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Comparison of the ω-Transaminases from Different Microorganisms and Application to Production of Chiral Amines

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Microorganisms that are capable of (*S*)-enantioselective transamination of chiral amines were isolated from soil samples by selective enrichment using (*S*)-α-methylbenzylamine ((*S*)-α-MBA) as a sole nitrogen source. Among them, *Klebsiella pneumoniae* JS2F, *Bacillus thuringiensis* JS64, and *Vibrio fluvialis* JS17 showed good ω-transaminase (ω-TA) activities and the properties of the ω-TAs were investigated. The induction level of the enzyme was strongly dependent on the nitrogen source for the strains, except for *V. fluvialis* JS17. All the ω-TAs showed high enantioselectivity ($E>50$) toward (*S*)-α-MBA and broad amino donor specificities for arylic and aliphatic chiral amines. Besides pyruvate, aldehydes such as propionaldehyde and butyraldehyde showed good amino acceptor reactivities. All the ω-TAs showed substrate inhibition by (*S*)-α-MBA above 200 mM. Moreover, substrate inhibition by pyruvate above 10 mM was observed for ω-TA from *V. fluvialis* JS17. In the case of product inhibition, acetophenone showed much greater inhibitions than L-alanine for all ω-TAs. Comparison of the enzyme properties indicates that ω-transaminase from *V. fluvialis* JS17 is the best one for both kinetic resolution and asymmetric synthesis to produce enantiomerically pure chiral amines. Kinetic resolution of *sec*-butylamine (20 mM) was done under reduced pressure (150 Torr) to selectively remove an inhibitory product (2-butanone) using the enzyme from *V. fluvialis* JS17. Enantiomeric excess of (*R*)-*sec*-butylamine reached 94.7% after 12 h of reaction.

Key words: ω-transaminase; enrichment culture; chiral amine; kinetic resolution; asymmetric synthesis

Enantiomerically pure chiral amines have great utilities not only as chiral synthons for pharmaceutical and agrochemical specialties but also as resolving agents for chiral acids.[1–5] Due to their industrial importance, many successful examples of biocatalytic as well as chemical production methods using chiral amines have been reported.[4–10] Biocatalytic methods of producing chiral amines usually comprise kinetic resolution of racemic amines using ω-transaminase (ω-TA)[4,9] or hydrolases such as lipase and subtilisin.[1,5–8]

Transaminase is ubiquitous in microorganisms and plays an important role in nitrogen metabolism.[4,11] It has been studied extensively since it was discovered over 60 years ago. However, few processes using the enzymes have been commercialized due to the low equilibrium constant of the transaminase reaction despite its high reaction rate, broad substrate specificity, no requirement for an external cofactor, and high stability. Unless the equilibrium problem is easily solved, high conversion cannot be obtained. Recently, removing one of the products by coupling other enzymes was successfully implemented to produce unnatural amino acids and D-amino acids.[12,13]

In the case of transamination between chiral amines and pyruvate using ω-TA, there is no such problem of unfavorable equilibrium position.[14] However, inhibition by the ketone product is a major drawback that should be overcome for an efficient resolution process. In previous reports, we demonstrated that removal of the inhibitory ketone product by extraction could increase the reaction rate dramatically.[9,14]

In this report, we compare of properties of three ω-TAs from different microorganisms, *i.e. Klebsiella pneumoniae* JS2F, *Bacillus thuringiensis* JS64, and *Vibrio fluvialis* JS17. This comparison would be helpful for the understanding general properties of the ω-TA and constructing a reaction set-up for kinetic resolution of racemic amines with ω-TA. We also present kinetic resolution of *sec*-butylamine using the ω-TA from *V. fluvialis* JS17.

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Materials and Methods

Isolation of microorganisms. Soil samples collected from different polluted sites around chemical and fertilizer plants were incubated in LB medium for approximately 10 h. The cell broth was transferred to a minimal medium in which (*S*)-α-MBA was a sole nitrogen source (50 mM of potassium phosphate buffer (pH 7.0), 30 ~ 50 mM of (*S*)-α-MBA, 100 mM of glycerol, 1 g/l of $MgSO_4 \cdot 7H_2O$, 0.2 mM of $CaCl_2$, and trace metal components as described previously),[9] and then enrichment of the microorganisms was done by repeated dilution of the culture broth with fresh medium (100 ~ 200-fold). After the enrichment, the culture broth was spread out on the same kind of minimal media agar plates. Each colony on the plate was cultivated in the same minimal media, and then specific ω-TA activities using whole-cell reactions were measured (α-MBA and pyruvate as substrates). Microorganisms showing the highest specific activity were stored at −70°C for further studies.

Cell cultivation and preparation of dialyzed cell extract. Colonies on LB agar plates were inoculated in 50 ml of LB medium and the culture broth was incubated for approximately 12 h at 37°C. In the case of the cultivation of *K. pneumoniae* JS2F and *B. thuringiensis* JS64, the seed culture broth was inoculated into 2 l of the same culture medium as for the enrichment culture except that 10 mM of (*S*)-α-MBA was used. In the case of *V. fluvialis* JS17, the seed culture medium was mixed into 2 l of LB medium, since the induction of the ω-TA did not require the use of minimal medium. The cultivation was done with a New Brunswick Bioflo IIc fermentor at 37°C and 500 rpm under pH control at 7.0.

Cells were harvested at late exponential phase by centrifugation, and washed two times with phosphate buffer (50 mM, pH 7.0). The cell pellet was resuspended in 10 ml of phosphate buffer (10 mM, pH 7.2) containing 20 μM pyridoxal 5′-phosphate, 2 mM EDTA, 1 mM PMSF, and 0.01% (v/v) 2-mercaptoethanol. The cells were then disrupted for 20 min with a sonicator and the resulting supernatant was dialyzed for 24 h against 10 mM phosphate buffer (pH 7.0) containing 20 μM pyridoxal 5-phosphate, 0.2 mM EDTA, 10 μM PMSF, and 0.001% (v/v) 2-mercaptoethanol. The dialyzed cell extract was stored at −20°C for further study. One unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmole of acetophenone in 1 min at 50 mM (*S*)-α-MBA and 50 mM pyruvate.

Enzyme assays. Unless otherwise specified, the activity assay was done at 37°C and pH 7.0 using dialyzed cell extract. A typical reaction volume was 200 μl. Adding enzyme solution of dialyzed cell extract in the reaction mixture started the reactions. After the predetermined reaction time, reactions were stopped by adding 75 μl of 16% (v/v) perchloric acid. The rate of the forward reaction, *i.e.* deamination of (*S*)-amine, was measured by analyzing the ketone product using HPLC. The rate of the reverse reaction, *i.e.* amination of achiral ketone resulting in the formation of (*S*)-amine, was measured by analyzing the (*S*)-amine produced using chiral HPLC.

Kinetic resolution of sec-butylamine. Kinetic resolution was done at 30°C under reduced pressure (150 Torr). Pressure was controlled by a vacuum regulator (NVC 1100, EYELA, Tokyo). Initial concentrations of *sec*-butylamine and sodium pyruvate were 20 and 50 mM, respectively. The enzyme concentration was 10 U/ml and total reaction volume was 5 ml. Periodically samples of the reaction mixture were taken and the *ee* of *sec*-butylamine was measured by HPLC.

Chemicals. Racemic α-MBA, 1-aminotetralin, 1-aminoindan, and 1-methyl-3-phenylpropylamine were from Aldrich Chemical Co. (Milwaukee, WI). Each enantiomer of α-MBA was also purchased from Aldrich Chemical Co. Pyruvate, glyoxylate, L-alanine, α-ketoglutarate, and oxalacetate were from Sigma Chemical Co. (St. Louis, MO). All other reagents were from Aldrich Chemical Co. and of the highest grade available.

Analytical methods. Quantitative chiral analysis of α-MBA was done by using a Crownpak CR (Daicel Co., Japan) column with a Waters HPLC system (Milford, MA) as described elsewhere.[9] Analysis of acetophenone, benzylacetone, 1-indanone, and α-tetralone was done using a Symmetry HPLC column (Waters, MA) with isocratic elution of acetonitrile/water (50/50 v/v) at 1 ml/min. All UV detection was done at 205 nm. Chiral analysis of *sec*-butylamine was done after derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate[17] using a Symmetry HPLC column (Waters, MA) with isocratic elution of methanol/10 mM potassium phosphaste buffer (pH 2.8) (30/70 v/v) at 1 ml/min. UV detection was done at 250 nm.

Results

Screening of microorganisms

Enrichment culture and selection of the microorganism showing the highest specific ω-TA activity were done three times with different soil samples. *K. pneumoniae* JS2F, *B. thuringiensis* JS64, and *V. fluvialis* JS17 were isolated at each round of screening. All the strains showed (*S*)-enantioselectivity for α-MBA, and *ee* values of (*R*)-α-MBA after whole cell reaction using racemic α-MBA and pyruvate were all above 99%.

We also isolated some strains that were capable of using (*R*)-α-MBA as a nitrogen source (data not shown). However, all of them showed similar growth rates in both minimal media containing each enantiomer of α-MBA as a sole nitrogen source. (*S*)-specific ω-TAs were identified from these strains, while enzymes responsible for the metabolism of (*R*)-α-MBA have not been identified yet.

Effects of nitrogen sources on enzyme induction

It was expected that the nitrogen source would strongly affect the induction level of ω-TA because the enzyme participates in nitrogen metabolism. Table 1 lists specific ω-TA activities in whole cell reactions according to the nitrogen sources in the culture media. Specific activities of *K. pneumoniae* JS2F and *B. thuringiensis* JS64 were greatly dependent on the nitrogen sources, and complex media such as LB medium gave lower ω-TA activities compared to the minimal medium containing (*S*)-α-MBA. Primary amines such as *n*-propylamine, isopropylamine, and (*S*)-α-MBA were good inducers for both *K. pneumoniae* JS2F and *B. thuringiensis* JS64. No amino acids were better inducers than (*S*)-α-MBA for either strain. In contrast, the enzyme induction in *V. fluvialis* JS17 was nearly independent of nitrogen sources and the highest specific activity was obtained with LB medium, suggesting that ω-TA of *V. fluvialis* JS17 is a constitutive enzyme.

Table 1. Effects of Nitrogen Source on the Relative Specific Activities[a] of the Microorganisms

Nitrogen source	*K. pneumoniae* JS2F	*B. thuringiensis* JS64	*V. fluvialis* JS17
(*S*)-α-MBA	100	100	100
n-propylamine	206	73.1	60.4
Isopropylamine	324	62.3	88.7
L-leucine	8.7	4.4	76.6
L-glutamate	41.6	12.0	70.0
L-alanine	0.6	15.9	91.7
L-valine	88.1	8.8	100
Glycine	6.1	46.5	93.2
L-arginine	0.8	31.5	86.7
L-phenylalanine	0.7	22.0	67.8
L-tryptophan	22.8	16.5	65.5
L-tyrosine	60.1	3.1	74.6
β-alanine	9.4	70.6	95.9
LB medium[b]	2.4	24.5	156

[a] Each cell type was cultivated at 37°C in 10 ml of a minimal medium containing 10 mM of each nitrogen source, 100 mM of glycerol, 50 mM of potassium phosphate (pH 7.0), and metal components. After 1 day of cultivation, cells were harvested and used in the whole-cell reaction at 50 mM of (*S*)-α-MBA and 50 mM of pyruvate.

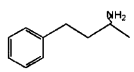
[b] No other components were added to the LB medium.

Substrate specificity

Amino donor specificities of the three ω-TAs for selected arylic amines are compared in Table 2 All the ω-TAs show lower activities toward 1-methyl-3-phenylpropylamine than α-MBA. The ω-TAs from *K. pneumoniae* JS2F and *V. fluvialis* JS17 show higher activities toward 1-aminotetralin and 1-aminoindan than α-MBA. All the ω-TAs show very low reactivities toward 3-amino-3-phenylpropionic acid and α-phenylglycinol which bear other functional groups attached to the β carbon relative to the amino group (data not shown). Compared to the arylic amines, aliphatic amines such as 3-aminoheptane, 1-cyclohexylethylamine, and *sec*-butylamine had low reactivities for all of the ω-TAs (data not shown).

Amino acceptor specificities were investigated using four keto acids and three aldehydes (Table 3). Among the keto acids, pyruvate showed the highest amino acceptor reactivities for all the ω-TAs. Besides pyruvate, considerable reactivities were observed for glyoxylate. All of them show little reactivities for oxalacetate and α-ketoglutarate, which are common amino acceptors for transaminases. In addition to pyruvate, aldehydes showed good reactivities. In the case of the ω-TA from *B. thuringiensis* JS64, propionaldehyde and butyraldehyde showed even higher reactivities than pyruvate. Unlike aldehyde, ketones such as 3-pentanone and methyl isobutyl ketone were found to be inert for all the ω-TAs (data

Table 2. Amino Donor Specificities of the Three ω-TAs

Amino donor		*K. pneumoniae* JS2F	*B. thuringiensis* JS64	*V. fluvialis* JS17[b]
α-MBA		170[a]	173	158
1-methyl-3-phenylpropyl-amine		73.6	100	43.7
1-aminotetralin		285	47.7	555
1-aminoindan		268	191	658

[a] All the values in this table are the initial rates (μM/min) at the following reaction conditions: 50 mM of racemic amine donor, 50 mM of pyruvate, 50 mM phosphate buffer (pH 7.0), and 0.22 U/ml of dialyzed cell extract.

[b] The data are from our previous paper (ref. 16).

Table 3. Amino Acceptor Specificities of the Three ω-TAs

Amino acceptor	*V. fluvialis* JS17	*B. thuringiensis* JS64	*K. pneumoniae* JS2F
Pyruvate	220[a]	222	227
Oxalacetate	0.3	2.4	3.0
α-ketoglutarate	1.0	3.0	3.3
Glyoxylate	34.9	23.1	31.1
Propionaldehyde	21.5	398	78.8
Butyraldehyde	102	323	156
Benzaldehyde	83.3	102	46.5

[a] All the values in this table are the initial rates (μM/min) at the following reaction conditions: 50 mM of (*S*)-α-MBA, 50 mM of amino acceptor, 50 mM phosphate buffer (pH 7.0), and 0.22 U/ml of dialyzed cell extract.

Table 4. Comparison of the Reaction Rates of Asymmetric Synthesis

Strains	Relative specific reaction rate[a]
K. pneumoniae JS2F	9.7
B. thuringiensis JS64	25.2
V. fluvialis JS17	100

[a] The reaction was done using dialyzed cell extract under the following conditions: 10 mM of acetophenone, 100 mM of L-alanine, and 50 mM phosphate buffer (pH 7.0). Concentrations of (*S*)-α-MBA produced were measured using chiral HPLC.

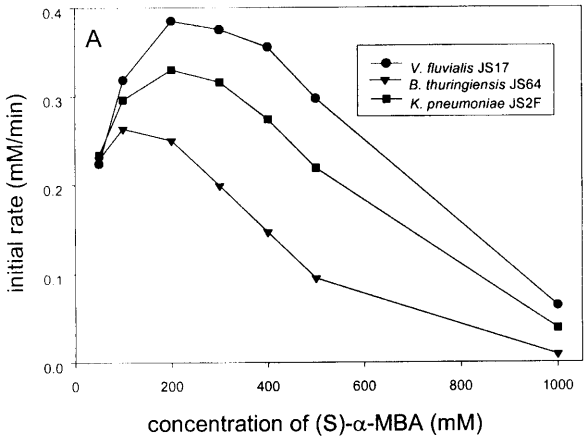
not shown).

Enantioselectivity

The three ω-TAs show (*S*)-enantioselectivity ($E>50$) for all the amino donors in Table 2. The ω-TA from *V. fluvialis* JS17 shows the highest E value, above 140. For all the ω-TAs, no detectable concentration of acetophenone was observed using (*R*)-α-MBA as an amino donor for the measurement of the initial rate. Due to the reversibility of the ω-transamination, the high enantioselectivities afford a stereoselective amination of achiral ketone yielding enantiopure (*S*)-amines.[15] Relative specific reaction rates of the asymmetric synthesis are compared in Table 4. The enantiomeric excess of (*S*)-α-MBA produced was above 99% with all the ω-TAs due to the high enantioselectivities. The ω-TA from *V. fluvialis* JS17 shows the highest reaction rate of the asymmetric synthesis.

Substrate inhibition

Substrate inhibition by (*S*)-α-MBA was not observed at all below 100 mM. However, when the concentration of (*S*)-α-MBA exceeded 100 mM, the initial rate decreased greatly as shown in Fig. 1(A). Initial rates begin to decrease at approximately 200, 100, and 200 mM for the ω-TAs from *V. fluvialis* JS17, *B. thuringiensis* JS64, and *K. pneumoniae* JS2F, respectively. Considering that the same concentrations of the enzymes were used, the ω-TA from *V. fluvialis* JS17 shows the lowest substrate inhibition



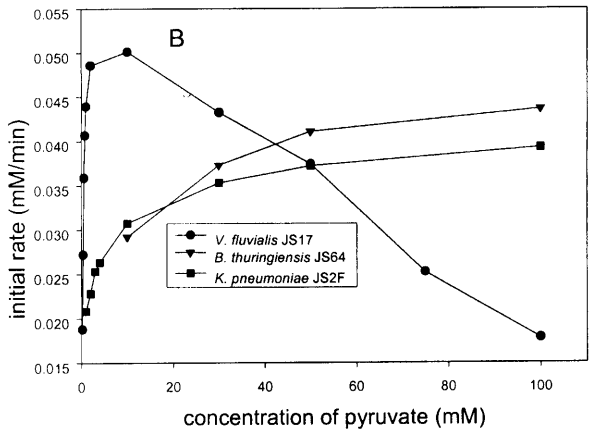


Fig. 1. The Substrate Inhibition of the ω-Transaminases from Different Sources.

(A) inhibition by (*S*)-α-MBA at 50 mM of pyruvate and 0.22 U/ml of enzyme (B) inhibition by pyruvate at 50 mM of (*S*)-α-MBA and 0.043 U/ml of enzyme.

because the reaction rate is higher than those of the other two ω-TAs at above 50 mM of (*S*)-α-MBA.

In the case of the amino acceptor, *i.e.* pyruvate, its substrate inhibition varies significantly depending upon the sources of the ω-TAs (Fig. 1(B)). The enzymes from *K. pneumoniae* JS2F and *B. thuringiensis* JS64 do not show substrate inhibition by pyruvate up to 100 mM. Unlike the enzymes, the enzyme from *V. fluvialis* JS17 shows severe substrate inhibition and initial rates begin to decrease at 10 mM. The substrate inhibition by pyruvate is much more severe than by (*S*)-α-MBA, suggesting that the control of pyruvate concentration in the reaction mixture is more significant to maintain the maximum enzyme activity.

Product inhibition

Figure 2 shows that activities of all the ω-TAs are inhibited much more severely by acetophenone than by L-alanine. Among the three, the ω-TA from *B. thuringiensis* JS64 shows the most severe product inhibition by acetophenone, and the one from *K. pneu-*

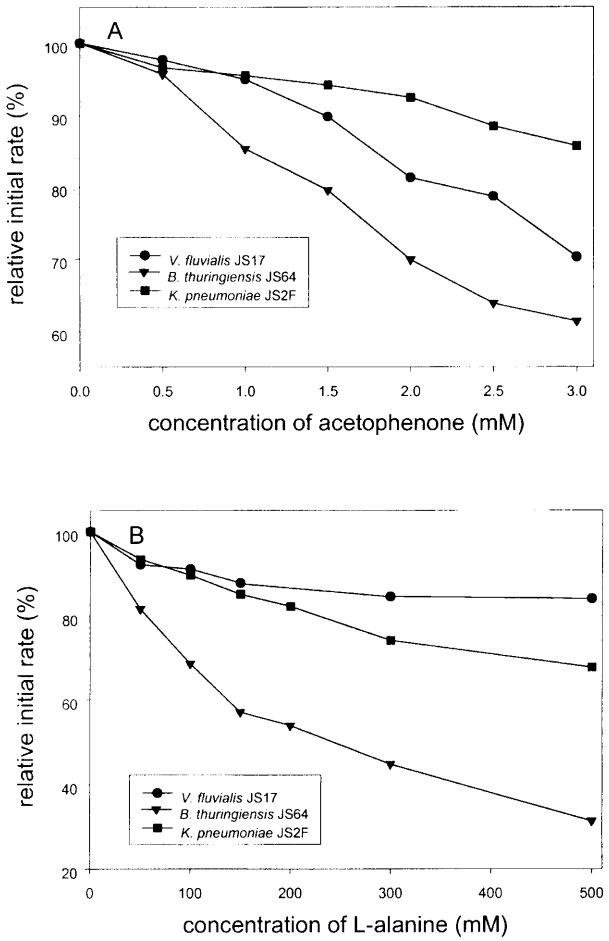


Fig. 2. The Product Inhibition of the ω-Transaminases from Different Souces.

Reaction conditions: 50 mM of (*S*)-α-MBA and 50 mM of pyruvate (A) inhibition by acetophenone (B) inhibition by L-alanine.

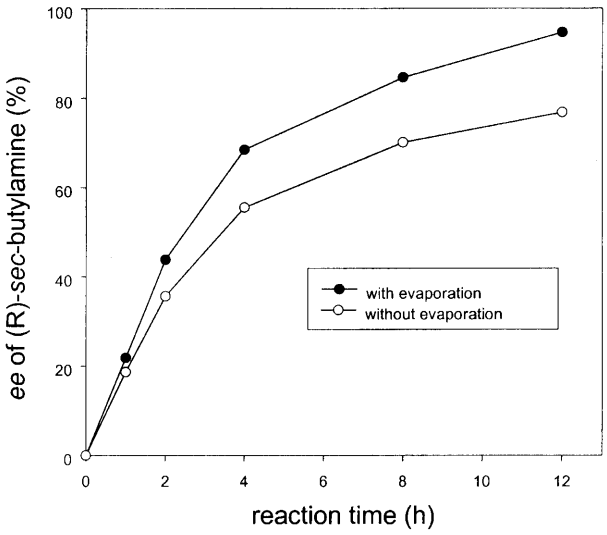


Fig. 3. Effects of the Removal of Inhibitory 2-Butanone on Kinetic Resolution of *sec*-Butylamine.

Reaction conditions: 20 mM of *sec*-butylamine, 50 mM of pyruvate, and 10 U/ml of dialyzed cell extract of *V. fluvialis* JS17.

monia JS2F is the least affected by product inhibition (Fig. 2(A)). The residual activity of the ω-TA from *B. thuringiensis* JS64 was 61% at only 3 mM of acetophenone. The same ω-TA also shows the most severe product inhibition by L-alanine (Fig. 2(B)). In this case, residual activity was 31% at 500 mM of L-alanine.

Kinetic resolution of sec-butylamine

We have demonstrated many examples of kinetic resolutions of arylic amines.[9,10,16] Besides the arylic amines in Table 2, our ω-TA strategy can be extended to aliphatic amines. We did kinetic resolution of *sec*-butylamine with ω-TA from *V. fluvialis* JS17 (the reactivity of *sec*-butylamine is 7% of that of α-MBA). As expected, we faced the product inhibition problem by 2-butanone (observed product inhibition constant = 5.6 mM, measured at 100 mM of *sec*-butylamine and 50 mM of pyruvate). Unlike the previous situation in which the product inhibitions could be selectively the removed by the extraction method due to high hydrophobicity of the ketone products, the high solubility of 2-butanone in water hindered us from using the same method. Therefore, we did the reaction under reduced pressure to facilitate evaporation of 2-butanone. The evaporation rate of 2-butanone at 150 Torr was approximately 5-fold higher than that at 760 Torr (data not shown). The reaction rate increased by applying the reduced pressure as shown in Fig. 3. The enantiomeric excess of (*R*)-*sec*-butylamine reached 94.6% at 150 Torr after 12 h of reaction, compared with 76.8% at 760 Torr.

Discussion

Judging from the screening results, microorganisms capable of (*S*)-specific ω-transamination appears to be ubiquitous, whereas ones showing the opposite stereoselectivity seem to be rare. All strains isolated after these enrichment cultures using (*S*)-α-MBA as a sole nitrogen source exclusively showed (*S*)-specific ω-TA activity. In contrast, strains enriched with (*R*)-α-MBA showed (*S*)-specific ω-TA activity as well as (*R*)-specific unidentified enzyme activity. None of them showed sole (*R*)-specific ω-TA activity.

The three ω-TAs presented here showed broad amino donor specificities toward aliphatic as well as arylic chiral amines, which is interesting in the industrial viewpoint. In the case of amino acceptor specificity, the ω-TAs show strict substrate specificity toward α-keto acids. However, besides pyruvate and glyoxylate, several aldehydes show high reactivities for the ω-TAs, which is interesting from the commercial viewpoint due to low costs of the aldehydes. A major problem in using the aldehydes as amino ac-

ceptors in the kinetic resolution is low enzyme stability (data not shown).

Production of chiral amines using ω-TA can be done by either kinetic resolution[9] of racemic amine (*i.e.* deamination of the amine) or asymmetric synthesis[15] using an achiral ketone (*i.e.* amination of the ketone). In both cases, either substrate or product inhibition is the major problem to be overcome. The substrate inhibitions of the ω-TAs result from formation of abortive dead-end complexes, *i.e.* between amino donor and E-PMP or between amino acceptor and E-PLP, as reported previously.[14] Such complexes cannot undergo further reaction and inhibit the formation of the right Michaelis complexes. Due to the substrate inhibitions in the kinetic resolution reaction, the concentrations of substrates should be kept below the threshold concentrations at which initial rates begin to decrease. For example, maintaining the concentrations of racemic α-MBA and pyruvate below 400 mM and 10 mM, respectively is recommended to fully utilize the maximum reaction rate of ω-TA from *V. fluvialis* JS17 (see Fig. 1). Unlike the substrate inhibition, product inhibition results from the right formation of Michaelis complex but between product and enzyme. Due to the reversibility of the transaminase reaction, products can bind to the enzyme and reduce the chances that substrates occupy an active site of enzyme. In addition, formation of the Michaelis complex commences a reverse reaction and causes the ensuing reduction in net reaction rate. Product inhibition rather than substrate inhibition usually poses more complicated problems in many industrial processes because the substrate inhibition can be easily overcome by controlling the concentrations of substrates in the reaction medium. Much higher product inhibition by acetophenone than L-alanine appears to be a property common to ω-TAs, which suggests that removal of the inhibition by ketone product is essential for a successful kinetic resolution process. We already demonstrated that the removal of the inhibition by solvent extraction could greatly increase the reaction rate of the kinetic resolution.[9] In this report, we also presented an evaporation method to facilitate ketone removal when the ketone product has high solubility in water.

In closing, we isolated three strains showing ω-TA activities from soil samples by an enrichment method using the reaction substrate as a sole nitrogen source. Those ω-TAs show similar properties such as strict enantioselectivity and broad amino donor specificity for chiral amines, substrate inhibition by amino donor and acceptor, product inhibitions, and a much lower reaction rate of amination of ketone than that of deamination of the cognate (*S*)-amine. However, detailed comparison of the properties reveals that the trends and threshold concentrations of substrate and product inhibitions are somewhat different, and the examination allowed us to design a much better reaction system for the biocatalysis. In this case, the ω-TA from *V. fluvialis* JS17 is the most promising one for both kinetic resolution and asymmetric synthesis. Besides the enzyme properties, no need of using minimal media for the induction of the enzyme would be another advantage of using the *V. fluvialis* JS17 strain.

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