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Continuous Flow Synthesis of Chiral Amines in Organic Solvents: Immobilization of *E. coli* Cells Containing Both ω-Transaminase and PLP

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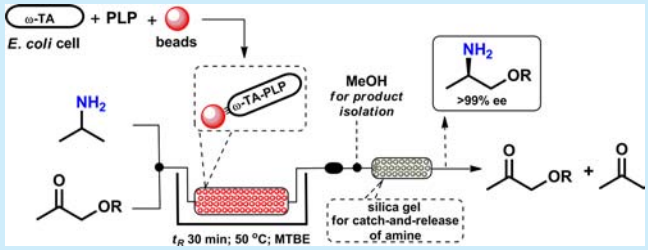
Supporting Information

ABSTRACT: *E. coli* cells containing overexpressed (*R*)-selective ω-transaminase and the cofactor PLP were immobilized on methacrylate beads suitable for continuous flow applications. The use of an organic solvent suppresses leaching of PLP from the cells; no additional cofactor was required after setting up the packed-bed reactor containing the biocatalyst (ω-TA-PLP). Non-natural ketone substrates were transformed in flow with excellent enantioselectivity (>99% ee). Features of this novel system include high-throughput (30−60 min residence time), clean production (no quench, workup, or purification required), high enzyme stability (the packed-bed reactor can be continuously operated for 1−10 days), and excellent mass recovery.



Optically pure amines enable the synthesis of several classes of biologically active compounds, such as agrochemicals and pharmaceuticals.[1] Numerous methods to prepare chiral amine derivatives have been described,[2] but the direct preparation of free amines themselves is still not a simple task, since most of the described methods do not provide such requirement. An efficient synthetic approach for this purpose is the use of enzymatic reactions. In particular, asymmetric amination of ketones catalyzed by ω-transaminases can produce chiral primary amines.[3−5] ω-Transaminases are pyridoxal 5′-phosphate (PLP)-dependent enzymes that can catalyze the transfer of amino groups from amine donors to ketone groups.

The need for enzyme recyclability and stability is an important goal in biocatalysis, and the use of a heterogeneous system is one possible means to this end. For example, ω-transaminases have been immobilized within sol−gel materials.[6,7] We and others have also described the entrapment of ω-transaminase and cells on sol−gel materials, prepared with poly(vinyl alcohol)s.[7]

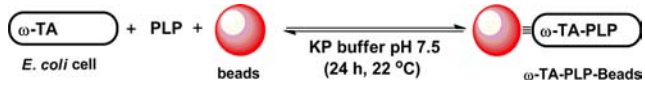
Flow chemistry is an important tool useful for increasing the product throughput of enzymatic processes, especially with lipases, which are not cofactor-dependent enzymes.[8] The immobilized system that we describe herein now enables the use of cofactor-*dependent* enzymes under flow conditions with organic solvents; an additional cofactor (PLP) beyond that which is already found in the naturally expressed enzyme is not required.

We envisioned the immobilization of a ω-transaminase together with its cofactor PLP on beads and its application under flow conditions using a packed-bed reactor in organic solvent (methyl *tert*-butyl ether, MTBE). The use of MTBE is desirable because transaminase, PLP, and cells are not soluble in MTBE, and thus, we can expect that neither enzyme nor PLP dissociation would be observed.

Having this concept in mind, we have developed an efficient system to produce chiral amines continuously by asymmetric amination of ketones catalyzed by ω-transaminase-PLP complex immobilized on methacrylated beads. *E. coli* cells containing the overexpressed (*R*)-selective ω-transaminase from *Arthobacter*,[9] was selected for this study.

Methacrylate polymeric resin (beads) was used as support for the *E. coli* cells containing (*R*)-selective ω-TA and PLP (Scheme 1). Despite the presence of naturally occurring PLP inside the cell, we added an additional amount (0.008 M) to the immobilization solution, only to ensure the presence of sufficient PLP in the system. We further reasoned that the peptidoglycan layer in the *E. coli* cell wall (containing functional groups such as amide, alcohol, and ether) could easily bind to the methacrylate polymer primarily via hydrogen bonding.

Scheme 1. Immobilization of *E. coli* Cells and PLP on Methacrylate Polymeric Resin



After *E. coli* and PLP immobilization, the enzyme activity (transaminase) for ω-TA-PLP-beads was evaluated by using the asymmetric amination of methoxyacetone (0.05 M) with

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isopropylamine (0.1 M) in MTBE at 30 °C for 24 h. (For details, see Table S1, Supporting Information.)[9]

The enantiopure (*R*)-amine **2a** was prepared in excellent conversion (96%) and enantiomeric excess (>99%). To explore enzyme recyclability, the beads were used four more times and the results were the same, indicating excellent enzyme stability under these nonaqueous conditions. As expected, the presence of an additional PLP (0.0005 M) did not increase the enzyme performance. As further addition of PLP to the reaction in organic solvent does not yield an active enzymatic system (*ω*-TA-PLP-beads), it would appear after attachment of *ω*-transaminase to the support that, under aqueous conditions, it is and remains saturated with PLP once organic solvents are used.

To reduce the reaction time (24 to 2 h), the temperature was increased to 50 °C. By applying these conditions, the chiral amine was obtained with 72% conversion and >99% ee. These results demonstrated that this system is efficient and stable at elevated temperature.

The design of the flow system is depicted in Table 1. By using two syringes, substrates (ketone and amino donor) in organic solvent (MTBE) can be injected into a packed-bed reactor (PBR) containing the immobilized enzymatic system (*ω*-TA-PLP) attached to methacrylate beads.

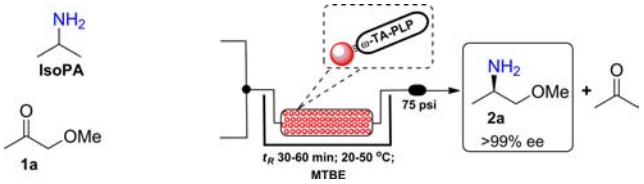
Two syringes were charged with solutions of methoxy ketone (0.1 M) and isopropylamine (0.4 M) and connected to the packed-bed reactor through a Y-adaptor. After mixing these two solutions, the final ketone concentration is 0.05 and 0.2 M for isopropylamine. The residence time (t_R) for the flow system was established at 30 min, and continuous flow asymmetric amination of methoxyacetone at 50 °C was efficiently achieved. The chiral amine was obtained with 85% conversion and >99% ee (Table 1, entry 1). By analyzing the reaction effluent every hour under continuous flow operation, it was possible to obtain, in all cases, the same conversion and enantioselectivity.

The ketone concentration was decreased to 0.01 M, and the conversion was excellent (95%); however, by increasing 5-fold the ketone concentration (0.05 M), an 85% conversion was observed (Table 1, entry 2).

The ratio between ketone concentration and isopropylamine was also evaluated under flow conditions (1:2, 1:4 and 1:10; Table 1), and the optimum ratio was found to be 1:4 (ketone/isopropylamine). To lengthen enzyme the lifetime, the continuous flow system was operated at 20 and 30 °C (Table 1, entries 4−10). At 20 °C and reduced ketone concentration (0.01 and 0.025 M), 42−65% conversion and >99% ee were observed (Table 1, entries 7−10). At 30 °C and 60 min, t_R comparable results (85% conversion, >99% ee; Table1, entry 5) to those obtained at 50 °C with a t_R of 30 min (85% conv, >99% ee; Table 1, entry 1). All reactions were carried out under continuous flow operation for at least 2 h. In all cases, the transaminase activity was evaluated every hour and maintained efficacy and selectivity, indicating a stable system.

To evaluate the reaction scope for the immobilized (*R*)-selective *ω*-transaminase (*ω*-TA-PLP-beads) under flow conditions in an organic solvent, different *α*-alkoxy and *α*-aryloxy acetones (**1b−d**) were investigated, with isopropylamine as the amine donor (Table 2). Reaction conditions were optimized to maximize amine production. The asymmetric

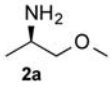
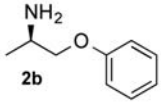
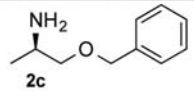
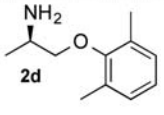
Table 1. Asymmetric Amination under Flow Conditions in Packed-Bed Reactor[a]



entry	**1a** (M)	isoPA (M)	cfo (h)	temp (°C)	(*R*)-**2a**[b] (%)
1	0.05	0.2	1, 2, 3, 4	50	85
2	0.01	0.04	1, 2	50	94
3	0.05	0.1	1, 2	50	77
4	0.05	0.2	1, 2, 3, 4	30	67
5[c]	0.05	0.2	1, 2, 3, 4	30	84
6	0.05	0.1	1, 2	20	42
7	0.025	0.1	1, 2, 3, 4	20	56
8	0.025	0.1	1, 2, 3	20	58
9	0.01	0.04	1, 2, 3, 4	20	65
10	0.01	0.1	1, 2, 3, 4	20	45

[a]Continuous flow operation (cfo): methoxyacetone (0.01, 0.025, or 0.05 M) and isopropylamine (isoPA: 0.040, 0.1, or 0.2 M) in MTBE (saturated with water); temp 20, 30, or 50 °C. Residence time (t_R): 30 min. *ω*-TA-PLP-beads (280 mg) were packed into a stainless steel tubing ($^1/_4$ in. × 4.6 mm × 10 cm; reactor volume with beads: 0.5 mL). Back pressure regulator (BPR): 75 psi. [b]Conversion and ee were determined by GC analysis after amine derivatization to acetamide. [c]t_R: 60 min.

Table 2. Asymmetric Amination of *α*-Alkoxy- and *α*-Aryloxy Acetones under Flow Conditions[a]

entry	**1a-d** (M)	isoPA (M)	cfo (h)	(*R*)-amine **2a-d**[b] conv (%)	ee (%)
				 **2a**	
1	0.05	0.2	1,2,3,4,20	85	>99
				 **2b**	
2	0.01	0.04	1,2,3,4	71	>99
3	0.05	0.2	1,2,3,4	67	>99
4[b]	0.05	0.2	1,2,3,4,20	81	>99
				 **2c**	
5	0.01	0.04	1,2,3,4	83	>99
6	0.05	0.2	1,2,3,4	76	>99
7[b]	0.05	0.2	1,2,3,4,20	90	>99
				 **2d**	
8	0.05	0.2	1,2,3,4,20	84	>99
9[b]	0.05	0.2	1,2,3,4	94	>99

[a]Continuous flow operation (cfo): ketones **1a−d** and isopropylamine (isoPA) in MTBE (saturated with water); temp: 50 °C. t_R: 30 min. *ω*-TA-PLP-beads (280 mg) were packed into a stainless steel tubing ($^1/_4$ in. × 4.6 mm × 10 cm; reactor volume with beads: 0.5 mL). BPR: 75 psi. [b]t_R: 60 min.

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amination of methoxyacetone **1a** (0.05 M) was also performed under continuous operation for 20 h, and transaminase activity remained constant (85% conv, >99% ee; Table 2, entry 1).

Phenoxyacetone **1b** at a 0.01 M concentration was transformed into the corresponding chiral amine with good conversion (71%) and high enantiomeric excess (>99%) (Table 2, entry 2), and increased ketone concentration (0.05 M) led to a slight reduction in conversion, but with a higher amine production per unit time (Table 2, entry 3). Increasing the residence time to 60 min increased the conversion (81% conv, >99% ee; Table 2, entry 4).

Another important feature of this enzymatic PBR system was that enzyme activity was maintained, even after 20 h of continuous operation (Table 2, entry 4). The same reaction conditions (t_R: 30 and 60 min) and ketone concentration (0.01 and 0.04 M) were applied for the asymmetric amination of benzyloxyacetone **1c**. Once again, the chiral amine was obtained in excellent enantiomeric excess (>99%) and with high conversion (76−90%) (Table 2, entries 5−7). (2,6-Dimethylphenoxy)acetone **1d** was also transformed to the corresponding chiral amine (mexiletine **2d**) with excellent results (Table 2, entries 8 and 9). It is noteworthy that the amine **2d** is a therapeutically important chiral drug used as antiarrhythmic, antimyotonic, and analgesic oral drug.[5e]

We also studied the stability of the immobilized cell during PBR continuous operation for several days (Figures 1 and 2).

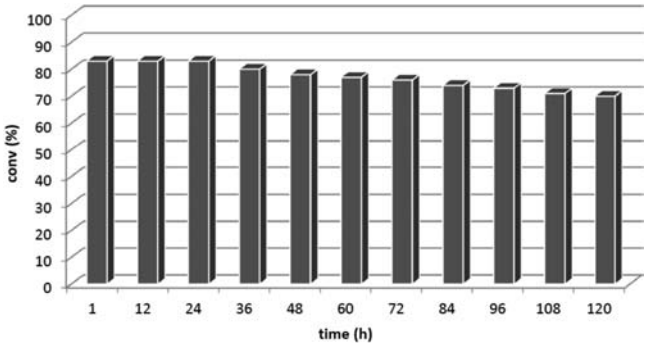


Figure 1. Continuous flow production of mexiletine **2d**. t_R: 30 min; temp: 50 °C.

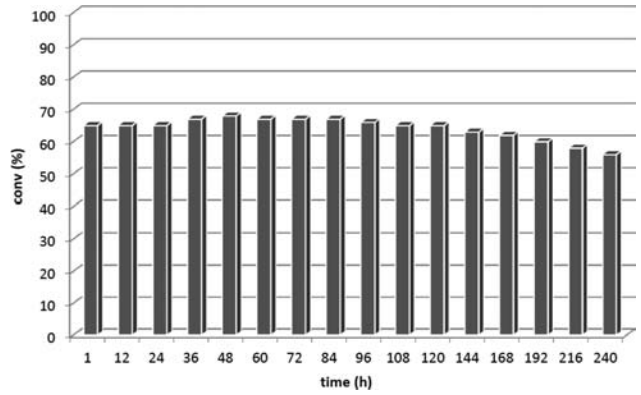


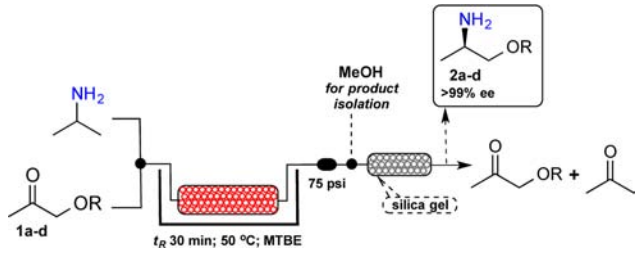
Figure 2. Continuous flow production of mexiletine **2d** (t_R: 60 min; temperature: 30 °C).

The first set of continuous operation was selected to have the same flow conditions described in Table 2 (t_R: 30 min; temp: 50 °C; ketone 0.05 M; isopropylamine 0.2 M). As shown in Figure 1, the immobilized cells were used for 5 days under continuous flow conditions with only a small decrease in enzyme activity (<10%), but with the same enantioselectivity (>99% ee for amine **2d**) (Figure 1). As this concept was applied for a small-scale process (reactor volume: 0.5 mL), after 5 days operation, the reaction effluent was 120 mL. Considering the small amount of *E. coli* cells used to prepare the packed bed reactor (50 mg/reactor), the chiral amine productivity was quite high.

Despite excellent performance of the system at 50 °C, we decreased the temperature to 30 °C and increased the residence time to 60 min to increase both enzyme lifetime under extended continuous operation (10 days, Figure 2) and productivity. As before, the chiral amine **2d** was produced with >99% ee, and only a small decrease in enzyme activity was observed (<10%) after 10 days of operation. Based on these results, we conclude that for higher amine production, a shorter residence time at 50 °C should be used.

To isolate the free chiral amine from the reaction product stream, we designed a catch-and-release system, which consists of a silica gel cartridge attached to the packed-bed reactor. As the reaction solvent is MTBE, the amines **2a−d** have higher retention factors in silica than do the starting materials (**1a−d**) under these conditions. Thus, the desired amine is retained, thus separating any remaining ketone continuously from the effluent (Scheme 2).

Scheme 2. Continuous Flow Production and Catch-and-Release System for Chiral Amines



We performed the continuous flow production of all chiral amines **2a−d** for 5 h, and then the silica gel cartridge was removed and, for amine release, the cartridge was rinsed with 4 mL of methanol. After evaporation of the solvent and the more volatile isopropylamine, it was possible to isolate the chiral amines in good yields [75% (amine **2a**), 78% (amine **2b**, t_R 60 min), 70% (amine **2c**), 79% (amine **2d**)].

In conclusion, a highly stable enzymatic system attached to methacrylate beads was developed to produce chiral amines under flow conditions using organic solvents. Immobilization of *E. coli* cells containing overexpressed ω-transaminase and the cofactor PLP on beads gave a biocatalyst (ω-TA-PLP-Beads) that was able to perform the asymmetric amination of non-natural ketones in flow with excellent enantioselectivity (>99% ee). The use of an organic solvent suppresses leaching of PLP from the cells; no additional cofactor was required after setting up the packed-bed reactor containing the biocatalyst. Other features of this novel system include high throughput (t_R 30−60 min), clean production (no quench, workup, or purification required), high enzyme stability (the packed-bed reactor can be used for several days), and excellent mass recovery.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, screening table, and continuous flow system pictures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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