported benzylamine (loading 20 mg/g, 1 g). The collected organic phase was dried over anhydrous Na_2SO_4 and the solvent evaporated. The desired compound was isolated in 90% yield and 99% e.e.

^{1}H-NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 2.85 (d, *J* = 5.9 Hz, 2H), 5.22-5.30 (m, 1H), 6.95-7.02 (m, 1H), 7.05-7.10 (m, 1H), 7.28-7.32 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 28.2, 66.2, 117.0, 124.7, 125.8, 127.1, 144. 5 ppm. IR m = 3429, 2922, 2254, 1413, 1040, 850, 709 cm^{-1}. MS (ESI) of C_7H_7NOS (m/z): calc. 153.2, found 175.8 [M^+Na^+]. HPLC analysis: Waters' Spherisorb ODS2 reverse phase column, eluent: water:MeCN:TFA= 60:40:0.1, flow rate = 1.0 mL/min and λ=210 nm; room temperature; rt: **1f** = 4.0 min, **2f** = 6.0 min.

Conclusions

The transfer of the catalytic efficiency shown by biocatalysts in nature to chemical processes is a key element to implement the sustainability of a reaction. However, the mere use of enzymes does not provide, *per se*, a sustainable process. Powerful synergies can be created when environmentally friendly media such as water and deep eutectic solvents are used and when biocatalysis is combined with a continuous flow approach. This combination is particularly attractive for developing greener synthetic protocols, in which biocatalyst stability is increased and the reuse is simplified. In the present work, immobilization of *R. rubra* whole cells using PVA/alginate beads, beyond the advantages of easier work up and potential catalyst reusability, was found to be an excellent strategy for improving the recyclability of the biocatalyst with respect to the use of cell free systems. The ChCl:Glc NADES resulted to be very efficient as both a co-solvent and efficient system to recycle the cofactor necessary to the reaction and is a greener alternative to DMSO. As further improvement, the enantioselective reduction of β-ketonitriles **1a-f** was performed under continuous-flow conditions exploiting a segmented air-water flow regime, which ensured high mass transfer between the gaseous and the liquid phase. The system resulted to be very stable; in fact, no decrease in the reaction outcome was observed after 5 days of continuous work. Notably, the immobilized whole cells recovered from the packed bed reactor were still highly performing when used in a batch biotransformation. Finally, a fully automated protocol including an in-line purification procedure was developed for the flow production of (*S*)-3-hydroxy-3-(thiophen-2-yl)propanenitrile **2f**, an intermediate useful for the synthesis of the antidepressant drug duloxetine.

Author Contributions

Conceptualization, L.T. and R.G.; methodology, F.A. and A.G.; investigation, F.A. and A.G.; resources, L.T. and R.G.; data curation, F.A. A.G. and P.C.; writing—original draft preparation, L.T., P.C. and R.G.; writing—review and editing, all authors; project administration, L.T. and R.G. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

References

1 D. Monti, G. Ottolina, G. Carrea and S. Riva, *Chem. Rev*., 2011, **111**, 4111.
2 G. W. Huisman, F. Hollmann, I. W. C. E. Arends and D. Holtmann, *Green Chem*., 2011, **13**, 2285.
3 R. Kratzer, J. M. Woodley and B. Nidetzky, *Biotechnol. Adv*., 2015, **33**, 1641
4 Y.-G. Zheng, H.-H. Yin, D.-F. Yu, X. Chen, X.-L. Tang, X.-J. Zhang, Y.-P. Zue, Y.-J. Wang and Z.-Q. Liu, *Appl. Microbiol. Biotechnol*., 2017, **101**, 987.
5 C. C. de Carvalho, *Microb. Biotechnol*., 2017, **10**, 250–263.
6 B. Lin and Y. Tao, *Microb. Cell Factories*, 2017, **16**, 106.
7 C. C. C. R. de Carvalho and M. M. R. da Fonseca, *Compr. Biotechnol*., 2nd edn, 2011, vol. 2, pp. 451–460.
8 P. Xu, G.-W. Zheng, M.-H. Zong, N. Li and W.-Y. Lou, *Bioresour. Bioprocess*, 2017, **4**, 34.
9 Á. Mourelle-Insua, I. Lavandera and V. Gotor-Fernández, *Green Chem*., 2019, **21**, 2946.
10 P. Xu, G.-W. Zheng, M.-H. Zong, N. Li and W.-Y. Lou, *Bioresour Bioprocess.*, 2017, **4**, 34.
11 M. Pätzold, S. Siebenhaller, S. Kara, A. Liese, C. Syldatk, and D. Holtmann, *Trends Biotech.*, 2019, **37**, 943.
12 Z. Maugeri and P. Domínguez de María, *ChemCatChem*, 2014, **6**, 1535-1537
13 Y. Liu, J. Brent Friesen, J. B. McAlpine, D. C. Lankin, S.-N. Chen, and G. F. Pauli, *J. Nat. Prod*., 2018, **81**, 679.
14 C. E. Paul and V. Gotor-Fernández, *J. Biotechnol.*, 2019, **293**, 24.
15 A. Paiva, R. Craveiro, I. Aroso, M. Martins, R. L. Reis, and A. R. C. Duarte, *ACS Sustainable Chem. Eng*., 2014, **2**, 1063.
16 L. Tamborini, P. Fernandes, F. Paradisi and F. Molinari, *Trends Biotechnol*. 2018, **36**, 73-88.
17 A. Pinto, M. L. Contente and L. Tamborini, *Curr. Opin. Green Sustain. Chem*., 2020, **25**, 100343.
18 N. Adebar, A. Nastke and H. Gröger, *React. Chem. Eng*., 2021, **6**, 977.
19 I. Rimoldi, G. Facchetti, D. Nava, M. L. Contente and R. Gandolfi, *Tetrahedron: Asymmetry*, 2016, **27**, 389–396.
20 P. Soni and U. C. Banerjee, *Appl. Microbiol. Biotechnol.* 2005, **67**, 771–777.
21 F. Wirth and Luciano Z. Goldani, *Interdiscip. Perspect. Infect. Dis*., 2012, Article ID 465717.
22 J. Zajc, C. Gostinčar, A. Černoša and N. Gunde-Cimerman, *Genes,* 2019, **10**, 42.
23 W. Tang, Y. Wang, J. Zhang, Y. Cai and Z. He, *J. Microbiol. Biotechnol*. 2019, **29**, 507–517.
24 R. Sharma and G. Ghoshal, *Biotechnol. Rep*., 2020, **25**, e00407.
25 J. M. Guisan, J. M. Bolivar and F. López-Gallego F, J. Rocha-Martín, Immobilization of enzymes and cells, methods and protocols. Springer; 2020

View Article Online
DOI: 10.1039/D1GC03786B

26 G.P. Borin, R. Rodrigues de Melo, E. Crespim, H. H. Sato and F. J. Contesini, An overview on polymer gels applied to enzymeand cell immobilization. In Polymer gels science and fundamentals. Edited by V. K. Thakur, M. K. Thakur, Eds, Springer; 2018, 63–86.
27 J. A. Trelles and C. W. Rivero, Whole cell entrapment techniques. In Immobilization of enzymes and cells, methods and protocols. Edited by J. M. Guisan, J. M. Bolivar, F. López-Gallego, J. Rocha-Martín, Eds, Springer; 2020, 385–394.
28 V. De Vitis, F. Dall'Oglio, F. Tentori, M. L. Contente, D. Romano, E. Brenna, L. Tamborini and F. Molinari, *Catalysts*, 2019, **9**, 208.
29 V. De Vitis, F. Dall'Oglio, A. Pinto, C. De Micheli, F. Molinari, P. Conti, D. Romano and L. Tamborini, *Chem. Open*, 2017, **6**, 668-673.
30 P. Zambelli, L. Tamborini, S. Cazzamalli, A. Pinto, S. Arioli, S. Balzaretti, F. J. Plou, L. Fernandez-Arrojo, F. Molinari, P. Conti, and D. Romano, *Food Chem.*, 2016, **190**, 607-613.
31 D. Romano, R. Gandolfi, S. Guglielmetti and F. Molinari, *Food Chem*., 2011, **124**, 1096-1098.
32 A. J. Smallridge, A. Ten, and M. A. Trewhella, *Tetrahedron Lett*., 1998, **39**, 5121–5124.
33 M. Cvjetko Bubalo, M. Mazur, K. Radošević and I. Radojčić Redovniković, *Process Biochem*., 2015, **50**, 1788–1792.
34 M. Panić, D. Delač, M. Roje, I. Radojčić Redovniković and M. Cvjetko Bubalo, *Biotechnol. Lett.*, 2019, **41**, 253–262.
35 P. Vitale, V. M. Abbinante, F. M. Perna, A. Salomone, C. Cardellicchio and V. Capriati, *Adv. Synth. Catal*., 2017, **359**, 1049.
36 L. Cicco, N. Ríos-Lombardía, M. J. Rodríguez-Álvarez, F. Morís, F. M. Perna, V. Capriati, J. García-Álvarez and J. González-Sabín, *Green Chem*., 2018, **20**, 3468.
37 D. Yang, M. Hou, H. Ning, J. Zhang, J. Ma, G. Yang and B. Han, *Green Chem*., 2013, **15**, 2261.
38 K. M. Reddy, V. Yamini, K. K. Singarapu and S. Ghosh, *Org. Lett*., 2014, **16**, 2658.
39 H. P. L. Gemoets, Y. Su, M. Shang, V. Hessel, R. Luque and T. Noel, *Chem. Soc. Rev.,* 2016, **45**, 83-117.
40 M. R. Chapman, S. C. Cosgrove, N. J. Turner, N. Kapur and A. J. Blacker, *Angew. Chem. Int. Ed.,* 2010, **49**, 2182-2184.
41 J. M. Bolivar, J. Wiesbauer and B. Nidetzky, *Trends Biotechnol.,* 2011, **29**, 333-342.
42 A. Idris, N. Azimah Mohd Zain and M. Suardi Suhaimi, *Process Biochem*., 2008, **43**, 331-338.
43 M.A.P. Nunes, H. Vila-Real, P. C. B. Fernandes and M. H. L. Ribeiro, *Appl. Biochem. Biotechnol*., 2010, **160**, 2129–2147.
44 T. Menegatti and P. Žnidaršič-Plazl, *Micromachines*, 2019, **10**, 867.
45 J. M. Flórez-Castillo, J .L. Ropero-Vega, M. Perullini and M. Jobbágy, *Heliyon*, 2019, **5**, e01872.
46 T. Menegatti and P. Žnidaršič-Plazl, *Front. Bioeng. Biotechnol*. 2021, **9**, 752064.
47 M. L. Contente, I. Serra, F. Molinari, R. Gandolfi, A. Pinto and D. Romano, *Tetrahedron,* 2016, **72**, 3974-3979.
48 J. M. Bolivar and F. López-Gallego, *Green Sustain. Chem.,* 2020, **25**, 100349.
49 P. Žnidaršič-Plazl, *Curr. Opin. Green Sustain. Chem.,* 2021, **32**, 100546.
50 Y. Liu, L. Sun, H. Zhang, L. Shang, and Y. Zhao, *Chem. Rev*., 2021, **121**, 7468−7529,