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# Simultaneous synthesis of enantiomerically pure (*S*)-amino acids and (*R*)-amines using α/ω-aminotransferase coupling reactions with two-liquid phase reaction system

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This article is dedicated to professor Jun-Shik Lee

**Abstract**

An efficient simultaneous synthesis of enantiopure (*S*)-amino acids and chiral (*R*)-amines was achieved using α/ω-aminotransferase (α/ω-AT) coupling reaction with two-liquid phase system. As, among the enzyme components in the α/ω-AT coupling reaction systems, only ω-AT is severely hampered by product inhibition by ketone product, the coupled reaction cannot be carried out above 60 mM substrates. To overcome this problem, a two-liquid phase reaction was chosen, where dioctylphthalate was selected as the solvent based upon biocompatibility, partition coefficient and effect on enzyme activity. Using 100 mM of substrates, the AroAT/ω-AT and the AlaAT/ω-AT coupling reactions asymmetrically synthesized (*S*)-phenylalanine and (*S*)-2-aminobutyrate with 93% (>99% $ee^S$) and 95% (>99% $ee^S$) of conversion yield, and resolved the racemic α-methylbenzylamine with 56% (95% $ee^R$) and 54% (96% $ee^R$) of conversion yield, respectively. Moreover, using 300 mM of 2-oxobutyrate and 300 mM of racemic α-methylbenzylamine as substrates, the coupling reactions yielded 276 mM of (*S*)-2-aminobutyrate (>99% *ee*) and 144 mM of (*R*)-α-methylbenzylamine (>96% *ee*) in 9 h. Here, most of the reactions take place in the aqueous phase, and acetophenone mainly moved to the organic phase according to its partition coefficient.


*Keywords:* Aminotransferase; Coupling reaction; Chiral amino acids; Amines; Two-liquid phase reaction

## 1. Introduction

The biosynthesis of optically active amino acids and amines has drawn great attention owing to their wide usages in chiral building blocks for the synthesis of various pharmaceuticals [1–4]. Among several enzymatic methods that have been employed for the synthesis of optically active amino acids and amines, aminotransferase (AT, EC 2.6.1.) is a promising one due to its high turnover number, broad substrate specificity and no requirement for external cofactor regeneration [5,6]. ATs catalyze pyridoxal 5′-phosphate dependent amino group transfer reaction between an amino acid (amine) and a keto acid (ketone) substrate pair [7]. The chiral amino acids and amines generally can be produced via two reaction schemes using AT. One is asymmetric synthesis starting from keto acids or ketones (i.e. backward reaction from amine transfer point

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of view) [23,28], and the other is kinetic resolution starting from racemic amino acids or amines (i.e. forward reaction) [22].

Both schemes have been well developed for the production of various chiral amino acids and amines using α-ATs, such as AroAT (EC 2.6.1.5), and D-AT (EC 2.6.1.21), and ω-AT (EC 2.6.1.18) [3,5,8–11], respectively. Due to the relaxed substrate specificity of microbial ATs, such as AroAT and branched-chain amino acid AT (BrAT, EC 2.6.1.42) of *E. coli*, these enzymes are efficient in the enantiospecific preparation of unnatural amino acids, such as (*S*)-homophenylalanine, (*S*)-2-aminobutyrate and (*S*)-*tert*-leucine from the corresponding keto acids [5,12]. Extending this approach to the synthesis of chiral amines has also been an important research topic in this field.

To carry out both the successful kinetic resolution and the asymmetric synthesis for the synthesis of enantiomerically pure amines and unnatural amino acids at high substrate concentration, several issues should be considered, such as enantioselectivity, reaction equilibrium, enzyme stability, product and/or substrate inhibitions, and product seperation [18]. In the case of α-AT, the reversible transamination reaction typically results in a 50% conversion. This low yield and low product purity are not attractive for economical large-scale production and have prompted various trials to overcome the limitation of an otherwise attractive biocatalytic process. Then et al. have described a possibility of using excessive amino donors for shifting the reaction equilibrium toward product formation [29]. The use of (*S*)-aspartate instead of (*S*)-glutamate as amino donor is another attempt, because (*S*)-aspartate yields the unstable β-keto acid product (i.e. oxaloacetate) instead of the relatively stable α-ketoglutarate. Rapid decarboxylation of the oxaloacetate to pyruvate accelerates (*S*)-aspartate consumption, thus increasing product yield significantly over 50% [5]. AroAT could catalyze the transamination between α-ketobutyrate and (*S*)-aspartate, and the α-ketobutyrate could be supplied by deamination of (*S*)-threonine. In the end, the transamination was driven to completion by an irreversible multi-step pathway comprised of transamination and deamination [30]. Although (*S*)-aspartate has been used as an amino donor to shift the equilibrium of transaminations as mentioned above, pyruvate accumulation is often undesirable because it can undergo a competitive transamination to form (*S*)-alanine, and the presence of this additional byproduct complicates product isolation [5]. α/ω-AT coupling reaction was introduced elsewhere for the simultaneous synthesis of enantiomerically pure unnatural (*S*)-amino acids and (*R*)-amines [13] to overcome the limitation of the low equilibrium constant of the α-AT reaction as well as to supply the substrate (i.e. (*S*)-alanine) for the α-AT reaction and remove the product for the ω-AT reaction.

However, still the product inhibition for the ω-AT exerted by ketone product prevents us from executing the reaction at high concentration of amines [10,13]. This was somewhat expected, because the ω-AT is seriously inhibited by such ketone products. To remove such product inhibition for the ω-AT, incorporation of extraction module or two-liquid phase reaction is well known [10,19,22]. Basically, the two approaches are the same in the respect of extracting the ketone product from the reaction mixture. As two-liquid (aqueous-organic) phase reaction is relatively easier and simpler to construct the system, we were interested in developing the system at high substrate concentrations. The two-liquid phase system has been widely used in biotransformations because it can possibly increase the solubility of immiscible substrate in aqueous media, shifts equilibrium to the direction favorable for products, and alleviates product inhibition at the same time. However, solvent toxicity to enzyme and mass transfer limitation attributed to restricted interfacial area would conversely become drawbacks [10,14–17]. Therefore, careful examination is essential for successfully applying the two-liquid phase system to the α/ω-AT coupling reactions. Here, as a following work of the α/ω-AT coupling reactions for the simultaneous synthesis of unnatural amino acids and chiral amines, we demonstrate that employing the two-liquid system allows us to perform the simultaneous synthesis at few hundred millimolar scale without any problem.

## 2. Materials and methods

### 2.1. Chemicals

The chemicals required for protein assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (CA,

USA). Restriction enzymes used for DNA manipulation were from Boehringer Mannheim GmbH (Mannheim, Germany). The oligonucleotides were purchased from Cosmo Genetech (Seoul, Korea). Amino acids, keto acids, amines, antibiotics, and other chemicals were from Sigma-Aldrich (MO, USA). Bacto-agar was purchased from Difco (MI, USA). HPLC grade methanol was obtained from Duksan Pure Chemical Co. (Ansan, Korea). All other reagents were of analytical grade.

### 2.2. Analytical methods

Quantitative analysis of phenylalanine, α-methylbenzylamine, and acetophenone were performed using Younglin HPLC system (Seoul, Korea) equipped with a C18 Symmetry® reverse phase column (Waters, USA). In the case of aliphatic amino acids, 2,3,4-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) derivatization method was applied to analyze their chirality [26]. After GITC derivatization, the analytes were eluted with a mixture of 40% methanol and 60% 10 mM phosphate buffer (pH 1.8) at a flow rate of 1.0 ml/min and detected at 254 nm. Other amino acids, amines and ketones were analyzed using methanol–water mixture with 0.1% TFA. The chiral analysis of aromatic amino acids and amines was carried out with Crownpak CR(+) column (Daicel, Japan). Separation of enantiomers was carried out using isocratic elution of water (pH 2, perchloric acid) at a flow rate of 0.7 ml/min, and they were analyzed using UV detector at 210 nm. In order to analyze α-keto acids, such as 2-oxobutyrate, the reaction mixtures were analyzed with HPLC using Aminex-87H column (Bio-Rad, USA) at 210 nm. The column was eluted with isocratic 5 mM sulfuric acid at 40 °C. Enantiomeric excess (*ee*), conversion ($\zeta$) and partition coefficient of acetophenone ($K_p$) were calculated from the following equations:

$$ee^R(\%) = \frac{C_t^R - C_t^S}{C_t^R + C_t^S} \times 100 \quad (1)$$

$$ee^S(\%) = \frac{C_t^S - C_t^R}{C_t^S + C_t^R} \times 100 \quad (2)$$

$$\zeta(\%) = \left\{1 - \frac{C_t^R + C_t^S}{C_0^R + C_0^S}\right\} \times 100 \quad (3)$$

$$K_P = \frac{[\text{Acetophenone}]_{org}}{[\text{Acetophenone}]_{aqu}} \quad (4)$$

### 2.3. Construction of plasmids and mutant strains

The plasmid pET23b(+) purchased from Novagen (Germany) and pET24ma donated by Dr. David Sourdive (Pasteur Institute, France) were used for the construction of the plasmids carrying AroAT (*tyr*B) gene and AlaAT (*avt*A) gene of *E. coli* K-12, and ω-AT (*ωta*A) gene of *V. fluvialis* JS17, respectively. The respective structural genes of *tyr*B and *avt*A were amplified from the genomic DNA of *E. coli* K-12 by PCR using appropriate oligonucleotide primers. Likewise, *ωta*A was amplified from the genomic DNA of *V. fluvialis* JS17. The *tyr*B, *avt*A and *ωta*A were cloned into the corresponding sites of pET23b(+) and pET24ma, and pET23b(+)-*tyr*B, pET23b(+)-*avt*A and pET24ma-*ωta*A were obtained, respectively. To obtain recombinant strains harboring both the α-AT and ω-AT, the plasmids pET23b(+) carrying *tyr*B or *avt*A were transformed into the recombinant *E. coli* BL21(DE3)/pET24ma-ω*ta*A, respectively, and selected for both ampicillin (100 μg/ml) and kanamycin (25 μg/ml) resistances. The transformations were carried out according to the heat shock method [27].

### 2.4. Expression of enzymes and preparation of whole cells

Transformed *E. coli* BL21(DE3) strains containing two AT genes were grown at 37 °C in 50 ml LB medium containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). At 0.6 $OD_{600\,nm}$, ATs were induced with 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG). After overexpressing both ATs for 6 h, the cells were harvested and pelleted by centrifugation. The pelleted cells were washed twice with 25 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.2). Finally, the cells were resuspended using 5 ml of the same buffer and kept in ice for further study. SDS-PAGE analysis was conducted to confirm the concomitant over expression of the soluble enzymes using the cell extract [27]. The whole cell reaction was carried out at 37 °C in 1 ml reaction. The AT activity in the whole cells was measured by analyzing L-amino acids and ketone produced. The α-AT and ω-TA activities were measured using reaction mixtures of

50 mM Tris–HCl buffer (pH 7.2), 10 mM amino donor (L-aspartic acid or L-alanine) and 10 mM α-keto acid, and 50 mM Tris–HCl buffer (pH 7.2), 10 mM pyruvate and 10 mM amine, respectively. The reaction was started by adding the whole cells to the reaction mixture, and stopped by adding 16% perchloric acid, and the samples were analyzed using HPLC.

### *2.5. Biocompatibility of organic solvents to the recombinant E. coli cells*

Biocompatibility of the several organic solvents to the recombinant *E. coli* cells harboring two plasmids was calculated from the change in optical density values at 600 nm in medium containing 20% (v/v) each organic solvent. 0.4 ml of the cultures of an early exponential growth phase was inoculated to 3.6 ml of new LB medium. Then, 1 ml of each organic solvent was added into the 4 ml culture. After 18 h of cell growth in each medium, the ratio of optical density value at 600 nm of aqueous phase in the culture containing 20% (v/v) organic solvent to that of control culture (4 ml) was calculated. In the case of water-immiscible solvents, the optical density value of aqueous phase was measured after emulsion was settled down.

### *2.6. Effect of organic solvents on the enzyme activity*

The prepared recombinant whole cells (0.1 ml) were preincubated at 4 °C in 0.8 ml of reaction medium saturated with the organic solvents (20%; v/v) for 1 h. For the measurement of enzyme activity of AroAT, AlaAT and ω-AT, reaction medium A (10 mM (*S*)-aspartate, 50 mM Tris–HCl (pH 7.2)), reaction medium B (10 mM (*S*)-alanine, 50 mM Tris–HCl (pH 7.2)) and reaction medium C (10 mM (*S*)-α-methylbenzylamine, 50 mM Tris–HCl (pH 7.2)) were used, respectively. After the preincubation, the reactions were started by adding 0.1 ml of 100 mM phenylpyruvate, 100 mM 2-oxobutyrate, and 100 mM pyruvate, respectively, into the reaction solution. Reaction medium for the control reaction used water instead of the organic solvents. After 10 min, the reactions were stopped by adding 16% perchloric acid and were centrifuged to remove the recombinant whole cells. Relative enzyme activities (%) were calculated on the basis of the enzyme activities in the buffer solution only.

### *2.7. Simultaneous synthesis of (S)-amino acids and (R)-amines using the coupled α/ω-AT reaction with two-liquid phase system*

To prevent volume changes of the two phases during the reaction, each phase was presaturated with the other phase. One volume of 200 mM Tris–HCl buffer and two volumes of dioctylphthalate were mixed using a magnetic stirrer for overnight at room temperature. Each phase was separated by settling down and used for further two-liquid phase reactions. In the AroAT/ω-AT coupled system, 100 mM phenylpyruvate, 100 mM (*S*)-aspartate, and 100 mM α-methylbenzylamine were added into Tris–HCl buffer presaturated with dioctylphthalate. In the case of AlaAT/ω-AT coupled system, 100 mM 2-oxobutyrate and 100 mM (*S*)-alanine were used instead of phenylpyruvate and (*S*)-aspartate, respectively. After adding 0.5 ml of dioctylphthalate into 2 ml of the reaction media, the reaction was started by adding the recombinant whole cell solution into the buffer phase at 37 °C. After appropriate reaction time intervals, the reaction was stopped by addition of 0.5 ml of 16% perchloric acid. In the case of the coupling reaction at high substrate concentration, 450 mg of wet cell weight of the recombinant cells harboring AlaAT and ω-AT was added into 4 ml reaction mixture composed of 100 mM of 2-oxobutyrate, 100 mM of (*S*)-alanine, 300 mM of racemic α-methylbenzylamine and 500 mM of Tris–HCl buffer (pH 7.2) with 20% dioctylphthalate as an organic phase. At the end of the each batch reaction, the solid state of 2-oxobutyrate (100 mM) was added into the reaction mixture exogenously. The aqueous phase in the reaction mixture was separated by centrifugation and analyzed by HPLC as described above.

## 3. Result and discussion

### *3.1. Substrate inhibitions in the α/ω-AT coupling reactions*

Although the α/ω-AT coupling reactions synthesized (*S*)-amino acids and (*R*)-amines simultaneously [13], full conversion of the two substrates (i.e. α-keto acids and racemic amines) was not achieved at concentrations above 60 mM (data not shown). These results

suggest that the substrate inhibition by α-keto acids and/or racemic amines, and/or the product inhibition by (*S*)-amino acids, α-keto acids and/or ketones appear to significantly limit the conversion. Conceptually, owing to the irreversibility of ω-AT reaction, the α/ω-AT coupling reactions (i.e. AroAT/ω-AT and AlaAT/ω-AT coupling reaction) remove or shuttle pyruvate to (*S*)-alanine, respectively, easily shifting the equilibrium of the α-AT reaction toward the product formation. Therefore, the shift of the reaction equilibrium is not feasible at high substrate concentration condition, because of the substrate and/or product inhibition.

To investigate factors limiting high conversion of substrate at high concentration, the simultaneous syntheses of (*S*)-aminobutyrate and (*R*)-α-methylbenzylamine and of (*S*)-phenylalanine and (*R*)-α-methylbenzylamine were selected as model cases. However, as the concentrations of the racemic α-methylbenzylamine and pyruvate, 2-oxobutyrate and (*S*)-alanine, and phenylpyruvate and (*S*)-aspartate increase, the decarboxylation rate of oxaloacetate to pyruvate and the shuttling rate of pyruvate to (*S*)-alanine may not catch up with the corresponding α-AT reaction rate. If the racemic α-methylbenzylamine, 2-oxobutyrate and phenylpyruvate are built up without conversion, respectively, the substrate inhibition may play a role. To examine this possibility of the substrate inhibitions in the α/ω-AT coupling reaction, each AT activity in the recombinant *E. coli* containing both α-AT and ω-AT was measured at various concentrations of 2-oxobutyrate, (*S*)-alanine, phenylpyruvate, (*S*)-aspartate, pyruvate and α-methylbenzylamine. The substrate inhibitions by 2-oxobutyrate, phenylpyruvate and α-methylbenzylamine on each AT in the coupling reactions were not observed up to 100, 100 and 200 mM, respectively, and the initial rates slowly start to decrease above those concentrations (data not shown). These results were consistent with other studies [20–22]. The substrate inhibitions of (*S*)-alanine and (*S*)-aspartate to AlaAT and AroAT were not observed at all over 500 mM (data not shown). However, although phenylpyruvate and 2-oxobutyrate are substrates for the AroAT and AlaAT,

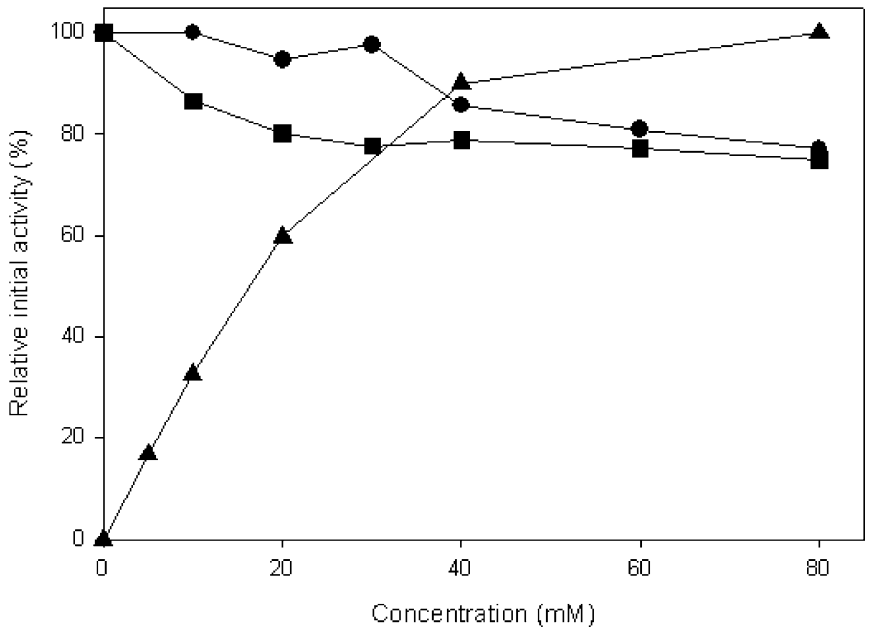


Fig. 1. Substrate inhibitions of ω-AT in the α/ω-AT coupling reactions. 11.1 mg wet cell weight of the recombinant *E. coli* cells harboring AroAT and ω-AT were added to a reaction mixture in 1 ml reaction volume. Reaction mixtures were composed of as follows: (▲) 200 mM pyruvate, the specified concentrations of α-methylbenzylamine and 200 mM Tris–HCl buffer (pH 7.2); (●) 5 mM α-methylbenzylamine, 20 mM pyruvate, the specified concentrations of 2-oxobutyrate and 200 mM Tris–HCl buffer (pH 7.2); (■) 5 mM α-methylbenzylamine, 20 mM pyruvate, the specified concentrations of phenylpyruvate and 200 mM Tris–HCl buffer (pH 7.2).

respectively, they also slightly inhibited ω-AT activity in the coupling reactions (Fig. 1). On the other hand, racemic α-methylbenzylamine could not inhibit AroAT and AlaAT activity at all (data not shown). To investigate whether or not α-keto acids, such as phenylpyruvate could be used as amino acceptor for ω-AT, the coupling reaction was carried out using racemic α-methylbenzylamine and phenylpyruvate. Based upon the reaction rate, it was concluded that phenylpyruvate could not become an amino acceptor, but become a weak inhibitor of the ω-AT (data not shown). In result, controlling concentration of the α-keto acids was one of the key factors for the coupling reactions at high substrate concentration. However, compared to product inhibition, substrate inhibition is relatively easy to handle for a desirable process via fed-batch type addition of substrate below inhibition concentration [23].

### 3.2. Product inhibitions in the α/ω-AT coupling reactions

To examine the product inhibitions, the above products were exogenously added into the reaction mixture before the reaction. As shown in Fig. 2A, the slightly reduced activity of AlaAT was observed when initial pyruvate concentration was varied. On the other hand, the AroAT activity was severely reduced by oxaloacetate and was almost completely lost at 100 mM (Fig. 2B), and the ω-AT activity was strongly inhibited by acetophenone at even low concentrations and was completely lost at 30 mM (Fig. 2C). These results agree well with other studies [10,20]. The effect of acetophenone on the AlaAT and AroAT activity was not observed at all. In addition, the decreases in the reaction rate of AlaAT, AroAT and

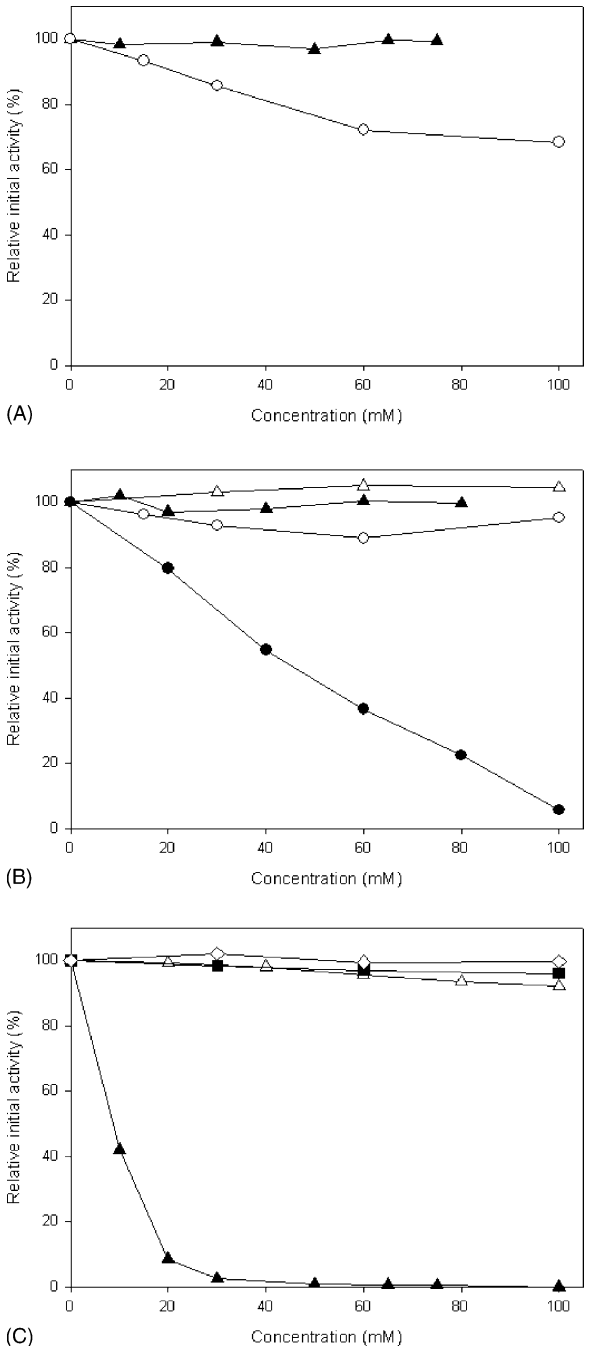


Fig. 2. Product inhibitions of AlaAT (A), AroAT (B) and ω-AT (C) in the α/ω-AT coupling reactions. 11.1 mg and 10.1 mg wet cell weight of the recombinant *E. coli* cells harboring AroAT and ω-AT, and AlaAT and ω-AT, respectively, were added to a reaction mixture in a 1 ml reaction volume. Reaction mixtures were composed of as follows: (A) 10 mM 2-oxobutyrate, 10 mM (*S*)-alanine, the specified concentrations of pyruvate and acetophenone in 200 mM Tris–HCl buffer (pH 7.2), (B) 10 mM phenylpyruvate, 10 mM (*S*)-aspartate, the specified concentrations of oxaloacetate, pyruvate, (*S*)-alanine and acetophenone in 200 mM Tris–HCl buffer (pH 7.2), (C) 5 mM α-methylbenzylamine, 10 mM pyruvate, the specified concentrations of acetophenone, oxaloacetate, pyruvate, (*S*)-alanine, (*S*)-aminobutyrate and (*S*)-phenylalanine in 200 mM Tris–HCl buffer (pH 7.2). Symbols: (○) pyruvate; (●) oxaloacetate; (△) (*S*)-alanine; (■) (*S*)-phenylalanine; (◇) (*S*)-aminobutyrate; (▲) acetophenone.

ω-AT by (*S*)-aminobutyrate, (*S*)-phenylalanine and (*R*)-α-methylbenzylamine, respectively, were nearly negligible compared with those by pyruvate, oxaloacetate and acetophenone, respectively (data not shown). Pyruvate is shuttled in AlaAT/ω-AT coupling reaction and oxaloacetate is spontaneously decarboxylated to pyruvate in AroAT/ω-AT coupling reaction [5,13]. Therefore, acetophenone come from the ω-AT reaction at high concentrations would directly cause the severe production inhibition of ω-AT. It also suggests that the reaction yield of AlaAT/ω-AT or AroAT/ω-AT coupling reaction cannot go to a completion above 60 mM of the substrates in aqueous buffer.

Fig. 3 represents the two-liquid phase reaction system composed of aqueous buffer and water-immiscible organic solvent. In this system, the α/ω-AT coupling reactions would take place only in aqueous phase. (*S*)-Amino acids, α-keto acids and racemic amines can be easily protonated in reaction buffer (pH 7.2), however, ketones, such as acetophenone cannot be,

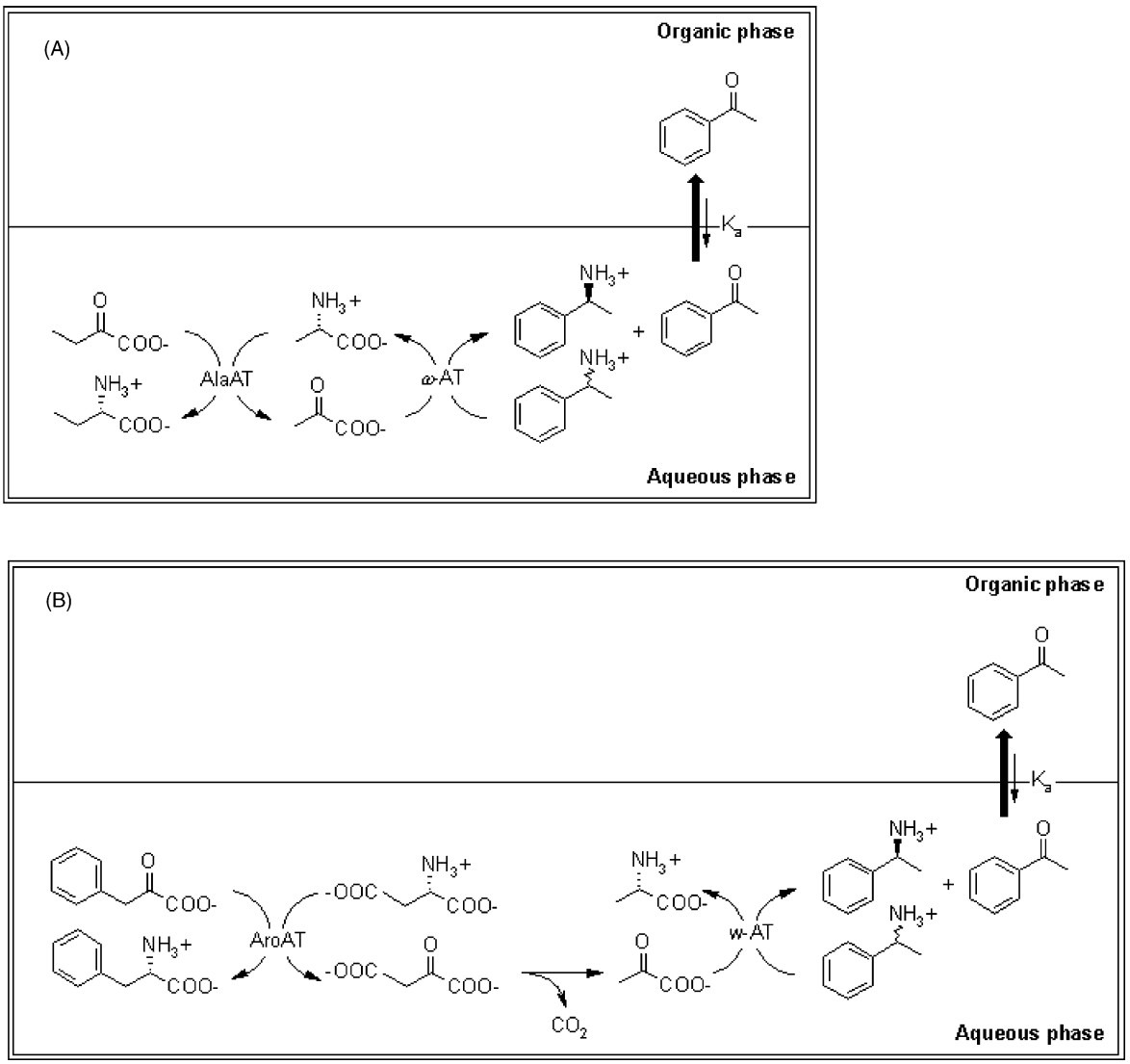


Fig. 3. Schematic diagram of simultaneous synthesis of (*S*)-amino acids and (*R*)-amines using the α/ω-AT coupling reactions in two-liquid phase system. (A) AlaAT/ω-AT system, (B) AroAT/ω-AT system.

resulting that the protonated substrates and products mostly reside in aqueous phase only. The ketone concentration in aqueous phase is inversely proportional to partition coefficient of organic phase, and can be controlled below its inhibition concentration during the reaction.

### 3.3. Organic solvents screening

Lanne et al. [24] mentioned that the biocompatibility and partition coefficient of organic solvent were major criteria in solvent selection due to relationship with enzyme stability and product inhibition. Eight organic solvents including ethyl acetate, cyclohexanone, cyclohexane, *n*-hexane, diphenyl ether, *iso*-octane, *n*-octane and dioctylphthalate were used to compare their biocompatibility against the recombinant *E. coli* harboring AroAT and ω-AT. As shown in Table 1, *iso*-octane, *n*-octane, dioctylphthalate, and diphenyl ether in order showed the higher biocompatibility against the recombinant *E. coli*, while cyclohexanone, ethyl acetate, and cyclohexane showed relatively low biocompatibility, suggesting that the organic solvents of log *P* around 4.5 are good for the biocompatibility against the *E. coli*. The presence of different genes, such as AlaAT/ω-AT or AroAT/ω-AT appears not to affect the biocompatibility of the organic solvents. Consequently, it was concluded that the organic solvent of low log *P* is quite toxic to the *E. coli* cells [31,32].

Table 1
Partition coefficients of acetophenone and biocompatibilities of organic solvents to the recombinant *E. coli* cells

| Organic solvent | log *P*[a] | $K_p$[a] | Biocompatibility (%)[b] |
|---|---|---|---|
| Ethyl acetate | 0.64 | 84.0 | 8.5 ± 0.4 |
| Cyclohexanone | 0.95 | 95.6 | 4.1 ± 0.3 |
| Cyclohexane | 3.20 | 18.3 | 10.9 ± 0.8 |
| *n*-Hexane | 3.52 | 16.4 | 10.8 ± 0.4 |
| Diphenyl ether | 4.06 | NM[c] | 44.2 ± 2.1 |
| *iso*-Octane | 4.50 | 12.9 | 101.1 ± 5.8 |
| *n*-Octane | 4.58 | 19.6 | 86.4 ± 3.2 |
| Dioctylphthalate | 9.60 | 68.0 | 63.5 ± 6.1 |

[a] log *P* values and partition coefficients ($K_p$) of acetophenone in several organic solvents were from Shin and Kim (1997).

[b] The biocompatibility was calculated from the change in optical density values at 600 nm in the culture containing 20% (v/v) of each organic solvent (See Section 2).

[c] NM: not measured.

As another selection criterion of the organic solvent, the partition coefficient of acetophenone indicating solvent's extraction capacity was chosen. Among the selected organic solvents, cyclohexanone, ethyl acetate and dioctylphthalate showed the highest $K_p$ value, 95.6, 84.0, and 68.0, respectively. However, cyclohexanone and ethyl acetate were solvents of low log *P* values showing low biocompatibility [10]. In result, dioctylphthalate was the best organic solvent among the selected organic solvents for the α/ω-AT coupling reaction with the recombinant *E. coli* cells.

Finally, to investigate the effect of the organic solvents on the AT activities, the α/ω-AT coupling reactions were carried out (Fig. 4). The relative activity of the ω-AT was not greatly affected by the kinds of organic solvent, whereas the relative activity of the α-ATs varied a lot depending upon the solvents. This means that the ω-AT does not have a close correlation with biocompatibility, but the α-ATs do. In the previous study, when the cell extract from *B. thuringiensis* was used, its ω-AT showed the similar trend [10]. However, in the case of the AroAT and AlaAT, *iso*-octane and dioctylphthalate showing high biocompatibility did not reduce their relative initial activities, but cyclohexanone lowered their activities by 24 and 12%, respectively. In result, considering biocompatibility, partition coefficient and enzyme activity, dioctylphthalate was selected as the most suitable organic solvent for further development of the α/ω-AT coupling reactions using two-liquid phase reaction system. Although dioctylphthalate of high viscosity might limit mass transfer of acetophenone, which causes the increase in product inhibition, it could be overcome by controlling the enzyme reaction rate and degree of mixing. Because the ω-AT reaction rate in the coupled reactions is controlled by pyruvate come from the α-AT reaction, the amount of acetophenone produced can be controlled below mass transfer rate limit by the control of α-AT activity.

In the two-liquid phase reaction system organic/aqueous phase volume ratio ($V_{org}/V_{aqu}$) is one of the important factors [25], and at a fixed $V_{org}/V_{aqu}$ ratio, the increase in the product yield is explained by the increase in the partition coefficient of acetophenone toward the organic phase. However, the increase in $V_{org}/V_{aqu}$ has a reciprocal relationship with the enzyme activity. Therefore, there should be an optimal ratio of $V_{org}/V_{aqu}$. In this study, 20% (v/v) of dioctylphthalate

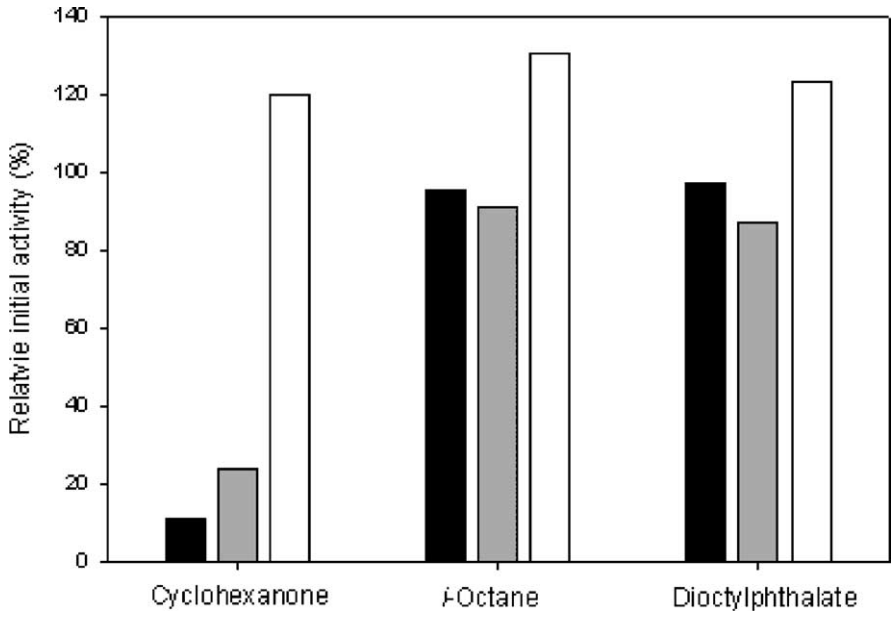


Fig. 4. Effect of organic solvents on the relative AT activities in the α/ω-AT coupling reactions. Reaction conditions were as follows: 10.1 mg wet cell weight of the recombinant *E. coli* cells harboring AlaAT and ω-AT and 11.1 mg wet cell weight of the recombinant *E. coli* cells harboring AroAT and ω-AT were added into 4 ml reaction mixture containing 20% (v/v) organic solvents, respectively. (■) 10 mM 2-oxobutyrate and 20 mM (*S*)-alanine in 50 mM Tris–HCl buffer (pH 7.2); (▒) 10 mM phenylpyruvate and 20 mM (*S*)-aspartate in 50 mM Tris–HCl buffer (pH 7.2); (□) 5 mM α-methylbenzylamine and 20 mM pyruvate in 50 mM Tris–HCl buffer (pH 7.2).

to aqueous phase was identified as an optimal point for the α/ω-AT coupling reactions (data not shown).

### *3.4. Coupling reactions at high substrate concentration*

As depicted in Fig. 5A, in the two-liquid phase reaction system, the recombinant *E. coli* harboring the AroAT and ω-AT converted 100 mM of phenylpyruvate and 100 mM of racemic α-methylbenzylamine to (*S*)-phenylalanine and (*R*)-α-methylbenzylamine with 93% (>99% $ee^S$) and 54% conversion yield (95% $ee^R$) in 20 h, respectively. Here, the conversion yields change depending upon the supply of one substrate for ω-AT, i.e. pyruvate. In the previous study, it was confirmed that as pyruvate is supplied by the subsequent decarboxylation of oxaloacetate, the conversion of (*R*)-amine would depend on the yield of the decarboxylation [13]. In other words, the pyruvate supply limits the ω-AT reaction, hence the α/ω-AT coupling reaction. The reaction profiles in Fig. 5A and B also indicate that the mass transfer of acetophenone resulting from the ω-AT reaction to dioctylphthalate occurs efficiently, and the α-AT reaction rate is not rate-limiting for this coupling reaction, rather the inhibition of the ω-AT by acetophenone limits the conversion yield. It also suggests that in the two-liquid phase reaction, the concentration of acetophenone in the aqueous phase becomes below the product inhibition concentration along the reaction. Here, (*S*)-phenylalanine, pyruvate, (*S*)-aspartate and phenylpyruvate are likely to take charges in the aqueous solution at the reaction pH. In contrast, most of α-methylbenzylamine exists in the organic phase as its neutral amine form [10]. As the coupling reaction proceeds, the amine diffuses into the aqueous phase by the concentration difference, and is charged to reach an equilibrium concentration. Consequently, all the reaction substrates and products exist in aqueous phase except α-methylbenzylamine and acetophenone, and the concentration of α-methylbenzylamine and acetophenone depend on the volumetric ratio of the two phases resulting changes in their equilibrium constant.

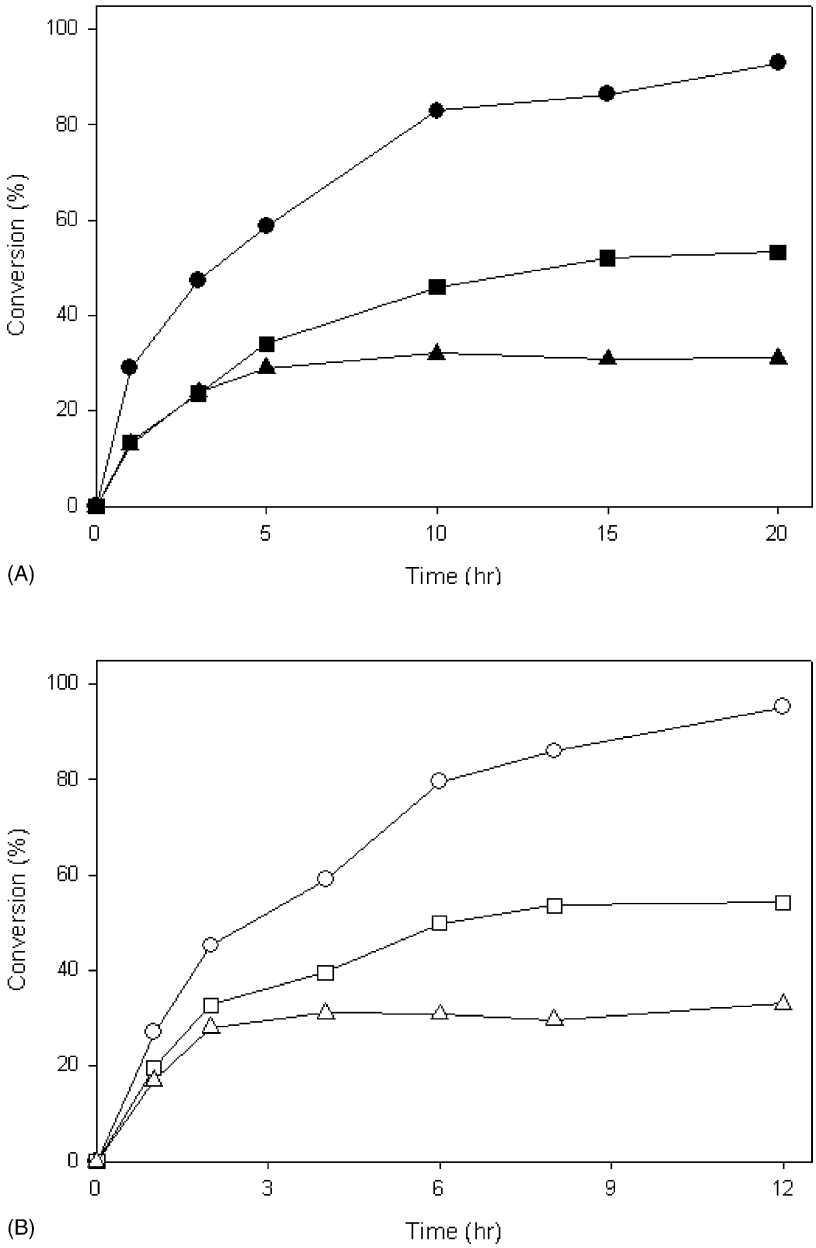


Fig. 5. Simultaneous synthesis of (*S*)-amino acids and (*R*)-amines using the α/ω-AT coupling reactions with two-liquid phase system. (A) 44 mg wet cell weight of the recombinant *E. coli* harboring AroAT and ω-AT were added into a reaction mixture containing 100 mM phenylpyruvate, 100 mM racemic α-methylbenzylamine and 100 mM (*S*)-aspartate in 4 ml of 500 mM Tris–HCl buffer (pH 7.2) with 20% (v/v) dioctylphthalate. (B) 40 mg wet cell weight of the recombinant *E. coli* harboring AlaAT and ω-AT were added into a reaction mixture containing 100 mM 2-oxobutyrate, 100 mM racemic α-methylbenzylamine and 100 mM (*S*)-alanine in 4 ml of 500 mM Tris–HCl buffer (pH 7.2) with 20% (v/v) dioctylphthalate. Symbols: (●) (*S*)-phenylalanine; (□, ■) acetophenone with the two-liquid phase system; (△, ▲), acetophenone without the two-liquid phase system; (○) (*S*)-aminobutyrate.

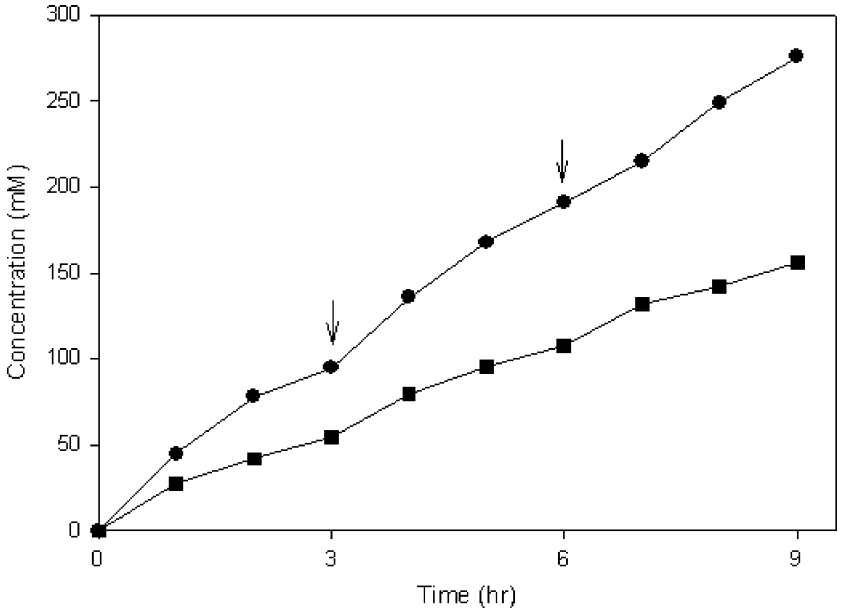


Fig. 6. The α/ω-AT coupling reaction at high substrate concentration (300 mM) with two-liquid phase system using 450 mg wet cell weight of the recombinant *E. coli* harboring the AlaAT and ω-AT. Symbols: (●) (*S*)-2-aminobutyrate; (■) acetophenone.

Likewise, 100 mM of 2-oxobutyrate and racemic α-methylbenzylamine could be converted to (*S*)-2-aminobutyrate and (*R*)-α-methylbenzylamine with 95% (>99% $ee^S$) and 55% (96% $ee^R$) conversion yields in 12 h, respectively (Fig. 5B). In this case, the decarboxylation of oxaloacetate is not involved, but the resulting pyruvate from the AlaAT is directly consumed by the ω-AT, and reproduced as (*S*)-alanine. Due to the efficient alanine-pyruvate shuttling in the coupled reaction, it appears that (*S*)-alanine is quite rapidly supplied for the α-AT reaction, and the yield of aliphatic (*S*)-amino acid is improved to a certain extent compared to that of the aromatic (*S*)-amino acid. The reaction profile again clearly showed that our reaction scheme of using the two ATs works nicely for the production of aliphatic (*S*)-amino acids and (*R*)-amines at high concentrations owing to the ketone extracting capacity of organic phase. To execute these reactions at further higher concentrations, we have carried out the reaction at 300 mM of 2-oxobutyrate and racemic α-methylbenzylamine for the asymmetric synthesis of (*S*)-2-aminobutyrate and the kinetic resolution of (*R*)-α-methylbenzylamine using 450 mg of wet recombinant cells harboring the AlaAT and ω-AT. Due to the substrate inhibition, 2-oxobutyrate was added into the reaction mixture repeatedly (i.e. three times repeated batching of 100 mM of 2-oxobutyrate). As depicted in Fig. 6, 276 mM of 2-oxobutyrate and 154 mM of α-methylbenzylamine could be efficiently consumed, and >92% of final (*S*)-2-aminobutyrate yield (>99% *ee*) and >55% of final (*R*)-α-methylbenzylamine yield (>96% *ee*) were obtained in 9 h. The reaction profile shows that the α/ω-AT coupling reaction works well in the two-liquid phase reaction system.

## 4. Conclusions

Unnatural amino acids and chiral amines are important chemicals for the preparation of chiral pharmaceuticals. Transamination would become a promising enzymatic method for the synthesis of optically active amino acids and amines, due to its broad substrate specificity and no requirement for external cofactor regeneration. For the application of this enzyme, there are two drawbacks to overcome. One is low equilibrium constant of α-AT reaction, which limits to obtain a high conversion yield. The other is product inhibition of ω-AT reaction, which prevents us

from using the enzyme at high concentrations above 60 mM. The α/ω-AT coupling reaction is one way of overcoming the equilibrium constant of α-AT reaction [13]. Aromatic (*S*)-amino acids and (*R*)-amines could be synthesized using AroAT and ω-AT and aliphatic (*S*)-amino acids and (*R*)-amines could be prepared using AlaAT and ω-AT. That is, pyruvate produced from the α-AT reaction was converted to (*S*)-alanine by ω-AT, concomitantly the racemic amines was resolved to (*R*)-amines. In the case of AlaAT and ω-AT coupled reaction, (*S*)-alanine and pyruvate could be successfully shuttled by the recombinant *E. coli* harboring the enzymes. However, due to the intrinsic product inhibition of ω-AT by ketone product, the coupled reactions at high substrate concentrations could not be achieved.

A two-liquid phase reaction system has been used to remove the inhibitory reaction product via partitioning between aqueous and organic solvent phase. To achieve the best two-phase system, dioctylphthalate was chosen based upon biocompatibility and high extraction capacity of ketone product, i.e. acetophenone. Although cyclohexanone showed the largest partitioning coefficient for acetophenone, it significantly decreased the α-AT activities. Using the dioctylphthalate as the organic phase, 300 mM of phenylpyruvate and 2-oxobutyric acid could be converted into (*S*)-phenylalanine and 2-aminobutyric acid, respectively, and racemic α-methylbenzylamine was simultaneously resolved into (*R*)-α-methylbenzylamine with high conversion yield and enantiomeric excess in the coupled reactions.

The two-liquid phase system with the α/ω-AT coupling reactions has several advantages over aqueous phase only. Firstly, the low activity of ω-AT due to product inhibition in the aqueous system can be avoided, therefore the coupled reactions can be performed at high substrate concentration. Secondly, the low conversion yield of α-AT resulting from intrinsic low equilibrium constant can be greatly improved. Our result shows that direct screening of right solvent based upon biocompatibility and extraction capacity of inhibiting compounds allows a construction of very simple and easy two-phase reaction system, and it leads to produce enantiomerically pure chiral amine and amino acid compounds at high concentrations. However, the two phase system also shows a certain limit above 300 mM of substrate (or product) concentration, so that other process development is further needed. Nonetheless, this study showed that the α/ω-AT coupling reactions can be easily carried out at high substrate concentrations enough to satisfy industrial production of chiral amine and amino acid compounds.

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