



Short communication

Flow-based stereoselective reduction of ketones using an immobilized ketoreductase/glucose dehydrogenase mixed bed system



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ABSTRACT

A robust two-enzyme system composed of an immobilized ketoreductase (KRED1-Pglu) and a glucose dehydrogenase (*BmGDH*) was developed via immobilization on aldehyde agarose for the stereoselective reduction of different ketones. The immobilized ketoreductase/glucose dehydrogenase system was continuously used in a flow reactor for weeks, even in the presence of concentrations of DMSO up to 20%.

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1. Introduction

Biocatalytic processes can be improved with the use of different and complementary strategies, including protein or metabolic engineering and immobilization techniques [1]. Besides, biocatalysis in flow chemistry reactors, where rates and productivities can be largely enhanced, seems a logical option [2]. Flow-based applications have potential advantages, such as: increased mixing efficiency, controlled scaling factors, improved safety ratings, in-line control and product recovery, easy set-up of cascade reactions, and continuous processing capabilities [3–5].

Flow-based biocatalysis has recently attracted interest for screening, optimizing or setting up preparative biotransformations [6,7]. Biotransformations such as hydrolysis and formation of esters [8–12], formation of C–C bonds using transketolases [13], preparation of monosaccharides [14] and oligosaccharides [15], synthesis of nucleosides [16], interconversion of carbonyls and amines using transaminases [17,18], and peptide condensation [19] have been reported in the last few years. Stereoselective enzymatic carbonyl reduction with immobilized systems has been poorly investigated [20–23] and, to our knowledge, only one example has been reported using an immobilized ketoreductase in a plug flow reactor [24]. Here we report the use of a mixed bed system composed of immobilized ketoreductase from *Pichia*

glucozyma (KRED1-Pglu) [25,26] and a glucose dehydrogenase from *Bacillus megaterium* (*BmGDH*) [27] for the stereoselective reduction of ketones in a flow reactor.

2. Experimental

2.1. General procedure for batch biotransformations

Reactions were carried in 2 mL volume of 50 mM Tris HCl buffer pH 8.0 containing NADP⁺ (0.1 mM), substrate (0.5 g/L), glucose (4 eq × eq of substrate) at 30 °C. The reaction was started by the addition of KRED1-Pglu and *BmGDH*, maintained under orbital shaking (180 rpm), and was monitored by HPLC.

2.2. General procedure for flow biotransformations

Solutions of the substrates at different concentrations were prepared in Tris HCl buffer pH 8.0 (50 mM) with DMSO (variable percentages depending on the substrate, for details see Table 2), 0.1 mM NADP⁺ and glucose (4 eq × eq of substrate). An Omnifit glass column (6.6 mm i.d. × 100 mm) was packed with pre-mixed KRED1-Pglu (540 mg) and *BmGDH* (540 mg), in order to obtain a KRED1-Pglu/*BmGDH* ratio of 1/25. The volume of the reactor was 0.90 mL. The packed column was pre-washed by flowing through it a solution of Tris HCl buffer pH 8.0 (0.050 mL/min for 15 min) followed by a solution of Tris HCl buffer pH 8.0 with DMSO (0.050 mL/min for 15 min). Then, the

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Table 1
Immobilization of KRED1-Pglu and *BmGDH*.^a

Entry	Enzyme	Load (mg·g ⁻¹)	T (°C)	Additive	Immobilized protein (%) ^b	Yield (%)
1	KRED1-Pglu	7	4	–	98	40 ^c
2	KRED1-Pglu	7	25	–	97	14 ^c
3	KRED1-Pglu	14	4	–	98	40 ^c
4	KRED1-Pglu	14	25	–	92	12 ^c
5	KRED1-Pglu	7	25	20% glycerol	99	12 ^c
6	KRED1-Pglu	14	25	20% glycerol	97	9 ^c
7	<i>BmGDH</i>	5	25	20% glycerol	92	32 ^d
8	<i>BmGDH</i>	5	25	20% PEG600	87	5 ^d
9	<i>BmGDH</i>	10	25	20% glycerol	90	28 ^d
10	<i>BmGDH</i>	10	25	20% PEG600	84	5 ^d

^a Experimental conditions: pH 10.0, 3 h.

^b Determined by Bradford method.

^c Determined by comparison of the loaded and recovered activity towards 4-nitroacetophenone (**1a**) [32].

^d Determined by comparison of the loaded and recovered activity assayed towards glucose.

substrate solution was pumped through the column maintained at 30 °C. The exiting solution was collected and analyzed at different times. See Supplementary materials for other experimental details.

3. Results and discussion

Ketoreductase from *Pichia glucozyma* (KRED1-Pglu) and glucose dehydrogenase from *Bacillus megaterium* (*BmGDH*) were prepared as previously reported [26,27] (see Supplementary materials for details). The first support taken into account was the commercially available epoxy-activated Relizyme 403/S. Although KRED1-Pglu was completely bound to the support, the resulting biocatalyst was poorly active (4% of expressed activity). Moreover, on the same support only 2% of the offered *BmGDH* was bound to the matrix. Aldehyde activated agarose, a support suitable for the immobilization/stabilization of several enzymes [28,29], including ketoreductases, was alternatively employed for immobilization. Immobilization on aldehyde agarose relies on the reaction between non protonated ε-amino groups of surface lysines and aldehyde groups on the support. Since alkaline conditions (pH ≥ 10) are required to ensure that the ε-amino groups are not protonated, stability studies of both soluble enzymes were carried out at this pH. *BmGDH* lost >50% of the initial activity after 1 h of incubation at pH 10.0 both at 25 and 4 °C. The addition of stabilizing agents (glycerol or PEG600) allowed to maintain 100% and 70% of the initial activity after 3 h of incubation at pH 10 [30,31]. On the other side, KRED1-Pglu retained 100% of its initial activity over 3 h at pH 10.0 under all the conditions tested (25 °C or 4 °C with 20% glycerol as additive). On the basis of these stability tests, immobilization of KRED1-Pglu was assayed under different conditions. The best results for the immobilization of KRED1-Pglu were obtained at 4 °C without additives and independently from protein loading (Table 1, entry 1 and 3). For *BmGDH*, as a consequence of the stability requirements, it was necessary to add stabilizing additives to the immobilization mixture. The addition of 20% of glycerol allowed to retain 32% and 28% of activity upon immobilization at 25 °C (Table 1, entry 7 and 9) while the addition of PEG600 was not beneficial to the immobilization outcome (Table 1, entry 8 and 10).

The stability of the free and immobilized enzymes was compared at 30 °C in the presence of different concentrations of DMSO (Figs. S1 and S2), a water-soluble solvent often employed for solubilizing hydrophobic substrates in biocatalytic reactions. Immobilization remarkably improved the enzymatic stability towards DMSO: 60–65% residual activity was found for immobilized KRED1-Pglu after 24 h in the presence of 5–20% DMSO, and only 4–6% of the original activity was lost in the case of immobilized *BmGDH*. It should be noted that both the free enzymes were mostly inactivated after 6 h even at the lowest DMSO concentration (Figs. S1A and S2A).

The two immobilized proteins were blended together to obtain a homogeneous agarose gel system suited for catalyzing the stereoselective

reduction of ketones at 30 °C with an efficient regeneration system for NADPH/NADP⁺. This system was able to convert 4-nitroacetophenone **1a** with yields between 78 and 83% in the presence of DMSO ranging from 4 to 8% while lower yields were observed at higher co-solvent concentrations (Fig. 1); **2a** was always recovered with an ee > 98%. Sluggish reactions could be observed with free enzymes in the presence of DMSO concentrations above 4%. Compound **1a** is mostly insoluble in water at 30 °C, while it is largely soluble in aqueous solutions with DMSO concentrations above 4%.

The re-usability of the immobilized system was investigated in a sequence of reaction cycles performed in the presence of 5% DMSO, where the biocatalysts were recovered by filtration after every cycle and used for a new batch reaction. The immobilized biocatalysts lost 20% of the original activity after one cycle and were totally inactive after 4 reaction cycles (see Fig. S3).

The reduction of **1a** was then carried out with the immobilized enzymes in a continuous packed bed flow reactor. Different amounts of the immobilized enzymes were mixed to find the best KRED1-Pglu/*BmGDH* ratio (see Fig. S4). The best results (>97% conversion with a residence time of 180 min) were obtained using a KRED1-Pglu/*BmGDH* ratio of 1/25, corresponding to 55 mU for KRED1-Pglu (100 mU/g), and 1375 mU for *BmGDH* (2750 mU/g).

The continuous flow reaction was carried out for 15 days in the presence of 5% DMSO with no significant change of the chemical composition of the outflow solution, which contained 97–98% of optically pure (S)-1-(4-nitrophenyl)ethanol (**2a**) (Fig. 2). Noteworthy, after 6 months

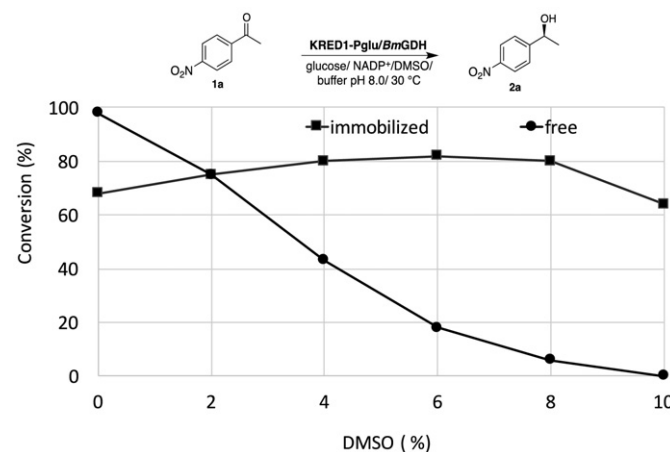


Fig. 1. Reduction of **1a** into **2a** by free and immobilized KRED1-Pglu/*BmGDH* in the presence of different concentrations of DMSO (batch reactions).

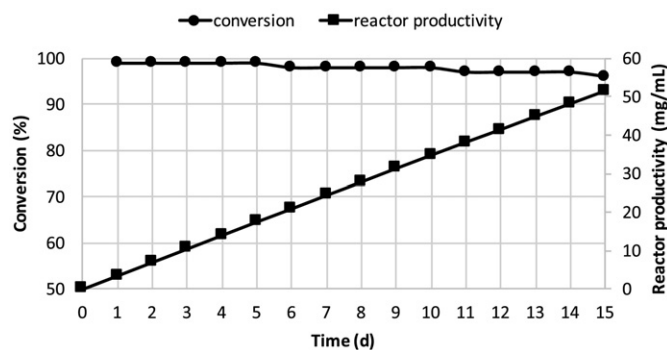


Fig. 2. Continuous reduction of **1a** in flow reactor.

of operation, the flow reactor only lost 30–32% of the original activity. The different operational stability shown by the immobilized system in the flow reactor and in shaken flasks may be due to the reduced mechanical stress under flow conditions. It should be underlined that biotransformations with immobilized enzymes are multi-phase systems needing continuous agitation in conventional batch reactors; agitation (orbital shaking in our case) can damage or grind the biocatalyst to fine particles [33].

The flow reaction was also applied to the stereoselective reduction of other ketones (**1b–d**). These substrates have a different solubility in water: compounds **1b** and **1c** are mostly soluble in 10% DMSO aqueous solutions, whereas ethyl secodione **1d**, a key intermediate for the synthesis of a number of hormonal contraceptives [34,35], is soluble only at 20% DMSO concentration. Flow reactions were carried out by pumping aqueous–DMSO solutions containing the substrate (3.0 mM), NADP⁺ (0.1 mM), and glucose (12.0 mM) through the packed bed reactor. The highest yield was achieved at relatively low residence times (Table 2). 1-Phenylpropane-1,2-dione **1b** (3 mM) gave optically pure (*S*)-2-hydroxy-1-phenylpropan-1-one (Table 2, entry 1) with a total conversion in correspondence of a very low residence time (7 min); the same reaction catalyzed by the free enzymes in batch mode gave a total conversion of the substrate (3 mM) in 60 min. 1-Phenylbutane-1,3-dione **1c** was reduced to (*S*)-3-hydroxy-1-phenylbutan-1-one **2c** by the free enzymes in pure aqueous solution with a maximum yield

of 60% (ee > 98%) after 24 h; the reduction in flow by immobilized enzymes and 10% DMSO allowed for a very high yield (95%) with a residence time of 120 min (Table 2, entry 2). A less pronounced improvement of the productivity was observed for the enantioselective reduction of the highly water-insoluble secodione **1d**. In fact, the reaction of **1d** with the free enzymes in batch gave yields up to 65% after 8 h starting from 6.5 mM substrate concentration, under conditions where the substrate was largely insoluble [35]; when the bioconversion was transferred in the flow mode, it was necessary to use a flow stream containing lower substrate concentration (3 mM) and relatively high DMSO concentration (20%) to have a homogeneous system. Under these conditions, yields of 65% were reached with a residence time of 180 min (Table 2, entry 3).

The flow biotransformation of ketones **1b–d** was carried out in a continuous mode without significant changes of the composition of the outflow stream for 15 days; details about the continuous reaction in the flow reactor of the three substrates (residence times, conversions, stability, enantiomeric excesses, space time yields, and isolated yields) are given in Table 2. The stability of the biocatalysts under these conditions enabled the obtainment of millimolar amounts of the desired products in a 0.90 mL reactor, using the same amount of enzyme that produced only micromolar quantities in batch reactions.

In conclusion, an immobilized system composed of a ketoreductase (KRED1-Pglu) and a glucose dehydrogenase (*BmGDH*) was successfully used to perform the continuous stereoselective reduction of ketones in a flow reactor for weeks; the prolonged operational stability in the flow reactor makes the set-up of the biotransformation attractive for preparative (bio)catalysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.catcom.2017.01.025>.

Table 2

Continuous stereoselective reduction of diketones **1b–d** (3 mM) with immobilized KRED1-Pglu/*BmGDH* in flow reactor.^a

Entry	Subst.	DMSO (%)	Residence time (min)	Conversion day 1 (%) ^b	Conversion day 15 (%) ^b	Space time yield (mg·d ⁻¹)	Catalyst productivity (mmol·U ⁻¹)	Product recovered (mg) ^c
1	1b	10	7	>98	96–97	81.5	150.1	1199
2	1c	10	120	95–96	93–94	5.0	8.4	67
3	1d	20	180	64–65	62–63	4.4	3.8	60

^a Flow solutions contained [**1b–d**] 3.0 mM, [NADP⁺] 0.1 mM, [glucose] 12.0 mM in Tris HCl buffer pH 8.0 (50 mM) in the presence of different amounts of DMSO.

^b Determined by HPLC.

^c After purification by flash chromatography.

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