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Biocatalytic asymmetric hydrolysis of (±)-β-hydroxy nitriles by *Rhodococcus* sp. CGMCC 0497

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Abstract

The asymmetric hydrolysis of several 3-hydroxy-4-aryloxybutanenitriles using *Rhodococcus* sp. CGMCC 0497 was studied. It was revealed that the reaction temperature of 20 °C was more effective than that conventional incubation temperature such as 30 °C. The products, (*R*)-amides and (*S*)-acids, were obtained in enantiomeric excesses of up to 87%.
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Keywords: Nitrile hydratase; Amidase; β-Hydroxy nitriles; *Rhodococcus*; Kinetic resolution

1. Introduction

It has been known for several decades that nitriles can be metabolized by a diverse range of microorganisms to amides and acids [1,2]. Furthermore, organonitriles can be readily prepared by a number of methods and are important intermediates in organic synthesis [3–5]. Nonetheless, the substrates studied for nitrile-converting enzymes are still very limited [6–10]. As for kinetic resolution, there have been papers focusing on the enantioselective hydrolysis of α-alkyl nitriles, α-hydroxy nitriles, α-acyloxy nitriles and α-amino nitriles [11–17], but as far as we know, few examples have been reported so far on the kinetic resolution of β-substituted nitriles [18] though there have been several reports on the enzymatic conversion of prochiral 3-hydroxyglutaronitrile derivatives [19,20]. As for the extension of microbial nitrile-converting reaction, we would like to report our results on some β-hydroxy nitriles.

Enzyme-catalyzed hydrolysis of the β-hydroxy substituted nitriles results in chiral β-hydroxy acids and β-hydroxy amides, which are precursors of β-blockers and 1,3-amino alcohols, intermediates for a large number of natural products, antibiotics and chiral auxiliaries [21–23]. For example, Banfi et al. reported the utility of monoprotected β,γ-dihydroxyesters in the synthesis of pharmacologically important β-lactam antibiotics [24].

We have reported the enantioselective hydrolysis of various racemic α-substituted arylacetonitriles using *Rhodococcus* sp. CGMCC 0497, a strain screened from soil by our group [25,26]. Excellent enantiomeric excesses were achieved in most cases. In this paper, the asymmetric hydrolysis of several 3-hydroxy-4-aryloxybutanenitriles was examined at 20 °C rather than conventional incubation temperature such as 30 °C. The products, (*R*)-amides and (*S*)-acids, were obtained in enantiomeric excesses of up to 87%.

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2. Experimental

2.1. Materials and methods

The commercially available reagents were used without further purification. Melting points were determined on a Mettler FP62 and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AMX-300 (300 MHz) spectrometer at room temperature with TMS as internal standard. Chemical shifts in ppm were positive for upfield shifts. IR spectra were recorded neat or in KBr and measured in cm^{-1}, using a Shimadzu IR-440 IR spectrophotometer. EI-MS spectra were recorded on an HP 5989A. High resolution mass spectra were obtained on a Finnigan MAT8430. Microanalyses were carried out on an Italian Carlo-Erba 1106. Polarimetry was carried out using an optical activity Perkin-Elmer 241 ML polarimeter and the measurements were made at the sodium D-line with a 10 cm pathlength cell. Concentrations (*c*) are given in g/100 ml. Enantiomeric excesses: chiral HPLC was conducted with a PE NELSON NCI900 using Chiralcel OD or Chiralpak AS, AD column at a flow rate of 0.7 ml/min with 2-propanol/hexane as the mobile phase. The A254 was detected by a Waters 2487 dual λ absorbance detector.

Racemic nitriles were prepared following the literature methods [27,28].

2.2. Microorganism and cultivation

The strain *Rodococcus* sp. CGMCC 0497 is available in CGMCC (China General Microbiological Culture Colletion Center). *Rodococcus* sp. CGMCC 0497 was subcultured at 30 °C for 24 h in a 100 ml shaking flask containing 20 ml of a medium consisting of 0.5 g of polypepton, 0.5 g of beef extract and 1 g of glucose per 100 ml of tap water, pH 7.0. Then the subculture was inoculated into a 5 l shaking flask containing 1 l of the rich medium consisting of 1 g of glucose, 0.5 g of beef extract, 0.25 g of methacrylamide, 100 mg of $K_2HPO_4{\cdot}3H_2O$, 75 mg of KH_2PO_4, 10 mg of NaCl, 0.1 ml of mineral medium per100 ml of tap water with methacrylamide added 24 h later. The pH of each medium was adjusted to around 7.0–7.2 by addition of 2 N NaOH or 3 N HCl. After incubation at 30 °C with reciprocal shaking for 48 h. The organism was harvested by centrifugation using an HIMAC centrifuge CR20B2 (Hitachi, Japan) with a RPR9-2 rotor (6800 g, 30 min, 10 °C). Cells were washed with 100 mM potassium phosphate buffer (pH = 7.0) and centrifugated.

2.3. General procedure with whole cells

A suspension of 5 g washed wet cells and 40 ml 0.1 mM potassium phosphate buffer (pH = 7.0) was incubated at 20 °C for 30 min with continuously magnetic stirring before the addition of the substrate, a solution of 100 mg 3-hydroxy-4-aryloxybutanenitriles dissolved in 200 μl acetone (for substrate **8a**, DMSO was used instead). The reaction, monitored by thin layer chromatography, was quenched after a period of time by centrifugation using an HIMAC centrifuge CR20B2 (Hitachi, Japan) with a RPR20-4-154 rotor (7800 g, 30 min, 20 °C). The resulting supernatant was basified with 2 N NaOH to pH = 12, and extracted with ethyl acetate. The organic solutions, after drying ($MgSO_4$) and concentration, gave the amide and unreacted nitrile. Separation of amide and nitrile was effected by column chromatography. The aqueous solution was then acidified using 3 N HCl to pH = 2 and extracted with ethyl acetate. Acid was obtained after removal of the solvent under reduced pressure.

Amides series **b** and acids series **c** in methanol were treated with catalytic concentrated sulfuric acid under gentle reflux (70 °C) for 3 h to yield the corresponding methyl esters and the esters were subjected to chiral HPLC. The acid **1c** was converted to its methyl ester using diazomethane [29] instead and the e.e. value of the ester proved the same as that achieved from the above-mentioned method.

2.3.1. *(S)-(−)-3-hydroxy-4-phenoxybutanenitrile (−)-**1a***

White solid, mp 53–54 °C [30]: 51–52 °C; e.e., 29%, $[\alpha]_D^{25}$ −1.93 (*c* 0.89, $CHCl_3$) [30]: 82.7% e.e., $[\alpha]_D$ −6.4 (*c* 1.0, $CHCl_3$), *S*; IR (KBr): 3400 (OH), 2274 (CN), 750, 696 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$): δ 7.35–7.27 (m, 2H, ArH), 7.04–6.99 (m, 1H, ArH), 6.94–6.91 (m, 2H, ArH), 4.37–4.31 (m, 1H, CH), 4.06 (d, 2H, J = 5.5 Hz, CH_2), 2.87 (s, 1H, OH), 2.83–2.86 (m, 2H, CH_2); MS *m/z*: 178 (M^+ + 1, 17%), 177 (M^+, 82), 160 (M^+ − OH, 7), 94 (100).

*2.3.2. (R)-(+)-3-hydroxy-4-phenoxybutanamide **1b***

White solid, mp 111–112 °C; 49% e.e., $[\alpha]_D^{23}$ +7.71 (*c* 0.58, EtOH); 76% e.e., $[\alpha]_D^{25}$ +11.71 (*c* 0.52, EtOH); IR (KBr): 3439 (br, OH), 3364 and 3204 (NH), 1673 (CO), 748, 694 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 7.37–7.26 (m, 2H, ArH), 6.96–6.86 (m, 3H, ArH), 4.22–4.17 (m, 1H, CH), 3.92–3.88 (m, 2H, CH_2), 3.37 (br s, 2H, NH_2), 2.40–2.24 (m, 2H, CH_2); MS *m/z*: 195 (M^+, 1%), 177 ($M^+ - H_2O$, 14), 133 (177-$CONH_2$, 20), 102 (100). Anal. calcd for $C_{10}H_{13}NO_3$: C, 61.5; H, 6.7; N, 7.2. Found: C, 61.2; H, 6.8; N, 7.1%.

*2.3.3. (S)-(−)-3-hydroxy-4-phenoxybutanoic acid **1c***

Colorless oil; 74% e.e., $[\alpha]_D^{23}$ −9.55 (*c* 1.00, $CHCl_3$); 59% e.e., $[\alpha]_D^{25}$ −7.54 (*c* 1.11, $CHCl_3$); IR (film) 3472 (br OH), 2780–3250 (br, OH), 1716 (CO), 754, 689 cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$): δ 7.32–7.25 (m, 3H, ArH), 7.0–6.9 (m, 2H, ArH), 4.49–4.41 (m, 1H, CH), 4.05–3.98 (m, 2H, CH_2), 3.5 (br s, 1H, OH), 2.80–2.73 (m, 2H, CH_2); MS *m/z*: 197 ($M^+ + 1$, 11%), 196 (M^+, 48), 179 (M^+ − OH, 17), 161 (18.4), 94 (100).

*2.3.4. (R)-(−)-3-hydroxy-4-(2-methoxyphenoxy)-butanamide **2b***

White solid, mp 140–141 °C; 23% e.e., $[\alpha]_D^{19}$ −0.70 (*c* 1.33, CH_3OH); 64% e.e., $[\alpha]_D^{19}$ −1.89 (*c* 0.91, CH_3OH); IR (KBr): 3388 (br, OH, NH), 3193, 1650 (CO), 773, 739 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 7.00–6.85 (m, 4H, ArH), 4.23–4.17 (m, 1H, CH), 3.92–3.83 (m, 2H, CH_2), 3.78 (s, 3H, OCH_3), 3.37 (s, 2H, NH_2), 2.37 (dd, 1H, J_1 = 15.3 Hz, J_2 = 4.8 Hz, CH), 2.28 (dd, 1H, J_1 = 15.3 Hz, J_2 = 8.7 Hz, CH); MS *m/z*: 225 (M^+, 1%), 207 ($M^+ - H_2O$, 1), 124 (16.2), 109 (26.3), 102 (100). Anal. cacld for $C_{11}H_{15}NO_4$: C, 58.66; H, 6.71; N, 6.22. Found: C, 58.60; H, 6.70; N, 6.04%.

*2.3.5. (S)-(+)-3-hydroxy-4-(2-methoxyphenoxy)-butanoic acid **2c***

White solid, mp 88–89 °C; 64% e.e., $[\alpha]_D^{18}$ +2.60 (*c* 1.96, $CHCl_3$); 46% e.e., $[\alpha]_D^{22}$ +1.93 (*c* 1.61, $CHCl_3$); IR (KBr): 3554 (br, OH), 3245 (br, OH), 1752 (CO), 1255, 1124, 765, 747 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 7.03–6.87 (m, 4H, ArH), 4.44–4.36 (m, 1H, CH), 4.02 (d, 2H, J = 4.8 Hz, CH_2), 3.84 (s, 3H, OCH_3), 3.60 (br, s, 2H, 2OH), 2.73 (dd, 1H, J_1 = 15.9 Hz, J_2 = 4.5 Hz, CH), 2.56 (dd, 1H, J_1 = 15.9 Hz, J_2 = 8.1 Hz, CH); MS *m/z*: 226 (M^+, 7%), 209 (M^+ − OH, 2), 157 (6.7), 124 (100), 109 (53). Anal. calcd for $C_{11}H_{14}O_5$: C, 58.40; H, 6.24. Found: C, 58.18; H, 6.10%.

*2.3.6. (R)-(+)-3-hydroxy-4-(4-methoxyphenoxy)-butanamide **3b***

White solid, mp 140–141 °C; 76% e.e., $[\alpha]_D^{24}$ +4.20 (*c* 0.58, CH_3OH); 29% e.e., $[\alpha]_D^{22}$ +1.77 (*c* 1.17, CH_3OH); IR (KBr): 3433 (br, OH), 3364, 3213 (NH), 1670 (CO), 1240, 1039, 827, 748 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 6.86 (s, 4H, ArH), 4.18–4.12 (m, 1H, CH), 3.81 (d, 2H, J = 5.7 Hz, CH_2), 3.69 (s, 3H, OCH_3), 3.37 (s, 2H, NH_2), 2.35–2.21 (m, 2H, CH_2); MS *m/z*: 226 ($M^+ + 1$, 1%), 225 (M^+, 1), 207 ($M^+ - H_2O$, 1), 124 (22.3), 123 (18.2), 109 (30.8), 102 (100). Anal. calcd for $C_{11}H_{15}NO_4$: C, 58.66; H, 6.71; N, 6.22. Found: C, 58.69; H, 6.83; N, 6.11%.

*2.3.7. (S)-(−)-3-hydroxy-4-(4-methoxyphenoxy)-butanoic acid **3c***

White solid, mp 85–86 °C [31]: 81–82 °C; 34% e.e., $[\alpha]_D^{18}$ −3.21 (*c* 1.36, $CHCl_3$); 40% e.e., $[\alpha]_D^{20}$ −3.70 (*c* 0.94, $CHCl_3$) [31]: 99% e.e., $[\alpha]_D^{20}$ −9.48 (*c* 0.80, $CHCl_3$), *S*; IR (KBr): 3424 (br, OH), 3250–2800 (br, OH), 1698 (CO), 1239, 1041, 828, 748 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 6.90, 6.84 (AB, 4H, J = 9.3 Hz, ArH), 4.41–4.31 (m, 1H, CH), 3.95 (d, 2H, J = 5.1 Hz, CH_2), 3.73 (s, 3H, OCH_3), 2.70 (dd, 1H, J_1 = 16.5 Hz, J_2 = 4.5 Hz, CH), 2.53 (dd, 1H, J_1 = 16.5 Hz, J_2 = 8.1 Hz, CH); MS *m/z*: 226 (M^+, 11%), 137 (5), 124 (100), 109 (53).

*2.3.8. (R)-(+)-3-hydroxy-4-(4-bromophenoxy)-butanamide **4b***

White solid, mp 155–156 °C; 65% e.e., $[\alpha]_D^{22}$ +3.96 (*c* 0.66, CH_3OH); 22% e.e., $[\alpha]_D^{22}$ +1.38 (*c* 1.56, CH_3OH); IR (KBr): 3352 (br, OH), 3200 (br, NH), 1684 (CO), 1249, 1047, 820, 698 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 7.38, 6.86 (AB, 4H, J = 9.0 Hz, ArH), 4.20–4.05 (m, 1H, CH), 3.85–3.80 (m, 2H, CH_2), 3.30 (s, 2H, NH_2), 2.35–2.15 (m, 2H, CH_2); MS *m/z*: 187 (5%), 185 (M^+ − $CH(OH)CH_2CONH_2$, 6), 174 (21), 172 ($[BrC_6H_4OH]^+$, 22.3), 157 (14.3), 155 (13.2), 102 (100). Anal. calcd for $C_{10}H_{12}NO_3Br$: C, 43.82; H, 4.41; N, 5.11; Br,

29.15. Found: C, 44.03; H, 4.69; N, 4.99; Br, 29.30%.

*2.3.9. (S)-(−)-3-hydroxy-4-(4-bromophenoxy)-butanoic acid **4c***

White solid, mp 133–134 °C; 26% e.e., $[\alpha]_D^{22}$ −4.02 (*c* 1.13, $CHCl_3$); IR (KBr): 3383 (br, OH), 3200–2780 (br OH), 1702 (CO), 1246, 1034, 835, 654 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 10.7(br s, 1H, COOH), 7.43, 6.93 (AB, 4H, J = 9.0 Hz, ArH), 4.41 (s, 1H, CH), 4.02 (d, 2H, J = 5.1 Hz, CH_2), 2.90 (br s, 1H, OH), 2.70 (dd, 1H, J_1 = 15.9 Hz, J_2 = 4.8 Hz, CH), 2.54 (dd, 1H, J_1 = 15.9 Hz, J_2 = 7.5 Hz, CH); MS *m/z*: 276 (M^+ + 2, 11%), 274 (M^+, 12), 256 (M^+ − H_2O, 1), 187 (6), 185 (6), 174 (97), 172 (100); Anal. calcd for $C_{10}H_{11}O_4Br$: C, 43.66; H, 4.03; Br, 29.05. Found: C, 43.66; H, 4.23; Br,28.89%.

*2.3.10. (R)-(−)-3-hydroxy-4-(4-nitrophenoxy)-butanamide **5b***

Pale yellow solid, mp 163–164 °C; 37% e.e., $[\alpha]_D^{22}$ −7.15 (*c* 0.35, CH_3OH); 87% e.e., $[\alpha]_D^{22}$ −16.02 (*c* 0.27, CH_3OH); IR (KBr): 3300 (br, OH), 3183 (br, NH), 1685 (CO), 1510, 1341, 844, 751 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 8.19, 7.14 (AB, 4H, J = 9.3 Hz, ArH), 4.23–4.15 (m, 1H, CH), 4.08 (dd, 1H, J_1 = 9.9 Hz, J_2 = 3.9 Hz, CH), 4.01 (dd, 1H, J_1 = 9.6 Hz, J_2 = 6.3 Hz, CH), 3.33 (s, 2H, NH_2), 2.34–2.28 (m, 2H, CH_2); MS *m/z*: 241 (M^+ + 1, 2%), 222 (M^+ − H_2O, 4), 152 (M^+ − $CH(OH)CH_2CONH_2$, 9), 102 (100). Anal. calcd for $C_{10}H_{12}N_2O_5$: C, 50.00; H, 5.04; N, 11.65. Found: C, 49.99; H, 5.08; N, 11.74%.

*2.3.11. (S)-(+)-3-hydroxy-4-(4-nitrophenoxy)-butanoic acid **5c***

Pale yellow solid, mp 127.5–128.5 °C; 35% e.e., $[\alpha]_D^{25}$ +2.41 (*c* 1.55, MeOH); IR (KBr): 3485 (br, OH), 3200–2800 (br, OH), 1710 (CO), 1513, 1346, 848, 753 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 8.20, 7.17 (AB, 4H, J = 9.3 Hz, ArH), 4.44–4.38 (m, 1H, CH), 4.21–4.14 (m, 2H, CH_2), 3.55 (br s, 2H, 2OH), 2.68 (dd, 1H, J_1 = 15.9 Hz, J_2 = 5.1 Hz, CH), 2.57 (dd, 1H, J_1 = 15.9 Hz, J_2 = 7.8 Hz, CH); MS *m/z*: 241 (M^+, 9%), 223 (M^+ − H_2O, 13), 178 (7), 123 (30), 109 (26), 103 (91), 43 (100). Anal. calcd for $C_{10}H_{11}NO_6$: C, 49.80; H, 4.60; N, 5.81. Found: C, 49.79; H, 4.62; N, 5.72%.

*2.3.12. (R)-(+)-3-hydroxy-4-(4-tert-butylphenoxy)-butanamide **6b***

White solid, mp 85.2–86.2 °C; 49% e.e., $[\alpha]_D^{20}$ +3.62 (*c* 0.90, CH_3OH); 46% e.e., $[\alpha]_D^{12}$ +3.56 (*c* 0.78, CH_3OH); IR (KBr): 3418 (br, OH, NH), 1663 (CO), 1247, 1047, 835 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 7.28, 6.85 (AB, 4H, J = 8.7 Hz, ArH), 4.20–4.12 (m, 1H, CH), 3.84 (d, 2H, J = 5.1 Hz, CH_2), 3.37 (s, 2H, NH_2), 2.36–2.21 (m, 2H, CH_2), 1.25 (s, 9H, $3CH_3$); MS *m/z*: 233 (M^+ − H_2O, 3%), 218 (8), 177 (7), 135 (26), 107 (12), 102 (100); HRMS(EI) calcd for $C_{14}H_{21}NO_3$: 251.15214. Found: 251.15486.

*2.3.13. (S)-(−)-3-hydroxy-4-(4-tert-butylphenoxy)-butanoic acid **6c***

White solid, mp 95–96 °C; 11% e.e., $[\alpha]_D^{11}$ −1.69 (*c* 0.82, $CHCl_3$); IR (KBr): 3400 (br, OH), 1722 (CO), 1184, 1114, 1044, 830, 815 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 7.33, 6.89 (AB, 4H, J = 8.9 Hz, ArH), 4.40–4.36 (m, 1H, CH), 3.99 (d, 2H, J = 5.4 Hz, CH_2), 3.48 (br s, 2H, 2OH), 2.69 (dd, 1H, J_1 = 15.6 Hz, J_2 = 4.8 Hz, CH), 2.54 (dd, 1H, J_1 = 15.6 Hz, J_2 = 8.0 Hz, CH), 1.29 (s, 9H, $3CH_3$); MS *m/z*: 252 (M^+, 6%), 237 (7), 234 (8), 219 (51), 177 (13) and 135 (100). Anal. calcd for $C_{14}H_{20}O_4$: C, 66.65; H, 7.99. Found: C, 66.59; H, 7.86%.

*2.3.14. (R)-(+)-3-hydroxy-4-(1-naphthyloxy)-butanamide **7b***

White solid; mp 144–145 °C; 12% e.e., $[\alpha]_D^{22}$ +2.97 (*c* 0.82, MeOH); 35% e.e., $[\alpha]_D^{22}$ +8.99 (*c* 0.71, MeOH); IR (KBr): 3441 (br, OH), 3300 (NH), 3192 (NH), 1677 (CO), 800, 775 cm^{-1}; ^1H NMR (300 MHz; DMSO): δ 8.30–8.27 (m, 1H, ArH), 7.88–7.85 (m, 1H, ArH), 7.56–7.38 (m, 4H, ArH), 6.95 (d, 1H, J = 7.5 Hz, ArH), 4.30–4.41 (m, 1H, CH), 4.08 (d, 2H, J = 5.4 Hz, CH_2), 3.38 (br s, 3H, NH_2, OH), 2.47–2.38 (m, 2H, CH_2); MS *m/z*: 245 (M^+, 2%), 227 (M^+ − H_2O, 1), 144 (26), 127 (17), 115 (39), 102 (100); HRMS(EI) cacld for $C_{14}H_{15}NO_3$: 245.1051942. Found: 245.10134.

*2.3.15. (S)-(−)-3-hydroxy-4-(1-naphthyloxy)-butanoic acid **7c***

White solid, mp 100–101 °C [31]: 69 °C; 12% e.e., $[\alpha]_D^{22}$ −3.12 (*c* 1.47, $CHCl_3$); 4% e.e., $[\alpha]_D^{22}$ −1.32 (*c* 2.02, $CHCl_3$) [31]: 98% e.e., $[\alpha]_D^{20}$ −15.76

(*c* 1.225 in $CHCl_3$), *S*; IR (KBr): 3491 (br, OH), 2780–3250 (br, OH), 1711 (CO), 1274, 1074, 873, 796, 770 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$) δ 8.37–8.34 (m, 1H, ArH), 7.88–7.83 (m, 1H, ArH), 7.56–7.38 (m, 4H, ArH), 6.98 (d, 1H, J = 7.5 Hz, ArH), 4.64–4.51 (m, 1H, CH), 4.25–4.18 (m, 2H, CH_2), 3.64 (br s, 2H, 2OH), 2.85 (dd, 1H, J_1 = 15.9 Hz, J_2 = 4.8 Hz, CH), 2.71 (dd, 1H, J_1 = 15.9 Hz, J_2 = 8.4 Hz, CH); MS *m/z*: 246 (M^+, 12%), 157 (6), 144 (100).

2.3.16. 3-hydroxy-4-(2-naphthyloxy)-butanenitrile ***8a***

White solid, mp 144–145 °C [30]: 134–138 °C; IR (KBr): 3401 (br, OH), 2274 (CN), 1258, 1185, 919, 841, 742 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 7.86–7.82 (m, 3H, ArH), 7.50–7.45 (m, 1H, ArH), 7.40–7.24 (m, 2H, ArH), 7.22 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.4 Hz, ArH), 4.43–4.39 (m, 1H, CH), 4.24 (dd, 1H, J_1 = 9.8 Hz, J_2 = 5.3 Hz, CH), 4.17 (dd, 1H, J_1 = 9.8 Hz, J_2 = 5.9 Hz, CH), 3.24 (br s, 1H, OH), 2.94 (dd, 1H, J_1 = 17.3 Hz, J_2 = 4.7 Hz, CH), 2.84 (dd, 1H, J_1 = 17.3 Hz, J_2 = 6.8 Hz, CH); MS *m/z*: 227 (M^+, 62%), 209 ($M^+ - H_2O$, 12), 157(10), 145 (19), 144 (100).

2.3.17. (S)-(+)-3-hydroxy-4-(2-naphthyloxy)-butanoic acid ***8c***

White solid, mp 133.6–134.6 °C; 41% e.e., $[\alpha]_D^{16}$ +5.53 (*c* 0.48, $CHCl_3$); 53% e.e., $[\alpha]_D^{15}$ +7.02 (*c* 0.69, $CHCl_3$); IR (KBr): 3422 (br, OH), 2800–3250 (br, OH), 1706 (CO), 1256, 1184, 841, 811, 742 cm^{-1}; ^1H NMR (300 MHz; $(CD_3)_2CO$): δ 10.70 (br s, 1H, OH), 7.83–7.79 (m, 3H, ArH), 7.47–7.42 (m, 1H, ArH), 7.37–7.31 (m, 2H, ArH), 7.21–7.17 (m, 1H, ArH), 4.52–4.45 (m, 1H, CH), 4.16 (d, 2H, J = 5.4 Hz, CH_2), 2.90 (br s, 1H, OH), 2.76 (dd, 1H, J_1 = 15.9 Hz, J_2 = 4.5 Hz, CH), 2.61 (dd, 1H, J_1 = 15.9 Hz, J_2 = 7.8 Hz, CH); MS *m/z*: 246 (M^+, 15%), 202 ($M^+ + 1 - COOH$, 21), 144 (100). Anal. calcd for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.01; H, 5.96%.

3. Results and discussion

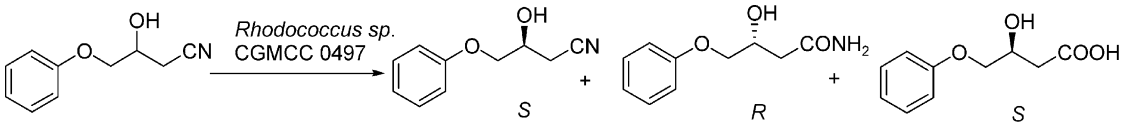
In order to gain reproducible results, the fresh microorganism cultivated on the optimal condition was used. The enantiomeric excesses of amides **b** and acids **c** were determined by means of chiral HPLC after conversion to the corresponding methyl esters. The enantiomeric excess of recovered nitriles **1a** and **8a** were determined directly by chiral HPLC. The absolute configurations of **3c**, **7c** [31] and the recovered **1a** [30] were obtained by comparing the direction of specific rotations with those in literature, and that of **1b** and **1c** were assigned by comparing the retention time of the corresponding methyl ester with the samples derived from **1a** using HPLC with chiral stationary phase. The absolute configurations of other compounds were assumed by the analogy with known enzyme-catalyzed reaction.

3.1. Hydrolysis of 3-hydroxy-4-phenoxybutanenitrile, the importance of temperature

In the study of hydrolyzing α-substituted arylacetonitriles using *Rhodococcus* sp. CGMCC 0497, the reactions were carried out at 30 °C. This condition was usually used in reactions catalyzed by nitrile-converting enzymes. But when 3-hydroxy-4-phenoxybutanenitrile was hydrolyzed in 30 °C (Scheme 1), the conversion was incomplete within 1.5 h and the e.e. value of the recovered substrate was low. So we carried out the reaction at 20 °C instead and found the decrease of reaction temperature resulted to complete conversion of the substrate within 1 h (Table 1).

The fact that temperature plays an important role in this reaction was not so surprising. Though seldom discussed in chiral molecular synthesis catalyzed by nitrile-converting-enzymes [32], this phenomenon has been mentioned in the production of acylamide. The first description on the low reaction temperature of NHase was reported by Asano et al. [33]. They deduced that the active site of NHase was possibly masked by the special substrate acrylonitrile at a higher temperature. The industrial production of acryamide using NHase is performed at an especially low temperature (2–4 °C) [34,35], which, as Kobayashi et al. explained, reduces the amidase activity and exerts little effect on the NHase activity [35] and thereby avoids the formation of by-product acrylic acid.

As it is well known that biotransformation of nitrile compounds to the corresponding acids and ammonia proceeds by two distinct routes: by nitrilase or by a combination of nitrile hydratase and amidase through



Scheme 1. Asymmetric hydrolysis of 3-hydroxy-4-phenoxybutanenitrile using *Rhodococcus* sp. CGMCC 0497.

Table 1
Biocatalytic asymmetric hydrolysis of 3-hydroxy-4-phenoxybutanenitrile by *Rhodococcus* sp. CGMCC 0497 at different temperature

Entry	Temperature (°C)	Time (h)	(*S*)-**1a** yield (%)	(*R*)-**1b** e.e. (%)	(*S*)-**1c** yield (%)	e.e. (%)	Yield (%)	e.e. (%)
1	30	1.5	18	29	44	59	30	76
2	20	1	–	–	66	49	18	74
3	20	3.5	–	–	40	76	51	59

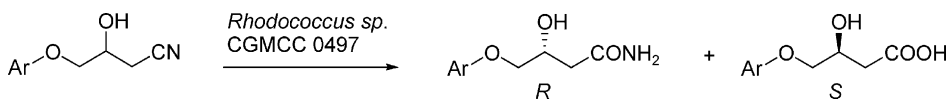
an intermediate amide [36], *Rhodococcus* sp. CGMCC 0497 appears to act mainly by the later one. At 30 °C, amidase activity was not inhibited and the accumulation of acid may do some unfavorable effect to NHase activity, thus resulted in the incomplete conversion of nitrile, while at 20 °C, amidase activity was reduced and the decreased acid production accelerated nitrile conversion. In fact, this phenomenon can also be observed in the hydrolysis of α,α-disubstituted malononitriles catalyzed by *Rhodococcus* sp. CGMCC 0497 [37]. Further rationalization of this phenomenon is currently in progress.

3.2. Hydrolysis of various 3-hydroxy-4-aryloxybutanenitriles

To examine the efficacy of this biocatalytic process with regards to substrate structure, seven other β-hydroxy substituted nitriles were tested at 20 °C (Scheme 2). As shown in Table 2, except substrate **8a**, the hydrolysis of all nitrile compounds proceeded smoothly and completely, and (*R*)-amides and (*S*)-acids were obtained within less than 6 h.

It seemed that **1a** without any other substitutions on the benzene ring gave the best enantioselectivity. Any substitution lead to the decrease of selectivity, but steric effects from the aromatic ring exerted little influence on this reaction because of the relatively long distance between the cyano group and the benzene ring. Hydrolysis of *ortho*-substituted **2a** proceeded with approximately the same enantioselectivity as the corresponding *para*-substituted substrate **3a** except that the reaction time was a little longer (Table 2, entries 1–4). With a bulky substituted group on the *para*-position, **6a** was still hydrolyzed smoothly and the yield of acid **6c** reached 63% within 4 h (Table 2, entries 7 and 8). Electron-withdrawing group substituted **4a** and **5a** were also hydrolyzed at a similar rate (Table 2, entries 5 and 6), but with enantioselectivity lower than **2a** and **3a** did.

Hydrolyzing substrates **7a** and **8a** with naphthene nucleus (Table 2, entries 9–12). The nitrile **8a** could not be converted completely within 8 h and prolonged reaction time hardly had any effect. It is surprising for there seemed no significant steric or electronic effects on this molecule. Probably other factors such as polarity or solubility played a role in this process because 100 mg of **8a** could not dissolve completely in 0.2 ml acetone like any other substrates tested and DMSO was used instead as a co-solvent.



Scheme 2. Asymmetric hydrolysis of 3-hydroxy-4-aryloxybutanenitriles using *Rhodococcus* sp. CGMCC 0497.

Table 2
Biocatalytic asymmetric hydrolysis of various 3-hydroxy-4-aryoxybutanenitriles by *Rhodococcus* sp. CGMCC 0497

Entry	Substrate **a**	Ar	Time (h)	Amide **b**		Acid **c**	
				Yield (%)	e.e. (%)	Yield (%)	e.e. (%)
1	**2**	*o*-OMe C_6H_4	2	64	23	26	64
2	**2**	*o*-OMe C_6H_4	5	35	64	52	46
3	**3**	*p*-OMe C_6H_4	1	57	29	35	40
4	**3**	*p*-OMe C_6H_4	3	24	76	69	34
5	**4**	*p*-Br C_6H_4	1	58	22	39	26
6	**4**	*p*-Br C_6H_4	3	9	65	82	5
7	**5**	*p*-NO_2 C_6H_4	1.5	43	37	53	35
8	**5**	*p*-NO_2 C_6H_4	6	12	87	81	2
9	**6**	*t*-Bu C_6H_4	4	29	46	63	11
10	**6**	*t*-Bu C_6H_4	5.5	21	49	69	3
11	**7**	1- $C_{10}H_7$	1	46	12	46	12
12	**7**	1-$C_{10}H_7$	3	20	35	71	4
13[a]	**8**	2-$C_{10}H_7$	4	–	–	8	53
14[b]	**8**	2-$C_{10}H_7$	8	–	–	11	41

[a] Substrate recovered with a yield of 80% and e.e. of <1%.
[b] Substrate recovered with a yield of 75% and e.e. of <1%.

3.3. Hydrolysis of O-substituted 3-hydroxy-4-phenoxybutanenitrile

To improve the resolution effects, 3-benzoyloxy-4-phenoxybutanenitrile **9** and 3-benzyloxy-4-phenoxybutanenitrile **10**, were prepared and subjected to the microbial transformation catalyzed by *Rhodococcus* sp. CGMCC 0497 at 20 °C. It has been reported that different protecting groups play an important role on the enantioselective hydrolysis of prochiral 3-hydroxyglutaronitriles [19,27,38]. We wonder if changing the electronic and steric factor on the β-position could enhance the enantioselectivity. But to our disappointment, **9** was hydrolyzed much faster by esterase than nitrilase or nitrile hytratase and **10** with the enantiomeric excess below 1% was recovered in 77% yield after 39 h probably due to the steric hindrance.

4. Conclusion

As we know, it is often a truth that a chiral carbon atom at the β-position to the reaction center would be recognized with much more difficulty than the one at the α-position. It is the first report on the resolution of β-hydroxy nitriles using nitrile-converting enzymes. The biocatalytic asymmetric hydrolysis of several (±)-β-hydroxy nitriles were studied. Though excellent enantiomeric excesses as that in the hydrolysis of α-substituted arylacetonitriles have not been achieved, yet (*R*)-β-hydroxy amides and (*S*)-β-hydroxy acid can still be obtained with moderate to high enantiomeric excess. The study of the reaction temperature shed more light on this kind of two-enzyme-catalyzed reaction and afforded new viewing angle to investigate the biotransformation of other nitriles.

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