Investigation of high molecular weight amine donors for the process intensification in chiral amine synthesis with transaminases

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Contents

List of abbreviations

α-MBA	α-Methylbenzylamine
3DPPA	3,3-Diphenylpropionic acid
3LP	Three-liquid-phase
AD	Amine donor
ADH	Alcohol dehydrogenase
AE	Atom economy
AlaDH	Alanine dehydrogenase
ALS	Acetolactate synthase
AmDH	Amine dehydrogenase
API	Active pharmaceutical ingredient
(A)TA	(Amine) transaminase
BA	Benzylacetone
BY	Biocatalyst yield
c	Conversion
DIPE	Diisopropyl ether
DKR	Dinamic kinetic resolution
DMSO	Dimethyl sulfoxide
DSP	Downstream processing
ee	Enantiomeric excess
EMC	Enzyme membrane contactor
EMR	Enzyme membrane reactor
FDH	Formate dehydrogenase
FS	Flat sheet
GDH	Glucose dehydrogenase
HF	Hollow fiber
HMW	High molecular weight
ISPR	*In situ* product recovery/ removal
IPA	Isopropylamine
IRED	Imine reductase
KR	Kinetic resolution
L-L	Liquid-liquid
LDH	Lactate dehydrogenase
MAO	Monoamine oxidase
MPPA	1-Methyl-3-phenylpropylamine
MTBE	Methyl tert-butyl ether
MW(CO)	Molecular weight cut-off
NADH	Nicotinamide adenine dinucleotide
NCS	Norcoclaurine synthase
NF	Nanofiltration
OSN	Organic solvent nanofiltration
P	Product concentration
PDC	Pyruvate decarboxylate
PEA	α-Phenylethylamine
PEG	Polyethylene glycol
PMP	Pyridoxamine-5'-phosphate
PLP	Pyridoxal-5'-phosphate
PV	Pervaporation
RedAm	Reductive aminase
SFS	Solvent-free system
SLM	Supported liquid membrane
STY	Space time yield
WT	Wild type
Y	Yield

Summary and list of publications

The synthesis of several bioactive compounds and active pharmaceutical ingredients relies on the development of general and efficient methods to prepare optically pure amines. Transaminases are industrially relevant enzymes and are useful for synthesizing a large number of compounds that contain a chiral amine functionality. Although the immense potential associated to the use of these biocatalysts, the equilibrium position is often unfavorable for amine synthesis. The use of an excess of amine donor, compared to the ketone substrate, combined with selective removal of the formed product, can help in overcoming this limitation.

This work mainly focused on broadening the application of membrane-based *in situ* product recovery (ISPR) techniques for the transaminase-catalyzed synthesis of chiral amines. The overall work was designed around the implementation of amine donors, possessing considerably larger molecular 'size' compared to commonly used amine donors. To clearly distinguish these molecules from traditional donor amines, we designate them as High Molecular Weigh amine donors. With a molecular weight between 400 and 1500 g/mol, in contrast to traditional donor amines, HMW amine donors enable a size-based separation between amine donor and amine product molecules. HMW amines, provided in excess for thermodynamic equilibrium shifting can thus be simply retained by a size-exclusion mechanism by commercial membranes, while the smaller product amines are permeated. Therefore, a selective recovery of the desired chiral amine product is possible. The implementation of ISPR techniques using HMW amine donors can theoretically lead to (i) equilibrium shifting, (ii) alleviation of product inhibition, and (iii) a highly pure product stream.

The feasibility of using HMW amine donors in aqueous, organic solvent and solvent-free media for the transaminase-catalyzed synthesis of 1-methyl-3-phenylpropylamine (MPPA) was proven in this thesis. The latter two approaches were investigated with the aim to achieve higher product concentrations. Along with that, we demonstrated two membrane-assisted ISPR proof of concepts. Specifically, nanofiltration was coupled with the enzymatic reaction performed in aqueous media (**Article I**), while liquid-liquid (L-L) extraction in a contactor was applied for transamination in organic solvent media (**Article II**). As an alternative to membrane-based strategies we also designed a spinning reactor concept for the integrated chiral amine synthesis (in organic solvent) and recovery (**Article III**).

Article I **Application of novel High Molecular Weight amine donors in chiral amine synthesis facilitates integrated downstream processing and provides in situ product recovery opportunities.**

Claudia Matassa, Dominic Ormerod, Uwe T. Bornscheuer, Matthias Höhne and Yamini Satyawali. *Proc. Biochem*. **2019**, *80*, 17-25.

Article II **Jeffamine® ED-600: a polyether amine donor for enzymatic transamination in organic solvent/ solvent-free medium with membrane-assisted product extraction.**

Claudia Matassa, Alessandra Romani, Dominic Ormerod, Uwe T. Bornscheuer, Matthias Höhne and Yamini Satyawali. *J. Chem. Technol. Biotechnol*. **2020**, *95*, 604-613.

Article III **Three-liquid-phase spinning reactor for the transaminase-catalyzed synthesis and recovery of a chiral amine**

Claudia Matassa, Dominic Ormerod, Uwe T. Bornscheuer, Matthias Höhne and Yamini Satyawali. *ChemCatChem* **2019***,* DOI: 10.1002/cctc.201902056.

1 Background

1.1 Chiral amines, importance and synthesis

In 1841, the French chemist and biologist Louis Pasteur separated by hand the two isomers of sodium ammonium tartrate, hence discovering the phenomenon of chirality.[1,2] However, it was only in the early twentieth century that A. Cushny established the relevance of chirality in biology, particularly in pharmacology, by comparing the pharmacological effects of atropine and its enantiomer hyoscyamine in animal studies. [3,4]

Chirality is at the base of life on Earth, as chiral molecules and 'chiral biological architectures' compose any living organism. Most of the proteins, enzymes, amino acids, carbohydrates, nucleosides and a number of alkaloids and hormones are chiral compounds and occur in a single enantiopure form. The chiral nature of biological systems has important consequences, especially for the physiological activity of pharmaceuticals, as the desired activity of a drug is often associated to just one enantiomer. The other enantiomer might be inactive or can even show undesired biological side effects. In 1992, after the tragic experience of the thalidomide drug,* the US Food and Drug Administration (FDA) recommended the assessments of each enantiomer activity for racemic drugs in the body and introduced strict regulations for the development of new chiral drugs as single enantiomers.[5] Following the introduction of these rules, the demand for drugs as well as many other synthetic products in an enantiopure form has continuously increased. The development of different and successful methods to obtain molecules in a non-racemic form is now a top-class subject for academic research as well as for industry.

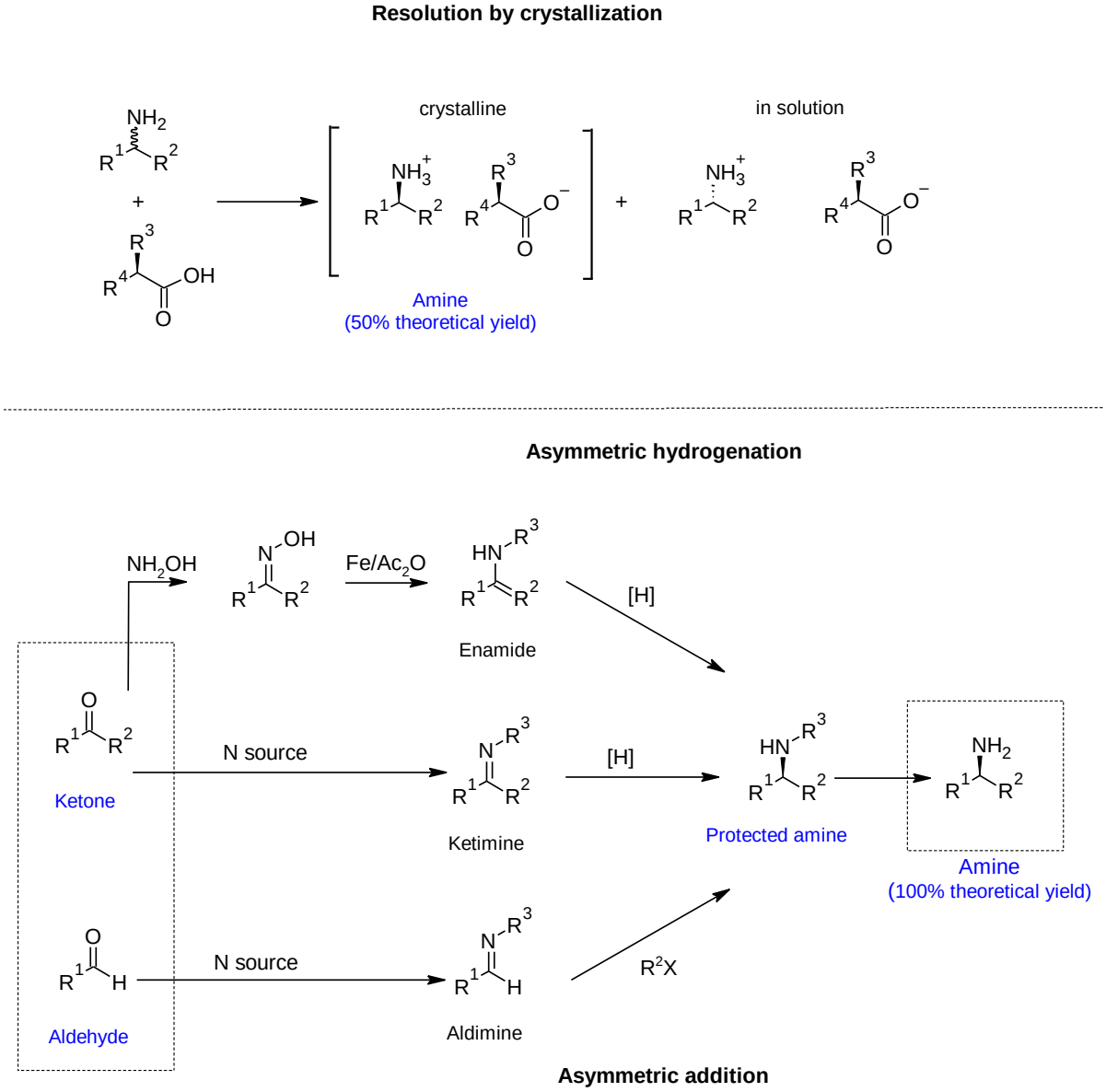
Chiral amines are key intermediate products in active pharmaceutical ingredients (API), agrochemicals and fine chemicals. It is estimated that approximately 40% of the new chemical entities contain one or more chiral amine building block.[6] Therefore, the development of broadly applicable strategies for their synthesis is of great interest.

1.2 Chemical and biocatalytic routes to optically active amines

Most of the chiral amines are produced chemically. The traditional route for accessing chiral amines is the resolution of racemates by precipitation of one enantiomer as diastereomeric salt (Scheme 1, top). Resolving agents such as chiral carboxylic acids are often used for this

* Only the (*R*)-enantiomer of this molecule possessed a sedative property while the opposite (*S*)-enantiomer had teratogenic effects.

purpose.[7] Although this technique is still of considerable importance, the maximum yield of the desired enantiomerically pure product is 50%, while the other 50% is generally discarded or has to be racemized.



Scheme 1. Chemical routes to chiral amines[8,9]

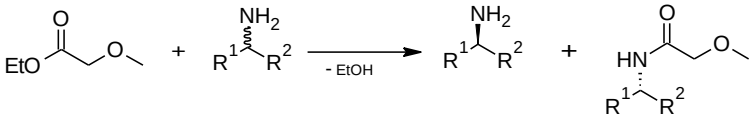
The alternative is to perform the asymmetric reduction of prochiral precursors to yield chiral products, with a theoretical yield of 100% (Scheme 1, bottom). Asymmetric hydrogenation and asymmetric addition are the two most established reduction methods. In the asymmetric hydrogenation, the ketone substrates are either converted to ketimines or enamides, which are then stereoselectively reduced to protected amines. In the asymmetric addition, the aldehydes are converted to imines. Protected amines are then formed by addition of carbanions or radicals to the aldimine.

From an industrial view point, the introduction of the nitrogen by a simple, preferably a one-step procedure, which features a high chemo-, regio-, diastereo-, and enantiocontrol is challenging.[10] Also the cleavage of the complex and expensive auxiliary groups (R^3, Scheme 1), often used to introduce the enantioselectivity, can be difficult.[9] Moreover, the enantioselectivity is often not perfect (products having < 95% ee) and therefore further purification steps are required to obtain compounds suitable for pharmaceutical applications. In contrast to chemical methods, enzymatic synthesis operates usually under mild conditions, avoiding the need of highly flammable metal-organic reagents or heavy metal contamination.[8] Enzymes, intrinsically able to differentiate between enantiomers of a racemic substrate, can impart high regio- and chemoselectivity to the transformation. New enzymes, able to catalyze an increasing number of reactions, are being continuously discovered either by protein engineering of known enzymes or by identifying wild-type (WT) enzymes possessing the desired specificity. Once characterized, protein engineering techniques are applied for broadening the often limited substrate scope of the biocatalysts or to engineer operating stability and tolerance to the used co-solvents and reagents.[11,12] Finally, the combination of protein engineering strategies and process engineering techniques are often applied for optimization of a developed biocatalytic process aiming at industrial scale applications.[13–17]

Enzymatic chiral amine synthesis is nowadays performed employing a large number of enzymes (Scheme 2) in both water and organic solvents as reaction media. Extensive research is conducted in both academy and industry sectors. For instance, BASF AG produces optically pure aliphatic amines, benzyl amines and amino alcohols on multiton scale employing *Burkholderia plantarii* lipase.[18] **Lipases** are hydrolytic enzymes, members of the hydrolase enzyme class. They catalyze the amide hydrolysis in aqueous systems or the acylation of amines in non-aqueous systems. The key success of the BASF process was the choice of the highly activated ethyl methoxyacetate as the acyl donor, which prevents the occurrence of non-enzymatic side reactions.[8] Although the BASF process is highly optimized, the reaction is conducted in kinetic resolution (KR) mode (Scheme 2, I). Therefore, the yield of the target chiral amine is limited to 50%. A racemization step could be included to facilitate a dynamic kinetic resolution (DKR). However, due to the harsh conditions needed to racemize the unwanted chiral amine, a DKR approach is still challenging.[8] **Monoamine oxidases** (MAOs), members of the oxidoreductase class of enzymes, selectively oxidize one amine enantiomer to the corresponding imine with simultaneous reduction of oxygen to hydrogen peroxide. The MAO-mediated enantioselective oxidation can be combined with a non-selective chemical reduction of the formed achiral imine. Over several cycles of enzymatic oxidation and chemical reduction, the starting racemate is converted to the target chiral amine enantiomer with yields up to 100% and with excellent enantiomeric purities (Scheme 2, II).[19]

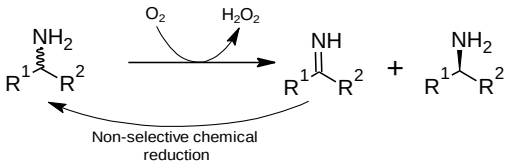
The most employed MAO originates from *Aspergillus niger* (MAO-N). Over the last decade, the group of Turner has combined directed evolution with rational design thus developing a tool-box of MAO-N variants which can generate enantiomerically pure primary, secondary and tertiary amines.[20] The applicability of MAO-N on an industrial scale has been also demonstrated. By applying protein engineering, Codexis and Merck obtained a new variant, employed for producing a key intermediate in the synthesis of boceprevir, a drug for the treatment of hepatitis C.[21] Studies for producing pharmaceutical building blocks and alkaloid natural products synthesis have also been reported.[19,22] **Amine dehydrogenases** (AmDHs) catalyze the NAD(P)H-dependent reductive amination of ketones to amines in the presence of ammonia as amine substrate (Scheme 2, III). The discovery and identification of AmDHs is the result of extensive protein engineering on the α-amino acid dehydrogenases leucine and phenylalanine dehydrogenase[23,24] as well as on lysine-ε-dehydrogenase.[25] For the cost-effective application of dehydrogenases, a cofactor regeneration is required. In the work of Li and co-workers, (*R*)-amphetamine and (*R*)-1-methyl-phenylpropylamine were produced with 95% conversion and each with >98% ee in reactions, in which NADH was recycled using glucose dehydrogenase (GDH).[26] Mutti and co-workers performed the reductive amination of a range of aromatic and aliphatic ketones by replacing GDH with formate dehydrogenase (FDH) for co-factor recycling.[27] Carbon dioxide formed as by-product is highly volatile and this leads to a favorable shift of the equilibrium. In addition, Mutti et al. combined alcohol and amine dehydrogenases in an elegant system for the one-pot transformation of racemic alcohols to chiral amines.[28] The limited substrate scope, main drawback of AmDHs, is being progressively overcome. The recent discovery of a family of AmDHs possessing significant activity towards ketones and aldehydes without a carboxylic acid group, expanded the biocatalytic toolbox of available enzymes for asymmetric reductive amination reactions.[29] While the use of AmDHs is limited to the preparation of primary amines, **Imine reductases** (IREDs) catalyze the NADPH-dependent asymmetric reduction of prochiral cyclic imines to chiral secondary cyclic amines (Scheme 2, IV).[30–33] The recently discovered **reductive aminases** (RedAms), a sub-group of IREDs, can also perform the single-step asymmetric reductive amination of ketones, hence generating primary, secondary, and tertiary chiral amines (Scheme 2, V).[33–37] An IRED possessing (i) high activity for imine formation from ketone and amine; (ii) high enantioselectivity for imine reduction; and (iii) broad substrate tolerance with respect to both amines and ketones, was recently discovered and characterized.[38] This work highlighted the unique and attractive properties of RedAms for the biocatalytic preparation of industrially important amines from prochiral ketones.

(I) LIPASES



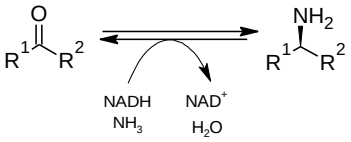
- Broad substate scope
- Industrially applied
- 50% maximum yield
- High solvent stability

(II) MONOAMINE OXIDASES (MAOs)



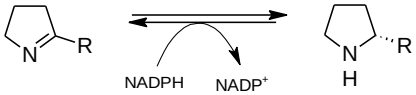
- Secondary and tertiary amines accessible
- No upscale because of inhibition
- 100% theoretical yield

(III) AMINE DEHYDROGENHASES (AmDHs)



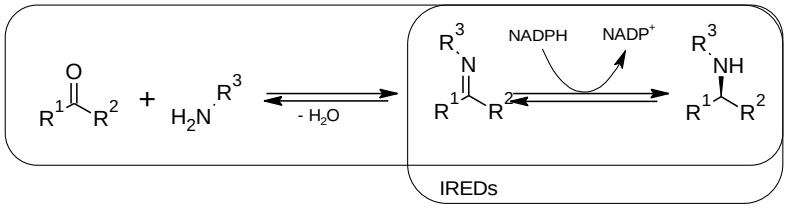
- Co-factor recycling necessary
- Cheap nitrogen donor
- 100% theoretical yield
- Often poor solvent stability

(IV) IMINE REDUCTASES (IREDs)



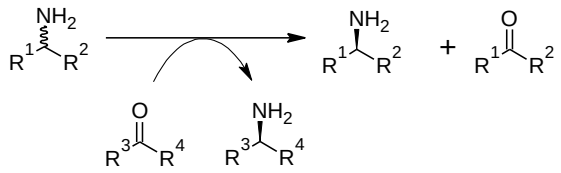
- Slow reaction for non-cyclic amines
- 100% theoretical yield

(V) REDUCTIVE AMINASES (IREDs)

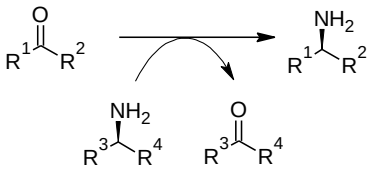


- Secondary and tertiary amines accesible from prochiral ketones
- 100% yield possible

(VI) AMINE TRANSAMINASES (ATA)



- Thermodynamic equilibrium unfavorable
- 50% yiled possible
- Prodcut and substrate inhibition



- Unfavorable thermodynamic equilibrium
- 100% yield possible
- Prodcut and substarte inhinbition

Scheme 2. Enzymatic routes to optically pure amines.[8]

Pictet-Spenglerases, novel biocatalysts for the synthesis of secondary amines, have been recently identified and applied. Specifically, Norcoclaurine synthase (NCS) from *Thalictrum flavum* has been employed in combination with carboligase and with a transaminase (TA) for

the synthesis of a pharmaceutically important alkaloid (1,3,4-trisubstituted THIQs with three chiral centers).[39,40] Furthermore, a biocatalytic cascade involving ω-TAs and the Pictet-Spenglerase enzyme Strictosidine synthase was studied for the chemo-enzymatic synthesis of C3-methyl-substituted enantiopure Strictosidine derivatives.[41] Finally, a last group of enzymes suitable for the biocatalytic synthesis of amines are derived from **P450 monooxygenases**: Protein engineering created catalysts that facilitate intramolecular C-H aminations.[42,43]

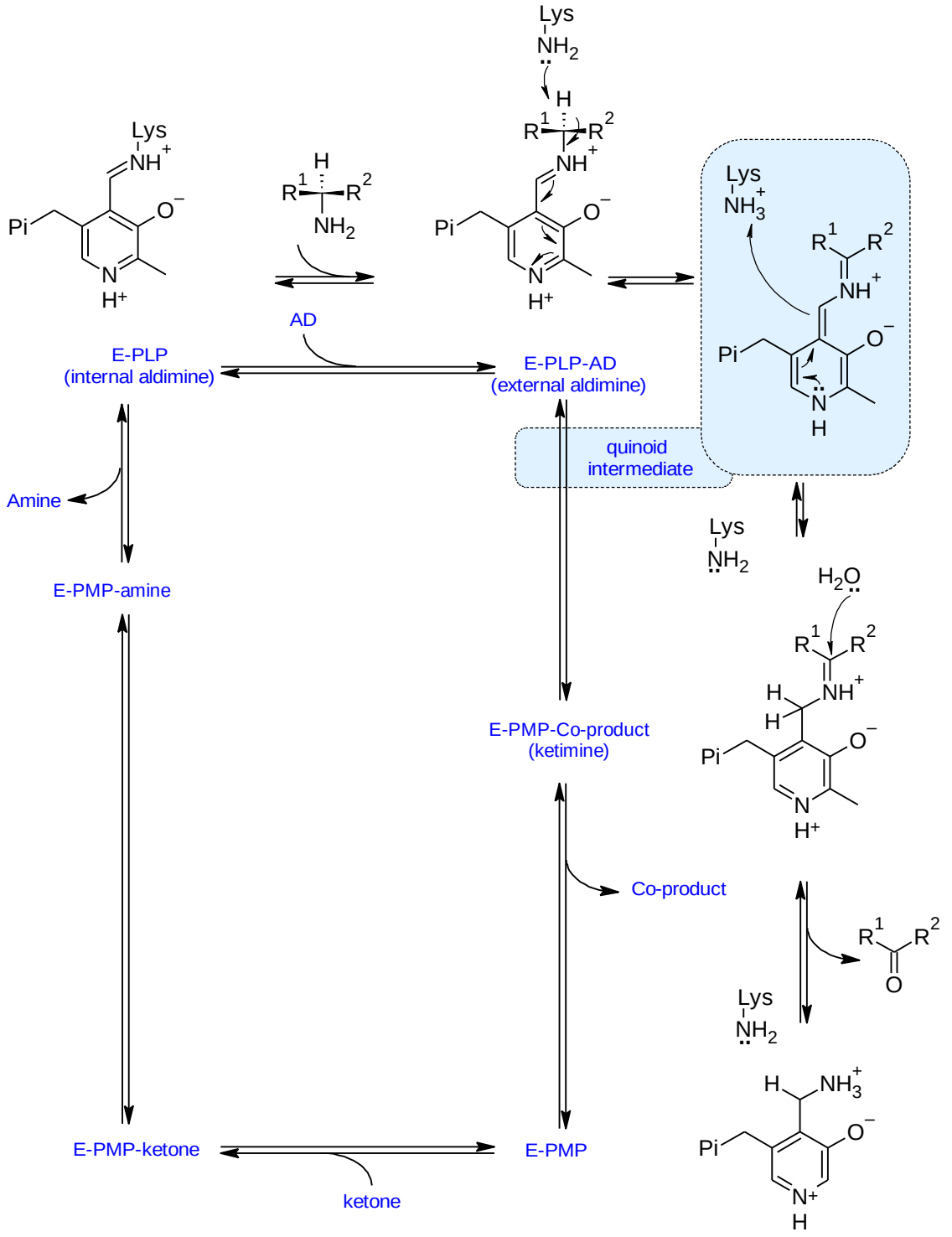
1.3 Transaminases

Transaminases (TAs) catalyze the transfer of an amino group of a donor amine to the carbonyl carbon atom of an α-keto acid, a ketone, or an aldehyde substrate employing the cofactor pyridoxal-5'-phosphate (PLP), one of the most relevant and versatile cofactors in nature. Based on the distance (number of carbon atoms in the substrate structure) between the amino group and the carboxylate function, transaminases have been grouped into α-TAs (catalyze the transfer of the amino group at the α-carbon) and ω-TAs (the amino group transferred in the reaction is located further away from the carboxylic moiety). Of particular interest and use in chemo-enzymatic routes for the production of chiral amines are the amine transaminases (ATA).[44–46] In contrast to α- and most ω-TAs, ATA are able to accept a large variety of carbonyl compounds – both aldehydes and ketones – as substrates and do not require the presence of a carboxylic group in the substrate molecule. As ATAs are the protagonists of this work, the following sections will specifically focus of ATA-catalyzed chiral amine synthesis.

1.3.1 Reaction mechanism

All transaminases follow a ping-pong bi-bi reaction mechanism[47–49] which involves two half reactions. In the first half reaction the amino group of the amine donor (AD) is transferred to PLP to form pyridoxamine-5'-phosphate (PMP). The ketone co-product is therefore released in the reaction environment. In the second half reaction the amino group is transferred from PMP to the amine acceptor (the ketone substrate) producing the corresponding amine product and regenerating the PLP cofactor (Scheme 3). Substrate and product inhibition are direct consequences of the reaction mechanism.[50] The substrate may bind to the 'wrong' free form of the enzyme forming abortive dead-end complexes (e.g., E-PLP-ketone or E-PMP-amine). Furthermore, every step of the catalytic cycle is reversible, therefore the equilibrium has to be pushed towards products to ensure high product yields. Possible protein and process

engineering solutions for circumventing product/substrate inhibition and for shifting the thermodynamic equilibrium will be discussed in the coming paragraphs.



Scheme 3. Reaction scheme of the transamination. The first half catalytic cycle is illustrated.

1.3.2 Synthetic approaches for ATA-mediated chiral amine synthesis

Transamination can be performed either as a KR of a racemic amine or as an asymmetric synthesis starting from a ketone (Scheme 2, VI). A combination of both strategies is also possible: the ketone generated by kinetic resolution can be aminated employing an ATA with opposite enantiopreference.[8] **The kinetic resolution** of primary amines requires stoichiometric amounts of an amino acceptor, as the reaction equilibrium favors product formation. The first large scale synthesis of enantiopure aliphatic and aromatic amines in KR mode was reported by Celgene Corporation in the early 1990s.[51] Since then, the KR approach has been extensively explored.[52–56] The main disadvantage of this strategy is the low atom efficiency, with a maximum theoretical yield of 50%. In addition, the low value formed ketone-product from corresponding amines often causes severe inhibition of the enzyme. Thus, the removal of the formed ketone to circumvent product inhibition is highly desirable. Various process-based strategies, to remove the ketone products and thus to shift the equilibrium of the reversible reaction towards product formation have been implemented (e.g. biphasic systems,[57–59] enzyme-membrane reactors[60,61] or reduced pressure systems[62]). Notably, very few transaminases display very little susceptibility to product inhibition.[13] For example, the ATA from *Ochrobactrum anthropi* enabled efficient kinetic resolution of α-methylbenzylamine (α-MBA) on 500 mM substrate loading without application of product removal strategies.[63] Also the ATA from *Mycobacterium vanbaalenii* showed very low product inhibition by ketone product. Specifically, this ATA was employed for the KR of aliphatic and aromatic amines on 100 mM substrate loading using pyruvate as amino acceptor. Moreover, the KR of α-MBA was successfully carried out by using acetone, an amino acceptor which is cheaper than pyruvate.[64] Improving the conversions in terms of atom economy and yield efficiency requires a distinct approach which allows theoretical yields of 100% to be obtained. This can be achieved by a **dynamic kinetic resolution** or deracemization approach, by employing two enantiocomplementary ATAs in a one-pot two-step deracemization process. In the first step, the entioselective deamination of the racemic amine gives ≤50% enantiopure amine and the corresponding ketone co-product. In the second step, the ketone is stereoselectively aminated by an enantiocomplementary ATA, forming the optically pure amine in up to 100% yield. Shin et al. performed simultaneous deamination/transamination steps for the deracemization of a series of aromatic amines affording conversions above 70%.[65] Koszelewski et al. reported a one-pot, two-step deracemization procedure employing (*R*) and (*S*)-selective commercial transaminases, purchased from Codexis for the DKR of pharmacologically relevant chiral amines.[66] The system was further improved by combining the first KR step with an amino acid oxidase for the in situ recycling of pyruvate and applied for the synthesis of mexiletine, an antiarrhythmic agent.[67] Single transaminase DKR

processes were also investigated. The synthesis of (*R*)-4-phenylpyrrolidin-2-one derivatives, key intermediates for the synthesis of 3-phelyl-γ-aminobutyric acid (3-phenyl-GABA), was conducted via a DRK by stereoselective amination, utilizing the commercially available ATA-117.[68] The synthetic strategy investigated in this work allowed to prepare the desired compounds within only three steps starting from commercial substrates, hence avoiding the cumbersome synthesis of racemic amine derivatives and reducing the number of reaction steps required in other approaches. A similar strategy was adopted for the synthesis of β-chiral 2-phenyl-1-propylamine derivatives, starting from the corresponding styrene derivatives.[69]

Similar to DKR, **asymmetric synthesis** of enantiopure amines can generate a theoretical yield of 100%. In asymmetric synthesis, prochiral ketones are aminated to the corresponding chiral amines. Undoubtedly, asymmetric synthesis is the most favored approach in biocatalytic amine synthesis. Nevertheless, deracemization is also attractive, especially when access to racemic amines as substrates is easier than to the corresponding prochiral ketone.[40] The first ATA-catalyzed asymmetric synthesis of chiral amines was described by Shin and Kim.[59] This study represents also the first *in situ* (co-)product removal application for thermodynamic equilibrium shifting and for preventing the accumulation of the inhibitory co-product pyruvate. Since then, a large number of chiral amines have been synthesized in an asymmetric synthesis fashion using transaminases.[13,40,70] The asymmetric synthesis of a large range of aliphatic and aromatic chiral amines has been recently carried out using diamine transaminases (SpuC ATA) with *n*-butylamine as amine donor, in small excess.[71] Aryl-amines with various boron-functionalities[72] and pyridylalkylamines, bearing different size alkyl chains on the C-2 position of the aromatic ring,[73] have been successfully synthesized employing a variety of (*R*)-and (*S*)-selective transaminases. Furthermore, studies focusing on the amination of functionalized cyclic ketones revealed the feasibility of performing the one-step asymmetric synthesis of 2-methylcyclohexylamine, a chiral amine with two chiral centers, starting from racemic starting material.[74] The first transaminase-catalyzed synthesis of an amine containing a bicyclic bridged moiety was recently reported. It involves the use of an evolved mutant of the ATA from *Ruegeria sp*., 3FCR.[75] Combination of rational protein design with random mutagenesis by error-prone PCR was required to identify the most suitable variants, similar to the findings reported by Savile et al. for the development of the (*R*)-transaminase employed for the synthesis of Sitagliptin.[76] Rational protein design was also applied to the (*S*)-selective ATA from *Vibrio fluvialis* to catalyze (1*S*)-1-(1,1'-biphenyl-2-yl)ethanamine, using isopropylamine (IPA) donor. While the wild type enzyme showed no activity, the best variant converted 42 % of 1 g/L of the substrate yielding the corresponding (*S*)-amine product with an enantiomeric excess (ee) value of > 99% ee.[77] Furfurylamines,

important as monomers in biopolymer synthesis and for the preparation of pharmacologically active compounds, have been synthesized by direct amination of furfural derivatives using IPA as donor. The (*S*)-ATA from *Chromobacterium violaceum*, the (*R*)-ATA *Arthrobacter sp.* variant ArRMut1115 and *Mycobacterium vanbaalenii* (Mv-TAm), were selected as they had already been used with a range of cyclic and aromatic substrates and demonstrated tolerance towards the low cost amine donor IPA.[78] Other recent successful examples of chiral amine synthesis performed in asymmetric synthesis mode include the synthesis of a key intermediate of the antiallergic drug Ramatroban, used for the treatment of asthma and coronary artery diseases,[79] and serinol-derivatives.[80] Moreover, substituted aminotetralins, potential agents used to treat Parkinson's disease and cardiovascular disorders, have also been successfully synthesized using an engineered (*S*)-ATA derived from *Arthrobacter citreus*[81] and a novel (*S*)-selective ATA from *Pseudomonas fluorescens* KNK08-18.[82]

The main limitation of accessing chiral amines by asymmetric amination of prochiral ketones is the unfavorable thermodynamic equilibrium. Consequently, various physical and chemical strategies have been developed to displace the equilibrium towards the product side. This topic is discussed in detail in section 1.4.1.

1.3.3 Choice of the amine donor

The widespread acceptance by ATAs and the various options to remove the co-product pyruvate makes alanine one of the most popular amine donor in TA-catalyzed reactions.[83] However, it is the amine donor resulting in the most unfavorable reaction equilibrium of most asymmetric synthesis reactions.[83] Usually, an excess of this amine donor, compared to the ketone substrate, does not lead to an efficient thermodynamic equilibrium shift. As alternative, IPA can be used. Its achiral nature, its acceptable chemical price, its excellent water solubility and the ease with which the by-product acetone can be removed, made IPA the most desired sacrificial amine donor for industrial applications.[70] However, some limitations associated to the use of this amine donor have to be taken into account. (i) A large excess of IPA is still required for driving most reactions towards completion.[45,84] (ii) The often structural similarity between the desired amine product and IPA may pose a problem during downstream processing.[85–87] (iii) Besides the commercially available powerful toolbox of engineered (*R*)- and (S)-selective ATA of Codexis, only a limited number of WT ATA are efficient with IPA.[88] Therefore, extensive enzyme engineering is required, to improve the substrate acceptance and the enzyme stability for industrial use.[76,89–95] Finally, it is important to mention that the basicity of IPA can lead to unwanted side reactions, as observed in the production of the antiallergic drug Ramatroban.[79] Aromatic ketone substrates are often aminated employing α-

MBA as amine donor. Among the previous mentioned amine donors, this amine donor results in the most thermodynamically favored reaction equilibrium. Although the acetophenone co-product can cause severe inhibition on ATAs, a number of strategies for either its physical removal or its chemical conversion have been developed (see paragraph 1.4.1).[57,96,97]

Besides the most frequently used α-MBA, IPA and alanine, less common amine donors, such as the 'smart' amine donors developed by the O'Reilly group, have been recently explored. Compared to the already mentioned amines, these more reactive amine donors are able to drive the reaction equilibrium to completion very easily, what reduces the required amine donor excess employed in the biocatalytic reactions. Advantages and drawbacks of these new generation of amines are also reported in paragraph 1.4.1.

1.3.4 Examples of large scale applications

The optimization of enzymatic reactions for industrial applications is a challenging task as many process and reaction parameters need to be optimized. Therefore, combination of process engineering and protein engineering strategies is often required. The TA-catalyzed process implementation approaches described in this thesis have been mainly investigated at a laboratory scale. However, in some cases, TA-catalyzed reactions have also been tested in an industrial environment for the synthesis different bioactive molecules.[57,98–101] The subsequent paragraph therefore focuses on successful upscaling of several TA-catalyzed reactions for chiral amine synthesis.

The first large scale TA process to produce optically active amines was devised at Celgene at the end of the 1980s. (*S*)- and (*R*)- TAs were employed for the kinetic resolution of various aliphatic and aromatic amines on a 2.5 m^3 scale. The reactions were performed in aqueous solutions or in mixtures of water and an organic solvent. Therefore, low product concentrations were usually achieved with hydrophobic substrates. Moreover, product mixtures from racemate solutions could only be separated by cumbersome methods.[9] More recent examples of large-scale application of ATAs are listed in Table 1. For example, ATA 117 (Codexis) has been employed to produce (6*R*)-methyl-6-methyl-2-oxo-piperidine-3-carboxylate (Table 1, Entry 4), a useful building block in the synthesis of the orexin antagonist MK-6096, a candidate for the treatment of insomnia. This biocatalytic reaction could be well integrated in the kg-scale nine-step chemoenzymatic synthesis of MK-6096 with 13% overall yield. Also the aryl-alkyl amine intermediate, required for the synthesis of a Janus kinase 2 (JAK2) inhibitor, developed by Astra Zeneca for the treatment of idiopathic myelofibrosis and polycythaemia rubra vera, was synthesized enzymatically (Table 1, Entry 1 and 2). Process conditions were optimized on gram scale. α-MBA was selected as amine donor and of a

biphasic system with 20% (v/v) toluene was built to increase the substrate loading and to avoid enzyme inhibition by the co-product acetophenone. In these conditions, a space time yield (STY) of 50 g l^{-1} d^{-1} was obtained, employing the wild-type ATA from *V. fluvialis*. After scaling-up on kg scale, the reaction was then implemented in the chemoenzymatic synthesis of JAK2 on 100 L scale with a >30% overall yield.[99] In a more recent study, a building block for the production of the antibiotic besifloxacin has been synthesized (Table 1, Entry 8) using a commercial wild-type TA. The study covers all the process development steps: from enzyme to reaction optimization and scale-up.[102] Although notably examples of upscaled processes employ wild-type transaminases, protein engineering interventions for adapting the biocatalyst to the processes requirements are often needed.[103] Particularly, Merck and Codexis had to engineer ATA 117 to catalyze the synthesis of the antidiabetic Sitagliptin (Table 1, Entry 6). Twelve mutations were needed to accept the bulky substrate. The best variant, carrying 27 mutations, was employed in the kg-scale synthesis of Sitagliptin, using IPA as the amine donor.[76] In comparison to the conventional chemical synthesis – a Rh-catalyzed asymmetric hydrogenation under high pressure – the TA reaction system resulted in a 13% higher overall yield and a 19% reduction of total waste.[7] Reaching a STY value above 150 g l^{-1} d^{-1}, this process currently represent the 'benchmark' TA-catalyzed process for industrial scale implementation.[83] The Sitagliptin synthesis was also performed in water saturated isopropyl acetate: immobilizing an engineered (*R*)-ATA of Codexis, a STY, similar to that achieved by the Codexis and Merk process was afforded. This simplifies the workup and enables reusability of the catalyst (Table 1, Entry 5).[83,100] Other interesting examples have been recently reported using commercially available ATAs, although on a smaller scale (g scale). For instance, the variant of ATA 117 (Armut11), known for possessing high tolerance towards high concentrations of DMSO and IPA and high temperatures, was applied in the asymmetric synthesis of the aminated precursor of the drug Ramatroban, a thromboxane receptor antagonist for the treatment of asthma or coronary artery diseases (Table 1, Entry 7). Compared to the chemical 3-step synthesis, a 1.5-fold higher yield and a 12.5-fold faster one-step reaction was achieved.

Table 1. Summary of TA-upscaled reactions or TA systems suitable for industrial scale applications.[46,83]

No.	Product	*c* substr. [mM]	Amine donor	Enzyme	C (y), *ee* [%]	Comment	Ref.
1	(*S*)-1-(5-Fluoropyrimidine-2-yl)-ethyl-amine	289	1 equiv. PEA	(*S*)-ATA *V. fluvialis**	66, 97	Two-phase system, toluene to avoid co-product inhibition	[57]
2	(*S*)-1-(5-Fluoropyrimidine-2-yl)-ethyl-amine	350	1.15 equiv. PEA	(*S*)-ATA TA-P1-A06 (Codexis)	98, >99	0.45 kg of substrate converted; 2-phase system, toluene	[58]
3	(*S*)-5-(4-Bromophenyl)-piperidine-2-one	255	4 equiv. IPA	(*S*)-ATA ATA-302 (Codexis)	84, >99	IPA feeding to keep pH constant	[101]
4	(6*R*)-Methyl-6-methyl-2-oxo-piperidine-3-carboxylate	446	3.6 equiv. ALA	(*R*)-ATA ATA-117* (Codexis)	74, 99	Bioconversion performed on 100 L scale; LDH/GDH cascade system applied for equilibrium shifting	[98]
5	Sitagliptin	492	2 equiv. IPA	(*R*)-ATA CDX-017 immobilized (Codexis)	91, 99	Reaction in water saturated isopropylacetate	[100]
6	Sitagliptin	492	2 equiv. IPA	(*R*)-ATA Armut11 (Codexis)	92, >99	Reaction in water with 50% DMSO. Stripping of acetone co-product was performed	[7,76]
7	Ramatroban precursor	50	2 equiv. PEA	(*R*)-ATA Armut11 (Codexis)	96, >97	1.5-fold higher yield and 12.5-fold faster compared to the chemical 3-step synthesis	[79]
8	(3*R*)-3-Aminoazepane	465.1	8.6 equiv. IPA	(*R*)-ATA-01*	80, >99	0.3 kg of substrate converted; deprotection is necessary for accessing the final product	[102]
9	(*R*)- and (*S*)-Valinol	200	1.25 equiv. ALA	(*R*)-BM-ATA	99 (*R*), >99	AlaDH/FDH cascade system applied for equilibrium shifting	[104]

*Wild-type (wt-TA)

1.4 Process intensification strategies for ATA-catalyzed chiral amine synthesis

Transaminases (TAs) are one of the most promising biocatalysts in organic synthesis for the preparation of chiral amino compounds. However, in order to optimize their performance, several challenges have to be overcome including substrate specificity, stability under non-physiological conditions, substrates and/or products inhibition, and unfavorable thermodynamic equilibrium. Subsequently, various strategies have been applied to prevail over these limitations.

Protein engineering has proven its high potential to improve enzyme stability and substrate scope of TAs itself and systems involving TAs. However, protein and metabolic engineering techniques cannot directly improve physical and chemical properties such as low solubilities of reactants within aqueous systems or undesired unfavorable thermodynamics, which prevent high conversions.

This part of the thesis will focus more in detail on the physical and chemical process-based strategies developed for overcoming the challenge of all the TA-mediated transformations: the unfavorable thermodynamic equilibrium.

1.4.1 Equilibrium displacement techniques

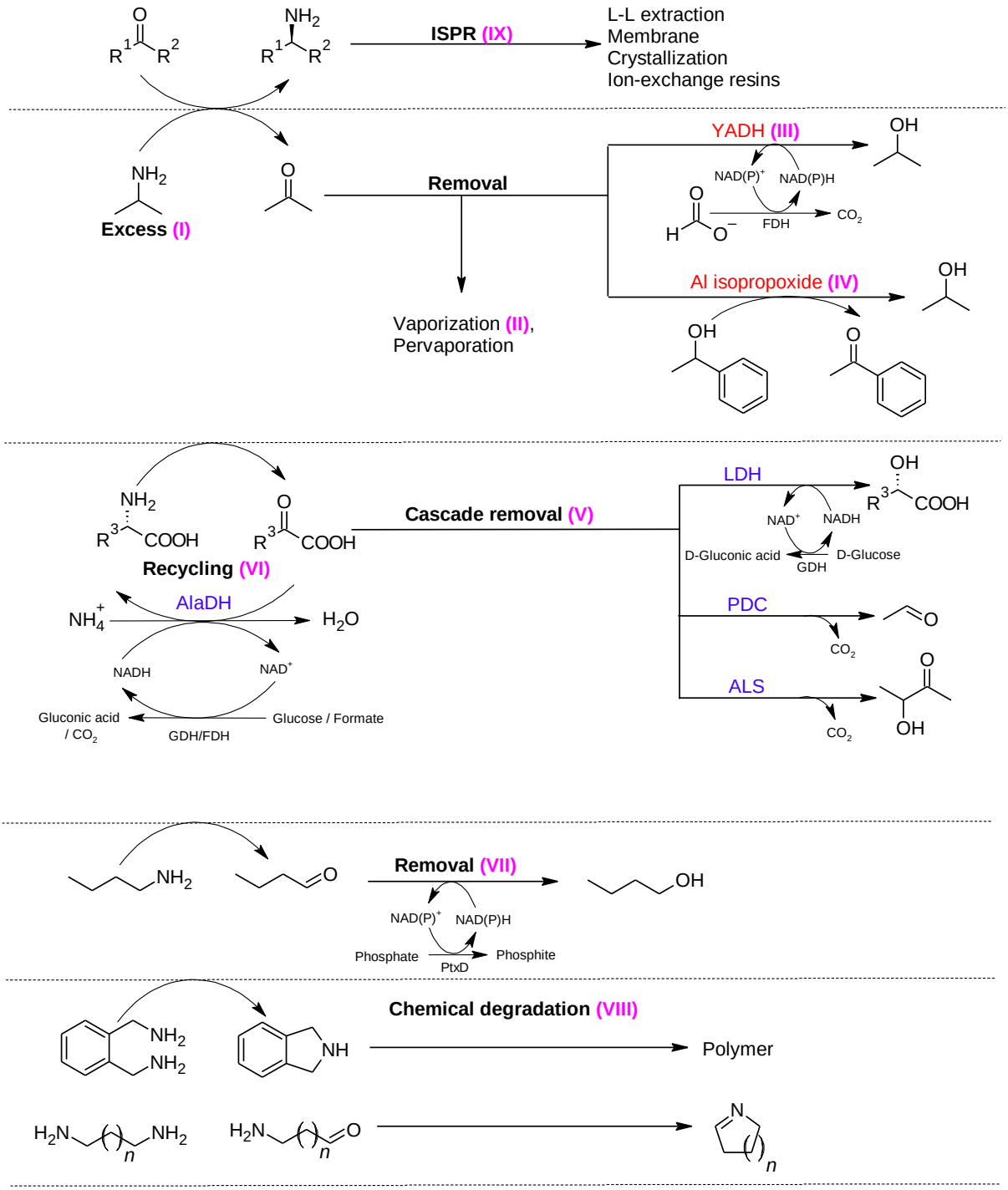
The easiest and a straightforward strategy for shifting the reaction equilibrium is to provide an excess of amine donor (Scheme 4, **I**). In order to push transamination reactions towards complete conversion, a 50-fold excess of the amine donor IPA was applied in the transamination of the thermodynamically challenging acetophenone (20 mM) to 1-phenylethylamine (α-MBA).[84] Removal of the co-products is another valid strategy to push reversible reactions toward completion. Ketone co-products, which usually hamper transamination, can be removed and/or recycled via physical processes or chemo/enzymatic cascades. There is no generally applicable strategy; it often depends on the choice of the amine donor. If IPA is used, the volatile acetone co-product formed can be easily removed by vaporization, due to its low boiling point (Scheme 4, **II**). Reduced pressure or nitrogen sweeping at regular temperature have been indeed proven to be sufficient for the removal of acetone.[62,76] However, as shown by Tufvesson et al., the K_{eq} and the volatility of the ketone substrate are critical parameters to consider in designing this process strategy.[105] In a recent study, the feasibility of applying pervaporation (PV) as acetone removal technique was investigated. Although feasible, PV cannot prevent the loss of acetophenone keto substrate in the permeate.[87] As alternative, the co-product acetone can be removed enzymatically, by

coupling the TA reaction with an alcohol dehydrogenases (ADH) and formate dehydrogenase (FDH) for cofactor recycling (Scheme 4, **III**).[106] Last, the chemical catalyst aluminium isopropoxide has been recently investigated and introduced into a transamination system for recycling acetone to IPA (Scheme 4, **IV**).[107]

When alanine, the natural substrate for most transaminases, is employed, the resulting pyruvate co-product must be removed from the system (Scheme 4, **V**) or recycled back to alanine (Scheme 4, **VI**) due to its inhibitory effect on most of the transaminases. Shin and Kim reported the first *in situ* (co-)product removal application for preventing the accumulation of the inhibitory co-product.[59] Pyruvate was converted to lactate, using a lactate dehydrogenase (LDH), coupled with a glucose dehydrogenase (GDH), for the NADH cofactor recycling, at the expense of glucose. To date, this cascade is one of the most frequently used system for the conversion of various ketones to the corresponding chiral amines.[44,108–112] Other cascade systems for pyruvate removal employ pyruvate decarboxylase (PDC)[113] or acetolactate synthase (ALS).[114] As alternative to removal, pyruvate can be recycled using of alanine dehydrogenase (AlaDH), coupled either with GDH[84] or formate dehydrogenase (FDH)[115] for cofactor recycling. Notably, the use of AlaDH for the recycling of L-alanine, has reduced the cost of the amine donor by 97%, compared to the LDH removal system, while simultaneously decreasing the E-factor (environmental factor; the ratio of the mass of waste per mass of product) and improving the atom efficiency.[108]

A cost-effective and environmentally benign three-enzyme cascade system, which employs near stoichiometric concentration of *n*-butylamine as the amine donor, has been recently reported (Scheme 4, **VII**).[71] The biotransformation was catalyzed by a putrescine transaminase from *Pseudomonas putida* (Pp-SpuC),[116] in combination with an aldehyde reductase and a phosphite dehydrogenase for the removal of the reactive co-product butanal as inert *n*-butanol. In this way, 92% substrate conversion – 4-fold higher than that of the system without a co-product removal strategy – was achieved.

Besides enzymatic cascades, chemical degradation of co-products (Scheme 4, **VIII**) is one of the more modern approaches to overcome the equilibrium problem. Diamines such as *o*-xylylene diamine[117] or *cis*-but-2-ene-1,4-diamine,[118] also called smart amine donors, were recently suggested as effective amine donors, as the produced co-products undergo cyclization and tautomerization and thus cannot be utilized in the reverse reaction. Because of their high price and the formation of insoluble precipitates, these amine donors are rather only suitable for laboratory scale applications. The biogenic terminal diamines, which require near stoichiometric donor loadings are a suitable alternative, being affordable and readily available.[119–122]



Scheme 4. Strategies for reaction equilibrium shifting in TA reactions.[70]

Process-based strategies, to remove the ketone co-products include the use of biphasic systems such as buffer/organic solvents.[57–59,123] The different polarity between amines and ketones can be taken as advantage, however, the application of this strategy is limited due to drawbacks including the similar properties between substrate and product or the destabilization of proteins by organic solvents. Enzyme-membrane reactors[60,61] or reduced pressure systems[62] for co-product removal have also been investigated.

In order to reduce downstream processing, instead of ketone co-product conversion/removal, *in situ* product removal (ISPR), (Scheme 4, **IX**) is of interest. ISPR techniques have been investigated in the framework of this project. The following part of this thesis will therefore focus on this topic. Aside product/ co-product removal, immobilization of ATAs for continuous flow applications is emerging as promising alternative.[124–126] Enzyme immobilization, which often enhances protein operational stability, can be combined with an in-line purification system for amine recovery[127] or with ISPR steps and devices for analysis.[128]

1.4.2 In situ product removal techniques

In situ product removal (ISPR), involves actions taken for the immediate separation of a product from its producing environment (Scheme 4, **IX**). Hence, the goal of this approach, as an alternative to batch processing characterized by product accumulation, is to remove the product as it is formed.[129] ISPR strategies are designed to increase the yield and productivity of bioprocesses via four effects: (a) overcoming inhibitory or toxic effects resulting from product accumulation; (b) minimizing of product losses; (c) shifting the thermodynamic equilibrium and (d) reducing the number of subsequent downstream processing steps.[130]

In situ product removal techniques such as liquid-liquid and solid-liquid extraction have proven to be effective in ATA-catalyzed processes. To overcome product inhibition, Yun *et al.* demonstrated the use of a solvent bridge, connecting two aqueous phases differing in their pH values.[114] Once formed, the amine product was extracted from the aqueous reaction phase into an acidic buffer via travelling through an organic solvent phase. The selective removal of the amine product improved the synthetic yield by a factor of 2.5. A two-step liquid-liquid extraction ISPR concept was recently tested using scaled-down unit operations.[128] In such a device, it was possible to continuously feed the main substrate benzylacetone (BA) and extract the main product 1-methyl-3-phenylpropylamine (MPPA). Overcoming the challenges of low substrate solubility and product inhibition, the tested ISPR concept achieved a product concentration of 26.5 g · L^{-1} and a purity up to 70% $g_{MPPA} \cdot g_{TOT}^{-1}$. As alternative to product extraction, the product amine can be selectively crystallized while all other reactants remain in solution.[131,132] This novel crystallization-ISPR approach has been recently applied for the asymmetric synthesis of (*S*)-1-phenylethylamine using IPA amine donor. Product crystallization – achieved by adding to the reaction environment 1.5 equivalents of 3,3-diphenylpropionic acid (3DPPA) – improved the conversion of acetophenone from 19% to 75%.[133] Another possibility for amine extraction is the use of ion-exchange resins. Truppo *et al.* achieved full conversion of acetophenone, working at 50 g/L substrate concentration, by

combining a cascade reaction (LDH/GDH) with extraction of the amine.[134] Due to the lack of selectivity, however, the method was ineffective when using excess of the amine donor.

Membrane technology for the ISPR of chiral amines

Separation processes play a remarkable role in the chemical and pharmaceutical industries, where they account for 40–70% of both capital and operating costs.[135] Water, the basic solvent for production processes in the chemical industry, and organic streams produced by organic synthesis in the chemical and pharmaceutical industry, need to be treated at the end of the process to avoid the associated environmental problems. Membrane processes, mainly applied to purification, separation and concentration of aqueous streams, have been widely used in a broad range of applications including water treatment, gas purification, energy and pharmaceutical industry. For industrial applications, membranes are nowadays combined with or used as alternatives of traditional purification and separation processes (such as distillation, evaporation, adsorption, extraction, and chromatography). This has been motivated by the benefits that membrane technology offers over conventional techniques, in terms of economy, environment, and safety.[135–137]

Membrane processes have been used for bioseparations since well before the start of the modern membrane industry. In 1936, John D. Ferry described the use of membrane technology (in analytical scale) for enzyme concentration, analysis of bacteriophages, preparation of cell- and protein-free ultrafiltrates from biological solutions, and sterile filtration.[138] Today, it is hard to imagine industrial biotechnology without membranes. Membranes are used in several ways and in different parts of a process, e.g. for retaining biocatalysts and cofactors, aerating reactors, ISPR and downstream processing.[139] In the field of biocatalysts, enzyme membrane reactors (EMR) are widely applied as techniques for process intensification. EMR is a specific mode for running continuous processes in which enzymes are separated from end products with the help of a selective membrane.[140] Such devices, employing enzymes in free or immobilized form , can function in different operational modes (Figure 1). Nevertheless, membrane technology applied to enzymatic processes has to ensure the compete rejection of the enzyme to maintain the full activity inside the volume. In combination, the simultaneous selective permeation of the product can enhance thermodynamic equilibrium shifting of reversible reactions. Lowering the concentration of the product in the vicinity of the enzyme, EMR can also avoid product inhibition. These factors enhance enzymatic processes resulting in higher conversions and increased process productivities and/or stability of the enzymes.[141] A list of general advantages and disadvantages of EMR compared to batch enzymatic processes is presented in (Figure 1).

Examples of EMR applications that resulted in enhanced enzymatic processes compared to batch operations have been recently summarized.[141] EMR has been mainly applied for the production and separation of (1) low-molecular weight peptides from protein hydrolysis and (2) oligo- and monosaccharides from different polysaccharides such as starch, cellulose, pectin and lactose. Depending on the application, different membrane types including nano-, micro- and, ultrafiltration, have been employed. The membrane choice was often associated to the size of molecules that have to be retained in the reaction environment. In addition, it has also been reported by various authors that electrostatic or hydrophobic interactions between the biological molecules and the membrane could also influence the process performance.[140]

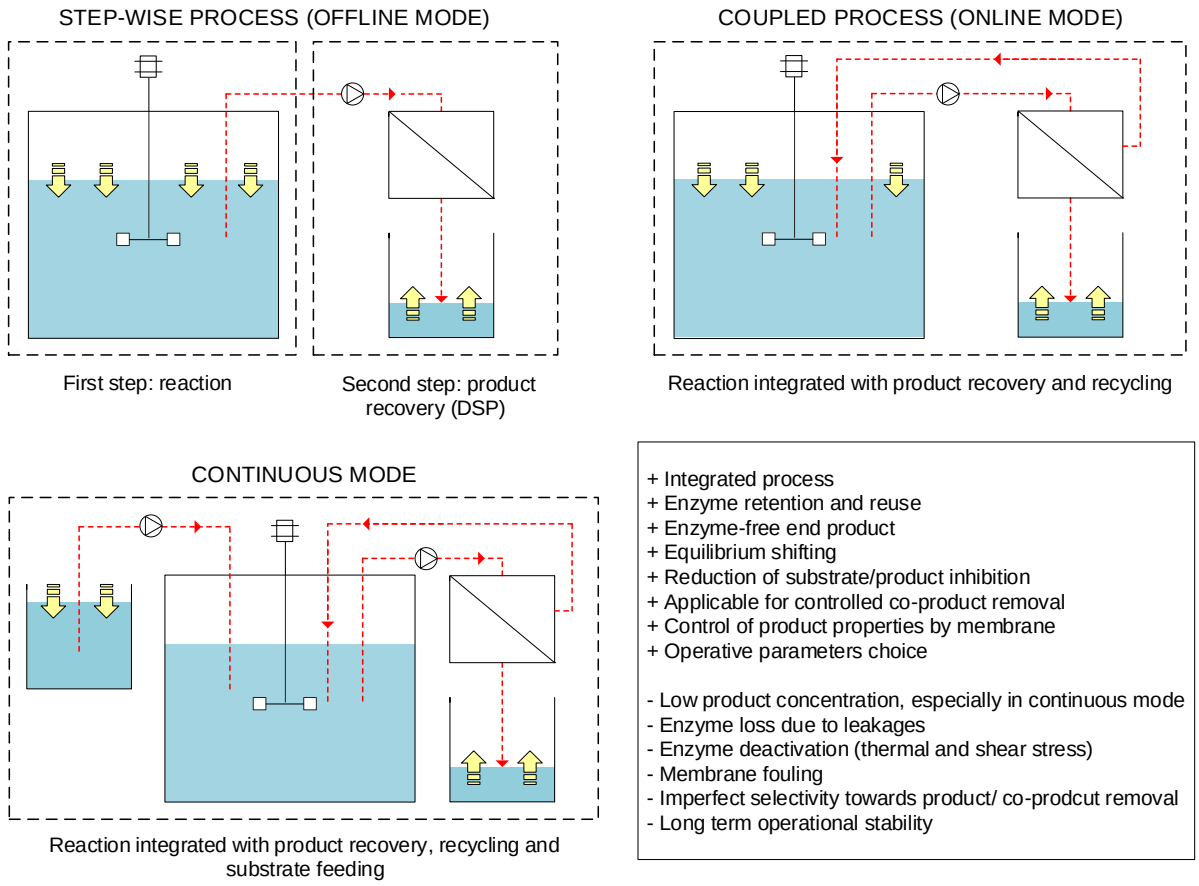


Figure 1. Operational modes of EMR systems.[141] Yellow arrows indicate volume changes in the vessels. Enzymes can be employed in free or immobilized form. Advantages (+) and inconveniences (-) of this technology compared to batch enzymatic reactions are also listed.

2 Filling the gap: High Molecular Weight amine donors for process intensification in chiral amine synthesis

Transaminases are industrially relevant enzymes and are useful for synthesizing a large number of compounds that contain a chiral amine functionality. As summarized in the background chapter, transaminases have shown a rapid development over the past few decades in both academic and industrial sectors. Recent attention has focused on overcoming limitations, including unfavorable reaction equilibrium and substrate and/or product inhibition. Specifically, the unfavorable reaction equilibrium, which cannot be tackled by enzyme engineering, remains an issue that needs process engineering intervention. The easiest strategy to shift the thermodynamic equilibrium is to use an excess of the amine donor (5 to 100 times higher), compared to the ketone substrate. Due to similar properties/characteristics between amine donor and product, it is often difficult to apply selective ISPR approaches. Consequently, the excess of amine donor is either wasted or requires further downstream processing (DSP), thus leading to high process costs. Membrane technology, a well-established separation technique, can help in ISPR of transaminase-catalyzed systems, but hasn't been explored to its full potential yet. For instance, size exclusion separation has not been applied because the amine donor, usually possessing a smaller or similar molecular weight compared to the amine product, would be lost in the permeate stream. The only examples of application of membrane technologies in transamination are membrane-based extractions. Although effective, these processes possess their own limitations and boundaries, which are mainly related to differences in characteristics of product and donor amines (different hydrophobicity and pK_a values). This leads to a limited choice of the amine donor.

In view of the above line of reasoning, this work mainly focuses on broadening the application of membrane-based ISPR techniques for the transaminase-catalyzed synthesis of chiral amines. Applying a size-exclusion-based membrane technology would result in two advantages: retaining the enzyme in the reactor as well as selective removal of the product amine. However, as mentioned above, this direct application is impeded by the poor selectivity between substrates (ketone and amine donor) and products (chiral amine product and co-product). To prevail over this limitation, the overall work was designed around the implementation of alternative amine donors, possessing considerably larger 'size', compared to commonly used amine donors. To clearly distinguish these molecules from traditional donor amines, we defined them as High Molecular Weight (HMW) amine donors. With a molecular weight between 400 and 1500 g/mol, in contrast to traditional donor amines, HMW amine donors enable a size-based separation between amine donor and amine product molecules.

We envisioned that HMW amines, provided in excess for thermodynamic equilibrium shifting, can thus be simply retained by a size-exclusion mechanism by commercial membranes, while the smaller product amines are permeated. Therefore, a selective recovery of the desired chiral amine product is possible. The implementation of ISPR techniques using HMW amine donors can theoretically lead to (i) equilibrium shifting, (ii) alleviation of product inhibition, and (iii) a highly pure product stream.

The feasibility of using HMW amine donors in aqueous, organic solvent and solvent-free media for the transaminase-catalyzed synthesis of 1-methyl-3-phenylpropylamine (MPPA) should be investigated in this thesis. The latter two approaches intend to achieve higher product concentrations. As a first step, suitable HMW amines should be identified that are converted by at least one transaminase with a comparable efficiency to established amine donors. Along with that, different membrane-assisted ISPR approaches should be investigated at the proof-of-concept level (Figure 2). Specifically, nanofiltration should be coupled with the enzymatic reaction performed in aqueous media (results presented in **Article I**), while liquid-liquid (L-L) extraction in a contactor should be applied for transamination in organic solvent media (results presented in **Article II**). As an alternative to membrane-based strategies we also aimed to design a spinning reactor concept for the integrated chiral amine synthesis (in organic solvent) and recovery (**Article III**).

The final aim of this work is to open new possibilities and perspectives in transaminase-catalyzed synthesis and recovery of chiral amines, using HMW amine donors. Advantages and drawbacks of each developed technology, as well as possible optimization strategies should be evaluated for further industrial application and critically discussed (see chapter 3).

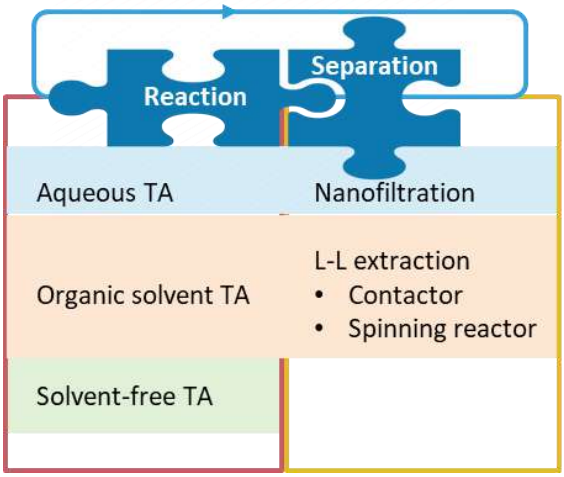


*Figure 2. ISPR approaches to be investigated in this thesis employing HMW amine donors. Nanofiltration was applied to an aqueous reaction system (**Article I**). Product extraction from organic solvent was performed either by membrane-based L-L extraction (**Article II**) or by using the spinning reactor concept (**Article III**).*

2.1 Nanofiltration for integrated downstream processing and ISPR (Article I)

Nanofiltration (NF) is a pressure-driven membrane process used for removing solutes with molecular weight in the range of 200–1000 g · mol^{-1}, typically from aqueous streams. As shown in Figure 5, separation mostly occurs by a sieving effect. Molecules with a size larger than that of the pore size of the membrane are retained while smaller molecules are permeated. Based on this concept, enzymatic synthesis of chiral amines combined with product recovery can be performed employing differently sized amine donor and product molecules (Figure 3).

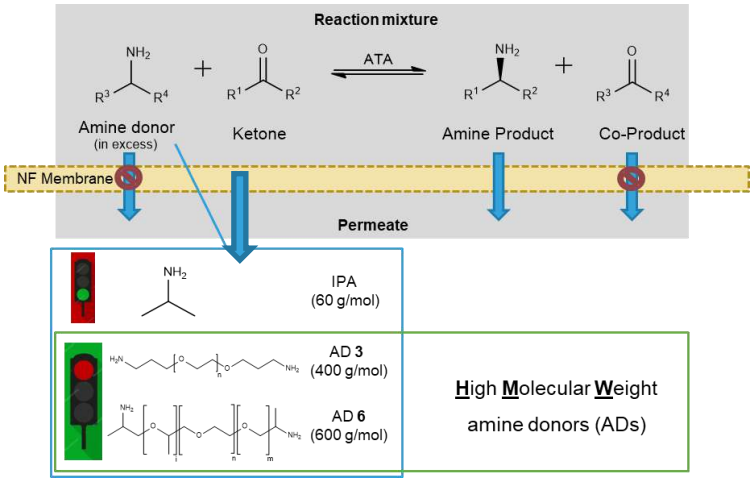
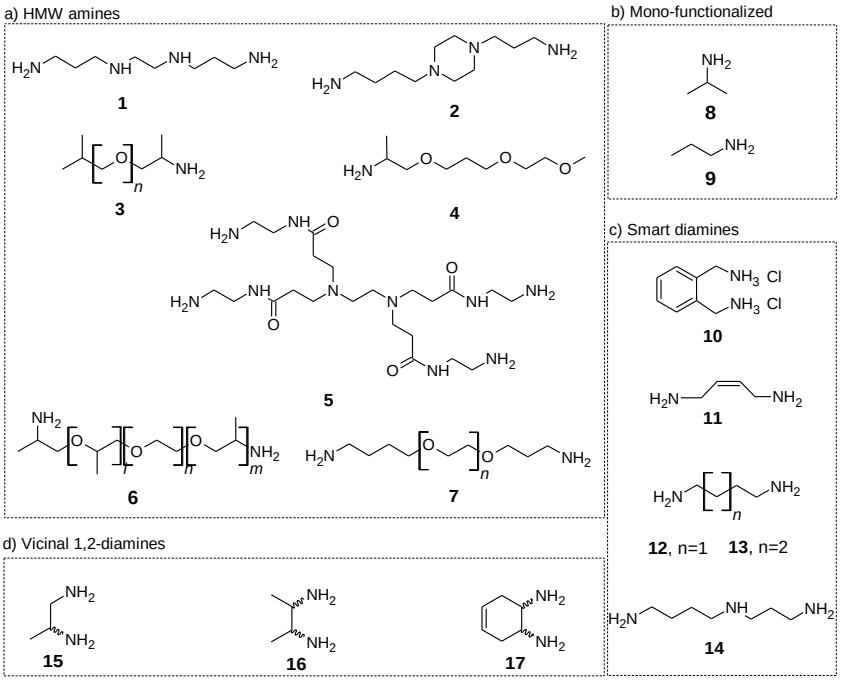


Figure 3. Application of high molecular weight (HMW) amine donors for the TA-catalyzed synthesis and nanofiltration-based recovery of chiral amines. Due to their large size, HMW amine donors are retained by nanofiltration (red ample light), while the common amine donor isopropyl amine (IPA) can permeate through the membrane (green ample light).

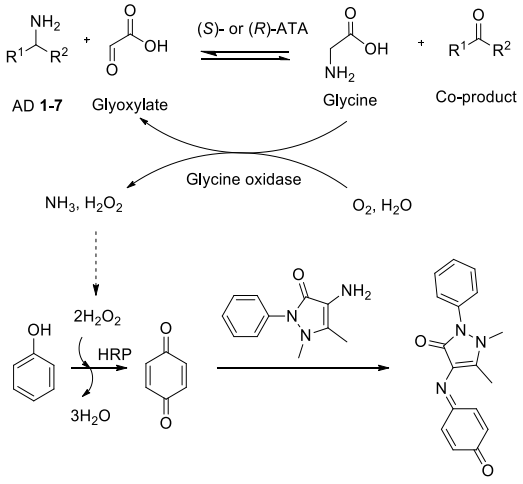
In contrast to the amine donors commonly employed in transamination, we envisioned that larger molecules – provided in excess for thermodynamic equilibrium shifting – should be retained by commercial nanofiltration membranes. A selective permeation of the desired smaller product amines while retaining the enzyme in the reaction media will therefore be possible (Figure 3). To the best of our knowledge this study represented (1) the first application of large amine donors (HMW amine donors) in transaminase-catalyzed reaction and (2) the first nanofiltration-based EMR system for the integrated production and separation of chiral amines.

To demonstrate the feasibility of this approach, the first requirement was to identify ATA that would convert a suitable HMW amine donor. Therefore, diverse HMW amine donors (**1–7**, Scheme 5) were selected for initial evaluations because of their commercial availability and their relative low cost. Notably, amine donors **3**, **4** and **6**, (Scheme 5) possess a typical

polyethylene glycol (PEG) backbone, which is known to prevent enzyme denaturation.[142,143] To identify the best ATAs accepting the selected HMW amine donors, a preliminary enzyme screening was performed. The glycine oxidase assay[144] was used as rapid and convenient method for the screening of (*R*)- or (*S*)-selective ATAs with amine donors **1**-**7** in microtiter plates (Scheme 6). The wild-type ATA from *Silicibacter pomeroy* – TA_3HMU[145] – and the solvent resistant TA_v2 from *Pseudomonas fluorescens*[95] were selected for further reaction design and optimization. Details regarding reaction investigations can be found in the main manuscript text.[146]

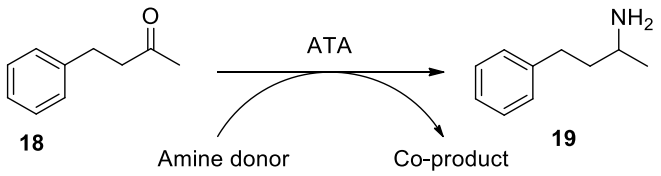


Scheme 5. Categories of amine donors employed for the asymmetric amination of 4-phenyl-2-butanone (18).



*Scheme 6. Photometric glycine oxidase microtiter plate assay for (R)- or (S)-selective amine transaminase (ATA) screening towards HMW amine donors (AD) **1-7** (Scheme 5).*

The asymmetric synthesis of 1-methyl-3-phenylpropylamine (MPPA) (**19**) from benzylacetone (**18**) with the HMW amine donors **3** and **6** was selected as model reaction (Scheme 7). Under the optimized reaction conditions, without any membrane strategy applied, significant substrate conversions, ranging between 45% and 66%, were achieved. Notably, when using the wild-type TA_3HMU, the substrate conversions reached in our study resulted higher than those achieved employing smart amine donors **9**, **15**-**17**, depicted in Scheme 5.[121] This reflects that the different approaches cannot be generalized to all available transaminase enzymes: TA_3HMU is not as suitable in accepting the selected smart amine donors, but on the other hand, we observed that only few selected ATA accepted our investigated HMW-amine donors.



*Scheme 7. Model reaction selected for the process investigations. Amine donors **3**, **6** and **8** (Scheme 5) were investigated in this study. Amine donor **6** was selected for further process development.*

Besides the identification of a suitable ATA accepting at least one HMW-amine donor, the second requirement for establishing a proof-of-concept of our envisioned ISPR approach is the discovery of a selective membrane. Therefore, in parallel to reaction investigation, separation performances of thirteen commercially available nanofiltration membranes (Table 2) were investigated.

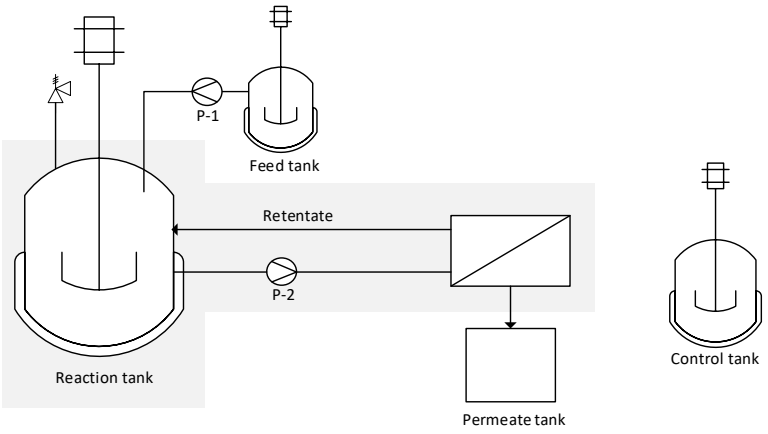


Figure 4. Schematic set-up of the in-house NF system. P-1 is the feed pump, P-2 is the circulation pump. The reaction tank can hold up to 0.9 L of solution. Everything in the shaded zone is under pressure during operation. In the control tank (0.9 L), a typical batch reaction was performed. Everything in the shaded zone is under pressure during operation.

Table 2. Parameters of selected polymeric flat sheet nanofiltration (NF) membranes for process development in the aqueous environment.

Entry	Membrane	Nom. Cut-off (Da)	Material	pH range	Application
1	Synder NFX	150-300	TFC/PA[a]	3-10.5	Aqua[c]
2	Synder NFW	300-500	TFC/PA	3-10.5	Aqua
3	Duramem200	200	PI[b]	n.a.	OSN[d]
4	SolSep-10206	Rejection (95%)~300	Propr	n.a.	OSN
5	GE KH Duracid	200	TFC/PA	0-9	Aqua
6	Desal DK GE	150	TFC/PA	3-9	Aqua
7	Alfalaval NF99	<200	TFC/PA	3-10	Aqua
8	Dow NF90	200	TFC/PA	3-10	Aqua
9	Puramem Flux	n.d.	TFC/PA	n.a.	OSN
10	Puramem Performance	n.d.	TFC/PA	n.a.	OSN
11	Puramem Selective	n.d.	TFC/PA	n.a.	OSN
12	MPF-34	200	TFC/PA	n.a.	n.d.
13	B4022	n.d.	n.d.	n.a.	n.d.

[a] Thin film composite (TFC) membrane /selective polyamide (PA) layer
[b] Integrally skinned asymmetric (ISA) membrane / Polyimide (PI)
[c] Operation possible in water-based solutions
[d] Organic Solvent Nanofiltration (OSN)
n.d.: not defined; n.a.: not applicable

To calculate the rejection† profiles (Table 3), aqueous solutions of either amine **3** or **6** were prepared and subjected to NF, using the set-up shown in Figure 4. As shown in Table 3, most of the tested membranes retained HMW amines. Ideally, amine donor rejection values of >99% are required to minimize further downstream processing. Additional purification steps would definitely be required, if the targeted product purity is not already obtained during nanofiltration. As such, the retention of HMW amine donors is not 'perfect' enough for fulfilling the product purities, often required for industrial implementation. Nevertheless, nanofiltration resulted beneficial for retaining >80 % of HMW amine donors in the reaction environment, therefore sufficient for further investigation and optimization of the system.

† Rejection measures the tendency of a certain compound to permeate across a membrane. It is function of the solute concentration in the permeate C_p and retentate C_r and it is calculated as follows:

$$R = \left(1 - \frac{C_p}{C_r}\right) \cdot 100$$

*Table 3. Membrane rejections toward HMW amine donor (AD) **3** (400 g/mol) and **6** (600 g/mol). Synthetic solutions containing 250 mM of **3** or **6**, 0.1 mM PLP, 100 mM CHES buffer, pH 9.5 were used.*

Membrane		**Amine donor rejection %**	
Commercial name	**Nom. Cut-off (Da)**	AD **3**	AD **6**
Synder NFX	150-300	86	86
Synder NFW	300-500	82	85
Duramem200	200	80	86
SolSep-10206	300	<30	84
GE KH Duracid	200	n.a.	62
Desal DK GE	150	85	88
Alfalaval NF99	<200	n.a.	83
Dow NF90	200	n.a.	83
Puramem Flux	n.d.	<30	n.a.
Puramem Performance	n.d.	<30	n.a.
PuraMem selective	n.d.	<30	n.a.
MPF-34	200	92	98
B4022	n.d.	80	85

n.a.: not tested due to very low fluxes; n.d.: not determined

The hydrophilic Desal DK (Osminics) and Duramem 200 (Evonik) membranes (Table 2, entries 6 and 3) were selected for further screenings with solutions containing also the small keto substrate and amine product solutes. The permeation of these small size molecules across the membrane is governed by a diffusion mechanism as opposed to the retention/rejection of HMW donors, which is determined by size exclusion. The keto substrate **18** was partially retained by both membranes while the amine product **19** was transported across the membrane preferentially into the permeate, hence achieving the desired product removal. From this result we suggest that there is a correlation between the hydrophilicity of the membrane and its capacity to permeate the product amine while retaining the substrate. The used ketone substrate is indeed hydrophobic compared to the product amine, which is more hydrophilic. Therefore, the hydrophilic membrane surface favors the permeation of amine product while partially retaining the ketone substrate.

Having proven the effectiveness of both – reaction and separation unit operations – we turned our investigations to process integration. Specifically, the enzymatic reaction, performed in batch mode, was combined with two sequential nanofiltrations. For details, the reader is referred to the paragraph 2.5.1 of the main manuscript text.[146] In accordance with the preliminary membrane screening, more than 80% of the unreacted excess of amine donor **6** was retained in the reaction environment during the nanofiltration operations, hence reducing

– but not avoiding – amine donor contamination of the product stream. Nevertheless, sequential nanofiltrations had a beneficial effect on the thermodynamic equilibrium. An overall increase of 25% yield was observed, compared to the control batch reaction system, where no NF was applied. Furthermore, product removal occurred faster than the transport of the buffer across Desal DK membrane. Therefore, the product concentration raised from 5.9 mM to 7.3 mM in the permeate while the partial retention of substrate **18** reduced the product stream contamination from 7.31 mM to 2.17 mM.

The last step of this process development was to integrate the enzymatic reaction and product recovery in an continuous fashion, including retentate recycling, and substrate feeding (Figure 1, continuous mode). Although this approach clearly has potential, this strategy did not fulfill the expectations. Continuous substrate feeding caused accumulation of the unreacted ketone substrate in the reaction tank. In turn, this resulted in partial substrate permeation across the membrane, and therefore product stream contamination. On the one hand some of the tested membranes, including Desal DK, resulted incompatible with high concentrations of the ketone substrate and DMSO. On the other hand, organic solvent resistant membranes such as Duramem 200, in addition to substrate permeation, showed lower product selectivity at high ketone substrate concentrations. Due to the above-mentioned reasons, at this stage, the system could not be practically run in continuous mode.

For further investigations in continuous mode, a long-term stable nanofiltration membrane, able to withstand high substrate and product concentrations is required. Additionally, the selected membrane should ideally possess high rejection values towards the unreacted substrates (ketone **18** and HMW amine donor) and DMSO. Besides these requirements, there are a large number of parameters both on the enzymatic reaction and the membrane filtration part that have an impact on the overall efficiency of the process and that further need to be optimized. On the enzyme reaction side, the most influential parameters are: enzyme loading, substrate concentration and amine donor excess. On the separation aspect, membrane selectivity toward product permeation, amine product concentration, and permeate flow rate influence the performance immensely. Moreover, matching reaction and permeation rates is another key challenge that needs to be addressed. This could be achieved by optimizing the process design, which would involve a ketone substrate feeding strategy and the use of immobilized enzymes in a packed bed reactor that would be coupled to nanofiltration via a reaction vessel in between. The best approach to study the effect of all the parameters, and to evaluate which requirements should be fulfilled to optimize the system, would be to assess a mathematical model, employing the collected experimental data.

2.2 Membrane contactor for chiral amine ISPR

Membrane contactors, also known as two-phase enzyme membrane reactors (EMC), make possible to accomplish gas-liquid or liquid-liquid mass transfer operations without dispersion of one phase within another. In contrast to the EMR previously described, the separation of the compounds in a two liquid phase EMC is mainly based on the principle of phase equilibrium. The contactor acts as a physical support for the interface and does not contribute to the separation through its selectivity.[147] Membrane contactors can have a hydrophilic or hydrophobic character. In this latter case, the membrane pores, which have a narrow pore size (typically in the range 0.02-0.2 μm), are filled by the non-polar phase (or by the gas). To avoid dispersion of the non-polar phase into the aqueous phase, a slight overpressure, lower than the breakthrough pressure‡, has to be kept on the aqueous phase. Membrane contactors in the form of hollow fibers provide well defined interface areas, significantly greater than traditional devices, leading to more compact systems. Moreover, being physically separated by the membrane, the two phases do not need to be separated downstream to the process.

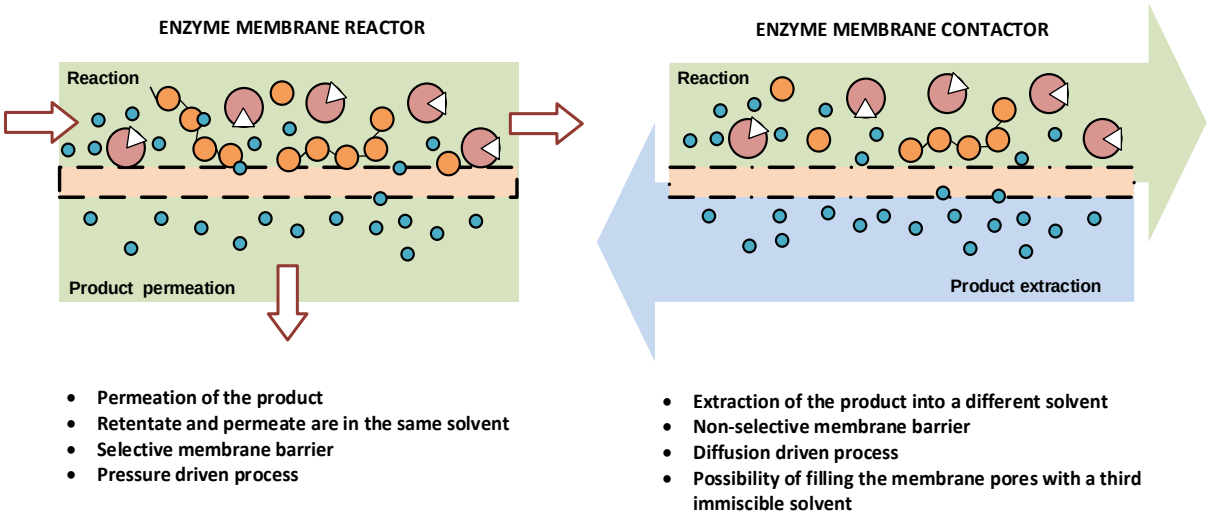


Figure 5. Conceptual diagram of enzyme membrane reactor, EMR (left) and two-phase enzyme membrane reactor, or membrane contactor (right). The enzyme, present in its free form, can also be immobilized on the membrane.

The use of membrane contactors had been reported about 18 years ago in a kinetic resolution process to produce chiral amines. The substrate solution containing racemic amine and

‡ The breakthrough pressure is the pressure required to force water to enter the pore and can be calculated through the Young-Lapace equation modified for use with hydrophobic membranes.[180] It depends on the maximum pore size, the interfacial tension between the two phases, the contact angle between the membrane and the two fluids, and it is valid for cylindrical pores.

pyruvate was recirculated through the enzyme membrane reactor while the inhibitory ketone product was selectively extracted into the organic solvent by the membrane contactor.[60] In the optimized reactor configuration, the enzyme was immobilized in a packed bed reactor.[61] In a more recent example, a hollow fiber membrane contactor was employed for the asymmetric synthesis and ISPR of MPPA. The reaction was conducted in an organic solvent and a non-miscible acidic aqueous solution was used for amine product extraction.[86]

If the hydrophobic porous matrix of a hollow fiber membrane contactor is soaked with a hydrophobic solvent, a supported liquid membrane (SLM) can be prepared. This operation allows recirculating two aqueous solutions on both sides of the contactor by using a solvent, immobilized and dispersed into the hollow fiber matrix, as physical barrier. There are many possible variants of SLM systems and many possible applications.[148] Hollow fiber or flat sheet modules can be used and the membrane phase can be filled with an organic solvent or an ionic liquid. Also, carrier substances can be added to the membrane phase to form a complex with the target molecules (e.g., amino acids), for enhancing the selective extraction.[149] A SLM contactor has been recently employed for the in situ product recovery of chiral amines. For instance, Börner et al. described the amination of the poorly water-soluble ketone BA employing alanine as amine donor in a combined ISPR approach using SLM together with an enzyme cascade. The hollow fiber contactor pores, filled with undecane, constituted a physical barrier between the reaction environment (aqueous buffer pH 9) and the extracting acidic aqueous solution. The ISPR strategy facilitated very high product purity (more than 98%) without any additional purification step, and eliminated product as well as co-product inhibition.[150] The versatility of this approach is, however, limited by the choice of the keto substrate and amino donor system. In order to be separated, amine donor and product must have differences in pK_a and hydrophobicity, which is not often the case. In a similar ISPR strategy proposed for the production of α-MBA employing IPA as amino donor, the author achieved 98% product yield compared to 50%, when no product extraction was conducted.[85] However, IPA amine donor was extracted together with the chiral amine donor, thus requiring further downstream operations to yield similar purity as achieved with alanine. In a further study, the factors affecting the SLM system in terms of membrane stability and separation efficiency were discussed in detail.[151]

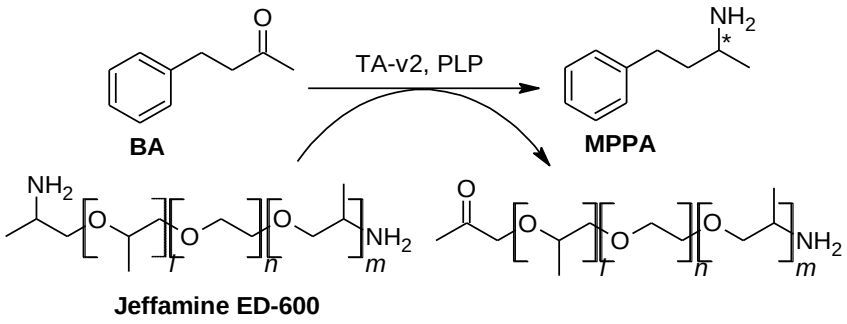
2.2.1 Transamination in organic solvent combined with membrane-based ISPR (Article II)

Amine transaminases have been extensively used for synthesizing various pharmaceutically relevant compounds, mainly in aqueous media. However, their applications are often limited

by poor substrate solubility, low productivity and difficult product separation. Consequently, it is be desirable to perform transamination reactions in organic solvents. The first attempt of non-aqueous transamination was the asymmetric synthesis of *trans*-(1*R*,2*R*)-1-amino-2-indanol using alanine as the amine donor. The crude preparation of the ATA from *V. fluvialis* partially retained enzyme activity in water-saturated ethyl acetate, although reaction rates were lower than in aqueous buffer.[152] In a further study, nine ATAs, most of them wild-type enzymes, proved to be highly active in methyl tert-butyl ether (MTBE). Enzyme activities were shown to be up to 17-fold higher compared to those of the aqueous buffer and ATAs efficiently accepted IPA as the amine donor, which is not the case when applied in aqueous medium.[153] Transamination in MTBE was again reported for the large scale synthesis of (*R*)- or (*S*)-valinol[104] (Table 1, entry 9). Asymmetric synthesis of 3-substituted cyclohexylamine derivatives from prochiral diketones via three biocatalytic steps in diisopropyl ether (DIPE) was also performed. The simultaneous one-pot approach cascade reaction was catalyzed by two hydrolases (C-C hydrolase and a lipase) in DIPE containing 2.5% (v/v) water and 1% (v/v) methanol, followed by transamination in DIPE.[154] Successful immobilization of a transaminase, capable of operating in organic solvent has been also reported. The immobilized (*R*)-ATA CDX-017, subjected to 10 consecutive recycles over a 200 hour time period, proved to be active and stable in water-saturated isopropyl acetate (Table 1, entry 5). This result enabled a >90% reduction in the amount of TA enzyme required, compared to the soluble enzyme process.[100] Furthermore, Andrade *et al.* described an immobilized system enabling the use of transaminases for the synthesis of aliphatic and aromatic amines under flow conditions with an organic solvent (MTBE).[155] To isolate the product amines, a catch-and-release system, which consists of a silica gel cartridge attached to the packed bed reactor, was designed. Although the separation between amine and ketones was achieved, the unreacted IPA, often provided in excess compared to the ketone substrates, was retained together with the products into the cartridge.[155] As already mentioned, the asymmetric synthesis of MPPA using IPA amine donor in *n*-heptane combined with ISPR was also recently investigated.[86] However, product purity was hampered by amine donor co-extraction.

In the above presented process development (see paragraph 2.1), we have introduced a novel class of HMW amine donors, in the range of 400 to 1500 g/mol. These molecules were retained by commercial nanofiltration membranes by a size exclusion mechanism. Performing transamination with HMW amine donors reduced the contamination of the product stream, thus simplifying the membrane-assisted downstream processing and potentially providing ISPR opportunities.[146] However, a low product concentration was achieved, mainly due to the limited solubility of the ketone substrate in aqueous environment (10 mM). Evidently, performing the reaction in an organic solvent would be beneficial. Based on this concept, the

transamination in non-aqueous media using the HMW amine donor Jeffamine® ED-600 (MW of 600 g/mol) was investigated (Scheme 8). The asymmetric synthesis of MPPA using the Jeffamine® ED-600 was performed in the presence of a non-polar organic solvent (*n*-heptane), in which the selected amine donor is not soluble, thus the organic solvent and the Jeffamine® ED-600 amine donor formed a two-liquid-phase system, schematically depicted in Figure 6.



Scheme 8. Transaminase-mediated synthesis of (S)—1-methyl-3-phenylpropylamine (MPPA) from 4-phenyl-2-butanone (BA), using Jeffamine ED-600 amine donor (I +m~6; n~39).

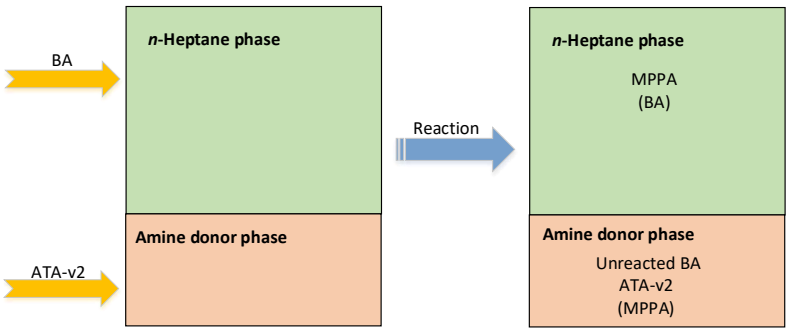


Figure 6. Composition of the two-liquid-phase system before (left) and during (right) the transamination reaction. Being heavier and not soluble in n-heptane, Jeffamine ED-600 amine donor and the enzyme form a layer on the bottom of the reaction vial. The substrate ketone BA, initially supplied to the n-heptane (indicated by the yellow arrow), partially diffuses from the n-heptane to the amine donor phase. The reaction takes place in the amine donor phase and the formed MPPA product is progressively released from the amine donor to the upper n-heptane phase.

Preliminary product and substrate partitioning investigations revealed that the *n*-heptane upper phase acted as a substrate feeding medium and at the same time as a reservoir for the product amine. The geometry of the reaction system and the intensity of mechanical stirring of the system showed to have an impact on the rate and amount of product released from the lower amine donor layer to the upper *n*-heptane phase. Along with that, different amine donor

loadings were tested to identify the optimal reaction conditions for further membrane-assisted process development.

In parallel to reaction investigation, polymeric membranes were tested with synthetic solutions composed of substrate (BA), amine donor (Jeffamine ED-600), and amine product (MPPA) in the set-up presented in Figure 7.

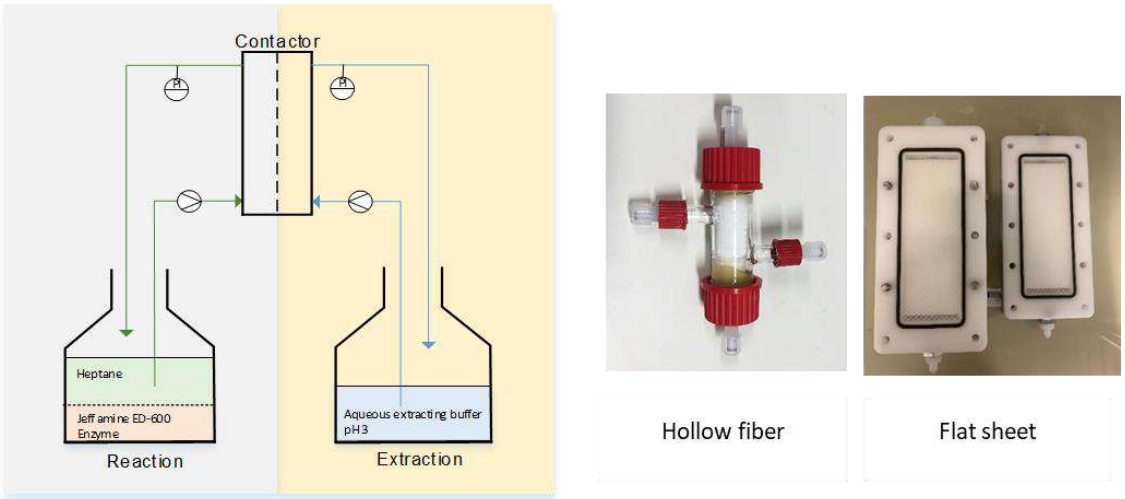


Figure 7. Set-up for membrane-assisted product (MPPA) extraction (left) and contactor modules (right). When the hollow fiber contactor was used, the solvent solution was pumped on the lumen side of the contactor while the aqueous extracting buffer was recirculated across the shell of the module.

A hollow fiber with modified housing and a flat sheet membrane contactor were used to physically separate two immiscible solutions: the *n*-heptane solution (upper phase of the of the reactor side), and the aqueous extracting solution (Figure 7). The aqueous extracting solution (100 mM sodium citrate buffer, pH 3.0) was chosen in such a way to enhance the selective MPPA extraction and to prevent its back extraction.[86] The system was tested employing an in-house constructed rectangular flat sheet with two different polymeric organic solvent nanofiltration (OSN) Puramem membranes. According to the supplier (Evonik-MET Ltd), these new membranes are integrally skinned[§] OSN membranes based on P84 polyimide, available in a wide range of MWCOs (280–600 g–mol^{-1}). They possess excellent chemical stability in a range of solvents, including apolar, hydrocarbon-based solvents. They are stable in solvents including *n*-hexane, *n*-heptane, toluene, and ethyl acetate and they are not recommended in most polar aprotic solvents, chlorinated solvents, and strong amines.[156] In

[§] Integrally skinned asymmetric membranes (ISA) possess a skin layer on top of a more porous sublayer with the same composition. The thin skin layer affects the final membrane selectivity and permeance. ISA membranes are most commonly prepared by the phase inversion immersion precipitation process, using stable polymeric materials such as polyimide (PI) (P84).[156] ISA membranes have been used as both NF and ultrafiltration UF membranes.

addition to Puramem membranes, a commercial membrane contactor was in-house modified by replacing the polycarbonate membrane with a glass housing to enhance its resistance towards the used solvent. Properties of the used modules and membranes are summarized in Table 4.

Table 4. Properties of the hollow fiber (HF) contactor with modified housing and the flat sheet (FS) membrane contactor.

Module	**HF***	**FS ****	
Configuration	Parallel flow	Parallel flow	
Membrane name, supplier	1 × 5.5 Mini Module Liqui-Cel®, 3M	Puramem Selective®, Evonik	Puramem Performance®, Evonik
Active surface area	0.10 m^2	0.01 m^2	
Membrane type	Polypropylene HF	ISA*** membrane based on P84 polyimide	
Potting material	Polyurethane	No potting required	

*Commercially available hollow fiber (HF) contactor with modified housing
**in-house constructed rectangular flat sheet (FS) module
***Integrally skinned asymmetric membrane (ISA)

An efficient and selective product removal was achieved with synthetic solutions. As expected, when using the hollow fiber, product MPPA extraction resulted faster compared to the flat sheet module (Figure 8, ■). As already mentioned, PuraMem® Selective and Performance are composite flat sheet OSN membranes. The solubility of each compound in solvents on both sides of the membrane, the acidic pH of the extracting phase and the membrane surface material are the dominating factors determining membrane performance and consequently the separation. The solubility of the ketone substrate BA is limited in aqueous environment (10 mM). Up to 5-fold higher concentration of BA has been dissolved in *n*-heptane. In accordance with previous studies,[86,150] no BA was detected in the aqueous extracting phase with both flat sheet and hollow fiber modules. The product amine MPPA is highly soluble in both *n*-heptane and extracting aqueous buffer. Almost 90% of the MPPA was extracted into the aqueous solution in 4 h operation with all the membranes tested (Figure 8). This phenomenon is due to the acidic pH value of the buffer that traps amines in their charged state, thus preventing back extraction into the organic solvent phase (as long as the stripping phase pH is kept sufficiently below the pK_a of the amine product). As such, the molecular weight cut-off (MWCO) is not characteristic for this type of membrane separation, especially since solvent–solute–membrane interactions can lead to a change in the membrane rejection from that observed under the conditions to determine the MWCO. The permeate flux of pure *n*-heptane, defined as the volume of solvent flowing through the membrane per unit area per time, resulted to be 60 and 15 $L \cdot h^{-1} \cdot m^{-2}$ when Puramem® Performance and Selective were

tested (data provided by the supplier). Therefore, Puramem® Performance behaves as a more open membrane compared to Puramem® Selective, hence, enhancing MPPA transfer from the solvent to the acidic buffer solution. This resulted in a faster MPPA extraction when using Puramem® performance (Figure 8, ●) compared to Puramem® Selective (Figure 8, ▲). The amine donor selected for this study (Jeffamine ED-600), not soluble in *n*-heptane, formed a second phase on the bottom of the *n*-heptane (Figure 6). Ensuring gentle mixing of the system during the start-up of the system (thus minimizing the contact between the membrane and lower amine donor phase), less than 3% of Jeffamine ED-600 was co-extracted during 6 h operation. This demonstrated the advantage of using this donor amine as opposed to IPA where the co-extraction of donor amine could not be avoided.[86]

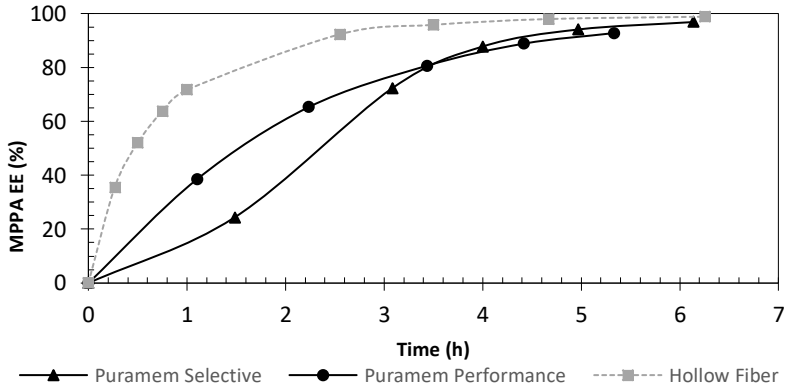


Figure 8. Product (MPPA) extraction efficiency (EE) over the time when using the flat sheet module with Puramem Selective (▲) or Puramem Performance (●), and when using the hollow fiber made in-house module (■). The reactor biphasic solution consisted of 100 g of Jeffamine ED-600 (amine donor phase) and 0.35 L of n-heptane. BA (2.3 g) and MPPA (1.3 g) were added to the reactor biphasic solution prior to the membrane extraction.

Having investigated reaction and separation systems, the two operations were combined to explore the effect of ISPR product removal. A detailed description and explanation of the experimental procedure is reported in our recent publication (Article II).[157] The following main outcomes were obtained in this study: the reaction system was combined intermittently with the membrane-assisted extraction set-up. This enabled (i) simultaneous recovery of product, without any consistent contamination of the unreacted substrates, (ii) shifting of the partition equilibrium of the formed MPPA, and (iii) shifting of the thermodynamic equilibrium. As it can be deducted from Figure 9, a product yield of 60% was reached, compared to 15% without product extraction. After three intermittent extractions, the product concentration in the acidic buffer amounted to 3.82 g/L. Less than 3% of the unreacted amine donor (Jeffamine ED-600), and no BA was co-extracted into the buffer. To achieve high product purities, along with amine donor and BA, also co-product extraction has to be avoided. Being structurally similar to the

amine donor, we expect that the ketone co-product was not co-extracted into the acidic buffer phase. This aspect needs to be analytically confirmed.

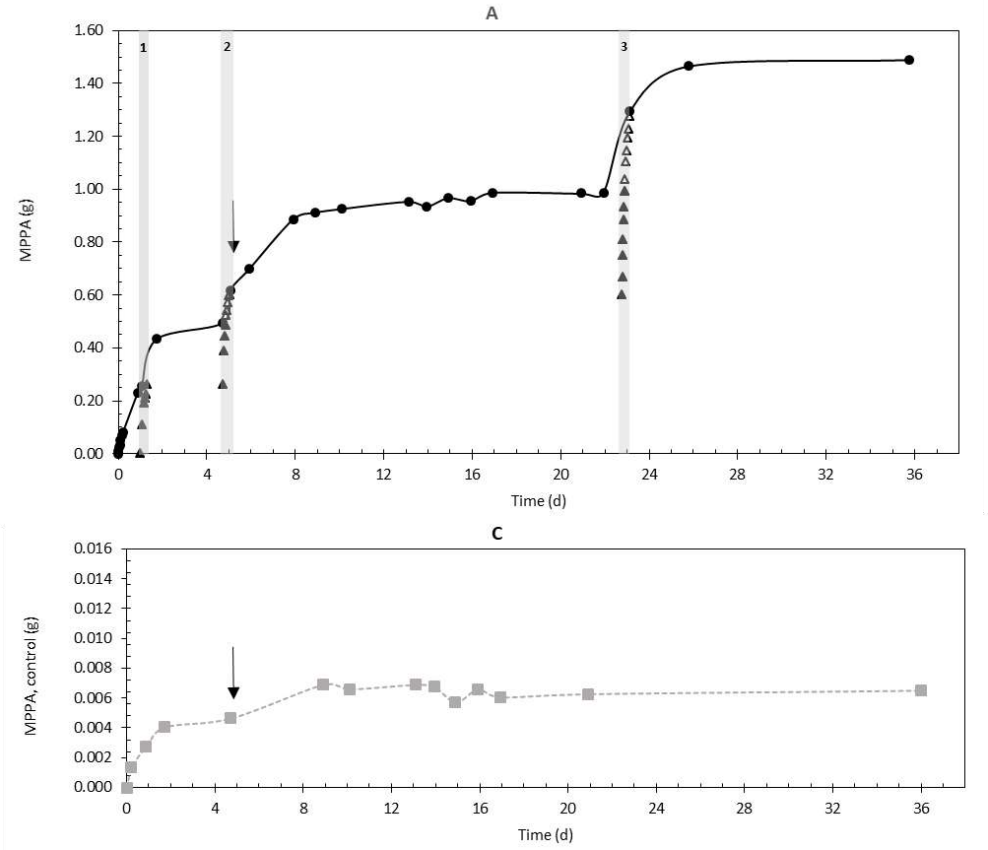


Figure 9. A) Production of MPPA during 36 days with 3 sequential extractions (grey areas 1-3). The amount of product extracted into the buffer (▲) and the total amount of product detected (●) are shown. C) Production of MPPA during 36 days in the control, where no sequential extractions were applied. Experiments were performed at 30°C. Initial amounts of amine (AD) lower phase and BA, dissolved in 0.35 L of n*-heptane upper phase, were 105 g (175 mmols) and 1.3 g (8.8 mmols), respectively. The AD lower phase contained 3.5 g of TA-v2 enzyme. Additions of BA (1.3 g) was made after 5 days of reaction (arrow). Solvent-free and control transamination (TA) were performed in 70-fold smaller scale compared to the system where separations were applied.*

The major advantage of this study is the selective isolation of the desired product (MPPA) from the other reaction components (unreacted BA and Jeffamine ED-600), without any additional purification step. Normally, the large excess of amine donor required for thermodynamic equilibrium shifting, does not facilitate the application of ISPR strategies. The unreacted excess of the commonly used IPA donor is easily lost into the product stream, hence causing contamination of the product solution and loss of the donor substrate.[86] Being heavier and not soluble in *n*-heptane, the Jeffamine® ED-600 amine donor is the key for overcoming this issue. Moreover, the intrinsic nature of this bifunctional primary amine,

characterized by repeating oxypropylene units in the backbone, can enhance the enzyme stability.[146]

Although showing the above-discussed potential, however, the choice of an amine donor/organic solvent biphasic system with strict characteristics such as solubility and density, could limit the wide applicability of this ISPR strategy. Furthermore, working with a two-liquid phase reaction system hampers a fast product extraction (mass transfer limitation). The produced chiral amine has to diffuse from the lower amine donor phase to *n*-heptane, prior extraction. This aspect needs attention since a slow product partitioning would negatively affect the space time yield of the entire process. Nevertheless, the mentioned system showed potential for further improvement. Optimization of the reactor geometry and of the stirring regime would facilitate the diffusion of the formed product from the lower amine donor layer to the upper *n*-heptane phase. Consequently, product extraction and therefore further reaction proceedings would be enhanced. Finally, a higher product concentration, compared to that reported in our study (3.82 g/L), could be achieved by minimizing the volume of acidic extracting buffer.

2.3 Solvent-free transamination (Article II)

Enzymatic catalysis is commonly applied in aqueous solvents. However, excellent catalytic activity can also be achieved in organic solvent media, as reported in several lipase- and transaminases-catalyzed processes. In parallel, the research trend within the biocatalysis community has been directed towards the utilization of 'green solvents' that are environmentally benign and recyclable.[158] This includes including ionic liquids, supercritical fluids and flourous solvents.[159] As alternative, running the reaction only in the presence of the reagents and the enzyme eliminates the use of any aqueous or organic solvents, thus resulting in solvent-free systems (SFSs).[158] In this case, the enzymatic reaction is performed at very high substrate concentrations. Such conditions generate the most challenging environments in terms of biocatalyst protein stability. Enzyme denaturation can be prevented by applying protein engineering strategies and/or enzyme immobilization techniques. Nevertheless, as demonstrated in many SFS applications, biocatalysis can run at higher rates with enhanced yields compared to conventional solvent systems.[159] For instance, the lipase-catalyzed kinetic resolution of ketoprofen in a SFS was 10-100 times more productive than in *n*-dodecanol and ethanol.[160] Furthermore, the elimination of solvent minimizes the required volume of reactor, simplifies the downstream processing hence reducing the associated costs. In the recent years, SFSs have gained attention for applications where solvent use is severely limited as, for instance, in the food industry.[161,162] Besides lipase-catalyzed processes,[159,160,163] a SFSs

has been recently employed for the cutinase-catalyzed synthesis of aliphatic polyesters.[164] To the best of our knowledge, there are no SFS reported beyond the class of hydrolases. The first application of a transaminase-catalyzed SFS, was studied in the framework of this PhD project.

As mentioned in paragraph 2.2.1, the asymmetric synthesis of MPPA using the Jeffamine® ED-600 was conducted in a two-liquid system where the upper organic solvent phase served as a medium for ketone substrate addition and as a reservoir for the product amine. We therefore decided to investigate the reaction system eliminating the organic solvent phase by adding the ketone substrate to vials containing only the transaminase TA_v2, PLP and the Jeffamine® ED-600 amine donor (Figure 10)

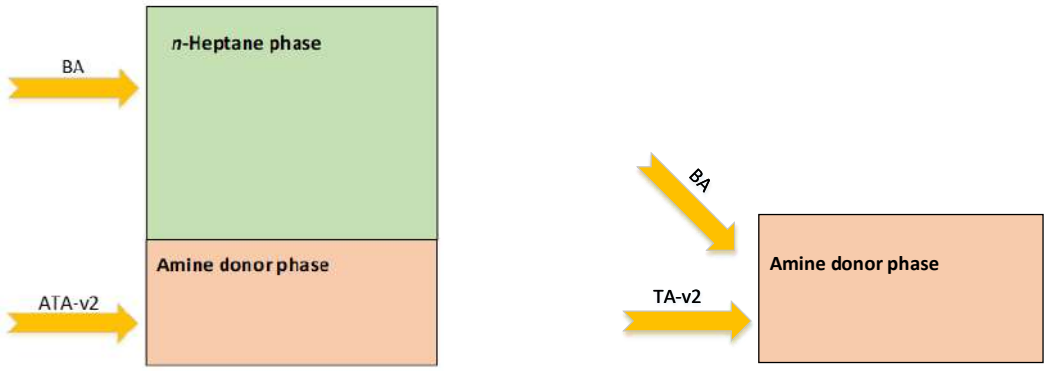


Figure 10. Comparing the concepts of the two-liquid-phase system (left) with the solvent-free system (right). Yellow arrows indicate substrate/catalyst additions to the respective phase.

Enzymatic reactions were performed fixing the enzyme loading, and testing different substrate amounts (■,□ Figure 11) and different amine donor/ketone substrate ratios (from 20:1 to 1:1, x axis, Figure 11). In accordance with previous studies,[86] increasing the BA concentration drastically decreased reaction performances. The inhibitory effect of the product amine MPPA, reported elsewhere,[150] could explain these results. Enzyme stability could also have affected the reaction proceeding, especially when high substrates loadings were tested (Figure 11 A, ■). Solvent-free transamination led to MPPA yields comparable to that observed in the biphasic batch reaction system (Article II, Table 1).[157] However, up to 6-fold higher product concentrations were achieved when running solvent-free transamination, compared to the

two-liquid-phase transamination. As shown in Figure 11 B, product concentrations up to 30 g/L were achieved working at high BA loadings.

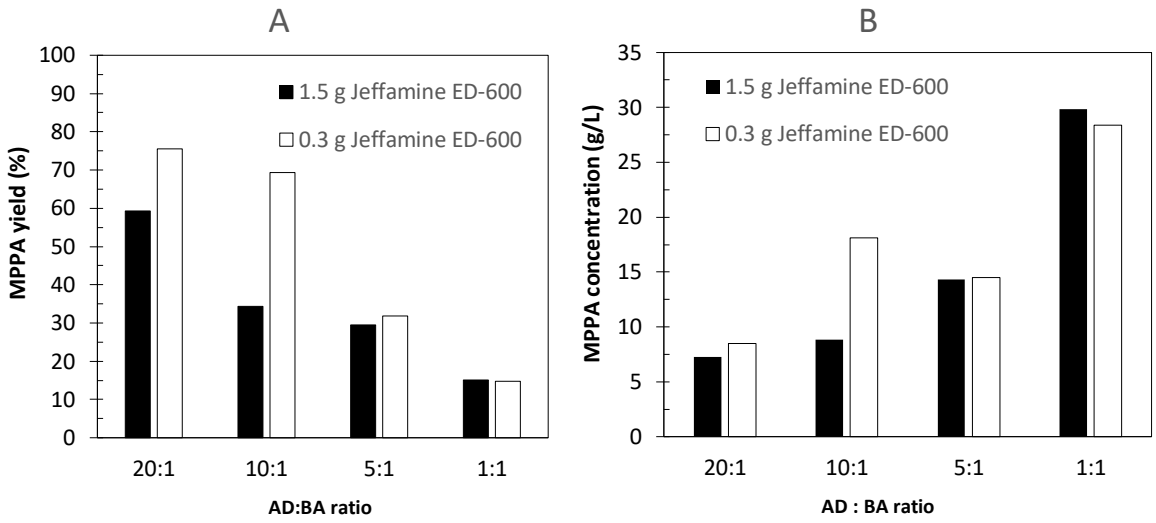


Figure 11. Effect of substrate (BA) amount on solvent-free transamination reaction in terms of product yield (A) and product concentration (B) when using 1.5 g (2.5 mmol) (■) or 0.3 g (0.5 mmol) (□) of Jeffamine ED-600 amine donor (AD). Reactions were performed at 30 ˚C, using 0.05 g TA-v2 enzyme. AD:BA ratio is expressed as mol_{AD}/mol_{BA}. Reaction time was 7 d.

Although having a potential in terms of achieving high product concentrations and reduced reaction volumes, SFSs employing Jeffamine ED-600 cannot to be combined with ISPR strategies. Jeffamine ED-600, characterized by repeating of polyethylene glycol units, is a very viscous amine donor. On the one hand its nature has shown to prevent enzyme denaturation.[146] On the other hand, the high viscosity of the reaction system limits the application of membrane-based strategies for ISPR. Assuming to work with a thermo-stable enzyme, the system could be operated at higher temperatures. This could, to some extent, reduce the viscosity of the polyethylene glycol amine donor, allowing to combine the solvent-free system with membrane-based ISPR techniques.

Overall, this study represents a proof of concept for application of SFSs in transaminase catalyzed reactions. For our preliminary investigations, we have run all the reaction for a total time of 7 days. For further studies, it would be useful to collect also data about the kinetics of the reaction and to investigate enzyme stability at different AD:BA ratios. To prevent enzyme denaturation, substrate concentration could be kept below a certain level by applying multiple step substrate additions. Extension to other substrates/ enzyme SFSs could open new possibilities and perspectives in transaminase-catalyzed chiral amine synthesis. For instance, membrane-based strategies for thermodynamic equilibrium shifting could be applied on SFSs, which employ less viscous amine donors.

2.4 An alternative device for the ATA-catalyzed synthesis and separation of chiral amines: the three-liquid-phase spinning reactor (Article III)

In the above presented results, we demonstrated the feasibility of performing the asymmetric synthesis of (*S*)-1-methyl-3-phenylpropylamine (MPPA) using HMW donor amines in aqueous (paragraph 2.1), organic solvent (paragraph 2.2.1) and solvent-free medium (paragraph 2.3). These systems realized benefits that are inherent in membrane-based ISPR. As final experimental step of this PhD project we focused on designing and exploring an alternative set-up for the synthesis and recovery of chiral amines: the three-liquid-phase (3LP) spinning reactor (Figure 12).

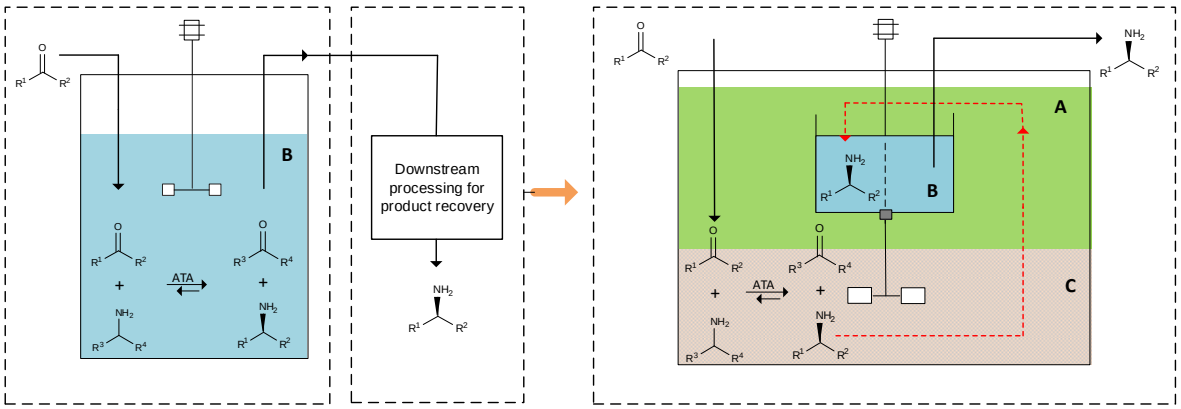


*Figure 12. Three-liquid-phase (3LP) spinning reactor for the one-step synthesis and recovery of chiral amines. Phase **A** has to be non-miscible with the other two phases **B** and **C.***

Figure 13. Three-liquid-phase (3LP) spinning reactor set-up.

This equipment can be considered as an evolution of a standard stirred 1.5 L double-jacketed glass reactor (Figure 13). The motor driven central shaft supported a stainless steel inner tubular cylinder and one radial flow impeller. Being mounted on the shaft, the designed inner tubular cylinder rotated together with the impeller. The feasibility of developing a three-liquid-phase system, employing the mentioned device, relies on the selection of a phase **A**, non-miscible with the other two phases **B** and **C**. The transaminase-mediated synthesis of (*S*)-1-methyl-3-phenylpropylamine (MPPA) was employed as model reaction (Scheme 8).

Based on the previous studies,[86,157] n-heptane was selected as organic solvent phase A. The substrate 4-phenyl-2-butanone (BA), initially supplied to phase **A**, progressively moved to phase **C**. The enzymatic reaction occurred in phase **C**, (reaction phase), consisting of the enzyme TA-v2[95] and the AD (Jeffamine ED-600), not soluble in n-heptane. Once formed, the product moved from the reaction phase **C** to the extracting phase **B** via travelling through *n*-heptane, due to partitioning. The acidic pH of the extracting phase traps the amines in their charged state, thus preventing back extraction into the organic phase and allowing the enrichment of the amine product. Depending on the design of the system, and on the working volumes employed, the device was tested in two different configurations, depicted in Figure 14.

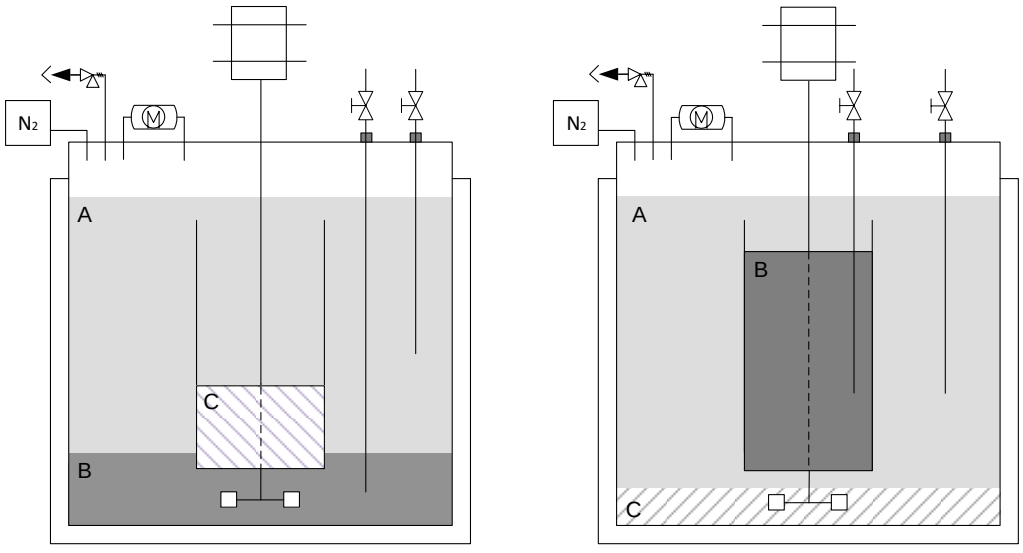


Figure 14. Principle of the three-liquid-phase (3LP) spinning reactor. A, B and C are the organic solvent phase, the reaction phase and the extraction phase, respectively. Depending on the design of the system, the device can operate in two configurations. The transaminase-mediated synthesis of (S)-1-methyl-3-phenylpropylamine (MPPA) was employed as model reaction (Scheme 8).

A preliminary partitioning test, aiming at proof of concept, was performed by preparing synthetic solutions of known concentrations of the ketone substrate and amine product. The first transaminase-mediated synthesis combined with continuous product recovery was carried out in the same configuration of the preliminary partitioning experiment (Figure 14, configuration 1), while the second experiment was run in the opposite configuration (Figure 14, configuration2). By placing the enzyme on the bottom of the vessel (diameter 2.6-fold larger than the inner tubular cylinder), the thickness of the phase containing the amine donor was considerably reduced. In this configuration product release from the amine donor layer to *n*-heptane was faster compared to configuration 1. In 5 days of continuous operation, a product yield of 52% was achieved. Compared to the control, a conventional batch experiment, where no MPPA extraction was applied, 2.6-fold higher product yield was achieved. Moreover, 85% of the formed product was extracted from the *n*-heptane into the buffer. During the operation, the MPPA concentration constantly increased in the stripping phase, despite the much lower concentration in the reactor (0.1 g/L). Minimizing the volume of the stripping phase a product concentration of 9 g/L was achieved in 5 days (Figure 15).

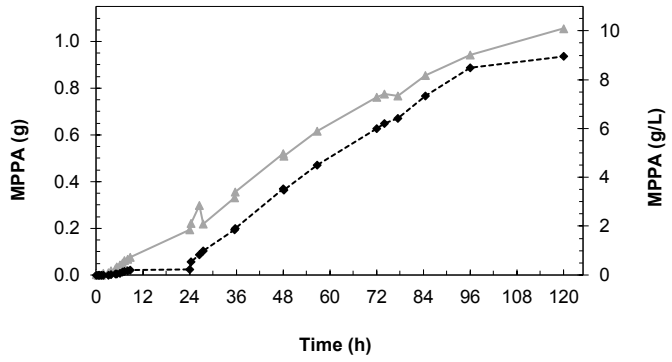


Figure 15. Production of MPPA for 5 days using the 3LP spinning reactor, configuration 2. Total product amount (g) detected in n*-heptane and in the extracting buffer, (▲, left axis) and product concentration in the extracting buffer (●, right axis) are shown.*

A higher product concentration could probably be achieved by prolonging the reaction time (Figure 15). On the one hand, the stirring of the reaction phase, imparted by the impeller, enhanced product release from the reaction phase to *n*-heptane and thus proved beneficial for simultaneous product extraction. On the other hand, stirring led to increased amine donor co-extraction into the extraction buffer (17% of the initial amount added was found in the extracting phase). Therefore, the system was stopped after 5 days of operation.

The main limitation of this extraction strategy for product removal was the amine donor co-extraction, which hampered the final product purity in the extraction phase. By comparing the performance of the 3LP reactor with the membrane-based L-L extraction set-up of our

previous work (Article II), this latter set-up was shown to be superior. Using Puramem Selective OSN membrane as physical barrier between the extraction phase and *n*-heptane phase, only 3% of amine donor was co-extracted.[157] In the 3LP system, co-extraction of the amine donor (17%) could not be avoided. To achieve higher product purities in the 3LP set-up, enzymatic reactions could be performed in aqueous environment, using alanine or another zwitter ionic amine donor. In this way, neither the amine donor nor pyruvate co-product would partition to the hydrophobic organic solvent phase.[150,165]

More in general, 3LP systems have been mainly reported for the separation and recovery of metals from complex mixtures.[166] In addition, working with three phases offers relevant technological solutions in oil recovery processes, in industrial processes such as ε-caprolactam production,[167] for the rapid isolation of organic macromolecules like cellulose enzymes and proteins[168] and for the straightforward separation of organic compounds e.g. during extraction of natural products from plants.[169] The major difficulty to tackle for a 3LP process is the physical separation of the three different phases. This can be achieved with the classical separation funnel, for batch applications. For conducting countercurrent and continuous operations, more complex devices, have been developed. The recently proposed mixer-settler-mixer three chamber integrated extractor was used for the separation of *p*-nitrophenol and *o*-nitrophenol. The separation of the two isomers was achieved by continuous mixing and separation of three non-miscible liquid phases: nonane (organic top-phase); polyethylene glycol (PEG 2000), (polymer middle-phase); and $(NH_4)_2SO_4$ aqueous solution, (aqueous bottom-phase).[170] In contrast to this existing technologies, our herein devised 3LP spinning reactor does not require special laboratory equipment, and allows to perform a separation of multicomplex mixtures between two miscible phases separated by a third immiscible phase. Hereof, it introduces more freedom for the selection of the three phases. The performance of the 3LP device can be exploited by varying the rotor speed, and the position, type, size and numbers of the impellers. Moreover, the geometry, the size and the position (height) of the tubular cylinder inside the reactor can be changed, depending on the working volumes of each phase. As already mentioned, the main limitation for product removal is often the poor selectivity between substrates and products. This aspect needs to be specifically addressed for each application. Moreover, the performance of the 3LP set-up could be affected by mass transfer limitations, due to the lower contact area between the phases. In this regards, membrane-based techniques are superior since they easily allow to increase/decrease the surface area. Finally, an additional limitation, especially for large-scale implementation, could be the design of the stainless steel inner tubular cylinder, fixed to the motor driven central shaft.

3 Chiral amine synthesis and recovery with HMW amine donors: future perspectives

The need for optically pure chiral molecules, combined with high reaction yields and minimization of side reactions has driven industries' interest towards biocatalytic process development. Due to the mild conditions frequently used in biocatalytic reactions (ambient temperature and pressure, neutral pH and aqueous media), biocatalytic processes often claim to be environmentally friendly and cost-effective. However, the only evaluation of the overall process parameters and performances, in particular during process development, can justify such conclusions.[171] The definition of the process targets, represents the first step towards process evaluation.[172] **Atom efficiency (AE)**, also known as atom economy, is nowadays the one of the most commonly used metrics for measuring the "greenness" of a reaction system, in the preliminary stage of route selection. It is based on reaction stoichiometry and mechanism, and measures how much of the starting materials end in the desired product in terms of molecular weights. In early development stages, metrics are mainly used for benchmarking biocatalytic processes for (1) assisting in the selection of different process alternatives and (2) evaluating the benefit and drawbacks of a particular technology. One of the crucial factors influencing the success and the economic feasibility of an industrial biocatalytic process in the **biocatalyst cost**. It depends on variables, such as expression level, efficiency of the fermentation protocol, enzyme specific activity and biocatalyst formulation (e.g., whole cell, cell-free extract (crude enzyme), purified or immobilized enzyme). Excluding the costs required for the biocatalysts development and optimization, a likely cost for an efficiently produced in house biocatalyst used for pharmaceutical production is calculated to be around 10–35 €/kg for whole cells (dry cell weight), 100–250 €/kg crude enzyme (cell- free extract) and 100–1000 €/kg for an immobilized preparation.[15,173] This in turn puts requirements on the **biocatalyst yield (BY)**, which indicates the product produced per amount of biocatalyst used. In a technological process, the enzyme cost should be less than 5-10% of the total process costs. Hence, in order to estimate costs, the amount of enzyme required to reach the maximal **product yield (Y)** within a given time must be determined.[174] This aspect is summarized by the **space-time yield (STY)** parameter, defined as substrate amount reacted or product produced per unit of time and volume. Another key parameter is the **product concentration (P)** also called process intensity. It determines the equipment cost and ease of downstream separation and recovery for an economical process. An economically viable biocatalytic process usually requires product concentrations of 50-100 g/L. Process environmental performance is generally quantified in an early development stage by using process-related

green chemistry metrics. If the interested reader desires more information, further reading is recommended.[171,175,176]

Table 5. Metrics commonly used to benchmark biocatalytic processes.[15,171]

Name	Expression	Target values
Atom economy (AE)	$\frac{\text{molecular weight of the product}}{\text{sum of the molecular weights of all the stochiometric reagents}} * 100$	100%
Biocatalyst yield (BY)	$\frac{\text{mass of the product}}{\text{mass of the enzyme}}$	10-35 kg $_{\text{product}}$/kg $_{\text{dry cell weight}}$ 100-250 kg $_{\text{product}}$/kg $_{\text{free enzyme}}$ 50-100 kg $_{\text{product}}$/kg $_{\text{immobilized enzyme}}$
Product yield (Y)	$\frac{\text{mass of the product}}{\text{total mass of the ketone substrate}} * 100$	>90%
Space-time yield (STY)	$\frac{\text{mass of the product}}{\text{total time} * \text{reactor volume}}$	>100 g/(L*d)
Product concentration (P)	$\frac{\text{mass of the product}}{\text{reactor volume}}$	50-100 g/L

The use of the above-mentioned process metrics allowed to evaluate the competitiveness of each proof-of concept developed in this project, compared to industrial requirements and to the other existing technologies. Advantages and drawbacks of each developed technology, as well as possible optimization strategies have been already discussed in detail. Here, an overall comparison in terms of product (MPPA) yield (Y) and concentration (P), is shown in Figure 16: Y and P of the different approaches show opposite trends. This can be explained by considering two main factors: the reaction medium and the product removal strategy applied. The highest product yield was achieved when coupling the reaction in aqueous medium with intermittent NF. However this membrane-based strategy resulted in a very low product concentration stream. By performing reactions in organic solvent, ketone substrate solubility limitations could be overcome. Therefore, the substrate (BA) loading was increased. This, combined with product extraction into a less diluted phase, resulted in enhanced product concentrations.

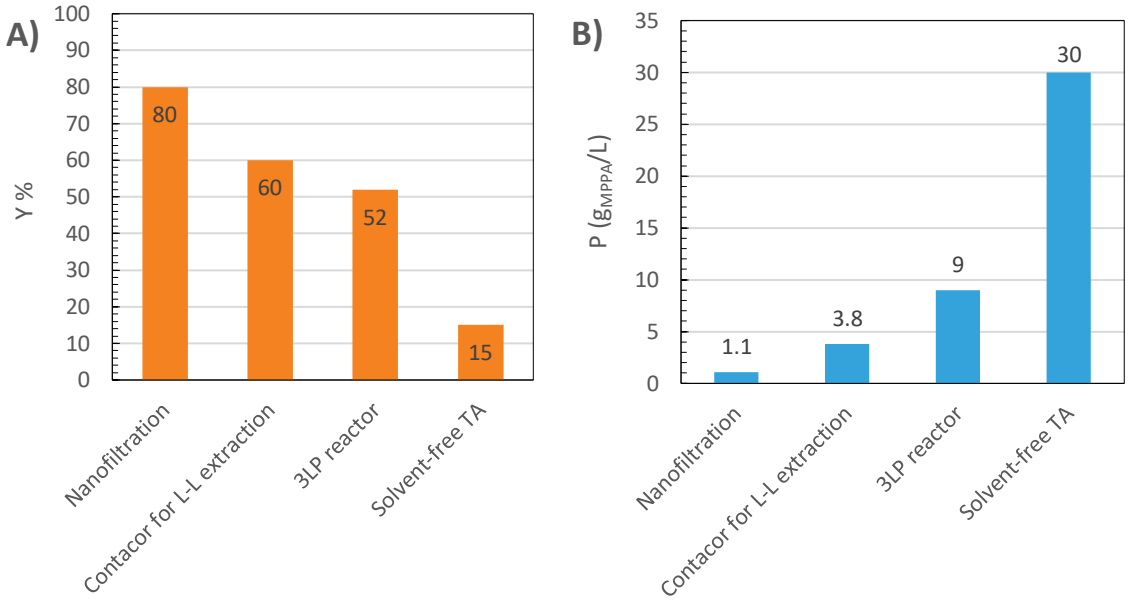


*Figure 16. Effect of different reaction media and process intensification techniques on prodcut yield (Y) ($g_{MPPA}/g_{BA,\ employed}$*100) **A**) and product (MPPA) concentration (P) **B**). Experimental details are provided in Table 6.*

Table 6. Experimental data employed for the calculation and evaluation of the process metrics.

		Total amount employed			Enzyme amount (g)	AD loss in the product stream (%)
		BA (g)	Jeffamine ED-600 (g)	Solvent (mL)		
1	**Nanofiltration**	1.3	90	600	1.2	20
2	**Contactor for L-L extraction**	2.6	105	350	3.5	< 3
3	**3LP reactor**	2	105	1400	3.5	17
4	**Solvent-free TA**	0.37	1.5	/	0.05	n.a.*

*n.a., not applicable

Highest product concentrations were achieved running SFSs (Figure 16). The highest value (30 g/L) was achieved by performing the asymmetric synthesis of MPPA from BA at an 1:1 AD:BA ratio. However, this reaction condition lead to the lowest RY observed (15%). All reactions were run for a total of 7 days and neither a kinetic nor an enzyme stability studies were conducted. Therefore, at this preliminary stage, it is difficult to evaluate the STY and the BY of solvent-free systems. As explained in paragraph 2.3, investigation on the enzyme stability and on the amine donor choice, as well as application of ISPR techniques, could reveal whether there can be or not room for further improvement of TA-catalyzed SFSs for chiral amine synthesis.

The highest product yield was achieved performing the reaction in an aqueous environment, in combination with intermittent nanofiltrations (Article I). The product yield could be further enhanced by operating the system in continuous mode. However, a nanofiltration membrane, possessing long-term stability and high rejection towards unreacted substrates is required. Nanofiltration is known to be an industrially relevant technology, well established for large-scale applications.[141,177,178] Although adaptations required for TA-catalyzed processes, it may be feasible to overcome the issues encountered in this study, including membrane stability and online process parameter control. The real limitation, which cannot be addressed by nanofiltration, is the low product concentrations of the permeate stream (Figure 16 A), compared to the target (50 g/L) and to other ISPR strategies (Table 8). NF can hardly lead to highly concentrated product streams and high productivities, unless the enzymatic reaction is performed at higher ketone substrate concentrations, in the presence of co-solvents or organic solvents. However, in this case, protein engineering techniques may be required for enhancing the stability of the enzyme at high ketone substrate/co-solvent concentrations. In contrast, product can be overconcentrated if extracted from an organic solvent (reaction medium) into a less diluted water solution (acidic buffer). This principle was applied to the spinning reactor set-up (Article III),[179] where the buffer volume was reduced by a factor of 3.5, compared to buffer volume used in the membrane contactor experiment (Article II). In this latter set-up, a product concentration of 13.3 g/L would have been achieved, by reducing the acidic buffer volume from 0.35 L to 0.1 L. Both ISPR proof-of-concepts suffer from mass transfer limitations. At this stage, this represents the main bottleneck for further process optimization. The slow diffusion of the formed product from the amine donor reaction phase to *n*-heptane limits the product extraction rate and negatively affects the final STY of the process. Therefore, this aspect needs investigations for further process optimization. For larger scale and eventually industrial applications, the membrane contactor device is recommended, compared to the 3LP spinning reactor. Contactors are compact systems, possessing a well-defined interface area, which is considerably higher compared to the designed 3LP reactor. The membrane contactor was shown to be superior also in terms of product purity, compared to all the other ISPR techniques of this study. Moreover, the enzymatic reaction could be practically combined with continuous product extraction. In contrast to the first process development (Nanofiltration, Article I), for this study, we found commercial OSN membranes possessing excellent stability in *n*-heptane (Puramem® performance and selective). In our publication, we only reported the results of experiments where the reaction was coupled with intermittent extractions. However, in our last experiment ─ performed after article acceptance ─ the feasibility of running the flat sheet contactor set-up in continuous extraction mode for 24h was proven (unpublished results).

Coupling the two-liquid-phase reaction system, with membrane extraction in continuous mode, instead of coupling the reaction with extraction intermittently (Figure 9), would improve the STY and the product yield of the overall process. Operating the system in continuous mode, assuming 100% of ketone substrate conversion in 24 h, would lead to a STY value of 7.4 g l^{-1} d^{-1}, 60-fold higher than the STY achieved running membrane product extraction in intermittent mode, as depicted in Figure 9.

Table 7. Summary of Advantages, limitations and potential of each technology investigated.

	Advantages	Limitations	Recommendations	Potential (based on the results of this study)
NF	- AD retention	- BA loss - Membrane compatibility	- Continuous mode - Enzyme immobilization	- Only with stable NF membranes. - Only for not-diluted reaction systems
Contactor	- AD retention	- Mass transfer	- Continuous mode	- Yes
3LP reactor	- No membrane required	- AD loss cannot be prevented - Mass transfer	- Optimization of the reactor geometry - Test zwitterionic ADs	- Only for small scale applications
SFS	- Minimization reactor volumes - No solvents used	- ISPR technique not applicable	- Test less viscous ADs - Kinetic study - Enzyme stability test	N.a., at this early stage

Besides the advantages and limitations of each technology ─ summarized in Table 7 ─ the use of HMW amine donors leads to a low AE reaction system. The highest MW has the amine donor, the lowest AE can be achieved. However, retention of these amines can considerably enhance this environmental metric. For instance, coupling the two-liquid phase reaction system with intermittent extractions, < 3% of HMW amine donor co-extraction was observed (Table 6, entry 2). Assuming to operate the system continuously, retaining the unreacted excess of donor amines, the AE of the process can ideally be improved from 2.4% (conventional batch reaction) to 20%. Although the improvement due to amine donor retention, the AE of a system which employs an amine donor of 600 g/mol results 3.5-fold lower compared to the AE of a conventional batch reaction with IPA amine donor (59 g/mol).

Table 8. State of the art in transaminase reaction for chiral amines synthesis. APH, acetophenone; IPA, isopropyl amine; Met, methoxyacetophenone; BA, benzylacetone; ALA, alanine; (s)-α-MBA , methyl benzylamine; PYR, pyruvate; PBR, packed bed reactor application; SLM, supported liquid membrane; LLE, liquid-liquid extraction; TD eq: thermodynamic equilibrium

Substrate - AD	Catalyst formulation	Catalyst immobilization. PBR	Product Conc. (g/L)	ISPR applied	Benefit ISPR vs. no ISPR	References
APH - IPA	Whole cells	√	55 g/L[85] 121g/L[151]	SLM	TD eq. shifted 98% vs. 50% conversion	Rehn et al., 2014[85] Rehn et al., 2016[151]
BA - IPA	Free enzyme	√	26.5	2 step LLE	Higher BA solubility Lower Prod inhibition	Heintz et al., 2017[128]
BA - IPA	Free enzyme in solvent	no	3.7 g/L	LLE using a contactor	99% vs. 70% conversion	Satyawali et al., 2017[86]
BA - Ala	Whole cells	√	Data missing	SLM	Product purity > 98%	Börner et al.,2015[150]
MBA - PYR	Free enzyme	√ (80% initial productivity retained over 21 days of continuous operation)	4.6 g/(L*min)	no	X	Bajić et. al, 2017[124]
Met - IPA	Free enzyme	no	31 g/L	Product crystallization	TD eq. shifted, high product purity	Hülsewede et al., 2019[132]
APH - IPA	Free enzyme	no	1.8 g/L	Pervaporation for co-product removal	TD eq. shifted 75% vs. 62% conversion	Satyawali et al., 2018[87]
Prositagliptin ketone - IPA	Free enzyme	no	200 g/L	Co-product evaporation	By removing 70% of the formed acetone, TD eq. shifted to > 90% conversion	Savile et al., 2010[76]

4 Conclusion

In Transaminase-catalyzed chiral amine synthesis, the choice of amine donor is highly relevant for reaction and process design and in terms of techno-economic industrial feasibility. To date, the preferred amine donor is isopropylamine (IPA), as the produced acetone can be removed easily under low pressure or slight heating, without complicating the downstream processing. However, IPA is not widely accepted from wild-type ATAs, and this fact compromises its wide applicability.[88]

In this work the feasibility of using novel, commercially available and inexpensive High Molecular Weight amine donors for the transaminase-catalyzed synthesis of chiral amines was demonstrated. Compared to the low molecular weight IPA, these molecules exhibit potential for their effective retention by commercially available nanofiltration membranes. Specifically, the application of two intermittent nanofiltrations on an enzymatic reaction performed in aqueous media enhanced an overall increase of the yield, compared to the batch reaction (**Article I**). Although the potential, the system could not be further improved due to membrane stability issues. Along with that, low product concentrations were achieved, due to the poor solubility of the ketone substrate in water environment. Therefore, the second process development focused on transamination in organic solvent (*n*-heptane). Compared to nanofiltration, higher product concentrations and purities were achieved coupling the reaction with intermittent membrane-based L-L extractions (**Article II**). The system showed potential for further optimization. For instance, coupling the reaction with product extraction in continuous mode could enhance the overall product yield and space-time yield of the process. As an alternative to membrane-based strategies we also designed a spinning reactor concept for the integrated chiral amine synthesis (in organic solvent) and recovery (**Article III**). However, membrane-based L-L extractions resulted superior compared to the spinning reactor set-up, in terms of reaction and separation efficiencies. The final aim of this work was to open new possibilities and perspectives in transaminase catalyzed synthesis and recovery of chiral amines, using HMW amine donors. In addition to reaction in aqueous and organic solvent, we also investigated the feasibility of running the enzymatic reaction in the only presence of substrates and enzyme (i.e., under solvent-free conditions). As the solvent-free system does not use solvents, this process minimizes the environmental impact. Further investigations could reveal whether this technique can or not be relevant for industrial process development.

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Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde. Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

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Publications

C. Matassa, D. Ormerod, U. T. Bornscheuer, M. Höhne, and Y. Satyawali, "**Three-liquid-phase spinning reactor for the transaminase-catalysed synthesis and recovery of chiral amines**" *ChemCatChem* **2019***,* DOI: 10.1002/cctc.201902056.

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Claudia Matassa, M.Sc.

Author contributions

Article I **Application of novel High Molecular Weight amine donors in chiral amine synthesis facilitates integrated downstream processing and provides in situ product recovery opportunities.**

Claudia Matassa, Dominic Ormerod, Uwe T. Bornscheuer, Matthias Höhne and Yamini Satyawali. *Proc. Biochem*. **2019**, *80*, 17-25.

YS, DO and MH directed the project; CM designed and conducted the experiments; CM drafted the manuscript, which was revised and approved by all authors.

Article II **Jeffamine® ED-600: a polyether amine donor for enzymatic transamination in organic solvent/ solvent-free medium with membrane-assisted product extraction.**

Claudia Matassa, Alessandra Romani, Dominic Ormerod, Uwe T. Bornscheuer, Matthias Höhne and Yamini Satyawali. *J. Chem. Technol. Biotechnol*. **2019**, *95*, 604-613.

YS, DO and MH directed the project; CM designed and conducted the experiments with the help of AR; CM prepared the manuscript, which was revised and approved by all authors.

Article III **Three-liquid-phase spinning reactor for the transaminase-catalyzed synthesis and recovery of a chiral amine**

Claudia Matassa, Dominic Ormerod, Uwe T. Bornscheuer, Matthias Höhne and Yamini Satyawali. *ChemCatChem* **2019**, DOI: 10.1002/cctc.201902056.

YS, DO and MH directed the project; CM designed the equipment and conducted the experiments; CM drafted the manuscript, which was revised and approved by all authors.

Claudia Matassa, M.Sc. Matthias Höhne, Jun.-Prof. Dr.

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To my father, my reference. This work is as yours as mine

To my mother, for her immense love, encouragement and empathy. You are with me, always.

Articles

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Application of novel High Molecular Weight amine donors in chiral amine synthesis facilitates integrated downstream processing and provides *in situ* product recovery opportunities

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ABSTRACT

The immense potential of transaminase-catalyzed reactions for chiral amine synthesis is often hampered by unfavorable thermodynamic equilibrium positions and product inhibition issues. In the here presented proof of concept, we demonstrate a membrane assisted strategy for addressing these challenges. It involves a separation based on differently sized amine donor and amine product molecules. Novel High Molecular Weight (HMW) amine donors, provided in excess for thermodynamic equilibrium shifting, are successfully employed in transaminase-catalyzed reactions and are effectively retained by commercial nanofiltration membranes by a size exclusion mechanism. Retention of HMW amine donors combined with selective product removal, in batch mode, shifted the equilibrium enhancing substrate conversion by an additional 25% compared to the control reaction. Along with the potential of this approach, certain limitations were also revealed in this study. Only few of the investigated enzymes accepted the HMW donor molecules, and along with very efficient amine product removal, there was undesirable loss of ketone substrate. Therefore, a broader enzyme screening, and the selection of a selective and solvent stable membrane, is essential for better and broader applicability of the concept.

1. Introduction

Chiral amines are important building blocks in pharmaceutical drugs, natural products, fine chemicals and agrochemicals. During the last decade, the use of transaminases (TAs) has been identified as a very powerful method for chiral amine synthesis compared to the present chemical methods, which are often multi-step with challenging purification operations. TAs, which are probably the most important and ubiquitous enzymes for synthesis and degradation of chiral amino acids and amines in nature, belong to the largest group of pyridoxal-5′-phosphate (PLP)-dependent enzymes [1]. Most TAs catalyze the transfer of an amine group of a sacrificial amine donor to the carbonyl carbon of an α-keto acid. Amine transaminases (ATA), a subgroup of ω-transaminases, also accept ketones and aldehydes as substrates and are especially interesting for the synthesis of α- or β-chiral amines [2]. Despite the enormous synthetic potential, the limitations associated with the use of ATAs, have contributed to the relatively slow uptake of these biocatalysts in both academia and industry. Indeed, a number of challenges related to the implementation of biocatalytic transamination processes have to be addressed for each target substrate, which include an unfavorable thermodynamic equilibrium of the reaction, substrate and product inhibition, restricted substrate scope and low productivity and stability of the biocatalyst. Developing novel equilibrium shift strategies is one of the ongoing fields of research [3,4]. To the best of our knowledge, the most commonly used amine donors in transamination reactions are alanine (Ala) and isopropylamine (IPA). 1-Phenylethylamine (PEA) is also applied in some processes. On the one hand, it is a relatively “strong” amine donor, but on the other hand, its applications might not be cost-efficient due to the higher price of the enantiopure PEA and complication of down-stream processing. Being less expensive than Ala or especially PEA, IPA is usually preferred for industrial process development [5,6]. In both cases, the unfavorable

Abbreviations: Ala, Alanine; ATA, Amine transaminase; DSP, Downstream processing; HMW, High molecular weight; IPA, Isopropylamine; ISPR, *In situ* product recovery; LMW, Low molecular weight; NF, Nanofiltration; OSN, Organic solvent nanofiltration; PEA, 1-Phenylethylamine; PEG, Polyethylene glycol; PLP, Pyridoxal-5′-phosphate; TA, Transaminase

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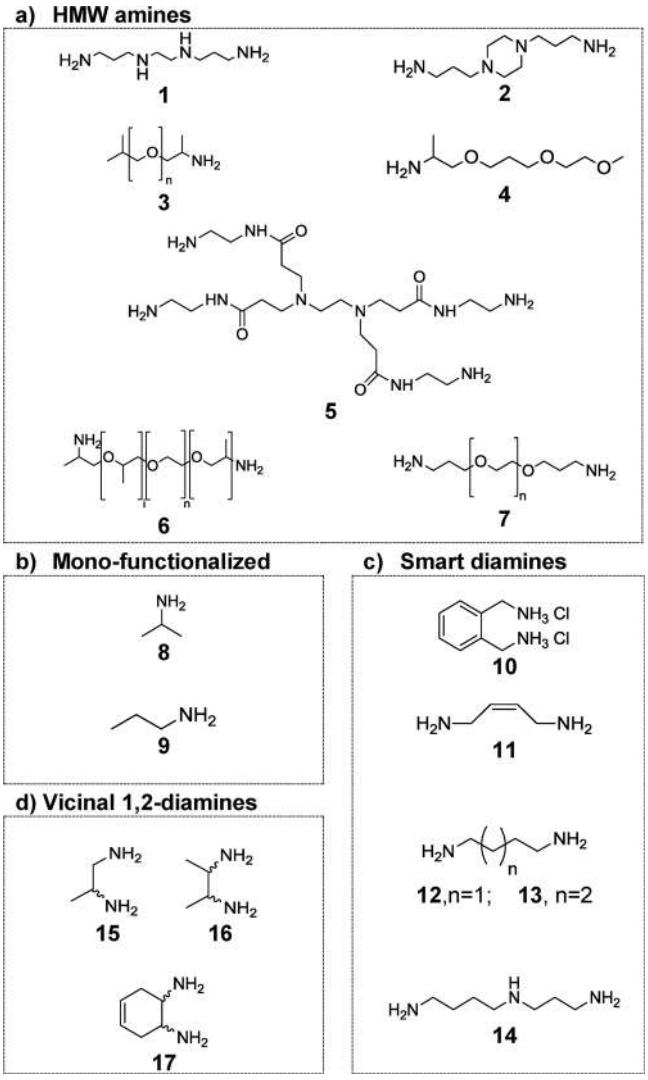

Fig. 1. Categories of amine donors employed for the asymmetric amination of 4-phenyl-2-butanone (**18**).

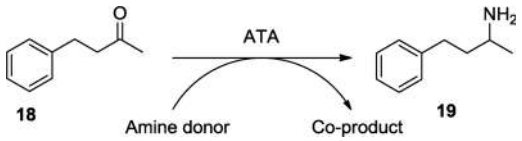

Fig. 2. Model reaction selected for the process investigations. Amine donors **3**, **6** and **8** (Fig. 1) were investigated. Amine donor **6** was selected for further process development.

reaction equilibrium (Keq) hampers high product yields, that is one of the main benchmarks required for pharmaceutically interesting chiral amines. Diamines such as *o*-xylylene diamine (**10**, Fig. 1) [7] or but-2-ene-1,4-diamine (**11**, Fig. 1) [8], also called smart amine donors, were recently suggested as effective amine donors, as the produced co-products undergo cyclization and tautomerization and thus cannot be utilized in the reverse reaction. Because of their high price and the formation of insoluble precipitates, these amine donors are rather only suitable for laboratory scale applications. The alternative biogenic terminal diamines (**12-14**, Fig. 1), which require near stoichiometric donor loadings, were found to afford high conversions of *ortho*-substituted ketone substrates. However, considerably lower conversions were achieved when using non-activated ketones such as 4-phenyl-2-butanone (**18**, Fig. 2) [9]. The easiest and straightforward strategy to counteract the unfavorable Keq, is to provide an excess of the amine donor (5–100 times or higher) compared to the keto-substrate and thus to push the reaction to high conversions. Moreover, an excess of amine donor can be beneficial to achieve a high productivity in cases of a high Km. As a result, the excess (unreacted) amine donor is either wasted, or requires further downstream processing thus leading to high process costs. Alternatively, applying *in situ* product recovery (ISPR) strategies can shift the equilibrium to the amine product side and avoid the losses of excess amine donor. In addition, ISPR strategies also address the product inhibition issues. Though membrane based technologies exhibit immense potential for process intensification in transamination reactions, to date, there are only a limited number of reported investigations in this field [10–13].

Alternatively to these reported solutions, we envisioned a strategy based on differently sized amine donor and amine product molecules. Particularly, this research focuses on the application of novel High Molecular Weight (HMW) amine donors (**1-7**, Fig. 1) in transamination reactions. In contrast to Ala or IPA, these large molecules, provided in excess for thermodynamic equilibrium shifting, can be easily retained by commercial nanofiltration membranes, thus a selective permeation of the desired smaller product amines is possible. This provides integrated downstream processing (DSP) as well presents ISPR opportunities. In order to demonstrate the feasibility of this approach, the here presented study focused on (i) screening of an ATA collection to identify enzymes accepting HMW amine donors, (ii) investigating membrane selectivity against the HMW amine donors with model reaction solutions, and (iii) conducting enzyme reactions together with nanofiltration membranes using the most suitable ATA and membrane identified before.

2. Material and methods

2.1. Chemicals and enzymes

The chemicals used in this study including 4-phenyl-2-butanone (**18**) (98% purity), 4-phenyl-2-butylamine (**19**) (98%), commercially available amines (**1-8**, Fig. 1), and pyridoxal 5′-phosphate (PLP), were purchased from Merck.

The amine transaminase TA_3HMU was purchased from Enzymicals AG, Greifswald, Germany. TA_3HMU enzyme was supplied as crude extract freeze dried powder. The activity of this enzyme was 970 mU/mg (photometric assay based on acetophenone detection [14]). The amine transaminases TA_v2 was purchased from c-LEcta GmbH, Leipzig, Germany. The purified enzyme was supplied as freeze dried powder. The activity of TA_v2 was 1.73 U/mg (value provided by the supplier, based on acetophenone detection [14]). Other amine transaminases tested (Table S1) were recombinantly expressed and purified using metal affinity chromatography as described previously [15–19].

2.2. Screening of the ATA collection

The glycine oxidase assay [20] was used as qualitative tool for the screening of ATAs for the acceptance of HMW amine donors **1-7**. Detailed experimental procedures for expression, preparation of ATAs crude lysate and screening are provided in the supporting information.

2.3. Model reaction investigations

The reaction used to investigate the potential of the HMW amine donors **3** and **6** was the asymmetric amination of 4-phenyl-2-butanone **18** to yield the chiral amine **19** (Fig. 2). In addition to the HMW amine donors **3** and **6**, the low molecular weight (LMW) isopropylamine (IPA) **8** (Fig. 1) was used as benchmark donor. Reactions were performed in 2 mL volume containing 0.78–12.5 mg/mL of either TA_3HMU [16] or TA_v2 [21], 10–500 mM donor amine **3**, **6** or **8** (Fig. 1), 10 mM model substrate **18** (dissolved in DMSO), and 0.1 mM PLP in 100 mM 2-

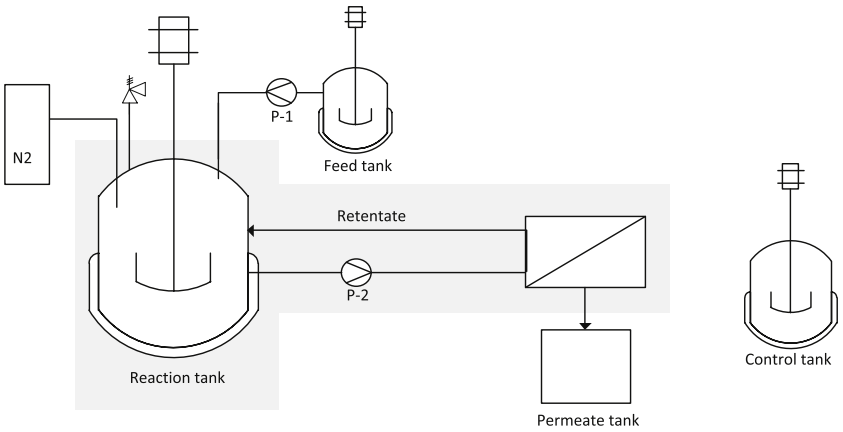

Fig. 3. Schematic setup of the in-house NF system. P-1 is the feed pump, P-2 is the circulation pump. Everything in the shaded zone is under pressure during operation.

(Cyclohexylamino)-ethanesulphonic acid (CHES) buffer, pH 9.5. The final DMSO concentration was 5% (v/v). While incubating at 30 °C and 1000 rpm, samples (200 µL) were taken after distinct time periods, supplemented with 10 µL of trifluoroacetic acid (TFA) and prepared for Ultra-Performance Liquid Chromatography (UHPLC).

2.4. Membrane screening

Nanofiltration (NF) tests were performed in a cross-flow filtration unit made in-house, specifically designed to work with both aqueous and organic solvent based streams, pressurized with nitrogen gas (Fig. 3). A cross flow velocity of 2 m/s and a transmembrane pressure of 20 bar were used. The test unit consisted of a 1 L thermostat-controlled reaction vessel (reaction tank, Fig. 3), a gear pump (P-2, Fig. 3) for circulation and a rectangular membrane housing, membrane surface area 100 cm^2. The permeate solution was collected in a recipient placed on a scale (permeate tank, Fig. 3).

First screening of thirteen, previously conditioned, commercial polymeric membranes (Table S2) was performed employing 300 mL aqueous solutions containing 250 mM **3** or **6**, pH 9.5. Out of these thirteen, two membranes were further tested with model aqueous solutions having the composition of a biocatalytic reaction expected after 24 h reaction. Specifically, 5 mM ketone substrate **18**, 5 mM amine product **19**, 250 mM HMW amine donors **3** or **6** and 0.1 mM PLP were dissolved in 100 mM CHES buffer, pH 9.5. Membrane screening was performed at three pressures, *viz.* 10, 20 and 30 bar and at room temperature. Permeate and retentate samples were taken at steady-state conditions (stable flux), and rejections were calculated as explained below

$$R\ (\%) = \left(1 - \frac{C_p}{C_r}\right)*100$$

where C_p and C_r denote concentrations in the permeate and retentate, respectively.

2.5. Combination of enzymatic reaction and membrane separation

2.5.1. Reaction in batch mode combined with sequential separation

The reaction (Reaction-1, Fig. 8a) was performed in 600 mL volume containing a reaction mixture of 2 mg/mL of TA_v2 powder, 250 mM HMW donor amine **6**, 10 mM substrate **18** (dissolved in DMSO) and 0.1 mM PLP in 100 mM CHES buffer, pH 9.5 at 30 °C. After 16 h reaction time, 550 mL of the reaction mixture was transferred into the NF system (Fig. 3) and NF was performed. The remaining 50 mL of the reaction mixture was used as control without filtration (control tank, Fig. 3). After the first NF (NF-1, Fig. 8a), the collected permeate and the retentate were mixed, and 5 mM of substrate **18** was manually added to the reaction tank (Reaction-2, Fig. 8a). Additional substrate was added also to the control condition (Control-2, Fig. 8a). After 15 h reaction time, the second nanofiltration was performed on the reaction mixture (NF-2, Fig. 8a). NF-1 was performed using Desal DK membrane while NF-2 was performed using Duramem 200 membrane. Reaction, permeate and retentate samples were taken after distinct time periods and prepared for quantitative analysis.

2.5.2. Reaction in continuous mode (constant substrate feeding) coupled with separation

The substrate solution (200 mM **18** dissolved in DMSO), placed in the feed tank (Fig. 3), was continuously added into the NF loop, charged with 2 mg/mL of TA_v2 powder, 0.1 mM PLP and 250 mM amine donor **6**. In this operational mode, substrate was slowly added to the reaction mixture by a constant volume diafiltration process, which was defined as a process in which solvent (in our study the substrate solution) is added to the filtration unit at a rate that is equivalent to the rate at which solvent permeates the membrane.

2.6. Analytical methods

Substrate (**18**, Fig. 2) and product (**19**, Fig. 2) concentrations were quantified by UHPLC (Thermo Scientific™) with UV detection at 194/210 nm. The chromatographic separation was achieved using 1 µL injection onto a C18 reversed-phase column (Waters Acquity UPLC® BEH C18 1.7 µm, 2.1 mm x 50 mm). The column temperature was kept at 40 °C. The gradient elution program is using a mobile phase A 0.1% formic acid (FA) in water (dH_2O) and a mobile phase B 0.1% FA in acetonitrile (ACN). The elution program was as follows: 0–4 min 99-30% A; 4–5 min 30-1% A; 5–5.1 min 1–99% A; 5.1–7.5 min 99% A; all at a flow rate of 400 µL/min. Amine donors **3** and **6** were analyzed by HPLC with ELSD detector on Alltima HP C18 HL 5 µm, 4.6 mm x 250 mm (Alltech Grace). The eluents for amine donors **3** and **6** were: A) dH_2O, 0.1% FA; B) ACN, 0.1% FA. The elution program was as follows: 0–3 min 100-95% A; 3–15 min 95-50% A; 15–18 min 50% A; 18–19 min 50–100% A; 19–24 min 100% A; all at a flow rate of 900 µL/min.

The acetophenone assay [14] and the glycine oxidase assay [20] were performed as described earlier in 96-well micro titer plates for assaying activity for PEA and HMW amine donors.

3. Results and discussion

3.1. Biocatalyst investigations

Diverse High Molecular Weight amine donors (**1–7**, Table 1) were selected for initial evaluations because of their commercial availability and their relative low cost. Structural similar diamines with lower

Table 1
Molecular weight of the commercial available HMW amine donors selected for this study. Amines **3**, **4** and **6** are Jeffamines®.

HMW donor amines	Molecular weight (g/mol)
1	174
2	200
3	400
4	600
5	516
6	600
7	1500

molecular weight have been successfully employed for transaminase mediated biotransformations [7–9,22–25]. For a proof of concept, we aimed to identify at least one ATA capable of accepting bulky HMW amines as donor substrates. For preliminary evaluations, the glycine oxidase assay (Figure S1) was used as rapid and convenient method for the screening of (*R*)- or (*S*)-selective ATAs with interesting amine donors in microtiter plates [20]. The ATA selection was made on the basis of ATAs availability, stability, broad substrate and amine donor scope. The first screening focused on evaluating eight wild-type biocatalysts with amine donors **1**, **2**, **5** and **7**. The photometric assay employed was semi-quantitative: the increasing absorbance correlates with enzyme activity. Under the experimental condition tested, the (*S*)-selective ATA

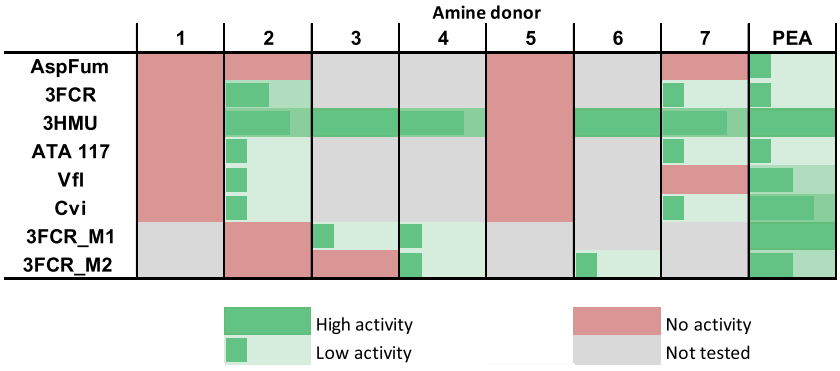

Fig. 4. Heat map: ATA screening towards HMW amine donors **1**–**7** (Fig. 1). Preparation of crude lysate of ATAs, glycine oxidase expression and purification, and the glycine oxidase assay were performed as described elsewhere [20]. Final substrate concentration amounted to 2.5 mM glyoxylate substrate, 20 mM HMW amine donors **1**–**7**, 7.28 U/mL horseradish peroxidase, 0.12 mg/mL purified glycine oxidase, 3 mM 4-amino antipyrine and 4.7 mM vanillic acid in CHES buffer (100 mM) pH 9.5 in total reaction volumes of 150 µL per well. 2.5 mM PEA was applied as positive control. TA_3HMU enzyme with the positive control PEA was taken as reference (high activity, full green bar). Low activity was defined as ¼ of the activity of the reference (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

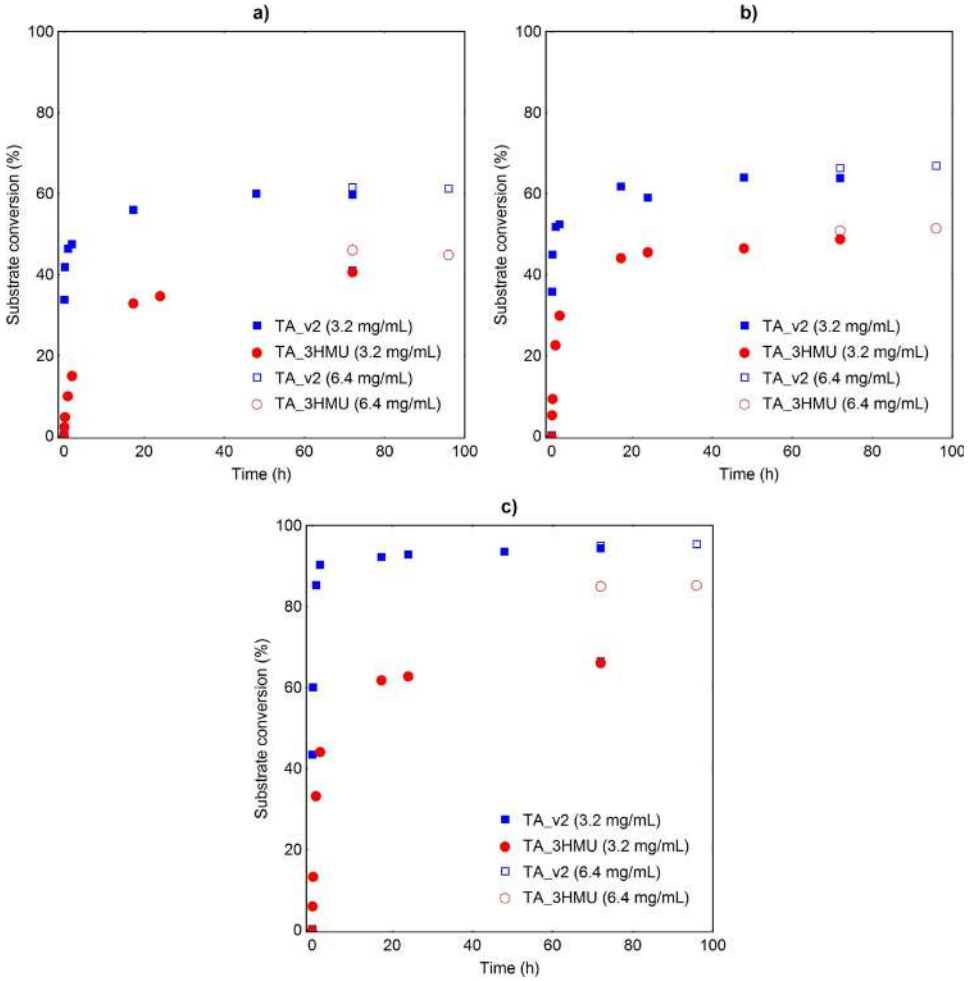

Fig. 5. Enzyme TA_3HMU (circle) and TA_v2 (square) stability with the HMW amine donors **3** a), and **6** b) or with the benchmark low molecular weight (LMW) amine donor **8** c). Experimental conditions: 10 mM **18**; 250 mM amine donor (**3**, **6** or **8**); 0.1 mM pyridoxal-5′-phosphate (PLP); 5% DMSO; 100 mM CHES buffer, pH 9.5, 30 °C, 1000 rpm. 3.125 mg/mL enzyme was used. The same amount of fresh enzyme was added after 72 h asymmetric synthesis reaction (empty dots).

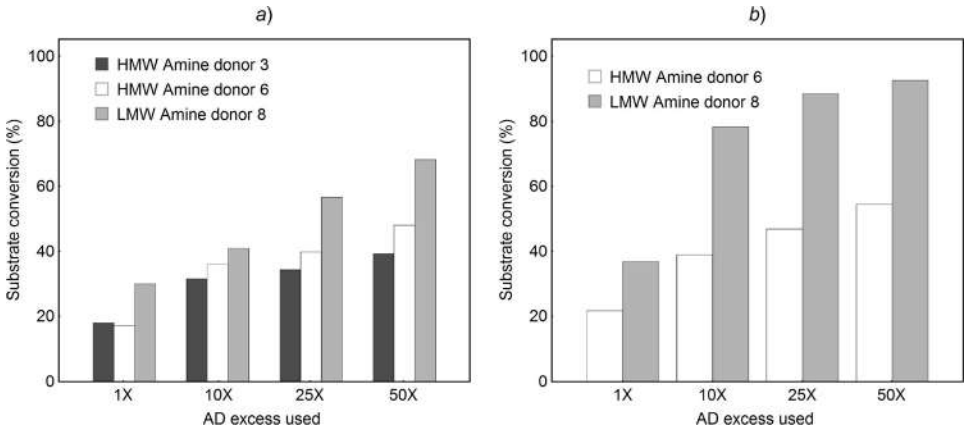

Fig. 6. a) 24 h conversion of substrate **18** when using different amine donor (AD) loadings. Experimental conditions: 3.125 mg/mL crude extract lyophilized TA_3HMU was used, b) 48 h conversion of substrate **18** when using different AD loadings. Experimental conditions: 0.5 mg/mL heat purified TA_v2 was used.

from *Silicibacter pomeroy* (TA_3HMU) [16] exhibited unexpected high activity in the presence of amines **2** and **7** as donor. Only modest activities were observed with the other tested enzymes (Fig. 4) The results achieved with the positive control PEA, were in-line with literature reports [20].

Having identified one enzyme able to well accept HMW amine donors, further investigations focused on the screening of TA_3HMU and three recently engineered 3FCR enzymes [5,26–28] with three commercial available HMW Jeffamines® (amine donors **3**, **4** and **6,** Fig. 1). In contrast to the other tested HMW amine donors, these molecules possess a typical polyethylene glycol (PEG) backbone, which is known to prevent enzyme denaturation [29,30]. The 3FCR mutants poorly converted glyoxylate substrate. In contrast, TA_3HMU outperformed the mutants in the presence of the selected Jeffamines® **3**, **4** and **6**.

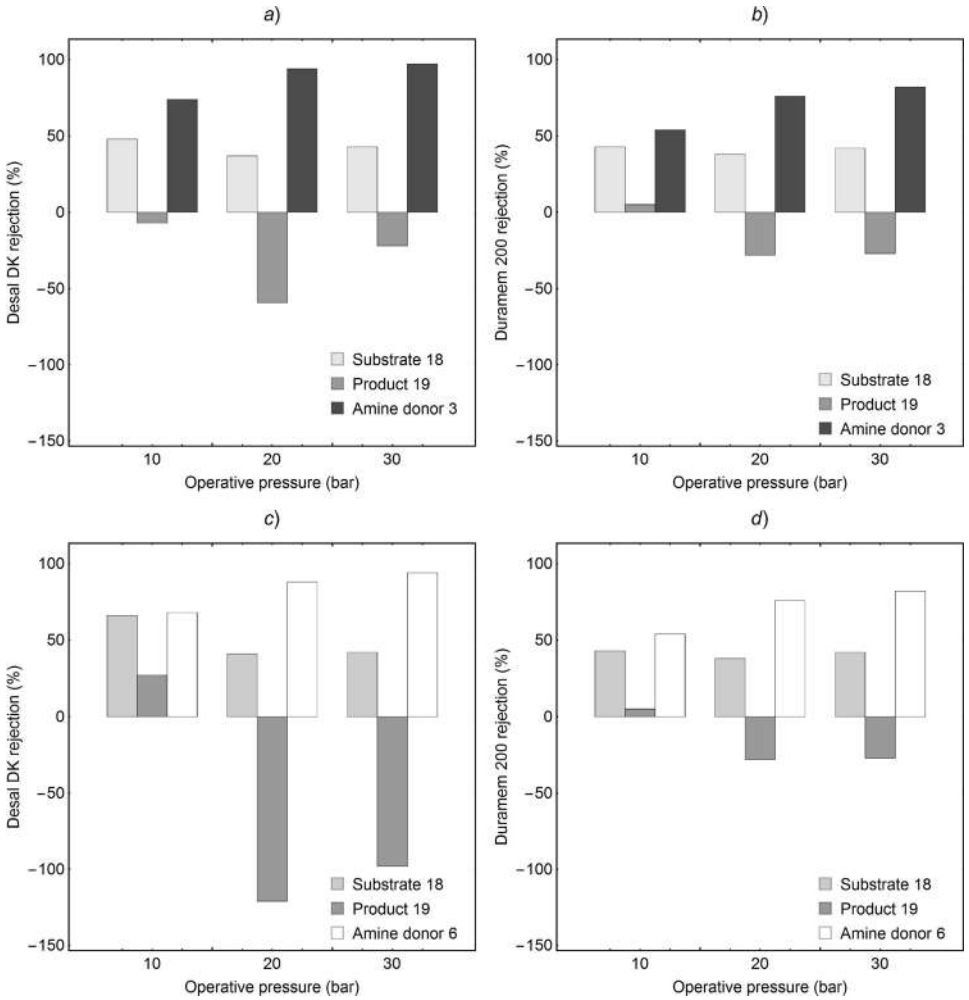

Fig. 7. Retention (rejection) of substrate **18** (light grey bars), product **19** (dark grey bars) and amine donor **3** (black bars) or amine donor **6** (white bars) in function of the operative pressure (bar) when using a), c) Desal DK or b), d) Duramem 200 membranes. Synthetic solutions containing 5 mM **18**, 5 mM **19**, 250 mM donor amine **3** a), b) or 250 mM donor amine **6** c), d), 0.1 mM pyridoxal-5′-phosphate (PLP), 100 mM CHES buffer, pH 9.5 were used.

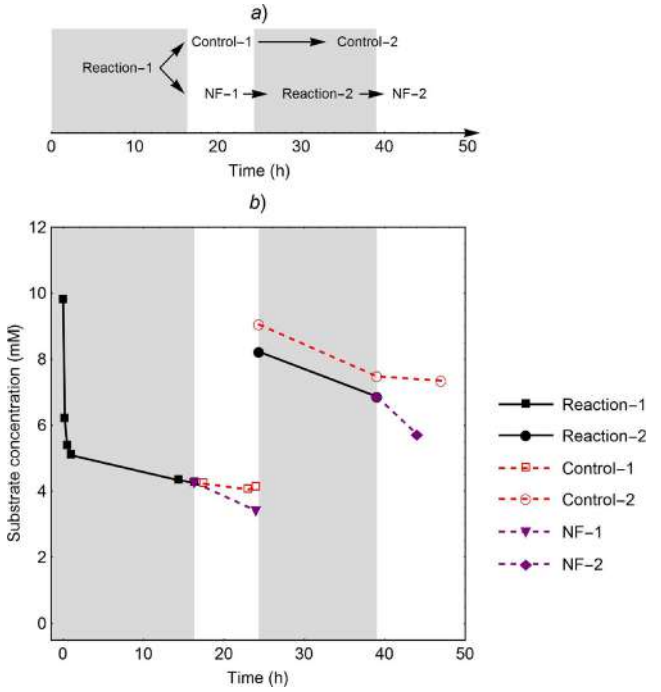

Fig. 8. Enzymatic reaction (grey areas) followed by separation (white areas): a) summary of each step and b) substrate consumption in function of time. Initial concentrations of substrate **18** and HMW amine donor **6** were 10 and 250 mM, respectively. After running the reaction for 16 h, the first nanofiltration (NF-1), using Desal DK membrane, was applied to 90% (v/v) of the reaction mixture. The remaining reaction mixture was used as control and no NF was applied (Control-1). After a total reaction time of 24 h, substrate **18** (5 mM) was added to both NF-1 and control-1 reaction mixtures (Reaction-2 and Control-2, respectively). After a total reaction time of 39 h, a second nanofiltration was applied to the NF-treated reaction mixture (NF-2) using Duramem 200 membrane. NF-2 was run for 5 h.

Moreover, no severe inhibition effects of large amine donors could be observed at the concentrations used in the biocatalysis experiments. The activities observed with TA_3HMU are indeed comparable to those achieved with the positive control PEA amine donor (Figure S2, supporting information).

3.2. Selection of the model reaction and optimization of the reaction parameters

The acceptance of different keto substrates by TA_3HMU enzyme in asymmetric synthesis reaction has already been described in literature [16,25,31,32]. For the process development of this study, three thermodynamically challenging keto substrates were rationally selected and investigated (Figure S3, Figure S4). The ketone substrate **18** (Fig. 2) was then chosen due to its relative ease of transamination. In addition to HMW donor amines **3** and **6**, IPA (**8**, Fig. 1) was used as benchmark donor. An enzyme concentration of 3.2 mg/mL was found to be optimal for further investigations (Figure S5, supporting information). The benchmark amine IPA **8** is a highly favored donor among the tested amine donors. 82% ketone substrate conversion was achieved when using this LMW amine while only ~40% substrate was converted when using the HMW amine donors **3** or **6** (Figure S5). As demonstrated on industrial scale, **8** is indeed a valuable amine donor [18]. However, the 50–100 fold excess often employed, complicates downstream processing and might hamper the stability and activity of especially wild-type ATAs [19,23,32,33]. TA_3HMU stability towards amine donors **3**, **6** and **8** was therefore explored.

The wildtype TA_3HMU appears to have inactivated in the presence of amine donor **8** before reaching the equilibrium. Indeed, conversion further increased when fresh enzyme was added to the reaction mixture (Fig. 5c, empty circle). In contrast to **8**, in the presence of HMW donor amines, the conversion did not further increase when fresh enzyme was added (Figs. 5a and 5b). Enzyme stability in the presence of the tested Jeffamines® **3** and **6** could be connected to the intrinsic nature of these bifunctional primary amines, characterized by repeating oxypropylene units in the backbone. In addition to TA_3HMU, the solvent resistant TA_v2 was also investigated. Surprisingly TA_v2, which has been recently engineered for working at high concentrations of **8**, [21] resulted to be active and stable also when using amine donors **3** and **6**. As shown in Fig. 5, TA_v2 enzyme (square) outperformed TA_3HMU (circle) in terms of final conversions and reaction rates. Moreover, as expected, an excess of HMW amines on both TA_3HMU and TA_v2 enzymes shift the equilibrium to the product side (Fig. 6) and lead to higher reaction rates (data not shown). For subsequent studies, a 250 mM (25 times excess) amine donor concentration was used, in order to achieve shifted equilibrium and to avoid any viscosity issues (which occurred with further increasing the concentrations of **3** and **6**).

Under the optimized reaction conditions, without any membrane strategy applied, significant substrate conversions were achieved (Table 2). As described in the introduction section, a range of smart amine donors have been designed with the intention to shift the equilibrium by cyclization of the formed co-products. With the exception of amine donors **10** and **11**, only moderate to good conversions were obtained (Table 2, **9-17**).

Table 2
Conversion (c) of **18** with different amine donors (ADs) after 24 h.

AD	AD excess	c (%)	Enzyme	References
3	25	45	TA_3HMU	This study
3	25	61	TA_v2	This study
6	25	51	TA_3HMU	This study
6	25	66	TA_v2	This study
8	25	60	TA_3HMU	This study
8	25	95	TA_v2	This study
8	5	68	TA_3HMU	[25]
9	1	0	TA_3HMU	[25]
10	1	> 99	ATA 113	[7]
11	3	92	TA-P1-A6	[8]
12	5	34	SpuC	[9]
13	5	72	SpuC	[9]
14	1	47	SpuC	[9]
15	5	53	TA_3HMU	[25]
16	5	10	TA_3HMU	[25]
17	5	25	TA_3HMU	[25]

3.3. Membrane screening

In an initial screening, we investigated the separation performance of thirteen NF membranes by applying solutions that only contained the amine donor **3** and **6**. With the exception of Puramem membranes, all the membranes gave ≥80% rejection of the tested Jeffamines (Table 3). Based on these results, the two membranes (Desal DK and Duramem 200) were selected for further exploratory investigations on substrate/product separation by employing model aqueous solutions (containing all other components except enzymes). Although these two membranes have not the highest rejections for **3** and **6**, they were selected since the transport of the small substrate **18** and product **19** solutes across polymeric nanofiltration membranes is governed by a diffusion mechanism as opposed to the retention/rejection of HMW donors, which is determined by size exclusion mechanism. The keto substrate **18** was partially retained by both membranes (Fig. 7, light grey bars). In contrast, the amine product **19** was transported across the membrane preferentially to the solvent, hence achieving negative rejections (Fig. 7, dark grey bars). Surprisingly, Desal DK showed higher selectivity compared to Duramem 200. From this result we suggest that there is a correlation between the hydrophilicity of the membrane and

its capacity to permeate the product amine while retaining the substrate. The used ketone substrate is indeed hydrophobic compared to the product amine which is more hydrophilic. Therefore, the hydrophilic membrane surface favors the permeation of amine product while retaining the keto substrate.

Table 3
Membrane rejections toward amine donor **(AD) 3** (400 g/mol) and **6** (600 g/mol). Synthetic solutions containing 250 mM of **3** or **6**, 0.1 mM pyridoxal-5′-phosphate (PLP), 100 mM CHES buffer, pH 9.5 were used.

Membrane		Amine donor rejection %	
Commercial name	Nom.Cut-off (Da)	AD 3	AD 6
Synder NFX	150-300	86	86
Synder NFW	300-500	82	85
Duramem200	200	80	86
SolSep-10206	300	< 30	84
GE KH Duracid	200	n.a.	62
Desal DK GE	150	85	88
Alfalaval NF99	< 200	n.a.	83
Dow NF90	200	n.a.	83
Puramem Flux	n.d.	< 30	n.a.
Puramem Performance	n.d.	< 30	n.a.
PuraMem selective	n.d.	< 30	n.a.
MPF-34	200	92	98
B4022	n.d.	80	85

n.a.: not tested due to very low fluxes; n.d.: not determined.

Overall, this part of the study indicates that the membrane selectivity between product and substrate is not large enough to fulfill all the advantages of ISPR. However, by finding a stable and highly selective membrane, with right polarity under test conditions, it would be possible to address both the thermodynamic equilibrium as well as product inhibition caused by the chiral amine product, without any consistent contamination of the product stream.

3.4. Combination of enzymatic reaction and membrane separation

3.4.1. Reaction in batch mode combined with sequential separation

Having successfully proven the effectiveness of both reaction and separation operations, we turned our investigations to process implementation. The enzymatic reaction was scaled up from 1 mL to 600 mL scale with the same performance/conversion. In accordance with the stability experiments, ~55% of **18** was converted within 16 h (Fig. 8b, Reaction-1). We then transferred 90% (v/v) of the reaction solution to the NF system, and started the nanofiltration using Desal DK membrane (NF-1). The enzyme was still active under the NF separation setup, as we observed that the reaction started again (Fig. 8b, NF-1). Compared to the control, the reaction combined with sequential separation resulted in an additional 10% product formation during the 8 h of nanofiltration, which clearly demonstrates the desired thermodynamic equilibrium shifting of the transamination. Indeed, after 16 h, the thermodynamic equilibrium was almost reached in the control since only 2% of substrate **18** was further converted in the following 8 h when no NF was applied (Fig. 8b, Control-1). To investigate whether the enzyme gets deactivated by the NF setup, we removed the reaction solution from the NF system and added 5 mM substrate ketone **18**. Additional 2 mM product was formed during 15 h in both the NF-treated reaction mixture and the control (Fig. 8b, Reaction-2 and Control-2). This result clearly shows that the TA_v2 enzyme was still active in both conditions, and can withstand the mechanical stress of the NF unit in the presence of the HMW amine donor **6** (high pressure and high stirring rate). We then conducted a second NF-step but this time with Puramem 200 membrane (NF-2): this step shifted the equilibrium towards the product and allowed the formation of additional 15% product during 5 h nanofiltration (Fig. 8b, NF-2). No further substrate conversion was observed in the control (Fig. 8b, Control-2) without NF.

In accordance with membrane screening and offline tests, more than 80% amine donor **6** was retained when using both membranes in this batch mode combined with sequential separation setup (Fig. 9). Desal DK selectively over-concentrated the product, however, together with the product, ~58% of unreacted substrate was lost in the permeate stream (Fig. 9a, dark grey bars). A permeate flux decline was observed when using Desal DK. This phenomenon could be connected to interaction of the membrane with **19** and **18** solutes [34], but more likely, flux decline was due to shrinking of the membrane matrix [35]. Desal DK is a nanofiltration membrane designed for aqueous systems therefore it probably lost its structural integrity upon prolonged exposure to the co-solvent DMSO, employed for enhancing substrate solubility. In contrast to Desal DK, no changes to the active surface of Duramem 200 membrane were visually observed. On the contrary, this organic solvent nanofiltration (OSN) membrane resulted to be stable even when exposed to higher substrate and DMSO co-solvent concentrations.

In addition to amine donor retention and thermodynamic equilibrium shifting, a highly selective membrane would enhance concentration of the product in a less diluted water stream. Due to the selective removal of the product, faster than the transport of the solvent (buffer) across Desal DK membrane, the concentration of **19** raised from 5.9 mM to 7.3 mM in the permeate while the partial retention of substrate **18** reduced the product stream contamination from 7.31 mM to 2.17 mM. Even if less selective, Duramem 200 enhanced the thermodynamic equilibrium shifting and product concentration in a less diluted stream as well (Table S3). Comparing the here presented results with other membrane assisted separations [10–13], it can be stated that in the present strategy the essential aspect being exploited is the difference in molecular weight of the amine donor and product as opposed the differences in pK_a and hydrophobicity between various substrates and products that could limit its general application.

3.4.2. Reaction in continuous mode (constant substrate feeding) coupled with separation

High reaction rate combined with relatively low permeate fluxes and selective product removal suggested coupled NF with continuous substrate feeding through the P-1 feed pump (Fig. 3) as a smart feeding strategy for further process optimization. Continuous feeding of high substrate concentrations can be obtained by diafiltration since it occurs at the same rate of permeation. However, when a solution of highly concentrated **18** (200 mM in DMSO) was subjected to diafiltration, the substrate started accumulating in the reaction tank. Moreover, Desal DK membrane resulted to be incompatible with high concentrations of **18**, **19** and DMSO while the solvent stable Duramem 200 showed lower product selectivity at high substrate concentrations. Therefore, the product concentrations obtained in the retentate and permeate of these coupled approaches are very low. Detailed results are presented in the supplementary information (Figures S6 and S7).

Compared to the promising results of the reactions in batch mode combined with sequential separations, the constant substrate feeding approach did not fulfill the expectations. There are indeed a number of parameters both on the enzymatic reaction and the membrane filtration part that have an impact on the overall efficiency of the process. On the enzyme reaction side, the most influential parameters are: enzyme loading, substrate concentration and amine donor excess. On the filtration aspect, membrane selectivity and stability, amine product concentration, permeate flow rate and diafiltration control influence the performance immensely. Moreover, matching reaction and permeation rates is another key challenge that needs to be addressed. In order to investigate the effect of all the parameters, on the basis of the experimental results achieved, a mathematical model was assessed. The main outcomes achieved by reaction and separation modeling, combined with a techno economic analysis of the process, will be reported elsewhere.

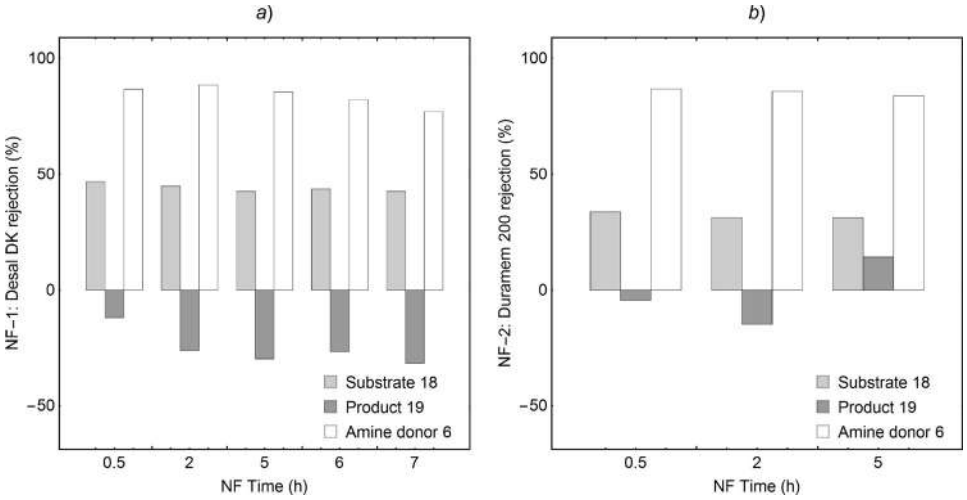

Fig. 9. Reaction in batch mode combined with sequential separation: a) rejection of substrate (light grey), product (dark grey) and HMW amine donor **6** (white) in function of time when using Desal DK membrane (NF-1) and b) rejection of substrate (light grey), product (dark grey) and HMW amine donor **6** (white) in function of time when using Duramem 200 membrane (NF-2).

4. Conclusions

This study presents a concept for process intensification in transaminase-catalyzed synthesis of chiral amines. In summary, the feasibility of using novel, commercially available and inexpensive HMW amine donors for the synthesis of chiral amines was demonstrated. Compared to low molecular weight amine donors such as IPA, these bulky donor amines exhibit potential for their effective retention by commercial available nanofiltration membranes by a size exclusion mechanism. Therefore, HMW amine donors provide a promising tool for integrated DSP and possibilities for membrane assisted ISPR. The potential of the process concept was demonstrated by using a synthetic model solution where a large amount of HMW donors were retained while permeating of the product amine took place. This result was further substantiated by two subsequent nanofiltration steps on a reaction solution where an overall increase of 25% yield was observed compared to the control system. Although our innovative approach clearly exhibited its potential, certain limitations were also revealed in this study. For instance, only few of the investigated enzymes accepted the HMW donor molecules and the large excess of amine donor used led to a low atom economy. Moreover, the selection of a solvent stable membrane, able to retain the keto substrate while selectively permeating the product, is essential for achieving high conversions, high product concentrations and for enhancing the atom economy of the process. Further optimization in operational mode is also needed to improve the overall system performances. Therefore, future efforts will mainly focus on the identification of ATAs that accept alternative HMW amine donors and on membrane functionalization. Despite the optimization required for the presented study, nanofiltration employing HMW amine donors can be potentially developed for other reaction systems, which face unfavorable thermodynamic equilibrium and/or are product inhibited.

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Supporting information

Enzyme and amine donor screening

Table S1. Overview of the recombinantly expressed transaminases tested with HMW amine donors **1-7** (Figure 1). Transaminases were available as glycerol stocks.

Name	Species	Mutation
AspFum	*Aspergillus fumigatus*	
ATA117	*Arthrobacter* sp.	
3FCR	*Ruegeria* sp. TM1040	
3FCR_M1	*Ruegeria* sp. TM1040	Y59W/Y87F/Y152F/T231A/I234M
3FCR_M2	*Ruegeria* sp. TM1040	Y59W/Y87L/T231A/L382M/G429A
3HMU	*Ruegeria pomeroyi*	
Vfl	*Vibrio fluvialis* JS17	
Cvi	*Chromobacterium violaceum*	

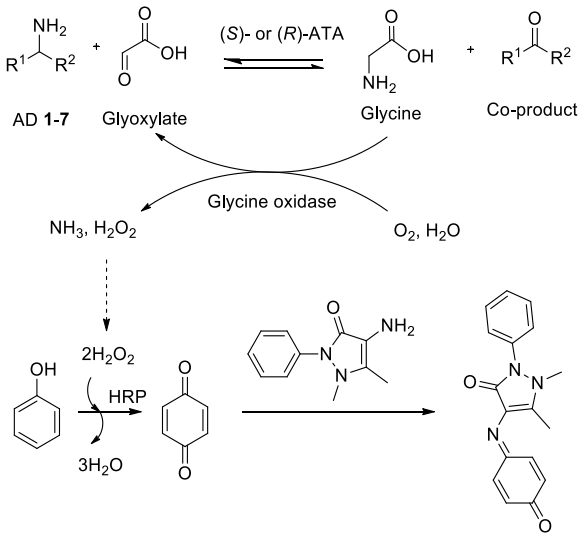


Figure S1. Photometric glycine oxidase microtiter plate assay for (*R*)- or (*S*)-selective amine transaminase (ATA) screening towards HMW amine donors (AD) **1-7** (Figure 1).

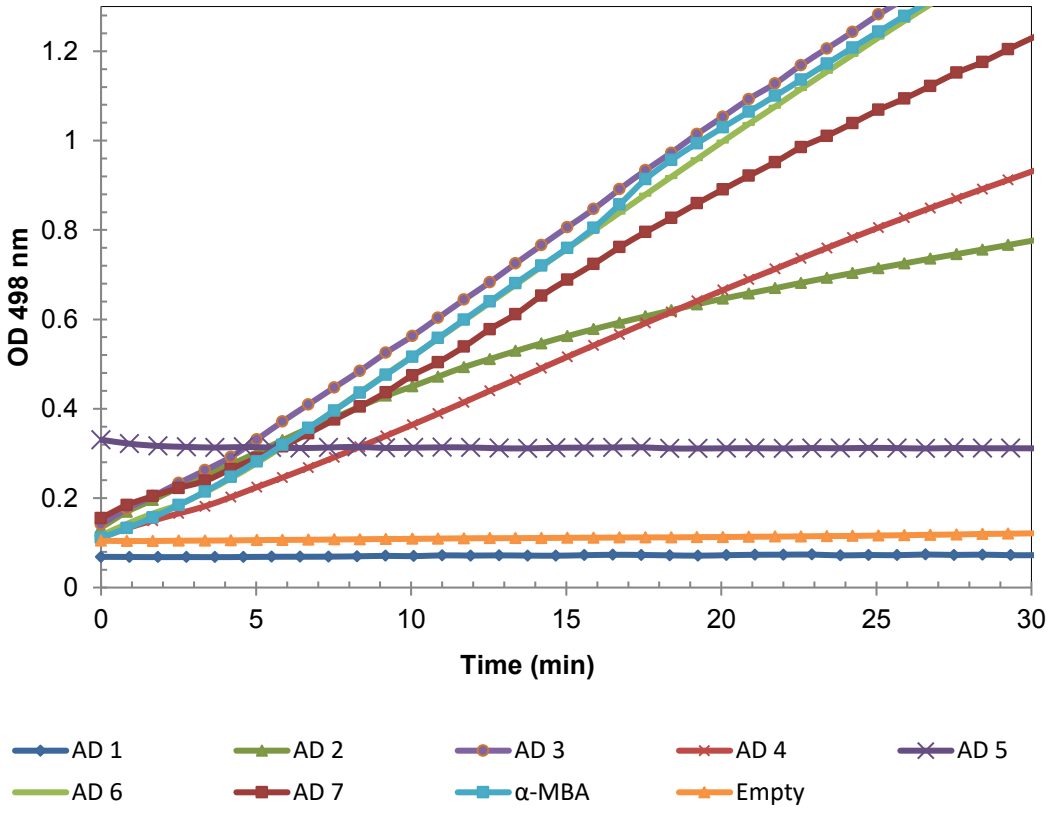
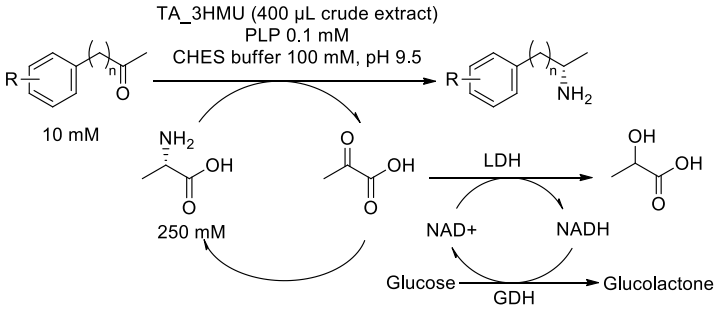


Figure S2. Increase of absorbance at 498 nm at 37°C over time for TA_3HMU enzyme when using amine donors (AD) **1–7** in the photometric glycine oxidase microtiter plate assay [2]. Cells with empty pET28a(+) vector were applied as negative control. PEA was applied as positive control.

Keto substrate screening: asymmetric synthesis using the LHD/GDH cascade system



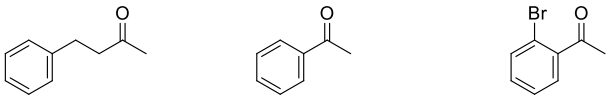


Figure S3. Preliminary screening of the selected keto substrates (bottom) employing the LDH/GDH cascade system (top). TA_3HMU crude extract was recombinantly expressed as described elsewhere [1]. Experimental coditions: 400 µL TA_3HMU crude extract, 90 U mL^{-1} LDH, 15 U mL^{-1} GDH, 250 mM L-alanine, 150 mM D-glucose, 1 mM NADH, 0.1 mM pyridoxal-5'-phosphate (PLP) and 10 mM keto substrate (dissolved in DMSO) in 100 mM CHES buffer, pH 9.5. The final DSMO concentration was 5 %(v/v). The solutions were shaken at 30°C and 1000 rpm. At distinct time points, 150 µL samples were taken for TLC analysis.

Thin layer chromatography (TLC) analysis for asymmetric synthesis experiments.

Two different mobile phases were found to be suitable for the analysis of the amines and the ketones. For amine separation, a mixture of *n*-butanol:acetic acid:H_2O = 4:1:1 was used as mobile phase and aluminum oxide foils were employed as stationary phase. The mobile phase for the analysis of the ketones was petrolether:ethyl acetate = 3.5:1. The amines were stained with ninhydrin solution (1.5 g ninhydrin, 5 ml acetic acid in 500 mL ethanol 95%, heating for color development necessary), while the ketones were observed under UV light at 254 nm.

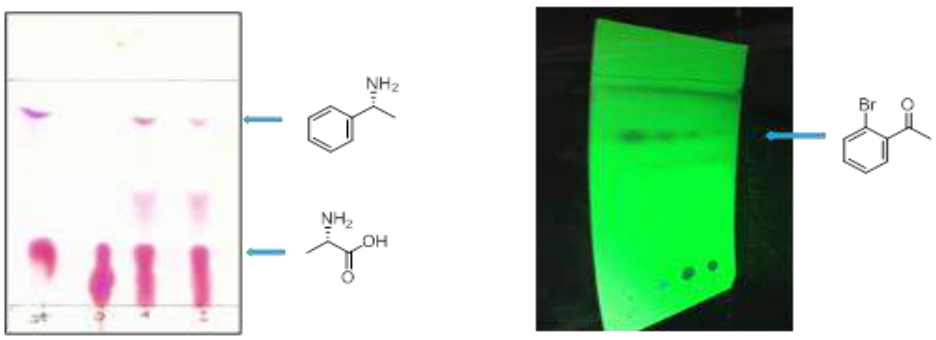


Figure S4. Exemplary TLC plates showing the two different detection methods employed for amine and ketone detection. Production of PEA from acetophenone substrate was visualized after ninhydrin staining on the left; depletion of 2'-bromo-acetophenone (**21**) on the right side (detection in UV light by quenching of a fluorescence dye). The first lane contains just the pure standard compounds. The second lane shows the reaction before enzyme addition while the third and the fourth lanes show the reaction progress after 15 and 25 h, respectively.

Additional model reaction investigations

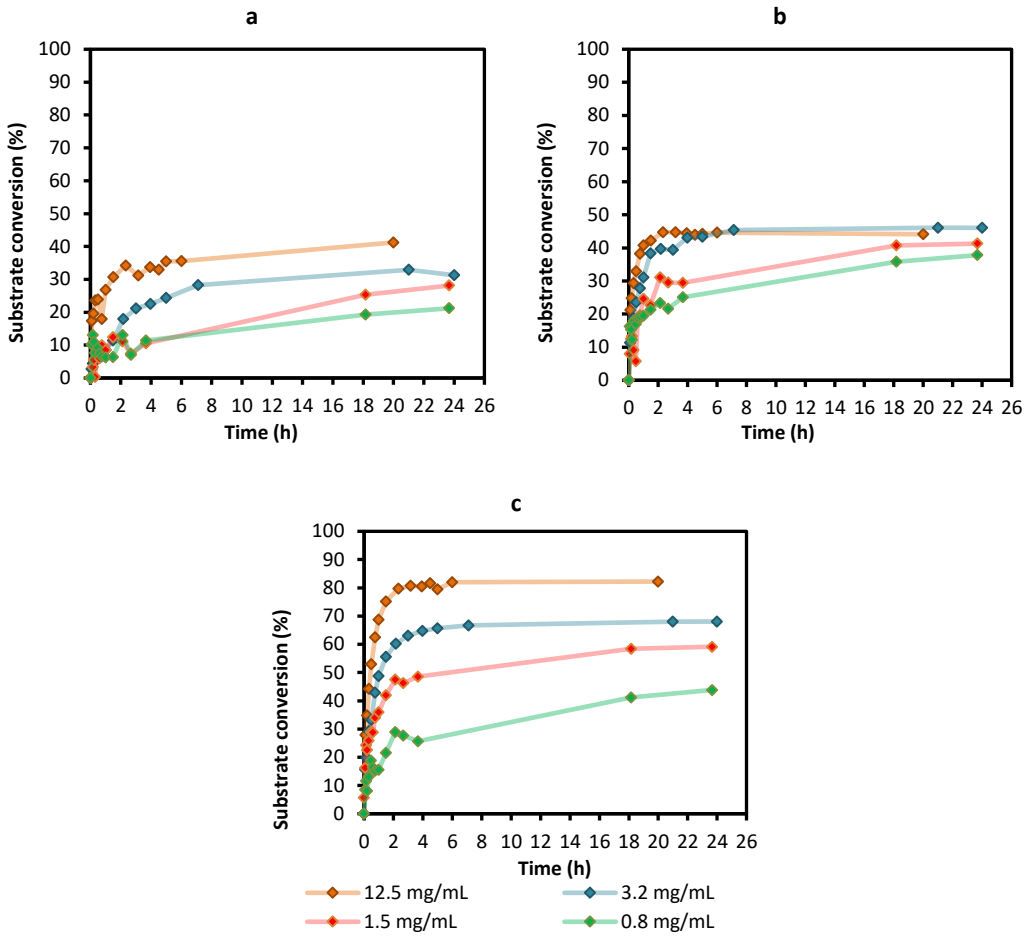


Figure S5. Substrate conversion in function of time when employing amine donors **3** a), **6** b) or **8** c) with different TA_3HMU enzyme loadings. Experimental conditions: 10 mM **18**; 250 mM AD; 0.1 mM pyridoxal-5'-phosphate (PLP); 5% DMSO; 100 mM CHES buffer, pH 9.5, 30°C, 1000 rpm.

Membrane screening

Table S2. Parameters of selected polymeric flat sheet nanofiltration (NF) membranes for process development in aqueous environment.

Entry	Membrane	Nom. Cut-off (Da)	Material	pH range	Application
1	Synder NFX	150-300	TFC/PA[b]	3-10.5	Aqua
2	Synder NFW	300-500	TFC/PA	3-10.5	Aqua
3	Duramem200	200	PI[c]	n.a.	OSN[d]
4	SolSep-10206	Rejection (95%)~300	Propr	n.a.	OSN
5	GE KH Duracid	200	TFC/PA	0-9	Aqua/Process
6	Desal DK GE	150	TFC/PA	3-9	Aqua/Process
7	Alfalaval NF99	<200	TFC/PA	3-10	Aqua
8	Dow NF90	200	TFC/PA	3-10	Aqua
9	Puramem Flux	n.d.	TFC/PA	n.a.	
10	Puramem Performance	n.d.	TFC/PA	n.a.	
11	Puramem Selective	n.d.	TFC/PA	n.a.	
12	MPF-34	200	TFC/PA	n.a.	

13	B4022	n.d.	n.d.	n.a.

[b] Thin film composite (TFC) membrane /selective polyamide (PA) layer
[c] Integrally skinned asymmetric (ISA) membrane / Polyimide (PI)
[d] Organic Solvent Nanofiltration (OSN)
n.d.: not defined; n.a.: not applicable

Table S3. Concentration of substrate **18** and product **19** before nanofiltration (reaction mixture) and after nanofiltration (permeate solution) with Desal DK (NF-1) and Duramem 200 (NF-2).

	19 (mM)	**18** (mM)
Reaction-1	5.93	3.53
Permeate (NF-1)	7.31	2.17
Reaction-2	6.85	6.26
Permeate (NF-2)	7.69	4.33

Reaction in continuous mode (constant substrate feeding) coupled with separation

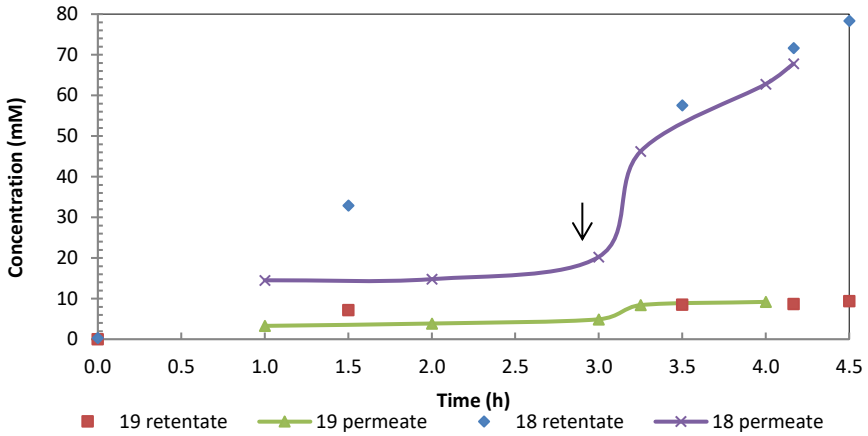


Figure S6. On-line nanofiltration (Desal DK membrane) coupled with enzymatic reaction. Since the permeate flux was very low, **18** was manually added by diafiltration (40 mM). After 1.5 h, 18% of **18** was converted. To increase the substrate concentration, after 3 h of reaction (see arrow), 200 mM **18** feed solution was automatically added at the same rate of permeation by diafiltration resulting in a drastic increase in the concentration of **18** in the feed tank (blue diamonds) and so in the retentate. However, at that same moment, the flux suddenly increased resulting in a drastic decrease in the retention of **18** (purple line); since the substrate **18** was lost from the reaction tank, no product was produced and the concentration of product in the retentae (red square) and permeate (green triangle) was very low.

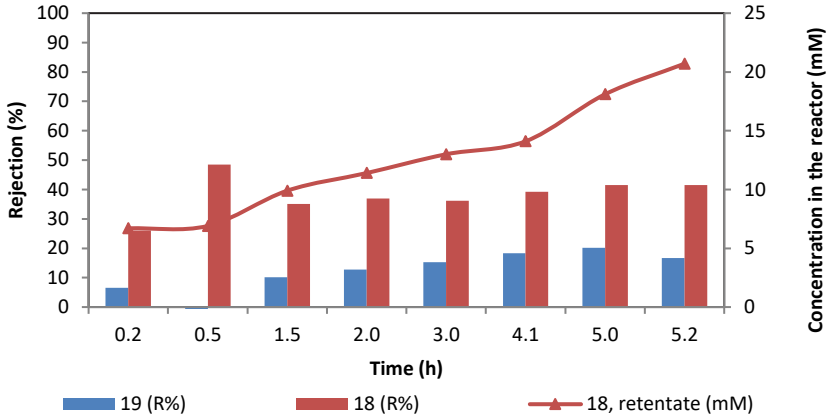


Figure S7. On-line NF (Duramem 200 membrane) coupled with enzymatic reaction. Product rejection (blue bars) increased with the increase of substrate concentration (red line). Since permeation and reaction rates did not match, **18** accumulated in the reaction tank (Figure 3) hence showing the limitation of the system.

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Article II

Research Article

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Jeffamine® ED-600: a polyether amine donor for enzymatic transamination in organic solvent/solvent-free medium with membrane-assisted product extraction

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Abstract

BACKGROUND: Amine transaminases have been extensively used for synthesizing various pharmaceutically relevant compounds, mainly in aqueous media. However, their applications are often limited by poor substrate solubility, low productivity and difficult product separation. This paper reports the use of Jeffamine® ED-600, a novel polyether amine donor, for the transaminase-catalyzed synthesis of 4-phenyl-2-butylamine in non-aqueous media.

RESULTS: Enzymatic transamination was performed in the presence of a non-polar organic solvent (*n*-heptane), in which the selected amine donor is not soluble, thus a two-liquid-phase system was achieved. Coupling the reaction system with membrane-assisted extraction resulted in simultaneous recovery of product, without any consistent contamination of the unreacted substrates. Moreover, a product yield of 60% was reached, compared with 15% without product extraction. The reaction was also successfully conducted without addition of any organic solvent, thus providing the first example of a solvent-free transamination system. In the presence of only enzyme and substrates, up to 6-fold higher product concentrations were achieved compared with the reaction performed in organic solvent.

CONCLUSION: The use of the Jeffamine® ED-600 in non-aqueous media resulted beneficial for 4-phenyl-2-butylamine synthesis. Enzymatic transamination in organic solvent with membrane-assisted product extraction enabled shifting of the equilibrium and selective product extraction. Solvent-free transamination minimized the required volume of the reactor and minimized the environmental impact. Extension to other substrate/enzyme solvent-free systems could open new possibilities and perspectives in transaminase-catalyzed chiral amine synthesis.

Supporting information may be found in the online version of this article.

Keywords: solvent-free transamination; two-liquid-phase transamination; amine donor; chiral amines; *in situ* product removal

NOMENCLATURE

AD	amine donor
Ala	alanine
ATA	amine transaminase
BA	4-phenyl-2-butanone
DIPE	diisopropyl ether
EE	extraction efficiency
IPA	isopropylamine
ISPC	*in situ* product crystallization
ISPR	*in situ* product removal
MPPA	4-phenyl-2-butylamine
MTBE	methyl *tert*-butyl ether
MW	molecular weight
MWCO	molecular weight cut-off
NF	nanofiltration
OSN	organic solvent nanofiltration
PLP	pyridoxal-5′-phosphate
SFS	solvent-free system

INTRODUCTION

The synthesis of several bioactive compounds and active pharmaceutical ingredients relies on the development of general and efficient methods to prepare optically pure amines.[1] It is therefore of

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great importance to develop efficient biocatalytic routes for chiral amine synthesis as environmentally friendly alternatives to the existing chemical routes.

The synthetic potential of amine transaminases (ATAs) for the synthesis of chiral amines is enormous, especially when the transamination is carried out in the asymmetric synthesis mode.[2] Although research in both academia and industry has been extensively performed, general challenges including substrate/product inhibition, instability of the enzyme and unfavorable thermodynamic equilibrium[3–6] need to be overcome. Such limitations imply that the process parameters (substrate concentration, biocatalyst performance and product yield, productivity) of ATA-catalyzed processes are not completely industrially competitive yet, despite a few successful industrial applications.[7–10]

The use of an excess of isopropylamine (IPA) or alanine (Ala) as amine donor is the easiest strategy for shifting the thermodynamic equilibrium of ATA-catalyzed reactions. As alternatives to the widely employed IPA and Ala, amine donors such as 1-phenylethylamine[11] and *o*-xylylene diamine[12] are thermodynamically attractive on the one hand but complicate the downstream processing on the other hand. Product isolation by membrane *in situ* product removal (ISPR)[13–16] and *in situ* product crystallization (ISPC)[17,18] have proven to be effective process intensification techniques, but their applicability remains still limited. Removal of the often inhibiting co-products such as acetone by stripping,[8,19] by co-solvent addition[11] or by cascade reactions[20] is also possible. Aside product/co-product removal, immobilization of ATAs for continuous flow applications[21–23] is emerging as a promising alternative, especially if the transamination is conducted in an organic solvent.[24] Operating transaminations in organic solvents would indeed enhance the solubility of poorly water-soluble substrates, leading to higher volumetric productivities and lowering the costs of equipment and downstream processing for product recovery.[25,26] Additionally, transaminations in organic solvents simplify the work-up procedure (i.e. no basification and extraction required) and give the possibility to recycle the solid catalyst.

The first attempt at non-aqueous transamination was the asymmetric synthesis of *trans*-(1*R*,2*R*)-1-amino-2-indanol using Ala as the amine donor. The crude preparation of the ATA from *Vibro fluvialis* partially retained its activity in water-saturated ethyl acetate, although reaction rates were lower than in aqueous buffer.[27] In a further study, nine ATAs, most of them wild-type enzymes, proved to be highly active in methyl *tert*-butyl ether (MTBE). Enzyme activities were shown to be up to 17-fold higher compared with those of the aqueous buffer, and ATAs efficiently accepted IPA amine donor, which is not the case when applied in aqueous medium.[26] Transamination in MTBE was again reported for the synthesis of (*R*)- or (*S*)-valinol.[28] Asymmetric synthesis of 3-substituted cyclohexylamine derivatives from prochiral diketones via three biocatalytic steps in diisopropyl ether (DIPE) was also performed. The simultaneous one-pot approach cascade reaction was catalyzed by two hydrolases (C–C hydrolase and a lipase) in DIPE containing 2.5% v/v water and 1% v/v methanol, followed by transamination in DIPE.[29] Asymmetric synthesis of 4-phenyl-2-butylamine (1-methyl-3-phenylpropylamine, MPPA) using IPA amine donor in *n*-heptane combined with ISPR was also recently investigated.[16] However, product purity was hampered by amine donor co-extraction.

In our previous study, we introduced a novel class of high-molecular-weight amine donors with a molecular weight (MW) in the range 400–1500 g mol^{-1}. These molecules, possessing a higher MW compared with commonly used amine donors, were effectively retained by commercial nanofiltration membranes by a size exclusion mechanism. Performing transamination with these amine donors reduced the contamination of the product stream, thus simplifying the membrane-assisted downstream processing and potentially providing ISPR opportunities.[30] However, low product concentration was achieved, mainly due to the limited solubility of the ketone substrate in aqueous environment (10 mmol L^{-1}). Evidently, performing the reaction in an organic solvent would be beneficial.

Based on this concept, the transamination in non-aqueous media using the polyether amine donor Jeffamine® ED-600 (MW of 600 g mol^{-1}) is reported here. The asymmetric synthesis of MPPA using the Jeffamine® ED-600 was performed in the presence of a non-polar organic solvent (*n*-heptane), in which the selected amine donor is not soluble, thus the organic solvent and the Jeffamine® ED-600 amine donor form a two-liquid-phase system. The above reaction system was coupled to a membrane contactor. The product amine was therefore extracted into the aqueous phase to investigate the potential of downstream processing and *in situ* product recovery approaches. Furthermore, as often seen in lipase-catalyzed processes,[31–33] and more recently in the cutinase-catalyzed synthesis of aliphatic polyesters[34] and in the cellulase-catalyzed hydrolysis of cellulose into glucose,[35] enzymatic reactions can be run in the presence of only the reactants and biocatalyst, without bulk solvents or other aqueous components, thus resulting in solvent-free systems (SFSs). This approach minimizes the required volume of the reactor, simplifies the downstream processing and reduces the associated costs. In this study, we thus have also investigated the solvent-free transamination of MPPA using Jeffamine® ED-600 as amine donor without any additional solvent phase. To the best of our knowledge, this represents the first example of solvent-free transamination for chiral amine production.

MATERIALS AND METHODS

Chemicals and enzyme

The chemicals used in this study, including 4-phenyl-2-butanone (benzyl acetone, BA) (98% purity), 4-phenyl-2-butylamine (1-methyl-3-phenylpropylamine, MPPA) (98%), the amine donors *O*,*O*′-bis(2-aminopropyl) polypropylene glycol-*block*-polyethylene glycol-*block*-polypropylene glycol (Jeffamine® ED-600, MW 600 g mol^{-1}) and polypropylene glycol bis(2-aminopropyl ether) (MW 400 g mol^{-1}), *n*-heptane (>97%) and pyridoxal-5′-phosphate (PLP), were purchased from Merck (Kenilworth, NJ 07033 U.S.A). The amine transaminase (TA-v2) was purchased from c-LEcta GmbH (Leipzig, Germany). The purified enzyme was supplied as a freeze-dried powder. The activity of TA-v2 was 1.73 U mg^{-1} (value provided by the supplier, based on acetophenone detection[36]). One unit is the amount of enzyme that produces 1 µmol acetophenone min^{-1} from α-methyl-benzylamine (MBA) at 30 °C in 50 mmol L^{-1} potassium phosphate buffer pH 7.4, 0.1 mmol L^{-1} PLP, 10 mmol L^{-1} sodium pyruvate and 10 mmol L^{-1} racemic MBA.

Two-liquid-phase transamination

Transamination was performed in 10 mL glass vials (diameter 2.5 cm), in which a biphasic system was built (Fig. 1, left side). According to our previous studies,[16] 0.05 g of TA-v2 enzyme was wetted with 100 µL of 0.5 mmol L^{-1} PLP/water mixture. The amine donor (AD) Jeffamine ED-600 was then added to the vials (either

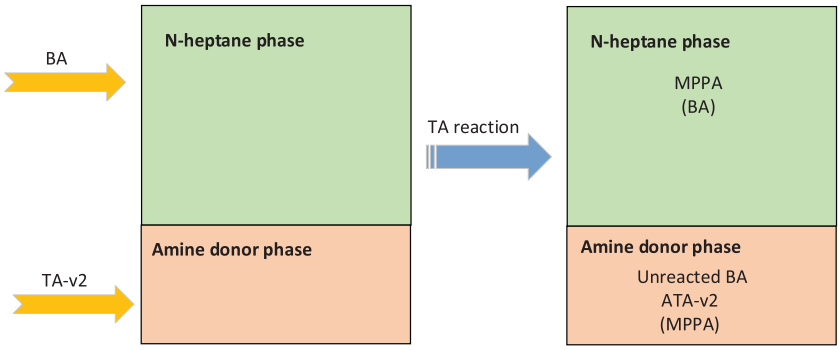

Figure 1. Composition of the two-liquid-phase system before (left) and during (right) the transamination reaction. Being heavier and not soluble in *n*-heptane, Jeffamine ED-600 amine donor (AD) and the enzyme form a layer on the bottom of the reaction vial. The substrate ketone BA, initially supplied to the *n*-heptane, partially diffuses from the *n*-heptane to the AD phase. The reaction takes place in the AD phase and the formed MPPA product is progressively released from the AD to the upper *n*-heptane phase.

1.5 or 0.7 g), followed by the addition of 5 mL of heptane and 0.04 g of ketone substrate BA. The ratio between amine donor and substrate (mol_{AD}/mol_{BA}) amounted to 10 or 5 when 1.5 or 0.7 g of AD respectively was used. Being heavier and not soluble in heptane, the AD forms a layer on the bottom of the vials together with the enzyme (lower layer), where the reaction takes place. The organic solvent (*n*-heptane) forms an upper layer, where substrate is supplied (Fig. 1, left side) and where product is released (Fig. 1, right side). The concentration of initial BA dissolved in heptane (upper layer) amounted to 50 mmol L^{-1}. Vials were incubated at 30 °C in a thermoshaker and either gently shaken or continuously stirred. In the latter case, to promote the diffusion of the formed MPPA from the lower layer to the upper layer, a magnet was added to each vial. Reactions were performed in duplicate and the results are presented as the average of two independent experiments. To investigate the effect of the geometry on the rate of product release from the lower phase to the upper phase, reactions were also performed in 2 mL Eppendorf tubes (diameter 1 cm). For this purpose, the reaction volumes were scaled down by a factor of 2.5. Reactions in Eppendorf tubes were performed in triplicate and the results are presented as the average of three independent experiments.

Samples of the upper *n*-heptane phase were taken after distinct time periods, analyzed as described elsewhere[16] and the product yield was calculated as follows:

$$\text{MPPA observed yield } (\%) = (g_{MPPA,hept}/g_{BA,initial}) \times 100$$

where $g_{MPPA,hept}$ is the amount of MPPA released in the *n*-heptane upper layer and $g_{BA,initial}$ is the amount of substrate initially added to the biphasic system.

Solvent-free transamination

The solvent-free asymmetric synthesis of MPPA from BA was performed in 10 mL glass vials. The enzyme TA-v2 (0.05 g) was wetted with 100 µL of 0.5 mmol L^{-1} PLP/water mixture. The amine donor (AD) Jeffamine ED-600 was then added to the vials (either 1.5 or 0.3 g), followed by substrate BA addition. The amount of water added to the system contributed less than 0.7 or 3% to the total weight of the system when 1.5 or 0.3 g of Jeffamine ED-600 respectively was used. The ratio between amine donor and substrate (mol_{AD}/mol_{BA}) was varied from 20 to 1 by changing the amount of substrate added. Vials were incubated at 30 °C in a thermoshaker and gently shaken. After 7 days, the reaction was stopped by adding 5 mL of either methanol or an aqueous solution of 5% v/v trifluoroacetic acid. Enzyme precipitation, leading to inactivity of the enzyme, was observed in both cases. Vigorous shaking followed by enzyme filtration was applied prior to chiral analysis and ultrahigh-performance liquid chromatography (UPLC) analysis for product and substrate quantification.

Membrane-assisted extraction

System configuration

The set-up for the product extraction is presented in Fig. 2. The contactor physically separates two immiscible solutions: the *n*-heptane solution (upper phase of the reactor side) (Fig. 2, right) and the aqueous extracting solution (Fig. 2, left). The aqueous extracting solution (100 mmol L^{-1} sodium citrate buffer pH 3.0) was chosen in such a way as to enhance the selective MPPA extraction and to prevent its back extraction.[13,14] Extraction was conducted using polymeric membranes with both flat sheet (in-house constructed rectangular flat sheet module) and hollow fiber contactors (supporting information Fig. S4). For investigating the extraction performances with hollow fibers, the commercial membrane contactor 1 × 5.5 Mini Module, Liqui-Cel®, purchased from 3M, was in-house modified. The polycarbonate membrane housing was replaced with a glass housing to enhance its resistance towards the used solvent. Properties of the used modules and membranes are summarized in Table 1. When the hollow fiber contactor was used, the solvent solution was pumped on the lumen side of the contactor while the aqueous extracting buffer was recirculated across the shell of the module. Having selected hydrophobic membranes, the organic phase filled the membrane pores. To avoid leakage of solvent into the aqueous side, the pressure on the right side was kept at least about 0.1 bar above the pressure of the left side, during the whole experiments.

Membrane and module investigations

A preliminary screening of five commercially available solvent-stable polymeric membranes was conducted (supporting information Fig. S3). The two best performing flat sheet membranes and the in-house modified hollow fiber contactor (Table 1) were further investigated using the membrane extraction set-up shown in Fig. 2. The simulated reactor biphasic solution consisted of all reaction components except the enzyme and PLP. Essentially, 100 g of Jeffamine ED-600 (AD lower phase), 0.350 L of *n*-heptane (upper phase), BA (2.3 g) and MPPA (1.3 g) were

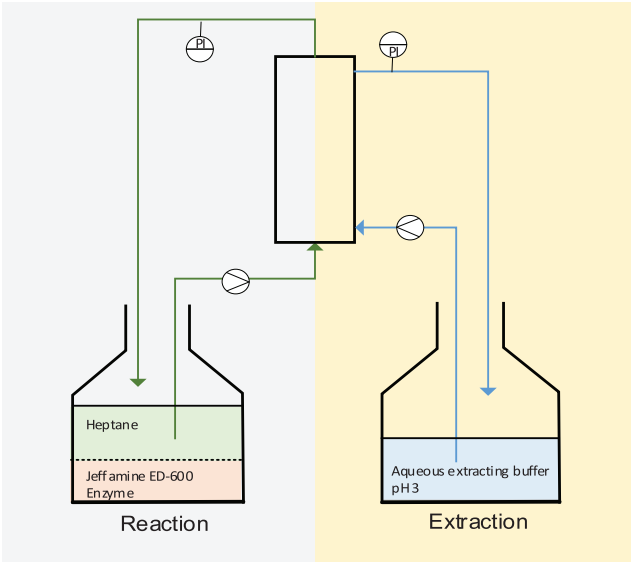

Figure 2. Set-up for membrane-assisted product (MPPA) extraction. Reaction occurs in the lower phase of the two-liquid-phase system (reaction, left side). Being released from the lower amine donor (AD) layer (orange phase) into the upper *n*-heptane (green phase), MPPA is simultaneously extracted into the aqueous acidic buffer (extraction, right side) and accumulated in the buffer solution (blue phase).

added to the left side of the system (Fig. 2). Extraction was performed employing 0.35 L of 100 mmol L^{-1} sodium citrate buffer pH 3.0. All tests were performed at 30 °C. At frequent intervals, samples of the aqueous phase (Fig. 2, right side) and upper phase of the simulated reactor biphasic solution (Fig. 2, left side) were taken for quantification. The MPPA extraction efficiency (MPPA EE) and the BA and AD losses (BA and AD loss) were calculated as follows:

$$\text{EE}\ (\%)\ \text{or loss}\ (\%) = (g_{i,\text{aq.buffer}}/g_{i,\text{initial}}) \times 100$$

where $g_{i,\text{aq.buffer}}$ is the amount of component *i* extracted into the aqueous buffer (Fig. 2, right side) and $g_{i,\text{initial}}$ is the amount of component *i* initially added to the reaction system (Fig. 2, left side).

Two-liquid-phase transamination combined with intermittent extractions

The experiments with simulated solutions were followed by extraction with actual reaction mixtures. The two-liquid-phase system employed for preliminary reaction investigations and depicted in Fig. 1 was scaled up by a factor of 70. The reaction was performed in a Scott Duran bottle, which was gently shaken in a thermoshaker at 30 °C. Initial amounts of AD lower phase and BA (initially dissolved in 0.35 L of *n*-heptane upper phase) were 105 and 1.3 g respectively. The AD lower phase contained 3.5 g of TA-v2 enzyme, previously wetted with 7 mL of 0.5 mmol L^{-1} PLP/water solution. The reaction was run for a total of 42 days. Additions of BA (1.3 g) were made after 3 h, 4 and 17 days of the reaction. Product extraction, employing a Puramem Selective membrane, was performed by coupling the reaction with the membrane-assisted extraction set-up (Fig. 2) for 6–8 h. Extractions were performed intermittently and were constantly monitored by an operator, for technical and safety reasons. The reaction was combined daily with the membrane-assisted extraction set-up for the first 4 days of the experiment. Two additional intermittent extractions were conducted after 8 and 32 days of the start of the reaction. Owing to product extraction, the pH of the aqueous extracting buffer rose slightly (from 3.0 to ~3.4) during each extraction. Therefore, after each extraction, the pH value was adjusted to 3.0 by HCl addition.

A second experiment was run for 36 days using the same system described above with the following modifications: three intermittent extractions were performed after 1, 5 and 22 days of the reaction. The first extraction was performed using the in-house modified hollow fiber instead of the Puramem Selective flat sheet membrane. Addition of BA (1.3 g) was made after the second extraction (day 5 of reaction).

Control transaminations were performed without extractions, at 70-fold smaller scale compared with the system where product extraction was applied. Substrate additions in the controls were performed in accordance with the tests with intermittent product extraction.

Analysis

Substrate (BA) and product (MPPA) in the solvent phase (*n*-heptane upper reaction phase) were analyzed with gas chromatography (GC) as described by Satyawali *et al.*[16] When intermittent extractions were applied, BA, MPPA and Jeffamine ED-600 extracted into the aqueous buffer were analyzed as described in our previous study.[30] Chiral analysis was performed by GC using a heptakis-(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethyl-silyl)-β-cyclodextrin column (25 m × 0.25 mm). The experimental procedure for sample preparation (amine extraction and derivatization to trifluoroacetamide) and details of chiral analysis are reported elsewhere.[37]

Table 1. Properties of the hollow fiber (HF) contactor with modified housing and of the flat sheet (FS) membrane contactor

Module	HF[a]	FS[b]	
Configuration	Parallel flow	Parallel flow	
Membrane name, supplier	1 × 5.5 Mini Module Liqui-Cel®, 3M	Puramem Selective®, Evonik	Puramem Performance®, Evonik
Active surface area	0.10 m^2	0.01 m^2	
Membrane type	Polypropylene HF	ISA[c] membrane based on P84 polyimide	
Potting material	Polyurethane	No potting required	

[a] Commercially available HF contactor with modified housing.
[b] In-house constructed rectangular FS module.
[c] Integrally skinned asymmetric.

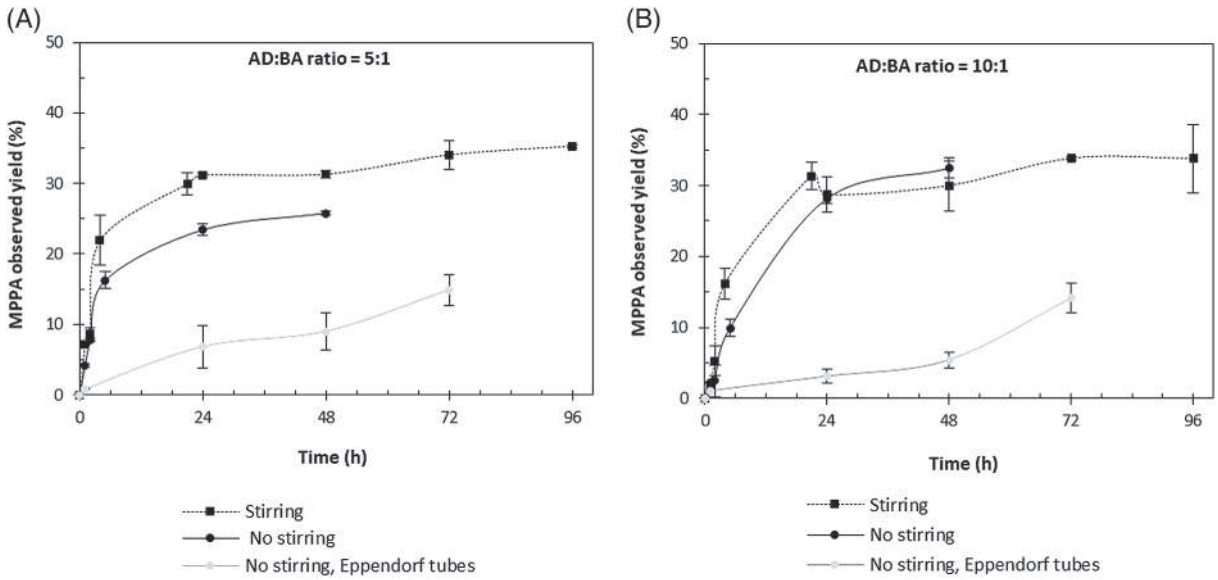

Figure 3. Product yield in *n*-heptane upper phase (MPPA observed yield) over time when using (A) 5:1 amine donor (AD)/ketone substrate (BA) ratio or (B) 10:1 AD/BA ratio. AD/BA ratio is expressed as mol_{AD}/mol_{BA}. Transamination was conducted in glass vials and the AD lower phase was either stirred with a magnet (■) or not (●). When transamination was conducted in Eppendorf tubes (●), no stirring was applied. Reactions were performed at 30 °C using 0.05 g of TA-v2 enzyme, 0.04 g of substrate BA dissolved in 5 mL of *n*-heptane (upper phase) and (A) 5-fold or (B) 10-fold excess (mol_{AD}/mol_{BA}) of Jeffamine ED-600 as amine donor (lower phase). Reactions in Eppendorf tubes were 2.5-fold scaled down.

RESULTS AND DISCUSSION

Two-liquid-phase transamination

The asymmetric synthesis of MPPA from BA in the two-phase system consisting of the amine donor (AD) Jeffamine ED-600 (lower phase) and *n*-heptane (upper phase) is schematically depicted in Fig. 1. Preliminary studies on product partitioning revealed that 30% of the initial MPPA added to the *n*-heptane upper phase diffused into the lower AD layer (supporting information Fig. S1). Further investigation on substrate partitioning showed that about 80% of the substrate initially added to the *n*-heptane upper phase moved from *n*-heptane to the amine donor layer (result not shown), where the reaction took place. Therefore the *n*-heptane phase acted as a substrate-feeding medium on the one hand and as a reservoir of the produced (*S*)-chiral amine on the other hand. Substrate BA and product MPPA could only be analyzed in the upper *n*-heptane phase, thus the results present an underestimation of the total amount of BA converted into MPPA.

Investigations, employing 5- and 10-fold (mol_{AD}/mol_{BA}) excess of Jeffamine ED-600 compared with the ketone substrate BA led to an observed product yield in the *n*-heptane phase of 26 and 32% in 48 h respectively (Figs 3A and 3B, ●). As expected, continuous stirring of the AD lower phase enhanced the rate of product release from the lower phase, where the reaction was taking place, to *n*-heptane (Figs 3A and 3B, ■). Along with the stirring, also the geometry of the vials influenced the rate of product diffusion from the lower AD to the upper *n*-heptane phase. When the reaction was performed in Eppendorf tubes (diameter 2.5-fold smaller than glass vials), the product slowly accumulated in the *n*-heptane phase (Figs 3A and 3B, ●). MPPA was mainly produced on the bottom of the Eppendorf tubes, where enzyme was settled. Therefore, when running reactions in Eppendorf tubes, MPPA had to diffuse through a 2.5-fold thick AD layer before being released into the *n*-heptane upper phase. Evidently, this factor affected the rate of product release from the AD layer to the heptane phase, thus showing the influence of the geometry on this biphasic reaction system.

When the transamination reaction was conducted employing a 5-fold higher enzyme concentration, and consequently increasing the amount of water necessary for wetting the enzyme, lower yields were observed (supporting information Table S1). Having significantly more water in the system, the formed amine probably stuck to the microaqueous water environment surrounding the enzyme, instead of travelling through the amine donor layer.

Solvent-free transamination

Transamination of BA was observed also when performing the reaction without the addition of any organic solvent. To the best of our knowledge, this is the first example of solvent-free transamination. Substrate conversions ranging from 15 to 75% were achieved when different AD loadings (1.5 or 0.3 g) and different AD/BA (mol_{AD}/mol_{BA}) ratios were explored (Fig. 4). In accordance with previous studies,[16] increasing the BA concentration drastically decreased reaction performances. When solvent-free transamination was performed at high AD/BA ratios (Fig. 4, left region), by keeping the same AD/BA ratio and the same enzyme loading, higher product yields were achieved when the total amount of substrate load (AD and BA) was reduced (Fig. 4, white bars). The inhibitory effect of the product amine MPPA, reported elsewhere,[14] could explain these results. As Table 2 shows, solvent-free transamination led to MPPA yields comparable to that observed in the biphasic batch reaction system. However, up to 6-fold higher product concentrations were achieved when running solvent-free transamination compared with the two-liquid-phase transamination (Table 2). The enantioselectivity for (*S*)-MPPA was 94% enantiomeric excess (ee) for both two-liquid-phase and solvent-free reaction systems.

In addition to these results, solvent-free transamination was explored also employing a different amine donor (MW 400 g mol^{-1}). The use of this amine donor resulted in lower yields but higher product concentrations compared with the Jeffamine ED-600 (supporting information Fig. S2).

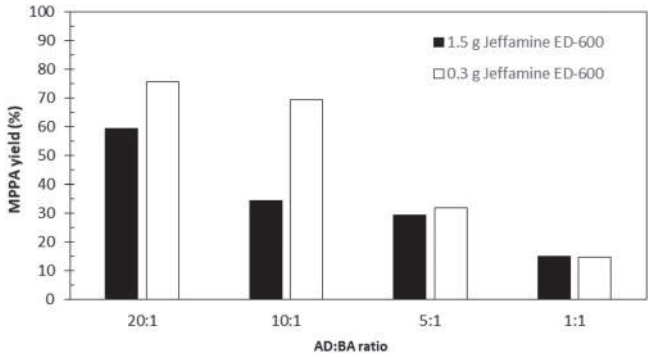

Figure 4. Effect of substrate (BA) amount on solvent-free transamination reaction when using 1.5 g (2.5 mmol) (black bars) or 0.3 g (0.5 mmol) (white bars) of Jeffamine ED-600 amine donor (AD). Reactions were performed at 30 °C using 0.05 g of TA-v2 enzyme. AD/BA ratio is expressed as mol_{AD}/mol_{BA}. Reaction time was 7 days.

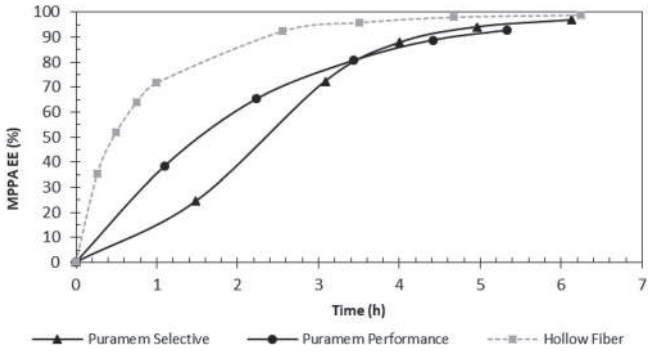

Figure 5. Product (MPPA) extraction efficiency (EE) over time when using the flat sheet module with Puramem Selective (▲) or Puramem Performance (●) and when using the hollow fiber made in-house module (●). The reactor biphasic solution consisted of 100 g of Jeffamine ED-600 (AD phase) and 0.35 L of *n*-heptane. BA (2.3 g) and MPPA (1.3 g) were added to the reactor biphasic solution prior to the membrane extraction.

Membrane extraction for product recovery

Membrane and module investigations

Using the membrane-assisted extraction set-up shown in Fig. 2, a highly efficient and selective product removal was achieved with simulated solutions. As expected, when using the hollow fiber, MPPA extraction resulted faster compared with the flat sheet module (Fig. 5, ●). Puramem® Selective and Performance are composite flat sheet membranes. The solubility of each compound in solvents on both sides of the membrane, the acidic pH of the extracting phase and the membrane surface material are the dominating factors determining membrane performance and consequently the separation. The solubility of the ketone substrate BA is limited in aqueous environment (10 mmol L^{-1}). Up to 5-fold higher concentration of BA has been dissolved in *n*-heptane.[16] In accordance with previous studies,[14,16] no BA was detected in the aqueous extracting phase with both flat sheet and hollow fiber modules. The product amine MPPA is highly soluble in both *n*-heptane and extracting aqueous buffer. Almost 90% of the MPPA was extracted into the aqueous solution in 4 h of operation with all the membranes tested (Fig. 5). This phenomenon is due to the acidic pH value of the buffer that traps amines in their charged state, thus preventing back extraction into the organic solvent phase (as long as the stripping phase pH is kept sufficiently below the pK_a of the amine product). As such, the molecular weight cut-off (MWCO) is not characteristic for this type of membrane separation, especially since solvent–solute–membrane interactions can lead to a change in the membrane rejection from that observed under the conditions to determine the MWCO. The permeate flux of pure heptane, defined as the volume of solvent flowing through the membrane per unit area per time, resulted to be 60 and 15 L h^{-1} m^{-2} when Puramem Performance and Selective respectively were tested (data provided by the supplier). Therefore Puramem Performance behaves as a more open membrane compared with Puramem Selective, hence enhancing MPPA transfer from the solvent to the acidic buffer solution. This resulted in a faster MPPA extraction when using Puramem Performance (Fig. 5, •) compared with Puramem Selective (Fig. 5, ▲). The amine donor selected for this study, not soluble in *n*-heptane, formed a second phase on the bottom of the *n*-heptane (Fig. 2). Ensuring gentle mixing of the system during the start-up of the system (thus minimizing the contact between the membrane and the lower AD phase), less than 3% of Jeffamine ED-600 was co-extracted in 6 h of operation. This demonstrated the advantage of using this donor amine as opposed to IPA, where the co-extraction of donor amine could not be avoided.[16]

Two-liquid-phase transamination combined with intermittent extractions

To investigate the efficiency and effectiveness of product extraction, the two-liquid-phase reaction system was scaled up 70-fold. Six intermittent extractions using the Puramem Selective membrane were applied. For comparisons, we refer to amounts (g) or content (%, $g_{MPPA}/g_{BA,total}$) of product and substrate rather than concentrations. As can be seen in Fig. 6A, during the first 4 days, daily extractions were performed. The total amount of MPPA detected (Fig. 6A, ˆ), defined as the sum of product extracted into the acidic buffer and product accumulated in the upper *n*-heptane phase of the biphasic reaction system, increased linearly over time. Then 25 mmol L^{-1} BA was added to the biphasic reaction mixture

Table 2. Observed product yields *Y* and product concentrations C_{MPPA} in heptane when running a two-liquid-phase transamination (TA) compared with product yields and product concentrations in solvent-free TA. The reactions were performed using 0.04 g of BA, 5- or 10-fold excess (mol) of Jeffamine ED-600 amine donor (AD) and 0.05 g of TA-v2 enzyme

	Two-phase TA				Solvent-free TA	
	No stirring		Stirring			
AD/BA ratio	*Y* (%)	C_{MPPA} (mg_{MPPA} g_{tot}^{-1})	*Y* (%)	C_{MPPA} (mg_{MPPA} g_{tot}^{-1})	*Y* (%)	C_{MPPA} (mg_{MPPA} g_{tot}^{-1})
5	25.8	2.3	32.5	2.8	32.4	14.9
10	31.3	2.5	30.0	2.4	34.4	8.6

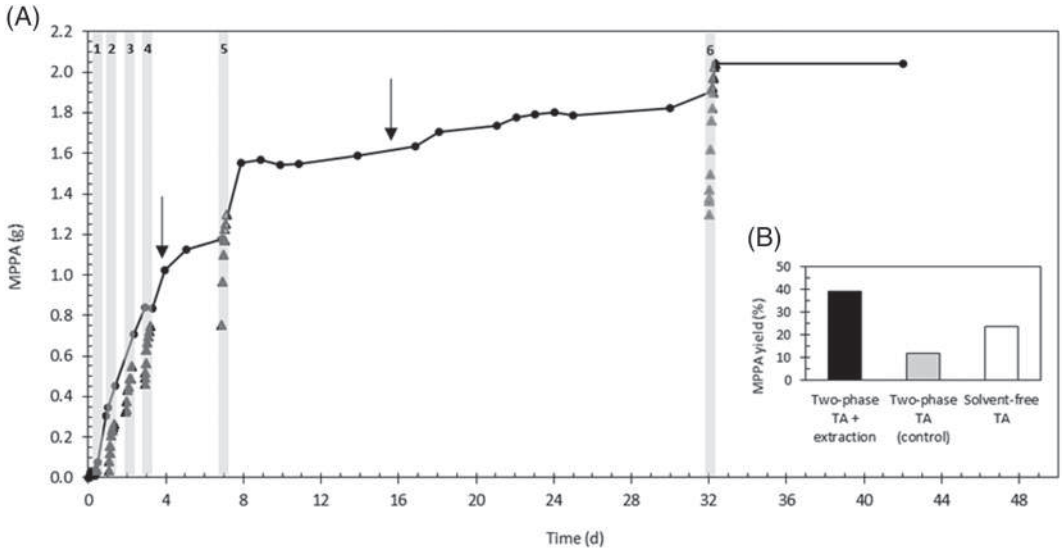

Figure 6. (A) Production of MPPA for 42 days with six sequential extractions (grey areas 1–6) using Puramem Selective membrane. The amount of product extracted into the buffer (▲) and the total amount of product detected (●) are shown. Initial amounts of amine (AD) lower phase and BA, dissolved in 0.35 L of *n*-heptane upper phase, were 105 g (175 mmol) and 1.3 g (8.8 mmol) respectively. The AD lower phase contained 3.5 g of TA-v2 enzyme. Additions of BA (1.3 g) were made after 3 h (not shown), 5 and 17 days (arrows). (B) Comparisons in terms of final product yield. Solvent-free and two-phase transaminations were performed at 70-fold smaller scale (compared with the system where the separations were applied) using 0.08 g of BA, 5-fold excess (mol) of Jeffamine ED-600 amine donor (AD) and 0.05 g of TA-v2 enzyme. Two-phase transamination with sequential extractions was performed as described above (A).

on day 5 (Fig. 6A, black arrow) and an additional extraction was run 2 days later (Fig. 6A, grey area 5). All the MPPA available in the upper phase of the reaction system was extracted in 3 h (Fig. 6A, grey area 5, ▲) and the remaining 3 h of extraction enhanced MPPA release from the lower amine donor phase to the *n*-heptane upper phase (Fig. 6A, grey area 5, ▲), leading to increased MPPA recovery. Poor production was observed between day 8 and day 32. No further product accumulation in the *n*-heptane phase of the reactor side was observed after the last extraction (Fig. 6A, grey area 6). This result indicated that all the residual product present in the upper heptane phase (Fig. 6A, grey area 6, ˆ) and in the lower AD phase (Fig. 6A, grey area 6, ▲) was extracted into the buffer when the final extraction was performed. After six intermittent extractions, amine product concentration in the acidic buffer amounted to 5.7 g L^{-1}. Less than 4% of the initial amine donor and almost no unreacted BA were co-extracted into the buffer.

Figure 6B shows the performance of the system in terms of product yield. The five intermittent extractions performed during the first 8 days of operation led to a product yield of 40%. This value resulted 3.2-fold higher than that of the control system (Fig. 6B, grey bar), where extractions were not applied, thus proving the additional benefit of product removal in the two-phase system in extracting the product trapped in the AD phase to the upper *n*-heptane phase. Moreover, the reaction system coupled with intermittent extractions achieved 1.6-fold higher yields than the solvent-free system (Fig. 6B, white bar), hence demonstrating that combining the enzyme reaction with product removal can shift the thermodynamic equilibrium of the model reaction under investigation.

A second experiment was carried out using a lower substrate amount to reach a higher product yield value. The reaction was monitored for 36 days. For the first extraction, performed on day 2 of the reaction, the hollow fiber module was used. After 1.5 h, extraction was stopped owing to technical problems. The contactor that outperformed the polymeric membranes when tested with simulated solutions (Fig. 5) proved to be incompatible with the real reaction solution. It was suspected that the ketone co-product caused swelling of the potting material, leading to cracks in the contactor housing. After 5 days of the reaction, product extraction was performed again using the Puramem Performance membrane. The simultaneous removal of product available in the upper *n*-heptane phase enhanced the diffusion of the remaining product from the lower AD phase to the upper *n*-heptane phase. Therefore the MPPA trapped in the lower AD layer was also extracted (Fig. 7A, grey area 2, △). The MPPA removed from the lower AD layer amounted to 33% of the total product extracted from the biphasic reaction system. After 8 h, the extraction was stopped and 1.3 g of BA (corresponding to 25 mmol L^{-1} of BA in *n*-heptane) was added to the reaction system (reactor side). Poor production was observed between day 8 and day 22 (no accumulation observed in the reactor side). When the final extraction was performed, the MPPA available in the upper phase (Fig. 7A, grey area 3, ˆ) and in the lower AD phase (Fig. 7A, grey area 3, ▲) was extracted into the buffer. The last extraction enhanced even further MPPA release into the heptane until equilibrium was reached. The final product concentration of the acidic buffer was 1.5-fold lower than that achieved in the first experiment (5.73 g L^{-1} was achieved in the first experiment). However, by reducing substrate dosing by a factor of 2, the final yield of this latter approach was almost 60% (1.7-fold higher compared with the final yield achieved in the first experiment). In accordance with the first experiment, the combination of the reaction with three sequential extractions (Fig. 7B, black bar) proved to be beneficial. Intermittent product removal led to a 3.6-fold higher product yield value compared with that achieved in the two-liquid-phase control system, where no sequential extractions were applied (Fig. 7B, grey bar). As it can been deduced from Fig. 7A, product yield tremendously increased, especially when the second and third extractions were performed (Fig. 7A, grey areas 2 and 3). In contrast, the MPPA detected in the control, where no product recovery was applied, remained constant (Fig. 7C). Besides shifting the partition equilibrium of the formed MPPA, intermittent extractions shifted the

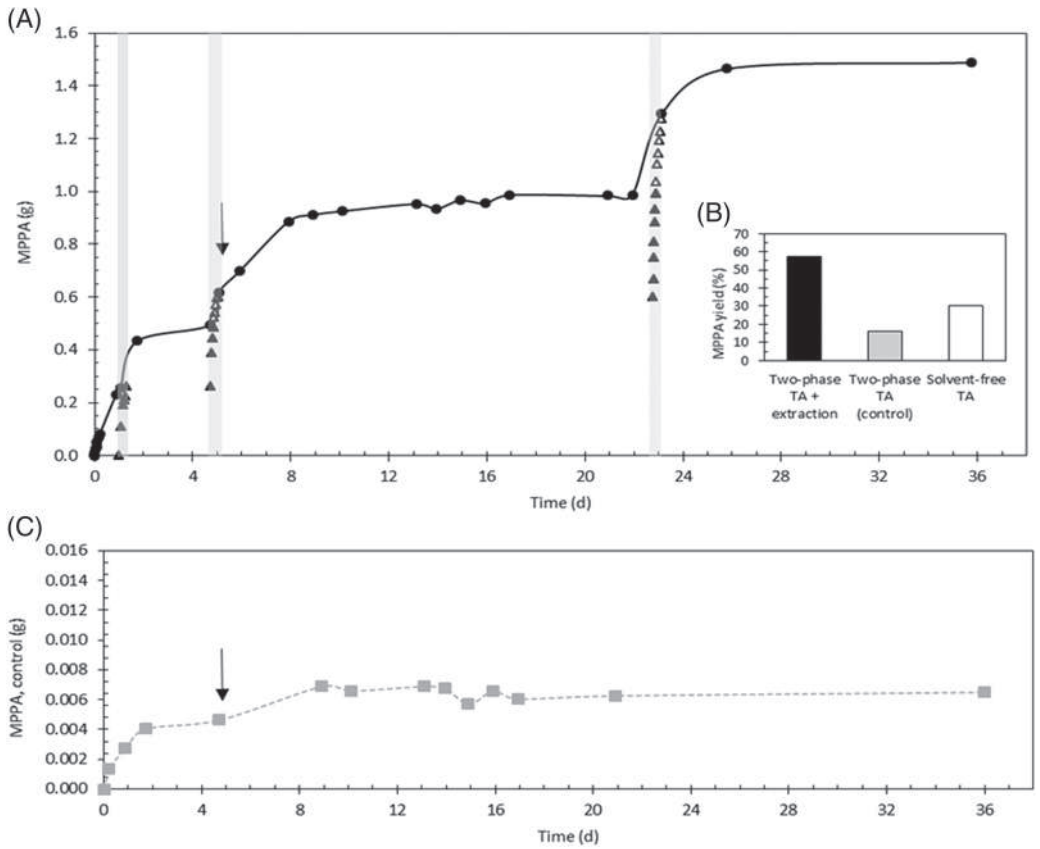

Figure 7. (A) Production of MPPA during 36 days with three sequential extractions (grey areas 1–3). The amount of product extracted into the buffer (▲) and the total amount of product detected (●) are shown. (B) Comparison between final product yield when combining the reaction (TA) with three sequential extractions (black bar), when running the reaction control without product extraction (grey bar) and when running the solvent-free reaction (white bar). (C) Production of MPPA during 36 days in the control, where no sequential extractions were applied. Experiments were performed at 30 °C. Initial amounts of amine (AD) lower phase and BA, dissolved in 0.35 L of *n*-heptane upper phase, were 105 g (175 mmol) and 1.3 g (8.8 mmol) respectively. The AD lower phase contained 3.5 g of TA-v2 enzyme. Addition of BA (1.3 g) was made after 5 days of reaction (arrow). Solvent-free and control transamination (TA) were performed at 70-fold smaller scale compared with the system where separations were applied.

thermodynamic equilibrium of the reaction system, resulting in an almost doubled yield in the solvent-free transamination system (Fig. 7B, white bar).

The major advantage of the study presented here is the selective isolation of the desired product from all other reaction components without any additional purification step. Normally, the large excess of amine donor required for thermodynamic equilibrium shifting does not facilitate the application of ISPR strategies. The unreacted excess of the commonly used IPA donor is easily lost into the product stream, hence causing contamination of the product solution and loss of the donor substrate.[13,16] Being heavier and not soluble in *n*-heptane, the Jeffamine ED-600 amine donor is the key for overcoming this issue. Moreover, the intrinsic nature of this bifunctional primary amine, characterized by repeating oxypropylene units in the backbone, can enhance the enzyme stability.[30] Although the potential is obvious, however, the choice of an amine donor/organic solvent biphasic system with strict characteristics such as solubility and density could limit the wide applicability of this ISPR strategy. In addition, it would be interesting to test the acceptance of Jeffamine ED-600 by other ATAs and also to investigate the substrate scope of TA-v2. To achieve high product purities, along with amine donor, also co-product extraction has to be avoided. Being structurally similar to the amine donor, we expect that the ketone co-product was not co-extracted into the acidic buffer phase, but this aspect needs to be analytically confirmed.

There are several ways to further optimize our approach. High product concentrations could be achieved by minimizing the volume of acidic extracting buffer. Moreover, the implementation of online monitoring and control of the pH of the extracting buffer would prevent amine product back extraction. Having found excellent flat sheet and hollow fiber solvent-stable membranes, instead of performing intermittent extractions, the system could be operated in continuous mode combined with continuous BA feeding. However, at this stage, the membrane set-up needs to be constantly controlled by an operator for technical and safety reasons. As shown in the membrane screening, performing extraction by using a membrane contactor with higher surface area would enhance the rate of product extraction and therefore further substrate conversion. However, such a module could not withstand the aggressiveness of the system towards the potting material. Therefore more work is required to increase the extraction speed and match the reaction rates with substrate feeding.

CONCLUSIONS

The novel polyether amine donor Jeffamine® ED-600, previously investigated in aqueous media,[30] was employed for the asymmetric synthesis of MPPA from BA in the presence of *n*-heptane as organic solvent. The choice of Jeffamine ED-600, an amine donor (AD) not soluble in the selected organic solvent,

resulted in the development of a two-liquid-phase system consisting of the enzyme and AD lower phase and the *n*-heptane upper phase. Poor MPPA production was observed, therefore membrane-assisted product extraction was performed. Intermittent extractions enhanced the product yield by a factor of 3.6 compared with the control two-liquid-phase system, where no extractions were applied. This outcome showed the influence of membrane-assisted product extraction for thermodynamic equilibrium shifting. In addition, product extraction without consistent contamination of the unreacted substrates was proven. However, it is important to mention that the selective product recovery, which can be achieved by only few other methods at present,[14,30] is strictly related to the choice of the combination of the solvent/amine donor system. System performances can be potentially improved. Optimization of the reactor geometry and of the stirring regime would facilitate the diffusion of the formed product from the lower AD reaction layer to the upper *n*-heptane phase. Consequently, product extraction and therefore further reaction proceedings would be enhanced.

In addition, the reaction was also successfully conducted in the presence of only the substrates and enzyme, hence providing the first example of solvent-free transamination for chiral amine synthesis. As the solvent-free system does not use organic solvents, this process minimizes the environmental impact. Compared with the two-phase system, solvent-free transamination resulted in higher product concentration (16 g L^{-1}). However, the product yield was 1.7-fold lower compared with the two-liquid-phase system combined with intermittent extractions. Additional strategies for thermodynamic equilibrium shifting and investigations on reaction kinetics and enzyme stability should be considered for this solvent-free reaction. Extension to other substrate/enzyme solvent-free systems could open new possibilities and perspectives in transaminase-catalyzed chiral amine synthesis.

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Supporting Information

Supporting information may be found in the online version of this article.

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Supporting information

Product partitioning

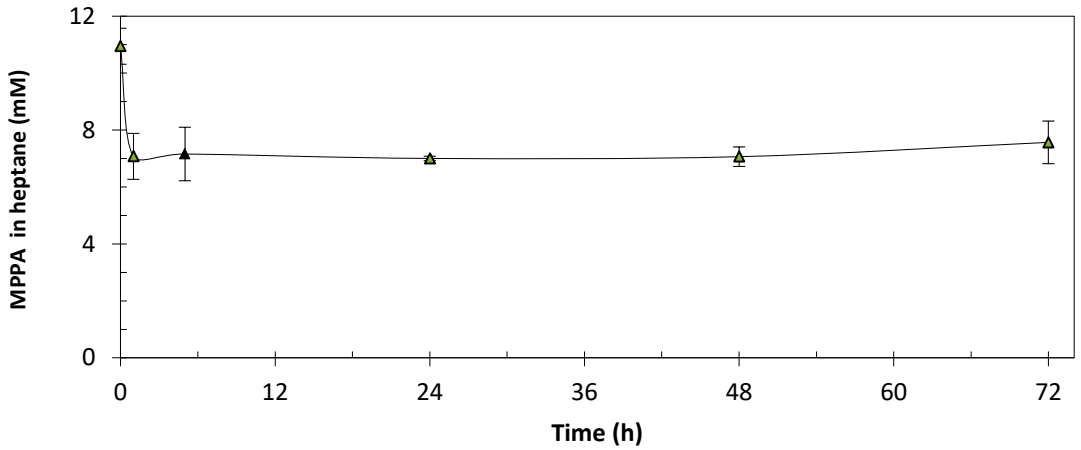

Figure S1. Product (MPPA) concentration in the upper n-*heptane phase over the time. The experiment was performed in 10 mL glass vials at 30 ˚C, using 0.05 g TA-v2 enzyme (wetted with 100 µL of 0.5 mM PLP-water mixture), 1.5 g of Jeffamine ED-600 (lower phase) and 5 mL n-heptane (upper phase). The concentration of MPPA initially dissolved in heptane amounted to 10 mM. No substrate was added. Tests were performed in triplicates and the results are represented as the average of three independent experiments.*

Effect of enzyme loading on the two-liquid phase transamination

Table S1. *Observed product yields (%) in heptane when running two-liquid phase transamination varying the substrates loading and the amine donor (AD)/ substrate (BA) ratio. Reactions were performed in glass vials, at 30 degrees, using 0.25 g of TA-v2 enzyme (previously wetted with 500 µL of 0.5 mM PLP-water mixture).*

BA (g) (g)	**Jeffamine ED-600** (g) (g)	**AD/BA ratio** mol $_{AD}$/mol $_{BA}$	**observed yield** (%)
0.008	0.3	10	9.2
0.04	0.3	2	22.3
0.04	1.5	10	27.2*

*When using 0.05 g of TA-v2 enzyme (wetted with 100 µL of 0.5 mM PLP-water mixture), the observed product yield after 48 h reaction was 32%

Solvent free transamination

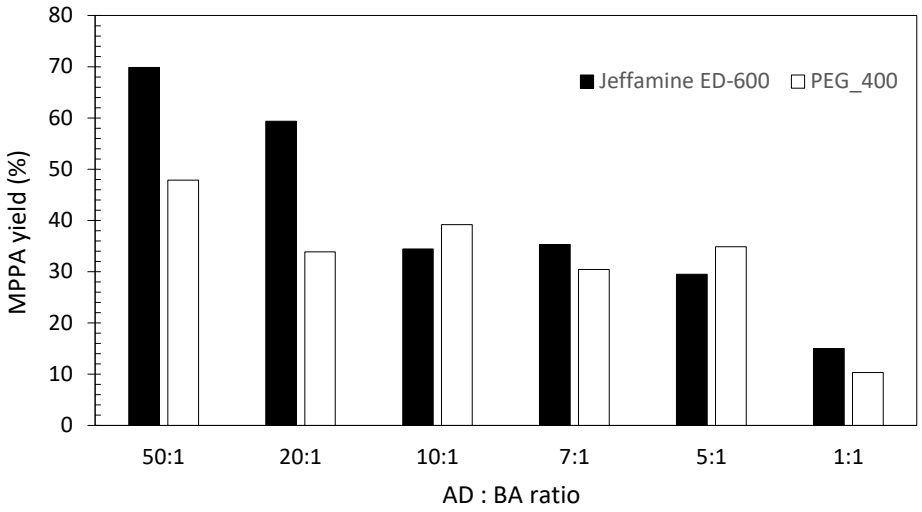

Figure S2. Effect of substrate (BA) amount on solvent-free transamination reaction when using either 1.5 g (2.5 mmols) of Jeffamine ED-600 amine donor (AD)(blue bars) or 0.9 g (2.5 mmols) of PEG_400 (Poly(propylene glycol) bis(2-aminopropyl ether)) amine donor. Reactions were performed at 30 ˚C, using 0.05 g TA-v2 enzyme. AD:BA ratio is expressed as mol_{AD}/mol_{BA}.

Preliminary membrane screening

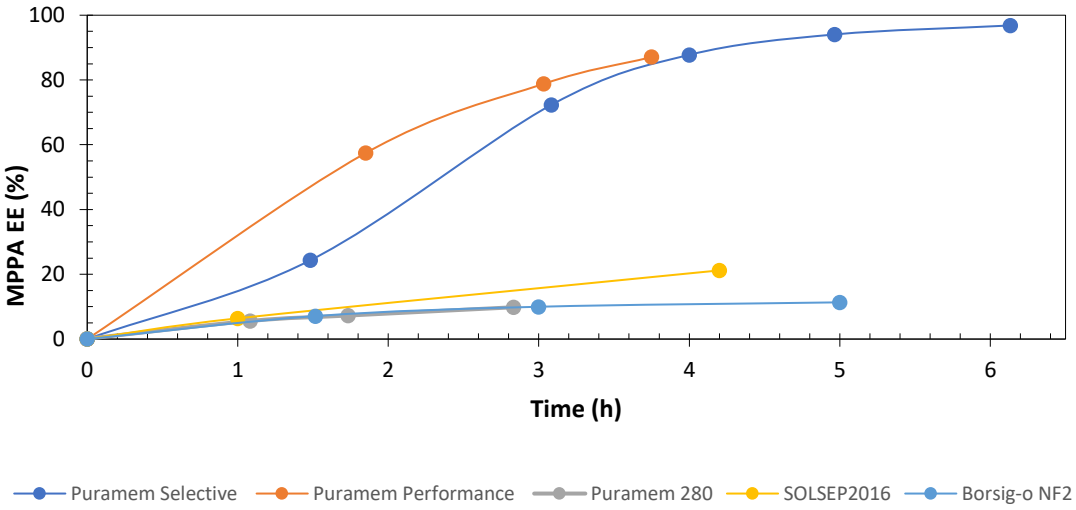

Figure S3. Product (MPPA) extraction efficiency (EE) over the time when using the flat sheet module with the polymeric membranes: Puramem Selective (Evonik) (blue line), Puramem Performance (Evonik) (orange line), Puramem 280 (Evonik) (grey line), oNF-2 (Borsig) (light blue line) and Solsep 2016 (Solsep BV) (yellow line). Membrane screening was performed using the membrane extraction set-up shown in the manuscript (Figure 1). The left side of the system was filled with a solution consisting of BA (2.3 g) and MPPA (1.3 g) dissolved in 0.350 L of n-heptane. No amine donor was added. Extraction was performed employing 0.35L of 100 mM Sodium citrate buffer, pH 3.0.

Contactor modules

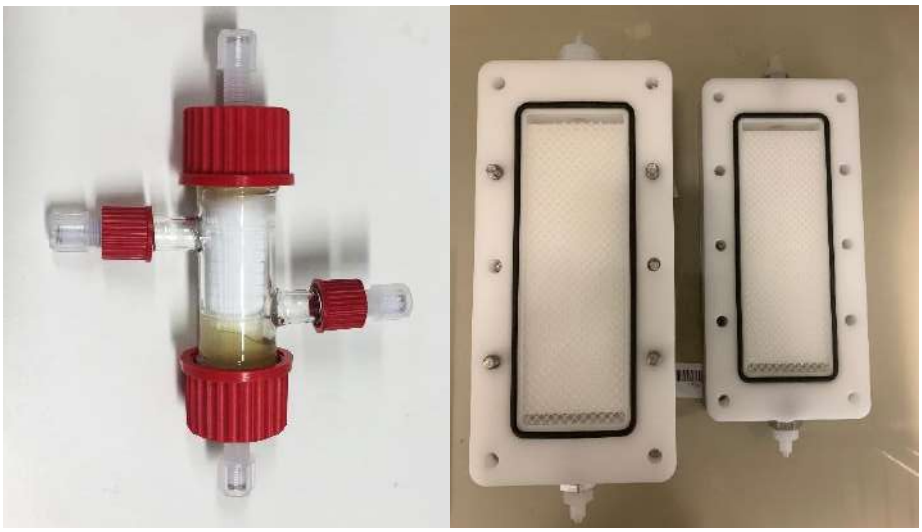

Figure S4. Hollow fiber (HF) contactor with modified housing and the flat sheet (FS) membrane contactor.

Article III

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CHEMCATCHEM Communications

Three-liquid-phase Spinning Reactor for the Transaminase-catalyzed Synthesis and Recovery of a Chiral Amine

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A device for the transaminase-catalysed synthesis combined with continuous recovery of chiral amines was designed. The system enabled the separation of the reaction components in three liquid phases: a reaction phase, an organic solvent phase (where the poorly water soluble ketone substrate was supplied), and an aqueous extraction phase for continuous product recovery. The transaminase-mediated asymmetric synthesis of (*S*)-1-methyl-3-phenylpropylamine was employed as model reaction. Factors influencing the performance of the system, such as reactor geometry, working volumes and operating parameters, were investigated. Specifically, reaction yield and product recovery were enhanced by i) reducing the thickness of the reaction phase, while continuously stirring and ii) reducing the volume of the extraction phase. Under the optimal condition tested, 85% of the product formed was extracted and a product concentration value of 9 g/L was reached. However, co-extraction of the unreacted amine donor (17%) was observed. Advantages and drawbacks of this process compared to existing technologies, as well as possible optimization strategies are discussed.

Biocatalytic transamination has attracted significant interest in recent years as an efficient method for the synthesis of chiral amines. These compounds are key building blocks in the agrochemical, fine-chemical and pharmaceutical industries. Transamination can be carried out either by direct amination of prochiral ketones (asymmetric synthesis mode) or by the thermodynamically favourable kinetic resolution of racemic amines. Due to a theoretical yield of 100%, the asymmetric synthesis is often preferred.[1–3] However, physical and chemical strategies for counteracting the unfavourable thermodynamic equilibrium and/ or product inhibition are needed.[4,5] Besides the use of an excess of amine donor (AD), methods such as co-product or product cyclization[6–8] or polymerization,[9,10] evaporation of the volatile co-product[11,12] and enzymatic cascades[4,13] for co-product removal have been developed. Membrane-assisted techniques for *in situ* product removal have been also investigated.[14–17] Specifically, membrane-based three liquid phase (3LP) systems were developed by filling the pores of an hydrophobic hollow fibre membrane contactor with an hydrophobic solvent. This operation allowed to physically separate the reaction and the extraction aqueous solutions by using a supported liquid membrane.[18–20] The only 3LP system developed without membranes was reported by Yun and Kim, 2008. They employed isooctane as an organic solvent bridge for the selective extraction of the inhibiting (*S*)-α-methylbenzylamine product. More general, 3LP systems have been mainly reported for the separation and recovery of metals from complex mixtures.[21] In addition, working with three phases offers relevant technological solutions in oil recovery processes, in industrial processes such as ε-caprolactam production,[22] for the rapid isolation of organic macromolecules such as cellulose enzymes and proteins[23] and for the straightforward separation of organic compounds e.g. during extraction of natural products from plants.[24]

We have previously demonstrated the feasibility of performing the asymmetric synthesis of (*S*)-1-methyl-3-phenylpropylamine using high molecular weight (HMW) donor amines in aqueous,[15] organic solvent and solvent-free media.[16] With a molecular weight (MW) between 400 and 1500 g/mol, these large molecules were effectively retained by commercial nanofiltration membranes, when employed in an aqueous environment.[15] Transamination using HMW ADs was also performed in the presence of a non-polar organic solvent (*n*-heptane). Specifically, the HMW AD Jeffamine ED-600 (MW of 600 g/mol), commercialized by Huntsman corporation, was insoluble in n-heptane, thus, resulting in a two-liquid-phase system.[16] Coupling the two-liquid phase reaction system with membrane-assisted product extraction, the reaction equilibrium was successfully shifted to reach 60% conversion compared to 15% without product extraction. Although product extraction without consistent contamination of the unreacted substrates was proven, the long term operational stability of the membrane set-up was found to be one of the main limitations for further process optimization.

As alternative to the mentioned membrane-assisted strategy, in this study we propose a 3LP spinning reactor (Figure 1)

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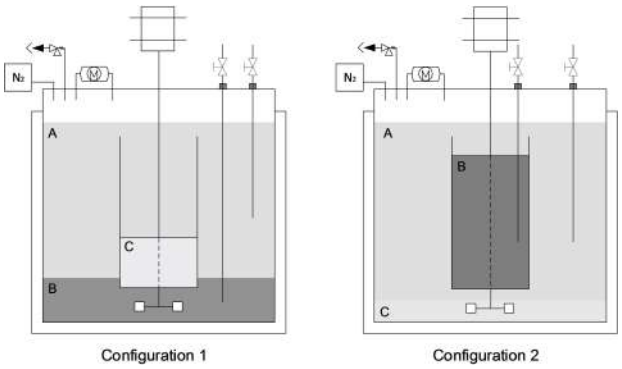


Figure 1. Principle of the three-liquid-phase (3LP) spinning reactor. A, B and C are the organic solvent phase, the reaction phase and the extraction phase, respectively. Depending on the design of the system, the device can operate in two configurations. The transaminase-mediated synthesis of (*S*)-1-methyl-3-phenylpropylamine (MPPA) was employed as model reaction (Scheme 1).

for the synthesis and recovery of chiral amines. The 3LP spinning reactor can be considered an evolution of a standard stirred 1.5 L double-jacketed glass reactor. The motor driven central shaft supported a stainless steel inner tubular cylinder and one radial flow impeller. Being mounted on the shaft, the designed inner tubular cylinder rotated together with the impeller. The feasibility of developing a three-liquid-phase system, employing the aforementioned device, relies on the selection of a suitable phase **A**, non-miscible with either phase **B** or **C** (Figure 1). The transaminase-mediated synthesis of (*S*)-1-methyl-3-phenylpropylamine (MPPA) was employed as model reaction (Scheme 1).

Based on the previous studies,[14,16] *n*-heptane was selected as organic solvent phase **A**. The substrate 4-phenyl-2-butanone (BA), initially supplied to phase **A**, progressively moved to phase **C**. The enzymatic reaction occurred in phase **C**, (reaction phase), consisting of the enzyme TA-v2[25] and the AD (Jeffamine ED-600), not soluble in n-heptane. Once formed, the product moved from the reaction phase **C** to the extracting phase **B** via diffusion through *n*-heptane, due to partitioning. The acidic pH of the extracting phase traps the amines in their charged state, thus preventing back extraction into the organic phase and allowing the enrichment of amine product. Depending on the design of the system, and on the working volumes employed, the device was tested in two different configurations, depicted in Figure 1.

To investigate the performances of the 3LP spinning reactor, a preliminary partitioning experiment using a synthetic solution was carried out. The device was tested in configuration 1, (Figure 1). In 6 h of operation, 3.5 g of MPPA, equivalent to 71% of the initial amount added to the system, was extracted (Figure 2). The content of Jeffamine co-extracted amounted to 9.7%. When the stirring rate, initially set to 150 rpm, was increased to 200 rpm, the rotation rate of the inner tubular cylinder, fixed to the agitator shaft, increased. The AD phase, placed in the inner tubular cylinder, spilled over the inner tubular cylinder, where it diffused through the *n*-heptane phase and was extracted into the buffer phase. Consequently, the pH of the buffer increased (Figure 3) and back extraction of MPPA was observed (Figure 2).

The first transaminase-mediated synthesis combined with continuous product recovery was performed employing the same configuration of the preliminary partitioning experiment (Figure 1, configuration 1). Having already proven in our recent work the reproducibility of the reaction system,[16] a single experiment at 1L scale was carried out. Out of 5 g of ketone

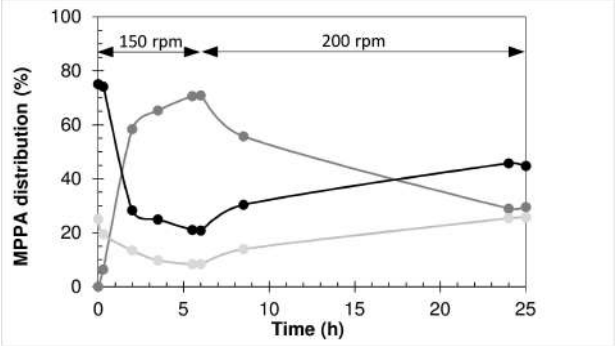


Figure 2. Preliminary partitioning experiment: distribution of 4-phenyl-2-butylamine (MPPA) in Jeffamine ED-600 (reactor phase, ●), *n*-heptane (●) and citric acid buffer solution (extraction phase, ●) over the time. The three-liquid-phase (3LP) spinning reactor was tested in configuration 1 (Figure 1). Experimental details are provided in the supplementary information, section 1.3.

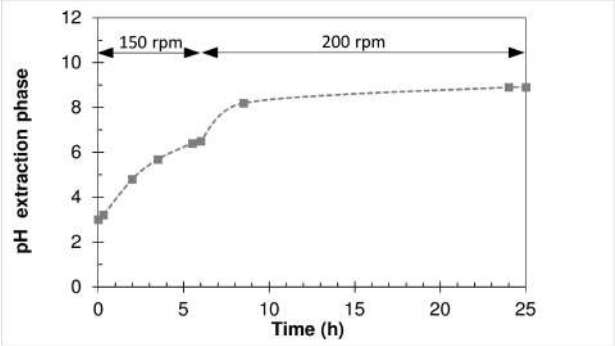
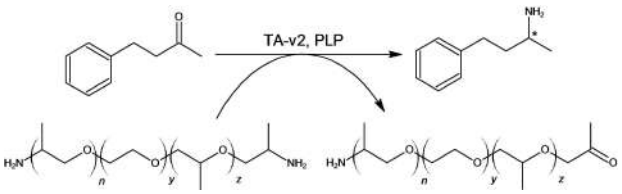


Figure 3. Preliminary partitioning experiment: pH of the extraction phase over the time. The three-liquid-phase (3LP) spinning reactor was tested in configuration 1 (Figure 1).



Scheme 1. The transaminase-mediated synthesis of (*S*)-1-methyl-3-phenyl-propylamine (MPPA) from 4-phenyl-2-butanone (BA), using Jeffamine ED-600 amine donor.

substrate, initially added in *n*-heptane, only 0.8 g of MPPA was extracted and isolated after 17 days operation (Figure 4). It is possible that more MPPA could be formed in the reactor phase but was not released into the *n*-heptane thus escaping extraction. Remarkably, the diffusion of product and therefore product extraction increased when continuously stirring the system at 100 rpm (Figure 4, day 8–14). A continuous motion of the inner tubular cylinder, imparted by the stirrer, enhanced product release and thus improved the product extraction. As demonstrated in our previous study,[16] the geometry of the reaction system affects the rate of product diffusion from the AD to the upper *n*-heptane phase. Working with a larger diameter cylinder would decrease the thickness of the AD layer thus facilitating the diffusion of the formed product from the reactor phase to the *n*-heptane phase. In accordance with the partitioning experiment, less than 2% of unreacted substrate BA was co-extracted in the acidic buffer. AD co-extraction could not be avoided and depended on the stirring. Less than 1% of the initial AD Jeffamine added in the inner tubular cylinder was found in the *n*-heptane middle phase during the entire test. However, the stirring and the constant contact/mixing between *n*-heptane and the acidic extracting buffer resulted in AD co-extraction, thus affecting the product purity of the buffer phase. After 6 days of operation, the concentrations in the extracting phase of MPPA and Jeffamine ED-600 were 8 mM and 44 mM, respectively. Although 25% of the AD was co-extracted, the AD was present in lower concentrations compared to that would be the case if performing a batch reaction in aqueous environment, without any product removal strategy.

High reaction rates combined with fast product release from the reaction phase to *n*-heptane are essential for optimizing the ratio between the extracted amines. With this aim, the second experiment was carried out in a different configuration (Figure 1, configuration 2). By placing the enzyme on the bottom of the vessel (diameter 2.6-fold larger than the inner tubular cylinder), the AD thickness was considerably reduced. As expected, in this configuration product release from the AD layer to *n*-heptane was faster. In 5 days of continuous operation, a product yield of 52% was achieved. Compared to the control, a conventional batch experiment, where no MPPA extraction was applied, 2.6-fold higher product yield was achieved. Moreover, 85% of the formed product was extracted from the *n*-heptane into the buffer. The MPPA concentration constantly increased in the stripping phase, despite the much lower concentration in the reactor (0.1 g/L). Minimizing the volume of the stripping phase has several benefits for the downstream processing (i.e. higher product concentration). Having reduced the buffer volume by a factor of 3.5, a product concentration of 9 g/L was achieved in 5 days (Figure 5, ●). A higher product concentration could probably be achieved by prolonging the reaction time. On the one hand, stirring enhanced product release from the reaction phase to n-heptane and thus proved beneficial for simultaneous product extraction. On the other hand, stirring led to increased AD co-extraction into the extracting buffer (17% of the initial amount added was found in the extracting phase). Therefore, the system was stopped after 5 days of operation.

The main limitation of the extraction strategy for product removal (using solvents, membranes or resins) is often the poor selectivity between substrates and products.[26] Higher product purity is probably achievable by performing the reaction in an aqueous environment, using alanine or another zwitterionic AD, as neither the AD nor pyruvate co-product would partition to the hydrophobic organic solvent phase.[20,27]

The major difficulty to tackle for a 3LP process is the physical separation of the three different phases. This can be achieved with the classical separation funnel, for batch applications. For conducting countercurrent and continuous operations, more complex devices have been developed. The recently proposed mixer-settler-mixer three chamber integrated extractor was used for the separation of *p*-nitrophenol and *o*-nitrophenol. The separation of the two isomers was achieved by continuous mixing and separation of three non-miscible liquid phases: nonane (organic top-phase); polyethylene glycol (PEG 2000), (polymer middle-phase); and $(NH_4)_2SO_4$ aqueous solution, (aqueous bottom-phase).[28] The 3LP spinning reactor here proposed, does not require special laboratory equipment, and allows separations of multicomplex mixtures between two miscible phases separated by a third immiscible phase to be

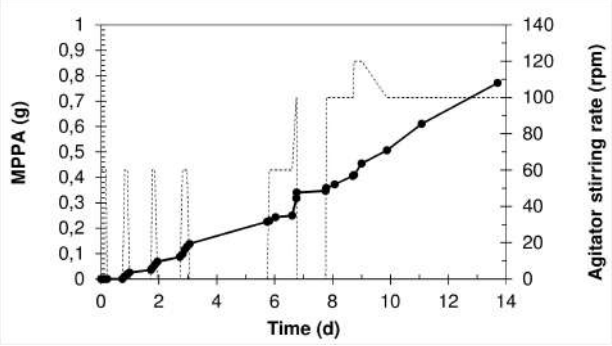


Figure 4. Effect of intermittent/discontinuous stirring rate (right axis —) on product extraction (left axis ●) in citric acid buffer, using the 3LP spinning reactor in configuration 1. Experimental details are provided in the supplementary information, section 1.4.

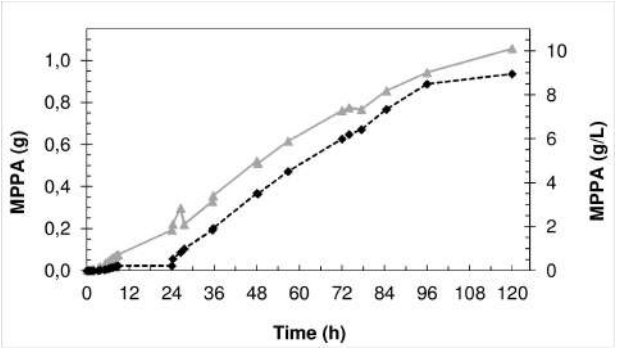


Figure 5. Production of MPPA for 4 days using the 3LP spinning reactor, configuration 2. Total product amount (g) detected in n-heptane and in the extracting buffer, (▲, left axis) and product concentration in the extracting buffer (●, right axis) are shown. Experimental details are provided in the Supplementary information, section 1.4.

performed. Additionally, it introduces more freedom for the selection of the three phases. The performance of the 3LP device can be exploited by varying the rotor speed, and the position, type, size and numbers of the impellers. Moreover, the geometry, the size and the position (height) of the tubular cylinder inside the reactor can be changed, depending on the working volumes of each phase.

In conclusion, the 3LP reactor concept, employed for transaminase-mediated synthesis of (*S*)-1-methyl-3-phenylpropylamine, was shown to be superior to the conventional set-up, where the reaction without product extraction was performed. Process engineering strategies for chiral amine synthesis have proven to enhance physical and chemical properties of transaminase-catalysed systems, such as low solubilities of reactants within aqueous media or undesired unfavourable thermodynamics.[26] Hereof, this study presents a step towards process intensification. Furthermore, the device should allow for the development of a continuous process, overcoming the limited lifetime of existing membrane-assisted three-liquid phase systems. The use of our device can be potentially extended to other product inhibited or thermodynamically unfavourable reaction systems or to different applications (e.g. separation of multi-component mixtures).

Keywords: Chiral amines · transaminases · biocatalysis · liquid-liquid extraction · asymmetric synthesis

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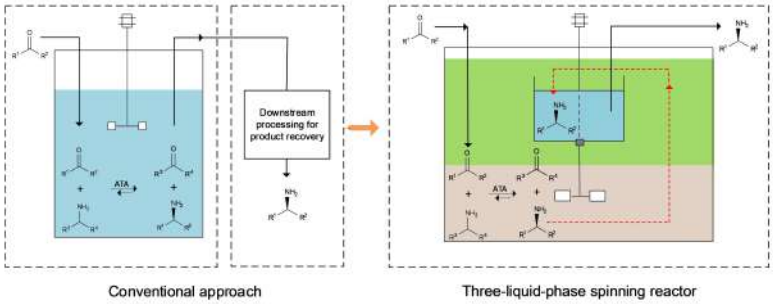

Conventional approach Three-liquid-phase spinning reactor

C. Matassa, Dr. D. Ormerod, Prof. U. T. Bornscheuer, Prof. M. Höhne, Dr. Y. Satyawali**

Reactor design: A device for the transaminase-catalysed synthesis and continuous recovery of chiral amines was designed. The system enabled the separation of the reaction components in three liquid phases: a reaction phase, an organic solvent phase (where the poorly water soluble ketone substrate was supplied), and an aqueous extraction phase for continuous product recovery.

Supporting material and method section

1.1 Chemicals and enzyme

The chemicals used in this study including 4-phenyl-2-butanone (benzyl acetone**,** BA) (98% purity), 4-phenyl-2-butylamine (1-methyl-3-phenylpropylamine, MPPA) (98%), the High Molecular Weight (HMW) amine donor *O*,*O*'-Bis(2-aminopropyl) polypropylene glycol-*block*-polyethylene glycol-*block*-polypropylene glycol (Jeffamine® ED-600, MW of 600 g/mol), *n*-heptane (>97%) and pyridoxal-5'-phosphate (PLP), were purchased from Merck. The (*S*)-selective amine transaminase (TA-v2) was purchased from c-LEcta GmbH, Leipzig, Germany. The purified enzyme was supplied as freeze dried powder. The activity of TA-v2 was 1.73 U/mg (value provided by the supplier, based on acetophenone detection).[1]

1.2 Equipment description

The three-liquid phase (3LP) reactor consisted of a 1.5-L double-jacketed glass reactor (INFORSHT) with a motor driven central shaft that supported the stainless steel inner tubular cylinder and one radial flow impeller. Being mounted on the shaft, the inner tubular cylinder rotated together with the impeller. The stirring rate was varied between 0 and 200 rpm. The reactor was kept at 30°C by hot water circulation. The headspace of the bioreactor was continuously flushed with N_2 to remove any residual oxygen. In order to minimize *n*-heptane evaporation, a condenser was installed. At specific times, samples from the aqueous extracting phase and from the *n*-heptane phase were taken out and prepared for analysis. Depending on the design of the system, the apparatus can operate in two different configurations (Figure 1). Depending on the configuration, two sampling ports were installed. The pH of the aqueous extracting phase was manually monitored by sampling and manually adjusted by HCl addition.

1.3 Chiral amine product extraction using a synthetic solution

A synthetic solution consisting of 5 g of 4-phenyl-2-butanone (BA) and 5 g of 4-phenyl-2-butylamine (MPPA) dissolved in 50 mL heptane was mixed with 28 mL of Jeffamine ED-600, placed in the inner tubular cylinder and was equilibrated for 30 min. A two-liquid phase system consisting of the Jeffamine ED-600 lower phase (phase **C**) and *n*-heptane was formed (Figure 1, configuration 1). The reactor was then filled with 220 mL of 100 mM citric acid buffer solution (Figure 1, configuration 1, phase **B**) and with *n*-heptane (Figure 1, configuration 1, phase **A**). The aqueous extracting solution (100 mM sodium citrate buffer, pH 3.0) was chosen in such a way to enhance the selective MPPA extraction and to prevent its back extraction.[2,3] The total amount of the *n*-heptane middle phase amounted to 920 mL. The 3LP spinning reactor was continuously stirred. After 6 h operation, the stirring rate was increased from 150 to 200 rpm. At frequent intervals, samples of the *n*-heptane and the extracting phase were taken for BA and MPPA and Jeffamine ED-600 quantification.

1.4 Enzymatic chiral amine synthesis and extraction

According to previous studies,[3] 1.5 g of TA-v2 enzyme was wetted with 3 mL of 0.5 mM PLP-water mixture. The mixture was placed in the inner tubular cylinder followed by addition of the HMW amine donor Jeffamine ED-600 (37 g). Enzyme and amine donor formed the reactor phase **C**. The reactor was filled with 350 mL of 100 mM citric acid buffer solution, pH 3.0 (extracting phase **B**), and with 950 mL of n-heptane (phase **A**) followed by substrate BA addition (5 g). The 3LP reactor was kept at 30°C for a total of 17 d. The stirring rate was varied from 0 to 120 rpm to investigate its influence on the

reaction and on the product extraction performances. The pH of the acidic extracting phase was constantly monitored and kept acidic by HCl addition.

A second experiment was run for 10 d using the same system described above with the following modifications: the apparatus was tested in configuration 2 (Figure 1). Therefore, the reactor phase, consisting of 3.5 g of TA-v2 enzyme wetted with 7 mL of 0.5 mM PLP-water mixture, and 105 g of HMW amine donor Jeffamine ED-600, was placed in the reactor vessel (Figure 1, configuration 2). A 3.5-fold smaller volume of citric acid buffer volume was used (100 mL) and placed into the inner tubular cylinder. The reactor was finally filled with 1.4 L of *n*-heptane. The initial ketone substrate BA amount added to *n*-heptane was 1 g. One further BA addition (1 g) was made after 1 d.

A control transamination was performed in glass vials, in 70-fold smaller scale compared to the 3LP system. A two-liquid phase system was built by adding to the vials containing the enzyme (0.05 g) and the AD (1.5 g), the organic solvent *n*-heptane. Substrate BA (0.015 g) was supplied to the *n*-heptane phase. Substrate additions in the controls were performed in accordance with the tests with intermittent product extraction. No product extraction was applied to the control vials.

1.5 Analysis

Product (MPPA) and amine donor (Jeffamine ED-600) in the organic solvent phase (*n*-heptane) were extracted with 100 mM citric acid buffer prior quantification and analyzed as described in our previous study [4]. Substrate (BA), MPPA and Jeffamine ED-600 concentrations in the extracting phase were also analyzed as previously described.[4] Chiral analysis were performed by gas chromatography using a heptakis-(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethyl-silyl)-β-cyclodextrin column (25 m x 0.25 mm). The experimental procedure for sample preparation (amine extraction and derivatization to trifluoroacetamide) and details of chiral analysis are reported elsewhere.[5]

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