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Use of whole cell culture of *Aeromonas* sp. as enantioselective scavenger: A facile preparation of L-amino acid derivatives in high enantiomeric excess

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ABSTRACT

The bacterium *Aeromonas* sp. (CGMCC 2226) can enantioselectively scavenge D-isomer, making L-amino acid derivatives (AADs) in high ee. The enantioselective scavenger (ES) has shown a broad substrate scope. Eleven L-AADs, Phe derivatives substituted with methyl-, mono- and dichloro-, bromo-, and nitro-group, were produced in high ee from corresponding racemates.

Inspired by Pasteur's early work in 1858, *Penicillium glaucum* was found to have specifically scavenged the D form of ammonium tartrate, leaving the L form unaffected,[1] we recently explored a strategy using enantioselective scavengers (ES) as means to discover new biocatalytic processes, biocatalysts (enzymes or strains), and their corresponding reaction types with respect to substrate specificity and enantioselectivity.[2] Ultimately, the ES strategy is expected to provide us with the tools necessary to get more insight into nature's rich realm of enzymatic catalysis, addressing fundamental questions such as the scope and usefulness of the radical mechanism in enzymes—a topic revitalized by the recent findings from Kim et al.[3]

In the course of our study, we found that the homogenate made from the yeast *Rhodotorula graminis* (*R. graminis*) was able to enantioselectively scavenge the L-enantiomer of a series of racemic (*rac*) mixture of non-proteinogenic amino acid derivatives. The ES contained a series of L-selective enzymes. Among these, some were redox enzymes that can smoothly use molecular oxygen in vitro under mild conditions. The L-ES demonstrated a broad substrate scope. It appears that the ES strategy can be used as an efficient method for the preparation of D-AADs in high ee. A series of D-AADs were obtained in enantiomerically pure form by removing L-isomer from their racemic substrates.[2]

In this letter, we wish to report, complementarily, a D-ES system comprised of D-selective enzymes with opposite enantioselectivity as compared to *R. graminis.* The D-ES also had a broad substrate scope and appeared to be an efficient method for preparation of L-AADs in high ee. A series of L-AADs were produced in enantiomerically pure form by removing D-isomer from their racemic substrates.

L-AAs are predominantly nature's choice for protein biosynthesis but D-AAs are present in small amounts practically in all living systems.[4] Thus, the AAs from natural sources contain inevitably a small amount of D-AAs as 'contaminants', either from the source organisms or by the racemization of their L-counterparts during the preparation process. Similarly, synthetic AAs and their derivatives (AADs) also contain more or less the undesired enantiomer 'contaminants'. They are either carried from precedent chiral pools and/or synthetic methods that (with a few exceptions) seldom reach enantiopure form.[5–9] In both cases, practical methods facilitating the removal of D-isomers from racemic or non-racemic mixtures in order to enrich the ee of L-AADs are in high demand.

In accordance with the predominance of L-AAs over D-AAs in nature, the number of L-selective enzymes predominate that of their D-selective counterparts. A few enzymes known to be D-selective include D-amino acid oxidase (D-AAO, EC 1.4.3.3), hydantoinase (EC 3.5.2.2),[10] *N*-carbamoyl-D-amino acid amidohydrolase (EC 3.5.1.77), and aminotransferase of *Escherichia coli* (*E. coli*).[11,12] Among these, D-AAO, a flavin dependant enzyme, has been the most studied prototype since its discovery by Krebs.[13] Even though

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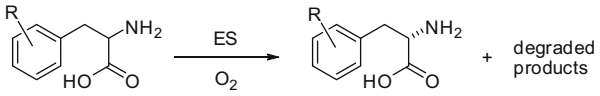
Table 1
ES of D-AADs in *rac*-AADs by *A.* sp.[a,b]



Entry	Substrate	Product	Time (h)	Yield (%)	Ee (%)
1	*Rac*-Phe	L-Phe	24	44	>99
2	*Rac*-4-chloro-Phe	L-4-Chloro-Phe	22	49	>99
3	*Rac*-4-bromo-Phe	L-4-Bromo-Phe	22	43	>99
4	*Rac*-2-bromo-Phe	L-2-Bromo-Phe	46	37	>99
5	*Rac*-4-methyl-Phe	L-4-Methyl-Phe	43	46	99
6	*Rac*-2,4-dichloro-Phe	L-2,4-Dichloro-Phe	22	46	>99
7	*Rac*-3,4-dichloro-Phe	L-3,4-Dichloro-Phe	22	50	>99
8	*Rac*-4-nitro-Phe	L-4-Nitro-Phe	42	51	95
9	*Rac*-2-chloro-Phe	L-2-Chloro-Phe	43	48	92
10	*Rac*-4-fluoro-Phe	L-4-Fluoro-Phe	53	41	72
11	*Rac*-(Tyr)	L-Tyr	53	60	40

[a] Ees are from HPLC analysis of the last sample.
[b] *E* values which describe the enantioselectivity of kinetic resolution were not calculated because the exact measure of conversions could not be performed in this range to meet the sensitivity of Sih's equation.

D-AAO is not as ubiquitous in nature, their presence is highly significant in biological systems. For example, D-AAO has high expression in human brain and it has been speculated to play a role in neurotransmission and aging,[14] Alzheimer disease,[15] etc. However, much of its biological role remains to be discovered.

Biocatalytically, D-AAO catalyzes the oxidative deamination of D-AAs to α-keto acids. The substrate scope of D-AAO is limited to the AAs with small hydrophobic side chains and polar, aromatic and basic group, excluding those with acidic group. For those acidic AAs, there exists a specific enzyme, D-aspartate oxidase (EC 1.4.3.1), which has a 50% identity in sequence with D-AAO. A microbial D-AAO from *Rhodotorula gracilis* was manipulated by rational design to have new and broader substrate specificity.[16] A nicotinamide cofactor dependant D-AAO from *E. coli* has been evolved through five rounds of directed evolution to accept a broader scope of substrates.[17] In all these cases, the enzymes exhibit high enantiospecificity toward the D-isomer of a limited number of substrates. Such features might find potential applications as biosensors and simple analytical tools used in the quantification of D-AAs.[18]

Generally, the enzyme functions may differ depending on their origin, structure, and homogeneity. More and more enzymes have recently been shown to be promiscuous with respect to their catalytic activity and substrate specificity.[19,20] Thus, with powerful enhancing technologies such as directed evolution, D-AAO may be expected to be a new addition in toolbox of biocatalysis.

In the course of our study of ES, we found that the bacterium *Aeromonas* sp. (*A.* sp. CGMCC 2226) (Note 1),[21] an isolate of soil sample from the Hangzhou area in China exhibited the D-selectivity in transforming *rac*-Phe, exactly opposite to that of yeast *R. graminis.* When incubated with *rac*-Phe under aerobic conditions, the bacterium selectively scavenged the D-Phe from *rac*-Phe. The transformation progressed until the D-Phe was completely scavenged beyond the detection limit, leaving the L-Phe in enantiopure form. We conducted a literature search and did not find any information related to this strain and the D-selective enzymes. We noticed that the system required aerobic conditions. Unlike *R. graminis*, however, when the cells of *A.* sp. were broken through ultra-sonication, the homogenate no longer functioned properly. The reactions stopped in less than 2 h. Therefore, the whole cell culture, which can remain active for more than two days, was employed as D-ES in all experiments in this study. We further explored the potential use of this microorganism as D-ES toward a range of non-proteinogenic amino acid derivative substrates. The experiments were carried out in parallel and repeatedly as to compare the differences between the substrates and to minimize the variations of reproducibility (Note 2).[22] The results are shown in Table 1.

The ES system showed excellent enantioselectivity toward a series of AADs, Phe derivatives substituted with methyl-, mono- and dichloro-, bromo-, and nitro-group. It selectively scavenged D-isomer of AADs from the corresponding racemate, leaving the remaining L-AADs (37–50%) in high ee (>99%) (entry 1–7). The slower conversion and lower ee of 4-nitro-Phe and 2-chloro-Phe (entry 8 and 9) may be due to the lower solubility of the substrate. The *A.* sp. cells remained active for about two days, after which point the enzyme activity decreased gradually. For the case of 4-fluoro-Phe (entry 10), the enzyme activity was lost before the conversion was completed. Exceptionally, for the case of Tyr (entry 11), the transformation of D-isomer of this natural AA was unexpectedly slow. An L-selective enzyme was involved which consumed the L-isomer, and decreased the ee. As both enzymes lost their activity before the completion of the conversion, this resulted in a non-racemic mixture of 40% ee (L). A typical set of chromatograms from our experiments with *rac*-AADs are shown in Figure 1.

The consumption of air indicated the organism's intake and use of molecular oxygen. Like all typical biooxygenation processes, it did not proceed in vitro without supplements or alternative method for regeneration of cofactors because the mechanisms for cofactor regeneration are lost once the cells are broken. At this stage it is still not known what cofactors are used in this system.

It was argued that the enrichment of ee of the resulting L-AADs might involve a D to L conversion. This possibility was excluded by experiments using single enantiomer of D-Phe and D-4-nitro-Phe as the sole substrates in the reaction. No D to L conversion was observed. However, the D-ES contains multiple enzymes. In case of Tyr, it was clearly seen that an L-selective enzyme was involved in the process, which consumed the L-isomer and caused the decrease of ee. Structurally, the substrates used in this study differ from Phe only by one or two substitutions on the aromatic ring. It was thus postulated that they would share the same scavenging pathways. Apparently, it seemed to be true in most cases in this study. However, the surprising behavior of Tyr indicated that the system might be more complex than it appears.

It would be desirable to know the exact enzymes that are responsible for the D-scavenging process, what their cofactor(s) are, if they are known enzymes in a different host, what is the sequence similarity between them and other known enzymes, and whether they are promiscuous in terms of catalytic activity and substrate specificity. To this end, the genome sequencing of the strains, purification of the enzymes, replication of our experiments

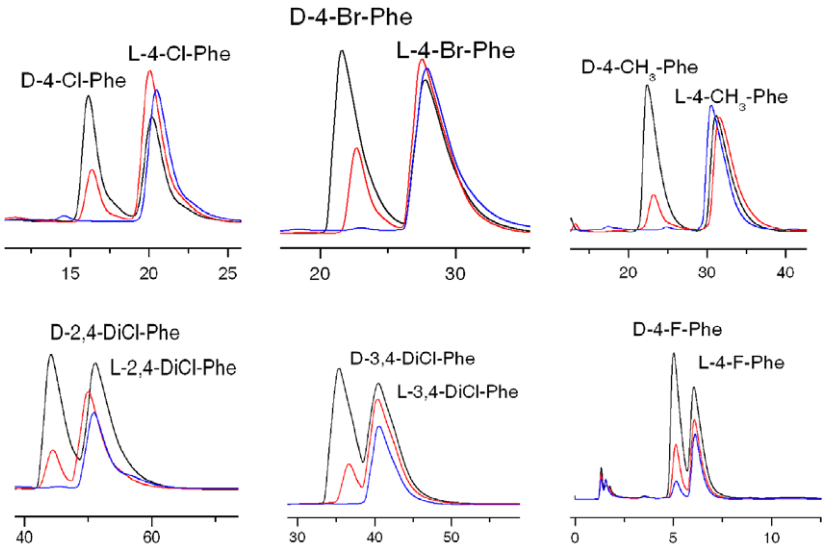


Figure 1. Depicted are chromatograms of the D-ES scavenging process corresponding to entry 2, 3, 5, 6, 7, and 10. The black, red, and blue lines are of the samples at the beginning, the middle, and the end of the reaction, respectively. The retention times (min) varies from one substrate to another. The D-enantiomer which elutes first was consumed.

using purified enzymes, and the isolation and determination of the products formed from these enzymes would certainly help in understanding the ES process. Nevertheless, this work uncovers a rich realm of discoveries. The practical usefulness of this work provides a valuable target for sequencing, cloning, expression, genetic engineering, biochemical study, biocatalytic exploration, as well as biomedical studies, etc.

In conclusion, the whole cell culture of the *A.* sp. containing multiple enzymes can be used as D-ES to remove the D-AADs in the *rac*-AADs, affording a series of L-AAD in high ee. This work presents a simple and efficient synthetic method that uses no solvents and requires no derivatization steps. Besides being immediately useful as a new purification method, this work opens doors to many new possibilities in the area of biocatalysis. We anticipate that the concept of ES can be applied to a broader range of uses in a variety of fields.

Ongoing work is being done in getting more insight on the process. They include mainly (1) the identification of the enzymes responsible for the reaction in this ES system; (2) the investigation of its mechanism and the scope of its application.

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21. *Note 1:* The bacterium strain was identified as *Aeromonas* sp. and deposited in China General Microbiological Culture Collection Center for preservation. The deposit number is: CGMCC No. 2226.
22. *Note 2:* A typical experimental procedure of the bioconversion is as follows: The bacterium *A.* sp. was inoculated under sterilized conditions in a 500-mL flask containing 100 mL of growth media (yeast extract 1.0%, peptone 1.0%, K_2HPO_4 0.5%, and L-phenylalanine 0.5%, the pH was not adjusted after sterilization) and incubated under 30 °C for 24–26 h. The culture was then harvested, centrifuged, washed twice with ice water, and the supernatant was decanted. The culture cake was put back into a shaking flask. The substrate (50 mg) was put into the flask after being dissolved in dilute sodium hydroxide. The pH was quickly adjusted to 7.3, and the total volume was adjusted to 50 mL by tris buffer. The flask was aerated before being closed by an air permeable stopper. The flask was then set in the shaker with a controlled speed (200 r/min) and temperature (30 °C). The progress of the bioconversion was monitored by regular sampling and HPLC analysis. The reaction mixture was allowed to react for 24–48 h and extended to 53 h in some cases depending on the conversion. For the samplings, 1 mL of reaction mixture was removed to an Eppendorff vial and centrifuged. The cell debris was decanted, and the supernatant was filtered. The filtrate was diluted by distilled water and used for injection into HPLC column for analysis. The HPLC system was equipped with a chiral column Crownpak CR (+) (Daicel, Japan). The standard conditions of elution for *rac*-Phe suggested by the column supplier were modified by adding 10% of methanol to isocratically elute all the AADs. In all cases, the clear (or baseline) separation of the two enantiomers was achieved. The detection wavelength was set at 200 nm. For the isolation of products, the experiment was carried out in duplicates (maximum in parallel with 28 flasks). At the end of transformation, the mixture was collected and centrifuged. The cell debris was decanted, and the supernatant was loaded onto Resin column and eluted by buffer. The elution conditions differ from one substrate to another with no optimization. The fractions were also analyzed by HPLC. L-AADs were collected in high purity after concentration.