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Journal of Molecular Catalysis B: Enzymatic 29 (2004) 249–258
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Control of the nitrile-hydrolyzing enzyme activity in *Rhodococcus rhodochrous* IFO 15564: preferential action of nitrile hydratase and amidase depending on the reaction condition factors and its application to the one-pot preparation of amides from aldehydes

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Received 19 November 2003; received in revised form 9 December 2003; accepted 15 December 2003

Abstract

The reaction conditions towards the preferential action of either nitrile hydratase or amidase in the harvested whole cells of *Rhodococcus rhodochrous* IFO 15564 were elaborated. The amidase showed higher heat tolerance than the nitrile hydratase and, at 45 °C the amidase worked exclusively. DMSO assisted the preferential action of nitrile hydratase, however, at more than 30% (v/v) addition of DMF, the nitrile hydratase activity was completely lost and only amidase worked. A one-pot chemo-enzymatic conversion of aldehydes to amides [(1) aq. NH_3, I_2, DMSO; (2) $Na_2S_2O_3$; (3) harvested cells of *R. rhodochrous*] was established. Under these reaction conditions, most of the amidase was lost, and the incubation of the firstly formed intermediates, nitriles in aq. NH_3 was responsible for the selective inhibition of amidase. The freezing of harvested cells in an exhaustively deionized environment provided a long-term preservable "ready to use" for the organic chemist.

Keywords: *Rhodococcus rhodochrous* IFO 15564; Nitrile hydratase; Amidase; Enzyme inhibition; One-pot synthesis of amides

1. Introduction

Among the biodegradation pathways of nitriles (cyano compounds), a representative class of non-natural industrial products, there has been clarified a two-step enzymatic sequential conversion. Nitrile hydratases catalyze hydration of nitriles into the corresponding amides, and amidases catalyze hydrolysis of amides into carboxylic acids [1–3]. These two processes are, from the standpoint of synthetic organic chemistry, often difficult to achieve under chemically mediated conditions such as heating at high temperature under either a strongly acidic or alkaline pH, in which molecules carrying other acid- or alkaline-labile functionalities hardly survive. As easily seen, enzymatic transformations are advantageous in that, they can be performed under such contrastingly mild conditions as neutral pH and room temperature. Accordingly, much effort has been devoted to enhance the catalytic activity of these enzymes towards a wide range of synthetic substrates in organic functional group transformations [4–8]. In recent years, microbial nitrile hydratases and amidases have been extensively developed, from *Rhodococcus equi* A 4 [9,10], *R.* sp. R 312 [11], *R. erythropolis* MP 50 [12,13], *R.* sp. AJ 270 [14–22], *Pantoea endophytica* [23], *R.* sp. CGMCC 0497 [24–28], and *Comamonas testosteroni* [29].

Among them, we have so far been engaged in study of *Rhodococcus rhodochrous* IFO 15564 [5,30]. The substrate specificity of this microorganism has extensively been examined in aromatic, aliphatic, alicyclic, heteroatom-containing, and carbohydrate-related compounds. The nitrile hydratases are metallic Lewis acid-promoting hydration enzymes [31], but in contrast, the amidases are a kind of serine proteases [32], and they work under completely different catalytic mechanisms. In the case of *R. rhodochrous* IFO 15564, the genes of two enzymes, however, locate very closely [33], and the isolation of enzyme proteins and the independent overexpression of the respective gene-cloned enzymes have remained an open question. Taking advantage of convenient

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doi:10.1016/j.molcatb.2003.12.014

availability of cultured and harvested microorganism cells, the selective "preferential action" of either nitrile hydratase or amidase in the whole cell is of great value in the selective functional group transformation of synthetic organic chemistry, as demonstrated in the well-established industrial productions of acrylamide and nicotinamide from acrylonitrile and 3-cyanopyridine by the action of the nitrile hydratase of *R. rhodochrous* J1 [8,34,35].

In this paper, we describe our efforts toward the goal of "preferential action" by elaboration of the reaction conditions involving the pH profile, heat tolerance, co-solvent, additives, and selective inhibitors. By integrating such information as above, we propose a newly developed chemo-enzymatic one-pot synthesis of various amides from aldehydes by applying the cultured whole cells. Moreover, a frozen form of preservable whole cells retaining the activity of both enzymes could be provided.

2. Experimental

2.1. General

All melting points are uncorrected. IR spectra were measured as thin films for oils or KBr disks of solids on a JASCO FT/IR-410 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ or DMSO-d_6 at 270 MHz on a JEOL JNM EX-270 or at 400 MHz on a JEOL JNM GX-400 spectrometer. HPLC analyses were performed with a SSC-5410 (Senshu Scientific Co. Ltd.) liquid chromatographs. Silica gel 60 (spherical, 100–210 μm, 37558-79) of Kanto Kagaku Co. was used for column chromatography. Peptone and yeast extract were purchased from Kyokuto Pharmaceutical Co., for the cultivation of microorganism.

2.2. Pre-cultivation of R. rhodochrous IFO 15564

2.2.1. Pre-cultivation with Fe ion: Method A

To a sterilized medium (pH 7.2, 95 ml) containing glucose (15 g/l), K_2HPO_4 (0.4 g/l), KH_2PO_4 (1.2 g/l), $MgSO_4{\cdot}7H_2O$ (0.5 g/l), yeast extract (1.0 g/l) and peptone (5.0 g/l) in a 500 ml baffled Erlenmeyer flask was added a solution of ε-caprolactam (0.1 g) and $FeSO_4{\cdot}7H_2O$ (28 mg) in deionized water (5 ml) via a pre-sterilized membrane filter (Advantec Toyo Ltd., 25CS020AS, 0.20 μm). A loopful of *R. rhodochrous* IFO 15564 was inoculated, and the flask was shaken at 30 °C on a gyrorotary shaker (180 rpm) for 60 h. The grown cells (ca. 3 g in wet weight) were harvested with 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0) and collected by centrifugation (1600 × *g*, for 10 min at 4 °C). All water used here was further deionized by Ultrapure Water System CPW-100 (Advantec Toyo Ltd.), until to show a resistance of 18.2 MΩ from a reverse osmotically pre-purified water.

2.2.2. Pre-cultivation without Fe ion: Method B

The whole cells were cultivated as the same manner as described in Section 2.2.1 except for the lack of $FeSO_4{\cdot}7H_2O$.

2.3. General procedure for measuring the enzyme activity

Nitrile hydratase and amidase activity were assayed in a reaction mixture with a total volume of 1.62 ml containing the substrate (2 mg), dissolved in DMF (60 μl; for DMSO, see Sections 2.4 and 3.2), 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0), and *R. rhodochrous* IFO 15564 prepared according to either Method A or B (wet cells, 46 mg) and incubated at 30 °C for 10–60 min. The substrates were 2-naphthylacetonitrile [**1**, mp 84.5–85.0 °C, recrystallized from MeOH (T [36], mp 85–86 °C)] for nitrile hydratase assay, and 2-naphthylacetamide [**2**, prepared according to the reported procedure [37], mp 207.5–208.0 °C, recrystallized from CH_3CN (T [38], mp 202–204.6 °C)] for amidase. The incubation was carried out in a tightly capped centrifugal tube (3.2 cm × 10 cm, 50 ml) to prevent evaporation of the solution. After appropriate reaction time, the reaction was quenched by the addition of HCl (0.2 M, 180 μl) and acetonitrile (880 μl). After the further incubation for 10 min at 30 °C, the cells were collected by centrifugation (1600 × *g*, for 10 min). The supernatant (500 μl) was added to internal standard solution (100 μl, see below) and monitored by HPLC analysis [Senshu Pak PEGASIL ODS, 25 cm × 4.6 mm (No. 0206266H, Senshu Scientific Co. Ltd.); solvent, 10 mM H_3PO_4–KH_2PO_4 buffer solution (pH 2.8)/acetonitrile = 3/2; flow rate, 1.0 ml/min; detection at 254 nm]. Retention time, 5.9 min (*o*-anisic acid, internal standard), 7.5 min (**2**), 15.4 min [2-naphthylacetic acid (**3**)] and 35.9 min (**1**). The internal standard solution was prepared as follows: *o*-anisic acid (41.8 mg, Kanto Co., 01400-30, analytically pure by HPLC) was dissolved in the HPLC eluent as above (25 ml).

Definition of activity is as follows: one (unit/mg dry cell weight, U/mg DCW) equals the amount of enzyme which converts 1 μmol of substrates per 1 min at pH 6.0, 30 °C in 1 mg of dry cell, toward 2-naphthylacetonitrile for nitrile hydratase and 2-naphthylacetamide for amidase, respectively. The relationship between the harvested wet cells (98 mg) and dry cells (26 mg) was determined by freeze-drying.

2.4. Influence of the reaction conditions on the enzyme activities

2.4.1. Effect of co-solvent at varied concentration

Pre-cultivated and harvested cells in Method B were used in this section. The experiments at varied concentration of DMF were performed and assayed as described in Sections 2.2 and 2.3 at pH 6.0, 30 °C. The reaction rate at 3.8% (v/v) addition of DMF [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The effect of DMSO was examined in a similar manner, at pH 6.0, 30 °C. In this case, the reaction rate at 3.8% (v/v) addition of DMSO [nitrile hydratase (57.6 mU/mg DCW), amidase (19.9 mU/mg DCW)] was defined as 100%. The results are summarized in Figs. 1 and 2.

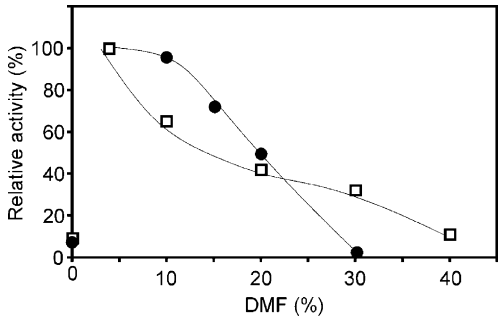


Fig. 1. DMF concentration dependence of nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate at 3.8% (v/v) addition of DMF was defined as 100%.

2.4.2. Effects of reaction temperature and pH

Pre-cultivated and harvested cells in Method B were used in this section. To determine the effect of temperature on the activity, the microorganism was pre-incubated for 20 min at varied temperature ranging from 5 to 60 °C in 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0). Then, substrate dissolved in DMF (60 μl, final concentration: 3.8% (v/v) in total reaction mixture) was added and incubated at each temperature for 10–60 min. The reaction rate at 30 °C [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The results are summarized in Fig. 3.

In the studies of pH dependence, 0.1 M solutions of the following buffers were used: citrate–Na_2HPO_4 (pH 3.0–6.0), NaH_2PO_4–Na_2HPO_4 (pH 6.0–8.4) and Na_2CO_3–$NaHCO_3$ (pH 9.0–10.0). Prior to the each assay, the harvested cells were washed twice with each above-mentioned buffer solution. The reaction rate at pH 6.0 [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The results are summarized in Fig. 4.

*2.5. Reaction of the mixture of 2-naphthylacetonitrile (**1**) and 2-naphthylacetamide (**2**)*

Pre-cultivated and harvested cells in Method A were used in this section. To a pre-heated (45 °C, 1 h) suspension of harvested cells of *R. rhodochrous* IFO 15564 (2.4 g) in phosphate buffer (78 ml, pH 6.0, 0.1 M), the mixture of **1**

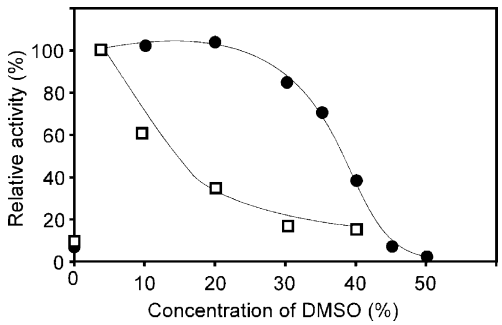


Fig. 2. DMSO concentration dependence of nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate at 3.8% (v/v) addition of DMSO was defined as 100%.

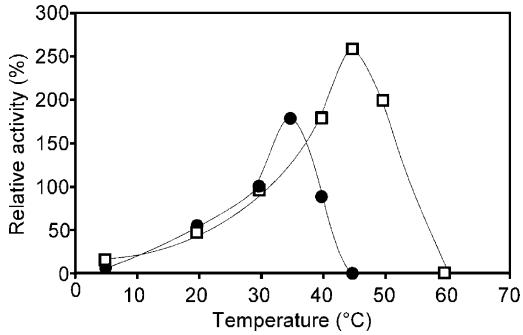


Fig. 3. Reaction temperature dependence of nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate at 30 °C was defined as 100%.

(50.9 mg, 0.304 mmol) and **2** (50.8 mg, 0.274 mmol) dissolved in DMF [3 ml, final concentration was 3.8% (v/v)] was added. After stirred at 45 °C for 1 h, the reaction mixture was acidified with 2 M HCl to be pH 1. The mixture was filtered through a Celite pad and the residual solid was washed with water and AcOEt. The filtrate was saturated with NaCl and extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was purified by silica gel column chromatography (3 g). Elution with hexane–AcOEt (10/1–2/1) afforded **1** (50.5 mg, 99%) and **3** (50.7 mg, 99%). (**1**) mp 84.5–85.0 °C; ^{1}H NMR ($CDCl_3$): δ 3.93 (s, 2H), 7.50–7.53 (m, 3H), 7.83–7.87 (m, 4H); IR: ν_{max} 2252 cm^{-1}. These spectra were identical with those of the authentic specimen (2.3) (Aldrich, 16,276-0). (**3**) mp 142.0–142.5 °C ([39], mp 143 °C); ^{1}H NMR (DMSO-d_6): δ 3.74 (s, 2H), 7.40–7.50 (m, 3H), 7.76–7.88 (m, 4H), 12.39 (s, 1H); IR: ν_{max} 3502, 1598 cm^{-1}. These spectra were identical with those of the authentic specimen [Tokyo Kasei Kogyo Co. Ltd. (TCI), N352].

2.6. One-pot synthesis of amides from aldehydes

2.6.1. General procedure

Pre-cultivated and harvested cells in Method A were used in this section. According to the reported procedure [40],

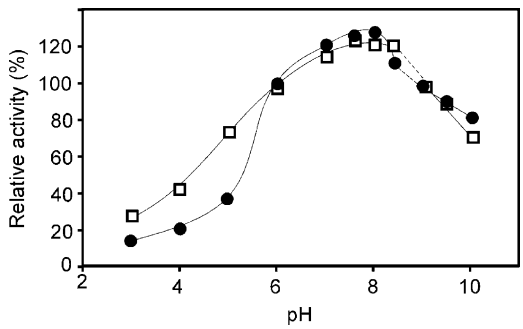


Fig. 4. pH dependence of nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate at pH 6.0 was defined as 100%.

an aldehyde (1 mmol) in DMSO (instead of originally reported THF, 1 ml) was added to aq. NH_3 (5 ml; 28% solution) and I_2 (280 mg, 1.1 mmol) at room temperature under Ar atmosphere and was stirred for 30 min. The consumption of the starting material was confirmed by TLC analysis, then $Na_2S_2O_3$ (24 mg, 0.1 mmol) was added to the reaction mixture and adjusted its pH to 8.0 with 12 M HCl. Then, harvested cells of *R. rhodochrous* IFO 15564 (1.0 g) in phosphate buffer (2 ml, pH 6.0, 0.1 M) were added and stirred at 30 °C for 1–24 h. The work-up and the subsequent purification were performed in the same manner as described in Section 2.5 to give corresponding amide.

2.6.2. 2-Furoamide (*4b*) from furfural (*4a*)

2-Furoamide (**4b**, 97.6 mg, 88%) was obtained from furfural (**4a**, 99.6 mg, 1.03 mmol) in the similar manner as described in Section 2.6.1; mp 140.0–140.5 °C, recrystallized from hexane–AcOEt ([41], mp 141–142 °C); ^{1}H NMR ($CDCl_3$): δ 6.03 (s, 1H), 6.29 (s, 1H), 6.52 (dd, J = 2.0, 3.4 Hz, 1H), 7.16 (d, J = 3.4 Hz, 1H), 7.47 (d, J = 1.5 Hz, 1H); IR: ν_{max} 3351, 3160, 1666, 1623 cm^{-1}. These spectra were identical with those reported previously [42].

2.6.3. 2-Thiophenecarboxamide (*5b*) from 2-thiophenecarbaldehyde (*5a*)

2-Thiophenecarboxamide (**5b**, 116 mg, 86%) from 2-thiophenecarbaldehyde (**5a**, 119 mg, 1.06 mmol); mp 176.0–176.5 °C, recrystallized from hexane–AcOEt ([43], mp 178–180 °C); ^{1}H NMR (DMSO-d_6): δ 7.10–7.12 (d, J = 3.4 Hz, 1H), 7.35 (s, 1H), 7.72–7.78 (m, 2H), 7.93 (s, 1H); IR: ν_{max} 3357, 3170, 1654, 1606 cm^{-1}. These spectra were identical with those of the authentic specimen (Avocado Research Chemicals, 15219).

2.6.4. Benzamide (*6b*) from benzaldehyde (*6a*)

Benzamide (**6b**, 115 mg, 94%) from benzaldehyde (**6a**, 107 mg, 1.00 mmol); mp 127.5–128.0 °C, recrystallized from EtOH ([44], mp 128–129 °C); ^{1}H NMR (DMSO-d_6): δ 7.34 (s, 1H), 7.41–7.52 (m, 3H), 7.83–7.88 (m, 2H), 7.95 (s, 1H); IR: ν_{max} 3367, 3170, 1656, 1623 cm^{-1}. These spectra were identical with those of the authentic specimen (TCI, B012).

2.6.5. Trans-cinnamamide (*7b*) from cinnamaldehyde (*7a*)

Trans-cinnamamide (**7b**, 119 mg, 78%) from cinnamaldehyde (**7a**, 137 mg, 1.04 mmol); mp 147.0–147.5 °C, recrystallized from AcOEt ([44], mp 145–147 °C); ^{1}H NMR (DMSO-d_6): δ 6.58 (d, J = 16.1 Hz, 1H), 7.09 (s, 1H), 7.33–7.42 (m, 4H), 7.52–7.56 (m, 3H); IR: ν_{max} 3374, 3170, 1662, 1608 cm^{-1}. These spectra were identical with those of the authentic specimen (Aldrich, C8,080-6).

2.6.6. 1-Naphthylamide (*8b*) from 1-naphthaldehyde (*8a*)

1-Naphthylamide (**8b**, 158 mg, 91%) from 1-naphthylaldehyde (**8a**, 158 mg, 1.01 mmol); mp 206.0 °C, recrystallized from EtOH ([45], mp 202 °C); ^{1}H NMR ($CDCl_3$): δ 5.95 (bs, 2H), 7.23–7.61 (m, 3H), 7.61 (d, J = 1.2 Hz, 1H), 7.89 (d, J = 7.2 Hz, 1H), 7.95 (d, J = 8.0 Hz, 1H), 8.43 (d, J = 8.4 Hz, 1H); IR: ν_{max} 3338, 3166, 1662, 1616 cm^{-1}. These spectra were identical with those reported previously [45].

2.6.7. 5-Hydroxymethyl-2-furoamide (*9b*) from hydroxymethylfurfural (*9a*)

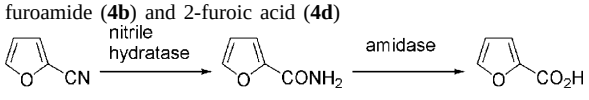
Hydroxymethylfurfural (**9a**, 128 mg, 1.01 mmol) was incubated as described in Section 2.6.1. Then the mixture was centrifuged (1600 × g, for 10 min at 4 °C) to remove the cells. The pellet was washed with water for another three times. Combined supernatant was filtered through a Celite pad and the residual cell debris was washed with water. The aqueous filtrate was desalted by AC-110-10 on Asahi Chemical Micro Acylyzer S2. At the initial stage, the conductivity was 85 mS, and after the desaltation at the 4.3 V (0.81 A), it reached 2.7 mS. The solution was concentrated in vacuo, mixed with water (0.5 ml) and Celite (0.6 g) and concentrated in vacuo again. The resulted powder was chromatographed on silica gel column (30 g). Elution with AcOEt–EtOH (30/1–4/1) afforded **9b** (127 mg, 89%); mp 150.5–151.0 °C, recrystallized from MeOH ([41], mp 135–136 °C); ^{1}H NMR (DMSO-d_6): δ 4.41 (d, J = 5.6 Hz, 2H), 5.33 (t, J = 5.6 Hz, 1H), 6.38 (d, J = 3.2 Hz, 1H), 7.01 (d, J = 3.6 Hz, 1H), 7.27 (s, 1H), 7.65 (s, 1H); IR: ν_{max} 3359, 3230, 1671, 1604 cm^{-1}. These spectra were identical with those reported previously [41].

2.7. Inhibitory effect of additives: 2-furoamide (*4b*) and 2-furoic acid (*4d*) from 2-furonitrile (*4c*)

2.7.1. Control experiment

Pre-cultivated and harvested cells in Method B were used in this section. The control experiment (Table 1, entry 1) was carried out as follows. The incubation of 2-furonitrile

Table 1
The effect of additives in the conversion of 2-furonitrile (**4c**) to 2-furoamide (**4b**) and 2-furoic acid (**4d**)



4c **4b** **4d**

Entry	Reaction time (h)	Additive (mmol/WCW)				Yield (%)	
		I^-	$Na_2S_2O_3$	I_2	NH_3	**4b**	**4d**
1	1	–	–	–	–	0	93
2	1	2.2	–	–	–	0	97
3	1	–	1	–	–	0	96
4	12	–	–	0.1	–	68	0
5	65	–	–	0.1	–	93	0
6	1	–	0.1	0.1	–	98	0
7[a]	1	–	0.22	0.1	–	0	95
8[b]	3	–	–	–	70	84	1
9[b]	3	–	–	–	70	85	9
10[a]	1	–	0.22	0.1	–	0	98

[a] See Section 2.7.6 for entry 7 and Section 2.7.9 for entry 10.
[b] See Section 2.7.7 for entry 8 and Section 2.7.8 for entry 9.

(**4c**, 58.2 mg, 0.625 mmol) in DMSO-buffer solution was performed in an almost similar manner as described for the one-pot conversion of furfural (**4a**) to furoamide (**4b**) (2.6.1) to give 2-furoic acid **4d** (65.6 mg, 95%); mp 129.5–130.0 °C, recrystallized from hexane ([46], mp 131–133 °C); ^{1}H NMR (DMSO-d_6): δ 6.49 (dd, J = 3.4, 2.0 Hz, 1H), 7.27 (d, J = 3.4 Hz, 1H), 7.57 (d, J = 1.5 Hz, 1H), 11.28 (s, 1H); IR: ν_{max} 3143, 1687 cm^{-1}. These spectra were identical with those of the authentic specimen (TCI, F0081).

*2.7.2. 2-Furoic acid (**4d**) from 2-furonitrile (**4c**) under the influence of NaI*

The incubation of 2-furonitrile (**4c**, 92.5 mg, 0.990 mmol) in DMSO-buffer solution was performed in an almost similar manner as described in Section 2.7.1 except for the addition of NaI (330 mg, 2.20 mmol, 2.20 mmol/WCW, WCW = wet cell weight, g, 147 mM of the final concentration, Table 1, entry 2) for 1 h resulted in **4d** (105 mg, 97%).

*2.7.3. 2-Furoic acid (**4d**) from 2-furonitrile (**4c**) under the influence of $Na_2S_2O_3$*

In the similar manner, 2-furonitrile (**4c**, 96.0 mg, 1.03 mmol) was treated with *R. rhodochrous* cells except for the addition of $Na_2S_2O_3{\cdot}5H_2O$ (248 mg, 1.00 mmol, 1.00 mmol/WCW, 40.0 mM of the final concentration, Table 1, entry 3) for 1 h to give **4d** (107 mg, 96%).

*2.7.4. 2-Furoamide (**4b**) from 2-furonitrile (**4c**) under the influence of I_2*

In the same manner, 2-furonitrile (**4c**, 50.1 mg, 0.538 mmol) was treated with *R. rhodochrous* cells except for the addition of I_2 (13.6 mg, 53.4 μmol, 0.100 mmol/WCW, 40.0 mM of the final concentration) for 12 h resulted in **4b** (37.8 mg, 68%, Table 1, entry 4). Prolonged incubation time to 65 h resulted in **4b** (55.5 mg, 93%, Table 1, entry 5).

*2.7.5. 2-Furoamide (**4b**) from 2-furonitrile (**4c**) under the combined use of I_2 and $Na_2S_2O_3$*

In the same manner, 2-furonitrile (**4c**, 32.5 mg, 0.348 mmol) was treated with *R. rhodochrous* cells except for the addition of a pre-mixture of I_2 (8.2 mg, 32 μmol, 0.100 mmol/WCW, 4.00 mM of the final concentration) and $Na_2S_2O_3{\cdot}5H_2O$ (8.2 mg, 32 μmol, 0.100 mmol/WCW, 4.00 mM of the final concentration, a half equivalent to I_2) for 1 h resulted in **4b** (34.9 mg, 98%, Table 1, entry 6).

*2.7.6. 2-Furoic acid (**4d**) from 2-furonitrile (**4c**) under the combined use of I_2 and $Na_2S_2O_3$*

In the same manner, 2-furonitrile (**4c**, 56.4 mg, 0.607 mmol) was treated with *R. rhodochrous* cells except for the addition of a pre-mixture of I_2 (15.2 mg, 60.1 μmol, 0.100 mmol/WCW, 4.00 mM of the final concentration) and $Na_2S_2O_3{\cdot}5H_2O$ (38.4 mg, 0.155 mmol, 0.250 mmol/WCW, 10.0 mM of the final concentration, 2.5 equivalent to I_2) for 1 h resulted in **4d** (63.3 mg, 95%, Table 1, entry 7).

*2.7.7. 2-Furoamide (**4b**) and 2-furoic acid (**4d**) from 2-furonitrile (**4c**) under the pre-treatment of **4c** with NH_3*

2-Furonitrile (**4c**, 101 mg, 1.09 mmol) was stirred in aq. NH_3 (5 ml, 28% solution) for 30 min and then treated with *R. rhodochrous* cells (Table 1, entry 8) for 3 h to give **4b** (100 mg, 84%) and **4d** (0.5 mg, 1%). Within 1 h, the nitrile was completely consumed.

*2.7.8. 2-Furoamide (**4b**) and 2-furoic acid (**4d**) from 2-furonitrile (**4c**) under the pre-treatment of **4c** with NH_4Cl*

Prior to the incubation with 2-furonitrile (**4c**, 93.1 mg, 1.00 mmol) as above, aq. NH_3 (5 ml, 28% solution) was neutralized with 2 M HCl to pH 8, and then the mixture was further treated with *R. rhodochrous* cells (Table 1, entry 9) for 3 h to give **4b** (94.5 mg, 85%) and **4d** (9.5 mg, 9%).

*2.7.9. 2-Furoic acid (**4d**) from 2-furonitrile (**4c**) under the pre-treatment of **4c** with I_2*

2-Furonitrile (**4c**, 92.3 mg, 0.991 mmol) was stirred with I_2 (25.3 mg, 0.100 mmol) in DMSO for 30 min, then quenched with the addition of $Na_2S_2O_3{\cdot}5H_2O$ (54.6 mg, 0.220 mmol, 2.2 equivalent of I_2), and treated with *R. rhodochrous* cells (Table 1, entry 10) for 1 h to give **4d** (105 mg, 98%).

2.8. The influence of the ingredients in one-pot synthesis on enzyme activities

2.8.1. Effect of ammonium ion on the enzyme activities

Pre-cultivated and harvested cells in Method B were used in this section. To determine the effect of ammonium ion on the activity, varied amount of NH_4Cl were added and assayed as described in Sections 2.2 and 2.3 at 30 °C, while its pH was adjusted to be 6.0. The reaction rate without any addition of the additive [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The results are summarized in Fig. 5.

2.8.2. Effect of iodide on the enzyme activities

Pre-cultivated and harvested cells in Method B were used in this section. To determine the effect of iodide on the

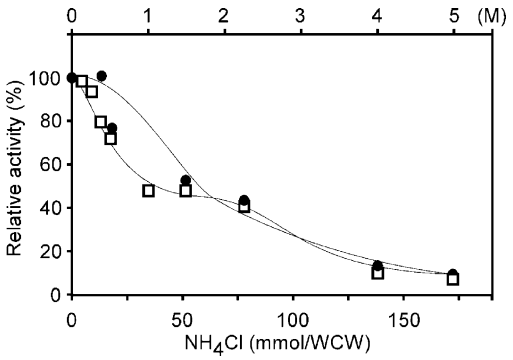


Fig. 5. NH_4Cl concentration dependence of nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate without any addition of NH_4Cl was defined as 100%.

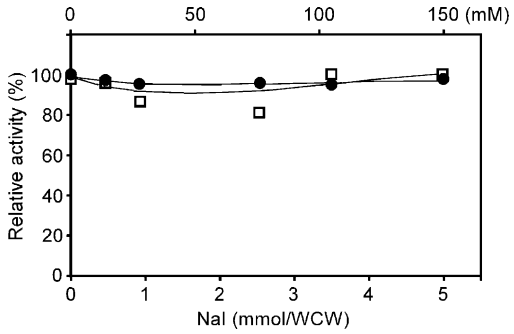


Fig. 6. NaI concentration dependence of nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate without any addition of NaI was defined as 100%.

activity, varied amount of NaI was added and assayed as described in Sections 2.2 and 2.3 at pH 6.0, 30 °C. The reaction rate without any addition of the additive [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The results are summarized in Fig. 6.

2.8.3. Effect of I_2 on the enzyme activities

Pre-cultivated and harvested cells in Method B were used in this section. To determine the effect of iodine on the activity, I_2 dissolved in DMF was added to the reaction mixture and assayed as described in Sections 2.2 and 2.3 at pH 6.0, 30 °C. Total amount of DMF was 10% (v/v). The reaction rate without I_2 but under 10% (v/v) DMF conditions [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The results are summarized in Fig. 7.

2.9. Storage of the whole cells

Pre-cultivated and harvested cells in Method B were used in this section. Harvested whole cells (1.0 g) were re-suspended with 10 ml of 0.1 M phosphate buffer, deionized water [18.2 MΩ], or a mixture of glycerol and 0.1 M phosphate buffer (4:1). In the second case, the cells were pre-washed twice with deionized water as above. The

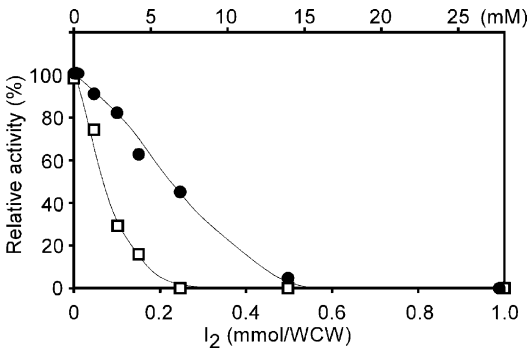


Fig. 7. Effect of I_2 on nitrile hydratase and amidase activities. (●) Nitrile hydratase; (□) amidase. The reaction rate without any addition of I_2 was defined as 100%.

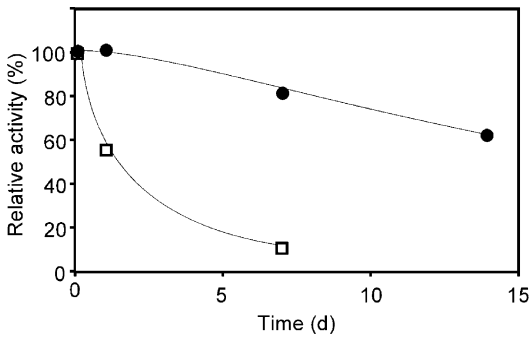


Fig. 8. The decrease of enzyme activities stored at 4 °C. (●) Nitrile hydratase; (□) amidase. The reaction rate of 0 day was defined as 100%.

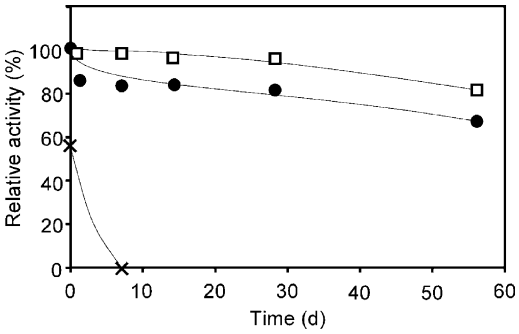


Fig. 9. Effect of storage temperature and solution on nitrile hydratase in which the whole cells were kept. (●) In 0.1 M phosphate buffer; (□) in deionized water (18.2 MΩ); (×) in the mixture of glycerol and 0.1 M phosphate buffer (4:1). The reaction rate of that immediately after the preparation in 0.1 M phosphate buffer was defined as 100%.

samples were stored at 4 °C or −20 °C and enzyme activities were measured after appropriate period of time (1, 7, 14, 28, 56 days). The reaction rate of the one immediately after the preparation in 0.1 M phosphate buffer [nitrile hydratase (45.4 mU/mg DCW), amidase (21.9 mU/mg DCW)] was defined as 100%. The results are summarized in Figs. 8–10.

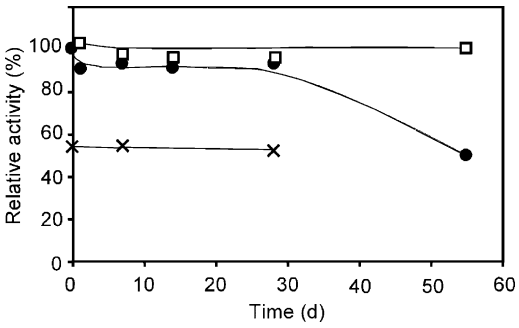
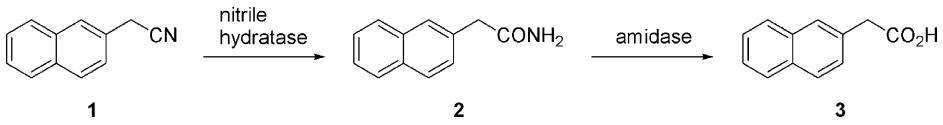


Fig. 10. Effect of storage temperature and solution on amidase in which the whole cells were kept. (●) In 0.1 M phosphate buffer; (□) in deionized water (18.2 MΩ); (×) in the mixture of glycerol and 0.1 M phosphate buffer (4:1). The reaction rate of that immediately after the preparation in 0.1 M phosphate buffer was defined as 100%.



Scheme 1. Action of nitrile hydratase and amidase in *R. rhodochrous* IFO 15564.

3. Results and discussion

3.1. Optimization of the amount of ε-caprolactam, the inducer

It has been believed that ε-caprolactam is the inducer for expression of both nitrile hydratase and amidase. Initially, to build up a firm basis of highly activated forms of both enzymes, optimization of the amount of ε-caprolactam at the stage of pre-cultivation of *R. rhodochrous* was attempted. We confirmed that ε-caprolactam is indispensable for the activity; however, there was no distinctive proportional correlation between the dose of ε-caprolactam ranged from 0 to 4 g/l and total enzyme activities. Moreover, an excessive amount (>2 g/l) showed inhibitory effect on the growth. In this way, 1 g/l of ε-caprolactam was concluded to be optimal, and *R. rhodochrous* was pre-incubated with this amount consistently throughout the studies.

3.2. Effect of co-solvents

A frequently faced, long-standing problem which prevents microbial catalysis from the introduction into synthetic organic chemistry is the low solubility of the substrates in aqueous solution. Taking this into account, a library of enzymatically benign, water-miscible co-solvents is of importance. Moreover, it would be possible to find the solvents which selectively and preferentially damage either of nitrile hydratase or amidase.

A preliminary screening suggested two candidates, DMF and DMSO. The concentration-dependent effect of DMF on enzyme activities in the conversion from 2-naphthylacetonitrile (**1**) to 2-naphthylacetamide (**2**) and 2-naphthylacetic acid (**3**, Scheme 1, Section 2.5), or from 2-naphthylacetamide (**2**) to 2-naphthylacetic acid (**3**) is summarized in Fig. 1. Addition of DMF to the final concentration of 3.8% (v/v) of DMF apparently enhance the activities of both enzymes; however, this phenomenon is only due to increasing solubility of the substrate. Amidase showed higher tolerance to DMF than nitrile hydratase. The activity of the latter decreased dramatically, and at more than 30% (v/v) concentration, only the activity of amidase was observed.

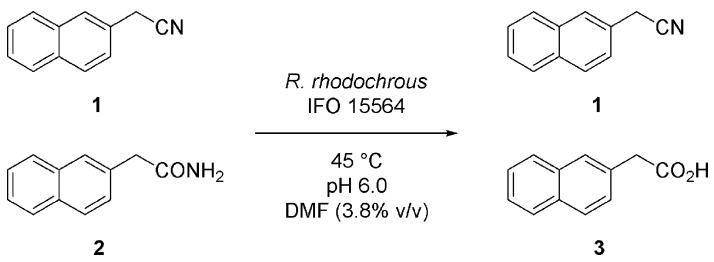
In contrast, as shown in Fig. 2 (Section 2.4), DMSO deactivated amidase rather than nitrile hydratase. In the range 20–30% (v/v) of DMSO, we can expect the preferential action of nitrile hydratase over amidase.

3.3. Effects of temperature and pH

As shown in Fig. 3, the optimal temperature for nitrile hydratase was 35 °C and that for amidase was 45 °C. Amidase is more stable at high temperature than nitrile hydratase, and at 45 °C, only the amidase works. In contrast to the heat tolerance, there was no clear tendency concerning the pH profile as shown in Fig. 4. Both enzymes were practically active in the pH range 6.0–9.0 and showed enhanced activities in the range 7.0–8.4.

*3.4. Preferential action of amidase: application to a mixture of 2-naphthylacetonitrile (**1**) and 2-naphthylacetamide (**2**)*

The above results suggested that the preferential action of amidase would be likely to proceed at high temperature, in the presence of other nitrile hydratase-susceptible compounds. So far, a very high activity of nitrile hydratase has been obtained in the presence of Fe ion during the pre-cultivation [47]. Even under those conditions (presence of Fe ion in pre-cultivation, Method A, Section 2.6.1), indeed, the selective conversion of a mixture of 2-naphthylacetonitrile (**1**) and 2-naphthylacetamide (**2**) was successful (Scheme 2). Under the conditions at 45 °C and 3.8% (v/v) addition of DMF, the nitrile hydratase was completely deactivated, and 99% of the nitrile (**1**) was recovered. On the other hand, amidase worked well to give the corresponding acid (**3**) in 99% yield.



Scheme 2. Preferential action of amidase of *R. rhodochrous* IFO 15564 on the mixture of a nitrile and an amide.

3.5. Inhibition of the activity of amidase

2-Furoamide (**4b**) serves as a precursor of 2-furylamine, which is a useful starting material of a variety of heterocyclic compounds [48] via Hofmann rearrangement. Fang and co-workers have proposed an excellent approach to **4b** from furfural (**4a**), available from a naturally abundant carbohydrate resource [49]. Their synthesis consists of successive two steps in one pot, which can avoid the loss of nitrile, the volatile intermediate through the isolation step: first, the treatment of an aldehyde with I_2 in aq. NH_3 to the corresponding nitrile (**4c**) [40]; second, the addition of 35% aq. H_2O_2 to an amide [50]. We expected that biocatalytic transformation, nitrile hydratase can be applied to the second step. The key to the success is the high activity of nitrile hydratase and the suppression of amidase, if possible.

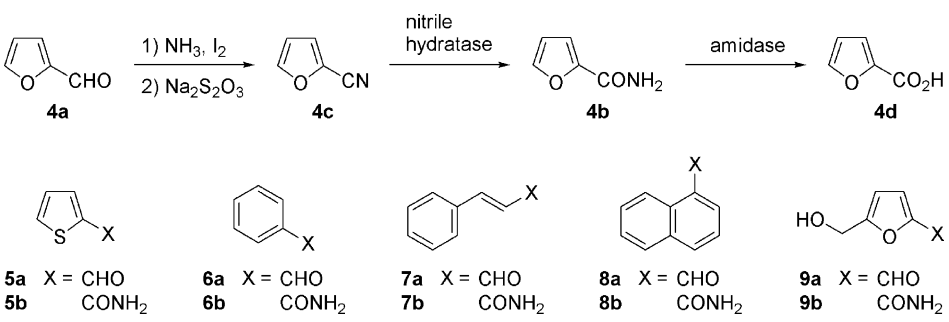
According to Fang's procedure, furfural was treated with aq. NH_3 and I_2. The use of DMSO as the co-solvent in our procedure instead of originally reported THF [40] is necessary to avoid the undesired hydrolysis of the amide to the acid. After the formation of 2-furonitrile (**4c**), confirmed by TLC analysis, the reaction mixture was neutralized to pH 8.0 and the excess I_2 was reduced with $Na_2S_2O_3{\cdot}5H_2O$, then the harvested cells of *R. rhodochrous* were added (Scheme 3). After working-up the reaction, we found that 2-furoamide (**4b**) was obtained in 90% yield, but there was absolutely no formation of carboxylic acid (**4d**). As the control experiment (Section 2.7.1), when 2-furonitrile (**4c**) was incubated under the conditions with DMSO (3.8%, v/v) as co-solvent at 30 °C and pH 8.0 for 1 h, the reaction proceeded in non-selective manner, to afford furoacid (**4d**) in 95% yield (Table 1, entry 1). DMSO has obvious no inhibitory effect on amidase at this concentration as shown in Section 3.2 (Fig. 2). This indicated that amidase was deactivated by some unknown factors involved in the above reaction conditions.

First, we suspected that large excess of the ammonium ion caused the product inhibition on amidase. To clear this problem, the enzyme activities of both nitrile hydratase and amidase were examined under a wide range of ammonium ion concentration. As shown in Fig. 5, the activities gradually declined, and ca. 45% of the initial activity of amidase still remained at the concentration of 75 mmol/WCW (= 2.4 M), which equals to that in the attempted one-pot synthetic reaction. These results do not account for the very selective inhibition on amidase.

At the stage of the formation of nitrile from aldehyde, from I_2 as an oxidant, iodide (I^-) is consequently generated. An examination of iodide at varied concentrations, however, showed no effect on either nitrile hydratase or amidase (Fig. 6), and indeed, the incubation of furonitrile (**4c**) in the presence of NaI resulted in the formation of furoic acid (**4d**, Table 1, entry 2, Section 2.7.2) in 97% yield. At the final stage of nitrile synthesis, $Na_2S_2O_3$ is introduced to reduce the excess amount (0.1 equiv) of I_2. An attempted experiment to incubate the nitrile (**4c**) with 1.00 mmol/WCW (= 40.0 mM) of $Na_2S_2O_3$ (Table 1, entry 3, Section 2.7.3), however, proved that this was not responsible for the deactivation, either.

A hint to solve this rather confusing situation was obtained from an accidental experiment. The so-far obtained results that either iodide (I^-) or thiosulfate ($S_2O_3^{2-}$) had no impact on the enzyme-catalyzed reaction misled us to omit the addition of $Na_2S_2O_3$ at the end of the transformation to nitrile and the reaction mixture was directly incubated with cells after adjustment to pH 8.0. Surprisingly, the subsequent biocatalytic reaction involving even the action of nitrile hydratase became very slow, and the yields of furoamide (**4b**) dropped to 43%. In turn, the quenching of a half-amount of the initially added I_2 brought about the restoration of the enzyme activities, and furoamide (**4b**) was obtained in 88% yield. To ascertain this result, the reaction of 2-furonitrile (**4c**) with 0.100 mmol/WCW (= 4.00 mM) of I_2 was performed and the reaction was found to be very slow. After 12 h, the yield of the amide was only 68% (Table 1, entry 4, Section 2.7.4). It took as long as 65 h for the consumption of nitrile (**4c**) (Table 1, entry 5).

At this stage, we became aware of the importance of the still existing trace amount of iodine (I_2 or I^+) in the mixture and examined the effect by varying its concentration. The results are shown in Fig. 7, and at the concentration between 0.25 and 0.50 mmol/WCW (= 7–14 mM), it is very clear that only the activity of amidase is considerably suppressed. The combined use of I_2 and the half-molar equivalent of $Na_2S_2O_3$ was effective to give amide in 98% yield



Scheme 3. Chemo-enzymatic one-pot two-step conversion of aldehydes to amides.

(Table 1, entry 6, Section 2.7.5). From the series of experiments, it was obvious that I_2 has some inhibitory effect on amidase; however, it is still difficult to explain the following independent result; there was no inhibition either on nitrile hydratase or amidase in the absence of I_2, by the quenching with 2.5 equiv of $Na_2S_2O_3$ (Table 1, entry 7, Section 2.7.6). This result is not consistent with the fact that furoamide was obtained in high yield in one-pot conversion.

Another possible factor responsible for the selective inhibition of amidase is due to some byproducts in the course of the formation of nitriles from aldehydes. Thus, we incubated furonitrile (**4c**) in aq. NH_3 solution for 30 min prior to the *R. rhodochrous* cell treatment (Table 1, entry 8, Section 2.7.7). As expected, the amidase activity was effectively suppressed, and the furoamide (**4b**) was obtained in 84% yield in 3 h. The formation of some inhibitors occurs certainly in the course of nitrile formation, as mixing of nitrile (**4c**) and NH_3, immediately followed by the pH adjustment to 8.0 and addition of the cells had no such effect (Table 1, entry 9, Section 2.7.8). Judging from the fact that a high concentration of NH_3 itself inhibits both enzymes (Fig. 5), the low yield of **4d** is understandable. The results suggest that under strongly basic conditions, adducts of NH_3 and the nitriles can be formed. It has been reported that some aromatic amidinium salts have inhibitory effects on serine protease activities [51–53]. In contrast, there was no effect on the co-incubation between furonitrile and I_2 (Table 1, entry 10, Section 2.7.9). These results concluded that the major reason for the selective formation of amide in one-pot synthesis is ascribable to the in situ generation of inhibitory substance from the intermediate, during the incubation in ammonia.

3.6. Application of one-pot synthesis of amides from aldehydes

The above-mentioned new one-pot synthesis of amide was satisfyingly reproducible on other aldehydes. Table 2 summarizes the results based on this protocol. Aromatic and heterocyclic amides (**4b**–**9b**) as shown in Scheme 3 were efficiently obtained in high yields. We confirmed that under the conventional incubation conditions, all the amides are good substrates for amidase and the in situ inhibition was very effective.

Table 2
The one-pot chemo-enzymatic conversion of aldehydes to amides

Entry	Substrate	Incubation time (h)	Yield (%)
1	**4a**	1	88
2	**5a**	24	86
3	**6a**	1	94
4	**7a**	5	78
5	**8a**	20	91
6	**9a**	5	89

For reaction conditions, see Section 2.6.1.

3.7. Activity of enzymes on the storage at low temperature

A ready-made biocatalyst reagent such as whole cells, which are kept and distributed in cold storage, is very important for synthetic organic chemistry, as contamination-free cultivation facilities of microorganisms are located far from those laboratories. In regard to the preservation, we need to pay attention to two important points: the high activity of enzymes and long-term stability. First, to examine the stability of enzymes in a refrigerator, the re-suspended harvested cells were kept in a 0.1 M phosphate buffer solution, and the enzyme activities were monitored occasionally (Fig. 8).

In contrast to the high heat tolerance, the decrease of the amidase activity (30% of the original, after 1 week) was greater than that of nitrile hydratase (60% of the original, after 2 weeks). Freezing temperature to as low as −20 °C was effective to maintain the enzyme activity of both nitrile hydratase (Fig. 9) and amidase (Fig. 10); even after 2 weeks, 90% of the original activities was preserved. Moreover, prior to freezing, removal of the contaminant inorganic salt remaining in the buffer solution in the harvested cells by repeatedly washing with deionized water further enhanced the preserved activity to as high as 97% of the freshly harvested cells. The addition of an anti-freezer such as glycerol (80%) had a deleterious effect, and the nitrile hydratase activity was lost within 1 week even under −20 °C. Establishment of conditions for the storage of whole cells would promote the availability of this biocatalyst in synthetic organic chemistry.

4. Conclusion

Some fundamental, but crucial aspects of the nitrile hydratase and the amidase activity in whole-cell use of *R. rhodochrous* IFO 15564 were clarified. The conditions for long-term storage were also established. By varying the reaction temperature and co-solvent (45 °C, DMF), amidase could preferentially work to leave the nitrile functionality completely intact. In contrast, a newly developed selective in situ formed inhibitor toward the amidase enabled the overwhelming action of nitrile hydratase, and a chemoenzymatic one-pot two-step synthesis of amides from aldehydes in high yields.

Acknowledgements

The authors thank Professor Hiromichi Ohta, Drs. Kenji Miyamoto and Yoichi Suzuki of Keio University, for their interest. Part of this work was accomplished as the collaborated program of “CREST: Creation of Functions of New Molecules and Molecular Assemblies” of Japan Science and Technology Corporation, and we express our sincere thanks to Professors K. Suzuki and T. Matsumoto for their discussions. This work was also supported both by a Grant-in-Aid

for Scientific Research (No. 14560084) and the 21st Century COE Program (KEIO LCC) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and acknowledged with thanks.

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