



Cite this: *Green Chem.*, 2017, **19**, 333

Transaminase biocatalysis: optimization and application

Fei Guo and Per Berglund*

Transaminases (TAs) are one of the most promising biocatalysts in organic synthesis for the preparation of chiral amino compounds. The concise reaction, excellent enantioselectivity, environmental friendliness and compatibility with other enzymatic or chemical systems have brought TAs to the attention of scientists working in the area of biocatalysis. However, to utilize TAs in a more efficient and economical way, attempts have to be made to optimize their performance. The demand for various substrate specificities, stability under non-physiological conditions and higher conversions in reversible reactions have been targeted and investigated thoroughly. A number of both protein- and process-based strategies have been developed to improve TAs and systems involving TAs. Moreover, by combination with other enzymes in cascade reactions or even in more complex systems, so called synthetic biology and systems biocatalysis, TAs can be biocatalysts with immense potential in the industrial production of high-value chemical products. This review will highlight strategies for optimization of TAs and will discuss a number of elegant systems for improving their performance. Transaminase biocatalysis has been, and will continue to be, one of the most interesting topics in green organic synthesis.

Received 20th August 2016,

Accepted 30th September 2016

DOI: 10.1039/c6gc02328b

www.rsc.org/greenchem

KTH Royal Institute of Technology, School of Biotechnology, Division of Industrial Biotechnology, AlbaNova University Centre, SE-106 91 Stockholm, Sweden.
 E-mail: per.berglund@biotech.kth.se



Fei Guo

Fei Guo was born in Baotou, China, in 1988. She obtained her Bachelor's degree in pharmaceutical engineering from Zhejiang University (Hangzhou, China) in 2010. Then she continued her study for a PhD in biological engineering there, supervised by Professor Hongwei Yu. After she obtained her PhD degree in 2015, she started a postdoctoral fellowship at KTH Royal Institute of Technology, Stockholm, Sweden, with

Professor Per Berglund. Her research focuses on computer-guided engineering of biocatalysts in asymmetric synthesis.

Introduction

Transaminases (TAs), or aminotransferases, are enzymes that mediate the transfer of an amino group from the amino donor to the acceptor for chiral amino acid or amine synthesis,



Per Berglund

Per Berglund originates from Sundsvall, Sweden, where he studied Chemistry and obtained his PhD in Organic Chemistry from Mid Sweden University in 1995. He then moved to University of Toronto, Canada, for a postdoc with Prof. J. Bryan Jones. Per returned to Sweden in 1997 and started at KTH Royal Institute of Technology, Stockholm, where he became Associate Professor and Docent in Organic Chemistry and finally

Full Professor in Biochemistry in 2009. His research interests include stereoselective biocatalysis and enzyme engineering and his group now focuses on transaminases in cascade reactions for industrial applications. In 2010, Prof. Berglund was elected Vice Dean of KTH and has been responsible for the educational structure and content at KTH since then.



providing a more concise route for reductive amination compared to its chemical counterparts.¹ This process consists of two steps (Scheme 1): (i) deamination of an amino acid or amine (amino donor, **a**) releasing amino donor product (**b**), and (ii) amination of a keto acid, ketone or aldehyde (amino acceptor, **c**) producing a new amino acid or amine (**d**).² A vitamin B₆-based cofactor, pyridoxal 5'-phosphate (PLP), bound to a catalytic lysine of the enzyme *via* an imine bond, assists the reaction by acting as a transient "custodian" of the amino group.^{3,4} By the end of the transamination, the cofactor releases the amino group and returns to its initial state. This automatic cofactor recycling is advantageous in enzymatic syntheses since no additional cofactor regeneration is required as is often the case for many other enzymes.^{5,6} Another appealing property of TAs is their extraordinary stereoselectivity.⁷ With the almost exclusive substrate-binding mode constrained by the relative position of PLP and two substrate-binding pockets of different sizes, the enantiomeric excess of the chiral products is remarkably high. These distinguished natural properties have brought TAs to the attention of the biocatalysis community.^{8–10}

Since their first applications in industry and academia at the end of the last century,^{11,12} an increasing number of attempts have been made to utilize TAs in a more efficient and economical fashion. The primary demand for the application of TAs is the practicability for various substrates other than α -amino acids. Of special interest are the transaminases that accept substrates with an amino group distant from the carboxylate (β - or other amino acids), or even lacking a carboxyl group (amines). The favourable substituent groups include not only small or medium aliphatic chains but also larger aromatic moieties. The development of biotechnology and protein engineering has facilitated the search for and generation of new TAs with diverse substrate specificities.^{13–15}

Even with appropriate substrate specificity, the TA stability may become an obstacle. Maintaining the quaternary and tertiary structures under non-physiological conditions (such as high concentrations of substrate and product, organic solvent, mechanical force, *etc.*) for a long period of time (10–100 h

working time and an even longer storage time) is a huge task for all biocatalysts. Attempts have been made by strategies of protein engineering, biocatalyst formulation and reaction medium engineering to solve the stability issue.^{16–18} These long-lived and even recyclable TAs are able to reduce the cost of enzymes prominently.

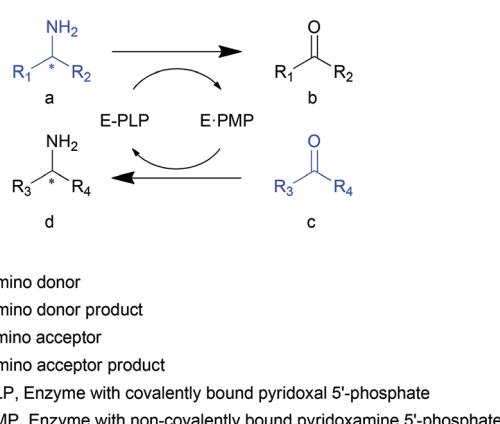
Nevertheless, organic synthesis by TAs suffer from the unfavourable reaction equilibrium, especially in the asymmetric synthesis of amines from an amino acid as amino donor.¹⁹ To maximize the productivity of TAs, shifting the equilibrium to the side of product is extremely important.^{20,21} Process control strategies have been developed in order to remove product *in situ*, among which cascade reactions employing additional biochemical reactions of the side products have gained much attention.²²

Afterwards, the optimized enzyme and process can be applied in the production of amino compounds, which are in most cases important synthons in pharmaceutical, food additive, cosmetic, agrochemical and material industries.^{8–10} Combining TAs with other enzymatic or chemical routes, even metabolic pathways in a one-pot fashion, has been demonstrated to be a smart method for practical applications, particularly in regard to shortening the reaction routes, avoiding protecting steps, reducing chemical waste and achieving a high atom-efficiency.^{22–24} In this process development, intelligent synthesis route design is the most critical and appreciable step. Besides this, metabolic flux control and systematic regulation are also able to improve the final production.

After nearly 20 years of study, TA has been proven to be a powerful biocatalyst in organic synthesis. A number of elaborate reviews have been published on this topic.^{2,7–10,20,22,25–28} In the current review, we focus on attempts to optimize the TA-mediated reactions in order to propose methodological options for the problems that may be encountered in the application of TAs. The elegant ways of using TAs in cascade reactions and in even more complex multi-step systems, so called synthetic biology and systems biocatalysis, are also presented to illustrate the immense potential of TAs in industrial applications.

Exploring and generating novel enzymes

The specificity for the chemicals of interest is the primary property to be considered and optimized when an enzyme is applied in organic synthesis. Basically, two strategies are practical in generating biocatalysts with the expected properties: gene mining to discover new enzymes and protein engineering to tailor-make enzymes. Gene mining is advantageous due to the vast database in nature to be searched in; however, the searching itself is a time-consuming process. Protein engineering, especially the rational strategy based on biochemical mechanisms and biological structures, is more efficient with a reduced number of designed mutants, although the efficiency of this process could be restricted by inappropriate starting-



Scheme 1 TA-mediated transamination.



point proteins. To accelerate the process of both these methods, high throughput screening strategies have been developed on the basis of diverse smart biochemical reactions. Combination of these techniques will provide effective and rapid methods for generating TAs with the expected substrate specificities.

Gene mining

As a tremendous library of proteins, nature has been employed for mining a large number of biocatalysis genes for enzymatic organic synthesis.^{29,30} With the development of biotechnology, the dominant strategy to discover new biocatalysts has varied from straightforward experimental assays for certain functions to deduction of protein functions from bioinformation databases. In this section, we will discuss the mining of TA genes with different strategies (Table 1 and Fig. 1).

Experimental assays for functions. Experimental assays for certain functions of microorganisms are straightforward methods of gene mining. Among them, enrichment cultivation has been demonstrated to be efficient, especially in tests of reactions involved in metabolism and biomass growth. The screening culture containing one amine as the sole nitrogen source has been employed for the identification of strains with transamination activity.³¹ After a few rounds of enrichment cultivation, the screened strains were further cultivated with an optimized growth broth and tested in biocatalysis assays. A number of microorganisms were identified through this strategy from soil sources with the corresponding enzymes being purified from the cell extracts afterwards. For example, Kim *et al.* identified 3 strains, *Klebsiella pneumonia* JS2F, *Bacillus thuringiensis* JS64 and *Vibrio fluvialis* JS17, with slightly different substrate preferences by taking 1-phenylethylamine (1-PEA, **1a**) as the sole nitrogen source.^{13,32} The corresponding TA in *V. fluvialis* was purified and characterized as an effective biocatalyst towards aromatic amines (**1a**, **7** and **10**),³³ and has become one of the most widely applied amine TAs in organic synthesis of amino compounds.^{34–36} In another example, two strains with complementary enantioselectivity, *Arthrobacter* sp. KNK168 with *R*-selectivity and *Pseudomonas* sp. KNK425 with *S*-selectivity towards the same amine (**3a**), were explored and characterized.³⁷ Notably, the *Arthrobacter* sp. KNK168 TA was until recently the only *R*-selective TA to be identified from experimental microorganism screening. Moreover, a number of microorganisms with substrate specificities corresponding to those of the screening culture including *Bacillus megaterium* SC6394,³⁸ *Moraxella lacunata* WZ34,³⁹ *Pseudomonas fluorescens* KNK08-18,⁴⁰ *Alcaligenes denitrificans* Y2k-2,⁴¹ *Mesorhizobium* sp. LUK⁴² and *Pseudomonas* sp. AAC^{43,44} were identified by enrichment cultivation by employing 1-cyclopropylethylamine (**18k**), alaninol (**24**), 7-methoxy-2-aminotetraline (SMA, **8b**), β -amino-*n*-butyric acid (**29b**), a substituted β -amino-3-phenylpropionic acid (**30c**) and 12-aminododecanoic acid (**28f**) as the sole nitrogen source, respectively. Despite the applicability of this enrichment-based assay for transaminase activity, a few drawbacks have also been shown. For example, only the enzymes responsible for biomass growth can be screened with

this strategy. Other drawbacks are the requirement for subsequent purification of the corresponding enzyme, limitations by low expression of the target enzyme and the interference from other enzymes in the system in the activity as well as in the enantioselectivity.

Experimental analysis of biosynthetic pathways for secondary metabolites may also provide TA candidates for biocatalysis. For example, by the investigation of the biosynthetic pathway of capsaicinoid in *Capsicum annuum* CH-19 sweet, a non-pungent pepper mutant derived from a pungent pepper strain, a T insertion in a putative vanillin TA gene catalysing the synthesis of vanillylamine (**5e**) was identified as the main reason for the phenotype alteration of this pepper.⁴⁵ Further *in vitro* investigation revealed that this vanillin TA can be employed in the biocatalysis of chiral amines due to its high stereoselectivity and activity.⁴⁶

Recently, another example of experimental screening was reported with a GC based transamination activity determination of more than 100 lyophilized cell preparations from diverse microorganisms.⁴⁷ Among them, *Janibacter terrae* DSM 13953, *Pseudomonas cichorii* DSM 50259 and *Pseudomonas fluorescens* ATCC 49838 were identified as highly enantioselective biocatalysts for the kinetic resolution of amine **1a**, albeit all strains showed *S*-enantiomer preference. The best strain *J. terrae* was further applied in a kinetic resolution of 143 mg *rac*-**1a**, resulting in 51% of conversion and product of >99% ee (*E* > 200) after 24 h of reaction. Some disadvantages of whole cell biocatalysis, indicated in this work, are that the side-products from the microorganism could disturb the results of screening; meanwhile, the strategies of equilibrium shifting may also be limited by other enzymes in the system.

With elegant substrate promiscuity, the TAs developed may exhibit novel activities towards new substrates. For the scientists in organic chemistry and biocatalysis, commercial collections available as “ready to use” screening toolkits could be another practical option. For example, TAs from Codexis, including ATA-103, ATA-113, ATA-114, ATA-117 and ATA-256, has been applied in a number of investigations.^{15,48–51}

From sequence to function. Aside from experimental assays, protein functions can also be deduced from its primary structure: the amino acid sequence. With the development of sequencing techniques, a vast number of genes have been deposited in databases, which promote sequence alignment to become an effective method for novel gene mining, resulting in single protein sequences to circumvent the purification process of microorganism screening (Table 1). For instance, *Chromobacterium violaceum* TA was explored by BLASTP with 38% identity to the template *V. fluvialis* TA, and exhibited outstanding performance and potential in amine transamination similar to the template protein.^{52–55} Both of these TAs have higher activities towards aromatic amines (**1a**, **2**, **5a** and **10**) than aliphatic amines (**18a, b, e and j**). Amino acids (**29b** and serine) are also unfavourable for them, except for alanine, which is a superb amino donor for *C. violaceum* TA. Another TA identified by homologous searching with *V. fluvialis* TA as a template was *Paracoccus denitrificans* (identity 94%), a putative



Table 1 Transaminases explored from gene mining

Strategy	Organism	Comment ^a	Ref.
Enrichment cultivation	<i>Klebsiella pneumoniae</i> JS2F	SNS, 1a	32
	<i>Bacillus thuringiensis</i> JS64	SNS, 1a	
	<i>Vibrio fluvialis</i> JS17	SNS, 1a	13 and 33
	<i>Arthrobacter</i> sp. KNK168	SNS, 3a ; <i>R</i> -selective	37
	<i>Pseudomonas</i> sp. KNK425	SNS, 3a	
	<i>Bacillus megaterium</i> SC6394	SNS, 18k	38
	<i>Moraxella lacunata</i> WZ34	SNS, 24	39
	<i>Pseudomonas fluorescens</i> KNK08-18	SNS, 8b	40
	<i>Alcaligenes denitrificans</i> Y2k-2	SNS, 29b ; β -TA	41
	<i>Mesorhizobium</i> sp. LUK	SNS, 30c ; β -TA	42
Metabolic function analysis	<i>Pseudomonas</i> sp. AAC	SNS, 28f ; ω -TA	43 and 44
	<i>Capsicum</i>	Vanillin TA for 3c	45 and 46
GC-based assay	<i>Janibacter terrae</i> DSM13953	TA for S-1a	47
	<i>Pseudomonas cichorii</i> DSM 50259	TA for S-1a	
	<i>Pseudomonas fluorescens</i> ATCC49838	TA for S-1a	
Homologous sequence searching	<i>Chromobacterium violaceum</i> DSM30191	38% identity to <i>V. fluvialis</i> TA	52
	<i>Paracoccus denitrificans</i>	94% identity to <i>V. fluvialis</i> TA	57
	<i>Ochrobactrum anthropi</i>	42% identity to <i>P. denitrificans</i> TA	58
	<i>Acinetobacter baumannii</i>	17% identity to <i>P. denitrificans</i> TA	
	<i>Acetobacter pasteurianus</i>	21% identity to <i>P. denitrificans</i> TA	
	<i>Caulobacter crescentus</i>	65% identity to <i>A. denitrificans</i> TA; β -TA	59
	<i>Polaromonas</i> sp. JS666	54% identity to <i>Mesorhizobium</i> TA; β -TA	60
	<i>Halomonas elongata</i>	38% identity to <i>V. fluvialis</i> TA; halo-adapted	104
	<i>Sphaerobacter thermophilus</i>	41% identity to <i>Polaromonas</i> TA; thermophile	18
	<i>Geobacillus thermodenitrificans</i>	51% identity to <i>V. fluvialis</i> TA; thermophile	103
Motif-based sequence searching	<i>Aspergillus terreus</i>	<i>R</i> -Selective	14 and 61
	<i>Penicillium chrysogenum</i>	<i>R</i> -Selective	
	<i>Aspergillus oryzae</i>	<i>R</i> -Selective	
	<i>Aspergillus fumigatus</i>	<i>R</i> -Selective	
	<i>Neosartorya fischeri</i>	<i>R</i> -Selective	
	<i>Gibberella zaeae</i>	<i>R</i> -Selective	
	<i>Hypomonas neptunium</i>	<i>R</i> -Selective	
	<i>Mycobacterium vanbaalenii</i>	<i>R</i> -Selective	
	<i>Mesorhizobium loti</i>	<i>R</i> -Selective	
	<i>Mesorhizobium loti</i> MAFF303099	10 putative TAs from protein family profile	63
	<i>Nectria haematococca</i>	<i>R</i> -TA from fungal source	62
	<i>Escherichia coli</i> K12	<i>ygiG</i> gene, TA for 21	64
	<i>Pseudomonas putida</i>	<i>spuC</i> gene, TA for 21	65
	<i>Pseudomonas chlororaphis</i>	<i>spuC</i> gene, TA for 21	
Sequence of known function	<i>Pseudomonas fluorescens</i>	<i>spuC</i> gene, TA for 21	
	<i>Pseudomonas aeruginosa</i>	β -TA	66
	<i>Arthrobacter citreus</i>	Aromatic amine TA	16
	<i>Rhodobacter sphaeroides</i>	Aromatic amine TA	67
Protein structure database	<i>Burkholderia graminis</i>	β -TA	68
	<i>Silicibacter pomeroyi</i>	PDB ID, 3HMU	69
	<i>Rhodobacter sphaeroides</i> KD131	PDB ID, 3I5T	
	<i>Ruegeria</i> sp. TM1040	PDB ID, 3FCR	
	<i>Mesorhizobium loti</i> MAFF30399	PDB ID, 3GJU	
	<i>Bacillus anthracis</i>	PDB ID, 3N5M	70

^a SNS, sole nitrogen source.

subgroup III TA⁵⁶ that was investigated to catalyse the transamination with amines as amino donor.⁵⁷ Subsequently, three TAs from *Ochrobactrum anthropi*, *Acinetobacter baumannii* and *Acetobacter pasteurianus* were explored based on the sequence of *P. denitrificans* TA.⁵⁸

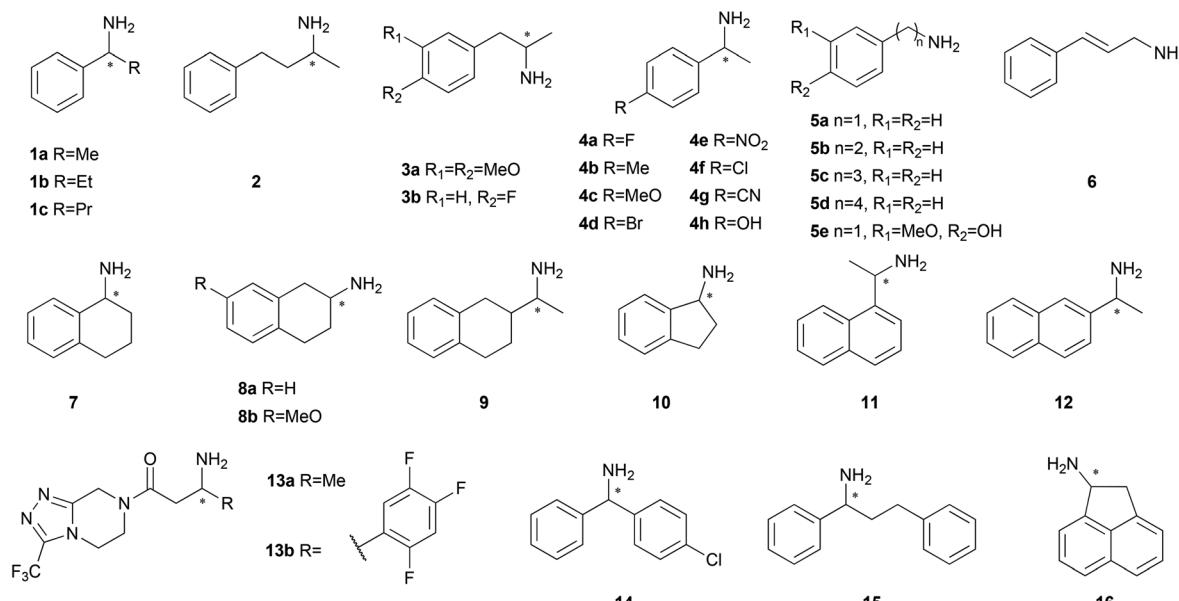
Taking the two β -TAs explored through microorganism screening as a template, *Caulobacter crescentus* TA⁵⁹ (65% identity to *A. denitrificans* TA) and *Polaromonas* sp. JS666 TA⁶⁰ (54%

identity to *Mesorhizobium* TA) were identified by homologous searching, exhibiting high activity towards β -amino acids.

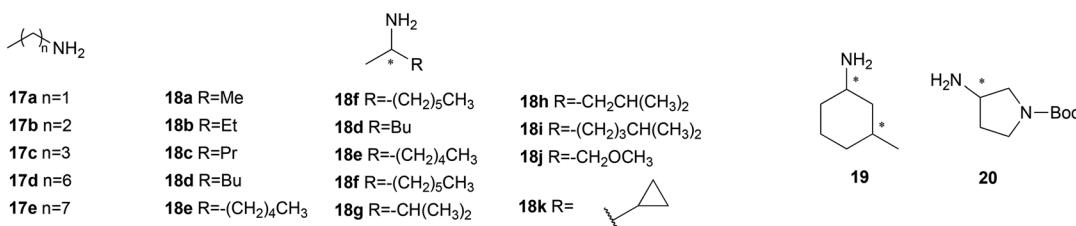
Nevertheless, the straightforward sequence alignment is only able to identify enzymes with similar specificities to the template enzymes. One severe issue of amine TAs has, until recently, been the fact that although *S*-selective enzymes have been largely explored and investigated, *R*-selective ones were barely reported. Bornscheuer *et al.*, at that time with no



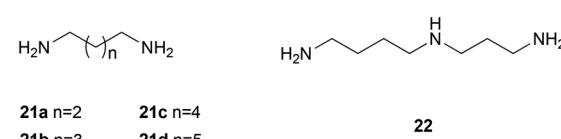
Aromatic amines



Aliphatic amines



Polyamines



Amino alcohols

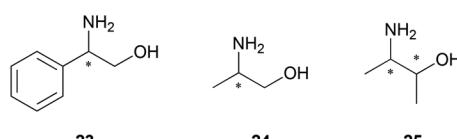
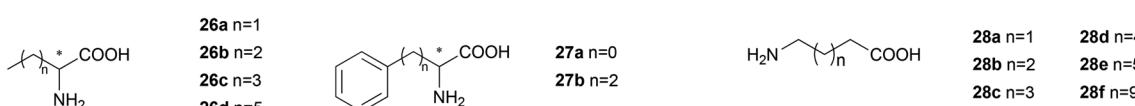
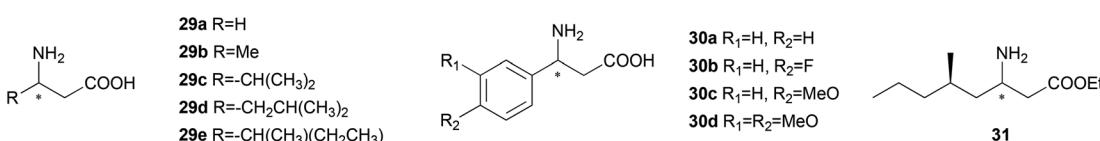
Non-protein α -amino acids β -Amino acids

Fig. 1 Amines as model substrates in gene mining and protein engineering.

R-selective amine TA sequence reported, developed a motif-based algorithm for enzyme function prediction and screening. PLP-dependent enzymes in fold class IV⁵⁶ was chosen as the protein database to be searched according to a hypo-

thesized evolution routine of R-selective amine TA. The sequences and structures of D-amino acid aminotransferases, L-branched chain amino acid aminotransferases and 4-amino-4-deoxychorismate lyase in this fold class were investigated

and a set of key motifs that are possible to use as markers for enzyme function and enantioselectivity were proposed. The algorithm was performed in the exclusion of template specificities and led to the subsequent identification of desired enzymes. As a result, seventeen *R*-selective TAs were identified by this strategy,¹⁴ seven of which were further characterized and proven to be efficient biocatalysts with excellent *R*-enantioselectivity.⁶¹ With a similar strategy, a novel *R*-selective TA was also identified from a fungal source, *Nectria haematoxocca*.⁶²

For a more complex motif analysis, a sequence set of proteins with certain functions is constructed with a seed enzyme in the protein family profile strategy. By this method, the genome of *Mesorhizobium loti* MAFF303099, a nitrogen fixation bacterium with more TAs than a normal strain, was analysed, from which 10 putative TAs were identified and characterized.⁶³

Multi-sequence alignment and functional motif analysis were also employed in the gene mining for TAs catalysing reactions with polyamines. Putrescine TA *yglG* from *Escherichia coli* K12 was firstly identified as a potential biocatalyst for terminal diamines (21), although it showed a strict preference for pyruvate over ketones.⁶⁴ Further alignments among amine TAs, gene cluster *yglG* and gene cluster *supC* (putrescine TA gene in *Pseudomonas* species) revealed a higher similarity between gene cluster *supC* and amine TA, indicating that putrescine TAs encoded by gene cluster *supC* show high potential in accepting both diamines and ketones as substrates.⁶⁵ Three *supC* genes were identified and characterized from *P. putida*, *P. chlororaphis* and *P. fluorescens* with obvious activity towards diamines (21a, 21b and 22) with ketones as amino acceptors.

Furthermore, although the strategy for bioinformation acquisition has not been reported, TAs from *Pseudomonas aeruginosa*,⁶⁶ *Arthrobacter citreus*,¹⁶ *Rhodobacter sphaeroides*⁶⁷ and *Burkholderia graminis*⁶⁸ were also cloned and applied in organic synthesis of amino compounds.

From structure to function. With the development of automated high throughput methods in crystallization and protein structure determination, a large number of uncharacterized structures of putative enzymes have been deposited in the protein database. Connecting these structures to their enzymatic functions is of significant importance. The crystal structures with unknown functions in the cluster of “ornithine-amine-otransferase (OAT)-like proteins” were investigated by Höhne *et al.* and four TAs from *Silicibacter pomeroyi*, *Rhodobacter sphaeroides* KD131, *Ruegeria* sp. TM1040 and *Mesorhizobium loti* MAFF303099 with a wide substrate spectrum and high specificity were identified.⁶⁹ Similarly, TA from *Bacillus anthracis* was also characterized based on the reported structure.⁷⁰ With the resolved structures, these enzymes can be easily involved in further engineering by structure-based protein design.

Protein engineering

Protein engineering, including directed evolution based on random mutagenesis and rational design based on sequence

and structure analysis, has been demonstrated extensively as a practical and efficient strategy for the optimization of biocatalysts.^{71,72} This strategy has also been employed in the improvement of TA. In this section, these studies will be discussed and their details are listed in Table 2 and the relevant compounds are shown in Fig. 1.

Random mutagenesis. Enzymes with specificities explored by gene mining as discussed above can be further improved or altered using various protein engineering approaches. One of these approaches is the error-prone based directed evolution method, which has been employed in the engineering of TAs. For instance, *V. fluvialis* TA was engineered into a mutant with 3-fold higher activity towards 3-amino-3-phenylpropionic acid (30a) although the mutated sites were not reported.⁷³ A follow-up investigation on the same enzyme resulted in a mutant (TAm1a (P233L, V297A)) with about 2-fold higher activities towards long chain aliphatic amines (18(e, f and j)).⁷⁴ In another example, the specific activity of *A. citreus* TA towards substituted *S*-aminotetralin (8) was improved from 5.9 to 1582.8 IU g⁻¹ by 6 rounds of random mutagenesis.¹⁶

Rational design: specificity for bulky substrates. To circumvent the large amount of screening work necessary in random mutagenesis, rational strategies based on the protein structure have been applied to TAs. Kim *et al.* firstly described the two binding pockets (large and small pockets) in the active site of *V. fluvialis* TA. In the large pocket, a residue (W57) with a bulky side chain was pointed out to be causing a steric hindrance against substrate binding.⁷⁵ Substitution with glycine resulted in a superb mutant, which exhibited up to 30-fold improvement of specificity towards a number of aromatic (1a, 2, 4(b, d and e), 5(a-d), 7, 8a and 10) and aliphatic (18(a, b, c, e, g and j)) amines. Engineering of the identified steric residues in the large pocket changed the substrate specificity from aromatic amines to aliphatic amines successfully. Guided by a recently resolved crystal structure of *V. fluvialis* TA (4E3Q.pdb), Bornscheuer *et al.* engineered the other substrate binding pocket, the small one, resulting in a TA mutant that is capable of mediating the transamination of α -hydroxyl ketones and aryl-alkyl ketones bearing an alkyl substituent larger than a methyl group.³⁶ Alleviation of steric hindrance in the small pocket by V153A improved the specificity by a factor of 4.2, 17.7 and 11 in the deamination of 1a, 1c and 23, respectively. Furthermore, the double mutant F85L/V153A exhibited a 26-fold improvement of specificity towards 1c while the double mutant Y150M/V153A was 53 times more active towards 23 than the wild type. The enhanced activity of these double mutants was also confirmed in asymmetric synthesis of 1c and 23, although with only up to 6-fold improvement.

Mutation of the residues in the small pocket was also investigated in the engineering of substrate specificity of other TAs. For instance, V153A in the small pocket of *P. denitrificans* TA was identified as a beneficial mutant for α -keto acid amino acceptors with a long linear alkyl side chain (26d for example).⁷⁶ The enzymatic activity in the transamination of 26d and 1a was improved 4 times, although the mutant did not exhibit enhanced specificities towards α -keto acids with



Table 2 Transaminases optimized by protein engineering

TA	Strategy ^a	Mutation	Result	Ref.
<i>V. fluvialis</i> TA	Random	—	3-Fold improvement of specificity towards 30a Decrease the inhibition from product of 2-butylamine (18b , 6-fold) and 2-aminoheptane (18e , 4-fold) 2-Fold higher activities towards long chain aliphatic amines (18e , f and j)	73 74
	Structure	W57G	Up to 30-fold improvement of activity for aromatic amines (1a , 2 , 4(b, d and e) , 5(a-d) , 7 , 8a and 10) and aliphatic amines (18(a, b, c, e, g and j))	75
		F85L, V153A Y150 M, V153A L56V	26-Fold improvement of specificity towards 1c 53-Fold improvement of specificity towards 23 Improvement of enantioselectivity towards 19 from 14% de (<i>1R,3S</i>) to 66% de (<i>1R,3S</i>)	36 85
		L56I	Reversion of enantioselectivity towards 19 from 14% de (<i>1R,3S</i>) to 70% de (<i>1S,3S</i>)	85
		M3 (W57F, R415L, L417 V)	3.4-Fold improvement of specificity of pentanal and loss of activity towards pyruvate	87
	Structure & sequence	r14 (F19 W, W57F, F85A, R88 K, V153A, K163F, I259 V, R415F)	60-Fold improvement of the specificity towards pre- 31 ketone	35
	Random	Mut6 (M46 T, D48G, Y60C, Y164F, Y185C, N186S, P195S, M197 T, C205Y, A242 V, A245 T, I252 V, F255I, N268S, T409R, K424E, C436A)	260-Fold improvement of activity towards substituted <i>S</i> -aminotetralin (8a)	16
	Structure	CNB05-01-V328A	Optimal temperature from 30 °C to 55 °C Reversion of enantioselectivity towards 3b from 98% ee (<i>S</i>) to 58% ee (<i>R</i>)	82
<i>C. crescentus</i> TA	Structure	N285A V227G	3-Fold improvement of specificity towards 30a 2-Fold improvement of specificity towards 30a	59
ATA-117 (<i>Arthrobacter</i> sp.)	Structure	Mut1a (S223P)	11-Fold improvement of activity towards pre- 13a ketone, but non-active for 13b	15
		Mut1b (adding V69G, F122I, A284G to Mut1a) Mut2 (adding H62 T, G136Y, E137I, V199I, A209L, T282S to Mut1b) Mut11 (adding S8P, Y60F, L61Y, G69 T, D81G, M94 T, I96L, I122 M, S124 T, S126 T, Y136F, I137E, Y150S, V152C, A169L, G215C, G217N, L269P, L273Y, P297S, S321P to Mut2)	Margin activity towards pre- 13b ketone 75-Fold improvement of activity to produce 13b compare to Mut1b Improvement of reaction conditions: substrate concentration 50 g l ⁻¹ , amino donor concentration 1 M, pH 8.5, temperature 45 °C and cosolvent DMSO concentration 50%	
<i>C. violaceum</i> TA	Structure	W60C	Improvement of enantioselectivity of 7 and 8a	83 and 84
	Sequence	A231F, F88A Y153 M W60Q F88R	29-Fold improvement of specificity towards 1a and about 5-fold improvement of specificity towards 4(b, c, e, g and h) Reversion of enantioselectivity of 7 and 8a	83 88
		V153A	4-Fold improvement of specificity towards 26d	76
	Structure	L57A	Up to 150-fold improvement of specificity towards aromatic amines (1(a-c) , 2 , 4(a-c) , 5a , 7 , 9 and 10)	77 and 78
<i>P. denitrificans</i> TA	Structure	W58L	Up to 5-fold improvement of specificity towards aliphatic amines (18(a-d, f-i and k)) Up to 48-fold improvement of specificity towards α -keto acids of corresponding amino acids (26(a-d)) At least 86-fold improvement of specificity towards aromatic ketones from corresponding amines (1(a-c) , 2 , 4(a-c) , 7 , 9 and 10)	79
		Y59W T231A	At least 40-fold improvement of specificity towards aliphatic ketones from corresponding amines (18(c, d, f, g, h and k))	
	Structure	Y59 W, Y87F, Y152F, T231A, P423H	22-Fold improvement of specificity towards 1a 4.3-Fold improvement of specificity towards 1a Up to 8900-fold improvement of specificity towards bulky amines 14 , 15 and 16	81 80
Ruegeria TA	Structure	Y59W T231A		

^a Protein engineering strategies. Random: random mutagenesis based directed evolution; structure: structure analysis based rational design; sequence: multi-sequence alignment based rational design.



shorter side chains (**26(a-c)**) that were readily accepted by the wild type. In another example, the structure of a β -TA from *C. crescentus* was investigated and the two-pocket model was also demonstrated in its active site.⁵⁹ To broaden its substrate specificity towards aromatic β -amines with a bulky phenyl ring supposed to bind in the small pocket, two residues were mutated into smaller ones to create a larger space in this pocket. As a result, the mutants N285A and V227G improved the specificity towards **30a** by 3- and 2-fold, respectively. Recently, Shin *et al.* investigated an amine TA from *O. anthropi* and expanded its substrate specificity to bulky substrates by engineering the residues in both the small and the large pocket. L57 has been identified as a residue which exhibits steric interference with the substrate substituent bound in the small pocket.^{58,59} An alanine mutation at this site removed this hindrance and resulted in a mutant with improved specificity towards a large amount of bulky substrates, including up to 150-fold improvement for aromatic amines (**1(a-c)**, **2**, **4(a-c)**, **5a**, **7**, **9** and **10**),⁷⁷ up to 5-fold improvement for aliphatic amines (**18(a-d, f-i and k)**)⁷⁷ and up to 48-fold improvement for α -keto acids of the corresponding amino acids (**26(a-d)**).⁷⁸ Besides, W58 in the large pocket was also mutated into a leucine with a smaller volume in order to reduce the steric barrier.⁷⁹ W58L has been proven to be a superb biocatalyst for amination of bulky acetones from the corresponding amines (**1(a-c)**, **2**, **4(a-c)**, **7**, **9**, **10** and **18(c, d, f, g, h and k)**) with isopropylamine (**18a**) as the amino donor.

As shown above, structure-based protein engineering has been utilized successfully to expand the TA substrate spectrum to substrates with bulky side chains. Among these compounds, sitagliptin (**13b**) is the most bulky one with large groups at both sides of the amino group. Its corresponding ketone is totally inert to the wild type ATA-117.¹⁵ Nevertheless, by structure-based engineering of both substrate-binding pockets, the specificity towards prositagliptin ketone was established and improved significantly. Twelve residues in the large pocket were firstly mutated and screened resulting in a variant (Mut1a) with a single mutation S223P, which improved the activity of the simplified product analogue (**13a**) with only one bulky side chain binding into the large pocket, although no sitagliptin activity was detected. Then the small pocket was opened by mutagenesis at four sites to accommodate the large trifluorophenyl group, establishing the activity to give **13b**. A mutant (Mut1b) with three mutations along with S223P was determined as a TA with marginal, but far from sufficient, activity. To enhance this activity, all the beneficial mutations from the two previous rounds of engineering were combined and tested. Finally, Mut2 with 6 added mutations was obtained with a 75-fold improvement of the activity to produce **13b**. In this research, the substrate binding pocket of ATA-117 was successfully opened up by structure-based protein engineering for a bulky substrate which was not accepted at all by the wild type enzyme.

Recently, another successful engineered *S*-selective TA for bulky substrates was also reported.⁸⁰ *Ruegeria* TA was chosen as the initial scaffold for engineering due to its potential

evolvability, which was based on a previous investigation, in which single-point mutations caused an improvement of the specificity towards **1a** (22-fold improvement by Y59W and 4.3-fold improvement by T231A).⁸¹ By combining these two sites with three more sites revealed from a structural analysis of the intermediate model, the mutant Y59W/Y87F/Y152F/T231A/P423H was constructed and determined to be a superb biocatalyst with up to 8900-fold improvement of specificity towards bulky amines (**14-16**). This motif of mutations was also validated in other protein scaffolds from *in silico* screening, resulting in more candidates for the biocatalysis of bulky amines.

Rational design: enantioselectivity. Aside from the specificity towards bulky substrates, enantioselectivity is one of the properties of major interest, which is possible to improve by protein engineering. Guided by molecular modelling, our group previously analysed the structure of *A. citreus* TA and identified key residues in the “phosphate-binding cup” which could have effects on the enzyme stereoselectivity in the production of 1-(4-fluorophenyl)propan-2-amine (**3b**).⁸² One mutation, V328A, was proposed to be more beneficial for the *R*-configuration of **3b** and was determined to reverse the stereoselectivity from 98% ee (*S*) of the parent variant CNB05-01 to 58% ee (*R*), which was of great significance due to the less commonly explored *R*-selective TAs compared to the *S*-selective ones at that time. In another example, W60 in the large substrate binding pocket of *C. violaceum* TA was selected as a mutation site to improve the enantioselectivity towards aminotetralin (**7** and **8a**)⁸³ enlightened by the previous protein engineering of *V. fluvialis* TA⁷⁵ and *A. citreus* TA.¹⁶ Cysteine substitution at this site was reported with both improved enantioselectivity by approximately 10-fold for both amines (**7** and **8a**). Additionally, this mutant was also found to be more active in the transamination of **1a** and 4'-substituted 1-phenylethylamines (**4(b, c, e, g and h)**).^{83,84} Meanwhile, a mutant (F88A/A231F) of *C. violaceum* TA with reversed enantioselectivity towards **7** as well as **8a** was obtained by the exchange of phenylalanine and alanine between the small and large pockets due to their determination of the substrate-binding conformations of the two enantiomers.⁸³ Recently, another investigation of the enantioselectivity of *V. fluvialis* TA towards cyclic ketones was also reported.⁸⁵ By protein–substrate complex structure analysis, L56 was identified as a key residue due to the vicinal position of its side chain to all the four amine (**19**) diastereomers. Subsequently, complementary enantioselectivity was obtained by two subtle residue substitutions, L56V with 66% de for (*1R,3S*)-**19** and L56I with 70% de for (*1S,3R*)-**19**. These mutants were applied in cascade reactions accompanied by enoate reductase to synthesize optically pure **19**.⁸⁵

Rational design: substrate promiscuity. Promiscuity⁸⁶ is another enzymatic property amendable by protein engineering. Unlike the specificity of bulky substrate and the enantioselectivity, reshaping and expanding the binding pockets may not be sufficient to switch the enzyme between different functions. Instead, the mutation of residues interacting with sub-

strate functional moiety may affect the results to a greater extent. Bornscheuer *et al.* reported an attempt to convert *V. fluvialis* TA from an amine:α-keto acid transferase into an amine:aldehyde transferase; the latter is more favourable in the organic synthesis.⁸⁷ W57, R415 and L417 in the large pocket were identified as the key residues that may distinguish between keto acids and aldehydes, especially the flipping arginine with both steric and electrostatic effects on the substrates. Substitution of R415 with hydrophobic phenylalanine together with a reduced volume of the other two residues (W57F and L417 V) resulted in a mutant with a 3.4-fold improvement of specificity of pentanal and loss of activity towards pyruvate. In another engineering study of *V. fluvialis* TA, a more complex variant with 8 mutations was established from combined approaches and exhibited a 60-fold improved amino acceptor specificity towards pre-31 ketone compared to pyruvate.³⁵ Mutations that have been reported before such as R415F and W57F have also been proven to be beneficial in this study of the amino acceptor specificity. Like the amino acceptor, the specificity for the amino donor can also be altered by protein engineering methods. Based on a hypothesized gradual progression of protein evolution from α-TA to amine TA, the sequence alignment and positional frequency analysis indicated three key residues that may affect the evolution pathway. Saturation mutagenesis of these sites resulted in several positive mutants that exhibited improved serine-pyruvate amino-transferase activity with the *C. violaceum* TA scaffold.⁸⁸

High throughput screening

Identifying the positive enzymes or variants in libraries from gene mining or protein engineering is a crucial step and the analytical methods used determine the efficiency of the whole process. The conventional analytical methods including GC, HPLC or MS are disadvantageous with low throughput, consequently consuming time and labour resources. Therefore, novel high throughput screening methods are of great interest to accelerate the identification of optimized proteins. A number of strategies have been developed and reported for transaminases, including enrichment cultivation, detection of the common substrates and employment of smart substrates. In this section, these approaches and their characteristics will be discussed. Among them, the screening assays based on common and smart substrates are summarized in Table 3 and Scheme 2 as **AI–AVII** and **BI–BIV**.

Enrichment cultivation. Enrichment cultivation can be employed as a high throughput approach to identify TAs and TA mutants corresponding to the biomass growth as described in the previous section.^{13,74} The specificity for different amines, the enantioselectivity and the product inhibition are all detectable by this method. Moreover, this strategy is advantageous since no additional requirement for equipment and reagent exists. Solid screening can be conducted by simply observing the colonies on the cultivation plates by the naked eye. However, the enzymatic activity and the biomass (mainly the diameter of the colony) of the strains are not correlated linearly, which limits the quantitative application of this strategy.

Common substrate-based assays. In most TA-mediated transaminations, only one of the two substrates (amino donor and amino acceptor) is the target compound, whereas the other one is the co-substrate. Some compounds have been commonly employed as a co-substrate, for instance, alanine with its corresponding keto acid pyruvate as well as 1-phenylethylamine (**1a**) with its corresponding ketone acetophenone. Detection of these common co-substrates or their corresponding co-products by various approaches is available to facilitate the screening of a variety of TA activities in a general and high throughput fashion.

As the natural substrates of TA, α-amino acids and the corresponding keto acids are the most commonly employed amino donors and acceptors. Especially in kinetic resolution of racemic chiral amines, using α-keto acid as the amino acceptor is advantageous due to the favourable reaction equilibrium towards the product side. Kim *et al.* reported a colorimetric high throughput screening method taking advantage of the blue colour generated from amino acids with CuSO₄ and MeOH, which could be detected at 595 nm (Method **AI** in Table 3 and Scheme 2).⁷³ The absorbance is linearly related to the concentration of amino acid generated in the reaction in 0.1 mM–20 mM with a sensitivity of 10 M⁻¹ cm⁻¹. The main drawback of this strategy is the strong background colour from other amino acids from the cell extract. Moreover, the chromogenic reagent (CuSO₄ and MeOH) is detrimental to the enzymes, which limits the application of this strategy in a continuous fashion.

Another approach is a colorimetric detection of alanine production by a combination of amino acid oxidase (AAO) and horseradish peroxidase (HRP) in a cascade reaction system (Method **AI**).^{89–91} In this system, alanine is deaminated by amino acid oxidase to produce hydrogen peroxide (H₂O₂), which can be detected by using horseradish peroxidase and some colorimetric indicators. Moreover, glycine is also able to be detected by deamination of glycine oxidase and the following H₂O₂ detection.⁹² This strategy can be used as solid phase selection and the co-product was recycled, which is beneficial to the reaction equilibrium. However, multiple enzymes involved in the system may cause operational problems and H₂O₂ may be detrimental to proteins. Another disadvantage is that this strategy can only be employed as a qualitative method due to the nonlinear correlation between the enzyme activity and the colour intensity as well as the obvious false positive results.

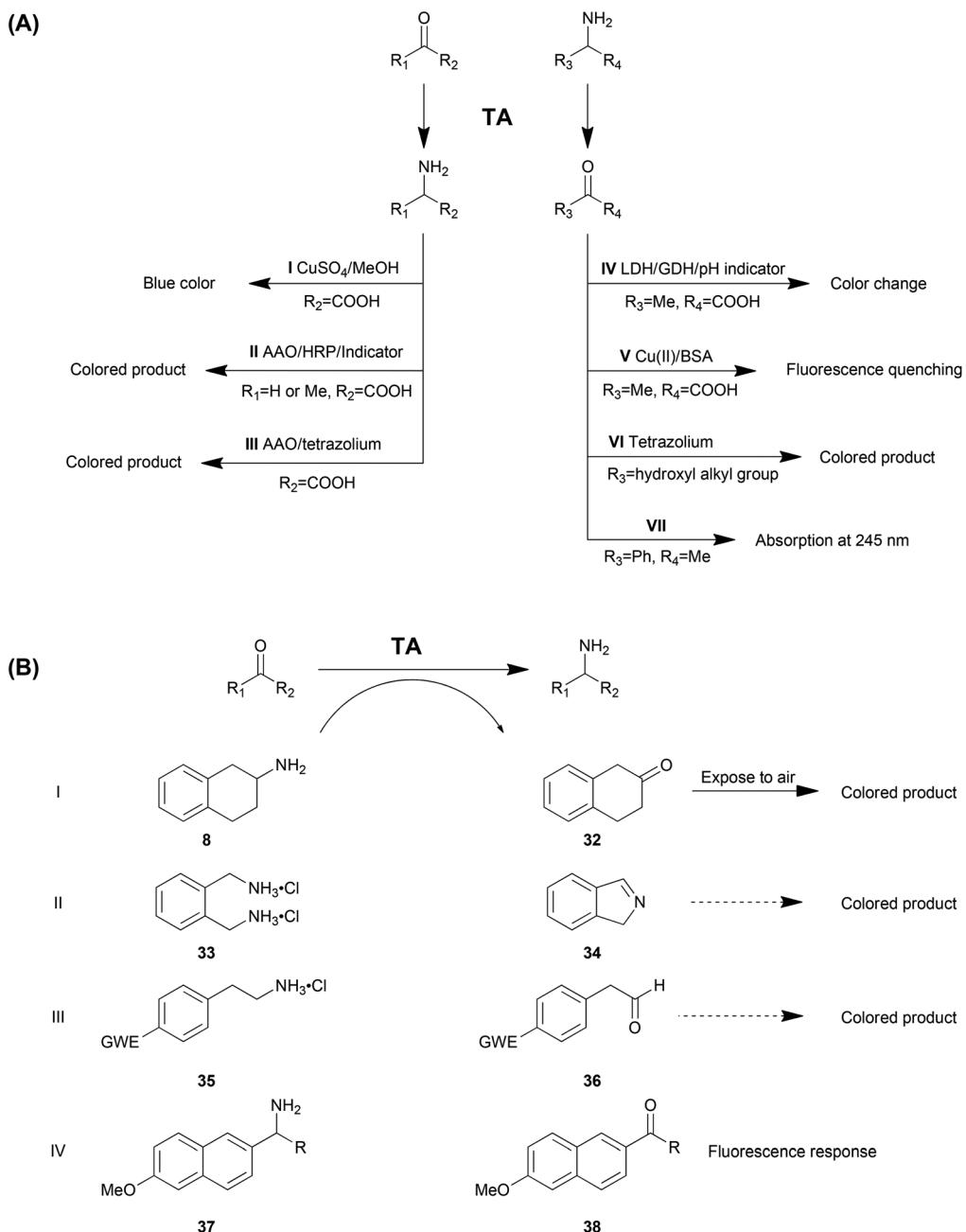
Similarly, another strategy based on AAO was employed for fast detection of TA activity (Method **AIII**).^{65,93} Produced in a TA-mediated reaction, alanine⁶⁵ or glutamate⁹³ can be oxidized by the specific oxidases AlaDH or GDH, resulting in a conversion from NAD⁺ to NADH. Simultaneously, tetrazolium salts in the system can be reduced into a coloured product, the amount of which was correlated to the concentration of NADH. The strategy with GDH was determined to be sensitive and linear with low glutamate concentrations (10–100 μM). Purified enzymes were required since amino acids and proteins in the cell extract resulted in a strong background colour.



**Table 3** High throughput screening assays for transaminase

	Substrate dependence	Additional reagent	Detection and equipment	Sensitivity	Background	Enantioselectivity	Continuous or end point	Liquid or solid	Ref.
AI	α -Amino acid	$\text{CuSO}_4/\text{MeOH}$	Blue colour	$\epsilon = 10 \text{ M}^{-1} \text{ cm}^{-1}$ linear in 0.1 mM–20 mM	Amino acids in cell extract	Y	E	L	73
AII	Alanine and glycine	Oxidase, HRP, colour indicators	Colour change	1% of initial activity, nonlinear	False positive ($<0.5 \text{ mU}$)	Y	C	Both	89–92
AIII	Alanine and glutamate	Oxidase, tetrazolium	Colour change	Linear in low product concentration (10–100 μM)	Amino acids in cell extract, PLP	Y	C	L	65 and 93
AV	Alanine	Oxidase, pH indicator	Colour change	n.r.	From pH disturbing substance	Y	C	L	94 and 95
AV	Complex of $\text{Cu}(\text{II})$ –Ala instead of alanine	$\text{Cu}(\text{II})$ and BSA	Fluorescence quenching with a fluorescence spectrometer	Linear, high sensitivity, LOD 3 U L^{-1} , 5–400 U L^{-1}	From $\text{Cu}(\text{II})$ –Ala complex degradation	N	C	L	96
AVI	2-Hydroxy ketone	Tetrazolium	Colour change	Linear in low activity, dependent on substrates	Amino acids in cell extract	Y	E	L	97 and 98
AVII	1-Phenylethylamine (1-PEA, 1a)	—	Absorption at 245 nm with a UV spectrometer	$\epsilon = 12 \text{ mM}^{-1} \text{ cm}^{-1}$	From protein	Y	C	L	67 and 99
BI	Substituted aminotetraline	—	Coloured product exposed to air	n.r.	n.r.	Y	C	L	16
BII	<i>ortho</i> -Xylylenediamine	—	Dark colour	High, nonlinear	Strong	N	C	Both	100
BIII	EWG-substituted aromatic amines	—	Coloured product	High quantitative LOD 1.2–30%	Low	N	C	Both	101
BIV	1-(6-Methoxynaphth-2-yl) alkylamines	—	Fluorescence at 450 nm with a fluorescence spectrometer	High LOD 0.35 nmol min^{-1} LOQ 1.2 nmol min^{-1}	Low	Y	C	L	102

n.r., not reported.



Scheme 2 High throughput screening strategies of TA.

Moreover, PLP for the transamination was determined to be detrimental to the GDH activity.

In asymmetric synthesis of chiral amines, alanine is one of the most commonly used amino donors. Therefore, the efficient detection of the amino donor product pyruvate is of great interest. A pH change-based method has been developed where the pyruvate produced is reduced by lactate dehydrogenase (LDH) accompanied by the oxidation of glucose to gluconic acid by glucose dehydrogenase (GDH), which causes a colour change of the pH indicator (Method AIV).^{94,95} The results of this method could be

affected by other pH disturbing substances in the reaction system.

Recently, a method for detecting transamination from alanine to pyruvate was developed based on fluorescence quenching of bovine serum albumin (BSA) in a reaction system containing the metal coordinate complex Cu(II)-Ala (Method AV).⁹⁶ Coordination of Cu(II) with L-alanine will be destroyed by conversion of L-alanine into pyruvate. Then the free Cu(II) ion will combine with BSA and cause the detectable fluorescence quenching. This method is highly sensitive with a limit of detection (LOD) of 3 U L⁻¹ and linear in 5-400 U L⁻¹.

However, it also suffers from drawbacks such as the background from the degradation of the Cu(II)-Ala complex and the requirement of additional equipment for fluorescence detection.

Tetrazolium salts have been demonstrated to be efficient reagents in the detection of 2-hydroxyl ketone (Method **AVI**). Colourless 2,3,5-triphenyltetrazolium chloride (TTC) can be reduced into a red-coloured formazan precipitate in the presence of 2-hydroxyl ketone, which has been employed as a strategy for detection of the TA activity for amino alcohols.⁹⁷ Recently, the application of a newer generation of tetrazolium reagents (WST-1) was proposed to improve the sensitivity of the alkaline 2-hydroxy ketone assay by 2 orders of magnitude, with the linear range changed from 1–10 mM (TTC) to 1–100 µM (WST-1).⁹⁸ Moreover, the coloured formazan product remains fully soluble, with increased dynamic range and reproducibility.

Another commonly used amino donor is 1-PEA (**1a**), used as a model structure of aromatic amines and a highly active amino donor in transaminations. A high throughput screening strategy was developed for the TA reaction of this compound due to the UV absorbance (245 nm) of its corresponding product acetophenone (Method **AVII**).⁶⁷ The significant ($\epsilon = 12 \text{ mM}^{-1} \text{ cm}^{-1}$) and exclusive absorbance (other components in the reaction system do not show a comparable absorbance at 245 nm) promotes this strategy to be an efficient high throughput screening method for quantitative analysis.⁹⁹ Moreover, this approach has other advantages including no need for additional reagents, the capability to determine the enantioselectivity and the capability to work in a continuous fashion. The main drawback aside from the substrate dependence is the requirement for a UV-detection spectrophotometer.

Smart substrate-based assays. Besides the common co-substrates discussed above, a number of smart compounds have been developed and employed for the high throughput screening of TA-mediated reactions. All the substrates discussed in this section are amino donors, which are called smart since their products have detectable properties, such as colour change or fluorescence absorbance. The main advantage of all the high throughput screening strategies based on smart substrates is the circumvention of additional reagents. The capability of these strategies to determine enantioselectivity depends on the chirality of the smart substrate.

Martin and co-workers reported the first example in a directed evolution study of TA for activity and thermostability. Substituted aminotetraline (**8**) gives the product ketone **32**, which turns into a coloured product after exposure to air (Method **BI**).¹⁶ In another report, *ortho*-xylylenediamine (**33**) was employed as the smart amino donor. After its deamination spontaneous polymerization of the isoindole by-product (**34**) generates dark-coloured derivatives (Method **BI**).¹⁰⁰ This strategy is sensitive but nonlinear, with a strong background colour observed. However, the advantage of the method is the capability to be applied in solid screening. Similarly, another smart amino donor 2-(4-nitrophenyl)ethan-1-amine (**35**) and several

other 4-nitroaryl amines were used in a HTS method for both solid and liquid screening (Method **BIII**). The 4-nitroaryl electron withdrawing group (EWG) in the smart co-product derivatives enhances its tendency to form an enamine which showed different colours.¹⁰¹ The variation of smart co-substrates provided a wider generality of this method. Moreover, the sensitivity was reported to be high with a LOD of 1.2–30% of the initial enzyme activity. This method is also applicable in both liquid and solid screening like Method **BIII**, although with the background colour much lower than the previous one.

Recently, our group in collaboration with Fessner developed a fluorescence-based method involving 1-(6-methoxynaphth-2-yl)alkylamines (**37**) as the amino donor (Method **BIV**). The produced acetonaphthone (**38**) can be monitored using its bright fluorescence at 450 nm with very high sensitivity (LOD 0.35 nmol min⁻¹) and selectivity (weak background response).¹⁰² The limitations are obviously the substrate dependence and the equipment requirement.

Constructing stable biocatalysts

In organic synthesis, TAs are practical and economical biocatalysts only if their stability under non-physiological conditions can be improved to a sufficient level. The stability includes enzyme operational stability, such as thermostability, organic solvent tolerance, chemical tolerance, and storage stability. A number of strategies have been employed to enhance the stability of TAs including gene mining, protein engineering, biocatalyst formulation/immobilization and medium engineering. In this section, these methods and their applications will be discussed.

Gene mining

Thermophilic strains provide a natural library of proteins with high thermostability due to their unique living environments. Therefore, gene mining of these microorganisms will result in thermostable proteins with optimal temperature over 60 °C. For example, an *S*-selective TA from *Sphaerobacter thermophilus* was identified by homologous sequence searching with *Polaromonas* TA as the template (Table 1).¹⁸ The optimal temperature of this TA was determined to be 60 °C, and after 1 h of incubation at this temperature, no loss of activity was observed. Similarly, TAs from the thermophilic *Geobacillus thermodenitrificans* was also identified as thermostable biocatalysts with an optimal temperature determined to be 65 °C (Table 1).¹⁰³ Moreover, an increase of activity was observed after the incubation in high temperature: a 2.5-fold increase after 8 h in 50 °C or 30 min in 60 °C, which was ascribed to temperature-related folding improving the enzyme stability.

Another microorganism which grows in special environments is the halophilic strain, consequently containing a library of halophilic proteins. These proteins are highly tolerant of high salt concentration and have potential to tolerate high substrate concentration. Recently, a TA was identified from *Halomonas elongate*, a moderate halophilic bacterium,

with a sequence identity of 38% to *V. fluvialis* TA and 55% to *C. violaceum* TA (Table 1).¹⁰⁴ The stability of this enzyme was not decreased on incubation in a high concentration of salt (KCl and NaCl) buffer (concentration from 1 M to 3 M) for 24 h, although the reactivity was repressed to some extent. More interestingly, the maintained stabilities were also observed in the presence of organic co-solvents (MeOH, DMSO, EtOH and *t*BuOH) up to 20%. With these stabilities, *H. elongate* TA was reported as a promising candidate for organic synthesis.

Protein engineering

Protein engineering has also been employed to improve the stability of TA. For example, by error-prone PCR-based directed evolution, Martin *et al.* engineered a mesophilic *A. citreus* TA into a thermostable mutant with 17 mutations after 5 rounds of screening (Table 2).¹⁶ The best mutant exhibited good activity at an optimal temperature of 55 °C, much higher than that of the wild type protein (30 °C).

A similar strategy has been employed in the protein engineering of TA for the production of sitagliptin (Table 2).¹⁵ To generate a practical and economical biocatalyst for industrial application, the TA-mediated reaction conditions were optimized, including substrate concentration, pH, temperature and organic co-solvent concentration. After 11 rounds of evolution, the optimized biocatalyst showed remarkable activity in 50 g l⁻¹ pro-sitagliptin ketone, 1 M amino donor IPA, pH 8.5, 45 °C and 50% co-solvent DMSO. This extraordinary success set up a milestone for protein engineering of TA.

Aside from the conventional protein engineering strategy, non-canonical amino acid substitution has also been demonstrated as an efficient method for stability improvement of TA. Global substitution by fluorotyrosine (FY) was conducted to *V. fluvialis* TA, resulting in a superior mutant TA[FY] with higher thermostability and more tolerance towards DMSO.¹⁰⁵ The half-life at 50 °C was prolonged from 3 h (WT) to 7 h (TA[FY]); the half-life at 50 °C with 20% DMSO was also prolonged from 7 h (WT) to 48 h (TA[FY]). This significant improvement of both thermostability and organic solvent tolerance was ascribed to the stronger interactions between two protein

monomers provided by the fluorotyrosine on the dimer interface.

Biocatalyst formulation

One enzyme in different biocatalyst formulations may exhibit diverse performance in the same reaction. Common biocatalyst formulations are summarized in Table 4 and compared in the biocatalyst production process, specificity to the target reaction, biocatalyst activity, stability, cost and application area. Purified enzyme is the most specific, which is required in most biochemical research projects. However, the production process and cost prevent it from large-scale usage. A crude cell extract may show comparable specificity and higher activity than purified enzyme only if the target protein is over-expressed in a sufficient amount. This formulation is the most widely used for its easy production and low cost. Nevertheless, neither purified enzyme nor crude cell extract is a stable formulation for long-time processing and storage. In this case, other biocatalyst formulations are needed.

For the application of TAs in organic solvents, lyophilization is an option that is commonly employed due to the easy production, superior organic solvent tolerance as well as long possible storage time.^{54,106,107} The process of lyophilization basically includes rapid freezing and water removal, which may maintain the protein conformation while preventing its degradation in the solvent.

Immobilization is the most widely used strategy for practical and economical biocatalysts in industry.^{108–110} Although additional immobilization reactions are required and in some methods the enzyme activity may be damaged to some extent, immobilized enzyme is still favoured due to its high operational and storage stability. In most cases, immobilized enzyme can be recycled by some physical separation from the reaction mixture, which largely reduces the cost of the biocatalyst. For instance, TA-117 was immobilized in a sol-gel/celite matrix resulting in the optimal temperature rising from 30 °C to 40–50 °C and almost no loss of activity after 5 reaction cycles.¹¹¹ In another example, cellulose-immobilized *V. fluvialis* TA showed a broad temperature range of 30–50 °C and maintained its activity after 4 cycles.¹¹² Besides, TAs have

Table 4 Biocatalyst formulations

	Production	Specificity	Activity	Stability	Cost	Application
Purified enzyme	+++	+++	++	+	+++	Biochemical
Crude cell extract	+	++/+ ^a	+++	+	+	Biochemical
Lyophilized enzyme	++	+++/++ ^b	++	++	++	Chemical
Immobilized enzyme	++	+++/++ ^b	+ ^c	+++	+ ^d	Industrial
Whole-cell biocatalyst	+	++/+ ^a	+++	++	+	Chemical
						Industrial

^a Specificity of this formulation depends on whether the favourable protein is over-expressed or not. ^b Specificity of this formulation depends on whether the enzyme is purified or not. ^c Some method of immobilization is detrimental for the enzyme activity. ^d The cost will be largely reduced if the enzyme could be well-recycled.



also been immobilized on other materials including resin,¹⁷ chitosan,¹¹³ Fe_3O_4 ¹¹⁴ and glass beads,¹¹⁵ resulting in stable and recyclable biocatalysts.

Furthermore, the use of whole-cell biocatalysts may also improve TA stability. For instance, the chemical tolerance of *V. fluvialis* TA was remarkably enhanced by this strategy. Used as crude cell extract, *V. fluvialis* TA was inactivated thoroughly by 80 mM of the substrate 2-aminoheptane, whereas the whole cell biocatalysts tolerated up to 200 mM of the same substrate.⁷⁴ Due to the short production process (fermentation only), this formulation is also favourable in industrial applications. However, the separation issue that is caused by the cell lysis in the reaction medium limits its application in organic synthesis.

Medium engineering

Due to its significant effects on protein structures, the reaction or storage medium is crucial for the biocatalyst stability. Organic solvents are generally regarded as detrimental for proteins; however some solvents have been reported to show positive effects on maintaining the enzyme structure. For instance, 20% DMSO prolonged the half-life of *V. fluvialis* TA at 50 °C from 3 h to 7 h and the half-life of the mutant TA[FY] even longer (from 7 h to 48 h).¹⁰⁵ In another example, addition of 20–50% glycerol, 10–20% methanol or 10–50% DMSO in the storage buffer improved the storage stability of *C. violaceum* TA.¹⁰⁶

Aside from the solvents, additives may also affect protein stability. As the cofactor of TAs, PLP is regarded as an efficient stabilizer for TA. The addition of 0.1 mM PLP improved the residual activity of *M. vanbaalenii* TA at 60 °C from 33% to 87%.¹¹⁶ Aside from the thermostability, the tolerance of chemicals may also be enhanced by adding PLP. With 80 mM of the substrate 2-aminoheptane, *V. fluvialis* TA lost all of its activity. However, only 5% of the activity was lost in the presence of 1 mM PLP.⁷⁴ The stabilization of TA may be ascribed to the important role of PLP in the dimerization of TA.¹⁰⁶ Release of PLP may accelerate the dissociation of the TA dimer and consequently destabilize the enzyme.

Shifting the reaction equilibrium

As reversible reactions, TA-mediated transaminations suffer from equilibrium issues. Especially in some asymmetric amine syntheses, the equilibrium can be far on the side of the substrates. To optimize TA performance in organic synthesis, a number of physical and chemical strategies have been developed and described to displace the reaction equilibrium to the product side,^{10,20–22} which will be discussed in this section (Scheme 3).

Excess of co-substrate

One of the conventional strategies is providing an excess amount of co-substrate (Scheme 3, Method I). Nearly 50-fold excess of the amino donor isopropylamine (1 M) was applied

in the transamination of acetophenone (20 mM) to 1-PEA (1a) in order to push transamination reactions towards complete conversion.⁹⁴ This strategy is straightforward and easy to be conducted albeit its applicability is limited by the initial equilibrium of the reaction, the substrate solubility and the enzyme inhibition.

Removal of product or co-product

Removal of the products is another straightforward method to shift the equilibrium of reversible reactions. Multiple strategies are available to remove the products, including physical vaporization/extraction methods and chemical reactions.

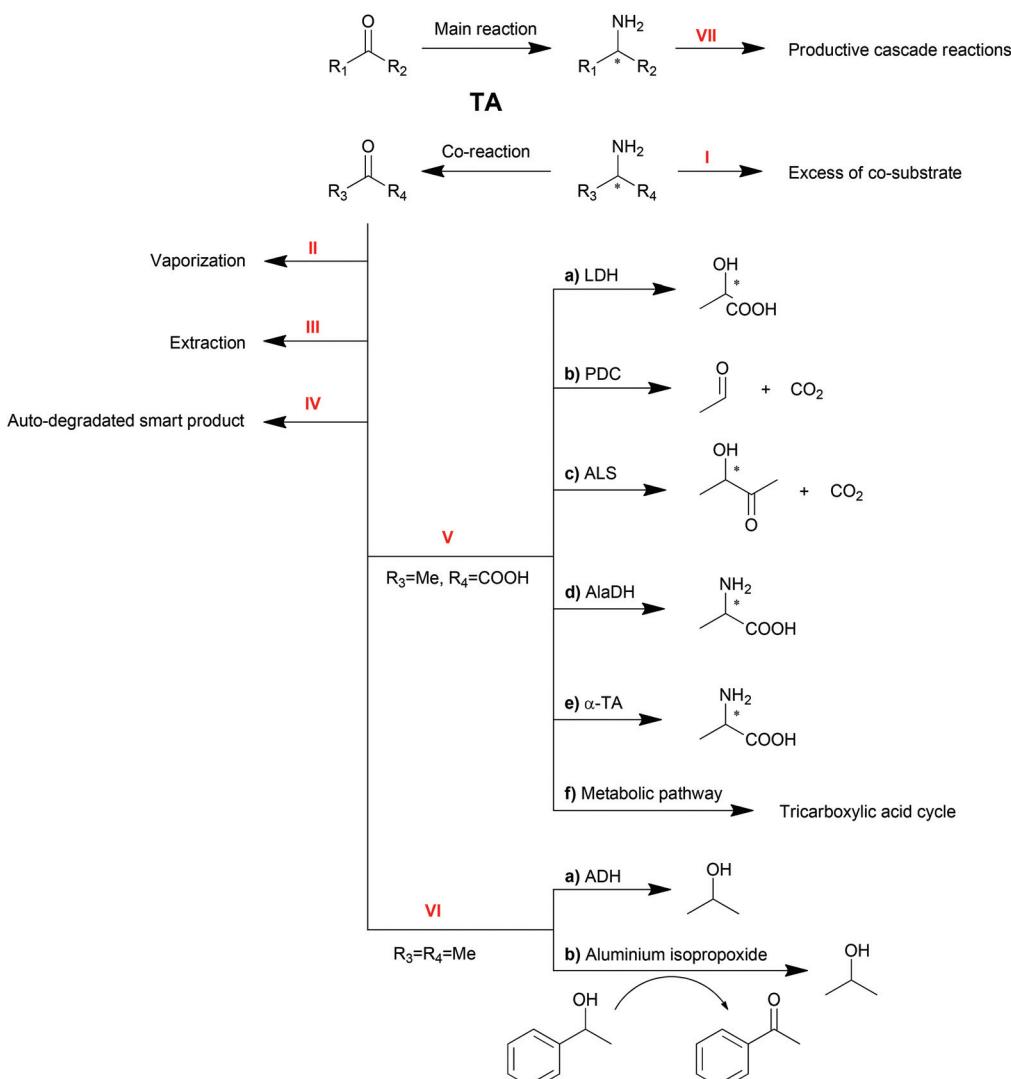
Physical separation. Ketone products are detrimental in transamination because of not only impeding the reaction equilibrium but also inhibiting the enzymes. As a consequence, isopropylamine has been regarded as an advantageous amino donor since its product acetone is volatile and easy to be removed.¹¹⁷ Reduced pressure or nitrogen sweeping at the regular temperature has been proven to be sufficient for the removal of acetone (Method II).^{15,118} Although this strategy has been utilized in a number of cases, its rationalization has not been discussed until recently.¹¹⁹ The equilibrium constant K_{eq} for the reaction and volatility of the ketones was identified as the critical parameters for the performance of the physical removal of ketones.

Because of the different polarities of amines and ketones, the extraction of ketone product by organic solvent is another option for product removal (Method III). Bi-phasic reaction systems, either liquid–solid^{120,121} or liquid–liquid,^{34,122} have been proven to be practical for ketone product extraction. Nevertheless, the application of this strategy is limited due to drawbacks including the poor distinction between substrate and product or the destabilization of proteins by organic solvents.

For the elimination of the amine product which is hydrophilic, a tri-phasic system has been developed consisting of an organic solvent bridge between two aqueous phases: an alkaline reaction phase and an acidic extraction phase.¹²³ With isooctane as the organic solvent, the inhibitive product 1-PEA (1a) moved from the reaction phase (pH = 8.0) to the extraction phase (pH = 3.0) leaving alanine and pyruvate in the reaction phase due to their ionization state. To further improve this tri-phasic system, a supported liquid membrane (SLM) contactor with a packed bed reactor was recently proposed in the production of 1-PEA (1a) with a yield of 98% compared to 50% with no product extraction.¹²⁴ This system avoids the contact of the biocatalyst with the organic solvent and has promising potential for continuous operation. Moreover, pH control and SLM regeneration can also be employed to optimize this system.¹²⁵ Besides, alanine was proposed as a better amino donor in this system because of its non-extractability.¹²⁶

Auto-degrading smart co-products. Chemical degradation of co-products is another option to shift the unfavourable equilibrium of transamination. Firstly, smart co-products which spontaneously transform have been employed (Method IV). For example, the deamination product of lysine can cyclize





Scheme 3 Strategies for TA reaction equilibrium shifting.

into 3,4,5,6-tetrahydropyridine-2-carboxylic acid, commonly known as Δ^1 -piperideine 2-carboxylic acid, spontaneously. Employing lysine as the amino donor, L-homophenylalanine was synthesized with 97% yield and more than 99.9% ee.¹²⁷ Similarly, spontaneously cyclizing into cyclic imines, smart co-products from diamines may also be employed to shift the equilibrium.^{51,64,65,100,128}

Furthermore, spontaneous tautomerization of an unstable co-product was utilized when a transamination system with 3-aminocyclohexa-1,5-dienecarboxylic acid as the amino donor was developed by our group.¹²⁹ The unstable ketone product from the amino donor spontaneously turned into its stable isomer 3-hydroxybenzoic acid, pushing the equilibrium to the product side with a theoretical yield of 100%.

Biochemical cascade reactions. In asymmetric synthesis of chiral amines, alanine is commonly employed as the amino donor, which transfers its amino group to the amino acceptor. However, it is much easier for pyruvate to receive the amino

group than for alanine to release it. Consequently, in most cases, the reaction equilibrium is on the side of alanine, which is unfavourable for chiral amine production. Therefore, the removal of pyruvate and even recycling it back into alanine is of great interest in the process engineering of these asymmetric syntheses. A number of enzymes degrading pyruvate or transforming pyruvate into alanine have been studied, including lactate dehydrogenase (LDH),^{12,48,60,61,94,130–132} pyruvate decarboxylase (PDC),¹³³ acetolactate synthase (ALS)¹²³ and alanine dehydrogenase (AlaDH).^{61,131,132,134–136} Accompanied by cofactor recycling by formate dehydrogenase (FDH) or glucose dehydrogenase (GDH), these strategies can be efficient and economical. Most of these strategies have been reviewed and discussed extensively.^{10,22} However, some recent updates will be discussed here.

A recent update of an AlaDH involved process is the combination with an alanine racemase.¹³⁷ For R-selective TA-mediated transaminations, the more expensive D-alanine is



Table 5 Common amino donor and acceptor pairs

Substrate pair	Chirality	Deamination	Model substrate	Inhibition	Detection	Elimination
Alanine	Chiral	Hard	α -Amino acid	—	—	Cascade reactions
Pyruvate	—	—	—	—	—	—
IPA	Achiral	Easy	—	—	—	Vaporization
Acetone	—	—	—	Severe	—	Cascade reactions
1-PEA	Chiral	Very easy	Aromatic amine	Observed	—	Extraction
Acetophenone	—	—	—	Severe	UV	Cascade reactions

required. Combining AlaDH and alanine racemase, the produced pyruvate was transformed firstly into *L*-alanine and then racemized. Therefore, the readily available *L*-alanine can be used as an amino donor, which significantly reduced the cost.

Due to its role in the carbon metabolic pathway, pyruvate can be removed by the innate pathway, acting as a strategy for shifting transamination equilibria in whole cell biocatalysts.¹³⁸ The production of *S*-sec-butylamine by *O. anthropi* TA was improved by carbon-starved cultivation without glucose. The cells were instead taking the produced pyruvate as the carbon source. However, given the complexity of the cell metabolic pathway, a practical application of this strategy is yet to be developed.

Another amino donor product, acetone, can also be removed by cascade reactions (Method VI). Alcohol dehydrogenase (ADH) can be one option to convert ketones or aldehydes into their corresponding alcohols, which will not be substrates for TAs.¹³⁹ Recently, a chemical catalyst mediated Oppenauer oxidation was introduced into the transamination system, which converted the ketone product back to the amino acceptor.¹⁴⁰ In the presence of aluminium isopropoxide, the catalyst of the Oppenauer oxidation, and 1-phenylethanol, the produced acetone is able to be transformed into isopropanol and concomitantly generate acetophenone, the substrate of the transamination. Both reactors are interconnected through a semi-permeable membrane, where multicomponent intra-membrane transport takes place by diffusion and viscous flow.

It is worth noting that the productive linear cascade reactions of the major product (Method VII) are also able to shift the equilibrium, which will be discussed in the next sections.

Substrate engineering

Despite a number of successful cases reported, the applicability of using an excess of substrate or removal of product has been restricted by the intrinsic equilibrium constant of the transaminase reactions. This constant depends on the physical conditions, initial component concentrations as well as the substrates pair: the amino donor and acceptor.¹⁹ In the reaction of α -TA, the amino acids and keto acids have almost the same ability for deamination and amination, which results in equilibrium constants approximately equal to 1. However, the situation is significantly different in the reaction of amine TA. For instance, the amino group transfer between alanine/pyruvate and 1-PEA/acetophenone performs quite differently depending on which is the amino donor. Alanine has great propensity to hold an amino group while 1-PEA (1a) is a

superb amino donor instead. Therefore, the matching pair of substrates is of great significance for the reaction equilibrium.^{141,142} Consequently, the theoretical and experimental calculation of the equilibrium constant is also of interest in the substrate engineering of TAs.¹⁴³

Three pairs of common substrates are listed and compared in Table 5. The difference in deamination free energy between alanine/pyruvate, which is the hardest to be deaminated, and 1-PEA/acetophenone, which is the easiest to be deaminated, is approximately 18 kJ mol⁻¹.¹⁴¹ In the purpose of shifting the transamination reaction to the product side, the amino donor should be easier to be deaminated than the amino acceptor product, for example, transamination from 1-PEA (1a) to pyruvate. Therefore, alanine is not a good amino donor in this aspect. However, being an easy and cheap source, acceptance by most TAs and hardly any inhibition to enzymes are the reasons why alanine has been employed anyway in many cases. Moreover, as previously discussed, the development of strategies for pyruvate elimination and alanine recycling further promotes the application of alanine.

IPA and acetone are a promising pair of substrates in the application of TAs in organic synthesis, although a limited number of TAs can accept them as efficient substrates,^{22,141} including *A. citreus* TA mutant,¹³⁹ ATA117 mutant¹⁵ and TAs from *P. aeruginosa*, *P. denitrificans* and *A. terreus*.¹⁴² The advantages include the achiral nature of IPA, which works with either *S*- or *R*-selective TAs, and the easy elimination of acetone by physical or chemical strategies.

1-PEA (1a) is widely used as the model substrate of aromatic amines. The great propensity to lose the amino group and the easy detection of its product acetophenone by UV absorbance increase the practicability of this pair of substrates. Although acetophenone shows severe inhibition of enzymes, a number of elimination approaches including extraction and chemical reactions have been developed.^{32,144}

Eliminating the substrate and product inhibition

TA suffers the inhibition from both substrates and products. An overdosed substrate may inhibit the enzyme by binding to alternative sites and destabilizing the protein. Product inhibition, as a strategy in most metabolic regulations, is ascribed to not only the effects on the reaction equilibrium but also the destabilization of the enzyme. To eliminate these inhibitions,



both process and protein perspectives should be taken into consideration.

Process engineering

Removal of product, as indicated above, is a practical method for product inhibition elimination as well as shifting the equilibrium.

For the TAs suffering the substrate inhibition, the fed-batch addition can be an option not only to ensure the total amount of substrate but also to prevent the detrimental effects of them. Employing a fed-batch strategy with a 300 mM 1-PEA (**1a**) added after 180 min in a constant velocity resulted in a final conversion yield of 62%, which was 25% higher than the corresponding yield using the one shot addition.¹⁴⁵ An alternative solution is to search for a less inhibitive substrate for the enzyme in use.

Protein engineering

With the aid of gene mining and protein engineering, TAs with decreased or eliminated inhibition are available to be further explored.

TAs from *O. anthropi*¹⁴⁶ and *M. vanbaalenii*¹¹⁶ were reported with low product inhibition (Table 1). The tolerance of acetophenone in both cases was reported up to 20 mM while *O. anthropi* TA can also tolerate 500 mM of 1-PEA (**1a**).

Moreover, by error-prone PCR mutagenesis and enrichment culture selection, the aliphatic ketone inhibition of *V. fluvialis* TA was reduced significantly (Table 2).⁷⁴ The mechanism of product inhibition of TAs has not yet been fully uncovered, so the analysis of these advanced proteins with low inhibition is of great interest for future work.

Enzymatic cascade reactions

Enzymatic cascade can be used as a smart synthesis strategy in which two or even more biocatalysts are combined for the production of a target compound. This approach has been demonstrated to be advantageous in shortening reaction routes, avoiding unstable or toxic intermediates, increasing the atom efficiency and reducing the amount of waste.^{147–150} Taking advantage of this method, TAs could be applied in organic synthesis in a more efficient fashion. A few elaborate reviews have been published on the topic of cascade reactions involving TAs, which provide a great amount of details.^{10,22,26} Therefore, in this section we will only discuss some possible cascade reaction routes and representative reactions (Scheme 4).

Unnatural amino acids

Unnatural amino acids have been utilized as building blocks in the synthesis of a number of chiral drugs. Unlike natural amino acids, fermentation and extraction strategies are not practical in the production of unnatural amino acids. That is why TA is a promising biocatalyst in the synthesis of unnatural amino acids. Moreover, in combination with other enzymes,

TA is also able to catalyse the synthesis of unnatural amino acids from accessible and cheap chemicals.

Unnatural L-homoalanine (**L-26a**) has been synthesized from natural L-threonine instead of from expensive keto acid by a cascade reaction of threonine deaminase (TD) and TA (Scheme 4a).⁵⁷ *E. coli* TD catalysed the dehydration and deamination of L-threonine, generating 2-oxobutyrate, which was then stereoselectively converted to L-homoalanine (**L-26a**) by *P. denitrificans* TA with amine **5a** as the amino donor. This reaction was conducted in a one-pot fashion with a 5-fold excess of TD to avoid inhibition from the final product. With this method, 91% conversion of L-threonine to L-homoalanine (**L-26a**) was achieved. A significant advantage in the application of amine TA to produce an amino acid is the fact that the reaction equilibrium lies on the side of amino acid product, which results in 100% theoretical conversion in the transamination step.

Another strategy for unnatural amino acid production is the combination of α -TA and amine TA.¹⁵¹ With the advantages found in synthesis of amino acids as described above, amine TA was employed as an “equilibrium shifter” for the transamination mediated by α -TA. The amino group was transformed from the amine into the keto acid by the sequential transamination of amine and α -TA with a pair of amino acid and keto acid as intermediates, which needs to be accepted by both of the two TAs. For example, after a screening of substrates, L-homoalanine (**L-26a**) and its keto acid product were chosen as the intermediate substrates for *E. coli* branched-chain transaminase and *O. anthropi* TA. Employing IPA as amino donor, 99% conversion of **40** resulted in **L-26c** (ee >99%) after 9 h (Scheme 4b). With this strategy, both enantioselective TAs were investigated, resulting in a number of successful examples of production of unnatural amino acids with different side chains.

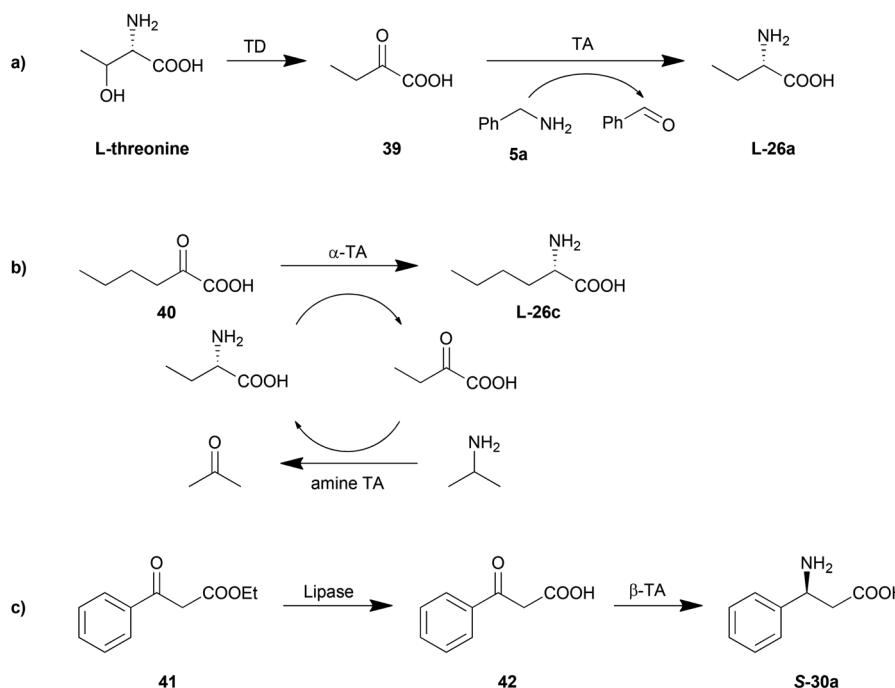
Aside from the unnatural α -amino acids, β -amino acids may also be important synthesis intermediates of chiral drugs. The asymmetric synthesis of β -amino acids was hampered by the unstable β -keto acid amino acceptor as well as the low β -activity of TAs.⁴² By gene mining and protein engineering, more TAs with β -activity have been explored (Tables 1 and 2).^{42,68} The challenge of the unstable β -keto acid has been overcome by cascade reactions of TAs combined with lipases. The accumulation of the unstable β -keto acid can be avoided by a one-pot reaction of lipase-mediated hydrolysis of a β -keto ester and the sequential TA-catalysed transamination.⁴² *S-30a* was produced through a cascade reaction of *Candida rugosa* lipase and *Mesorhizobium* TA in 20% yield and >99% ee (Scheme 4c). Further optimization can be conducted by manipulating the concentration of lipase.¹⁵² The total activity of TA should be slightly higher than that of the lipase in order to minimize the accumulation of intermediate β -keto acid.

Deracemization of amines

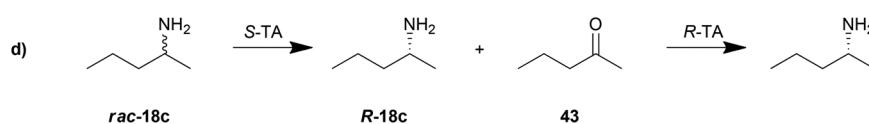
Deracemization has been shown to be an efficient strategy for the production of optically pure amines from their racemates. A straightforward route is the combination of two TAs with



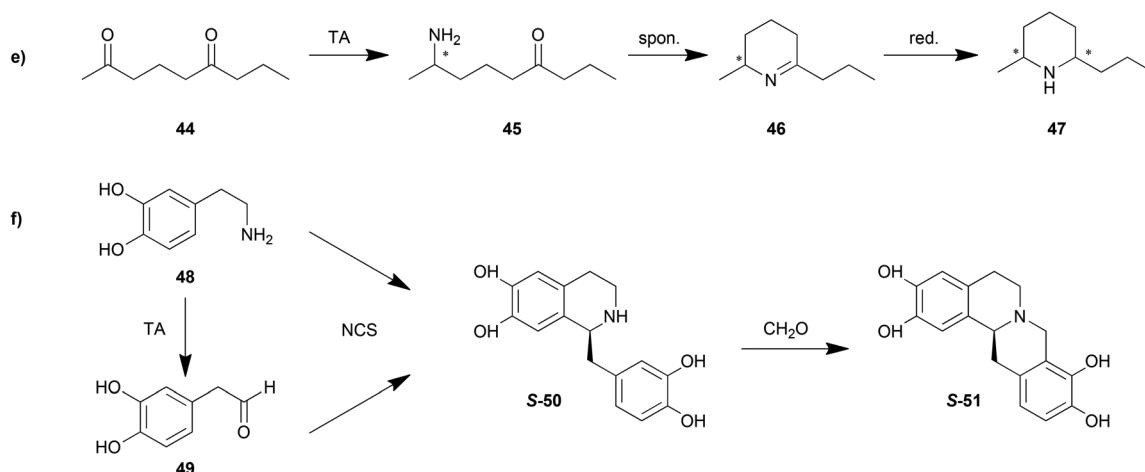
Unnatural amino acids



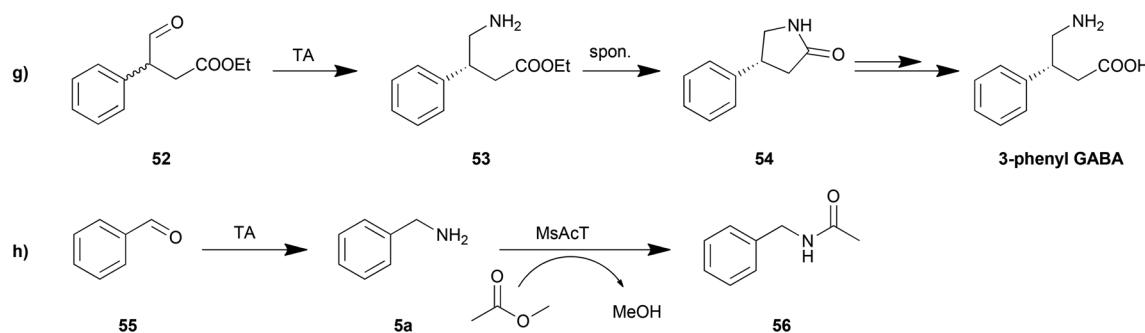
Deracemization of amines



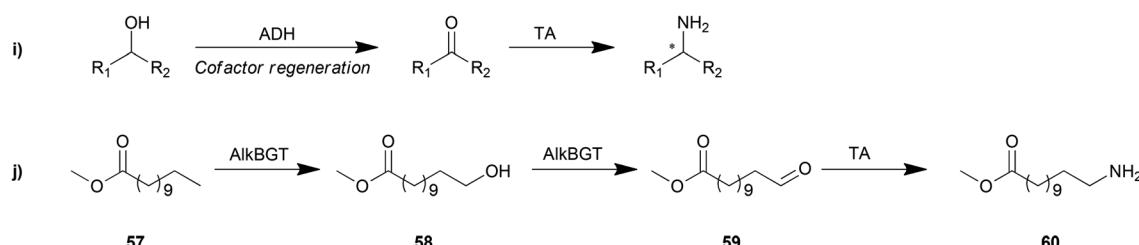
Imines, secondary amines and amides



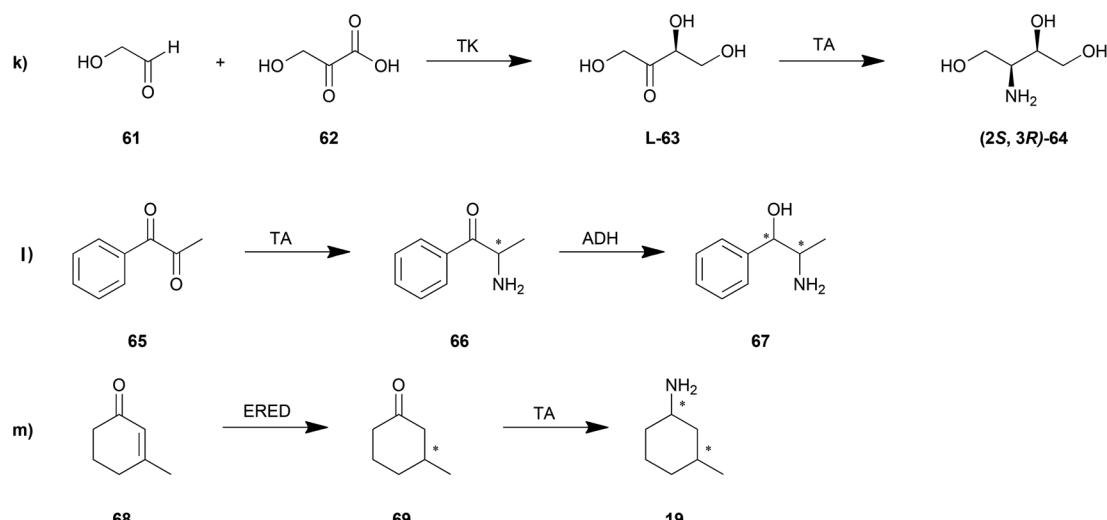
Scheme 4 Representative cascade reactions involving TA.



Amination of -OH or -CH₃



Amines with two stereo-centers



Scheme 4 (Contd.).

complementary enantioselectivity, turning one enantiomer into ketone and then switching this ketone into the other enantiomer.^{111,153–155} This method has been limited by the substrate compatibility of these two TAs, whereas the cascade reaction needs to be conducted in a stepwise fashion with the elimination of the first enzyme. For example, deracemization of *rac*-18c by a combination of ATA-113 (*S*-selective) and ATA-117 (*R*-selective) resulted in only 75% ee (*S*) or 29% ee (*R*), depending on the enzyme addition order (Scheme 4d).¹⁵³ However, if an additional heat inactivation step was conducted before addition of the second enzyme, the final ee of each enantiomer could be achieved over 99%. Further optimization

of this strategy was proposed using a smart amino acceptor in the first step which will not be accepted by the second TA so as to drive the reaction to the product side.¹⁵⁶ The inactivated step was also avoided with this smart substrate.

In another method avoiding the interference of two TAs, oxidases have been employed in the first step of deamination. Like TAs, enantioselective oxidases also transform one of the amine enantiomers into its keto product, followed by amination by a TA with complementary enantioselectivity. *D*-Amino acid oxidase¹⁵⁷ and monoamine oxidase¹⁵⁸ have been demonstrated to be promising candidates for this strategy. The disadvantage of this approach is the consumption of oxidant in



the first step, which could be compensated for by additional recycling of these compounds.

Imines, secondary amines and amides

Primary amines are the only products that can be directly obtained in the straightforward transamination catalysed by TAs due to the enzyme mechanism. However, with the aid of other reactions, for instance, spontaneous dehydration or enzyme-mediated condensation, imines, secondary amines or amides are possible to be produced.

Kroutil *et al.* reported a regio- and stereoselective mono-amination of 2,6-diketones (**44**) by both *S*- and *R*-selective TAs (Scheme 4e).⁵⁵ With five tested TAs, the ω -1 carbonyl moiety was exclusively aminated leading to a cyclic imine Δ^1 -piperideine (**46**) with either *S*- or *R*-product in an optically pure form (ee >99%). The generality of this strategy was also demonstrated by regio- and stereoselective synthesis of 2,6-diketones with a number of other substituents. Further reduction of the double bond provides chiral piperidines (**47**); all of the four diastereomers were obtained by utilizing *R*- and *S*-selective TAs with different chemical chiral reductants.¹⁵⁹ With an alternative strategy in the reduction step, monoamine oxidase (MAO-N) was employed in a one-pot cascade reaction system producing enantiomerically pure 2,5-disubstituted pyrrolidines.¹⁶⁰ Furthermore, this strategy may also be applied in the stereoselective monoamination of triketone compounds giving a chiral pyrrolizidine product.¹⁶¹ In a recent example, by combination with a carboxylic acid reductase at the beginning of the cascade reaction, piperidines and pyrrolidines can be synthesized from keto acids.¹⁶²

A novel triangular cascade reaction for cyclic amines was reported consisting of TA and norcoclaurine synthase (NCS) taking both the substrate and product of TA as substrates (Scheme 4f).¹⁶³ With a second Pictet-Spengler cyclisation by sequential addition of formaldehyde, pharmaceutically active tetrahydroprotoberberine alkaloids (**S-51**) can be produced with high enantiomeric purity (>95% ee). Another similar cascade route for cyclic amines was proposed as the combination of TA and strictosidine synthase for the synthesis of *C3*-methylated strictosidine derivatives from indolylketone and secologanin.¹⁶⁴

Aside from amines, cyclic amides can also be prepared by cascade reactions involving TA and spontaneous dehydration. Keto acids or keto esters were firstly aminated by TA and then turned into cyclic amides by spontaneous cyclization and dehydration. For example, a dynamic kinetic resolution route was developed with amination of a racemic 4-oxo-3-phenylbutyric acid ethyl ester (**52**) followed by spontaneous racemization into 4-phenylpyrrolidin-2-one (**54**), which after subsequent chemical reactions gives 3-phenyl-GABA (Scheme 4g).¹⁶⁵ A similar strategy has also been applied in the production of Niraparib, an orally active poly(ADP-ribose)polymerase inhibitor, by amination of a 5-carbon aldehyde ester.⁴⁹ Another example is the chemoenzymatic synthesis of the dual orexin receptor antagonist MK-6096. Amination of a keto ester followed by a cyclization provided a chiral piperidone.¹⁶⁶

Biocatalytic amidation may be achieved by a hydrolase only in organic solvents or by an acyl transferase in aqueous solutions. Recently, our group proposed a one-pot one-step cascade reaction combining TA and acyl transferase for the amidation of an aldehyde (Scheme 4h).¹⁶⁷ After optimization of pH, acyl donor and enzyme concentration, a conversion of 97% was reached in the synthesis of *N*-benzylacetamide (**56**).

Amination of -OH or -CH₃

Alcohols have gained a great amount of attention in the production of amines due to their oxidation state, which is the same as that of amines. Alcohols are firstly oxidized into ketones or aldehydes and then reductively aminated by TAs into amines (Scheme 4i). The combination of TAs with oxidants or oxidases has provided a number of successful examples in asymmetric amination of primary,^{168–170} secondary alcohols,^{171,172} diols¹⁶⁹ and saccharides with multiple hydroxyl groups.¹⁷³ One of the elegant strategies for the amination of alcohols is recycling of the cofactor NAD/NADH, which is consumed in the oxidation step, regenerating the cofactor in an AlaDH-mediated alanine recycling employed in the transamination step.^{169,171,174}

A terminal methyl group can also be aminated in a cascade reaction. This inert group in dodecanoic acid methyl ester (**57**) was oxidized into an aldehyde (**59**) in two steps by an alkane monooxygenase (AlkBGT), and then transformed into a terminal amine (**60**) by TA (Scheme 4j).¹⁷⁵ This cascade reaction was achieved in a single cell biocatalyst with the two involved enzyme genes recombined.

Amines with two stereo-centres

Working with other stereoselective enzymes, TAs are able to be applied in the synthesis of amines with two stereo-centres. The advantage of these routes is the circumvention of the protection step of the first stereo-centre before the production of the second stereo-centre.

Chiral amino alcohols have been produced in a cascade reactions together with transketolase (TK)/TA, acetohydroxyacid synthase I (AHAS-I)/TA, TA/ADH and keto reductase (KRED)/TA. Among them, the TK/TA cascade reaction is the most studied system. TK, important in the pentose phosphate pathway, mediates the C–C bond formation between aldehydes and keto-alcohols. The produced ketose containing one chiral hydroxyl group in the vicinity of a carbonyl group (**63**) is further catalysed by TA to produce chiral amino alcohols (**64**) with two stereo-centres (Scheme 4k).⁶⁶ An engineered variant of *E. coli* transketolase (D469 T) and *C. violaceum* TA were employed in this system with an isolated yield of 26% in the 100 ml reaction scale.¹⁷⁶ Recently, the routine was further optimized by adjusting the expression of the enzymes and reducing the substrate inhibition providing a 6-fold greater yield.¹⁴⁵ Moreover a deamination of serine was introduced in the reaction system for producing hydroxypyruvate from a common source.²⁴

Like TK, thiamine diphosphate (ThDP)-dependent acetohydroxyacid synthase I (AHAS-I) from *E. coli* can catalyse C–C



bond formation providing a keto alcohol to TA for further amination.¹⁷⁷ Pyruvate was reported to be decarboxylated and subsequently ligated to benzaldehyde yielding *R*-phenylacetylcarbinol, which is a substrate for TAs. Two diastereomers were produced by employing *R*- or *S*-selective TA. However, limited by the enantioselectivity of the first step, only the *1R*-product can be obtained. All four diastereomers were produced by involving another *S*-selective AHAS-I.¹⁷⁸ Meanwhile, starting from 1-phenylpropane-1,2-dione (65), another cascade reaction with amination and reduction turned two carbonyl groups into an amine and a hydroxyl group respectively (Scheme 4l). Similarly, all four diastereomers of 1,3-amino alcohol were accessed by the combination of a ketoreductase and TA.¹⁷⁹ The regio-selectivity in both cases is crucial for the purity of products.

Recently, other cascade reactions to obtain chemicals with two stereo-centres have been developed by the combination of TAs and other enzymes. Ene-reductase, catalysing the reduction of olefins, is one of the candidates. Both β -substituted cyclic enone⁸⁵ (Scheme 4m, 68) and open-chain α -alkyl- β -arylenones⁵⁰ were turned into amines with two stereo-centres. By employing enantio-complementary reductases and TAs, all four diastereomers can be obtained.

Transaminase in synthetic biology and systems biocatalysis

As important biocatalysts in nitrogen metabolism, TAs have been found to be involved in amino acid synthesis and degradation as well as in the biosynthesis of some secondary metabolites.^{180,181} Integration of cascade reactions involving TAs into a single microbial cell could be a new proposal in synthetic biology and the strategies of metabolic engineering may also be applicable in these cases. Alternatively, complex multistep synthesis pathways with multiple enzymes *in vitro* provide the examples of systems biocatalysis, which is a promising strategy in organic synthesis.¹⁸²

Natural biosynthetic pathway

The innate amino acid biosynthesis pathways in microorganisms can be regulated for the industrial production of these

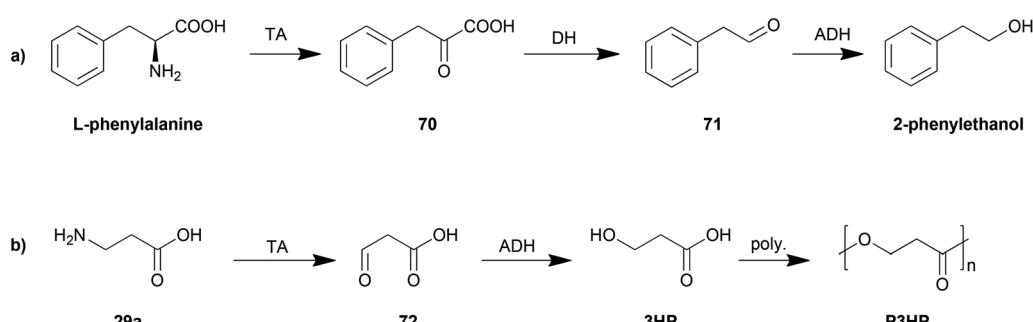
amino acids by manipulating the TAs involved. For instance, the production of *L*-tyrosine was improved significantly by combinatorial overexpression of aromatic amino acid biosynthesis genes in *E. coli*.¹⁸¹ Besides ydiB and aroK, coding for a shikimate dehydrogenase and a shikimate kinase, overexpression of tyrB encoding the last enzyme of the AAAB pathway, an aromatic amino acid transaminase, improved *L*-tyrosine production significantly when co-overexpressed together with ydiB or aroK. Similarly, overexpression of a putative class II aminotransferase increased the *L*-ornithine production in *Corynebacterium glutamicum* from 8.42 g l⁻¹ for the control to 12.48 g l⁻¹.¹⁸³

Aside from natural amino acids, unnatural ones have also been produced by construction of artificial biosynthetic pathways based on the innate ones. *L*-Phenylglycine, non-proteinogenic but important for the preparation of several antibiotics and taxol, was biosynthesized from glucose with an engineered *E. coli* containing an *L*-phenylglycine synthetic pathway from *Amycolatopsis orientalis* and *Streptomyces coelicolor*.¹⁸⁴ The production of *L*-phenylglycine was increased by 224-fold compared with the original strain by optimization of the heterologous pathway and attenuation of native *L*-phenylalanine transamination.

The control of the degradation of amino acids has also been proposed as an efficient strategy for production of other organic chemicals. The yeast Ehrlich pathway (Scheme 5a) has been investigated and employed as the biosynthesis route for 2-phenylethanol. This pathway including an aromatic transaminase was constructed in either *Saccharomyces cerevisiae*¹⁸⁵ or *E. coli*,¹⁸⁶ both of which achieved an improvement in 2-phenylethanol production.

Another example is the biosynthesis of 3-hydroxypropionic acid (3HP), a potential chemical building block for sustainable production of superabsorbent polymers and acrylic plastics, from the degradation of β -alanine (Scheme 5b). The pathway was engineered and optimized by using a β -alanine-pyruvate aminotransferase to convert β -alanine into 3HP.¹⁸⁷ By further employing polymerization steps, poly(3-hydroxypropionate) (P3HP) can be obtained.¹⁸⁸

Besides the pathways of amino acids, the biosynthesis of some secondary metabolites may also be achieved and optimized by engineering of TA-mediated processes. For instance,



Scheme 5 Biosynthesis based on degradation of amino acids.

in the biotin biosynthesis in *B. subtilis*, the bottleneck of the process was identified as the TA which catalyses the formation of the intermediate 7,8-diaminopelargonic acid (DAPA) from 7-keto-8-aminopelargonic acid (KAPA).¹⁸⁰ This limitation of this TA was ascribed to the unfavourable amino donor *S*-adenosylmethionine. As a consequence, substitution of this compound by a favourable L-lysine dramatically stimulated the conversion of KAPA to DAPA by the *B. subtilis* DAPA TA.

Artificial biosynthetic pathway

Cascade reactions involving TAs have been thoroughly investigated for organic synthesis, which has been discussed in the previous section. Based on these routes, expressing all of the needed enzymes in one whole cell generates a cell factory for the target product. An example of this cell factory is the construction of a whole-cell biocatalyst catalysing the terminal amino-functionalization of fatty acid methyl esters (e.g., dodecanoic acid methyl ester) and alkanes (e.g., octane) by heterologous pathway engineering (Scheme 4j).¹⁷⁵ Coupling oxygenase and transaminase catalysis *in vivo*, both substrates are converted with absolute regiospecificity to the terminal amine *via* two sequential oxidation reactions followed by an amination step. This route was further tuned by metabolic engineering methods recently.¹⁸⁹ Hydrophobic outer membrane porin AlkL was employed to improve the substrate uptake, consequently increasing the oxygenation and transamination activities by 8.3- and 7.6-fold, respectively. Moreover, a heterologous alanine dehydrogenase was introduced to construct an alanine self-sufficient whole-cell transamination. Finally, the introduction of an alcohol dehydrogenase enabled an increase in pathway flux and minimized the undesired over oxidation.

Similarly, another biosynthesis pathway was also monitored using strategies of metabolic engineering. To biosynthesize (2S,3R)-2-amino-1,3,4-butanetriol (ABT), Ward *et al.* established an artificial pathway containing *E. coli* transketolase (TK) and the *C. violaceum* TA in *E. coli* (Scheme 4k). The kinetic models and the analysis of parameters of each enzymatic node identified that TA is the bottleneck with a 25 times slower conversion rate than the upstream enzyme TK.¹⁴⁵ Increasing the expression amount of TA and decreasing the substrate inhibition of TA successfully scaled up the production of ABT to preparative scales. Furthermore, a modification of the pathway by adding a production of hydroxypyruvate (HPA) from serine by TAs made the biosynthetic pathway more economical.²⁴ This system may be further improved by constructing a recycling cascade where serine is used as the donor to aminate erythrulose (catalysed by a transketolase) for the simultaneous synthesis of ABT and HPA.

An increasing amount of interest has been attracted by this promising biocatalyst even though the application of TA is limited by some inherent problems as discussed above. The whole cell containing all the cascades reaction nodes can be a promising strategy in TA catalysis which may degrade the co-products, can improve the stability and even perform the synthesis of cofactors. With versatile enzymes from either gene mining or protein engineering, the current long organic syn-

thesis routes have possibilities to be transformed into artificial biosynthesis pathways and integrated into whole-cell factories.

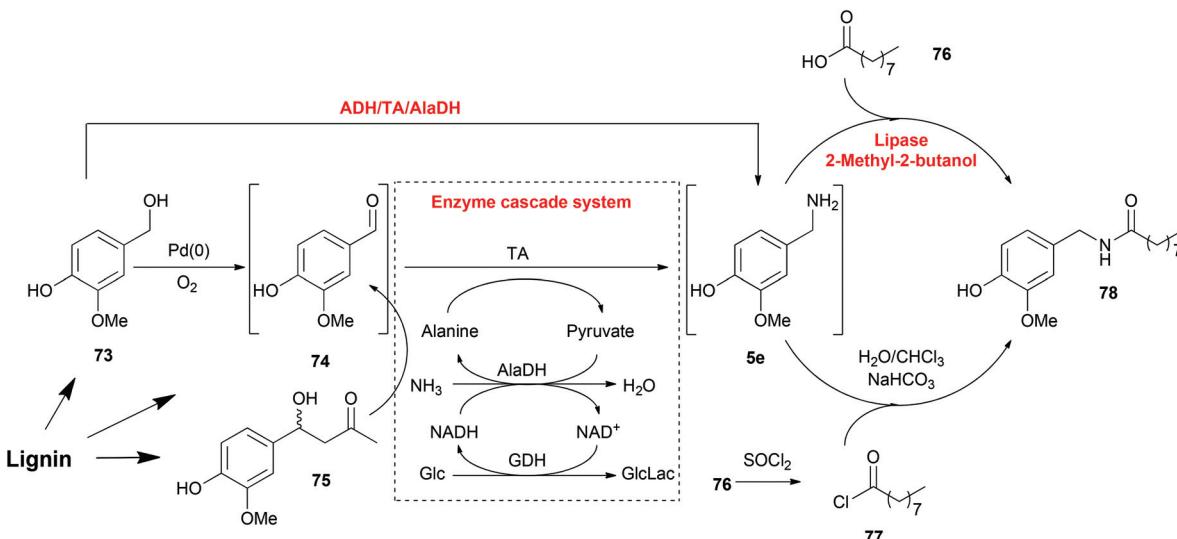
Systems biocatalysis

Working in a cell-free fashion, systems biocatalysis employs *in vitro* "artificial metabolisms" as pathways for the preparation of valuable chemicals.¹⁸² Even though it is a much smaller system than the total metabolisms of any host cell, it is still highly important that every reaction step and their compatibility should be carefully designed. Regardless of the complexity, systems biocatalysis has a great number of advantages including avoiding competing metabolic pathways, avoiding physical barriers and toxicity problems and, the most significant advantage, construction of novel systems for non-natural substrates.

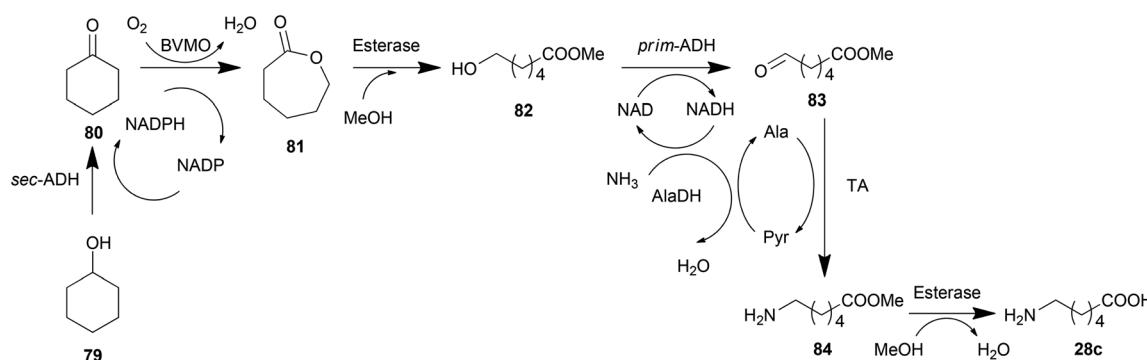
One example is the total synthesis of capsaicin analogues from lignin-derived compounds explored by our group involving heterogeneous metal, organocatalytic and enzymatic cascade routes (Scheme 6).¹⁷⁴ This synthesis process consisted of three steps: the production of vanillin (74), the amination of vanillin (74) and the production of amide 78, whereas each of them can be achieved by either chemo- or bio-catalysis. Vanillin (74) can be synthesized by either the oxidation of vanillyl alcohol (73) or the retro-aldol transformation of aldol compound 75. Each method is compatible with the TA mediated transamination afterwards. Especially, the ADH-catalysed oxidation of vanillyl alcohol (73) contributed to the regeneration of NAD/NADH in the transamination process, which resulted in an elegant one-pot one-step cascade reaction. As the last step, the amidation of amine 5e was also performed with either chemo- or bio-catalysts. Lipase-mediated amidation is a greener strategy avoiding the employment of halogenated organics, although the chemical method catalysed by SOCl_2 gave a higher conversion. This last step is connected to the previous reactions in a one-pot fashion without any purification steps, although the removal of water as solvent was required. In this total synthesis of capsaicin analogues, multiple steps with various catalysts were integrated in a one-pot fashion without any protection and purification steps, which resulted in a more efficient and economical route.

Another example is the biosynthesis of the nylon-6 monomer 6-aminohexanoic acid (28c) from cyclohexanol (79) (Scheme 7).²³ This synthesis route consisted of two cofactor self-sufficient cascade modules, the production of ϵ -caprolactone (81) from cyclohexanol (79) and subsequent production of 6-aminohexanoic acid (28c). In the first module, a NADP/NADPH-dependent alcohol dehydrogenase (ADH) and a Baeyer–Villiger monooxygenase (BVMO) were combined in a cascade reaction, resulting in a recycling of the cofactor. The reaction route in the second module is longer, with four enzymes combined in four steps: the ring-opening reaction by an esterase, the oxidation of the terminal hydroxyl group by ADH, the subsequent amination by TA together with AlaDH and the hydrolysis into the final product 28c. The oxidation and amination steps contributed to the regeneration of cofactor NAD/NADH, which is an elegant strategy for the amination of alcohols. Moreover, the methanol added in the ring-opening step resulted in the methyl ester product (82) instead





Scheme 6 Total synthesis of capsaicin analogues.



Scheme 7 Production of 6-aminohexanoic acid.

of its acid analogue, which is a dead end molecule for the following oxidation by ADH. Besides, methanol was more practical than ethanol due to its low activity for ADH afterwards. To conduct these two modules simultaneously, more details have been taken into consideration. Employed in both modules, the ADHs need to be highly specific towards the substrates, *sec*-alcohol in the first module while *prim*-alcohol in the other, to avoid the interferences. Similarly, the cofactors used were also different in the two modules. The concentration of methanol was also adjusted since it improved the production in the second module while it significantly destabilized BVMO in the first module. Therefore, further optimization of this route may be achieved by enhancing the MeOH tolerance of BVMO or improving the specificity of the esterase towards methyl ester product in the ring-opening reaction.

Conclusions

TAs have been proven to be promising biocatalysts in organic synthesis due to their extraordinary nature of producing highly

enantiomerically enriched amino acids and amines. Various strategies developed at every step from enzyme discovery to product elimination have played an increasingly significant part in the optimization of TAs and of processes involving TAs. These exquisite processes are practical and efficient in the production of chiral pharmaceuticals, giving a strong competition to conventional chemical routes.^{190,191} The greenness of these outstanding biocatalysts, including aspects such as shorter routes without protecting steps, lower cost of equipment and energy, less environment-detrimental waste, easier procedures for work-up and even lower cost of catalysts considering the recycling of enzymes, has made them one of the top options for industrial organic synthesis.¹⁹²

Acknowledgements

This work was funded by KTH Royal Institute of Technology. Henrik Land, KTH, is gratefully acknowledged for critical comments on the text.

References

- 1 F. Hollmann, I. W. C. E. Arends and D. Holtmann, *Green Chem.*, 2011, **13**, 2285–2314.
- 2 P. Berglund, M. S. Humble and C. Branneby, in *Comprehensive Chirality*, ed. E. M. Carreira and H. Yamamoto, Elsevier, Amsterdam, 2012, vol. 7, pp. 390–401.
- 3 A. C. Eliot and J. F. Kirsch, *Annu. Rev. Biochem.*, 2004, **73**, 383–415.
- 4 F. Steffen-Munsberg, C. Vickers, H. Kohls, H. Land, H. Mallin, A. Nobili, L. Skalden, T. van den Bergh, H. J. Joosten, P. Berglund, M. Höhne and U. T. Bornscheuer, *Biotechnol. Adv.*, 2015, **33**, 566–604.
- 5 S. Kara, J. H. Schrittwieser, F. Hollmann and M. B. Ansorge-Schumacher, *Appl. Microbiol. Biotechnol.*, 2014, **98**, 1517–1529.
- 6 W. F. Liu and P. Wang, *Biotechnol. Adv.*, 2007, **25**, 369–384.
- 7 D. Koszelewski, K. Tauber, K. Faber and W. Kroutil, *Trends Biotechnol.*, 2010, **28**, 324–332.
- 8 D. Ghislieri and N. J. Turner, *Top. Catal.*, 2014, **57**, 284–300.
- 9 H. Kohls, F. Steffen-Munsberg and M. Höhne, *Curr. Opin. Chem. Biol.*, 2014, **19**, 180–192.
- 10 M. Fuchs, J. E. Farnberger and W. Kroutil, *Eur. J. Org. Chem.*, 2015, 6965–6982.
- 11 G. Matcham, M. Bhatia, W. Lang, C. Lewis, R. Nelson, A. Wang and W. Wu, *Chimia*, 1999, **53**, 584–589.
- 12 J. S. Shin and B. G. Kim, *Biotechnol. Bioeng.*, 1999, **65**, 206–211.
- 13 J.-S. Shin and B.-G. Kim, *Biosci., Biotechnol., Biochem.*, 2001, **65**, 1782–1788.
- 14 M. Höhne, S. Schatzle, H. Jochens, K. Robins and U. T. Bornscheuer, *Nat. Chem. Biol.*, 2010, **6**, 807–813.
- 15 C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman and G. J. Hughes, *Science*, 2010, **329**, 305–309.
- 16 A. R. Martin, R. DiSanto, I. Plotnikov, S. Kamat, D. Shonnard and S. Pannuri, *Biochem. Eng. J.*, 2007, **37**, 246–255.
- 17 M. D. Truppo, H. Strotman and G. Hughes, *ChemCatChem*, 2012, **4**, 1071–1074.
- 18 S. Mathew, S. P. Nadarajan, T. Chung, H. H. Park and H. Yun, *Enzyme Microb. Technol.*, 2016, **87–88**, 52–60.
- 19 J. H. Seo, D. Kyung, K. Joo, J. Lee and B. G. Kim, *Biotechnol. Bioeng.*, 2011, **108**, 253–263.
- 20 P. Tufvesson, J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto and J. M. Woodley, *Biotechnol. Bioeng.*, 2011, **108**, 1479–1493.
- 21 R. Abu and J. M. Woodley, *ChemCatChem*, 2015, **7**, 3094–3105.
- 22 R. C. Simon, N. Richter, E. Bustos and W. Kroutil, *ACS Catal.*, 2014, **4**, 129–143.
- 23 J. H. Sattler, M. Fuchs, F. G. Mutti, B. Grischek, P. Engel, J. Pfeffer, J. M. Woodley and W. Kroutil, *Angew. Chem., Int. Ed.*, 2014, **53**, 14153–14157.
- 24 M. F. Villegas-Torres, R. J. Martinez-Torres, A. Cázares-Körner, H. Hailes, F. Baganz and J. Ward, *Enzyme Microb. Technol.*, 2015, **81**, 23–30.
- 25 M. S. Malik, E.-S. Park and J.-S. Shin, *Appl. Microbiol. Biotechnol.*, 2012, **94**, 1163–1171.
- 26 S. Mathew and H. Yun, *ACS Catal.*, 2012, **2**, 993–1001.
- 27 S. Mathew, G. Shin, M. Shon and H. Yun, *Biotechnol. Bioprocess Eng.*, 2013, **18**, 1–7.
- 28 W. John and W. Roland, *Curr. Org. Chem.*, 2010, **14**, 1914–1927.
- 29 J. S. Gong, Z. M. Lu, H. Li, Z. M. Zhou, J. S. Shi and Z. H. Xu, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 6603–6611.
- 30 L. P. Wackett, *Curr. Opin. Biotechnol.*, 2004, **15**, 280–284.
- 31 S. Dover and Y. S. Halpern, *J. Bacteriol.*, 1972, **109**, 835–843.
- 32 J.-S. Shin and B.-G. Kim, *Biotechnol. Bioeng.*, 1997, **55**, 348–358.
- 33 J. S. Shin, H. Yun, J. W. Jang, I. Park and B. G. Kim, *Appl. Microbiol. Biotechnol.*, 2003, **61**, 463–471.
- 34 R. E. Meadows, K. R. Mulholland, M. Schürmann, M. Golden, H. Kierkels, E. Meulenbroeks, D. Mink, O. May, C. Squire, H. Straatman and A. S. Wells, *Org. Process Res. Dev.*, 2013, **17**, 1117–1122.
- 35 K. S. Midelfort, R. Kumar, S. Han, M. J. Karmilowicz, K. McConnell, D. K. Gehlhaar, A. Mistry, J. S. Chang, M. Anderson, A. Villalobos, J. Minshull, S. Govindarajan and J. W. Wong, *Protein Eng., Des. Sel.*, 2013, **26**, 25–33.
- 36 A. Nobili, F. Steffen-Munsberg, H. Kohls, I. Trentin, C. Schulzke, M. Höhne and U. T. Bornscheuer, *ChemCatChem*, 2015, **7**, 757–760.
- 37 A. Iwasaki, K. Matsumoto, J. Hasegawa and Y. Yasohara, *Appl. Microbiol. Biotechnol.*, 2011, **93**, 1563–1573.
- 38 R. L. Hanson, B. L. Davis, Y. Chen, S. L. Goldberg, W. L. Parker, T. P. Tully, M. A. Montana and R. N. Patel, *Adv. Synth. Catal.*, 2008, **350**, 1367–1375.
- 39 D. Chen, Z. Wang, Y. Zhang, Z. Sun and Q. Zhu, *Bioprocess Biosyst. Eng.*, 2007, **31**, 283–289.
- 40 N. Ito, S. Kawano, J. Hasegawa and Y. Yasohara, *Biosci., Biotechnol., Biochem.*, 2014, **75**, 2093–2098.
- 41 H. Yun, S. Lim, B. K. Cho and B. G. Kim, *Appl. Environ. Microbiol.*, 2004, **70**, 2529–2534.
- 42 J. Kim, D. Kyung, H. Yun, B. K. Cho, J. H. Seo, M. Cha and B. G. Kim, *Appl. Environ. Microbiol.*, 2007, **73**, 1772–1782.
- 43 M. Wilding, E. F. Walsh, S. J. Dorrian and C. Scott, *Microb. Biotechnol.*, 2015, **8**, 665–672.
- 44 M. Wilding, T. S. Peat, J. Newman and C. Scott, *Appl. Environ. Microbiol.*, 2016, **82**, 3846–3856.
- 45 Y. Lang, H. Kisaka, R. Sugiyama, K. Nomura, A. Morita, T. Watanabe, Y. Tanaka, S. Yazawa and T. Miwa, *Plant J.*, 2009, **59**, 953–961.
- 46 N. Weber, A. Ismail, M. Gorwa-Grauslund and M. Carlquist, *BMC Biotechnol.*, 2014, **14**, 1–6.
- 47 D. Clay, D. Koszelewski, B. Grischek, J. Gross, I. Lavandera and W. Kroutil, *Tetrahedron: Asymmetry*, 2010, **21**, 2005–2009.

48 D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell and W. Kroutil, *Adv. Synth. Catal.*, 2008, **350**, 2761–2766.

49 C. K. Chung, P. G. Bulger, B. Kosjek, K. M. Belyk, N. Rivera, M. E. Scott, G. R. Humphrey, J. Limanto, D. C. Bachert and K. M. Emerson, *Org. Process Res. Dev.*, 2014, **18**, 215–227.

50 D. Monti, M. C. Forchin, M. Crotti, F. Parmeggiani, F. G. Gatti, E. Brenna and S. Riva, *ChemCatChem*, 2015, **7**, 3106–3109.

51 A. Gomm, W. Lewis, A. P. Green and E. O'Reilly, *Chem. – Eur. J.*, 2016, **22**, 1–5.

52 U. Kaulmann, K. Smithies, M. E. B. Smith, H. C. Hailes and J. M. Ward, *Enzyme Microb. Technol.*, 2007, **41**, 628–637.

53 M. S. Humble, K. E. Cassimjee, M. Hakansson, Y. R. Kimbung, B. Walse, V. Abedi, H. J. Federsel, P. Berglund and D. T. Logan, *FEBS J.*, 2012, **279**, 779–792.

54 F. G. Mutti and W. Kroutil, *Adv. Synth. Catal.*, 2012, **354**, 3409–3413.

55 R. C. Simon, B. Grischek, F. Zepeck, A. Steinreiber, F. Belaj and W. Kroutil, *Angew. Chem., Int. Ed.*, 2012, **51**, 6713–6716.

56 R. D. Finn, A. Bateman, J. Clements, P. Coggill, R. Y. Eberhardt, S. R. Eddy, A. Heger, K. Hetherington, L. Holm, J. Mistry, E. L. L. Sonnhammer, J. Tate and M. Punta, *Nucleic Acids Res.*, 2013, 1–9.

57 E. Park, M. Kim and J.-S. Shin, *Adv. Synth. Catal.*, 2010, **352**, 3391–3398.

58 E.-S. Park, M. Kim and J.-S. Shin, *Appl. Microbiol. Biotechnol.*, 2011, **93**, 2425–2435.

59 B.-Y. Hwang, S.-H. Ko, H.-Y. Park, J.-H. Seo, B.-S. Lee and B.-G. Kim, *J. Microbiol. Biotechnol.*, 2008, **18**, 48–54.

60 H.-S. Bea, H.-J. Park, S.-H. Lee and H. Yun, *Chem. Commun.*, 2011, **47**, 5894–5896.

61 S. Schätzle, F. Steffen-Munsberg, A. Thontowi, M. Höhne, K. Robins and U. T. Bornscheuer, *Adv. Synth. Catal.*, 2011, **353**, 2439–2445.

62 C. Sayer, R. J. Martinez-Torres, N. Richter, M. N. Isupov, H. C. Hailes, J. A. Littlechild and J. M. Ward, *FEBS J.*, 2014, **281**, 2240–2253.

63 J.-H. Seo, J.-Y. Hwang, S.-H. Seo, H. Kang, B.-Y. Hwang and B.-G. Kim, *Biosci., Biotechnol., Biochem.*, 2014, **76**, 1308–1314.

64 I. Slabu, J. L. Galman, N. J. Weise, R. C. Lloyd and N. J. Turner, *ChemCatChem*, 2016, **8**, 1038–1042.

65 J. L. Galman, I. Slabu, N. J. Weise, C. Iglesias, F. Parmeggiani, R. C. Lloyd and N. J. Turner, *Green Chem.*, 2016, DOI: 10.1039/c6gc02102f.

66 C. U. Ingram, M. Bommer, M. E. Smith, P. A. Dalby, J. M. Ward, H. C. Hailes and G. J. Lye, *Biotechnol. Bioeng.*, 2007, **96**, 559–569.

67 S. Schätzle, M. Höhne, E. Redestad, K. Robins and U. T. Bornscheuer, *Anal. Chem.*, 2009, **81**, 8244–8248.

68 S. Mathew, H. Bea, S. P. Nadarajan, T. Chung and H. Yun, *J. Biotechnol.*, 2015, **196–197**, 1–8.

69 F. Steffen-Munsberg, C. Vickers, A. Thontowi, S. Schätzle, T. Tumliorsch, M. Svedendahl Humble, H. Land, P. Berglund, U. T. Bornscheuer and M. Höhne, *ChemCatChem*, 2013, **5**, 150–153.

70 F. Steffen-Munsberg, P. Matzel, M. A. Sowa, P. Berglund, U. T. Bornscheuer and M. Höhne, *Appl. Microbiol. Biotechnol.*, 2016, **100**, 4511–4521.

71 U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore and K. Robins, *Nature*, 2012, **485**, 185–194.

72 J. M. Woodley, *Curr. Opin. Chem. Biol.*, 2013, **17**, 310–316.

73 B.-Y. Hwang and B.-G. Kim, *Enzyme Microb. Technol.*, 2004, **34**, 429–436.

74 H. Yun, B. Y. Hwang, J. H. Lee and B. G. Kim, *Appl. Environ. Microbiol.*, 2005, **71**, 4220–4224.

75 B.-K. Cho, H.-Y. Park, J.-H. Seo, J. Kim, T.-J. Kang, B.-S. Lee and B.-G. Kim, *Biotechnol. Bioeng.*, 2008, **99**, 275–284.

76 E.-S. Park, S.-R. Park, S.-W. Han, J.-Y. Dong and J.-S. Shin, *Adv. Synth. Catal.*, 2014, **356**, 212–220.

77 S.-W. Han, E.-S. Park, J.-Y. Dong and J.-S. Shin, *Adv. Synth. Catal.*, 2015, **357**, 2712–2720.

78 S.-W. Han, E.-S. Park, J.-Y. Dong, J.-S. Shin and S. J. Liu, *Appl. Environ. Microbiol.*, 2015, **81**, 6994–7002.

79 S.-W. Han, E.-S. Park, J.-Y. Dong and J.-S. Shin, *Adv. Synth. Catal.*, 2015, **357**, 1732–1740.

80 I. V. Pavlidis, M. S. Weiß, M. Genz, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding and U. T. Bornscheuer, *Nat. Chem.*, 2016, DOI: 10.1038/nchem.2578.

81 F. Steffen-Munsberg, C. Vickers, A. Thontowi, S. Schätzle, T. Meinhardt, M. Svedendahl Humble, H. Land, P. Berglund, U. T. Bornscheuer and M. Höhne, *ChemCatChem*, 2013, **5**, 154–157.

82 M. Svedendahl, C. Branneby, L. Lindberg and P. Berglund, *ChemCatChem*, 2010, **2**, 976–980.

83 M. S. Humble, K. E. Cassimjee, V. Abedi, H.-J. Federsel and P. Berglund, *ChemCatChem*, 2012, **4**, 1167–1172.

84 K. E. Cassimjee, M. S. Humble, H. Land, V. Abedi and P. Berglund, *Org. Biomol. Chem.*, 2012, **10**, 5466–5470.

85 L. Skalden, C. Peters, J. Dickerhoff, A. Nobili, H. J. Joosten, K. Weisz, M. Höhne and U. T. Bornscheuer, *ChemBioChem*, 2015, **16**, 1041–1045.

86 M. S. Humble and P. Berglund, *Eur. J. Org. Chem.*, 2011, 3391–3401.

87 M. Genz, C. Vickers, T. van den Bergh, H.-J. Joosten, M. Dörr, M. Höhne and U. Bornscheuer, *Int. J. Mol. Sci.*, 2015, **16**, 26953–26963.

88 D. Deszcz, P. Affaticati, N. Ladkau, A. Gegel, J. M. Ward, H. C. Hailes and P. A. Dalby, *FEBS J.*, 2015, **282**, 2512–2526.

89 J. Hopwood, M. D. Truppo, N. J. Turner and R. C. Lloyd, *Chem. Commun.*, 2011, **47**, 773–775.

90 J. E. B. Barber, A. M. Damry, G. F. Calderini, C. J. W. Walton and R. A. Chica, *Anal. Biochem.*, 2014, **463**, 23–30.

91 S. C. Willies, J. L. Galman, I. Slabu and N. J. Turner, *Philos. Trans. R. Soc., A*, 2016, **374**, 20150084.



92 M. S. Weiss, I. V. Pavlidis, C. Vickers, M. Höhne and U. T. Bornscheuer, *Anal. Chem.*, 2014, **86**, 11847–11853.

93 M. Bommer and J. M. Ward, *Enzyme Microb. Technol.*, 2013, **52**, 218–225.

94 M. D. Truppo, J. D. Rozzell, J. C. Moore and N. J. Turner, *Org. Biomol. Chem.*, 2009, **7**, 395–398.

95 M. D. Truppo and N. J. Turner, *Org. Biomol. Chem.*, 2010, **8**, 1280–1283.

96 M. Chen, L. Rong and X. Chen, *RSC Adv.*, 2015, **5**, 103557–103562.

97 T. Sehl, R. C. Simon, H. C. Hailes, J. M. Ward, U. Schell, M. Pohl and D. Rother, *J. Biotechnol.*, 2012, **159**, 188–194.

98 M. Bommer and J. M. Ward, *Anal. Biochem.*, 2016, **493**, 8–10.

99 K. E. Cassimjee, M. S. Humble, V. Miceli, C. G. Colomina and P. Berglund, *ACS Catal.*, 2011, **1**, 1051–1055.

100 A. P. Green, N. J. Turner and E. O'Reilly, *Angew. Chem., Int. Ed.*, 2014, **53**, 10714–10717.

101 D. Baud, N. Ladkau, T. S. Moody, J. M. Ward and H. C. Hailes, *Chem. Commun.*, 2015, **51**, 17225–17228.

102 T. Scheidt, H. Land, M. Anderson, Y. Chen, P. Berglund, D. Yi and W.-D. Fessner, *Adv. Synth. Catal.*, 2015, **357**, 1721–1731.

103 Y. Chen, D. Yi, S. Jiang and D. Wei, *Appl. Microbiol. Biotechnol.*, 2016, **100**, 3101–3111.

104 L. Cerioli, M. Planchestainer, J. Cassidy, D. Tessaro and F. Paradisi, *J. Mol. Catal. B: Enzym.*, 2015, **120**, 141–150.

105 K. Deepankumar, M. Shon, S. P. Nadarajan, G. Shin, S. Mathew, N. Ayyadurai, B.-G. Kim, S.-H. Choi, S.-H. Lee and H. Yun, *Adv. Synth. Catal.*, 2014, **356**, 993–998.

106 S. Chen, H. Land, P. Berglund and M. S. Humble, *J. Mol. Catal. B: Enzym.*, 2016, **124**, 20–28.

107 H. Yun, J. Kim, K. Kinnara and B.-G. Kim, *Biotechnol. Bioeng.*, 2006, **93**, 391–395.

108 R. Bahulekar, N. R. Ayyangar and S. Ponrathnam, *Enzyme Microb. Technol.*, 1991, **13**, 858–868.

109 Y. J. Lu, P. J. Li, Y. L. Guo, Y. Q. Wang and G. Z. Lu, *Prog. Chem.*, 2008, **20**, 1172–1179.

110 U. Hanefeld, L. Gardossi and E. Magner, *Chem. Soc. Rev.*, 2009, **38**, 453–468.

111 D. Koszelewski, N. Müller, J. H. Schrittwieser, K. Faber and W. Kroutil, *J. Mol. Catal. B: Enzym.*, 2010, **63**, 39–44.

112 S. P. de Souza, I. I. Junior, G. M. A. Silva, L. S. M. Miranda, M. F. Santiago, F. Leung-Yuk Lam, A. Dawood, U. T. Bornscheuer and R. O. M. A. de Souza, *RSC Adv.*, 2016, **6**, 6665–6671.

113 H. Mallin, M. Höhne and U. T. Bornscheuer, *J. Biotechnol.*, 2014, **191**, 32–37.

114 J. Yang, K. Ni, D. Wei and Y. Ren, *Biotechnol. Bioprocess Eng.*, 2015, **20**, 901–907.

115 K. Engelmark Cassimjee, M. Kadow, Y. Wikmark, M. Svedendahl Humble, M. L. Rothstein, D. M. Rothstein and J. E. Bäckvall, *Chem. Commun.*, 2014, **50**, 9134–9137.

116 G. Shin, S. Mathew and H. Yun, *J. Ind. Eng. Chem.*, 2015, **23**, 128–133.

117 E.-S. Park, J.-Y. Dong and J.-S. Shin, *Org. Biomol. Chem.*, 2013, **11**, 6929–6933.

118 H. Yun, B.-K. Cho and B.-G. Kim, *Biotechnol. Bioeng.*, 2004, **87**, 772–778.

119 P. Tufvesson, C. Bach and J. M. Woodley, *Biotechnol. Bioeng.*, 2014, **111**, 309–319.

120 J.-S. Shin, B.-G. Kim, A. Liese and C. Wandrey, *Biotechnol. Bioeng.*, 2001, **73**, 179–187.

121 J.-S. Shin, B.-G. Kim and D.-H. Shin, *Enzyme Microb. Technol.*, 2001, **29**, 232–239.

122 B.-K. Cho, H. J. Cho, H. Yun and B.-G. Kim, *J. Mol. Catal. B: Enzym.*, 2003, **26**, 273–285.

123 H. Yun and B.-G. Kim, *Biosci., Biotechnol., Biochem.*, 2008, **72**, 3030–3033.

124 G. Rehn, P. Adlercreutz and C. Grey, *J. Biotechnol.*, 2014, **179**, 50–55.

125 G. Rehn, B. Ayres, P. Adlercreutz and C. Grey, *J. Mol. Catal. B: Enzym.*, 2016, **123**, 1–7.

126 T. Börner, G. Rehn, C. Grey and P. Adlercreutz, *Org. Process Res. Dev.*, 2015, **19**, 793–799.

127 H.-H. Lo, S.-K. Hsu, W.-D. Lin, N.-L. Chan and W.-H. Hsu, *Biotechnol. Prog.*, 2005, **21**, 411–415.

128 L. Martínez-Montero, V. Gotor, V. Gotor-Fernández and I. Lavandera, *Adv. Synth. Catal.*, 2016, **358**, 1618–1624.

129 B. Wang, H. Land and P. Berglund, *Chem. Commun.*, 2013, **49**, 161–163.

130 F. G. Mutti, C. S. Fuchs, D. Pressnitz, J. H. Sattler and W. Kroutil, *Adv. Synth. Catal.*, 2011, **353**, 3227–3233.

131 F. G. Mutti, C. S. Fuchs, D. Pressnitz, N. G. Turrini, J. H. Sattler, A. Lerchner, A. Skerra and W. Kroutil, *Eur. J. Org. Chem.*, 2012, 1003–1007.

132 D. Pressnitz, C. S. Fuchs, J. H. Sattler, T. Knaus, P. Macheroux, F. G. Mutti and W. Kroutil, *ACS Catal.*, 2013, **3**, 555–559.

133 M. Höhne, S. Kuhl, K. Robins and U. T. Bornscheuer, *ChemBioChem*, 2008, **9**, 363–365.

134 D. Koszelewski, I. Lavandera, D. Clay, G. M. Guebitz, D. Rozzell and W. Kroutil, *Angew. Chem., Int. Ed.*, 2008, **47**, 9337–9340.

135 M. D. Truppo, N. J. Turner and J. D. Rozzell, *Chem. Commun.*, 2009, 2127–2129.

136 D. Koszelewski, M. Göritzer, D. Clay, B. Seisser and W. Kroutil, *ChemCatChem*, 2010, **2**, 73–77.

137 N. Richter, J. E. Farnberger, D. Pressnitz, H. Lechner, F. Zepeck and W. Kroutil, *Green Chem.*, 2015, **17**, 2952–2958.

138 S.-W. Han and J.-S. Shin, *Biosci., Biotechnol., Biochem.*, 2014, **78**, 1788–1790.

139 K. E. Cassimjee, C. Branneby, V. Abedi, A. Wells and P. Berglund, *Chem. Commun.*, 2010, **46**, 5569–5571.

140 T. Esparza-Isunza, M. González-Brambila, R. Gani, J. M. Woodley and F. López-Isunza, *Chem. Eng. J.*, 2015, **259**, 221–231.

141 E.-S. Park, M. S. Malik, J.-Y. Dong and J.-S. Shin, *ChemCatChem*, 2013, **5**, 1734–1738.

142 K. Fesko, K. Steiner, R. Breinbauer, H. Schwab, M. Schürmann and G. A. Strohmeier, *J. Mol. Catal. B: Enzym.*, 2013, **96**, 103–110.

143 P. Tufvesson, J. S. Jensen, W. Kroutil and J. M. Woodley, *Biotechnol. Bioeng.*, 2012, **109**, 2159–2162.

144 H. Yun, Y.-H. Yang, B.-K. Cho, B.-Y. Hwang and B.-G. Kim, *Biotechnol. Lett.*, 2003, **25**, 809–814.

145 L. Rios-Solis, P. Morris, C. Grant, A. O. O. Odeleye, H. C. Hailes, J. M. Ward, P. A. Dalby, F. Baganz and G. J. Lye, *Chem. Eng. Sci.*, 2015, **122**, 360–372.

146 E.-S. Park and J.-S. Shin, *Appl. Environ. Microbiol.*, 2013, **79**, 4141–4144.

147 P. A. Santacoloma, G. R. Sin, K. V. Gernaey and J. M. Woodley, *Org. Process Res. Dev.*, 2010, **15**, 203–212.

148 E. Ricca, B. Brucher and J. H. Schrittewieser, *Adv. Synth. Catal.*, 2011, **353**, 2239–2262.

149 I. Oroz-Guinea and E. Garcia-Junceda, *Curr. Opin. Chem. Biol.*, 2013, **17**, 236–249.

150 R. Sigrist, B. Z. D. Costa, A. J. Marsaioli and L. G. de Oliveira, *Biotechnol. Adv.*, 2015, **33**, 394–411.

151 E.-S. Park, J.-Y. Dong and J.-S. Shin, *ChemCatChem*, 2013, **5**, 3538–3542.

152 S. Mathew, S.-S. Jeong, T. Chung, S.-H. Lee and H. Yun, *Biotechnol. J.*, 2016, **11**, 185–190.

153 D. Koszelewski, D. Clay, D. Rozzell and W. Kroutil, *Eur. J. Org. Chem.*, 2009, 2289–2292.

154 D. Koszelewski, D. Pressnitz, D. Clay and W. Kroutil, *Org. Lett.*, 2009, **11**, 4810–4812.

155 D. Koszelewski, B. Grischek, S. M. Glueck, W. Kroutil and K. Faber, *Chem. – Eur. J.*, 2011, **17**, 378–383.

156 G. Shin, S. Mathew, M. Shon, B. G. Kim and H. Yun, *Chem. Commun.*, 2013, **49**, 8629–8631.

157 Y.-M. Seo, S. Mathew, H.-S. Bea, Y.-H. Khang, S.-H. Lee, B.-G. Kim and H. Yun, *Org. Biomol. Chem.*, 2012, **10**, 2482–2485.

158 E. O'Reilly, C. Iglesias and N. J. Turner, *ChemCatChem*, 2014, **6**, 992–995.

159 R. C. Simon, F. Zepeck and W. Kroutil, *Chem. – Eur. J.*, 2013, **19**, 2859–2865.

160 E. O'Reilly, C. Iglesias, D. Ghislieri, J. Hopwood, J. L. Galman, R. C. Lloyd and N. J. Turner, *Angew. Chem., Int. Ed.*, 2014, **53**, 2447–2450.

161 S. E. Payer, J. H. Schrittewieser, B. Grischek, R. C. Simon and W. Kroutil, *Adv. Synth. Catal.*, 2016, **358**, 444–451.

162 S. P. France, S. Hussain, A. M. Hill, L. J. Hepworth, R. M. Howard, K. R. Mulholland, S. L. Flitsch and N. J. Turner, *ACS Catal.*, 2016, **6**, 3753–3759.

163 B. R. Lichman, E. D. Lamming, T. Pesnot, J. M. Smith, H. C. Hailes and J. M. Ward, *Green Chem.*, 2015, **17**, 852–855.

164 E.-M. Fischereder, D. Pressnitz and W. Kroutil, *ACS Catal.*, 2016, **6**, 23–30.

165 D. Koszelewski, D. Clay, K. Faber and W. Kroutil, *J. Mol. Catal. B: Enzym.*, 2009, **60**, 191–194.

166 M. Girardin, S. G. Ouellet, D. Gauvreau, J. C. Moore, G. Hughes, P. N. Devine, P. D. O'Shea and L.-C. Campeau, *Org. Process Res. Dev.*, 2013, **17**, 61–68.

167 H. Land, P. Hendil-Forssell, M. Martinelle and P. Berglund, *Catal. Sci. Technol.*, 2016, **6**, 2897–2900.

168 M. Fuchs, K. Tauber, J. Sattler, H. Lechner, J. Pfeffer, W. Kroutil and K. Faber, *RSC Adv.*, 2012, **2**, 6262–6265.

169 J. H. Sattler, M. Fuchs, K. Tauber, F. G. Mutti, K. Faber, J. Pfeffer, T. Haas and W. Kroutil, *Angew. Chem., Int. Ed.*, 2012, **51**, 9156–9159.

170 M. Pickl, M. Fuchs, S. M. Glueck and K. Faber, *ChemCatChem*, 2015, **7**, 3121–3124.

171 K. Tauber, M. Fuchs, J. H. Sattler, J. Pitzer, D. Pressnitz, D. Koszelewski, K. Faber, J. Pfeffer, T. Haas and W. Kroutil, *Chem. – Eur. J.*, 2013, **19**, 4030–4035.

172 C. Palo-Nieto, S. Afewerki, M. Anderson, C.-W. Tai, P. Berglund and A. Córdova, *ACS Catal.*, 2016, **6**, 3932–3940.

173 A. Lerchner, S. Achatz, C. Rausch, T. Haas and A. Skerra, *ChemCatChem*, 2013, **5**, 3374–3383.

174 M. Anderson, S. Afewerki, P. Berglund and A. Córdova, *Adv. Synth. Catal.*, 2014, **356**, 2113–2118.

175 M. Schrewe, N. Ladkau, B. Bühler and A. Schmid, *Adv. Synth. Catal.*, 2013, **355**, 1693–1697.

176 M. E. B. Smith, B. H. Chen, E. G. Hibbert, U. Kaulmann, K. Smithies, J. L. Galman, F. Baganz, P. A. Dalby, H. C. Hailes, G. J. Lye, J. M. Ward, J. M. Woodley and M. Micheletti, *Org. Process Res. Dev.*, 2010, **14**, 99–107.

177 T. Sehl, H. C. Hailes, J. M. Ward, R. Wardenga, E. von Lieres, H. Offermann, R. Westphal, M. Pohl and D. Rother, *Angew. Chem., Int. Ed.*, 2013, **52**, 6772–6775.

178 T. Sehl, H. C. Hailes, J. M. Ward, U. Menyes, M. Pohl and D. Rother, *Green Chem.*, 2014, **16**, 3341–3348.

179 H. Kohls, M. Anderson, J. Dickerhoff, K. Weisz, A. Córdova, P. Berglund, H. Brundiek, U. T. Bornscheuer and M. Höhne, *Adv. Synth. Catal.*, 2015, **357**, 1808–1814.

180 S. W. Van Arsdell, J. B. Perkins, R. R. Yocum, L. Luan, C. L. Howitt, N. Prasad Chatterjee and J. G. Pero, *Biotechnol. Bioeng.*, 2005, **91**, 75–83.

181 T. Lütke-Eversloh and G. Stephanopoulos, *Metab. Eng.*, 2008, **10**, 69–77.

182 W.-D. Fessner, *Nat. Biotechnol.*, 2015, **32**, 658–664.

183 D.-J. Kim, G.-H. Hwang, J.-N. Um and J.-Y. Cho, *J. Mol. Microbiol. Biotechnol.*, 2015, **25**, 45–50.

184 S. P. Liu, R. X. Liu, A. A. M. M. El-Rotail, Z. Y. Ding, Z. H. Gu, L. Zhang and G. Y. Shi, *J. Biotechnol.*, 2014, **186**, 91–97.

185 S. Yin, H. Zhou, X. Xiao, T. Lang, J. Liang and C. Wang, *Curr. Microbiol.*, 2015, **70**, 762–767.

186 J.-Y. Hwang, J. Park, J.-H. Seo, M. Cha, B.-K. Cho, J. Kim and B.-G. Kim, *Biotechnol. Bioeng.*, 2009, **102**, 1323–1329.

187 I. Borodina, K. R. Kildegard, N. B. Jensen, T. H. Blicher, J. Maury, S. Sherstyuk, K. Schneider, P. Lamosa, M. J. Herrgård, I. Rosenstand, F. Öberg, J. Forster and J. Nielsen, *Metab. Eng.*, 2015, **27**, 57–64.

188 Q. Wang, P. Yang, M. Xian, L. Feng, J. Wang and G. Zhao, *Biotechnol. Lett.*, 2014, **36**, 2257–2262.



189 N. Ladkau, M. Assmann, M. Schrewe, M. K. Julsing, A. Schmid and B. Buhler, *Metab. Eng.*, 2016, **36**, 1–9.

190 A. A. Desai, *Angew. Chem., Int. Ed.*, 2011, **50**, 1974–1976.

191 L. Frodsham, M. Golden, S. Hard, M. N. Kenworthy, D. J. Klauber, K. Leslie, C. Macleod, R. E. Meadows, K. R. Mulholland, J. Reilly, C. Squire, S. Tomasi, D. Watt and A. S. Wells, *Org. Process Res. Dev.*, 2013, **17**, 1123–1130.

192 D. J. C. Constable, P. J. Dunn, J. D. Hayler, G. R. Humphrey, J. J. L. Leazer, R. J. Linderman, K. Lorenz, J. Manley, B. A. Pearlman, A. Wells, A. Zaks and T. Y. Zhang, *Green Chem.*, 2007, **9**, 411–420.

