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Microbial Reactions for the Production of Useful Organic Compounds

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Introduction

In recent years, the most significant development in the field of synthetic chemistry has been the application of biological systems to chemical reactions. Reactions catalyzed by enzymes and enzyme systems display far greater specificities than conventional forms of organic reactions. Of all the reactions available, enzymatic synthesis has the greatest potential. Studies on the production of various biologically and chemically useful compounds such as amino acids, amides, acids, pyrogallol, theobromine, pantothenic acid, coenzymes, and polyunsaturated fatty acids using microbial enzymes as catalysts, have been carried out for more forty years in our laboratory in Kyoto University. An overview of the status of our work is presented in *Table 1*.

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Four successful examples will be discussed in detail, in comparison with conventional chemical processes.

1. Synthesis of D-Phenylglycine and Related D-Amino Acids

Dihydropyrimidinase is an enzyme that catalyzes the ring-opening reaction of dihydropyrimidines. A crystalline preparation of microbial dihydropyrimidinase has been obtained and characterized in some detail in our laboratory. We have proved that this enzyme catalyzes the hydrolysis of a variety of 5-monosubstituted D-hydantoins including not only those of naturally occurring amino acids, but also some of unnatural amino acids. For example, D-forms of 5-phenyl-, 5-hydroxyphenyl-, 5-chlorophenyl-, 5-methoxyphenyl-, and 5-thienyl-hydantoins are well hydrolyzed to the corresponding D-forms of the *N*-carbamoyl glycine derivatives [1–3]. The L-isomers of hydantoins are spontaneously racemized under mild alkaline conditions used for the enzymatic hydrolysis. Therefore, D,L-hydantoins are completely converted to the corresponding D-forms of *N*-carbamoyl amino acids. These *N*-carbamoyl amino acids are quantitatively transformed to D-amino acids on treatment with $NaNO_2$ under acidic conditions. As a result of these fundamental examinations of the production of D-amino acids, we have developed a new process for the production of D-*p*-hydroxyphenylglycine. This amino acid is an important component of semisynthetic penicillins and cephalosporins and has so far been synthesized chemically as a racemic mixture, which was then optically resolved through rather complicated processes. In our process, the starting substrate, D,L-5-(*p*-hydroxyphenyl)-hydantoin, is synthesized through a newly established aminoalkylation of phenol with glyoxylic acid and urea under acidic conditions [4]. The hydantoin is then hydrolyzed asymmetrically by the action of microbial cells with high enzyme activity. This process is followed by decarbamoylation with $NaNO_2$ (*Scheme 1*). The present process may be the most economical one for large scale production of D-*p*-hydroxyphenylglycine.

The production plant built by *Kaneka Singapore Pte. Ltd.* is located at the Jurong Industrial Estate, Singapore. The commercial production of D-*p*-hydroxyphenyl glycine started in 1981.

In a similar manner, D-phenylglycine and D-valine, which are important intermediates for the synthesis of semisynthetic penicillins and pesticides, respectively, have been commercially produced in the plant.

2. Synthesis of Aromatic and Sulfur-containing Amino Acids

β-Tyrosinase, tryptophanase, cysteine desulfhydrase, *O*-acetyl L-serine sulfhydrase and β-chloro-D-alanine dehydrochlo-

Table 1. *Forty Years of Studies in Enzymatic Synthesis*

Product	Enzyme (source)	Yield g/l	(mol%)
Amino Acids			
D-*p*-Hydroxyphenylglycine	Dihydropyrimidinase (*Bacillus* sp.)	5	(74)
D-Phenylglycine	Dihydropyrimidinase (*Bacillus* sp.)	6	(91)
L-Tyrosine	β-Tyrosinase (*Erwinia herbicola*)	61	
L-Dopa	β-Tyrosinase (*Erwinia herbicola*)	53	
L-Tryptophan	Tryptophanase (*Proteus rettgeri*)	100	(95)
L-Cysteine	Cysteine desulfhydrase (*Enterobacter cloacae*)	50	(86)
L-Cysteine	Cysteine synthase (*Bacillus sphaericus*)	70	(82)
D-Cysteine	β-Chloro-D-alanine dehydrochlorinase (*Pseudomonas putida*)	22	(88)
L-Cystathionine	Cystathionine γ-synthase (*Bacillus sphaericus*)	42	(92)
L-Serine	Serine hydroxymethyltransferase (*Hyphomicrobium* sp.)	52	
Ethyl (*R*)-4-chloro-3-hydroxybutanoate	Aldehyde reductase (*Sporobolomyces salmonicolor*)	88	(95)
Amides and Acids			
Acrylamide	Nitrile hydratase (*Pseudomonas chlororaphis*)	400	(100)
Acrylamide	Nitrile hydratase (*Rhodococcus rhodochrous*)	650	(100)
Methacrylamide	Nitrile hydratase (*Pseudomonas chlororaphis*)	200	
Crotonamide	Nitrile hydratase (*Pseudomonas chlororaphis*)	200	
Nicotinamide	Nitrile hydratase (*Rhodococcus rhodochrous*)	1465	(100)
Acrylic acid	Nitrilase (*Rhodococcus rhodochrous*)	80	(100)
Nicotinic acid	Nitrilase (*Rhodococcus rhodochrous*)	72	(100)
6-Hydroxynicotinic acid	Hydroxylase (*Comamonas acidovorans*)	120	(96)
6-Hydroxypicolinic acid	Hydroxylase (*Alcaligenes faecalis*)	116	(97)
D-Malic acid	Maleate hydratase (*Arthrobacter* sp.)	87	(72)
Pyrogallol	Gallic acid decarboxylase (*Citrobacter* sp.)	23	(100)
Theobromine	Oxygenase (*Pseudomonas putida*)	20	(92)
D-Pantoyl lactone	Carbonyl reductase (*Candida parapsilosis*)	100	(83)
D-Pantoic acid	Aldonolactonase (*Fusarium oxysporum*)	700	(95)
Coenzymes			
5'-IMP	Nucleoside phosphotransferase (*Pseudomonas trifolii*)	5.6	(80)
Coenzyme A	Multi-step enzyme system (*Brevibacterium ammoniagenes*)	115	(95)
Adenosylmethionine	AdoMet synthetase (*Saccharomyces sake*)	12	(45)
Adenosylhomocysteine	AdoHcy hydrolase (*Alcaligenes faecalis*)	74	(97)
FAD	FAD pyrophosphorylase (*Arthrobacter globiformis*)	18	(28)
Pyridoxal 5'-phosphate	PMP oxidase (*Pseudomonas fluorescens*)	0.15	(98)
NADH	Formate dehydrogenase (*Arthrobacter* sp.)	30	(90)
NADPH	Glucose dehydrogenase (*Gluconobacter suboxydans*)	73	(100)
Polyunsaturated Fatty Acids			
Dihomo-γ-linolenic acid	Multi-step conversion (*Mortierella alpina*)	4.1	
Arachidonic acid	Multi-step conversion (*Mortierella alpina*)	4.5	
Eicosapentaenoic acid	Multi-step conversion (*Mortierella alpina*)	1.8	

rinase have been crystallized in our laboratory. It was found with the crystalline enzyme preparations, that these enzymes catalyze a variety of reactions, α,β-elimination (I), β-replacement (II) and the reverse of α,β-elimination (III), through the coenzymatic action of pyridoxal phosphate (*Scheme 2*) [5][6]. We have proved that these enzymes catalyze the synthesis of L-tyrosine, L-tryptophan, L-cysteine, D-cysteine and their related amino acids in significantly high yields through reactions (II) and (III).

$$RCH_2CHNH_2COOH + H_2O \rightarrow RH + CH_3COCOOH + NH_3 \quad (I)$$

$$RCH_2CHNH_2COOH + R'H \rightarrow R'CH_2CHNH_2COOH + RH \quad (II)$$

$$R'H + CH_3COCOOH + NH_3 \rightarrow R'CH_2CHNH_2COOH + H_2O \quad (III)$$

3,4-L-Dopa and other hydroxy-L-tyrosines, and various alkylated or halogenated L-tyrosines are synthesized through reactions catalyzed by β-tyrosinase. 5-Hydroxyl-, 5-methyl-, and 5-amino-L-tryptophans are synthesized by tryptophanase. A variety of S-substituted cysteines of both L- and D-forms are respectively synthesized by cysteine desulfhydrase and β-chloro-D-alanine dehydrochlorinase [7][8].

To produce these amino acids, microbial cells were prepared by culturing them under conditions which accumulated sufficient enzymes in the grown cells, and the cells were added to the reaction mixtures directly as the enzyme catalyst. In the case of L-dopa synthesis, more than 90 g of L-dopa was synthesized in 1 l of reaction mixture with a significantly high yield for substrates, pyruvate and pyrocatechol.

3. Synthesis of Amides by Enzymatic Hydration of Nitriles

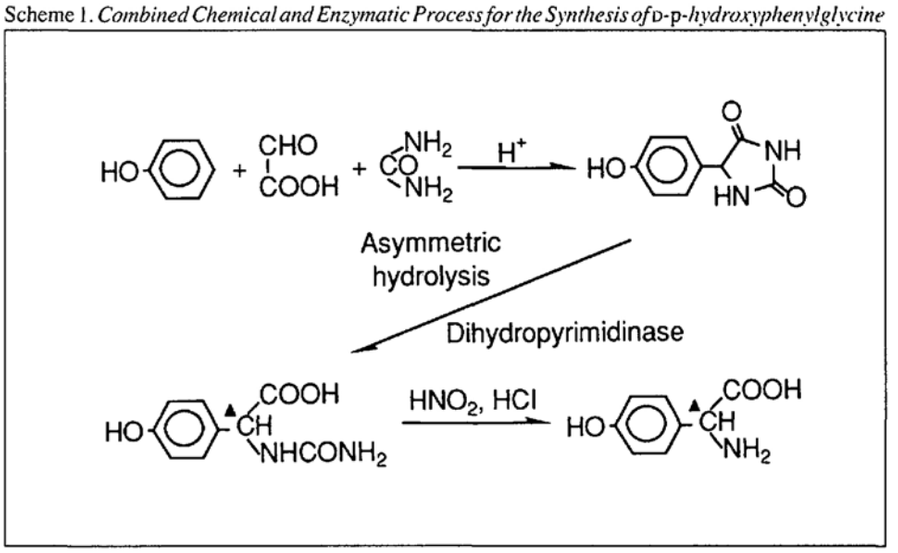
Acrylamide and methacrylamide are industrially produced as a monomer for synthetic fibers and flocculant agents. The synthetic process involves hydration of the nitriles with sulfuric acid or using copper or palladium complexes as the catalyst. We propose a new process using the microbial enzyme, nitrile hydratase, as a catalyst for the hydration of the nitriles.

During the course of the studies on the degradation of nitrile compounds by microorganisms, we found a new enzyme termed ‚nitrile hydratase' which catalyzes the hydration reaction of nitrile to amide [9] (*Eqn. 1*).

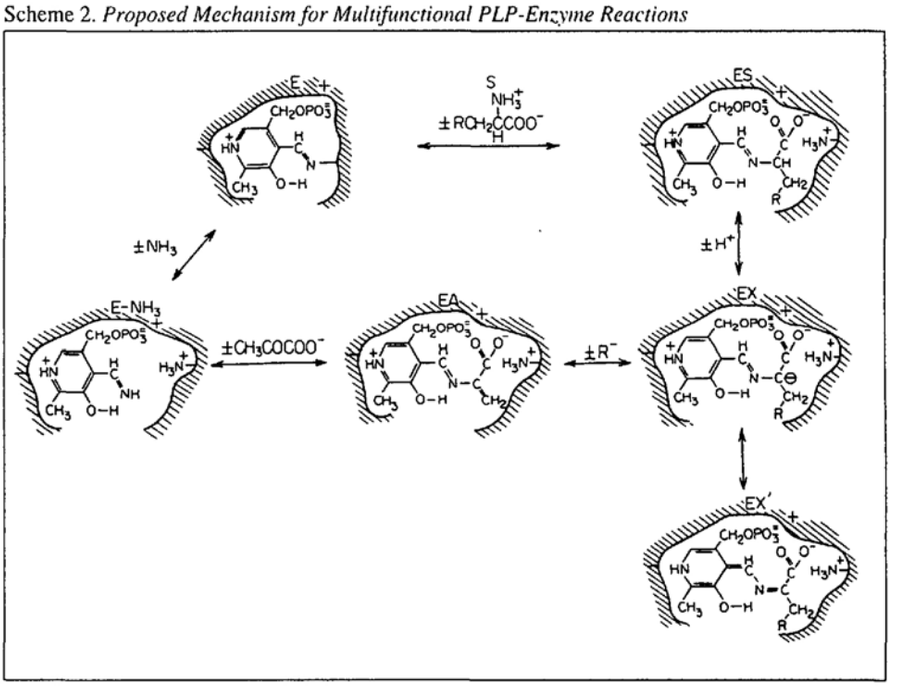
$$CH_2 = CHCN + H_2O \rightarrow CH_2 = CH\text{-}CONH_2 \qquad (Eqn.\ 1)$$

The enzyme was highly purified and characterized in our laboratory. We proved that this enzyme catalyzes the hydration of a variety of nitriles including acrylonitrile and methacrylonitrile. Screening of microorganisms with high enzyme activity showed that *Pseudomonas chlororaphis* has high hydratase activity when grown with isobutyronitrile as a major nitrogen source. Although the micro-organism pro-

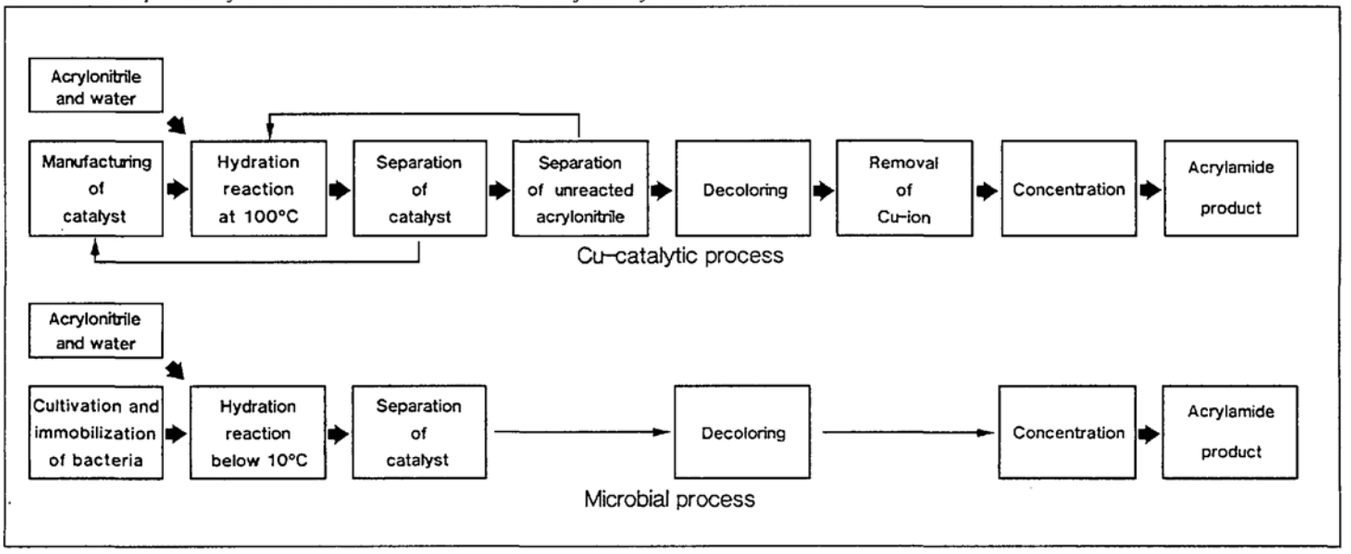
Scheme 1. *Combined Chemical and Enzymatic Process for the Synthesis of* D-p-*hydroxyphenylglycine*



Scheme 2. *Proposed Mechanism for Multifunctional PLP-Enzyme Reactions*



Scheme 3. *Comparison of Microbial and Conventional Process for Acrylamide*



duces an amidase together with the hydratase during growth, the amidase is inactive towards acrylamide and metacrylamide. Thus, theoretically stoichiometric conversion of the nitriles to the amides is possible even when hydratase contaminated with amidase is used as the catalyst. For practical purposes, the reaction is carried out by exposing the nitriles directly with the bacterial cells having high enzyme activity. Under suitable conditions, more than 400 g of acrylamide and 200 g of methacrylamide are produced in 1 l medium. The molar yields in both cases are nearly 100%. In order to develop this enzymatic reaction for industrial process, we optimized the culture conditions and isolated improved mutants of *P. chlororaphis* B23.

Optimization of culture conditions and mutation studies significantly improved nitrile hydratase activity [10], and lead to the development of the process for industrial application. A new bioreactor using cells entrapped in a cationic acrylamide-based polymer gel was designed [11] and constructed for a compact and efficient commercial plant [12].

In contrast with conventional chemical processes (*Scheme 3*), the recovery of unreacted acrylonitrile is not necessary because the conversion yield of the enzymatic hydration process is > 99.99%. The removal of copper ions from the product is also no longer necessary [12][13]. Overall, the enzymatic process is simpler and more economical. It is carried out below 10° under mild reaction conditions and requires no special energy source (*Table 2*). The immobilized cells are used repeatedly and a very pure product is obtained.

The industrial production of acrylamide was first started with *Rhodococcus* sp. N-774, a test strain, which was isolated by the research laboratory, *Nitto Chemical Industry*, in 1985. In 1988 it was replaced by the mutant of *P. chlororaphis* B23 [14][15]. By changing the biocatalyst, the productivity of acrylamide increased by *ca.* 50% (*Table 3*).

Compared to conventional organic synthesis, the biocatalysts have potential to improve productivity and yields. In order to enhance the productivity of acrylamide, the biocatalyst was further improved. Very recently we found that *Rhodococcus rhodochrous* J1 is capable of higher production of acrylamide compared to *P. chlororaphis* B23. Nitrile hydratases of *P. chlororaphis* B23 and *Brevibacterium* R312 contain ferric ions as a cofactor [16][18]. On the other hand, *R. rhodochrous* J1 nitrile hydratase was found to contain cobalt ions as a cofactor [19][20] (*Table 4*). When *R. rhodochrous* J1 was cultivated in nutrient medium supplemented with cobalt ions and urea as inducers, the enormous amount of nitrile hydratase which corresponds to more than 40% of total soluble protein, was found in cells of R. *rhodochrous* J1 [21].

R. rhodochrous J1 nitrile hydratase is more heat stable and more tolerant to high concentrations of acrylonitrile compared to *P. chlororaphis* B23 and *Brevibacterium* R312 enzymes. In particular, *R. rhodochrous* J1 nitrile hydratase exhibits strong tolerance to high concentrations of acrylamide. *R. rhodochrous* J1 cells also exhibited catalytic activity even in the presence of 50% acrylamide, *P. chlororaphis* B23 and *Brevibacterium* R312 nitrile hydratases did not show their full activity in the presence of 20% of acrylamide.

Using *R. rhodochrous* J1 resting cells, the accumulation reaction was carried out by feeding acrylonitrile to maintain a level of 6%. After 10 h incubation, 656 g/l (at

Scheme 4. *Reactions Involved in the Enzymatic Transformation to* D-*(−)-Pantoyl Lactone or* D-*(+)-Pantothenate.* D-PL, D-(−)-pantoyl lactone; L-PL, L-(+)-pantoyl lactone; KPL, ketopantoyl lactone; KPA, ketopantoic acid; D-PA, D-(−)-pantoic acid; L-PA, L-(+)-pantoic acid: KPA, 2'-ketopantothenate; D-PaA, D-(+)-pantothenate.

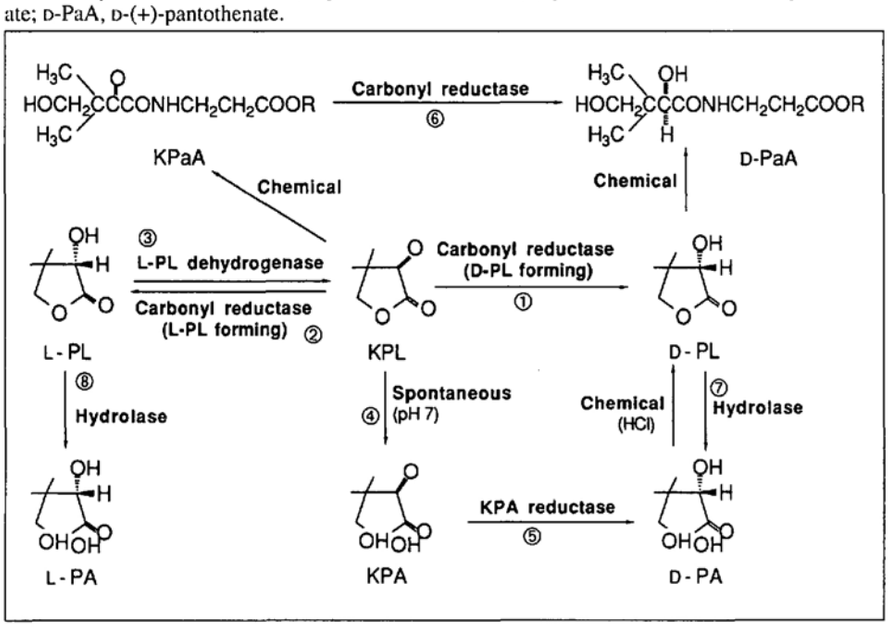


Table 2. *Operating Conditions for Acrylamide Production*

Reaction conditions	Productivity	
pH 7.5–8.5	Conversion of acrylonitrile	99.97%
Temperature 0–5°	Yield of acrylamide	99.99%
Acrylonitrile concentration in the reactor 1.5–2.0%	Acrylamide concentration from the reactor	27–30%

Table 3. *Comparison of Three Kinds of Biocatalysts*

	Rhodococcus sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodococcus rhodochrous* J1
Tolerance to acrylamide (%)	27	40	50
Acrylic acid formation	very little	barely detected	barely detected
Cultivation time (h)	48	45	72
Activity of culture broth (units/ml)	900	1 400	2 100
Specific activity (units/mg cells)	60	85	76
Cell yield (g/l)	15	17	28
Acrylamide productivity (g/g cells)	500	850	>7 000
Total amount of production (t/year)	4 000	6 000	30 000
Final concentration of acrylamide (%)	20	27	40
First year of production scale	1985	1988	1991

10°), 567 g/l (at 15°), and 560 g/l (at 20°) of acrylamide were accumulated, respectively [22]. The efficacy of the three kinds of biocatalyst in terms of acrylamide production, is summarized in *Table 3*.

Due to the high stability and high tolerance to acrylamide, at least 40% acrylamide accumulation can be attained by large scale production. When *R. rhodochrous* J1 cells are used, productivity of acrylamide up to 30 000 t/year can be achieved. In spite of the use of alternative biocatalyst, no change in the plant operation is required. Thus, the choice of biocatalyst is important. Since 1991, R. *rhodochrous* J1 was used for the industrial production of acrylamide. At present about 10 000 t of acrylamide is produced per year by *Nitto Chemical Industry Ltd.*

Three kinds of nitrile hydratases of *P. chlororaphis* B23 [16], *Rhodococcus* N-774 [23], and *R. rhodochrous* J1 [20] have been purified and characterized (*Table 4*). These enzymes are composed of two subunits of different sizes (α and β subunits). *R. rhodochrous* J1 nitrile hydratase has much higher molecular mass and is composed of 10 α and 10 β subunits. These genes have been cloned and the open reading frame for the β subunit was located just upstream of that for the α subunit in the *R. rhodochrous* J1 enzyme gene [24], but the arrangement of the coding sequences for two subunits is reverse order of that found in the nitrile hydratase genes of *Rhodococcus* N-774 [25] and *P. chlororaphis* B 23 [26]. The primary structure of each subunit of these nitrile hydratases has been determined. The amino-acid sequences of each subunit showed significant similarities. Each of the nitrile hydratase gene was expressed in *Escherichia coli* cells under the control of *lac* promoter, only when they were cultured in the medium supplemented with $CoCl_2$ or $FeCl_3$ [27].

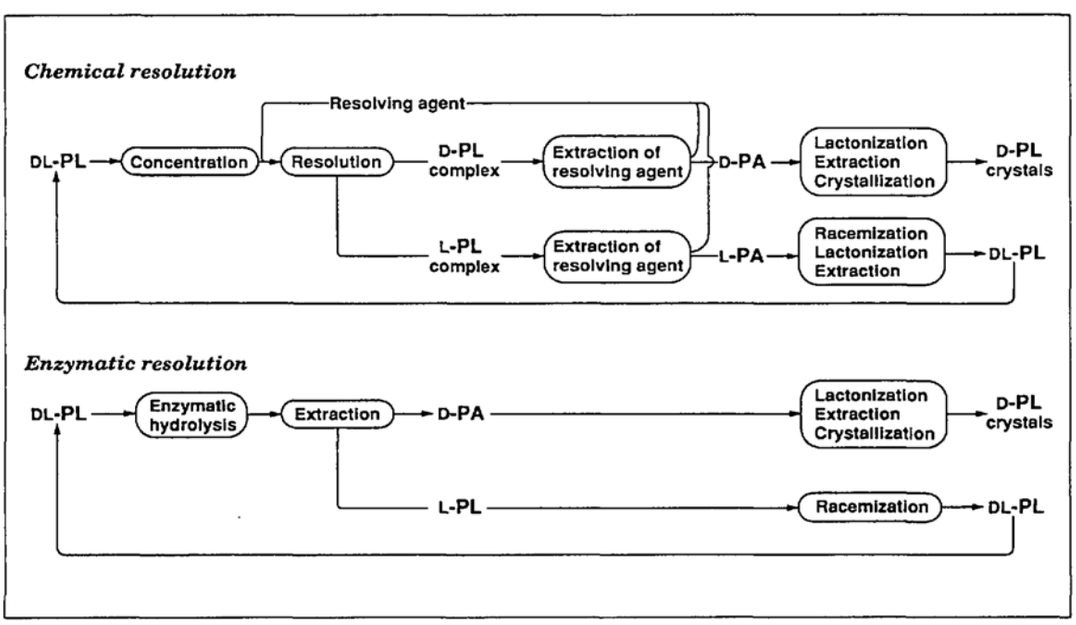
Table 4. *Comparison of Three Kinds of Nitrile Hydratases*

	Rhodococcus sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodococcus rhodochrous* J1
Molecular mass	70 000	100 000	505 000
Subunit molecular mass	α 27 000	α 25 000	α 26 000
	β 27 500	β 25 000	β 29 000
Metal	Fe(III)	Fe(III)	Co
Optimum temperature [°]	35	20	35–40
Heat stability [°]	30	20	50
Optimum pH	7.7	7.5	6.5
pH stability	7.0–8.5	6.0–7.5	6.0–8.5
Substrate specificity	aliphatic nitriles	aliphatic nitriles	aliphatic and aromatic nitriles
Activation by light irradiation	+	–	–
Formation type	constitutive	inducible (methacrylamide)	inducible (urea)

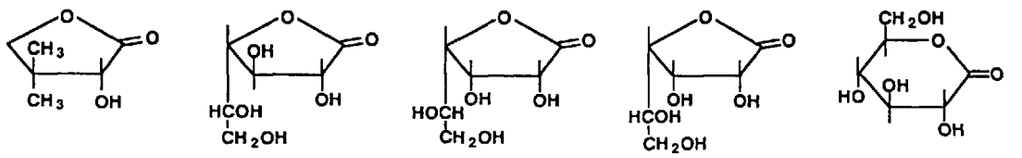
4. Optical Resolution of Pantoyl Lactone by a New Fungal Lactonase

Commercial production of pantothenate depends exclusively on chemical synthesis. The conventional chemical process involves the reaction yielding racemic pantoyl lactone from isobutyraldehyde, formaldehyde, and cyanide, optical resolution of the racemic pantoyl lactone to D-(–)-pantoyl lactone with compounds such as quinine, quinidine, cinchonidine, brucine, and condensation of D-(–)-pantoyl lactone with β-alanine. A problem of this chemical process is the troublesome resolution of the racemic pantoyl lactone and the racemization of the remaining L-(+)-isomer. Therefore, most of the recent studies in this area have been concentrated on the development of efficient methods to obtain D-(–)-pantoyl lactone. We have reported that there are several microbial reactions useful for the synthesis of chiral intermediates for D-pantothenate production [28][29] (*Scheme 4*).

Kinetic resolution of D,L-pantoyl lactone can be carried out by specific fungal hydrolases. We found that many molds belonging to the genera *Fusarium*, *Gibberella*, and *Cylindrocarpon* specifically hydrolyze D-(–)-pantoyl lactone to D-(–)-pantoic acid (*Scheme 4*, reaction Ⓐ). On the other hand, several yeast strains hydrolyzed only the L-(+)-isomer (*Scheme 4*, reaction Ⓑ). For practical purposes, the



Scheme 5. *Comparison of Enzymatic and Conventional Chemical Resolution Processes for* DL-*Pantoyl Lactone.* DL-PL, DL-pantoyl lactone; D-PL, D-(–)-pantoyl lactone; L-PL, L-(+)-pantoyl lactone; D-PA, D-(–)-pantoic acid; L-PA, L-(+)-pantoic acid.



	D-Pantoyl lactone	D-Galactonolactone	L-Mannonolactone	D-Gulonolactone	D-Gluconolactone
Relative activity (%)	100	207	264	345	221
Km (mM)	120	3.6	23	29	

Figure. *Substrate specificity of the aldonolactonase of* Fusarium oxysporum

former reaction is more advantageous than the latter, because, in the latter case, optical purity of the remaining D-(–)-pantoyl lactone is low unless the hydrolysis of L-(+)-pantoyl lactone is complete. Among various *Fusarium* strains tested, *Fusarium oxysporum* AKU 3702 showed the highest hydrolysis activity and gave D-(–)-pantoic acid of high optical purity (>95% ee). When *Fusarium oxysporum* cells were incubated in 70% (*w/v*) aqueous solution of D,L-pantoyl lactone for 24 hours at 30° with automatic pH control (pH 6.8–7.2), *ca.* 90% of the D-(–)-isomer was hydrolyzed. The resultant D-(–)-pantoic acid in the reaction mixture showed a high optical purity (96% ee) and the coexisting L-(+)-isomer remained without any modification [30][31].

The enzyme responsible for this hydrolysis was isolated from *Fusarium oxysporum* cells and crystallized. It is a kind of aldonolactonase with a molecular mass of 125 000. The enzyme is composed of two identical subunits, each of which contains one mole of Ca^{2+} and *ca.* 15% carbohydrate. Ca^{2+} is necessary for the enzyme activity. Ca^{2+} also plays an important role as a stabilizer of the enzyme. Mannose is a major component of the carbohydrate. The enzyme catalyzes reversible hydrolysis of several sugar lactones, such as D-galactonolactone, L-mannonolactone, D-gulonolactone, and D-gluconolactone (*Fig.*). The enzyme greatly favors the hydrolytic direction under neutral or mild alkaline conditions. Reaction equilibrium at pH 6.0 is *ca.* 50% when D-(–)-pantoyl lactone is the substrate. Several aromatic lactones, *i.e.*, dihydrocoumarin, homogentisic acid lactone and 2-coumaranone, are also good substrates of the enzyme. All the sugar lactones which serve as substrate have a downward hydroxyl group at the 2-position, when the lactone rings are drawn according to the *Haworth* system. The corresponding enantiomers are competitive inhibitors.

Practical hydrolysis of the D-(–)-isomer in a racemic mixture is carried out using immobilized mycelia of *Fusarium oxysporum* as the catalyst. Stable catalyst with high hydrolytic activity can be prepared by entrapping the fungal mycelia into calcium alginate gels. When the immobilized mycelia were incubated in a reaction mixture containing 300 g/l D,L-pantoyl lactone for 16 h at 30° under the conditions of automatic pH control, 90–95% of the D-(–)-isomer was hydrolyzed (optical purity, 90–95% ee). After repeated reactions for 100 times over 100 d, the immobilized mycelia retained more than 90% of their initial activity [31].

Comparison of the enzymatic process proposed by the present study and the conventional chemical process for the resolution of D,L-pantoyl lactone is shown in *Scheme 5.*

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