Application of nitrile converting enzymes for the production of useful compounds

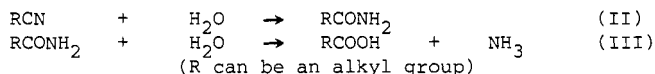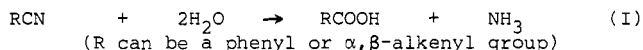TORU NAGASAWA and HIDEAKI YAMADA

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Abstract-The chemical industry has recently begun to look at enzymes as novel catalysts for many organic syntheses. Nitrile-hydrolyzing enzymes such as nitrile hydratase and nitrilase have great potential as catalysts in processing organic chemical because they can convert nitriles to the corresponding higher-value amides or acids. Recently, the use of bacterial nitrile hydratase for industrial production of the important chemical commodity, acrylamide, was pioneered in Japan. We review here the enzymatic production of acrylamide, and how other recent progress in nitrile biotransformations.

INTRODUCTION

Some microorganisms have the ability to utilize nitriles as carbon and/or nitrogen sources (ref. 1). Benzonitrile and related aromatic nitriles have been shown to be converted directly to the corresponding acids and ammonia catalyzed by nitrilase (Eq. I). On the other hand, aliphatic nitriles are catabolized in two steps, via conversion to the corresponding amide (Eq. II) and then to the acid plus ammonia catalyzed by amidase (Eq. III). We purified and characterized this new enzyme for the first time and termed the enzyme 'nitrile hydratase', because this enzyme is clearly distingushable from the nitrilase based on the mode of degradation of nitrile. It was particularly from the viewpoint of bioindustry that the finding of nitrile hydratase drew our attention. This was because nitrile hydratase seemd to be promising for the enzymatic transformation of nitrile into amide.

$$RCN \quad + \quad 2H_2O \quad \rightarrow \quad RCOOH \quad + \quad NH_3 \qquad (I)$$
$$(R \text{ can be a phenyl or } \alpha,\beta\text{-alkenyl group})$$

$$RCN \quad + \quad H_2O \quad \rightarrow \quad RCONH_2 \qquad (II)$$
$$RCONH_2 \quad + \quad H_2O \quad \rightarrow \quad RCOOH \quad + \quad NH_3 \qquad (III)$$
$$(R \text{ can be an alkyl group})$$

ENZYMATIC PRODUCTION OF ACRYLAMIDE

Acrylamide is industrially produced as a monomer for synthetic fibers and flocculant agents, etc. About 200,000 tonnes of acrylamide is produced per year in the world, and about 25% of its is produced in Japan. Thus acrylamide is one of the most important commodity chemicals and there is a great demand for it. Conventional synthesis involves the hydration of nitriles with the use of copper salts or paradium complexes as a catalyst. However, the preparation of the catalyst is laborious and the process requires high temperature.

A French group led by Dr. Galzy (ref. 2-6), and our Kyoto University group (ref. 7) proposed an enzymatic production process of acrylamide involving a nitrile hydratase as a catalyst for the hydration of acrylonitrile, independently. We isolated various nitrile-assimilating bacteria, and cultivated it in each nitrile-containing medium. Using these resting cells, we assayed the bioconversion activity of acrylonitrile into acrylamide. The strain B23 was isolated as an isobutyronitrile degrading strain from soil and it exhibited high acrylamide-producing activity (ref. 7). We identified it as Pseudomonas chlororaphis B23. The resting cells of P. chlororaphis B23 was added as a catalyst to the reaction mixture and substrate acrylonitrile was fed a little at a time, because a high concentration of acrylonitrile is inhibitory to the nitrile hydratase activity. In an 7.5 h reaction at 10°C, more than 400 g of acrylamide per liter was accumulated. The conversion of acrylonitrile into acrylamide was more than 99%. There was no formation of acrylic acid as a by-product. Such high productivity and high conversion yield suggested that this bioconversion could be applied for the industrial production of acrylamide.

In order to develop this enzymatic reaction into the industrial process, there was room to improve the culture conditions. We tried to optimize the culture conditions in detail. The P. chlororaphis B23 nitrile hydratase is inducibly formed by various nitriles, amides and

acid. Among them, methacrylamide gave the greatest induction. The addition of ferrous or ferric ions to the medium greatly increased the formation of nitrile hydratase. No other metal ions replace the effect of ferrous or ferric ions. The optimized culture medium contains sucrose, ferrous ions and a cheep soy been hydrolysate, and the addition of methacrylamide as an inducer greatly enhanced nitrile hydratase formation.

When P. chlororaphis B23 was cultivated, it produced a large amount of mucilage polysaccharides. Therefore, it was not easy to harvest the cells by brief centrifugation due to the high viscosity of the medium. This was an important technical disadvantage that we had to overcome. In addition, the high viscosity of the culture medium seemed to inhibit the aeration and the growth. Thus, we attempted to isolate the mutants which do not produce polysaccharides (ref. 9). The wild-type strain formed a large and swollen colony on the agar plate. This may have been due to the production of polysaccharide. Then, the small colony was picked up after NTG-mutagenization treatment of the wild strain. Mutants which do not produce polysaccharide are easily centrifuged and precipitated. Thus mutant Am-3 which did not produce polysaccharide was obtained. Next, to obtain the nitrile hydratase hyper-producing mutant, mutagenization treatment was carried out again. When the mutants which grow quickly on isobutyronitrile medium were picked up, the high nitrile hydratase-producing mutant Am-324 was obtained. By the improvement of the culture medium and the isolations of mutants, the specific activity increased about 200-times, and total activity increased 3,000-times compared with the initial obtained level. Thus, we established the basis for the industrial production of acrylamide by the enzymatic hydration process.

We cooperated with the Nitto Chemical Industry to establish this process for industrial use. The nitrile hydratase-containing cells is immobilized with polyacrylamide gel (ref. 10). A very efficient and sophisticated bioreactor plant was built by Nitto Chemical Industry (ref. 11). The industrial plant is very compact. In Fig. 1, the enzymatic hydration process and coventional process using copper-salt catalyst for the production of acrylamide are compared. In the case of enzymatic hydration, the conversion yield is almost 100%, and therefore this step to recover the remaining acrylonitrile can be omitted. The step to remove the copper ion from the product can also be omittted. All in all, this enzymatic process is simpler. Because the enzymatic process is carried out below 10°C, under mild reaction conditions, and as the immobilized cells are used repeatedly, it is more economical. The enzymatic process is already in use and at present about 6,000 tonnes of acrylamide is produced per year by Nitto Chemical Industry.

It was analyzed Why P. chlororaphis B23 accumulates so much acrylamide (ref. 12). The first reason is, P. chlororaphis B23 exhibits enormously powerful nitrile hydratase activity compared to the amidase activity. The nitrile hydratase activity for acrylonitrile is at least 3,000-times higher than amidase activity for acrylamide in the methacrylamide-induced

cells. Next, acrylonitrile, a powerful nucleophilic reagent attacks the active thiol residue of typical thiol-enzyme, amidase and inactivates amidase activity. On the other hand, nitrile hydratase is not so sensitive to acrylonitrile. Therefore, the inactivation of the amidase activity resulted in the accumulation of acrylamide and non-formation of acrylic acid under conditions used for the acrylamide production. In addition, the nitrile hydratase purified from P. chlororaphis B23 shows high resistance against the high concentration of acrylamide. The P. chlororaphis B23 enzyme acts on acrylonitrile even in the presence of 17.5% acrylamide. On the other hand, nitrile hydratase from Brevibacterium R312 which was isolated by the French group is not so tolerant to the high concentration of acrylamide and cannot act on acrylonitrile any more in the presence of 17.5% acrylamide. Therefore, P. chlororaphis B23 enzyme is a more suitable catalyst from the viewpoint of acrylamide accumulation.
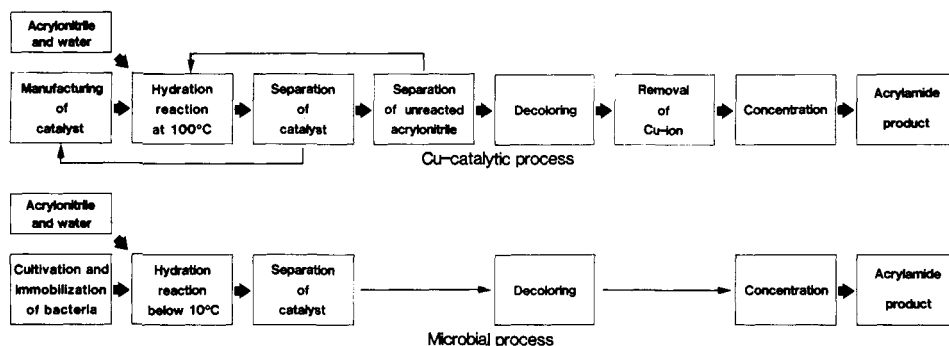

Fig. 1. Comparison of microbial and conventional process for acrylamide.

CHARACTERIZATION OF IRON-CONTAINING NITRILE HYDRATASE

We have purified and crystallinzed the nitrile hydratases from P. chlororaphis B23 (ref. 14) and Brevibacterium R312 (ref. 15). Both enzymes are active towards various aliphatic nitriles; nitriles with 3-6 carbon atoms serve as the most suitable substrates. The P. chlororaphis B23 enzyme has a molecular mass of about 100 kDa and consists of four subunits identical in molecular mass. The Brevibacterium R312 enzyme has a molecular mass of about 85 kDa and is composed of two types of subunit (26 kDa and 27.5 kDa). Concentrated solutions of both of the highly purified enzymes have a green colour and exhibit broad absorption peaks in the visible range with an absorption maximum at 720 nm though the two are immunologically different. These nitrile hydratases are the first non-haem iron enzymes found to contain typical low-spin ferric active sites. The electron spin resonance (ESR) features of the enzyme are characteristic of rhombic low-spin ferric type (g_{max}=2.284, g_{mid}=2.140, g_{min}=1.971)(ref. 15). Ferric ions are tightly coordinated, possibly by the four nitrogen atoms of the imidazole ring. One of the axial ligands is probably the thiolate donor. The other most likely axial ligand is probably water, because the sample in $H_2^{17}O$-water broadens the ESR spectrum, a change attributable to transferrred hyperfine interaction. The large ESR change brought about by the substrate suggests that the nitrile binds to the iron site of the enzyme.

In addition to the iron, recently we clarified that nitrile hydratase involves active carbonyl cofactor as a second prosthetic group, probably pyrroloquinoline quinon (PQQ)ref. 16). It was assumed that PQQ(or probably, a hydrated derivative) participates in the hydration of the nitrile group; interaction between the low-spin ferric site and PQQ was revealed by recent ESR experiments (ref. 17). A nitrile and its corresponding amide and acid are all at the same oxidation level, and the overall reaction in each case is a hydrolysis. Although a simple hydrolytic mechanism might be expected for the reaction catalyzed by nitrile hydratase, the presence of ferric ion and PQQ cofactors raises the possibility that a biological oxidation-reduction mechanism is involved. The chemical oxidation-reduction mechanism involved in the conversion of nitriles to amides by hydroperoxide anion under mildly alkaline conditions is well known (ref. 18).

ENZYMATIC PRODUCTION OF NICOTINAMIDE

The aliphatic nitriles are very good substrates for the P. chlororaphis B23 and Brevibacterium R312, however, these enzyme do not act on aromatic nitriles, such as benzonitrile and 3-cyanopyridine. Therefore, we tried the screening to find the nitrile hydratase which acts on aromatic nitrile. We isolated Rhodococcus rhodochrous J1 from soil which exhibits broad substrate specificity (ref. 19). Using crotonamide as an inducer for the enzyme and by adding cobalt ions to the culture medium, enormous amounts of nitrile hydratase can be produced in R. rhodocrhous J1 (more than 50% of all the soluble protein in the best cases). Ferric or ferrous ions cannot replace cobalt ions.

Nicotinamide is a useful vitamin and used in animal feed supplementation. 3-Cyanopyridine is chemically synthesized by ammoxidation reaction of picoline and it is converted to nicotinamide with alkali. However, when produced in this way, nicotinamide is contaminated by the by-product, nicotinic acid. Enzymatic processes could avoid this. Using R. rhodochrous J1 cells, up to 1456 gL^{-1} of nicotinamide was produced from 3-cyanopyridine in 9 h in a reaction mixture containing resting cells (1.17 g dry weight) with 100% molar conversion (ref. 19). The high yield of this process promises much for the industrial production of nicotinamide.

This enzymatic hydration process has also been used to produce isonicotinamide (1099 gL^{-1}), picolinamide (977 gL^{-1}), the tuberculostatic compound pyrazinamide (985 gL^{-1}), the agricultural chemical 2,6-difluorobezamide (306 gL^{-1}), thiphenecarboxamide (210 gL^{-1}), 3-indoleacetamide (1045 gL^{-1}), benzamide (489 gL^{-1}) and 2-furancarboxamide (522 gL^{-1})(ref. 20 and 21). Due to the high yields of this process, the easy cultivation of R. rhodochrous J1 cells and the stability of the resting cells, which are stable at -20°C for more than 6 months, the use of this enzymatic hydration process seems to be promising for the industrial production of various aromatic and heterocyclic amides. In addition, the R. rhodochrous J1 nitrile hydratase acts on aliphatic nitriles, too. Above all, we achieved very high accumulations of acrylamide due to its high tolerance to high concentration of acrylonitrile and acrylamide. Therefore, the R. rhodochrous J1 nitrile hydratase will probably be used for the industrial production of acrylamide, too.

CHARACTERIZATION OF COBALT-CONTAINING NITRILE HYDRATASE

We have already purified and crystallized the R. rhodochrous J1 nitrile hydratase (ref. 22). In Table 1, the properties are compared with the P. chlororaphis B23 and Brevibacterium R312 nitrile hydratase. The R. rhodochrous J1 nitrile hydratase is more heat stable than these nitrile hydratases. The R. rhodochrous J1 nitrile hydratase was found to contain cobalt ions, and does not contain any ferric or ferrous ions. The R. rhodochrous J1 nitrile hydratase is of red color.

The application of bioconversion process has been generally restricted to the production of fine-chemicals which are difficult to synthesize by chemical synthesis. However, the industrial production of acrylamide, a typical commodity chemical, was started by using biocatalysis, nitrile hydratase. This fact encourages us very much and confirms to us that the further application of biocatalysis is promising for not only fine chemicals but also commodity chemicals, too and will become more common for the industrial production of useful compounds.

TABLE 1. Properties of Nitrile Hydratase

	P. chlororaphis B23	Brevibacterium R312	R. rhodochrous J1
Molecular mass			
Sedimentation equilibrium	100,000	85,000	505,000
Toyosoda 3000SW	100,000	87,000	520,000
Subunit molecular mass	α 25,000	α 26,000	α 26,000
	β 25,000	β 27,500	β 29,000
Number of subunits	4	4	18-20
Absorption maxima (nm)	280, 720	280, 720	415
Metal (mol/mol)	Fe(III) 4	Fe(III) 4	Co 20
Optimum temperature (°C)	20	20	35
Heat stability (°C)	20	20	50
pH stability	6.0-7.5	6.5-8.5	6.0-8.5

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