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Review

Selection and Directed Evolution of New Microbial Biocatalysts and Their Application to Organic Synthesis

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As a typical example of the screening for a microbial biocatalyst from nature, isolation of nitrile-synthesizing microorganisms, characterization of a new enzyme aldoxime dehydratase, and its function in the aldoxime-nitrile pathway are introduced. Catalytic properties of some of our enzymes were improved through a direct evolutionary approach.

Key words: *microbial biocatalyst, aldoxime dehydratase, nitrile hydratase, molecular evolution.*

The recent success in microbial transformation has been based on the screening for microbial enzymes catalyzing new reactions,[1] or screening known enzymes for an unknown activity with synthetic substrates.[2] We screen microorganisms for an enzyme for many reasons, including when: a) no information is available on the desired reaction, as exemplified in the case where a new enzyme is discovered during physiological studies on microbial degradation of xenobiotics; b) nothing is known about the specific reaction, except that a homologous reaction occurs: c) it is known only in other biological source; d) not enough enzyme is produced by a certain organism for a practical transformation, and e) increased stability for practical use is required. Enrichment culture[3] is a technique for isolating microorganisms which have special growth characteristics. Some microorganisms grow faster than others in media with limited nutrients, high temperature, extreme pHs, etc. Microorganisms that grow faster than other species become dominant after several culture transfers. An acclimation technique[3] is applied when a toxic or unnatural compound is used as a substrate, and is usually run long-term to isolate microorganisms which cannot be easily isolated in an enrichment culture. An adaptation to a synthetic medium containing a target compound often results in the isolation of microorganisms possessing a new enzyme. Genetic changes in the microorganisms may be expected.

A typical example of the acclimation procedure was demonstrated through the successful isolation of an acrylonitrile-utilizing bacterium, *Rhodococcus rhodochrous* (formerly *Arthrobacter* sp.) I-9 (K-22, AKU 629).[4] Since acrylonitrile is a very toxic compound, it was not possible to isolate the degrader utilizing it as sole carbon and nitrogen sources through an enrichment culture technique. Activated sludge obtained from sewage disposal facilities was suspended in an acclimation medium containing 0.0008% acrylonitrile. After three months, *R. rhodochrous* I-9, which utilizes acrylonitrile as carbon and nitrogen sources, was isolated for the first time from the sludge. Through the enrichment culture technique, *R. rhodochrous* J-1[4] and *Pseudomonas chroloraphis* B23,[5] among others, were isolated as acetonitrile and isobutyronitrile utilizers. From *R. rhodochrous* J-1, nitrile hydratase was discovered, purified, and characterized.[6,7] Two pathways of nitrile hydrolysis were also discovered to co-exist in *R. rhodochrous* J-1, one combining nitrile hydratase and amidase[8] and the other involving nitrilase.[9] *P. chroloraphis* B23,[5] an industrial strain used to produce acrylamide from acrylonitrile and 5-cyanovaleramide,[10] was selected among the isobutyronitrile-utilizing microorganisms, based on the result that acrylonitrile was directly hydrolyzed to acrylic acid and ammonia in *R. rhodochrous* I-9 and by carefully screening for differences in the substrate specificities of nitrile hydratase and amidase among the nitrile degraders. The observation of high accumulation of amides using a new bacterial enzyme, "nitrile hydratase",[4-7] led to the successful application of the enzyme to the industrial production of various amides. Thus, the enzyme has become one of the most important industrial enzymes.[11] Recent progress includes a mechanical study on the structural formation of an Fe-containing enzyme through X-ray analysis.[12] It has been considered that the occurrence of the enzyme is rather rare,[6,7] although the distribution of the enzyme was later found to be broader than originally thought. Furthermore, the physiological meaning of the occurrence of the enzyme in the microbial world has never been studied. These situations have prompted us to study the microbial metabolism of aldoximes involving a new enzyme phenylacetaldoxime dehydratase, and its application to nitrile synthesis.

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A New Enzyme, Aldoxime Dehydratase, and Its Application

Recently, we have been successful in the enzymatic synthesis of nitriles from aldoximes using the new microbial enzyme aldoxime dehydratase. We isolated the enzyme for the first time and studied its enzymological properties, and found that the enzyme and nitrile-degrading enzymes such as nitrile hydratase and nitrilases are linked genetically as well as enzymatically. We acclimated soil samples for about 4 months to isolate *Bacillus* sp. strain OxB-1, a degrader of *Z*-phenylacetaldoxime.[13] The strain possessed an inducible enzyme catalyzing the formation of phenylacetonitrile from Z-phenylacetaldoxime, and showed a strong activity in hydrolyzing nitriles. Partially purified enzyme showed a stoichiometric formation of phenylacetaldoxime from *Z*-phenylacetaldoxime.

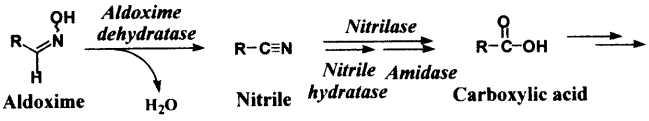
Although the enzyme activity was completely lost upon dialysis of the cell-free extract, it was restored through the addition of FMN. The enzyme was purified to homogeneity from the cell-free extract and was shown to be monomeric with a *Mr* of about 42 kDa. The enzyme was specific for *E/Z*-arylalkylaldoximes and *E/Z*-alkylaldoximes, giving the corresponding nitriles. The results of absorption spectrum analysis, atomic absorption spectrum analysis, and heme staining revealed that the enzyme contains loosely bound protoheme IX and requires FMN for its activity. It can be categorized into a group of enzymes that require flavin and yet catalyze a reaction involving no net oxidation or reduction. Based on the results, we tentatively named the enzyme "phenylacetaldoxime dehydratase (EC 4.2.1.-)".[14]

Dehydration of aldoxime, easily prepared from aldehydes and hydroxylamine, is a useful method for synthesizing nitriles, although many such chemical methods require harsh reaction conditions. On the other hand, there has been a report on a partially purified indole acetaldoxime hydratase from *Gibberella fujikroi*, though details of its enzymological properties are not yet known. We screened for microorganisms to convert *E*-pyridine-3-aldoxime to the corresponding nitrile and isolated *Rhodococcus* sp. YH3-3 from soil.[15] The cells containing the inducible enzyme catalyzed the stoichiometric dehydration of aldoximes to form nitriles. Because of the occurrence of nitrile hydratase and amidase in the cells, the nitrile, once synthesized through the action of the dehydratase, was hydrolyzed to benzoic acid. We treated the cells to inactivate nitrile hydratase. This improved the yield of nitrile up to 99%. Various nitriles, such as 3-cyanopyridine, 2-cyanopyridine, and cyanopyrazine, were synthesized at preparative scales from 10-100 mM aldoximes under optimized reaction conditions (pH 7.0, 30°C). This is the first report on the enzymatic synthesis of nitriles.[16]

There has been no previous report on the biosynthesis of nitriles and the physiological function of the enzymes. We investigated the distribution of aldoxime dehydratase and the relationship between aldoxime- and nitrile-degrading enzymes among different microorganisms.[17] All the aldoxime degraders possessed nitrile-hydrolyzing activities. On the other hand, all nitrile degraders described in the literature possessed aldoxime-degrading activities. Thus, we discovered the co-existence of aldoxime dehydratase and nitrile-degrading enzymes and elucidated the role of nitrile degrading enzymes in aldoxime metabolism in microorganisms. Nitrile hydratase, whose remarkable catalytic activity has been much stressed in the synthesis of amides, was found to be linked with aldoxime dehydratase both genetically and physiologically.[16,18]

The *oxd* gene coding for the enzyme was cloned, and an open reading frame coding for 351 amino acid residues was identified. A nitrilase, which participates in the aldoxime metabolism in the organism, was found to be coded by the region just upstream from the *oxd* gene. In addition an open reading frame (orf2), whose gene product is similar to the bacterial regulatory (DNA-binding) proteins, was found just upstream from the coding region of the nitrilase. These findings provide genetic evidences for a novel gene cluster that is responsible for aldoxime metabolism in this microorganism.[16]

We also found that the occurrence of aldoxime dehydratase is as wide as that for nitrile-degrading enzymes such as nitrile hydratase, amidase, and/or nitrilase. All nitrile degraders hitherto isolated contained aldoxime dehydratase activities.[17] The author would like to propose that the pathway in which aldoximes are successively degraded *via* nitrile be named as the oxime-nitrile pathway.



Directed Evolution of New Microbial Biocatalysts

Recently, the directed evolution[19,20] of enzymes has attracted much attention to optimize or fine-tune their properties by screening for particular properties after random mutagenesis,[21] error prone PCR,[22-24] and gene shuffling.[25,26] Random mutagenesis makes it possible to generate a large number of randomly distributed, nucleotide substitution mutations in cloned DNA fragments through treatments with chemicals such as nitrous acid, hydroxylamine, formic acid, and hydrazine, among others. Error-prone PCR has been developed to introduce random point mutations into the cloned genes. Modifications were made to decrease the fidelity of the PCR reaction by increasing the concentration of $MgCl_2$, adding $MnCl_2$, or unbalancing the concentrations of the four dNTPs. Gene shuffling enables molecular mixing of naturally similar or randomly mutated genes. In an error-prone PCR and gene shuffling, as well as in random mutagenesis, screening for

certain properties is very important. With the directed evolutionary technique, these mutation methods are repeatedly carried out to obtain desirable properties of the enzymes. Thus, the selection, conventional screening methods for new microbial enzymes, and optimization of biocatalysts by these DNA-based methods have a common feature in that they repeatedly screen for certain desirable activities. Combination of these methods enables us to obtain biocatalysts ideal for the enzymatic synthesis of useful compounds. Our recent results on the improvement of several of new enzymes can found elsewhere.[24,27]

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