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A New Enzymatic Method of Acrylamide Production†

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To produce acrylamide from acrylonitrile by use of a new enzyme, nitrile hydratase, a number of nitrile-utilizing microorganisms were screened for the enzyme activity by an intact cell system. An isobutyronitrile-utilizing bacterium, strain B23, showed the best productivity among 186 strains tested. The strain was identified taxonomically as *Pseudomonas chlororaphis*. The culture and reaction conditions for the production were studied for the strain. Under the optimum conditions, 400 grams/liter of acrylamide was produced in 7.5 hr. The yield was nearly 100% with a trace amount of acrylic acid. The cell-free extract of the strain showed strong activity of nitrile hydratase toward acrylonitrile and extremely low activity of amidase toward acrylamide.

Acrylamide is industrially produced as a monomer for synthetic fibers, floculant agents, *etc.* The process involves hydration of acrylonitrile with sulfuric acid. In this chemical hydration of nitrile, there is difficulty in controlling the reaction which proceeds as sequential formation of amide, carboxylic acid and ammonia. The process using sulfuric acid also yields ammonium sulfate as a by-product. Recently, catalysts of copper salts or paradium complexes[1,2] have been developed for the selective hydration of nitriles to yield amide without using acid or base. However, the preparation of these catalysts is laborious and the process requires a high temperature. On the other hand, in a series of studies on the microbial degradation of nitrile compounds, we found a new enzyme, "aliphatic nitrile hydratase," which catalyzed the stoichiometric hydration of nitrile to yield amide.[3,4] Therefore, we attempted to produce acrylamide from acrylonitrile using this microbial enzyme.

This paper describes the screening of microorganisms which produce acrylamide from acrylonitrile, identification of the strain, and the culture and reaction conditions for the production of acrylamide.

MATERIALS AND METHODS

Isolation of nitrile-utilizing microorganisms. Nitrile-utilizing microorganisms were isolated from soil samples by an enrichment culture technique using medium containing 0.2% (v/v) nitrile with or without 0.5% (w/v) glycerol in the basal medium which was described previously.[5] Microorganisms which utilized acetonitrile, propionitrile, acrylonitrile, isobutyronitrile, succinonitrile, glutaronitrile, adiponitrile and triacrylonitrile as a sole source of carbon and nitrogen or that of nitrogen were isolated.

Screening and assay method for acrylamide producing strains. The isolated strains were cultured aerobically at 28° C for 3 days on the isolation medium. The cells were centrifuged, washed with physiological saline and suspended in 0.1 M potassium phosphate buffer, pH 7.0. The reaction mixture for the screening of acrylamide-producing strains contained 100 μmol of potassium phosphate buffer, pH 7.0, 300 μmol of acrylonitrile as substrate, and washed cells from 3 ml of culture broth, in a total volume of 1.0 ml. The reaction was carried out at 30°C for 1 hr with moderate shaking and terminated by addition of 0.2 ml of 1 N HCl. The amount of acrylamide formed in the reaction mixture was determined with a Shimadzu gas-liquid chromatograph, Model GC-4CM, equipped with a flame ionization detector. The column used was a glass column of 3 mm inside diameter, packed with Porapack Q (80 to 100 mesh). Operational conditions

† Microbial Degradation of Nitrile Compounds. Part VII. For Part VI, see ref. 6.

were: column temperature, 210°C; injection and detector temperature, 240°C. The carrier gas was N_2 at 40 cm^3/min. The integration and calibration of peak areas were carried out with a Shimadzu Chromatopack C-R1A. One unit of acrylamide-forming activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of acrylamide per min.

Preparation of cell-free extract and enzyme assay. Suspended cells obtained as described above were disrupted for 10 min on ice with a Kaijo-denki 19 kHz ultrasonic oscillator. The disrupted cells were centrifuged at 17,000 × *g* for 20 min at 5°C. The supernatant solution was dialyzed overnight against 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol.

Nitrile hydratase and amidase activities were measured as described previously.[4,6]

Isolation of acrylamide. The reaction mixture containing acrylamide was centrifuged to remove the cells. The supernatant was lyophilized and then extracted with methanol. After removing the insoluble residue by centrifugation, the extract was evaporated *in vacuo* at room temperature. Crude crystals were dissolved in warm methanol and filtered. Recrystallization from warm methanol gave colorless crystals.

Chemicals. Triacrylonitrile was prepared as described in the previous paper.[7] Other chemicals were the usual commercial products and used without further purification.

RESULTS

Screening of acrylamide producing strains

The ability to produce acrylamide from acrylonitrile was tested among 186 strains which were isolated as utilizers of one of acetonitrile, propionitrile, acrylonitrile, isobutyronitrile, succinonitrile, adiponitrile and triacrylonitrile.

Table I shows the distribution of acrylamide producing strains among the nitrile-utilizers. High activity was shown by propionitrile- and isobutyronitrile-utilizing strains. Most of the di- and trinitrile utilizers did not accumulate either acrylamide or acrylic acid. About 10 acetonitrile-utilizing strains accumulated a higher amount of acrylic acid than that of acrylamide.

Identification of microorganisms

A bacterial strain, B23, which was isolated as an isobutyronitrile-utilizing microorganism and chosen as the best producer of acrylamide, was identified taxonomically as follows.

Rods, measuring 0.8 to 1.1 × 1.6 to 2.7 μm, occurring singly. Non-spore forming. Motile with one to three polar flagella. Gram-negative. Aminopeptidase-positive.[8]

Growth on nutrient agar: circular, convex, glistening, butyrous. Crystals of pigment are produced on older nutrient agar slants. Nitrate reduction: positive. Indole and hydrogen sulfide formation: negative. Catalase and oxidase: positive. Hugh-Leifson: oxidation. Pigment formation on King's A medium: negative; King's B medium: fluorescent green pigment. Acid without gas from glucose, galactose, and mannose. No acid and gas from fructose, sucrose, lactose, maltose, glycerol, raffinose, dextrin, starch, inulin, glycogen or mannitol. Assimilation of carbon compounds: acetate,

TABLE I. ACRYLAMIDE ACCUMULATION BY NITRILE-UTILIZING MICROORGANISMS

Growth substrate	Number of strains tested	Number of acrylamide accumulating strains		
		<0.01	0.01~0.1 (units/ml)	>0.1
Acetonitrile	55	43	12	0
Propionitrile	47	40	4	3
Acrylonitrile	3	3	0	0
Isobutyronitrile	11	6	2	3
Succinonitrile	20	20	0	0
Adiponitrile	26	26	0	0
Triacrylonitrile	24	24	0	0
Total	186	162	18	6

succinate, pyruvate, lactate, ethanol, glucose, arabinose, sucrose, sorbitol, trehalose, *meso*-inositol, L-alanine, L-valine, β-alanine, propylene glycol, α-ketoglutarate, L-glutamate and betaine are assimilated; DL-β-hydroxybutyrate, propionate, butyrate, methanol and geraniol are not assimilated. Growth temperature: between 5°C and 36.5°C, with the optimum at 31°C. Growth pH: between 6.0 and 9.9 with the optimum between 7.0 and 8.0. The G+C content of DNA was 64.6%, determined by the thermal denaturation method.

According to "Bergey's Manual of Determinative Bacteriology" 8th Ed.,[9] the strain was identical with *Pseudomonas chlororaphis*.

Identification of acrylamide

Acrylamide was isolated in crystalline form by the procedure described in MATERIALS AND METHODS. mp, 85.0°C, *Anal.* Calcd. for C_3H_5ON: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.68; H, 7.17; N, 20.01. The infrared spectrum of the crystals coincided well with that of authentic acrylamide as shown in Fig. 1. The ^{1}H-NMR spectrum of isolated acrylamide was identical with that of authentic acrylamide.

Culture conditions for the preparation of cells of P. chlororaphis B23 with high activity

Culture conditions to prepare cells having the higher activity were investigated as follows.

1) Optimum temperature. Figure 2 shows that the maximum activity was obtained when the strain was grown below 25°C, whereas the best growth was at 31°C. The cells had no activity when grown above 35°C, although they grew well at that temperature.

2) Effect of carbon and nitrogen source. Table II shows that the enzyme activity was the highest when dextrin was used as carbon source. As shown in Table III, isobutyronitrile

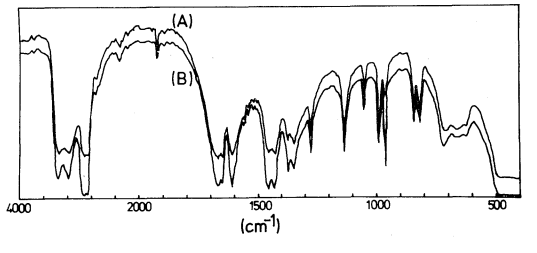


FIG. 1. IR Spectrum of Authentic (A) and Isolated (B) Acrylamide.

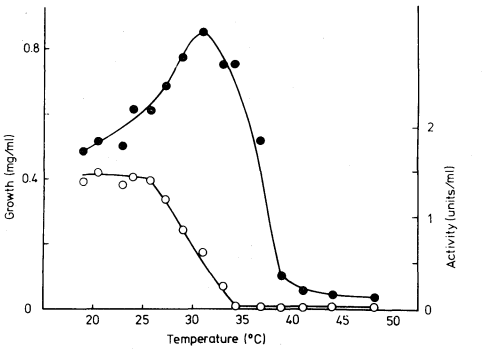


FIG. 2. Effect of Temperature on the Growth and Enzyme Formation of *P. chlororaphis* B23.

Washed cells (0.43 mg as dry weight) grown on the culture medium containing 0.15% isobutyronitrile and 0.5% glycerol were inoculated into 5 ml of the same medium. Cultivation was carried out for 2 days. The activity was measured as described in MATERIALS AND METHODS. Growth (●) and enzyme activity (○).

TABLE II. EFFECT OF CARBON SOURCE ON THE PRODUCTION OF ACRYLAMIDE

Washed cells (24 μg as dry weight) grown on the culture medium containing 0.15% (w/v) isobutyronitrile and 0.5% glycerol were inoculated into 5 ml of the culture medium containing 0.15% isobutyronitrile and 0.5% carbon compounds. Cultivation was carried out at 28°C for 3 days. The activity was measured as described in MATERIALS AND METHODS.

Carbon source	Cell growh (mg dry weight/ml culture broth)	Enzyme activity (units/ml)	Specific activity (units/mg)
Glycerol	0.90	0.13	0.14
Glucose	0.22	0	0
Fructose	0.03	—	—
Sucrose	0.21	0.05	0.21
Maltose	0.16	0.13	0.77
Galactose	0.38	0	0
Dextrin	0.55	0.40	0.72
Succinate·Na_2	0.11	0.09	0.14
Acetate·Na	0.11	0.03	0.07
Ethanol	0.40	0.03	0.07
None	0.20	0.17	0.85

—, no tested.

TABLE III. EFFECT OF NITROGEN SOURCE ON THE PRODUCTION OF ACRYLAMIDE

Washed cells (41 μg as dry weight) grown on the culture medium containing 0.15% isobutyronitrile and 0.5% glycerol were inoculated into 5 ml of the culture medium containing 0.2% (v/v) nitriles with 0.5% glycerol. The cultivation and assay were carried out as described in Table II.

Nitrogen source	Cell growth (mg dry weight/ml culture broth)	Enzyme activity (units/ml)	Specific activity (units/mg)
Acetonitrile	0.70	0	0
Propionitrile	1.46	0.08	0.05
n-Butyronitrile	1.78	0.11	0.07
n-Capronitrile	0.23	0.11	0.38
Methacrylonitrile	0.65	0.02	0.03
Isobutyronitrile	1.98	0.25	0.13
Glutaronitrile	0.73	0	0
Triacrylonitrile	0.18	0	0
Yeast extract	2.84	0.04	0.01
Malt extract	1.61	0.01	0.01
Polypepton	2.90	0.02	0.01
$(NH_4)_2SO_4$	1.07	0	0
$NaNO_3$	0.76	0	0

was the most favorable nitrogen source for the production. The activity was induced when grown on nitriles. The strain did not grow on acrylonitrile, *n*-valeronitrile, crotonitrile, lactonitrile, malononitrile, succinonitrile, benzonitrile or triacrylonitrile as the nitrogen source. It grew well on a medium containing up to 0.23% isobutyronitrile.

The total activity of acrylamide production was growth-associated and it was not lost even at the stationary phase of growth. Cells after 3-days cultivation had a high total activity and were suitable for the production of acrylamide.

Reaction conditions for the acrylamide production with P. chlororaphis B23

1) Optimum pH and temperature. Figures 3 and 4 show that maximum activity was obtained at pH 7.0 and between 10°C and 20°C. At a temperature higher than 30°C, the activity was decreased rapidly and the reaction did not proceed for a long time.

2) Effect of detergents. Twenty-one kinds of cationic, anionic and nonionic detergents were tested for the ability to increase the activity. However, none of them was effective for the production, and most of them inhibited the reaction.

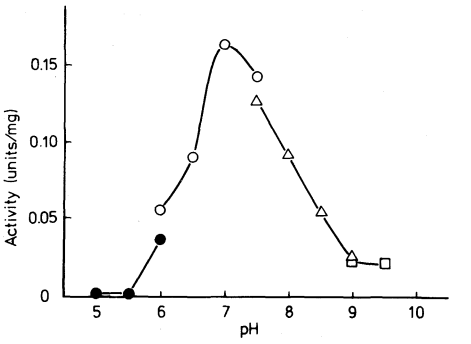


FIG. 3. Effect of pH on the Production of Acrylamide.

The reaction mxiture contained 300 μmol of acrylonitrile, 100 μmol buffers and 10 mg cells (as dry weight) in a total volume of 1.0 ml. The cells used were the same as described in Fig. 2. The reaction was carried out as described in MATERIALS AND METHODS, except that the reaction time was 30 min. The buffers used were: sodium acetate buffer (●), potassium phosphate buffer (○), Tris-HCl buffer (△), and glycine-NaOH buffer (□).

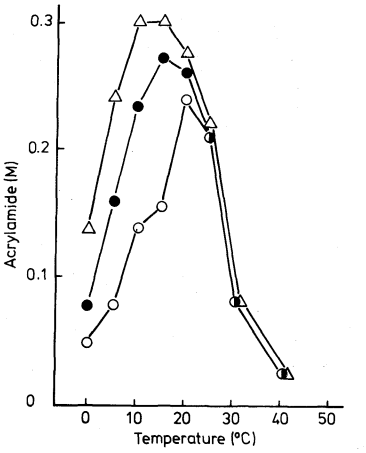


FIG. 4. Effect of Temperature on the Production of Acrylamide.

Cells were grown at 28°C for 3 days on the culture medium containing 0.15% isobutyronitrile and 0.5% dextrin. The activity was measured as described in MATERIALS AND METHODS at various temperatures using 0.8 mg cells (as dry weight). The reaction time was: 1 hr (○), 2 hr (●), and 3 hr (△).

Substrate specificity

The substrate specificity of the activity of

TABLE IV. SUBSTRATE SPECIFICITY OF NITRILE HYDRATASE OF *P. chlororaphis* B23

The reaction mixture contained 300 μmol nitrile, 100 μmol potassium phosphate buffer, pH 7.0, and 10 mg cells (as dry weight), in a total volume of 1.0 ml. The reaction was carried out as described in MATERIALS AND METHODS. Amides formed were assayed by gas-liquid chromatography.

Nitrile	Nitrile hydratase activity	
	(units/mg)	(%)
Acetonitrile	0.11	31
Propionitrile	0.41	117
Acrylonitrile	0.35	100
n-Butyronitrile	0.20	57
Isobutyronitrile	0.18	51
Methacrylonitrile	0.08	23
n-Valeronitrile	0	0
Benzonitrile	0	0

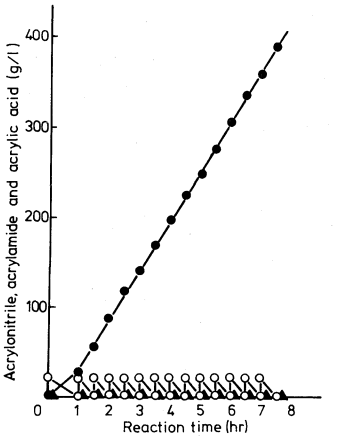


FIG. 5. Time Course of Acrylamide Production with *P. chlororaphis* B23 Cells.

The cells used were the same as described in Fig. 4. The reaction was carried out at 0°C to 4°C with stirring. Acrylonitrile (○), acrylamide (●), and acrylic acid (▲).

nitrile hydratase of the strain was investigated in an intact cell system. Table IV shows the results obtained for cells grown on dextrin and isobutyronitrile. Propionitrile was hydrated faster than isobutyronitrile. The enzyme acted on acetonitrile, propionitrile, acrylonitrile, *n*-butyronitrile, methacrylonitrile and isobutyronitrile. *n*-Valeronitrile and benzonitrile were not attacked by the enzyme. Only a trace amount of the corresponding carboxylic acid was produced from all the nitriles attacked by the enzyme.

Production of acrylamide by intact cells

Production of acrylamide through the hydration of acrylonitrile by cells of *P. chlororaphis* B23 was carried out. After the strain had been grown on dextrin and isobutyronitrile, the culture broth was directly used as an enzyme source and incubated at 5°C with moderate shaking. Acrylonitrile was added successively so as not to exceed a concentration of 0.4 M. Thus, 100 g/liter of acrylamide was produced in 84 hr. Figure 5 shows a typical time course of the reaction using washed cells of the strain. The reaction mixture contained 2 g (as dry weight) of washed cells of the strain, 10 mmol of potassium phosphate buffer, pH 7.0, and 560 mmol of acrylonitrile which was added in portion of 40 mmol at 30 min intervals, in a total volume of 100 ml. About 400 g/liter of acrylamide was produced after 7.5 hr reaction. The yield of acrylamide was more than 99% with a trace amount of acrylic acid (0.7%). Although the reaction seemed to proceed further, it was stopped because the reaction mixture became gradually viscous presumably due to the polyacrylamide formed.

Degradation of isobutyronitrile by P. chlororaphis B23

When the strain was grown on isobutyronitrile and glycerol, the successive formation of isobutyramide and isobutyric acid was observed with a decrease of isobutyronitrile, in concentration. The maximum concentrations of isobutyramide and isobutyric acid in the culture broth were 11 and 19 mM, respectively, when the concentration of isobutyronitrile at the start was 38 mM (0.15%, w/v).

Enzyme activities of nitrile hydratase and amidase in the cell-free extract of P. chlororaphis B23

To investigate the mechanism of the accumulation of acrylamide, activities of nitrile

TABLE V. SUBSTRATE SPECIFICITY OF NITRILE HYDRATASE AND AMIDASE IN CELL EXTRACT OF *P. chlororaphis* B23 AND *Arthrobacter* sp. J-1

Arthrobacter sp. J-1 was grown for 3 days on acetonitrile as described previously.[7] *P. chlororaphis* B23 was grown as described in Fig. 4.

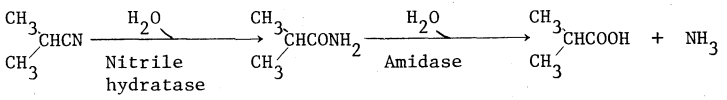
Substrate	Specific activity (units/mg) of Nitrile hydratase: *P. chlororaphis* B23	Nitrile hydratase: *Arthrobacter* sp. J-1	Substrate	Amidase: *P. chlororaphis* B23	Amidase: *Arthrobacter* sp. J-1
Acetonitrile	0.006	0.003	Acetamide	0.005	0.088
Acrylonitrile	0.076	0.011	Acrylamide	0.006	0.167
Isobutyronitrile	0.130	0	Isobutyramide	0.100	0

hydratase and amidase of isobutyronitrile-grown *P. chlororaphis* B23 and acetonitrile-grown *Arthrobacter* sp. J-1[7] were measured (Table V). Nitrile hydratase of *P. chlororaphis* B23 was not specific for isobutyronitrile. It also acted on acrylonitrile, about 60% the rate on isobutyronitrile. On the other hand, amidase of the strain was active on isobutyramide, but scarcely active on acrylamide. The spectrum of the substrate specificity did not changed at 0°C, indicating that the accumulation of acrylamide was not caused by the effect of the low reaction temperature. Amidase activity of *Arthrobacter* sp. J-1 was much higher than that of nitrile hydratase. The enzymes acted on acrylamide and acrylonitrile, respectively. In the other acrylamide producing strains which were found in the screening test, the specific activities of amidase toward acrylamide were also much lower than that of nitrile hydratase toward acrylonitrile.

DISCUSSION

We are the first to report the production of acrylamide by microbial enzyme system catalyzed by nitrile hydratase which was found and characterized by the authors.[3,4] We have established the cultural and reaction conditions for the production of acrylamide with *Pseudomonas chlororaphis* B23, which was isolated as an isobutyronitrile-utilizing strain. Nearly 100% of acrylonitrile was converted to acrylamide with only a trace amount of acrylic acid. The optimum temperature of the reaction was between 10°C and 20°C. This would be a promising method for the production of acrylamide as compared with the chemical hydration of acrylonitrile with respect to the selectivity, reaction temperature, and easiness of preparation of the enzyme.

There are two pathways in the microbial degradation of nitrile compounds. One is the direct hydrolysis of nitrile to carboxylic acid and ammonia, catalyzed by nitrilase.[10~13] The other is the two step degradation pathway of nitrile which involves nitrile hydratase and amidase, with amide as an intermediate.[3~7] When *P. chlororaphis* B23 was grown on isobutyronitrile, isobutyramide, isobutyric acid and ammonia were successively formed in the culture broth as isobutyronitrile was consumed. This shows that *P. chlororaphis* B23 degraded isobutyronitrile as follows:



The specific activities of nitrile hydratase and amidase toward isobutyronitrile and isobutyramide, respectively, in the cell-free extract of *P. chlororaphis* B23 grown on isobutyronitrile were almost the same, in agreement with the result that isobutyramide was not accumulated much in the culture broth. On the other hand, the latter enzyme scarcely attacked acrylamide which was formed from acrylonitrile by the former enzyme. The accumulation of a large amount of acrylamide might be due to the difference in the rate of hydration of acrylonitrile and hydrolysis of acrylamide. There is good evidence which explains the phenomenon, when acetonitrile-grown *Arthrobacter* sp. J-1 was incubated with acrylonitrile, acrylic acid was accumulated instead of acrylamide, because the specific activity of amidase was higher than that of nitrile hydratase.[4,6] With successive feeding of acrylonitrile, more than 100 g/liter of acrylic acid was accumulated in a short time (data not shown).

The low reaction temperature would be one of the advantageous characteristics of this enzymatic method, as compared with the chemical processes. The low optimum temperature for acrylamide production was also shown in 3 other acrylamide producing strains. As acrylonitrile is a specific modifier of the protein sulfhydryl group,[14,15] the active site of nitrile hydratase was possibly masked with acrylonitrile at higher temperatures.

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