Appl Microbiol Biotechnol (1993) 40:189–195

Applied and Microbiology Biotechnology

The superiority of the third-generation catalyst, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial production of acrylamide

Toru Nagasawa[1], Hitoshi Shimizu[2], Hideaki Yamada[3]

[1] Laboratory of Bioreaction Engineering, Department of Food Science and Technology, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464, Japan
[2] Nitto Chemical Industry Co., Ltd., 10.1 Daikoku-cho, Tsumuri-ku, Yokohama 230, Japan
[3] Department of Agricultural Chemistry, Kyoto University, Sakyoku, Kyoto 606, Japan

Received: 2 March 1993/Received revision: 7 May 1993/Accepted: 3 June 1993

Abstract. As the third-generation biocatalyst for industrial production of acrylamide, the superiority of *Rhodococcus rhodochrous* J1 nitrile hydratase was demonstrated in comparison with other acrylamide-producing bacteria. *R. rhodochrous* J1 enzyme is much more heat stable and more tolerant to a high concentration of acrylonitrile than *Pseudomonas chlororaphis* B23 and *Brevibacterium* R312 enzymes. The J1 enzyme is peculiar in its extremely high tolerance to acrylamide. The hydration reaction of acrylonitrile catalysed by J1 cells proceeded even in the presence of 50% (w/v) acrylamide. The tolerance of J1 enzyme to various organic solvents such as *n*-propanol and isopropanol was prominent. 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, the accumulation of acrylamide was approximately 65.6% (w/v) at 10° C, 56.7% (w/v) at 15° C, and 56.0 (w/v) at 20° C. The high stability, high catalytic efficiency and other outstanding features of the J1 enzyme are analysed and discussed.

Introduction

Bioconversion processes have generally been applied only to the production of fine chemicals that are difficult to synthesize by conventional organic synthesis. Recently, microbial processes in the industrial production of commodity chemicals are rapidly gaining practical significance from the viewpoint of preparation of high purity products, environmental acceptability and energy saving (Nagasawa and Yamada 1993). Our group and the Nitto Chemical Industry research group have pioneered co-operatively the use of bacterial nitrile hydratase for industrial production of the important chemical commodity, acrylamide.

The industrial production of acrylamide started in 1985 using the nitrile hydratase of *Rhodococcus* sp. N-774, the first-generation strain (Watanabe et al. 1987a, b). In 1988 it was replaced by our second-generation strain, *Pseudomonas chlororaphis* B23 (Nagasawa and Yamada 1989; Nagasawa et al. 1989). By changing the biocatalyst, the productivity of acrylamide was greatly enhanced. *Rhodococcus* sp. N-774 and *P. chlororaphis* B23 nitrile hydratases contain ferric ions as a cofactor (Nagasawa et al. 1986, 1987; Sugiura et al. 1987; Endo and Watanabe 1989). Thereafter we found that cobalt-induced and cobalt-containing nitrile hydratase was produced by *R. rhodochrous* J1 (Nagasawa et al. 1988b, 1991b).

When seeking to optimize the culture conditions, we found that a nutrient medium supplemented with cobalt ions and urea as an inducer was the most suitable for the preparation of cells exhibiting high nitrile hydratase activity (Nagasawa et al. 1991a). We found that *R. rhodochrous* J1 has a much more powerful ability to produce acrylamide than *P. chlororaphis* B23. In fact, since 1991, *R. rhodochrous* J1 cells have been used as the third-generation biocatalyst for the industrial production of acrylamide.

For more than 10 years, enormous efforts have been made to isolate hyper-producing strains of nitrile hydratase. The occurrence of nitrile hydratase was shown in *Arthrobacter* sp. J-1 for the first time (Asano et al. 1982a). However, this strain was not suitable for acrylamide production due to the low nitrile hydratase activity and high amidase activity. At present, *Brevibacterium* R312 (Commeyras et al. 1976, 1983), *Rhodococcus* sp. N-774 (Watanabe et al. 1987a, b), *P. chlororaphis* B23 and *R. rhodochrous* J1 are acknowledged to be the superior producers of nitrile hydratase and acrylamide. The nitrile hydratases from these strains have been purified and characterized (Nagasawa et al. 1986, 1987, 1991b). The genes coding these enzymes have been determined (Ikehata et al. 1989; Mayaux et al. 1990; Kobayashi et al. 1991; Nishiyama et al. 1991). The former two strains produce nitrile hydratase constitutively whereas the latter two produce the enzyme inducibly. The nitrile hydratases produced by the former three strains act preferentially on aliphatic ni-

Correspondence to: T. Nagasawa

triles, whereas the enzyme produced by the latest strain exhibits broad substrate specificity (Nagasawa et al. 1988a; Mauger et al. 1988, 1989).

In the present paper, the outstanding features of *R. rhodochrous* J1 nitrile hydratase are presented.

Materials and methods

Bacterial strains and cultivation. Brevibacterium R312, deposited as a patent microorganism, no. FERM-P2722 (Commeyras et al. 1976, 1983), was obtained from the Fermentation Research Institute, Ministry of International Trade & Technology, Japan, and used for this study. The cultivation of *Brevibacterium* R312 was carried out at 28° C for 45 h in a nutrient medium as described previously (Nagasawa et al. 1986). *P. chlororaphis* B23, isolated in our laboratory (Asano et al. 1982b), was cultivated at 25° C for 28 h in optimized medium containing methacrylamide as an inducer, as described previously (Yamada et al. 1986). *R. rhodochrous* J1 studied as a nicotinamide-accumulating bacterium in our laboratory (Nagasawa et al. 1988a), was cultivated at 28° C for 75 h in the optimized medium containing urea and cobalt ions, as described previously (Nagasawa et al. 1991a). Cell growth was determined turbidimetrically by means of each dry cell calibration curve of the absorbance at 610 nm.

The purified enzymes of *Brevibacterium* R312, *P. chlororaphis* B23 and *R. rhodochrous* J1 were prepared as described previously (Nagasawa et al. 1986, 1987, 1991b).

Enzyme assays. The nitrile hydratase activity was assayed in a mixture (2 ml) comprised of 10 μmol potassium phosphate buffer, pH 7.5, 377 mM acrylonitrile and an appropriate amount of enzyme or cells. The amounts of acrylamide in the reaction mixture were determined with a Shimadzu gas-liquid chromatograph (model GC-4CM) (Yamada et al. 1986). One unit of acrylamide-forming activity was defined as the amount of cells or enzyme that catalysed the formation of 1 μmol of acrylamide per minute. Specific activity was expressed as units per milligram of dry cells or protein. Acrylic acid formed was determined by analytical HPLC, which was performed with a Toyosoda CCPM equipped with a lichrosorb NH_2 column (Cica Merck) at the flow rate of 1.0 ml/min, using the solvent system of methanol/10 mM KH_2PO_4, pH 6.0, 7:3 (v/v), at 40° C.

Protein was determined by the Coomassie brilliant blue G-250 dye-binding method (Bradford et al. 1976) using dye reagent supplied by Bio-Rad.

Results and discussion

Many characteristic similarities have been found between the first-generation nitrile hydratase of *Rhodococcus* sp. N-774 and the *Brevibacterium* R312 enzyme (Nagasawa et al. 1986; Endo and Watanabe 1989). It has been reported, for example, that the nucleotide sequences of the α-subunit of *Brevibacterium* R312 enzyme is completely identical with those of *Rhodococcus* sp. N-774 (Ikehata et al. 1989; Mayaux et al. 1990). Despite the similarities of both nitrile hydratases, their morphological appearance on the agar plate seems to be different.

In the following experiments, the excellence and superiority of *R. rhodochrous* J1 enzyme were analysed by comparing it with *Brevibacterium* R312 and *P. chlororaphis* B23 enzymes as industrial biocatalysts.

Effect of temperature

Acrylonitrile is volatile and unstable, and acrylamide polymerizes easily at higher temperatures. Therefore, it is desirable that the hydration process by which acrylonitrile becomes acrylamide be carried out at a lower temperature. With the reaction at lower temperature, the durability of the immobilized cells generally improves. In fact, the enzymatic hydration of acrylamide is performed at 0–5° C in industrial plants (Nakai et al. 1988). Nevertheless, heat stability of the biocatalyst has always been required, particularly for its storage and transport.

The hydration process catalysed by three kinds of purified enzyme was followed at various temperatures (Fig. 1). The same unit of each enzyme was added to the reaction mixture. *Brevibacterium* R312 and *P. chlororaphis* B23 enzymes were inactivated completely at 30° C, whereas the *R. rhodochrous* J1 enzyme was entirely stable at 30° C. The J1 enzyme was stable even at 40° C, but a slight inactivation was observed at 40° C with prolonged incubation. Thus, the J1 enzyme is much more heat-tolerant than the R312 and B23 enzymes.

Effect of acrylonitrile concentration

Nitrile compounds are physically damaging to the cells. Acrylonitrile is an especially powerful nucleophilic reagent. Thus, during the course of the production of

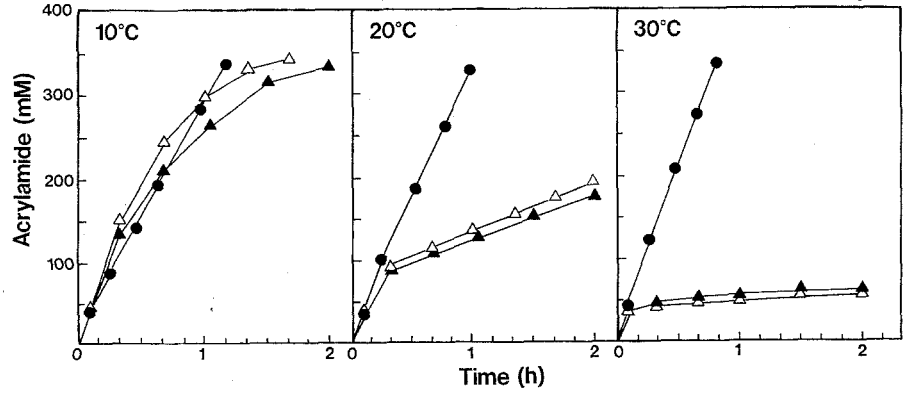

Fig. 1. The nitrile hydration reaction catalysed by three kinds of nitrile hydratase at 10° C, 20° C or 30° C. The reaction was carried out in a mixture (2 ml) comprising 100 μmol potassium phosphate buffer, pH 7.5, 377 mM acrylonitrile, and 12 units (U) of either of the three kinds of nitrile hydratase at 10° C, 20° C or 30° C: ▲, *Brevibacterium* R312; △, *Pseudomonas chlorographis* B23; ○, *Rhodococcus rhodochrous* J1

acrylamide, the concentration of acrylonitrile in the reactor should be kept low. In fact, its concentration was kept at 1.5–2.0% (w/v) in the reactor by controlling the feeding interval of acrylonitrile, using a sensor in the industrial process of acrylamide production with *Rhodococcus* sp. N-774 or *P. chlororaphis* B23.

The sensitivity of the R312, B23 and J1 enzymes to acrylonitrile was compared (Fig. 2). The hydration reaction was carried out in a reaction mixture containing various concentrations of acrylonitrile. The R312 and B23 enzymes were more sensitive to acrylonitrile. Their catalytic activity decreased in accordance with the increase of acrylonitrile from 2 to 6% (w/v), whereas the *R. rhodochrous* J1 enzyme exhibited almost full activity even in the presence of 7% (w/v) acrylonitrile. It is obvious that the J1 enzyme has a much higher tolerance to acrylonitrile than R312 and B23 enzymes.

Effect of acrylamide concentration

Commodity chemicals are generally synthesized on a large scale at low cost. Thus, one of the most important factors in the success of the industrial production of commodity chemicals through a bioconversion process may well be the high accumulation of the product, which results in energy saving and simplification of the downstream process. Thus an enormous accumulation of acrylamide must be attained for industrial application of the enzymatic hydration process of acrylonitrile. Compared to acrylonitrile, acrylamide is much less damaging to cells, but the tolerance of the biocatalyst to acrylamide is much more crucial for industrial applications.

To compare the tolerance of the R312, B23 and J1 enzymes to high concentrations of acrylamide, the hydration reaction of acrylonitrile was carried out in the presence of various concentrations of acrylamide (Fig. 3). The *R. rhodochrous* J1 enzyme acts on acrylonitrile even in the presence of 30% (w/v) acrylamide, and the acrylonitrile added was found to be completely converted into acrylamide after a 2-h incubation. On the other hand, the *P. chlororaphis* B23 and *Brevibacterium* R312 nitrile hydratases lost their catalytic activity in the presence of 30% (w/v) acrylamide after a 30-min incubation. The J1 purified nitrile hydratase acted on acrylonitrile even in the presence of 40% (w/v) acrylamide. When similar experiments were carried out using *R. rhodochrous* J1 cells, the cells exhibited much higher acrylamide-tolerance than the purified enzyme. For example, even in the presence of 50% (w/v) acrylamide, the hydration of acrylonitrile still proceeded without any prominent inhibition at 10° C (Fig. 4). Next, the tolerance of the J1 enzyme to acrylamide was compared at 5° C and 10° C. The J1 enzyme exhibited higher tolerance to 40% (w/v) acrylamide at 5° C than at 10° C. Thus, for higher tolerance to acrylamide, the hydration reaction is most suitably carried out at a lower temperature. From these results, it is clear that *R. rhodochrous* J1 is a very stable and powerful biocatalyst for the production of acrylamide.

Accumulation of acrylamide

The accumulation of acrylamide was attempted using *R. rhodochrous* J1 cells on a bench-scale with the periodic addition of acrylonitrile at 10° C, 15° C and 20° C. When the reaction was carried out for 7 h at 10° C, the accumulation of acrylamide reached 500 g/l of reaction mixture. When the feeding of acrylonitrile was continued further, white crystals of acrylamide appeared due to the lower solubility at 10° C. During the course of accumulation, the quantitative determination of acrylamide was difficult due to the appearance of crystalline acrylamide at 10° C. However, after stopping the

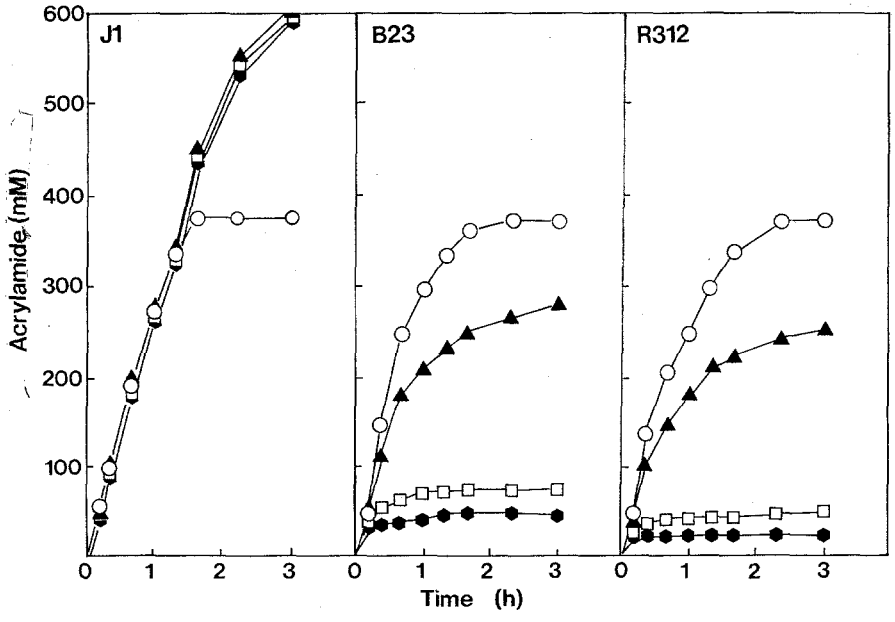

Fig. 2. Effect of various concentrations of acrylonitrile on three kinds of nitrile hydratase. The reaction was carried out at 10° C in a mixture (2 ml) comprising 100 μmol potassium phosphate buffer, pH 7.5, 12 U of nitrile hydratase and 2% (w/v) (○), 4% (w/v) (▲), 6% (w/v) (□) or 7% (w/v) (●) acrylonitrile

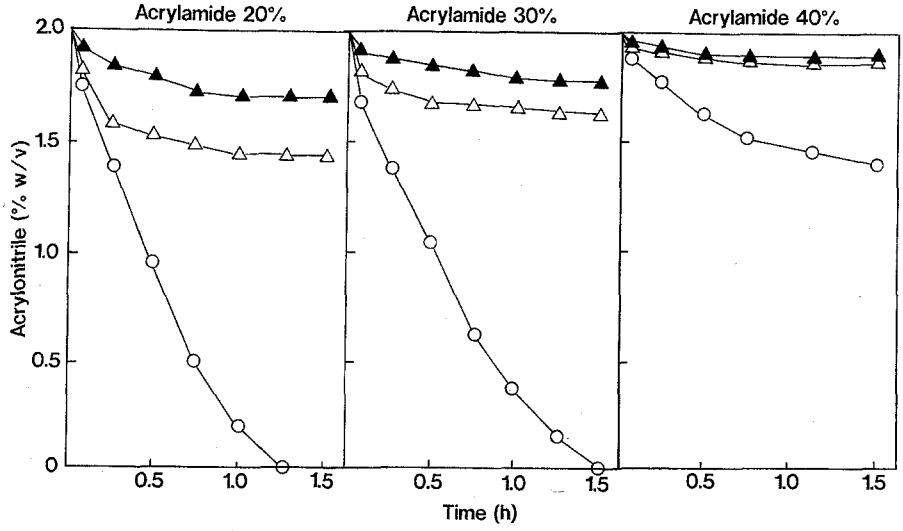

Fig. 3. Effect of acrylamide concentration on three kinds of nitrile hydratase. The reaction was carried out at 10° C in a mixture (2 ml) comprising 100 μmol potassium phosphate buffer, pH 7.5, 24 U of R312 (▲), B23 (△) or J1 (○) enzyme, 377 mM acrylonitrile and 20% (w/v), 30% (w/v) or 40% (w/v) acrylamide

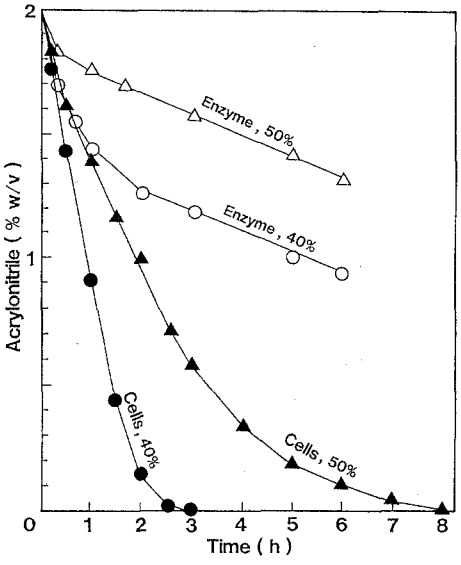

Fig. 4. Effect of high concentration of acrylamide on *R. rhodochrous* J1 cells or its purified enzyme. The reaction was carried out at 10° C in a mixture (2 ml) comprising 100 μmol potassium phosphate buffer, pH 7.5, 24 U of J1 nitrile hydratase or J1 resting cells, 377 mM acrylonitrile and 40% (w/v) or 50% (w/v) acrylamide. The consumption of acrylonitrile was followed

Table 1. Effect of various organic solvents on nitrile hydratases from *Brevibacterium* R312, *Pseudomonas chlorographis* B23 and *Rhodococcus rhodochrous* J1

Organic solvent (50%, v/v)	Relative activity (%)		
	Brevi-bacterium	*P. chloro-raphis*	*R. rhodo-chrous*
None	100	100	100
Methanol	6	8	89
Ethanol	8	10	94
Isopropanol	1.3	3	100
n-Propanol	8	10	100
Acetone	10	12	66
1,4-Dioxane	0	1.5	60
N,N'-Dimethylformamide	12	18	78
Tetrahydrofuran	26	32	44
Dimethyl sulphoxide	22	38	58
Ethylene glycol	64	55	136

Incubation was carried out for 20 min at 10° C in a mixture (2 ml) comprising 12 units of the purified nitrile hydratase, 2% (w/v) acrylonitrile and 50% (v/v) of various organic solvents. Potassium phosphate buffer was not added to the reaction mixture, to avoid precipitation of potassium phosphate due to the addition of organic solvent. The reaction was stopped by adding 0.2 ml of 3 M HCl and the acrylamide formed was measured by gas chromatography

reaction, the crystalline acrylamide was dissolved completely and its determination was carried out. The final accumulation of acrylamide exceeded approximately 65.6% (w/v) at 10° C. When the reaction was carried out at 15° C and 20° C, the accumulation of acrylamide reached 56.7% (w/v) and 56.0% (w/v), respectively (Fig. 5), and no crystals of acrylamide were formed. The formation of acrylic acid was barely detected in each case. When similar accumulation experiments were carried out using *Brevibacterium* R312, *Rhodococcus* sp. N-774 or *P. chlororaphis* B23 cells, the highest accumulation of acrylamide did not attain the level of J1 cells (data not shown). The use of *R. rhodochrous* J1 enables one to obtain much higher accumulation of acrylamide. The enormously high accumulation may be ascribed to the high tolerance of J1 enzyme to acrylamide.

Tolerance to organic solvent

The tolerance of nitrile hydratase to various organic solvents was examined (Table 1). *R. rhodochrous* J1 enzyme exhibited the highest tolerance among them. In particular the J1 enzyme was tolerant to isopropanol and *n*-propanol, and was fully active even in the presence of 70% (v/v) isopropanol and *n*-propanol. On the other hand, *P. chlororaphis* B23 and *Brevibacterium*

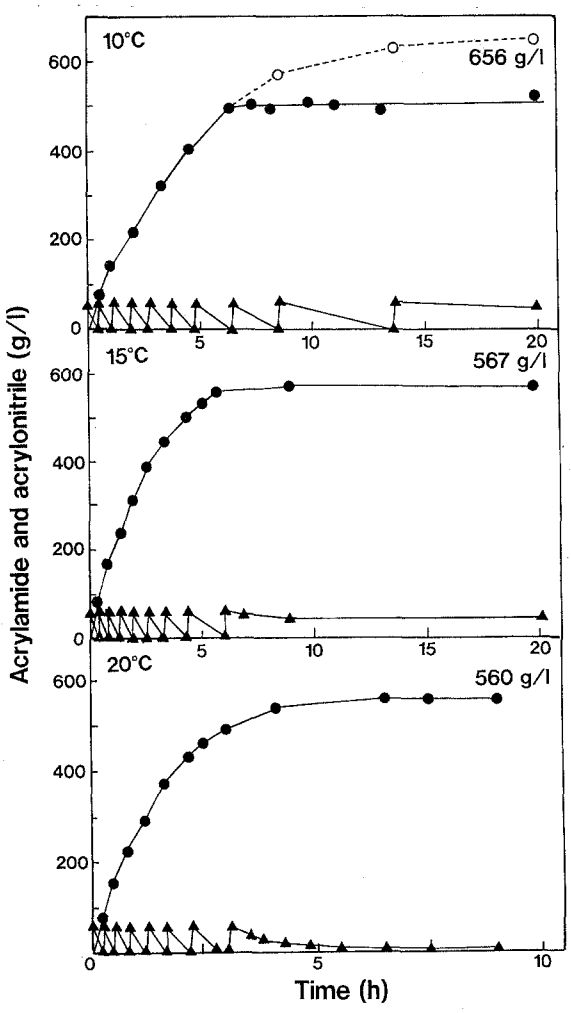

Fig. 5. Accumulation of acrylamide by *R. rhodochrous* J1 cells. The initial reaction mixture (30 ml) comprising the J1 resting cells (17.7 mg dry cell weight; specific activity 119 U/mg dry cell weight at 20° C), 6% (w/v) acrylonitrile and 1.5 mmol potassium phosphate buffer, pH 7.5, was incubated at 10° C, 15° C and 20° C. According to its consumption, acrylonitrile was fed periodically at the concentration of 6% (w/v). An aliquot (2 ml) of the reaction mixture was taken and after brief centrifugation, the supernatant solution was subjected to the determination of acrylamide (●), acrylonitrile (▲) and acrylic acid. The change in volume of the reaction mixture caused by the periodic feeding of acrylonitrile and the consumption of H_2O for the hydration reaction was taken into consideration for the calculation of acrylamide determination. The data shown as (O---O) were not quantitatively exact due to the appearance of crystalline acrylamide at 10° C. For the determination of these samples, sampling was carried out after thoroughly shaking the reaction mixture and then the reaction was stopped by adding 3-M HCl. The crystalline acrylamide was dissolved at 20° C, and then acrylamide was determined

R312 enzymes were sensitive to these solvents. Even when the J1 enzyme solution was shaken with the same volume of *n*-heptane, *n*-hexane or *n*-pentane, the catalytic activity was not damaged at all. Thus, these advantageous features of the J1 enzyme guarantees its broad application for the solvent-containing reaction system.

In addition, the nitrile hydratases of *P. chlororaphis* B23 and *Brevibacterium* R312 were very sensitive to reducing reagents, such as dithiothreitol and 2-mercaptoethanol, and to oxidizing reagents, such as H_2O_2 an ammonium persulphate. However, the J1 enzyme proved resistant to reducing and oxidizing reagents (Nagasawa et al. 1991b).

As described above, the J1 enzyme is much more stable than other nitrile hydratases from various standpoints. How, then, can the stability of the J1 enzyme be explained? The stability and tolerance may be ascribed to the difference in metal-ion cofactors, ferric ions and cobalt ions. The deduced amino acid sequences of each subunit of J1 enzyme, B23 and *Rhodococcus* sp. N-774 showed a significant similarity (Ikehata et al. 1989; Nishiyama et al. 1991; Kobayashi et al. 1991), and the difference in metal-ion cofactors could be ascribed to a small number of amino acids at their ligand-binding sites. In addition, we suspect the stability is due to the difference in molecular mass of nitrile hydratase. The molecular mass of the *R. rhodochrous* J1 enzyme is 505 kDa and consists of 10α and 10β subunits, whereas those of *Brevibacterium* R312 and *P. chlororaphis* B23 enzymes are 100 kDa and 85 kDa, respectively. Each consists of 2α and 2β subunits.

Recently, we isolated other cobalt-containing nitrile hydratases with a molecular mass of 100 kDa. Their stability is at the same level as *P. chlororaphis* B23 and *Brevibacterium* R312 enzymes. Thus, we assume the high stability of the J1 enzyme might be related to the association of 20 subunits in the J1 enzyme. Hence, the association of subunits can probably depress the flexibility of the protein and keep the structure more rigid by the interaction of subunits (Stellwagen and Wilgus 1978). However, further studies are required to clarify this point.

Maximum reaction velocity (V_{max}) and Michaelis constant (K_m) values

The high reaction rate and high specific activity are important for an industrial biocatalyst. The V_{max} and K_m values of three kinds of purified nitrile hydratase for acrylonitrile under the standard reaction conditions are summarized in Table 2. Acrylonitrile is one of the most suitable substrates for these enzymes. Compared to ordinary microbial enzymes, nitrile hydratase is characterized by its high specific activity (V_{max} values indicate 1470 to 1760 μmol/min per milligram protein at 20° C). On the other hand, the specific activity of

Table 2. Maximum reaction velocity (V_{max}) and Michaelis constant (K_m) values of three kinds of nitrile hydratase

Nitrile hydratase producing strains	V_{max} (μmol/min/mg)	K_m (mM)
Brevibacterium R312	1470	16.7
P. chlororaphis B23	1490	34.6
R. rhodochrous J1	1760	1.89

The V_{max} and K_m values were determined at 20° C under standard conditions

Table 3. Improvement of biocatalyst for the production of acrylamide

Parameters	*Rhodococcus* sp. N-774	*P. chlororaphis* B23	*R. rhodochrous* J1
Acrylic acid formation	Very little	Barely detected	Barely detected
Cultivation time (h)	48	45	72
Activity of culture (units/ml)	90	1400	2100
Cell yield (g/l)	15	17	28
Acrylamide productivity (g/g cells)	500	850	>7000
Total annual production (tons)	4000	6000	>30000
Final concentration of acrylamide (%)	20	27	40
First year of production scale	1985	1988	1991

several kinds of nitrilase that catalyse the direct hydrolysis of nitrile into acid and ammonia, ranged over 0.1–144 μmol/min per milligram protein (Harper 1977a, b, 1985; Bandyopadhyay et al. 1986; Goldlust and Bohak 1989; Kobayashi et al. 1989, 1990; Nagasawa et al. 1990; Yamamoto and Komatsu 1991) at 25° C or 30° C. Thus, in contrast to nitrilase, the hydration reaction catalysed by nitrile hydratase proceeds much more rapidly. Generally speaking, nitrile hydratase belongs to the category of a very efficient biocatalyst. In particular, the J1 enzyme exhibits the highest V_{max} values and the lowest K_m value for acrylonitrile among them.

The above advantages of the *R. rhodochrous* J1 enzyme make it a more suitable catalyst for acrylamide accumulation, and, with Nitto, we have developed a process applying J1 nitrile hydratase for industrial use. The *R. rhodochrous* J1 enzyme was not inactivated by immobilization in a polyacrylamide gel, and repeated use still yielded a product of high purity. Even in the change of biocatalyst, almost no change is required in the manufacturing plant.

Compared to conventional organic synthesis, a biocatalyst sometimes has a great potential for improved productivity and yields. By changing the biocatalyst, through replacement of *P. chlororaphis* B23 with *R. rhodochrous* J1, the productivity of acrylamide per cell weight and per year has increased greatly (Table 3). At present, a kiloton-scale production involving *R. rhodochrous* J1 cells is under way at Nitto.

References

Asano Y, Fujishiro K, Tani Y, Yamada H (1982a) Aliphatic nitrile hydratase from *Arthrobacter* sp. J-1; purification and characterization. Agric Biol Chem 46:1165–1174

Asano Y, Yasuda Y, Tani Y, Yamada H (1982b) A new enzymatic method of acrylamide production. Agric Biol Chem 46:1183–1189

Bandyopadhyay AK, Nagasawa T, Asano Y, Fujishiro K, Tani Y, Yamada H (1986) Purification and characterization of benzonitrilases from *Arthrobacter* sp. strain J-1. Appl Environ Microbiol 56:302–306

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Commeyras A, Arnaud A, Galzy P, Jallageas JL (1976) Manufacturing of amides through biological hydrolysis. Japanese patent publication 51-86186

Commeyras A, Arnaud A, Galzy P, Jallageas JL (1983) Manufacturing of organic acids through biological hydrolysis Japanese laid-open patent publication 58-15120

Endo T, Watanabe I (1989) Nitrile hydratase of *Rhodococcus* sp. N-774: purification and amino acid sequences. FEBS Lett 243:61–64

Goldlust A, Bohak Z (1989) Induction, purification, and characterization of the nitrilase of *Fusarium oxysporum* f. sp. *melonis*. Biotechnol Appl Biochem 11:581–601

Harper DB (1977a) Microbial metabolism of aromatic nitriles. Biochem J 165:309–319

Harper DB (1977b) Fungal degradation of aromatic nitriles. Biochem J 165:685–692

Harper DB (1985) Characterization of a nitrilase from *Nocardia* sp. (*Rhodochrous* group) N.C.I.B. 11215, using *p*-hydroxybenzonitrile as sole carbon sourse. Int J Biochem 17:677–683

Ikehata O, Nishiyama M, Horinouchi S, Beppu T (1989) Primary structure of nitrile hydratase deduced from the nucleotide sequence of a *Rhodococcus* species and its expression in *Escherichia coli*. Eur J Biochem 181:563–570

Kobayashi M, Nagasawa T, Yamada H (1989) Nitrilase of *Rhodococcus rhodochrous* J1: Purification and characterization. Eur J Biochem 182:349–356

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1990) Purification and characterization of a novel nitrilase of *Rhodococcus rhodochrous* K22 that acts on aliphatic nitriles. J Bacteriol 172:4807–4815

Kobayashi M, Nishiyama M, Nagasawa T, Horinouchi S, Beppu T, Yamada H (1991) Cloning, nucleotide sequence and expression in *Escherichia coli* of two cobalt-containing nitrile hydratase genes from *Rhodococcus rhodochrous* J1. Biochim Biophys Acta 1129:23–33

Mauger J, Nagasawa T, Yamada H (1988) Nitrile hydratase-catalyzed production of isonicotinamide, picolinamide and pyrazinamide from 4-cyanopyridine, 2-cyanopyridine and cynopyrazine in *Rhodococcus rhodochrous* J1. J Biotechnol 8:87–96

Mauger J, Nagasawa T, Yamada H (1989) Synthesis of various aromatic derivatives using nitrile hydratase of *Rhodococcus rhodochrous* J1. Tetrahedron 45:1347–1356

Mayaux J-F, Cerbelaud E, Soubrier F, Faucher D, Petre D (1990) Purification, cloning, and primary structure of an enantiomer-selective amidase from *Brevibacterium* sp. strain R312: structural evidence for genetic coupling with nitrile hydratase. J Bacteriol 172:6764–6773

Nagasawa T, Yamada H (1989) Microbial transformations of nitriles. Trends Biotechnol 7:153–158

Nagasawa T, Yamada H (1993) Microbial production of commodity chemicals. Pure Appl Chem (in press)

Nagasawa T, Ryuno K, Yamada H (1986) Nitrile hydratase of *Brevibacterium* R312: purification and characterization. Biochem Biophys Res Commun 139:1305–1312

Nagasawa T, Nanba H, Ryuno K, Takeuchi K, Yamada H (1987) Nitrile hydratase of *Pseudomonas chlororaphis* B23: Purification and characterization. Eur J Biochem 162:691–698

Nagasawa T, Mathew CD, Mauger J, Yamada H (1988a) Nitrile hydratase-catalyzed production of nicotinamide from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. Appl Environ Microbiol 54:1766–1769

Nagasawa T, Takeuchi K, Yamada H (1988b) Occurrence of a cobalt-induced and cobalt-containing nitrile hydratase in

Rhodococcus rhodochrous J1. Biochem Biophys Res Commun 155:1008–1016

Nagasawa T, Ryuno K, Yamada H (1989) Superiority of *Pseudomonas chlororaphis* B23 nitrile hydratase as a catalyst for the enzymatic production of acrylamide. Experimentia 45:1066–1070

Nagasawa T, Mauger J, Yamada H (1990) A novel nitrilase, arylacetonitrilase, of *Alcaligenes faecalis* JM3: purification and characterization. Eur J Biochem 194:765–772

Nagasawa T, Takeuchi K, Nardi-Dei V, Mihara Y, Yamada H (1991a) Optimum culture conditions for the production of cobalt-containing nitrile hydratase by *Rhodococcus rhodochrous* J1. Appl Microbiol Biotechnol 34:783–788

Nagasawa T, Takeuchi K, Yamada H (1991b) Characterization of a new cobalt-containing nitrile hydratase purified from urea-induced cells of *Rhodococcus rhodochrous* J1. Eur J Biochem 196:581–689

Nakai K, Watanabe I, Sato T, Enomoto K (1988) Development of acrylamide manufacturing process using microorganism. Nippon Nogeikagaku Kaishi 62:1443–1450

Nishiyama M, Horinouchi S, Kobayashi M, Nagasawa T, Yamada H, Beppu T (1991) Cloning and characterization of genes responsible for metabolism of nitrile compounds from *Pseudomonas chlororaphis* B23. J Bacteriol 173:2465–2472

Stellwagen E, Wilgus H (1978) Relationship of protein thermostability to accessible surface area. Nature 275:343–343

Sugiura Y, Kuwahara J, Nagasawa T, Yamada H (1987) Nitrile hydratase: the first non-heme iron enzyme with a typical low-spin Fe (III) active center. J Am Chem Soc 109:5848–5850

Watanabe I, Satoh Y, Enomoto K (1987a) Screening, isolation and taxonomical properties of microorganisms having acrylonitrile-hydrating activity. Agric Biol Chem 51:3193–3199

Watanabe I, Satoh Y, Enomoto K, Seki S, Sakashita K (1987b) Optimal conditions for cultivation of *Rhodococcus* sp. N-774 and for conversion of acrylonitrile to acrylamide by resting cells. Agric Biol Chem 51:3201–3206

Yamada H, Ryuno K, Nagasawa T, Enomoto K, Watanabe I (1986) Optimum culture conditions for production by *Pseudomonas chlororaphis* B23 of nitrile hydratase. Agric Biol Chem 50:2859–2865

Yamamoto K, Komatsu K (1991) Purification and characterization of nitrilase responsible for the enantioselective hydrolysis from *Acinetobacter* sp. AK226. Agric Biol Chem 55:1459–1466