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A Process Concept for High-Purity Production of Amines by Transaminase-Catalyzed Asymmetric Synthesis: Combining Enzyme Cascade and Membrane-Assisted ISPR

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Supporting Information

ABSTRACT: For the amine transaminase (ATA)-catalyzed synthesis of chiral amines, the choice of donor substrate is of high importance for reaction and process design. Alanine was investigated as an amine donor for the reductive amination of a poorly water-soluble ketone (4-phenyl-2-butanone) in a combined in situ product removal (ISPR) approach using liquid-membrane extraction together with an enzyme cascade. This ISPR strategy facilitates very high (>98%) product purity with an integrated enrichment step and eliminates product as well as coproduct inhibition. In the presented proof-of-concept alanine shows the following advantages over the other frequently employed amine donor isopropyl amine: (i) nonextractability of alanine affords high product purity without any additional downstream step and no losses via coextraction, (ii) higher maximum reaction rates, and (iii) broader acceptance among ATAs.

1. INTRODUCTION

Optically pure amines stand in high demand for the synthesis of biologically active compounds, such as agrochemicals and drugs.[1,2,2b] The biocatalytic synthesis of chiral amines with amine transaminases (ATA) affords high enantio- and regioselectivity and offers a sustainable and more environmentally friendly alternative to the traditional transition metal catalysis.[3−7] Amine transaminases belong to the pyridoxal 5′-phosphate (PLP) dependent enzymes and catalyze the amine group transfer from a donor molecule (e.g., amine or amino acid) to an amine acceptor substrate (e.g., ketone or keto acid).[8,9] Although other emerging enzymatic routes can yield chiral amines, for example, employing NADH-dependent amine dehydrogenases[10] or imine reductases,[11] this work will focus on ATA-catalyzed amine synthesis by presenting a conceptually unique process concept affording pure amines.

Overall, the production of chiral amines using ATAs as biocatalyst faces several challenges which need to be overcome in order to yield an attractive industrial process. The ATA-catalyzed reductive amination reaction is essentially reversible and prone to both substrate and product inhibition.[8] In addition, although ATAs are enzymes with a rather broad substrate scope (giving access to a variety of chiral amines),[12,13] the substrate specificity may vary depending on the source organism.[14] The most commonly used amine donating substrates are alanine (ALA) and isopropyl amine (IPA). In both cases, an unfavorable reaction equilibrium (K_{eq}) hinders high synthetic yields, which becomes even more pronounced for pharmaceutically interesting amine products.[15] Consequently, strategies have to be developed to displace the K_{eq} and avoid inhibition affording high yields and high reaction rates.

There exist several strategies to counteract the unfavorable K_{eq} in ATA reactions.[16−18] The easiest method is to supply the amine donor in excess thereby "pushing" the reaction to high conversion.[19] In situ coproduct removal (IScPR) renders an attractive concept and can be achieved by evaporation of acetone (ACE) in IPA reactions[20,21] or by enzymatic transformation of pyruvate (PYR) in ALA reactions. Various combinations of auxiliary enzymes targeting PYR have been proven to promote high synthetic yields and are summarized in recent reviews.[22,23] A well-established enzyme cascade reaction for PYR removal represents the combined use of ATA together with a lactate dehydrogenase (LDH) and a glucose dehydrogenase (GDH) for PYR removal and cofactor generation, respectively.[12,24] Alternatively, chemo-enzymatic synthesis steps may be an option to drive the reaction forward, but they remain case-specific and thus very limited to certain types of amine donors.[25] Moreover, IScPR methods not only affect the K_{eq} but can also prevent coproduct inhibition.

Contrary to IScPR, in situ product removal (ISPR) strategies represent a physical separation approach.[16,26] Practically, the method should realize amine product capturing or isolation. Ionic exchange resins have been employed in a continuous process to bind the product amine in situ, but for amine donor/product selectivity and capacity reasons, they were found to lack (so far) industrial practicality.[27] Similarly, a silica gel-based "catch-and-release" method (in 0.5 mL scale) was recently published exploiting the principle of flash-chromatography to simply separate amines form ketones.[28] Hence both amines (donor and product) are coreleased when flushing the silica cartridge with the eluent.

Generally, ISPR may have the benefit of an integrated downstream step compared to IScPR.[29] The principle of selective removal/recovery of the amine product renders

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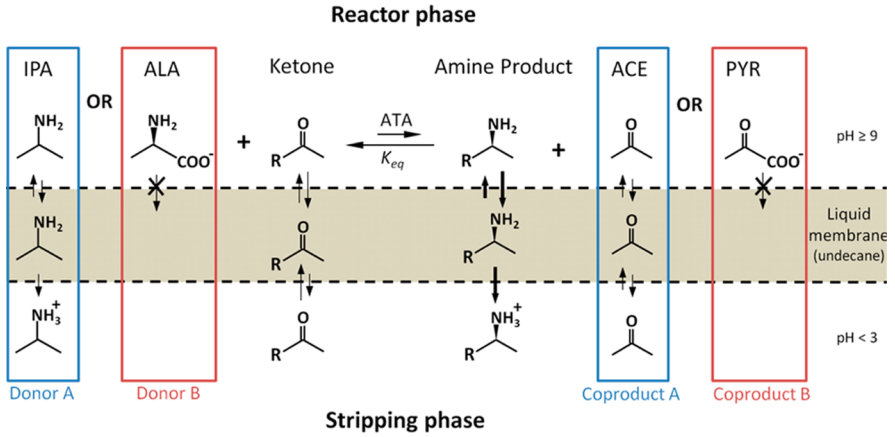
Scheme 1. Principle of in Situ Product Removal (ISPR) for Asymmetric Synthesis of Chiral Amines Using a Supported-Liquid Membrane (SLM)[a]



[a]Isopropyl amine (IPA) or L-alanine (ALA) can serve as amine donor in the ATA-catalyzed amination of ketone substrates. Amine donor and coproduct extraction into the stripping phase occurs only when using donor A (IPA, framed blue). Donor B (ALA) and coproduct B (PYR) allow high product purity (framed red). In both cases (red and blue) ISPR influences the reaction equilibrium towards chiral amine synthesis. For more explanations see text.

thereby perhaps an even more promising strategy in asymmetric amine synthesis.[30] Rehn et al. presented the production of α-methylbenzylamine from acetophenone together with IPA as an amine donor using supported-liquid membrane (SLM) extraction as ISPR method,[31] which affords high product concentrations (about 100 g/L).[32] The principle of SLM extraction is schematically illustrated in Scheme 1. The different pH of the reactor and stripping phase as well as the hydrophobic membrane solvent represent the main driving forces for selective extraction. Only the deprotonated amine species extracts into the solvent and is then trapped in the stripping phase (due to protonation). This resembles a dead-end extraction and theoretically all amines can so be extracted into the stripping phase, as long as the stripping phase pH is kept sufficiently below the pK_a of the amine product. Due to the usually high pK_a values of amines, the reactor phase should feature rather alkaline conditions to establish a large fraction as possible of extractable (deprotonated) amine species. ATAs are advantageous for this approach, because they remain catalytically active and stable also at higher pH.[13] If IPA serves as amine donor, partial coextraction is unavoidable (Scheme 1, blue frames).[31] However, if the amine donor features a permanently charged character at high pH such as the zwitter-ionic ALA (Scheme 1, red frames), coextraction would be avoided, and ALA is thus an ideal candidate in this respect. Furthermore, as depicted in Scheme 1, the coproduct (PYR) will not cross the membrane either, and hence the ALA reaction should facilitate a much higher amine product purity compared to IPA.

Due to the potential advantages of amine selectivity and higher acceptance of the natural substrate among ATAs,[33] the aim of this study was to investigate the feasibility of using ALA as an amine donor in combination with the SLM strategy for ISPR. Such an integrated process design would have a significant advantage to the existing methods, because in principle, it continuously yields a pure and enriched amine product without any additional downstream step. A model reaction was chosen, where the ketone (4-phenyl-2-butanone) features a poor water-solubility, which is often typical for pharmaceutical precursors and challenging for large-scale applications of ATA-catalyzed reactions.

2. MATERIALS AND METHODS

Materials. All chemicals such as 4-phenyl-2-butanone (benzylacetone, BA), (*S*)-(+)-1-methyl-3-phenylpropylamine, isopropyl amine, acetone, L-alanine, sodium pyruvate, NADH (β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate), pyridoxal 5′-phosphate, and all used solvents were purchased from Sigma-Aldrich Corporation. The amine transaminase (ATA-50, from metagenomic library) crude enzyme powder and freeze-dried cells were provided by c-LEcta GmbH, Leipzig, Germany. Lactate dehydrogenase (LDH) from hog muscle (Boehringer Mannheim GmbH, Germany) and from bovine heart type III (Sigma-Aldrich Corporation) was kindly provided by Prof. Per-Olof Larsson, Pure & Applied Biochemistry, Lund University, Sweden.

Transamination Reactions. In small scale, 4.5 mL glass vials (with Teflon septum sealed screw cap) were used containing 2 mL of reaction solution placed in a thermoshaker (HLC Biotech, Bovenden, Germany), shaken at 600 rpm, 30 °C. A typical substrate solution contained 450 mM of amine donor, either IPA or ALA, and 10 mM amine acceptor, BA. A 20 mM sodium phosphate buffer pH 8 including 0.1 mM PLP was used for kinetic experiments. The pH was adjusted with HCl or NaOH after all substrates and cofactor had dissolved. For initial rate experiments (specific activity determination, U/mg) 0.05−0.5 mg/mL of ATA-50 crude enzyme or cells was added to the reaction solution. The stopped-assay method (batch reaction) was used taking aliquots at different time intervals. The product concentration (of the quenched reaction in mobile phase of final pH 11) was analyzed by HPLC. Initial rates were determined by linear regression of product concentrations over time below 10% conversion (considering reaction equilibrium position, see Results). To obtain the half-maximal inhibitory concentration ($IC_{50\%}$), the inhibition data were fitted to the Michaelis−Menten equation for competitive inhibition. For conversion profile experiments 2 mg/mL ATA-50 crude powder or 4 mg/mL cells were added.

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Cascade reactions were conducted in 50 mM borax-HCl buffer pH 9.0 and 9.5, 0.2 mM PLP, 5 mM NADH, 0.5 M ALA, 10 mM BA, and 0.5 mg/mL (LDH) from hog muscle and bovine heart type III together with 2 mg/mL ATA-50 at 30 °C and shaken at 600 rpm.

Product (MPPA, ACP) formation was analyzed by HPLC (Dionex Ultimate 3000) provided with a reversed phase (C18) Gemini NX 3u 110 Å column (100 × 2 mm) equipped with a guard column purchased from Phenomenex (Denmark). The analytes were eluted in isocratic mode within 2.45 min (MPPA) and 1.9 min (ACP) using a mobile phase composition of 65% aqueous phase pH 11 (10 mM NaOH) and 35% acetonitrile.

s-ISPR System and Reaction. The setup of the simultaneous in situ product and coproduct removal (s-ISPR) system is given in Figure 5. Two Liqui-Cel MiniModule hollow fiber (polypropylene) membrane contactors with a surface area of 100 cm^2 was purchased from Membrana (Charlotte, NJ, USA). The stripping phase contained 100 mM citrate-HCL buffer (pH < 3). The reactor phase was filled with 50 mL of reaction solution (50 mM borax-HCl buffer, pH 9.5, 0.5 M ALA, 10 mM BA, 0.2 mM PLP) and LDH (5 mg) together with NADH (5 mM) was added initially and two more times during the process at 40 and 70 h. Immobilized ATA-50 cells were used in the SLM experiments: 250 mg ATA-50 cells were entrapped in chitosan and packed into a column together with Celite (0.2−0.5 mm, 30−80 mesh from BHD Laboratory Supplies, Poole, England) as a scaffold stabilizer (reducing compressibility). For detailed immobilization procedure, see Rehn et al.[34] Both phases were circulated with peristaltic pumps (3 mL/min, Alitea, Stockholm, Sweden) and passing the supported-liquid membrane contactor.

The isolated amine product was obtained through solvent extraction. For the single step isolation the pH of the stripping phase (10 mL) was adjusted to pH 12 (conc. NaOH), and MPPA plus BA was extracted using 3 × 10 mL ethyl acetate, pooled, and concentrated (under vacuum). For increased isolated purity BA was removed first from the stripping phase (pH < 3) by ethyl acetate (3 × 10 mL) extraction. Then MPPA was extracted as described above. Products were analyzed by a Varian gas chromatography (GC 430-GC-FID, Agilent Technologies Inc., Santa Clara, CA.) equipped with a flame ionization detector and a SGE analytical capillary column (BPX35, 15 m, i.d. 0.25 mm).

3. RESULTS AND DISCUSSION

3.1. Kinetics of Asymmetric MPPA Synthesis. In order to carry out process development in a rational way a basic kinetic analysis was performed, comparing amine donors ALA and IPA. In Figure 1, the kinetics of the ATA-catalyzed reductive amination reaction of benzylacetone (BA) is shown using IPA and ALA at different concentrations. In terms of initial reaction rate (U/mg) ALA resulted in approximately 30% higher activity than IPA. Although substrate inhibition in ATA-catalyzed reactions is known to occur,[8,35] no obvious substrate inhibition of either donor was detected within the tested concentration range (up to 0.8 M). The apparent Michaelis constant, K_m, for IPA and ALA were very similar, about 128 and 120 mM, respectively. Thus, a donor concentration of about 0.5 M may be sufficient for efficient synthesis allowing about 80% of the maximum reaction rate (V) of 0.05 and 0.07 U/mg for IPA and ALA, respectively (from fitted data in Figure 1). Donor concentrations at 0.5 M and 10 mM BA corresponds to a donor to acceptor ratio of 50, which is at the lower end of the values commonly reported for donor excess (50−100).[36] The specific activity of ATA-50 at 0.5 M IPA and 10 mM BA was determined to about 0.04 U/mg for the crude enzyme powder and 0.02 U/mg freeze-dried *E. coli* cells containing the same enzyme overexpressed. Compared to a previous study, which reported 0.003 U/mg activity for cells (with a different ATA) in a reaction using 6 mM BA and 1 M IPA,[8] the here obtained activity was almost 10-fold higher.

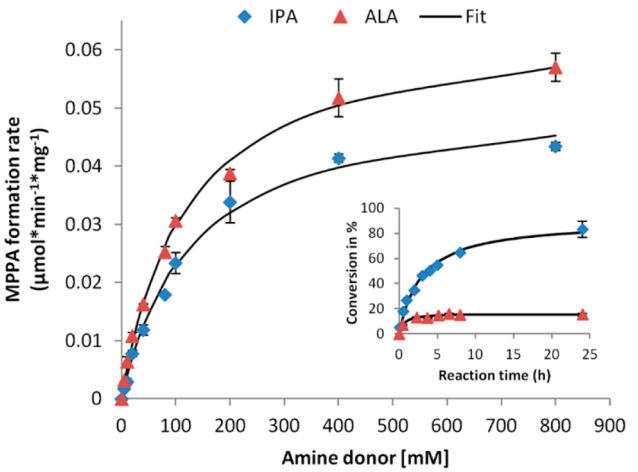

Figure 1. Kinetic comparison between two amine donors in the amine transaminase (ATA-50)-catalyzed amination of benzylacetone (BA). Although different in maximum reaction rates alanine (ALA) and isopropyl amine (IPA) feature a similar, apparent K_m value of 120 ± 8 mM and 128 ± 14 mM. The reaction progress curves (inset) show that about 90% and 16% of BA was converted into MPPA (1-methyl-3-phenylpropylamine) with IPA and ALA, respectively. Ketone concentrations were set to 10 mM at varying amine donor concentrations using 0.2 mg/mL ATA-50 crude powder.

The time courses of the reactions, depicted in the inset of Figure 1, show that for a simple batch reaction the highest conversion of the amine acceptor (BA) into the amine form (MPPA) was about 90% and 16% with IPA and ALA, respectively. Assuming that equilibrium was reached, the reaction equilibrium constants (K_{eq}) were calculated, and the IPA reaction featured the more favorable K_{eq} of 0.17 compared to only 0.0007 for ALA and both are consistent with literature values.[15]

When the BA concentration was varied at constant amine donor concentration (IPA or ALA), a relatively low apparent K_m of about 2.5 mM was determined (Figure 2). Fitting the data in Figure 2 to the Michaelis−Menten equation, the predicted maximum reaction rate (V) for 0.45 M donor was estimated to about 0.05 and 0.07 U/mg for IPA and ALA, respectively. Since higher BA concentrations above solubility (ca. 10.8 mM) are required to obtain V, the practical achievable rate is thus about 80% for both donors. The low K_m of the amine acceptor, however, may circumvent the necessity to increase the solubility of BA in the reaction phase. Normally, water miscible or immiscible solvents are employed to enhance substrate concentrations in ATA reactions.[37,33,38,39] In fact, cosolvent addition studies revealed poor ATA-50 stability (see SI, Figure S1). Among the tested water-miscible solvents ATA-50 exhibited the highest tolerance toward dimethyl sulfoxide (DSMO). The kinetic analysis given in Figure 3, however, shows that increased ketone solubility in the presence of 25% (v/v) DMSO had no beneficial effect on the rates. The reaction rates decreased drastically upon cosolvent addition, and the

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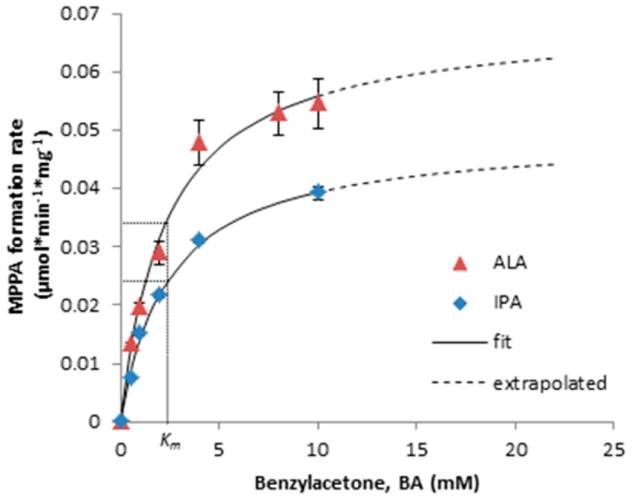

Figure 2. Influence of ketone substrate (BA) concentrations for the two different donors: isopropyl amine (IPA) and alanine (ALA). At the solubility limit of benzylacetone (BA, 10.8 mM) ATA-50 exhibited 80% of its theoretical maximum activity (1-methyl-3-phenylpropyl amine (MPPA) formation rate). For both donors a similar, apparent K_m value of about 2.5 mM was determined. Amine donor concentrations were set to 450 mM at varying ketone concentrations using 0.2 mg/mL ATA-50 crude powder.

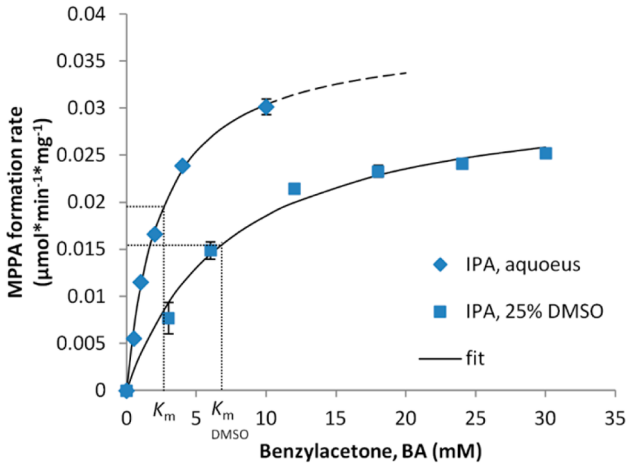

Figure 3. Effect of cosolvent addition on the ATA-50 kinetics. Increased ketone solubility was achieved upon dimethyl sulfoxide (DMSO, 25%, v/v) addition but associated with a drastic activity loss in MPPA (1-methyl-3-phenylpropyl amine) formation compared to no cosolvent (aqueous). Reaction conditions: 450 mM amine donor (isopropyl amine, IPA) in 20 mM sodium phosphate buffer, 0.1 mM PLP, pH 8 at 30 °C.

apparent K_m value (ca. 7.4 mM) for BA had more than doubled compared to no cosolvent (Figure 3). The use of organic solvents is not only problematic for the enzyme stability and kinetics but also complicates the downstream process as well as affecting the "greenness" of the process in total.[4] Hence, maintaining saturation of BA by feeding the ketone continuously the SLM methodology would allow for high conversions and high product concentrations despite low ketone solubility.

As expected, both product and coproduct inhibition were a serious problem. It was found that 2 mM PYR inhibited the reaction rate by about 50% ($IC_{50\%}$), and it was about 3 mM for the amine product (MPPA) (Figure 4). A similarly strong

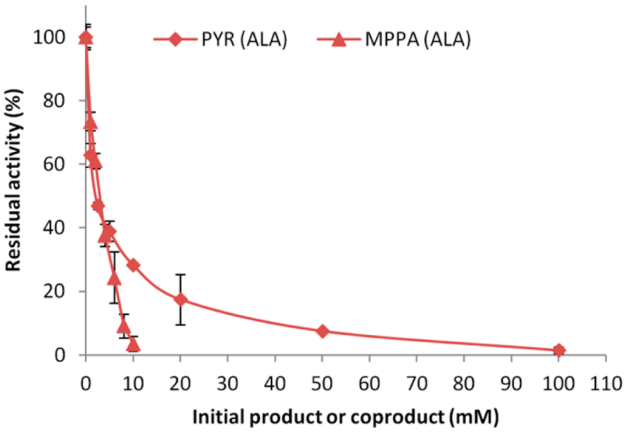

Figure 4. Product inhibition profiles when using alanine (ALA) and benzylacetone (BA) as a amine donor and acceptor, respectively. A similar half-maximal inhibitory concentration ($IC_{50\%}$) was determined for 1-methyl-3-phenylpropylamine (MPPA) and pyruvate (PYR) of about 2.5 mM and 3.0 mM, respectively. Varying concentrations of product and coproduct were added to constant amine donor (450 mM) and ketone (10 mM) concentration. The reaction pH was adjusted to 8 before 0.2 mg/mL ATA-50 crude powder was added.

inhibition ($IC_{50\%}$ of ~4 mM) was also determined for ATA-50 in whole cells (see SI Figure S2). The inhibition kinetics show that not only MPPA but also PYR removal is required to avoid a rapid decrease in reaction rate.

To achieve efficient removal of dual product inhibition, we attempted a new concept of the simultaneous in situ product and coproduct removal (s-ISPR) which is a combination of SLM-extraction of the product (MPPA) and an auxiliary enzyme for coproduct (PYR) removal.

3.2. Simultaneous in Situ Product and Coproduct Removal (s-ISPR). The process design of the s-ISPR system is schematically depicted in Figure 5 (see also SI Figure S7). The

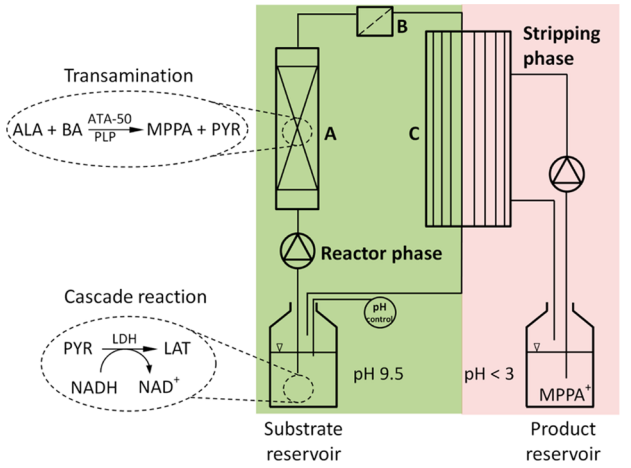

Figure 5. Simultaneous in situ product and coproduct removal (s-ISPR) system for the production of 1-methyl-3-phenylpropylamine (MPPA) from L-alanine (ALA) and benzylacetone (BA). The whole cell biocatalyst containing the amine transaminase (ATA-50) was immobilized in a packed-bed reactor (A). The reactor stream (reactor phase, green) was filtered (B) before entering the SLM extraction unit (C). MPPA was selectively extracted and enriched in the stripping phase (red). The coproduct pyruvate (PYR) was reduced in the reactor phase to lactate (LAT) by a lactate dehydrogenase (LDH) consuming the cofactor (NADH).

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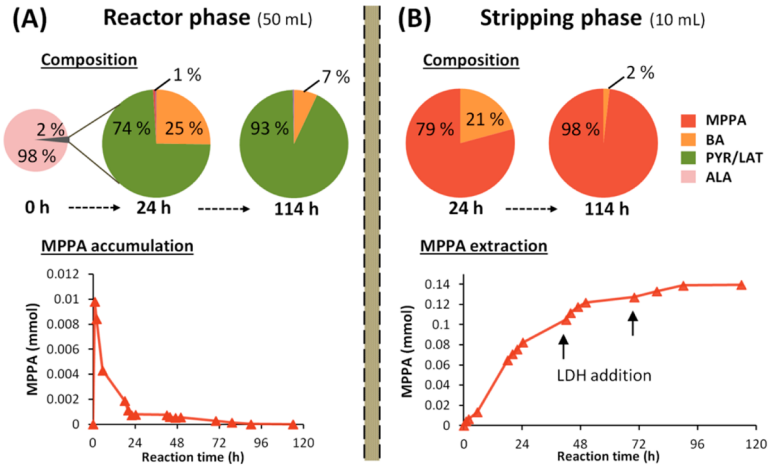

Figure 6. Amine production and extraction performance of the s-ISPR system. The composition of the (A) reactor and (B) stripping phase at different reaction times are given (pie charts). The amine substrate (alanine, ALA) was present in 50-fold excess (98%) compared to the ketone benzylacetone (BA). The product accumulation and extraction profile of MPPA in the (A) reactor and (B) stripping phase is given in mmol. 5 mg of LDH was added to the reactor phase initially as well as at 40 and 70 h.

whole cell biocatalyst (ATA-50 cells) was immobilized using chitosan flocculation.[34] This formulation, composed of 90% cells (10 g cells/g chitosan), retained nearly all of its activity compared to free cells (see SI Figure S3). Continuous ketone supply (as second phase) was initially tested but was rejected because the freely dissolved LDH enzyme (and also ATA-50 to some extent) appeared to be very sensitive to interfacial inactivation retaining hardly any activity (data not shown). In addition the resulting protein precipitates/aggregates caused clogging of the system. The pH induced inactivation (showing much less precipitations) was not causing any clogging, and a filter was sufficient to prevent particle entering the SLM module. In order to evaluate the feasibility of the s-ISPR system the reactor phase was initially saturated with BA, and no further BA was added.

Both enzymes ATA and cascade enzyme must remain catalytically active at alkaline conditions, because the efficiency of the SLM-extraction of the amine product MPPA is dependent on the difference between the pH of the reactor phase (9.5) and the pK_a of MPPA (10.5) (Scheme 1). ATA-50 remained catalytically active at pH 9.5 (data not shown). For the cascade reaction, however, two lactate dehydrogenases (LDHs) were chosen and tested under conditions related to the s-ISPR system. LDH from hog muscle exhibited activity at pH 9.5 and enabled roughly 30% conversion in a batch reaction together with crude ATA-50 enzyme (see SI Figure S4). Nonetheless, much higher conversion improvement was reported using LDH as cascade enzyme at around neutral pH.[40] But as the high pH caused protein precipitation, full inactivation was detected after a certain reaction time (data not shown). The little amount of precipitated LDH compared to the reactor volume could simply be retained through a filter. To compensate for the poor pH stability of the LDH, a fed-batch mode for the auxiliary enzyme supply was used in further experiments. Also, because sufficient for the purpose of demonstration, simple PYR removal was envisaged without any other enzyme recycling the cofactor NADH, which has been reported elsewhere.[23]

Using the s-ISPR setup shown in Figure 5, continuous extraction and enrichment of MPPA was achieved. Due to the volume difference in reactor (50 mL) and stripping phase (10 mL), MPPA was concentrated 5-fold during the process. For comparison, we refer to amounts (mmol) or content (%, n_i/n_{total}) of substrates and products rather than concentrations. As can be seen in Figure 6B, during a reaction time of 24 h, the amine product (MPPA) amount increased linearly in the stripping phase, suggesting constant MPPA production and extraction rates. About 50% of the final MPPA (0.14 mmol at 114 h) was produced within the first 24 h of the reaction (which was about 20% of the total operation time). Accumulation of MPPA on the reactor side was detected only at a very early stage of the reaction (Figure 6 A), when the reaction rate was high due to sufficient BA amount and low inhibition. After 24 h the extraction rate of MPPA decreased (Figure 6B). The MPPA amount in the reactor phase was orders of magnitude lower compared to the stripping phase and exhibited a further decrease until process termination (Figure 6A). Considering reaction rates, a decrease in MPPA production rate was probably caused by reduced ketone substrate (BA) availability and increasing concentration of PYR. The measured composition of the reactor phase given in Figure 6 at 24 h revealed that the BA content (25%) was lower than that of the coproduct (75%). In fact, due the hydrophobicity of BA a significant amount partitioned into the membrane solvent as well as into the plastic tubings. Thus, the membrane solvent acts as a substrate reservoir. Here is another strong benefit of the SLM strategy; since it utilizes a very small solvent volume, less BA would be captured compared to extraction alternatives including a separate organic phase.[41] In addition, back-extraction of BA was observed in the later stage of the reaction. The measured BA amount in both phases depleted while the MPPA amount almost doubled from 24 to 114 h (Figure 6B). When the experiment was terminated (at 114 h), some BA remained in the system. Conclusively, to achieve a high final purity, the feeding of BA should be stopped, and the residing ketone in the stripping phase and membrane phase then diffuses back into the reactor phase where it is converted.

Accumulation of the coproduct PYR was another important factor influencing the overall conversion rate. PYR was converted into lactate (LAT) by LDH and NADH added at the start of the reaction and twice later at 40 and 70 h. The

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further additions of LDH clearly enhanced the production of MPPA, shown as accumulation in the stripping phase (Figure 6B). Evident from Figure 6B, almost 20% of the total MPPA amount was produced and extracted upon the second LDH addition (40 h), which demonstrates the importance of PYR removal. However, it also shows that sequential addition of small LDH/NADH amounts could be an option. Once the reaction rate has decreased to a certain threshold, it can quickly be reestablished by LDH/NADH addition. It is likely that substantial improvements in the process can be achieved using auxiliary enzyme(s) with better stability than the LDH used here. The combination of continuous ketone supply and effective cascade reaction would maintain the initially (24 h) observed MPPA production/extraction rate shown in Figure 6B and thus yielding a much higher productivity.

Although far from an optimized process, the presented concept achieved very high product purity without any additional purification steps. The use of ALA in the s-ISPR approach facilitated a direct purity of ≥98% MPPA in the stripping phase (Figure 6B). This represents a major advantage in contrast to other existing ISPR strategies, because no other method allows such selective amine isolation of the desired product from the other components. This also includes the previously reported single ISPR method employing SLM extraction at a lower reactor pH (9.0) together with IPA and ACP (which has a 5-fold higher solubility than BA).[31] Even higher purity can be attained if the contaminating BA (here about 2%) is allowed to back-extracted completely before the amine product is harvested (SI, Figure S5). Thus, no further purification step may be needed, and a high isolated yield can be achieved.

It is worth mentioning that the stripping phase and so the purity remained unaffected by operating the s-ISPR with an excess of amine donor as long as it features a charged character. ALA was confirmed as excellent amine donor, because it remained on the reactor side (see SI, Figure S6). Since the maximum soluble amine acceptor (BA) concentration was about 10 mM (0.5 mmol, which is ∼2% of total substrate), the aqueous reactor phase mainly contained ALA (∼98%, 0.5 M, see Figure 6) throughout the entire course of reaction. Normally, a large excess of amine donor imposes separation and impurity problems using other processes, such as 2-phase extraction or ionic exchange resins,[41,27] which do not facilitate selective in situ amine product recovery strategies. With the s-ISPR concept, in fact, the donor excess can be minimized, because both the kinetic (inhibition) and the thermodynamic (equilibrium) barrier are controllable. Yet, the amine donor needs to be available in sufficient amounts, supplied either batch-wise or continuously, in order to maintain the reaction velocity.

3.3. s-ISPR Product Scope. This work is focused on the asymmetric MPPA synthesis in high purity from BA using the simultaneous in situ product removal (s-ISPR) approach. ALA represents the key to high purities since it is not extracted compared to using IPA as an amine donor.[31] In the following we give a brief description of the general applicability of the s-ISPR to a broad range of amine products and its extraction principle depicted in Scheme 1. In principle, any amine which is soluble in organic solvents could be extracted, purified, and enriched. In the s-ISPR approach both the reactor pH and the membrane solvent influence the extraction of the amines. Only the deprotonated form extracts from the reactor phase into the thin solvent film (Scheme 1). To achieve this, the reactor pH is selected to be relatively close to the pK_a value of the amine product. Note, the SLM extraction allows one-way extraction. The deprotonated amine becomes reprotenated once it diffuses from the solvent into the stripping phase (low pH), thus preventing back-extraction. The stripping phase should have a pH preferable three units below the pK_a, but needs to be maintained, for example, by high buffer concentrations or active titration (e.g., for continuous process and in large scale). Hence, the amine product determines the pH gradient from the reactor to the stripping phase required for sufficient extraction. Many modern drugs contain pyridines and other nitrogen containing heterocycles,[42] some of which might contain pH sensitive moieties. Generally, aromatic amines feature a lower basicity than aliphatic ones, for example, benzimidazole (pK_a = 5.5) or 2-aminopyridine (pK_a = 6.8) compared to *sec*-butylamine (pK_a = 10.6). Producing such class of amines with the s-ISPR strategy would also be possible, because the pH of the reactor and stripping phase can be selected according to the product pK_a to ensure sufficient extraction and mild enough conditions for the sensitive amines. A difference in pK_a can also be found among primary amines containing aromatic structures, such as MBA and MPPA. Due to the more closely adjacent phenyl ring the pK_a of MBA (9.6) is about one unit lower than that of MPPA (10.6). Previously, Rehn et al. have synthesized and extracted MBA at pH 9.0, which constituted a good compromise between reaction and extraction performance.[31] In this study, however, synthesizing MPPA a higher reaction pH (9.5) was required to achieve sufficient extraction.

4. CONCLUSIONS

The conceptually novel approach, simultaneous in situ product and coproduct removal (s-ISPR), employing ALA as amine donor in combination with supported-liquid membrane (SLM) extraction and a cascade reaction affords the amine product in high purity (≥98%) without any additional purification step. In contrast, for reactions using IPA as an amine donor coextraction causes contamination of the product solution and to a loss of donor substrate.[31] Thus, despite its often more favorable reaction equilibrium, IPA reactions require further downstream operations to yield similar purity as achieved with ALA. Another key point of the s-ISPR is that it allows the simultaneous removal of product and coproduct, which cannot be achieved by any other methods at present. Other advantages of employing ALA as amine donor represents its wide acceptance among ATAs, which is not the case for IPA,[33] and a faster reaction velocity compared to the unnatural amine donor (IPA). In principle, a single s-ISPR process setup could be used for the production of different types of chiral amines with high purity. Noteworthy, the (alkaline) reaction pH can be adjusted according to the pK_a of the amine product and, similarly, the acid pH of the stripping phase. This, in turn, permits the application of pH sensitive substrates and/or products, such as nitrogen containing aromatic heterocycles.

Overall, our study represents a proof-of-concept for the s-ISPR strategy using ATAs, but it requires certain optimization for increased performance. Two major bottlenecks were encountered: first, the pH stability of the auxiliary enzyme(s) and, second, a more sufficient supply of the ketone substrate. The development of a whole-cell system furnished with a transaminase and the entire cofactor regeneration system (e.g., LDH/GDH) is currently under investigation. Immobilized cells would then permit the supply of the poorly water-soluble ketone via a second phase. This targets both limitations

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mentioned above, and a direct comparison between IPA and ALA including further s-ISPR optimization will then be reasonable.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental information on solvent stability and immobilization of ATA, screening of LDH in batch, as well as a detailed setup of the s-ISPR system with illustrations and pictures are provided. This material is available free of charge via the Internet at http://pubs.acs.org/.The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.oprd.5b00055.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACE, acetone; ADH, amine dehydrogenase; ALA, L-alanine; ATA, amine transaminase (omega-transaminase); BA, benzylacetone (4-phenyl-2-butanone); DMSO, dimethyl sulfoxide; GDH, glucose dehydrogenase; IPA, isopropyl amine; ISPR, in situ product removal; IScPR, in situ coproduct removal; s-ISPR, simultaneous in situ product and coproduct removal; LDH, lactate dehydrogenase; MPPA, 1-methyl-3-phenypropyllamine; NADH, β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate; PLP, pyridoxal 5′-phosphate; PYR, sodium pyruvate

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