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ω-Transaminases for the Production of Optically Pure Amines and Unnatural Amino Acids

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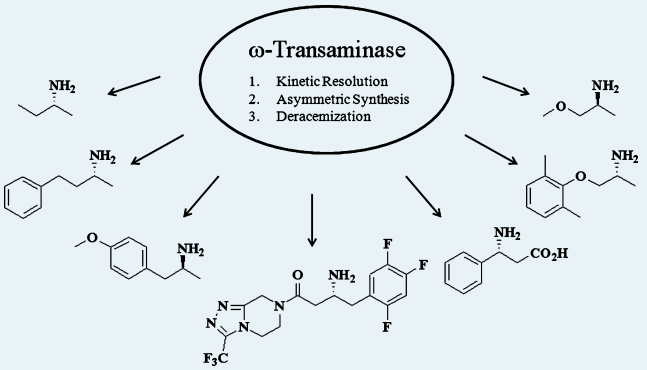
ABSTRACT: ω-Transaminases have been increasingly used as efficient biocatalysts due to their ability to produce a wide range of optically pure amine compounds. Several approaches have been adopted, including screening, engineering, and development of new techniques in reaction systems for different aspects of the enzymes. This review summarizes the various methodologies and approaches adopted to produce enantiomerically pure amines and unnatural amino acids using ω-transaminases.



KEYWORDS: *transaminase, biocatalysis, chiral amines, directed evolution, site directed mutagenesis, deracemization, kinetic resolution, asymmetric synthesis, unnatural amino acids*

1. INTRODUCTION

Until the late 1930s, inadequate experimental resources and data hindered the elucidation of metabolic pathways and characterization of individual reactions.[1] In 1939, Braunstein and co-workers demonstrated the transfer of an amino group between a donor amino acid and an acceptor α-keto acid using living animal tissues for the first time.[2] This generated a keen interest among researchers to investigate the role of transamination in metabolic reactions. Later, Schoenheimer revealed the transfer of amino groups among amino acids and thereby highlighted its key role in amino acid metabolism.[1] After Schoenheimer's discovery, transaminases (TAs) have been the most extensively studied pyridoxal-5′-phosphate (PLP) dependent enzymes.[3] The transfer of an amino group from α-amino acids to α-keto acids is the primary metabolic role of TAs and the ubiquitous occurrence of TA reactions play a vital role in nitrogen metabolism in all organisms.[4,5] The TA reaction mechanism consists of two main steps; in the initial step, PLP is used as a carrier to transport amines and electrons between the amino acceptor and the amino donor. During this reaction, a prototropical rearrangement followed by hydrolysis reversibly converts PLP to pyridoxamine-5′-phosphate (PMP). In the subsequent step, the amino group from PMP is transferred to the amino acceptor to regenerate PLP (Figure 1).

TAs can be broadly classified into α-TAs and ω-TAs based on the relative position of the amino group to be transferred with respect to the carboxyl group of the substrate.[6] ω-TAs transfer an amino group from an amino donor onto a carbonyl moiety of an amino acceptor, in which at least one of the two substances is not an α-amino acid or an α-keto acid.[7] In the case of α-TAs, it requires the presence of a carboxylic acid group in the α-position to the keto or amine functionality and hence only allow the formation of α-amino acids.[8] ω-TAs are more useful, as it is capable to aminate keto acids, aldehydes, and ketones.[9] Moreover, ω-TA reaction generates a high equilibrium constant in contrast to the low equilibrium reactions involving α-TA.[9,10] ω-TAs with different enantiopreference exist: (*R*)- and (*S*)-selective. And until recently, most of them were (*S*)-selective. ω-TA reaction has many desirable features when compared to other enzymes such as hydrolases and dehydrogenases, including broad substrate specificity, high enantioselectivity, high turnover number, and no requirement for regeneration of external cofactors.[11] The use of ω-TAs to produce optically pure chiral amine compounds has been a focus of major research interest, as these compounds are extensively used in pharmaceutical, agricultural, and chemical industries.[9,12]

2. IDENTIFICATION OF NOVEL TRANSAMINASES

The ability to generate optically pure compounds mainly depends on the activity and enantioselectivity of the enzyme toward the substrate. Therefore, screening appropriate enzymes is of vital importance to efficiently produce enantiomerically pure amines. Screening of ω-TAs can be broadly classified into classical and computational methods. The traditional approach of identifying ω-TAs by testing microorganisms for enzyme activity constitutes the classical method, whereas the computational method is based on detecting ω-TAs by analyzing biological databases using algorithms and tools.

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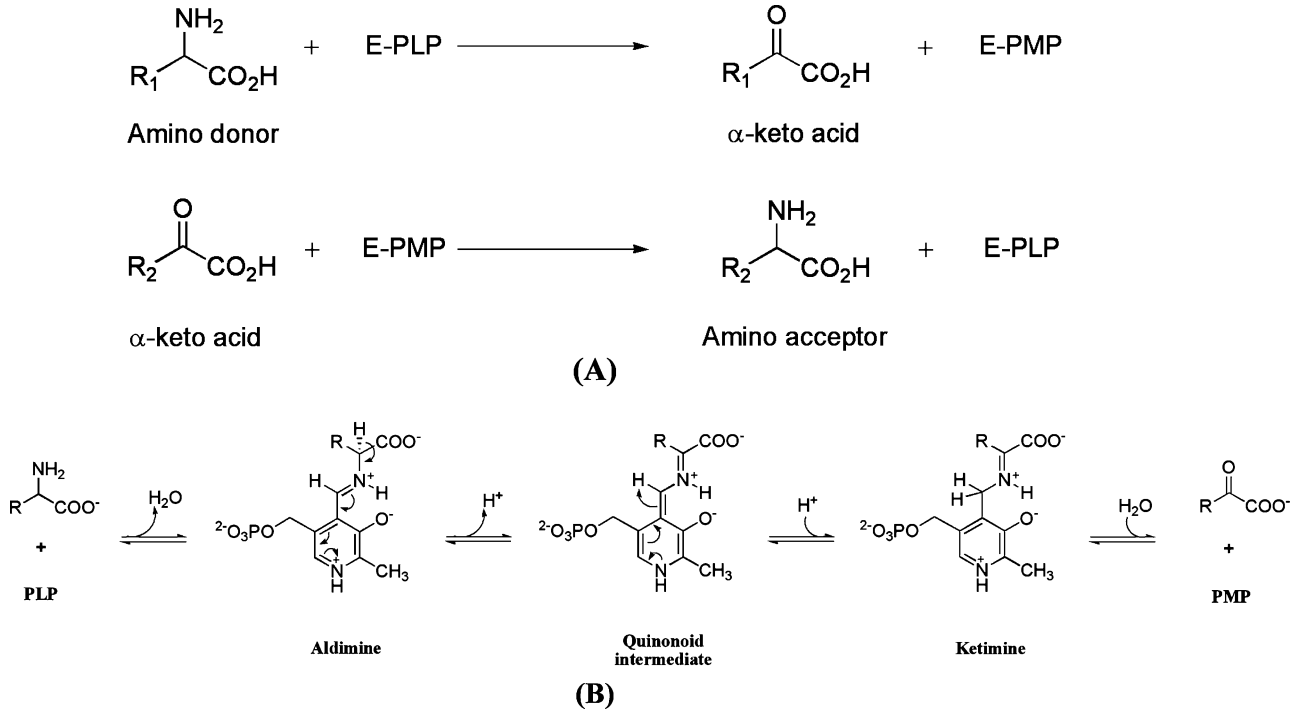

Figure 1. (A) Schematic diagram of transaminase reaction. (B) Detailed reaction mechanism of transaminase.

2.1. Classical Method. Microorganisms constitute an abundant repository for scores of enzymes because of their requirements to synthesize various compounds for metabolic activities. The conventional way of screening ω-TAs from microorganisms is by enrichment culture and later selecting the microorganism showing high enzyme activity and enantioselectivity. Microorganisms containing ω-TA has the capability to utilize amine compounds as nitrogen source for their metabolic activities. By employing this characteristic, (*S*)-α-methylbenzylamine (MBA) was used as the sole nitrogen source to identify ω-TA activity in *Klebsiella pneumonia* JS2F, *Bacillus thuringiensis* JS64, and *Vibrio fluvialis* JS17, among which *V. fluvialis* JS17 had the highest enzyme activity.[13] Later, ω-TAs were screened from *Rhodobacter sphaeroides,*[14] *Alcaligenes denitrificans* Y2k-2,[15] *Bacillus megaterium* SC6394,[16] *Mesorhizobium sp.LUK,*[17] and *Anthrobacter* sp. KNK 168[18] microorganisms using (*S*)-α-MBA, β-amino-*n*-butyric acid, (*R*)-1-cyclopropylethylamine, 3-amino-3-phenylpropionic acid, and *sec*-butylamine, respectively, as the sole nitrogen source.

Kroutil and co-workers have prepared more than 100 lyophilized whole cells of various bacterial species to identify strains with good ω-TA activity.[19] Kinetic resolution of *rac*-α-MBA was performed on these cells by employing pyruvate as amino acceptor. Among investigated strains, *Pseudomonas oleovorans* DSM 1045 exhibited the highest enzyme activity. The reaction produced (*R*)-α-MBA with 50% conversion and >99% ee. Other strains that showed good enzyme activity are *Janibacter terrae* DSM 13953, *Pseudomonas cichorri* DSM 50259, *Pseudomonas fluorescens* ATCC 49838, and *Pseudomonas* sp. NCIMB 11753. Recently, Turner and co-workers developed a novel method to rapidly screen transaminase activity and its enantioselectivity by using D- and L-amino acid oxidases.[20] In this method they have used amino acid oxidases (AAO) to oxidize alanine to imine; H_2O_2 which was formed during this oxidation process was detected colorimetrically by mixing it with pyrogallol red and horse radish peroxidase.

2.2. Computational Method. The last two decades has seen an exponential increase in biological data due to the successful completion of large genome and proteome sequencing projects. In contrast, the number of protein functions that has been discovered is very low as experimental investigation is costly and time-consuming. This scarcity has led to predicting protein functions based on sequence homology.[21] In recent times, computational techniques have been increasingly used to screen ω-TAs from databases and predict their functionalities using BLAST search.[22] ω-TAs screened using BLASTP are given in Table 1.

Table 1. ω-Transaminases Screened Using BLASTP

identified enzyme	source	query sequence	identity	ref
ω-TA-Po (ABE43415.1)	*Polaromonas species*	ω-TA-Ms	54%	12
ω-TA-Cc (AAK25105)	*Caulobacter crescentus*	ω-TA-Ad	65%	22
ω-TA-Cv (NP_901695)	*Chromobacterium violaceum*	ω-TA-Vf	38%	23
ω-TA-Pd (ABL72050.1)	*Paracoccus denitrificans*		94%	24
ω-TA-Oa (YP_001368759.1)	*Ochrobactrum anthropi*	ω-TA-Pd	42%	25
ω-TA-Ab (YP_002319938.1)	*Acinetobacter baumannii*		17%	
ω-TA-Acp (YP_003188652.1)	*Acetobacter pasteurianus*		21%	

Most of the ω-TAs screened until recently were (*S*)-selective, whereas only one (*R*)-selective enzyme was known: ω-TA from *Arthrobacter sp.* Recently, Bornscheuer and co-workers developed an in silico strategy to identify novel (*R*)-selective ω-TAs (Figure 2).[26] Initially structures of related enzymes, including branched chain aminotransferase (BCAT), D-amino acid transferase (DATA), and 4-amino-4-deoxychorismate

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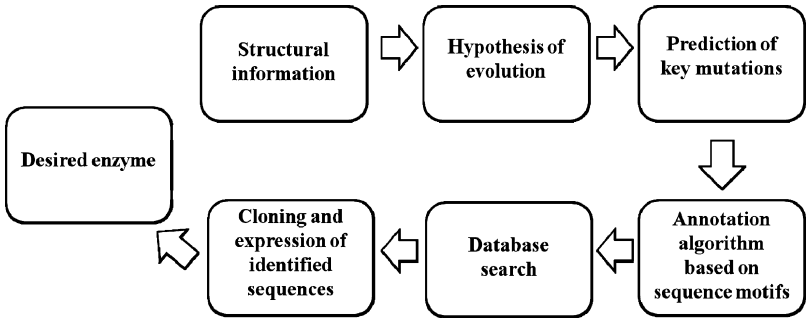

Figure 2. Strategy to computationally screen putative enzymes using in silico approach.[26]

lysase (ADCL) were analyzed to identify motifs of (*S*)- or (*R*)-selective TAs. The presence of DATA and BCAT in the same subgroup of TAs indicated flexibility of substrate recognition for TAs within this subgroup.[27] The opposite enantioselectivity of DATA and BCAT was due to the difference in substrate coordination at the active site, which is constituted by two binding pockets. If the alpha carboxyl group of the amino donor binds to the small binding pocket of the enzyme, (*S*)-enantiomeric compounds are generated. Conversely, (*R*)-enantiomeric compounds are produced if the alpha carboxyl group binds to the large binding pocket of the enzyme. The analysis of these enzymes led to the development of an annotation algorithm. This algorithm was later used to identify 21 putative (*R*)-ω-TAs from 5700 sequences that were present in the sub group by filtering out BCATs, DATAs, and ADCLs. Further investigations on the activity and enantioselectivity of putative (*R*)-ω-TAs showed positive results for 17 enzymes. Later, seven of these enzymes were used to produce enantiomerically pure aliphatic, aromatic, and arylaliphatic amines.[28] Kroutil and co-workers also used two of these enzymes to generate optically pure amines.[29]

3. ENZYME ENGINEERING

The efficiency of an enzyme reaction mainly depends on the availability of appropriate enzymes with high activity, good thermostability, desired substrate selectivity, and high enantioselectivity.[30] Naturally identified enzymes do not always give a good yield toward a wide spectrum of compounds because of poor enantioselectivity and reactivity. Moreover, these enzymes may also be unsuitable in an industrial scale due to its instability during processing conditions.[31] Enzyme engineering is an emerging field that involves designing new proteins with desired functions by mutating wild type enzymes.[32] These mutated enzymes are ideal candidates for industrial production as they not only provide enhanced enzyme activity but also provide better stability and enantioselectivity. The two main techniques employed for improving ω-TA characteristics are (i) random mutagenesis and (ii) site specific mutagenesis.[33] Mutational studies on ω-TAs and its effects are given in Table 2.

3.1. Random Mutagenesis. One of the major issues in the commercial production of compounds using biocatalysts is the incompatibility of enzyme synthesis under extreme reaction conditions. The random mutagenesis (directed evolution) method has been increasingly used as an efficient strategy to improve enzyme characteristics thus making it more suitable for industrial production. Unlike the rational design technique, directed evolution can be performed in the absence of detailed structural information.[30,34] Two main steps are involved in this methodology: (i) generating a genetic library by random mutagenesis and (ii) efficiently screening mutants with desirable properties such as higher enantioselectivity and improved activity. Depending upon the size of the genetic library, various techniques are employed for the high throughput screening (HTS) of variants: in vivo selection (10^8–10^{10} variants), in vitro detection (10^5–10^6 variants), and 96-well microtiter plate format (10^3–10^4 variants).[32]

Table 2. ω-Transaminase Mutants with Its Characteristic Features

wild-type	mutation	characteristic	ref
ω-TA-Vf	P233L/V297A	reduced product inhibition by aliphatic ketone	6
	W57G W147G	enhanced activity toward aliphatic amines	44
	R415K	enhanced activity toward aromatic α-amino acids	
ω-TA-Cc	N285A V227G	enhanced activity toward phenylpropionic acid	22
ω-TA-Ac	17 mutations	improved activity and thermostability for substituted aminotetralin	36
	E326D Y331C E326D/Y331C	enhanced enantioselectivity toward 4-fluorophenylacetone	45
	V328A	enantioselectivity shifted from (*S*) to (*R*) for 4-fluorophenylacetone	
ω-TA-117	27 mutations	enhanced activity toward prositagliptin ketone	46

Directed evolution strategy was used to identify mutant ω-TA-Vf with reduced product inhibition.[6] The mutant library generated by error-prone polymerase chain reaction (PCR) mutagenesis was later screened using a HTS method on enriched minimal media containing 2-aminoheptane as the sole nitrogen source and 2-butanone as an inhibitory ketone. The identified mutant ω-TAmla, showed significantly reduced product inhibition by aliphatic ketones such as 2-heptanone. Another ω-TA-Vf mutant was identified using random mutagenesis method; it showed a 3-fold increase in activity toward 3-amino-3-phenylpropionic acid compared to that of the wild type ω-TA-Vf.[35] However the mutated sites of the enzyme were not reported. A staining solution of CuSO4/MeOH was added to the reaction mixture, the α-amino acid that was generated during the reaction produced blue color complexes when reacted with copper ion. HTS method based on UV–vis spectrophotometer was used to detect this color and thereby identified mutants with enhanced activity from the mutant library. In an another work, mutant ω-TA from *Arthrobacter citreus* CNB05-01 (ω-TA-Ac) that showed improved enzyme activity and thermo stability were identified using directed evolution method with a combination of PCR mutagenesis and colorimetric screening.[36] The colonies were placed in nitrocellulose paper soaked with screening solution.

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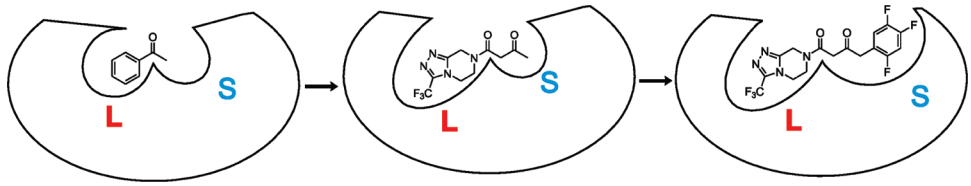

Figure 3. Altered substrate specificity of (*R*)-ω-transaminase by using substrate walking approach to synthesize sitagliptin: (1) Acetophenone bound to the active site of a ω-TA, which consists of a large binding pocket (L) and a small binding pocket (S). (2) The large binding pocket was first altered to generate an enzyme variant reactive toward methyl ketone analog. (3) Further mutagenesis was performed to generate mutant which was reactive toward prositagliptin ketone.[46]

The assay solution consisted of substituted aminotetralin and sodium pyruvate as substrates, which, in turn, produced alanine and substituted tetralone. The color that substituted tetralone acquired when exposed to air was used as a discriminating factor to select mutants with improved activity. The best mutant which contained 17 mutations was identified from the mutant library after three rounds of mutagenesis. The selected mutant showed a 3-fold reduction in biocatalyst loading for tetraline, almost a 5-fold increase in product concentration, 5-fold improvement in product reduction process cycle time, and enhanced thermal stability for an extended period of time (>50 °C).

One of the major constraints while using directed evolution strategy is the screening of a large mutant library.[37] Directed evolution can experimentally screen 10^3−10^6 mutants for each generation. Recent advancements in computational techniques have improved the construction of smaller efficient mutant libraries that contain fewer redundant sequences.[38] The efficiency of directed evolution can be boosted using in silico techniques to prescreen the mutant library. Voigt and co-workers devised a computational method to reduce the screening effort for directed evolution by predicting the enzyme region where mutations are likely to provide better characteristics.[39]

3.2. Site Specific Mutagenesis. Site directed mutagenesis is a very effective strategy to improve enzyme efficiency. However, detailed structural and functional information of the enzyme is required to generate the desired changes.[34] Once the PDB structure of the protein is available, a rational designing approach can be used to study various aspects of the enzyme such as substrate specificity,[40] cofactor binding affinity,[41] enantioselectivity,[42] and stability.[43] In the absence of experimental protein structure, homology modeling can be used to predict reasonably reliable 3D structures, provided a template PDB structure with good sequence similarity (>35%) with the concerned enzyme sequence is available.[30]

A homology model of ω-TA-Vf was effectively used to study its active site. This information was later used to redesign the substrate specificity of ω-TA-Vf by site directed mutagenesis.[44] The wild type ω-TA-Vf had a high activity toward aromatic amines, whereas the activity was poor toward aliphatic amines. The ω-TA-Vf homology model suggested the presence of a large and small binding site. Differences in enzyme activity between the aromatic and aliphatic amines were due to the active site of the enzyme. Two mutant enzymes were generated by introducing two site-directed mutations: W57G and W147G. These mutations contributed in reducing the hydrophobic interaction which in turn helped in overcoming the low activity toward aliphatic amines. This broadened substrate specificity toward aliphatic compounds did not alter the activity or enantioselectivity toward aromatic amines. Another site specific mutation, R415K, in the small binding pocket of ω-TA-Vf, enhanced the activity toward aromatic α-amino acids such as phenylglycine and phenylalanine.

Site specific mutagenesis was also introduced in enhancing the enantioselectivity of ω-TA-Ac toward 4-fluorophenylacetone.[45] A homology model of ω-TA-Ac was generated and point mutations were carried out in three specific sites: Glu326, Val328, and Tyr331. Three of the variants generated from these mutations, E326D, Y331C, and E326D/Y331C, enhanced the enantioselectivity of ω-TA-Ac toward 4-fluorophenylacetone. Interestingly another variant, V328A altered the enantiopreference from (*S*)-selective to (*R*)-selective. However, the altered enantioselectivity was substrate dependent, and the point mutation resulted in (*R*)-selectivity for 4-fluorophenylacetone while retaining (*S*)-selectivity for 4-nitroacetophenone.

A substrate walking approach employing a directed evolution and rational design was performed on (*R*)-ω-TA-117, to develop a mutant that would act as a biocatalyst for sitagliptin synthesis (Figure 3).[46] Docking studies of the modeled ω-TA-117 showed the inability of prositagliptin ketone to bind with the small enzyme binding cavity. Initially, mutations were performed in the large binding pocket to isolate the variant reactive toward truncated methyl ketone analog. The large binding cavity of the enzyme was enlarged using a point mutation: S223P. This variant enhanced the enzyme activity by 11 times toward truncated methyl ketone, compared to its parent. However, it did not show any activity toward prositagliptin ketone. Later, based on structural analysis of the modeled enzyme variant, four residues were selected for generating ω-TA activity toward prositagliptin ketone: Val69, Phe122, Thr283, and Ala284. ω-TA activity toward the targeted substrate was first detected when the mutations V69G, F122I, and A284G were introduced on the enzyme. Subsequently, 10 rounds of mutagenesis were performed on the enzyme to generate the best variant. The final variant contained 27 mutations and converted prositagliptin ketone to sitagliptin with an ee > 99.95%. When compared to the rhodium-based chiral catalyst, which was earlier used to synthesize sitagliptin, the final variant had an increase of approximately 13% in overall yield and a 19% reduction in total waste, which ultimately reduced the production cost. The final ω-TA variant also had broad substrate specificity and better tolerance toward high concentrations of isopropylamine and other organic solvents. Savile's work elegantly demonstrated the potentiality of protein engineering in designing enzymes with desired characteristics. This work was recently highlighted by Desai along with other methods that were used to produce sitagliptin.[47]

4. ENZYME REACTIONS

4.1. Production of Amines. Enantiomerically pure amines can be produced via three reaction schemes using ω-TA: (1)

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asymmetric synthesis from pro-chiral ketones, (2) kinetic resolution, and (3) deracemization from *rac*-amines (Figure 4).

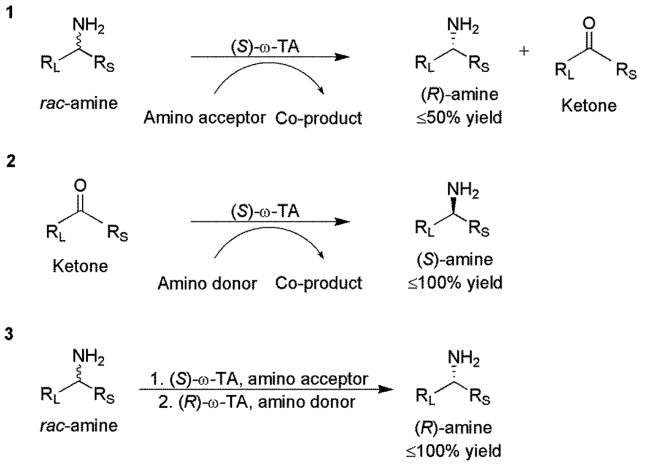

Figure 4. Transaminase reaction systems. (1) During kinetic resolution, a transaminase is used to convert a racemic amine into an optically pure enantiomer and ketone with a theoretical yield of ≤50%. (2) During asymmetric synthesis, pro-chiral ketones are converted into optically pure enantiomers with a yield ≤100% in the presence of a favorable equilibrium. (3) During the deracemization reaction, the racemic amine is converted into an optically pure enantiomer with a theoretical yield ≤100%.

Although the former reaction scheme is more challenging in terms of the equilibrium constant, the synthesis approach is more favored, as it can theoretically generate 100% yield in contrast to the 50% yield produced by the resolution approach.[48,49] Deracemization is the most complex reaction when compared to the other two reaction schemes. However, recently this approach have been increasingly used to generate a very high yield of amines using ω-TA. Some of the important compounds that have been synthesized by these reactions are given in Table 3.

4.1.1. Kinetic Resolution. This method converts *rac*-amines into enantiomerically pure amines with a theoretical yield of 50%. The use of biocatalytic kinetic resolution with hydrolytic enzymes such as proteases, lipases, and esterases has been extensively studied for this purpose.[50] Production of enantiomeric amines can be classified into two categories based on the reaction media: hydrolase-catalyzed aminolysis in nonaqueous medium and TAs in aqueous medium.[11] Enzyme activities in nonaqueous medium have several shortcomings such as low enzyme activity[11,51] due to heterogeneous catalysis, enzyme instability[52−54] due to solvent and phase toxicities, and the need for solvent screening.[55,56] Since the mid 1990s, ω-TA has been increasingly used for the kinetic resolution of *rac*-amines. Nevertheless, a main disadvantage of this process is product inhibition, which makes the reaction unsuitable at high concentrations.[11] This prompted researchers to devise new methods to eliminate ketones and increase product yield.

A number of techniques have been devised to remove ketones from the reaction media and thereby increase product yield (Figure 5a). In kinetic resolution, pyruvate is mainly used as amino acceptor.[7] During a kinetic resolution reaction, amine, pyruvate, ketone, alanine, and enzyme exist in aqueous reaction solution. The inhibitory ketones formed during this reaction can be removed from the aqueous solution using ketone extracting organic solvents. Biocompatibility and ketone extracting capacity are two key criteria in solvent screening, as they directly relate to enzyme stability and resolution yield of the reaction. Ideal organic solvent in terms of ketone extracting capacity can be selected based on its partition coefficients.[10] A biphasic (aqueous/organic) reaction system for kinetic resolution of α-MBA using ω-TA from *B. thuringiensis* successfully demonstrated reduced enzyme inhibition caused by ketones.[10,11] This biphasic reaction with ω-TA is particularly beneficial because of its simplicity and high conversion due to the removal of ketone products.[6,11] As enzyme stability during the reaction is of critical importance, care must be taken to avoid direct contact of the enzyme with the organic solvent, as it may inactivate the enzyme. However, it is difficult to create a kinetic resolution reaction without the enzyme contacting the organic solvent in biphasic system. To overcome this difficulty, a kinetic resolution process combining an enzyme-membrane reactor (EMR) with a membrane contactor was developed.[7,10] This process permits homogeneous catalysis with minimal loss of enzyme activity. The membrane contactor facilitates the rapid and convenient removal of aromatic ketones from the aqueous solution using a hydrophobic extractant. Later, the reaction was carried out using packed-bed reactor (PBR). The kinetic resolution of chiral amines with immobilized whole cells of *V. fluvialis* JS17 in 2% Ca-alginate gel beads was successfully demonstrated using a PBR process.[55] This method reduces substrate and product inhibition by using whole cells entrapped in hydrophilic matrix. However, the internal diffusion limitation in the PBR process affects the rate of the reaction. In situations where the purification of the enzyme is complex, the PBR process using immobilized enzyme is preferred over EMR.

In cases of low molecular weight amines such as *sec*-butylamine, removal using reduced pressure is an ideal option due to volatility of the ketone products. The kinetic resolution of *sec*-butylamine using ω-TA-Vf was carried out under reduced pressure (150 Torr) to selectively eradicate the inhibitory product 2-butanone.[56] The reaction was carried out at pH 7.0 to reduce the evaporation rate of *sec*-butylamine, even though the optimum pH of ω-TA activity for *sec*-butylamine is 9.0. This system is particularly suitable for producing chiral aliphatic amines with low molecular weight. Nevertheless, reducing the pressure may result in evaporation of the reaction media, which is a drawback of this process. Additionally, dehydrogenases can convert ketone into corresponding optically active alcohol.[58,59] This inherent property of alcohol dehydrogenase (ADH) was utilized by coupling ADH and glucose dehydrogenase (GDH) with ω-TA to simultaneously synthesize (*R*)-1-phenylethanol and (*R*)-α-MBA from *rac*-α-MBA.[12] This system is mainly suitable to overcome product inhibition of water-soluble ketones, which is difficult using a biphasic reaction. Yun and co-workers successfully used endogenous oxidoreductases from recombinant *Pichia pastoris* expressing ω-TA to overcome the product inhibition of acetophenone in the kinetic resolution of α-MBA.[59]

Studies have also been focused on carrying out kinetic resolution in a cost-effective manner by recycling the pyruvate used in the reaction. Two techniques are currently been employed for this purpose. Coupling ω-TA with an α-TA is particularly suitable if there is a need for simultaneous synthesis of α-amino acids along with the chiral amines.[60] Another method is by coupling ω-TA with amino acid oxidase.[6] The use of AAO has been efficient in enhancing the conversion of α-MBA from 7.5% to 50% and ee from 8% to 99%.[61] A key

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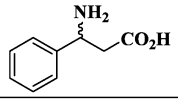
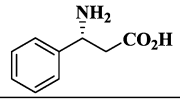
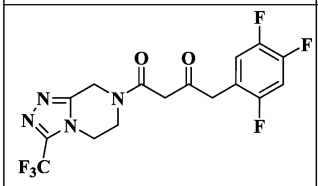
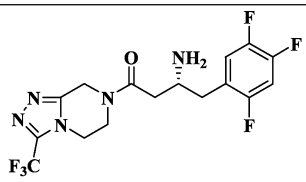
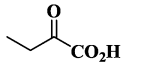
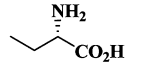
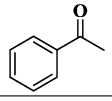
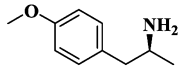
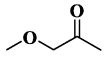
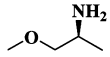
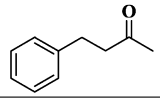
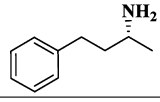
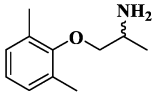
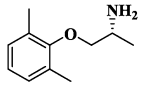
Table 3. Representative Examples of Amines and Unnatural Amino Acids Synthesized Using ω-Transaminases

Substrate	Product	Conversion (%)	*ee* (%)	Enzyme	Reaction	Reference
		50.7	>99	(*S*)-ω-TAPo	Kinetic resolution	12
		53	98	(*S*)-ω-TA-Vf	Kinetic resolution	56
		92	>99.95	(*R*)-ω-TA-117 mutant	Asymmetric synthesis	46
		96	>99	(*S*)-ω-TA-Vf	Asymmetric synthesis	77
		>99	>99	(*S*)-ω-TA-113	Asymmetric synthesis	85
		97	>99	(*S*)-ω-TA-Vf	Asymmetric synthesis	86
		98	>99	(*R*)-ω-TA-117	Asymmetric synthesis	
		>99	>99	(*S*)- ω-TA-Cv & (*R*)- ω-TA-117	Deracemization	70

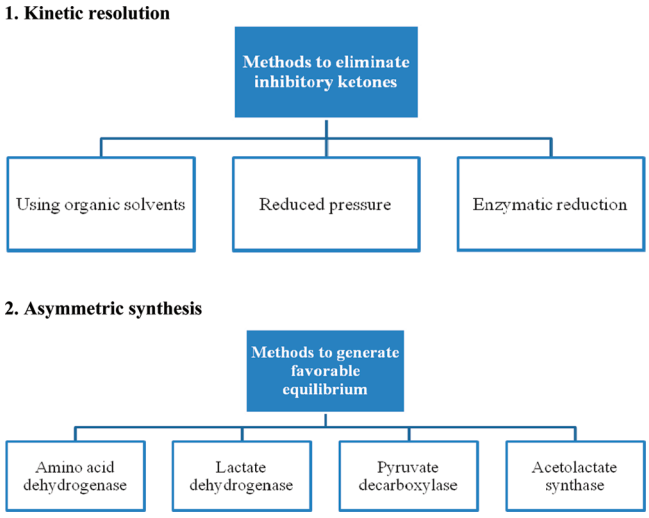


Figure 5. Strategies to improve the yield of transaminase reactions. (1) Kinetic resolution: different methods were devised to remove inhibitory ketones using organic solvents, reduced pressure, and enzyme reduction. (2) Asymmetric synthesis: a favorable equilibrium can be generated by two approaches such as recycling the amino donor from pyruvate and degrading pyruvate. Amino acid dehydrogenase is used to recycle the amino donor from pyruvate, whereas lactate dehydrogenase, pyruvate decarboxylase, and acetolactate synthase are used to degrade pyruvate.

advantage of this method is that it requires only catalytic quantities of pyruvate.

4.1.2. Asymmetric Synthesis. Asymmetric synthesis is one of the most important strategies employed in biotransformation. This technique is used to produce chiral amines by transferring an amino group to prochiral ketones. This process has been of keen interest among researchers, as it can theoretically generate two times the yield produced by the kinetic resolution method. However, an unfavorable thermodynamic equilibrium and acute product inhibition are major challenges facing this technique.[62,63]

Recent studies on the half reactions of ω-TA-Vf with purified PLP-enzyme and PMP-enzyme complexes demonstrated that an ideal pair of substrates prevents heightened product inhibition.[63] The amino donor and acceptor should have higher reactivity when compared to their corresponding ketone and amine product for ideal asymmetric synthesis reaction. Protein−ligand docking helps to analyze the potential reactivity between the enzyme and the substrate which can be effectively utilized to screen ideal pairs of substrates with good activity. The easiest way to shift equilibrium favorably toward the product side is to add excess amino donor. Amination of 4-methoxyphenylacetone using the bacterial microorganism *Brevibacterium linens IFO 12141* increased substantially by adding 16-fold excess alanine.[6,64] However, this strategy cannot be used in situations in which a highly unfavorable equilibrium exists. For example, yield of α-MBA was only slightly improved (from 0.5% to 3%) even though the L-alanine amino donor concentration was increased to 10 equiv.[65] Depending on the chemical properties of the amino donor, its accumulation in the reaction media beyond certain extent will be ineffective, as it may cross the threshold of amino donor solubility.[48]

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Isopropylamine has been increasingly used as an amino donor, as it is cost-effective and easily obtainable.[45,46,57,61,65−67] Moreover, the byproduct acetone formed during the synthesis reaction is highly volatile and can be removed easily. Recently, a number of pharmacologically relevant amine compounds such as sitagliptin have been synthesized using isopropylamine as the amine donor.[46]

Several enzyme coupling methods with ω-TA have been devised to improve the effectiveness of asymmetric synthesis by degrading pyruvate (Figure 5b). Degradation of the pyruvate using amino acid dehydrogenase (AADH) and lactate dehydrogenase (LDH) along with NADH cofactor is a convenient way to generate favorable reaction equilibrium. AADH and LDH convert pyruvate generated in the reaction to alanine and lactate respectively. One of the drawback of the above two methods is the requirement of expensive NADH cofactor. However, the system can be made more efficient by adding additional enzymes such as formate dehydrogenase (FDH) or GDH to recycle the NADH cofactor.[65,66] The degradation of pyruvate using AADH with ω-TA-117 and GDH was used for the production of (*R*)-MBA products from acetophenone with 96% conversion and ee > 99%.[65] This system is very economical as only a small amount of alanine is required, since the pyruvate generated is recycled using GDH and ammonia. The inclusion of LDH/GDH in ω-TA reaction system for the generation of (*S*)-α-MBA from acetophenone increased the conversion from <0.5% to 70%.[65] Recently, LDH/GDH system has been employed for the synthesis of many compounds.[11,28,29,48,49,61,63,65−67]

Another method that is used to degrade pyruvate formed in the reaction is by utilizing pyruvate decarboxylase (PDC).[48,68] The advantage of this method is that it does not require recycling of cofactor and the byproduct formed are highly volatile, which can be removed easily. Three PDCs from *Zymomonas mobiliz*, *Zymobacter palmae*, and a recombinant PDC were effectively used along with ω-TA-Vf to increase the yield of three amines: 1-*N*-Boc-3-aminopyrrolidine, 3-Boc-aminopiperidine, and 1-methyl-3-phenylpropylamine dramatically.[68] Pyruvate can also be decarboxylated to acetoin by coupling acetolactate synthase with ω-TA. A recombinant *E. coli* coexpressing ω-TA and acetolactate synthase as a whole-cell biocatalyst was used to remove pyruvate from the reaction media.[60] A solvent-bridge reaction system was used to overcome product inhibition and increase the yield of the whole cell reaction by 2.5-fold. The inhibitory (*S*)-α-MBA produced in the ω-TA-Vf reaction solution (pH 8.0) was transferred into the extraction solution (pH 3.0) via an organic solvent (iso-octane).

4.1.3. Deracemization. A deracemization technique is used to convert a racemic mixture into a single enantiomer with 100% theoretical yield. The production of enantiomeric amines by deracemization catalyzed by ω-TA has been achieved using two different approaches: (i) dynamic kinetic resolution (DKR) and (ii) a two-step one-pot process. Deracemization using ω-TA was first developed by Kroutil and co-workers to produce both (*R*) and (*S*) enantiomerically enriched amine from 4-phenylpyrrolidin-2-one using the DKR method.[69] ω-TA-Vf and three commercially available ω-TAs—ω-TA-113, ω-TA-114, and ω-TA-117—along with L- or D-alanine as amino donors were used for the reaction. Three ω-TAs such as ω-TA-113, ω-TA-114, and ω-TA-Vf exhibited (*S*)-selectivity, among which the ω-TA-113 catalyzed reaction showed the best result with 99% conversion and 45% ee. ω-TA-117 showed (*R*) preference with 99% conversion and 65% ee.

A one-pot two-step process was used to synthesize both enantiomers of mexiletine employing ω-TA.[70] In the first phase of the reaction, kinetic resolution of the racemic mixture was performed using ω-TA. This was followed by stereoselective amination using an opposite enantioselective ω-TA. Seven ω-TAs, including ω-TA-113, ω-TA-117, ω-TA-Vf, ω-TA-Cv, ω-TA-Ad, and ω-TA-Bm from *Bacillus megaterium* and mutant (CNB0501) ω-TA-As from *Arthrobacter sp*, were tested using this process. All enzymes except ω-TA-117 displayed (*S*)-selectivity. Using this approach, optically pure (*S*)-mexiletine with 98% conversion and ee > 99% was achieved using ω-TA-117 and ω-TA-113 in the two step process. Similarly, ω-TA-113 and ω-TA-117 enzymes were employed in the two step process to form (*R*)-mexiletine with 97% conversion and ee > 99%.

Racemization is a key step for successful deracemization using the DKR method. Metal and enzymatic catalysts used for DKR of secondary alcohols have been widely established.[71] However, efficient catalysis for the selective racemization of amines is scarce. Metal catalysis is not suitable when used with amines, as it has a tendency to bind with metal ions and hamper the reaction.[72,73] Higher chemoselectivity and intrinsic mild reaction conditions makes enzymes an ideal system for the amine racemization. (*S*)-1-Methoxy-2-propylamine has been catalyzed by a set of complementary enantioselective ω-TAs, ω-TA-117, and ω-TA-Vf, to produce a clean racemic mixture.[72] The presence of two opposite optically active biocatalyts and the addition of an external ketone to facilitate the amino group transfer between the substrates enhanced the speed of racemization.

4.2. Production of Unnatural Amino Acids. The industrial production of unnatural amino acids has become very important due to its requirement from diverse sectors such as the pharmaceutical, cosmetic, food, chemical, and agricultural industries.[74,75] Amino acids are traditionally produced using extraction and fermentation methods. However, the production of unnatural amino acids using the fermentation process has not been well established.[74,75] The enzyme catalysis approach has often been used for this purpose mainly due to its ability to produce customized and highly selective products in a cost-effective way. Among the different biocatalytic methods used to produce enantiomerically pure unnatural amino acids, TAs is one of the most prominent biocatalysts.[3,69,76] ω-TAs have been increasingly used to prepare both α and β unnatural amino acids using kinetic resolution and asymmetric synthesis.

4.2.1. α-Amino Acids. The enantiomerically pure unnatural amino acid L-homoalanine was asymmetrically synthesized from 2-oxobutyric acid and benzylamine using ω-TA-Vf.[77] However, severe product inhibition by benzaldehyde during the reaction drastically reduced conversion. Later, a biphasic reaction system was introduced to overcome the acute product inhibition of benzaldehyde. Hexane was selected as the solvent for the reaction based upon its partition coefficient. The biphasic system was able to reduce product inhibition to a great extent and thereby increase yield from 39% to 96% with an ee > 99%. A coupled enzyme reaction consisting of threonine deaminase (TD) and ω-TA-Vf were employed to produce an unnatural amino acid: L-homoalanine by one-pot conversion from L-threonine.[24] In this reaction, TD deaminates L-threonine to 2-oxobutyrate, which was later asymmetrically transformed to L-homoalanine by ω-TA. One of the major advantages of this

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system is to completely avoid the need for α-keto acid as a reactant and, thereby, the reaction can be conducted in a cost-effective manner. Recently, deracemization method was used to generate enantiomerically pure L-homoalanine from *rac*-homoalanine by combining D-AAO and ω-TA-Vf. A whole cell reaction using biphasic system converted 500 mM *rac*-homoalanine to 485 mM L-homoalanine (>99% ee).[78] ω-TA-Vf was also asymmetrically synthesize (*R*)-3-fluoroalanine from 3-fluoropyruvate and (*S*)-α-MBA with 95% conversion and an ee > 99%.[79]

Enantiomerically pure (*S*)-amino acids and (*R*)-amines were simultaneously synthesized using coupled (α/ω)-TA reactions. The coupling of α-TA with ω-TA is mainly used to overcome the low equilibrium constant of α-TA and increase the yield of the optically active amino acid and amines simultaneously. Three pairs of coupled reactions such as AlaTA/ω-TA, TyrTA/ω-TA, and AspTA/ω-TA were used to produce (*S*)-amino acids such as (*S*)-phenylalanine, (*S*)-homophenylalanine, and (*S*)-aspartate.[80] However, the ketone that was generated from the ω-TA reaction resulted in severe product inhibition at high concentrations, which resulted in a low yield. Later, a biphasic reaction system was introduced to overcome the product inhibition that was observed in the aqueous system and enhanced the product yield considerably. Dioctylphthalate was selected as the solvent for the reaction system based on the partition coefficient, enzyme activity, and biocompatibility. (*S*)-phenylalanine and L-homoalanine were asymmetrically synthesized using AroAT/ω-TA and AlaTA/ω-TA coupling reactions with a conversion of 93% and 95%, respectively. Concurrently, the reaction yielded (*R*)-α-MBA with 56% (95% ee) and 54% (96% ee), respectively. Another three aliphatic (*S*)-amino acids: (*S*)-valine, (*S*)-leucine, and L-homoalanine were also produced with the same system with a conversion of 73−90% and an ee > 99%. Simultaneously, *rac*-amines were also resolved with an ee of 83−99% (*R*).[59]

4.2.2. β-Amino Acids. β-amino acids are used in the preparation of synthetic drugs antibiotics, enzyme inhibitors, and other compounds with pharmacological properties.[12] For example, β-amino acids have been incorporated into a number of drugs such as cispentacin and taxol which are used for their antifungal and antitumor activities, respectively.[16,81−84] ω-TA has been successfully used to generate both aliphatic and aromatic β-amino acids. An enantiomerically pure aliphatic β-amino acid: D-β-amino-*n*-butyric acid was produced using ω-TA-Ap.[15] ω-TA-Ap was obtained from *A. denitrificans* Y2k-2 and was used to kinetically resolve racemic β-amino-*n*-butyric acid with 53% conversion and 99% ee (*S*).

Later, a β-TA gene, cloned from *Mesorhizobium* sp. strain LUK and coupled with lipase from *C. rugosa*, was used to asymmetrically synthesize optically pure aromatic β-amino acids from a ketocarboxylic acid ester substrate.[84] The D-β-amino carboxylic acid was synthesized with a 20% yield and 99% ee. Although this enzyme was named β-TA to emphasize its activity toward β-amino acids; the enzyme can be generally called ω-TA. Recently, the protein sequence of this enzyme was used to identify ω-TA-Po by BLAST search.[12] ω-TA-Po was employed to kinetically resolve five *rac*-aromatic β-amino acids such as 3-amino-3-phenylpropanoic acid, 3-amino-3-(4-fluorophenyl) propanoic acid, 3-amino-3-(4-methoxyphenyl) propanoic acid, 3-amino-3-(3,4-dimethoxyphenyl) propanoic acid, and 3-amino-3-benzo[1,3]dioxol-5-ylpropanoic acid into (*R*)-β-amino acids with an ee > 99% and a conversion of approximately 50%.

CONCLUSION

The recent spurt in research activities related to ω-TAs has clearly demonstrated that this group of enzymes has become important in the production of optically pure amine compounds. Besides the traditional approach of screening enzymes from microorganisms, the post genomic era has opened up new techniques to computationally screen novel enzymes by analyzing genomic data available in public databases. The recent synthesis of sitagliptin by combining random and site-directed mutagenesis methods has provided a new approach to generate desirable variants that are required for the commercial production of amine compounds. Optimizing reaction systems is also of critical importance to generate good yields. The development of deracemization techniques would be an interesting area for researchers due to its ability to produce optically pure amine compounds from racemic mixture with a theoretical yield that is double than that of kinetic resolution. Another promising area is the production of unnatural amino acids using ω-TAs. Recent advances in various aspects of ω-TAs promise immense potential to efficiently generate a wide range of enantiomerically pure amines and unnatural amino acids.

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Notes

The authors declare no competing financial interest.

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