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# SYNTHESIS OF VARIOUS AROMATIC AMIDE DERIVATIVES USING NITRILE HYDRATASE OF RHODOCOCCUS RHODOCHROUS J1

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**Abstract** - Nitrile hydratase, that is produced abundantly in cells of Rhodococcus rhodochrous J1, catalyses the conversion of various aromatic nitrile derivatives to the corresponding amides. Using Rh. rhodochrous J1 resting cells, the conditions for the production of benzamide, 2,6-difluorobenzamide, indoleacetamide, thiophenecarboxamide and furanecarboxamide were optimized. Under the determined conditions, 489 g of benzamide, 306 g of 2,6-difluorobenzamide, 1045 g of 3-indoleacetamide, 210 g of 2-thiophenecarboxamide and 522 g of 2-furanecarboxamide were produced, with 100% molar conversion, from the corresponding nitriles, per litre of reaction mixture.

## INTRODUCTION

Bioconversion is more and more commonly used as an alternative to classical organic synthesis. In our laboratory we are focusing our research on the hydration of nitrile compounds into either the corresponding amides or acid derivatives using microorganisms. We and a French group led by Galzy [1, 2] proposed a new enzymatic process for the production of acrylamide, a typical chemical commodity, on an industrial scale involving nitrile hydratase as a catalyst. Pseudomonas chlororaphis B23 and Brevibacterium R312, which have been selected as favorable strains, produced high amounts of acrylamide from acrylonitrile under suitable conditions. Thus, very recently, the use of the P. chlororaphis B23 nitrile hydratase as a catalyst for the industrial production of acrylamide began [3, 4]. We purified and characterized the nitrile hydratases from P. chlororaphis B23 [5] and

Brevibacterium R312 [6]. These nitrile hydratases contain ferric ions at their active centers [7]. Even though these iron-containing enzymes exhibited very high activity toward aliphatic nitriles, their activity toward aromatic nitriles was very low. Recently, in our laboratory, a cobalt-induced and cobalt-containing nitrile hydratase was found in Rhodococcus rhodochrous J1 and characterized [8]. Contrary to the above mentioned iron-containing enzymes, this enzyme showed a broader specificity since it exhibited remarkable activity toward not only cyanopyridines [9, 10], but also toward aliphatic nitrile derivatives [11].

In the present work we examined the reaction of Rh. rhodochrous J1 resting cells with various aromatic nitrile compounds in order to universalize the reaction, and consequently thus we have optimized the reaction conditions for the production of benzamide, 2,6-difluorobenzamide, 3-indoleacetamide, 2-thiophenecarboxamide and 2-furanecarboxamide from benzonitrile, 2,6-dibenzonitrile, 3-indoleacetonitrile, 2-thiophenecarbonitrile and 2-furonitrile respectively.

## RESULTS AND DISCUSSION

We previously examined the optimum conditions for the production of nicotinamide [9]. The optimum pH for the production of nicotinamide was found to be 8.0 and the optimum temperature to be around 25°C. Therefore, these conditions were selected for the production of benzamide, 2,6-difluorobenzamide, 3-indoleacetamide, 2-thiophenecarboxamide and 2-furanecarboxamide.

The aromatic nitrile compounds were added a little at a time to the reaction mixture during the course of the reaction to avoid the inhibitory effect of the substrate at high concentration, except for in

the case of 3-indoleacetonitrile, which could be added at one time.

The maximum production of benzamide was attained with seven feedings of 206 mg of benzonitrile. Thus, on 24 h incubation, 4.04 M benzonitrile was completely converted into benzamide, which corresponds to the production of 489 g of benzamide per litre of medium (Fig. 1 A).

In the case of the production of 2,6-difluorobenzamide, five feedings of 139 mg of 2,6-difluorobenzonitrile led to the total conversion of 2.5 M substrate on 22 h incubation, which is equivalent to the production of 306 g of 2,6-difluorobenzamide per litre of reaction mixture (Fig. 1 B). The lower production of 2,6-fluorobenzamide compared to that of benzamide can be explained by the steric hindrance due to the two atoms of fluorine.

In the case of 2-thiophenecarbonitrile, which seemed to be more inhibitory for the enzyme in the cells, only two feedings of 218 mg of substrate were possible, leading, nevertheless, to the hydration of 1.65 M 2-thiophenecarbonitrile on 5 h incubation (Fig. 1 C). With higher concentrations, even on long term incubation, cells showed hardly any activity and the substrate remained in the medium. However, the highest production corresponded to the formation of 210 g of 2-thiophenecarboxamide per litre of medium.

On the contrary, the lower inhibition by 2-furonitrile allowed eight feedings of 186 mg. Thus, on 30 h incubation, 3.93 M 2-furanecarbonitrile was completely converted into 2-furanecarboxamide, which is equivalent to the production of 522 g per litre of reaction mixture (Fig. 1 D).

Since 3-indoleacetonitrile exhibited low inhibition toward nitrile hydratase, one step addition of substrate was performed. However, because of the lower conversion rate observed in the case of 3-indoleacetonitrile compared to the other substrates, a higher amount of

cells was added (c.f. experimental). Under these conditions, 4 M 3-indoleacetonitrile was completely converted on 24 h incubation and almost 6 M on 48 h incubation. The trace of substrate remaining at the

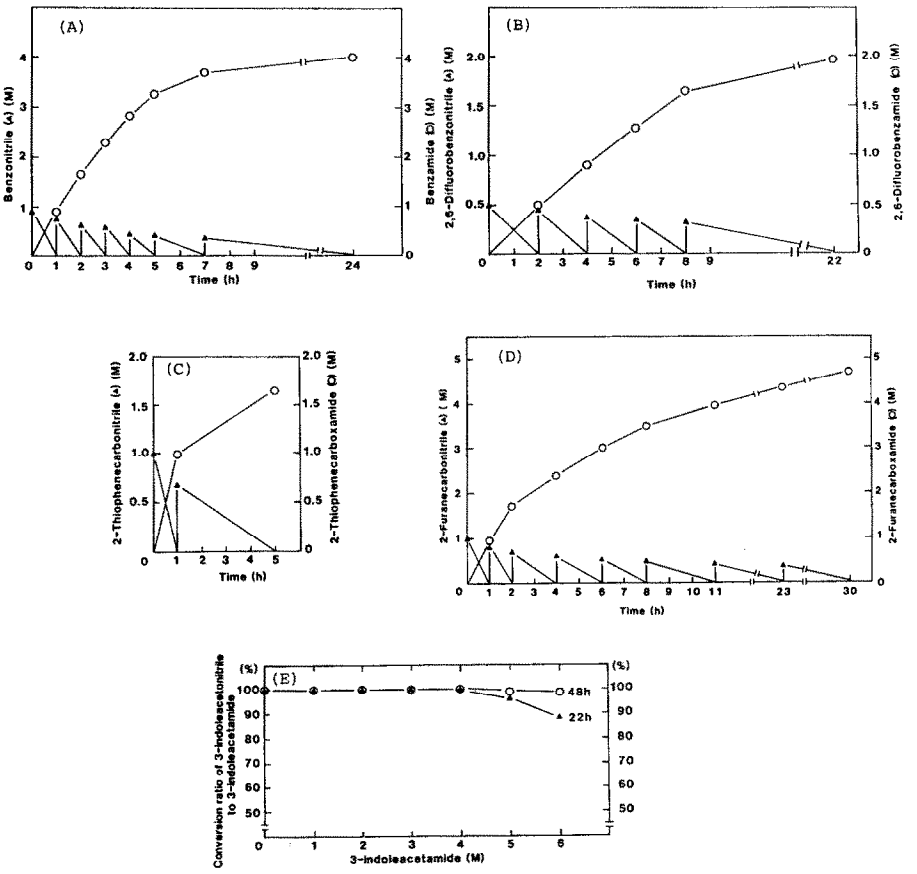


Fig. 1. Enzymatic conversion of benzonitrile (A), 2,6-difluorobenzonitrile (B), 2-thiophenecarbonitrile (C), 2-furonitrile (D) and 3-indoleacetonitrile (E) into benzamide, 2,6-difluorobenzamide, 2-thiophenecarboxamide, 2-furancarboxamide and 3-indoleacetamide, respectively.

latter time can be explained by the strong heterogeneity of the medium due to the rapid crystallization of the product. This production is equivalent to 1045 g of 3-indoleacetamide per litre of solution (Fig. 1 E).

In each case only the corresponding amide was isolated, except in the case of 3-indophenolacetamide production. No formation of the corresponding acids or other by-products was detected.

## CONCLUSION

Previously we showed the ability of Rh. rhodochrous J1 cells to convert, in large scale, cyanopyridines [9, 10] and various aliphatic nitriles to the corresponding amides. In the present study, we extended the process to various aromatic nitrile compounds, thus 489 g benzamide, 306 g of 2,6-difluorobenzamide, 210 g of 2-thiophenecarboxamide, 522 g of 2-furanecarboxamide and 1045 g of 3-indoleacetamide per litre of reaction mixture were produced at 25°C, with a molecular conversion yield of 100%, i.e., without the formation of the corresponding acids. In addition, since Rh. rhodochrous J1 can be easily prepared on a large scale [8] and can be stored for more than 6 months at -20°C, the use of Rh. rhodochrous J1 cells as an efficient catalyst for the hydration of nitrile compounds to the corresponding amides can be considered as a promising and widely applicable process.

## EXPERIMENTAL

### Analysis

Nitrile compounds and amides were detected by analytical HPLC, which was performed with a Shimadzu LC-5A equipped with an M&S Pack $C_{18}$ column (reversed-phase column, 4.6x150 mm; M&S Instruments Inc., Japan), at a flow rate of 1.0 ml $min^{-1}$, using the following solvent system: acetonitrile/10 mM $KH_2PO_4$-$H_3PO_4$ buffer (pH 2.8), 1/2 (v/v). The absorbance was measured at 230 nm.

$^{1}H$- and $^{13}C$-nuclear magnetic resonance (NMR) spectra were recorded in DMSO with a Nihondensi JNN-GX270 and a Perkin Elmer 1710-FTIR spectrophotometer, respectively. Chemical shifts are given in relation to internal standards (TMS and DMSO). Infrared and mass spectra were recorded, respectively, with a Shimadzu IR 27G and a Hitachi M-80. Elemental analysis was carried out with a Perkin Elmer 240B.

## Culture conditions and preparation of resting cells

Rh. rhodochrous J1, previously isolated from a soil sample and then identified in our laboratory [8], was used. The subculture was carried out at 28°C for 24 h with reciprocal shaking in a test tube containing 4 ml of medium (pH 7.0) consisting of 5 g of Polypepton (Daigo, Osaka), 5 g of meat extract (Mikunikagaku, Tokyo), 2 g of NaCl and 0.5 g of yeast extract (Oriental Yeast, Tokyo) per litre of tap water. Then the contents of two test tubes (8 ml) were added to 2-litre flasks containing 400ml of culture medium (pH 7.2) consisting of 3 g of yeast extract, 0.5 g of $K_2HPO_4$, 0.5 g of $KH_2PO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $CoCl_2.6H_2O$ and 2 g of crotonamide per litre of distilled water, followed by incubation with shaking at 28°C. After 30 h and 60 h, 0.2 % (w/v) crotonamide was added. The cultures (4.66 litres) were pooled after 80 h, followed by centrifugation at 10,000 x g for 20 min at 0°C. The cells obtained were washed with 0.85 % (w/v) NaCl, centrifuged at 10,000 x g and then suspended in 233 ml of the same solution (1.58 mg [dry weight] of cells per ml). This cell suspension was used for the resting-cell reaction.

## Assay for nitrile hydratase activity in the culture medium

The nitrile hydratase activity in the culture medium was assayed in a standard mixture (2 ml) containing 50 µmol of $K_2HPO_4/KH_2PO_4$ buffer (pH 8.0), 20 µmol of benzonitrile and an appropriate amount of the cell suspension. The reaction was carried out with shaking at 25°C for 5 min and stopped by adding 0.2 ml of 3 N HCl. One unit of nitrile hydratase activity was defined as the amount of cells that catalysed the formation of 1 µmol of benzamide per min under these conditions. The specific activity of the cell suspension corresponded to 119 units/mg dry cell weight.

## Assay for the production of benzamide, 2,6-difluorobenzamide, 2-thiophenecarboxamide, 2-furanecarboxamide and 3-indoleacetamide.

The standard reaction mixture (2 ml) for amide production contained various concentrations of the nitrile compounds, 20 µmol of $K_2HPO_4$-$KH_2PO_4$ buffer (pH 8.0) and 5.27 mg of cells, as dry cell weight, or 15.8 mg in the case of 3-indoleacetamide production. The reaction was carried out at 25°C with shaking and stopped by adding 1 ml of 3 N HCl.

## Isolation and identification of benzamide, 2,6-difluorobenzamide, 2-thiophenecarboxamide, 2-furanecarboxamide and 3-indoleacetamide

To isolate the amide derivatives, each reaction mixture was filtered with Millipore type AB filter and the filtrate evaporated in vacuo. The solid obtained was dissolved in hot water and then kept overnight in an ice bath. Recrystallization was carried out in the same manner. The crystals formed were filtrated and then dried in a vacuum desiccator over $P_2O_5$.

Identification of the products as benzamide, 2,6-difluorobenzamide, 2-thiophenecarboxamide, 2-furanecarboxamide and 3-indoleacetamide was performed through comparison with the infrared, $^{1}H$- and $^{13}C$-NMR and mass spectra of authentic samples. Elemental analysis of the synthesised products was also performed:

- Benzamide, $^{1}H$-NMR (DMSO): 7.60 (m, 7H) ppm; $^{13}C$-NMR (DMSO): 127, 128, 131, 134 ppm; IR: 1630, 1665, 3200, 3480 $cm^{-1}$; mass spectrum: m/z, 51, 105, 121; elemental analysis, Anal. Cald. for $C_7H_7NO$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.03; H, 5.85; N,11.81.

- 2,6-Difluorobenzamide, $^{1}H$-NMR (DMSO): 7.30 (m, 3H), 8.00 (d, 2H) ppm; $^{13}C$-NMR: 112, 116, 131, 157, 160, 161 ppm; IR: 1630, 1660, 3200, 3400 $cm^{-1}$; mass spectrum: m/z, 44, 113, 141, 157; elemental analysis, Anal. Cald. for $C_7H_5F_2NO$: C, 53.51; H, 3.21; N, 8.92. Found: C, 53.08; H, 3.06; N, 8.89.

- 2-Thiophenecarboxamide, $^{1}H$-NMR (DMSO): 7.45 (m, 3H); 7.60 (d, 2H) ppm; $^{13}C$-NMR: 128, 129, 131, 140, 163 ppm; IR: 1610, 1660, 3190, 3390 $cm^{-1}$; mass spectrum: m/z, 39, 111, 127; elemental analysis, Anal. Cald. for $C_5H_5NOS$: C, 47.22; H, 3,96; N, 11.01. Found: C, 46.86; H, 3.81; N, 10.99.

- 2-Furanecarboxamide, $^{1}H$-NMR (DMSO): 7.20 (m, 3H); 7.50 (d, 2H) ppm; $^{13}C$-NMR (DMSO): 111, 114, 145, 148, 160 ppm; IR: 1635, 1680, 3180, 3560 $cm^{-1}$; mass spectrum: m/z, 39, 95, 111; elemental analysis, Anal. Cald. for $C_5H_5NO_2$: C, 54.06; H, 4.54; N, 12.61. Found: C, 53.91; H, 4.19; N, 12.19.

- 3-Indoleacetamide, $^{1}H$-NMR (DMSO): 3.45 (s, 2H); 7.10 (m, 7H); 10.80 (s, 1) ppm; $^{13}C$-NMR (DMSO): 33, 109, 111, 118, 121, 124, 128, 138, 173 ppm; IR: 1620, 1645, 3200, 3400, $cm^{-1}$; mass spectrum: m/z, 44, 77, 130, 174; elemental analysis, Anal. Cald. for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found: C, 68.59; H, 5.75; N, 15.54.

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