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The Kinetic Resolution of Racemic Amines Using a Whole-Cell Biocatalyst Co-Expressing Amine Dehydrogenase and NADH Oxidase

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Abstract: Amine dehydrogenase (AmDH) possesses tremendous potential for the synthesis of chiral amines because AmDH catalyzes the asymmetric reductive amination of ketone with high enatioselectivity. Although a reductive application of AmDH is favored in practice, the oxidative route is interesting as well for the preparation of chiral amines. Here, the kinetic resolution of racemic amines using AmDH was first extensively studied, and the AmDH reaction was combined with an NADH oxidase (Nox) to regenerate NAD^+ and to drive the reaction forward. When the kinetic resolution was carried out with 10 mM *rac*-2-aminoheptane and 5 mM *rac*-α-methylbenzylamine (α-MBA) using purified enzymes, the enantiomeric excess (*ee*) values were less than 26% due to the product inhibition of AmDH by ketone and the inhibition of Nox by the substrate amine. The use of a whole-cell biocatalyst co-expressing AmDH and Nox apparently reduces the substrate and product inhibition, and/or it increases the stability of the enzymes. Fifty millimoles (50 mM) *rac*-2-aminoheptane and 20 mM *rac*-α-MBA were successfully resolved into the (*S*)-form with >99% *ee* using whole cells. The present study demonstrates the potential of a whole-cell biocatalyst co-expressing AmDH and Nox for the kinetic resolution of racemic amines.

Keywords: amine dehydrogenase; biocatalysts; chiral amine; kinetic resolution; oxidative deamination

1. Introduction

Recently, considerable efforts have been made to economically obtain enantiomerically pure intermediates for drugs and agrochemicals. Among them, the chiral amines are of great interest to the pharmaceutical and fine-chemical industries, since enantiopure amines are important building blocks for pharmaceutical manufacturing [1]. Given the significance of chiral amines, their efficient synthesis in enantiopure form has become an attractive challenge to organic chemists and biologists in recent years [2–4]. Recently, many biocatalytic methods for the production of chiral amines have been developed using biocatalysts, such as lipase [5], amine oxidase [6], imine reductase [7], transaminase [8], ammonia lyases [9], Pictet-Spenglerase [10], barberine bridge enzyme [11], engineered P450 monooxygenase [12], and amine dehydrogenase (AmDH) [3]. Each enzyme has its own advantages and disadvantages for the synthesis of chiral amines ([13] and references therein for more details).

Among them, as AmDH catalyzes the asymmetric reductive amination of ketones, and uses cheap ammonia as the reagent and generates only water as by product, with high atom efficiency and enantioselectivity [14], AmDH is highly attractive and possesses tremendous potential for the synthesis of chiral amines [13]. Until now, only one NAD^+-dependent AmDH found in nature has been reported, in *Streptomyces virginiae*, but it showed poor enantioselectivity and its DNA information is not available [15]. Recently, AmDHs have been created through protein engineering using the existing L-amino acid dehydrogenase. First, AmDH was engineered by the Bommarius group using leucine dehydrogenase (LeuDH) from *Bacillus stearothomophilus* to accept ketone substrates through the introduction of 2–4 point mutations [16]. Subsequently, a second amine dehydrogenase was developed based on the scaffold of phenylalanine dehydrogenase (PheDH) from *Bacillus badius* by the same group using a similar approach [17]. Further, a chimeric enzyme was generated by combining the N-terminal section (residues 1–149) of the second AmDH (PheDH variant) with residues 140–366 of the first AmDH (LeuDH variant), and the chimeric enzyme displayed good activity towards various amines, such as acetophenone and adamantyl methyl ketone, and converted them to (*R*)-amine products with excellent enantioselectivity [18]. Since then, different AmDHs have been developed using L-amino acid dehydrogenases from *Rhodococcus* sp. M4, *Exiguobacterium sibiricum*, and *Caldalkalibacillus thermarum* [14,19,20]. A systematic investigation of substrate acceptance, optimal reaction conditions, and the chemo- and stereoselectivity of AmDHs has been carried out [13,20]. In addition, AmDHs have been used in elegant hydrogen borrowing dual-enzyme cascade reactions for the synthesis of chiral amines from alcohols with "closed-loop" recycling of the cofactor [19,21]. Due to the importance of the reductive amination of ketones by AmDH, an extensive and detailed review on relevant studies has recently been published [3].

Although a reductive application of dehydrogenases is favored in practice, the oxidative route is interesting as well for the preparation of asymmetric compounds from prochiral substrates, such as D-amino acids with L-amino acid dehydrogenases, (*S*)-alcohols with (*R*)-specific alcohol dehydrogenases, or the conversion of hydroxy acids, amino acids, and alcohols into the corresponding keto compounds [22]. Until now, all reported AmDHs exhibit (*R*)-enantioselectivity because they were generated from an L-amino acid dehydrogenase scaffold. Therefore, (*R*)-amines are produced through reductive amination by AmDHs from ketones (for certain compounds, (*S*)-forms were generated due to the Cahn–Ingold–Prelog priority rules for naming chiral centers). Since (*S*)-specific AmDH is not available, (*S*)-amines cannot be synthesized via reductive amination. Although (*S*)-amines can be obtained through the kinetic resolution of racemic amines using oxidative deamination by (*R*)-specific AmDH, the kinetic resolution of amines using AmDH has not been extensively studied until recently.

We describe herein the production of (*S*)-amines through the oxidative deamination of racemic amines by AmDH with help of an NADH oxidase (Nox) (Figure 1). One of the main advantages of using Nox for NAD^+ coenzyme regeneration is that it catalyzes an irreversible reaction, which then shifts the equilibrium of the primary reaction to the side of the product formation. Nox is a flavoprotein which oxidizes NADH to NAD^+ via a coupled reaction of molecular oxygen to either water in a four-electron reduction or to hydrogen peroxide in a two-electron reduction [22]. Due to the toxicity of hydrogen peroxide [23], water-forming Nox from *Lactobacillus brevis* was used in this study [24]. Among the reported AmDHs, the chimeric enzyme [18] generated by the domain shuffling of the LeuDH and PheDH variants was used in this study.

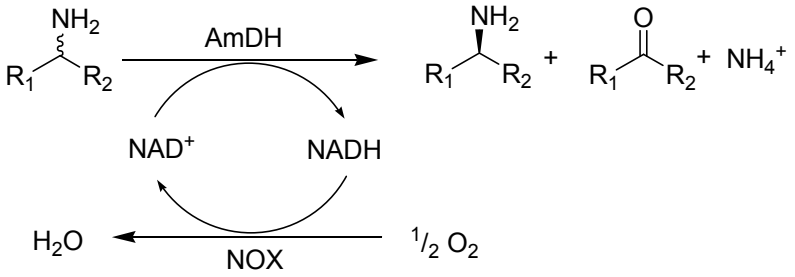
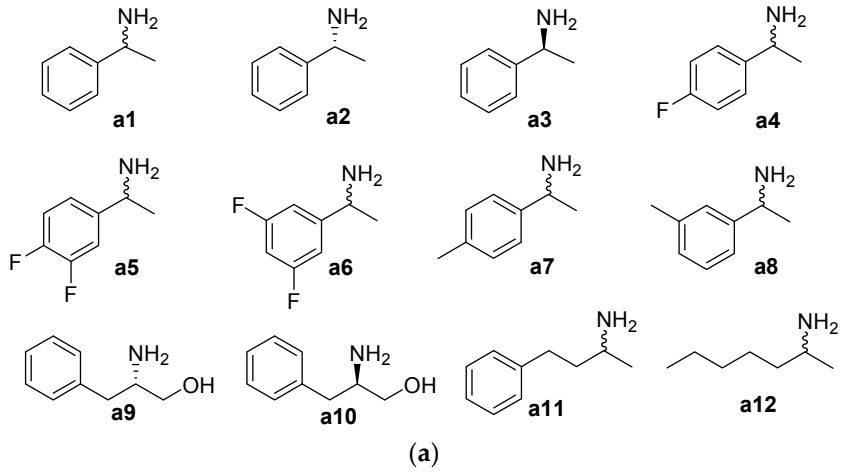


Figure 1. The kinetic resolution of amine by (*R*)-specific AmDH in combination with NADH oxidase (Nox). (*R*)-amine is oxidized into the corresponding ketone by AmDH utilizing NAD^+. Additionally, NAD^+ is regenerated by Nox, and enantiomerically pure (*S*)-amine is obtained.

2. Results and Discussion

2.1. Substrate Specificity and Kinetic Parameters of AmDH

The AmDH gene was cloned into a pET24ma vector with a C-terminal His6-tag, effectively expressed in the *Escherichia coli* BL21 cell (DE3), and subsequently purified on a Ni-NTA affinity column. As mentioned above, the main interest of this study is the oxidative deamination of amines by AmDH. The oxidative deamination activity of AmDH towards various amines was examined in order to explore the applicability of the enzyme to the kinetic resolution of various amines (Figure 2). The enzyme showed considerable activity towards the tested amines. The enzyme showed good activity towards (*R*)-α-methylbenzylamine (α-MBA, **a2**) and (*S*)-phenylalaninol (**a9**), but gave negligible activity for (*S*)-α-MBA (**a3**) and (*R*)-phenylalaninol (**a10**). It suggested that the enantioselectivity of the enzyme is high, and the result is consistent with that reported previously [18]. The enzyme's reactivity towards a broad range of substrates and high enantioselectivity indicate the applicability of the enzyme in the production of chiral amines. After that, *rac*-α-MBA (**a1**) and *rac*-2-aminoheptane (**a12**) were selected as representative aromatic and aliphatic amines for further study. To determine the kinetic parameters of the enzyme, the initial rate of the enzyme was analyzed with various concentrations of **a1** or **a12** (0–25 mM) in the presence of 1 mM NAD^+ (Figure 3 and Figure S2). The kinetic parameters were determined based on the Lineweaver–Burk plot. The K_m and k_{cat} values of AmDH for **a1** were 3.99 (±0.39) mM and 0.96 (±0.043) min^{-1}, respectively. The K_m and k_{cat} values for **a12** were 8.17 (±0.25) mM and 11.88 (±0.53) min^{-1}, respectively.



(**a**)

Figure 2. *Cont.*

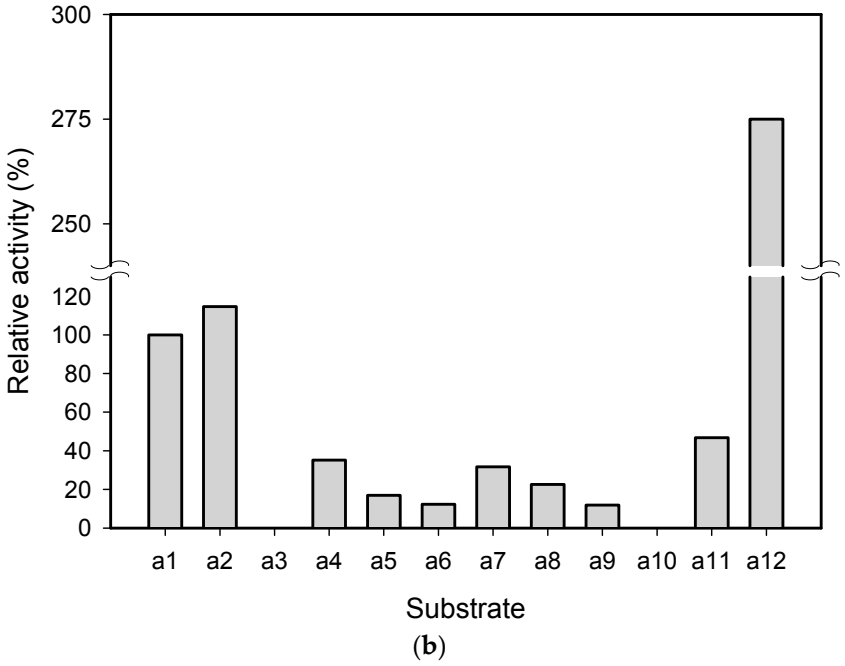


Figure 2. (**a**) Substrate spectrum; (**b**) Substrate specificity of AmDH. Oxidative deamination was carried out in 100 mM glycine buffer (pH 10.0) containing AmDH (0.15 mg/mL), 5 mM substrate, and 1 mM NAD^+ at 25 °C. One hundred percent (100%) corresponds to 44 mU/mg.

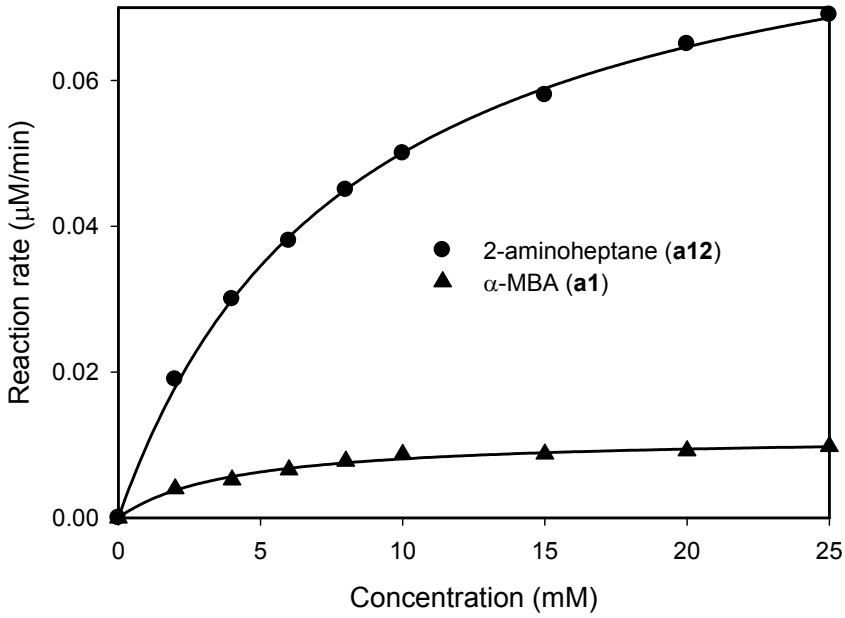


Figure 3. Michaelis–Menten plot of the reaction rate depending on the substrate concentration. The reactions were carried out in 100 mM Tris/HCl (pH 8.5) containing substrate (0–30 mM), AmDH (0.3 mg/mL for **a12**, 0.45 mg/mL for **a1**), and 1 mM NAD^+ at 25 °C.

2.2. Kinetic Resolution of Amines Using a Purified AmDH/Nox Coupling System

In the kinetic resolution of amines by AmDH, the regeneration of cofactor NAD^+ is of importance, as the reaction requires a stoichiometric amount of NAD^+ (Figure 1). In addition to reducing the cost of the enzymatic reaction, NAD^+ cofactor regeneration simplifies product isolation, prevents problems of product inhibition by the cofactor, and can favorably drive the reaction forward. For NAD^+

regeneration, Nox from *Lactobacillus brevis* was used [24]. The reduced byproduct of Nox is H_2O, and this reaction is considered irreversible, which means that it is the driving force to move the reaction forward in a coupled AmDH/Nox reaction [25]. In the coupled enzyme reaction, especially when the optimum pH of the two enzymes is different, the pH of the reaction should be carefully determined to balance the two enzyme activities. Therefore, the pH dependence of the activities of AmDH and Nox was examined (Figure 4). The AmDH and Nox showed the highest activity at pH 10.0 and 7.5, respectively. The results are in good agreement with previously reported results [18,26]. The specific activity of AmDH and Nox was 48 mU/mg and 95 U/mg at the optimum pH, respectively.

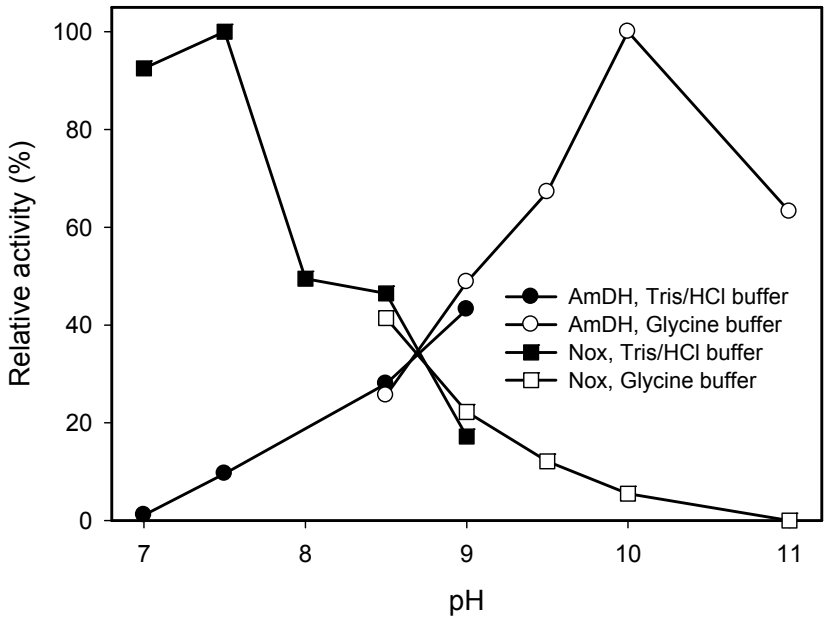


Figure 4. Effect of pH on the activity of AmDH and Nox. For the AmDH activity assay, the reactions were performed in 100 mM Tris/HCl buffer (pH 7.0–9.0) or 100 mM glycine buffer (pH 9.0–11.0) containing 20 mM **a1**, 1 mM NAD^+, and AmDH (0.15 mg/mL). For the Nox activity assay, 0.2 mM NADH and 0.01 mg/mL of Nox were used.

Next, the kinetic resolution of 10 mM *rac*-2-aminoheptane (**a12**) was carried out in 100 mM Tris/HCl buffer (pH 8.0–9.0) containing 1 mM NAD^+ with AmDH (1 mg/mL) and Nox (1 mg/mL). The enantiomeric excess (*ee*) value of a12 was 22%, 26%, and 19% at pH 8.0, 8.5, and 9.0, respectively (Figure 5). Also, when the kinetic resolution was carried out with 5 mM *rac*-α-MBA (**a1**) at the condition described above at pH 8.5 for 24 h, the *ee* value of **a1** was only 20%. The results suggest that the activities of AmDH and Nox were affected by the substrate and/or product. Several amino acid dehydrogenases have been observed to suffer from strong product inhibition, often by their native L-amino acid product [20]. In addition, in the asymmetric synthesis of amines from ketones by AmDH, product inhibition has been previously suggested by the use of a biphasic reaction system to ostensibly overcome inhibition in AmDH-catalyzed reactions [27].

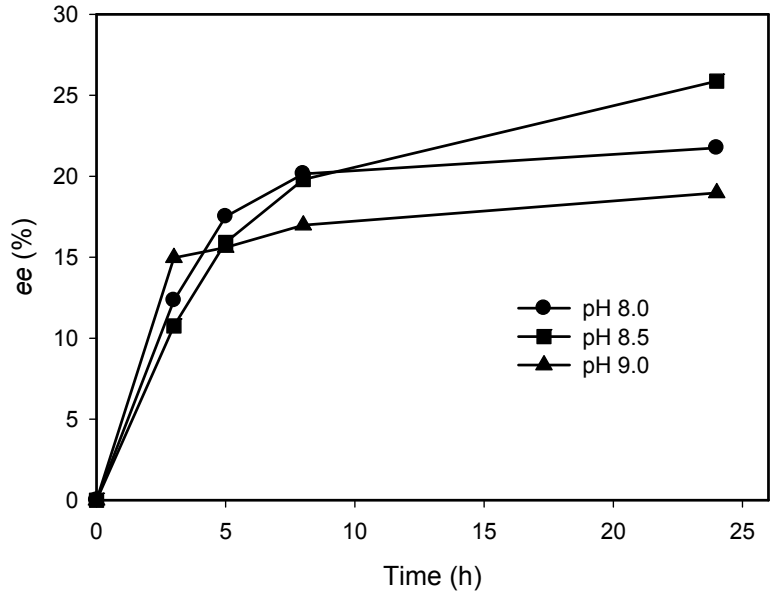
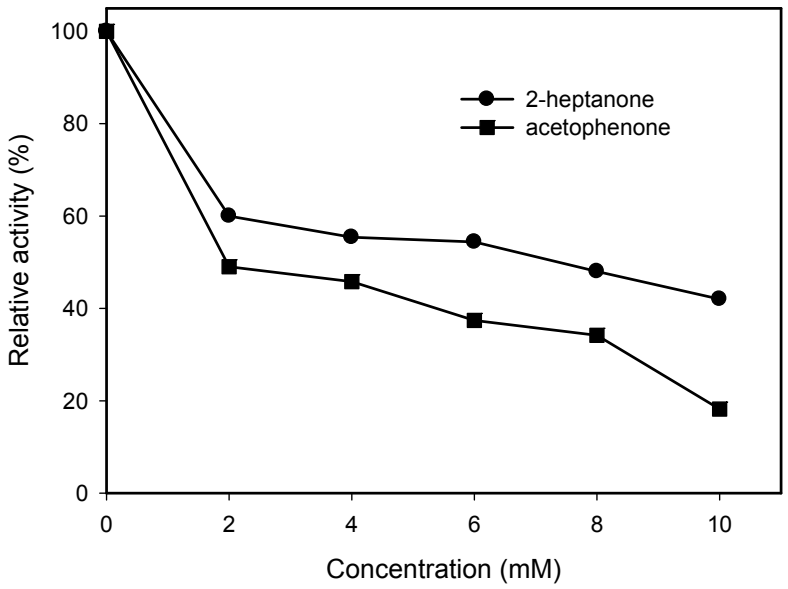


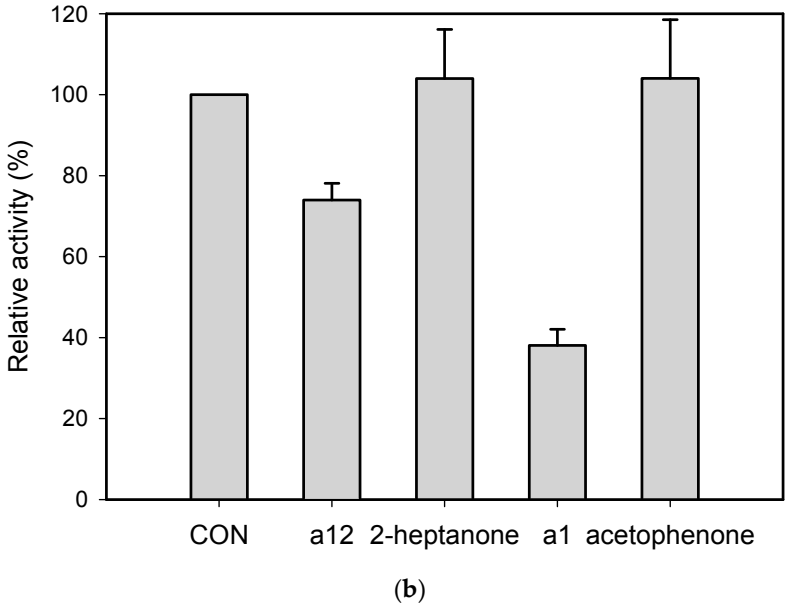
Figure 5. The kinetic resolution of 10 mM **a12**. Reaction conditions: 1 mM NAD^+, 100 mM Tris/HCl buffer (pH 8.0–9.0), 1 mg/mL of AmDH, and 1 mg/mL of Nox.

Therefore, the product inhibition of AmDH by ketones (2-aminoheptanone or acetophenone) was examined in the presence of 10 mM **a1** and **a12** (Figure 6a). In the presence of 2 mM 2-aminoheptanone and acetophenone, the enzyme showed only 60% and 49% of its original activity. These results indicate that the continuous removal of ketone in the reaction mixture could improve the degree of conversion. Also, the activity inhibition of Nox by the substrate and product was examined (Figure 6b). The ketones did not significantly affect the activity of Nox, but the activity was dramatically reduced in the presence of amine (5 mM). The relative activities of Nox in the presence of **a1** and **a12** were only 38% and 74% of its original activity, respectively. The unexpected poor conversion of the coupled AmDH/Nox reaction is partly due to product inhibition of AmDH by ketone and activity inhibition of Nox by the amine.



(**a**)

Figure 6. *Cont*.



(**b**)

Figure 6. Substrate and product inhibition of AmDH and Nox; (**a**) Effect of ketone concentration on AmDH activity. Reaction conditions: 100 mM Tris/HCl buffer (pH 8.5), 1 mM NAD^+, 10 mM **a12** (or **a1**), 2-heptanone (or acetophenone) (0–25 mM), 0.3 mg/mL of AmDH; (**b**) Effect of amine and ketone on Nox activity. Reaction conditions: 0.2 mM NADH, 5 mM amine or ketone, 100 mM Tris/HCl buffer (pH 8.5), and 0.05 mg/mL of Nox. (CON: control, the Nox activity in the absence of amine and ketone.)

2.3. Kinetic Resolution of Amines Using E. coli Co-Expressing AmDH and Nox

The immobilization of enzymes on a solid support system improves catalyst stability, recycling, and longevity under industrially relevant process conditions [28]. For example, polyethyleneimine-activated agarose beads were used to non-covalently attach phosphorylated versions of the cofactors NAD^+, FAD^+, and PLP, creating an association/dissociation equilibrium of phosphorylated and non-phosphorylated cofactors on support [29]. An increase in turnover number was observed in comparison to non-immobilized enzymes or non-immobilized cofactors. However, in this study, substrate and product inhibitions of enzymes were the major hurdles for carrying out the efficient kinetic resolution of racemic amines using AmDH and Nox. Therefore, recombinant *Escherichia coli* cells co-expressing AmDH and Nox were used as biocatalysts (*vide infra*). The use of whole-cells instead of isolated enzymes in biological processes has a simple and economical advantage, as the cost for cell lysis and purification is reduced [30]. Another advantage of a whole-cell reaction system is that cofactor-dependent enzymatic reactions can be effectively performed by using endogenous cofactors without adding costly cofactors [30]. Sometimes, a whole cell reaction is less susceptible to enzyme inhibition by substrate and product due to the diffusion barrier of the cell membrane [31].

In order to develop a recombinant *E. coli* system expressing both enzymes in a single cell, the AmDH and Nox genes were cloned into pET24ma and pETDuet vectors, respectively. After overnight induction at 20 °C, the cells were harvested, washed twice with 100 mM Tris/HCl buffer (pH 8.5), and used for the whole-cell reaction. When kinetic resolutions of 10 mM **a12** and 5 mM **a1** were carried out in the 100 mM Tris/HCl buffer (pH 8.5) with whole-cell (20 mg dry cell weight (DCW)/mL) without adding cofactor NAD^+ for 24 h, both of the amines were successfully resolved into the (*S*)-form with a high *ee* (>99%). These results clearly showed that the use of whole-cell was much more efficient than the reaction using purified enzymes that gave 26% *ee* (Figure 6b). The Shell group reported that in the asymmetric synthesis of chiral amine by AmDH, the lyophilized whole-cell gave better conversions both in the absence and presence of co-solvent heptane than lyophilized lysate [20]. The use of whole-cell apparently reduces the substrate/product inhibition, and/or it

increases the stability of the enzymes. When the reactions with 20, 30, 40, and 50 mM **a12** were carried out at pH 8.5 using whole-cell (25 mg DCW/mL) for 24 h, the *ee* values of (*S*)-**a12** were >99%, 87%, 48%, and 30%, respectively (Figure 7). At initial substrate concentrations of 20, 40, and 50 mM, the produced 2-heptanone, determined by the consumed **a12**, was 14.0, 12.9, and 11.6 mM, respectively. When 2-heptanone is accumulated to some extent (11–14 mM), the reaction does not seem to proceed any further. The main reason for the lower *ee* at higher substrate concentrations seems to be the inhibition of AmDH by 2-heptanone.

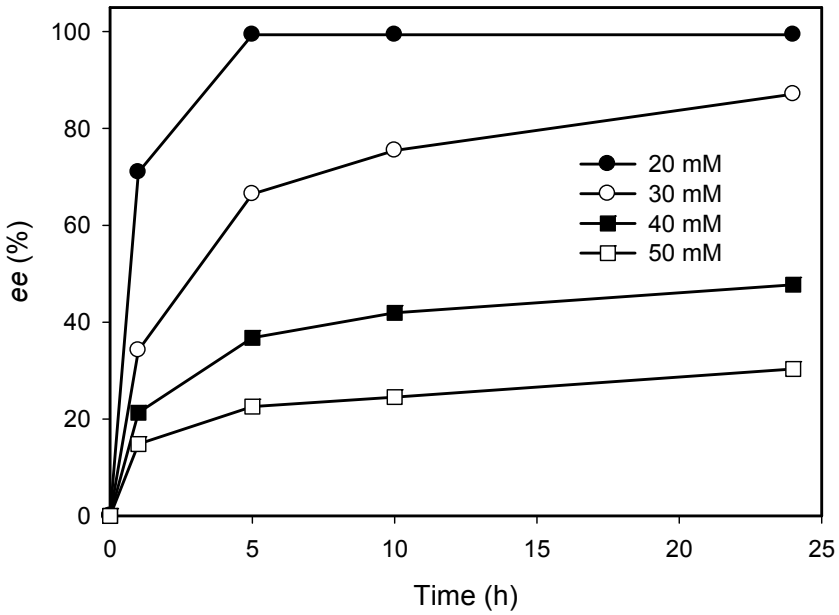


Figure 7. Reaction profiles of the kinetic resolution at different concentrations of **a12**. Reaction conditions: **a12** (0–50 mM), 100 mM Tris/HCl buffer (pH 8.5), whole-cell (25 mg dry cell weight (DCW)/mL).

The next step was to increase the final product's concentration, which could be accomplished by enhancing the biocatalyst concentrations in the reaction medium. Compared to **a12**, the kinetic resolution of **a1** is much more difficult due to the poor reactivity of AmDH for **a1**, a more severe product inhibition by the corresponding ketone, acetophenone, and a stronger inhibition of Nox by **a1** (Figure 6). After the concentrations of **a1** and **a12** were set at 20 mM and 50 mM, respectively, the reaction was performed to determine the amount of whole-cell catalyst required to obtain the optically pure (*S*)-amine (Figure 8). The minimum amounts of whole-cell required to resolve 50 mM **a12** and 20 mM **a1** into (*S*)-form (>99% *ee*) were 60 and 100 mg of DCW/mL, respectively. Next, the reaction profiles for the kinetic resolution of **a1** and **a12** were determined (Figure 9). In the 50 mM **a12** reaction, 40 mg DCW/mL of whole-cell gave only 77% *ee* for 24 h (Figure 8), but 60 mg DCW/mL of whole-cell gave >99% *ee* in 1 h (Figure 9). Also, the *ee* value of a1 was 95% in 24 h of reaction with 80 mg DCW/mL of whole-cell; however, the *ee* value became >99% after 5 h of reaction with 100 mg DCW/mL of whole-cell. This result is consistent with the observation that the enzyme activity of AmDH is inhibited by the ketone product.

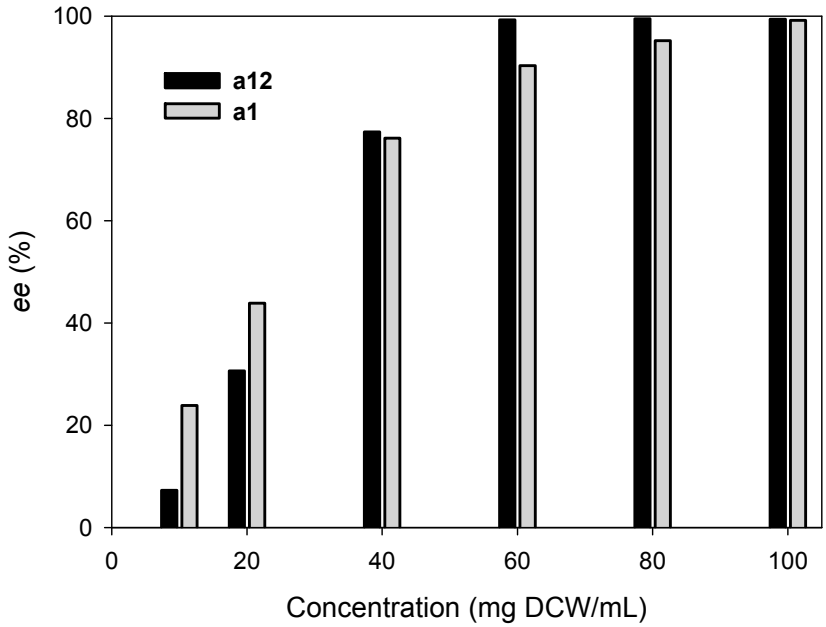


Figure 8. The effect of the amount of whole-cell on the kinetic resolution of 50 mM **a12** or 20 mM **a1**. Reaction conditions: 100 mM Tris/HCl buffer (pH 8.5), 50 mM **a12** (or 20 mM **a1**), and whole-cell (10–100 mg DCW/mL).

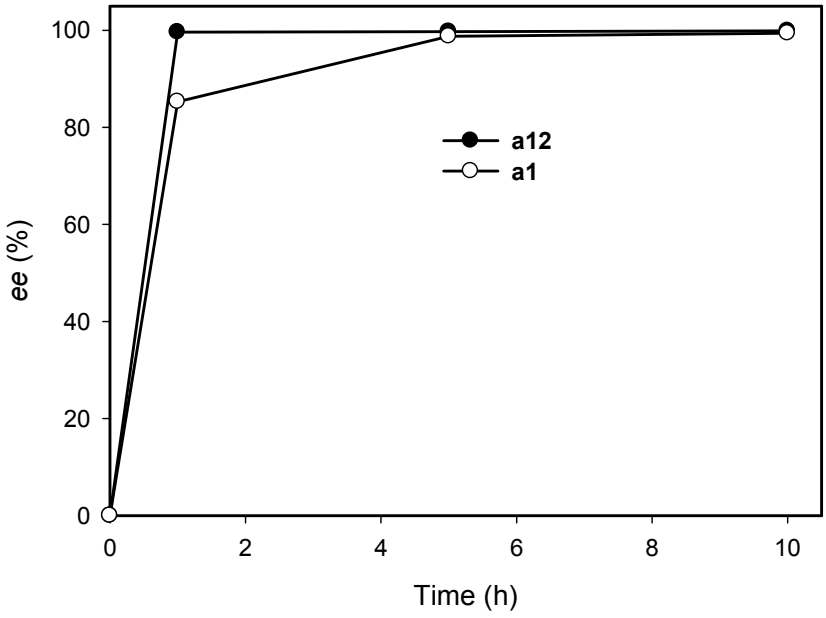


Figure 9. Reaction profiles of the kinetic resolution of 50 mM **a12** or 20 mM **a1**. Sixty milligrams (60 mg) and 100 mg DCW/mL of whole-cell was used for the reaction of 50 mM **a12** or 20 mM **a1**, respectively.

To investigate the utility of a whole-cell system expressing AmDH and Nox, the kinetic resolutions of various *rac*-amines (10 mM) were performed (Table 1). **a5**, **a7**, and **a8** were successfully resolved into (*S*)-form with a high *ee* (>99%). However, the kinetic resolutions of **a4**, **a6**, and **a11** gave 66%, 28%, and 33% *ee*, respectively. Among the fluorinated amines (**a4**, **a5**, and **a6**), **a4** is the most reactive substrate, but only **a5** was converted into an optically pure form. Also, although **a11** was the most reactive substrate in the tested substrates (Figure 2), the *ee* value of **a11** was only 33%. In general, the more reactive substrates are expected to have higher conversion rates into product. However, it is likely that there was no clear correlation between final conversion and substrate reactivity because of

an unclear correlation between the reactivity and product inhibition of AmDH by the corresponding ketone. In the case of **a4**, **a6**, and **a11**, the reactions were carried out at a lower concentration (5 mM) with the same condition, and the *ee* values were 77%, 78%, and 51%, respectively (Table 1). Although the *ee* value increased more than when 10 mM substrate was used, the optically pure form could not be obtained.

Table 1. Kinetic resolution of various amines using AmDH/Nox [a].

Substrate	Conc. (mM)	Conv. (%)	ee^S (%)
a4	10	40	66
a4	5	44	77
a5	10	50	>99
a6 [b]	10	22	28
a6 [b]	5	44	78
a7	10	50	>99
a8	10	50	>99
a11	10	25	33
a11	5	34	51

[a] The reaction conditions: 5 mL of reaction volume, 120 mg DCW/mL of whole-cell, Tris/HCl buffer (100 mM, pH 8.5), and 5 or 10 mM racemic substrate (see Figure 2) for a 24 h reaction at 37 °C. Conversion and *ee* were determined by Crownpak CR(+) column; [b] Conversion and *ee* were determined by C_{18} Symmetry column after GITC derivatization.

3. Materials and Methods

3.1. Chemicals

All amines (**a1**–**a12**), GITC (2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocynate), cofactors (NADH and NAD^+), and isopropyl-β-D-thiogalactopyranoside were obtained from Sigma-Aldrich Yongin, Korea.

3.2. Enzyme Expression and Purification

The AmDH and Nox genes [18,24] were synthesized by Bioneer Corporation (Daejeon, Korea), and each gene was then inserted into IPTG-inducible pET24ma vectors. The plasmid was then introduced into *E. coli* (BL21) cells and the transformants were grown at 37 °C in 1 L LB-containing kanamycin (100 μg/mL) [32]. When the OD_{600} reached 0.5, IPTG was added to a final concentration of 0.5 mM. After overnight induction at 20 °C, the cells were harvested and washed twice with 100 mM Tris/HCl buffer (pH 8.5). After centrifugation, the cell pellets were resuspended in 10 mL volume of 20 mM Tris/HCl buffer (pH 8.5). The suspension was then subjected to ultrasonic disruption. The disruption period was 5 s with 10 s intervals in an ice bath for a duration of 30 min. The C-terminal His6-tagged enzyme was purified at 4 °C on a Ni-NTA agarose resin obtained from Qiagen (Hilden, Germany). The eluted solution containing purified protein was dialyzed against 50 mM potassium phosphate buffer (pH 8.5) and concentrated using an Amicon PM-10 ultrafiltration unit. Glycerol was added to the purified enzyme solution (25%) and it was stored at −20 °C for further study.

For the co-expression of AmDH and Nox, the AmDH gene was amplified and subsequently inserted into the pETDuet-1 (Novagen, Madison, WI, USA). The plasmid was then introduced into the *E. coli* (BL21) bearing pET24ma:Nox. Then, enzymes were overexpressed in the presence of kanamycin (100 μg/mL) and ampicillin (100 μg/mL) as describe above. After harvesting, the cells were washed twice with 100 mM Tris/HCl buffer (pH 8.5) and they were immediately used for the whole-cell reaction.

3.3. Enzyme Assay

The activities of AmDH and Nox were determined using an NADH-dependent (340 nm, λ_{340} = 6220 M^{-1} cm^{-1}) spectrophotometric assay. Activity is calculated from the stoichiometric

reduction of NAD^+ or oxidation of NADH as measured by the change in absorbance over time. One unit of activity is defined as the amount enzyme that catalyzes the formation of 1 μmol of NADH or formation of 1 μmol of NAD^+ in 1 min. A standard AmDH activity assay was carried out in 100 mM glycine buffer (pH 10.0) containing AmDH (0.15 mg/mL), 5 mM substrate, and 1 mM NAD^+ at 25 °C [18]. The standard solution for the assay of Nox contained 0.2 mM of NADH and an appropriate amount of the enzyme [24].

3.4. Analysis of Amines

The conversion and *ee* analysis of amines were performed by HPLC using a Crownpak CR (Daicel Co., Osaka, Japan) column at 210 nm with elution with a perchloric acid solution of pH 1.5 (0.6 mL min^{-1}) [33]. Each enantiomer was separated by these analytical conditions except **a6** and **a12**. A quantitative chiral analysis of **a6** and **a12** was performed using a C18 symmetry column (Waters, Milford, MA, USA) with a Waters HPLC system at 254 nm after the derivatization of the sample with GITC [33]. The separation of each enantiomer of **a6** and **a12** was achieved through isocratic elution with a mixture of 50% methanol and 50% water (0.1% TFA) at a flow rate of 1.0 mL min^{-1}.

4. Conclusions

To produce chiral amines, the stereoselective reductive amination of ketones by AmDH is favored in practice. To prepare chiral amines with opposite chirality, the oxidative deamination of racemic amines by AmDH is as important as reductive amination. Here, the kinetic resolution of racemic amines was performed using AmDH in combination with Nox, in which Nox was used to regenerate NAD^+ and to drive the reaction forward. Compared to the purified enzyme system, the whole-cell system showed much better performance, probably due to the reduction of the inhibitory effect of substrate/product on the enzymes and the increased stability of the enzymes in the whole-cell system. By using the whole-cell system expressing AmDH and Nox, 50 mM 2-aminoheptane and 20 mM α-MBA were successfully resolved into (*S*)-form with >99% *ee*, respectively. The kinetic resolution of amines at a high concentration by AmDH can be carried out effectively by the following approach. First, inhibitory ketones in the reaction mixture should be removed by using ketone-extracting organic solvents (biphasic reaction) or ketone-converting enzymes (e.g., alcohol dehydrogenase) to convert them into non-inhibitory compounds [32]. Second, alkaliphilic enzymes, such as Nox from *Brevibacterium* sp. KU1309 [34], should be used for the coupling reaction, then the coupling reaction can be carried out at a pH that is close to the optimal pH (10.0) of AmDH. The further development of the kinetic resolution of amines using AmDH is currently being investigated with these approaches.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/7/9/251/s1, Figure S1: SDS-PAGE analysis of purified AmDH (42.37 kDa), Figure S2: Lineweaver–Burk (double-reciprocal) plot of 1/v against 1/[S]title, Figure S3: SDS-PAGE analysis of recombinant *E. coli* expressing AmDH (42.37 kDa) and Nox (50.47 kDa). Table S1: Retention times of chiral amines in HPLC analysis.

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