Biocatalytic Hydrolysis of 3-Hydroxyalkanenitriles to 3-Hydroxyalkanoic Acids

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Abstract: Microbial catalysts having a combination of nitrile hydratase and amidase activities had a significantly-higher specific activity for hydrolysis of 3-hydroxyalkanenitriles than microbial nitrilase catalysts. *Comamonas testosteroni* 22–1, *Dietzia* sp. ADL1 and *Comamonas testosteroni* 5-MGAM-4D nitrile hydratase/amidase biocatalysts each hydrolyzed 3-hydroxyvaleronitrile to 3-hydroxyvaleric acid (as the ammonium salt) in 99–100% yields, but in consecutive batch reactions with catalyst recycle, alginate-immobilized *C. testosteroni* 5-MGAM-4D had superior enzyme stability and volumetric productivity. In a series of 85 consecutive batch reactions with biocatalyst recycle for the production of 1.0 M 3-hydroxyvaleric acid, the recovered nitrile hydratase and amidase activities in the final reaction were 29% and 40%, respectively, of the initial activities. The catalyst productivity for this series of reactions was 670 g 3-hydroxyvaleric acid/g dry cell weight (50 g 3-hydroxyvaleric acid/g biocatalyst bead), and the volumetric productivity of the initial reaction in the series was 44 g 3-HVA/L/h. Similar results were obtained with alginate-immobilized *C. testosteroni* 5-MGAM-4D for the hydrolysis of 3-hydroxybutryonitrile and 3-hydroxypropionitrile to the corresponding 3-hydroxyalkanoic acid ammonium salts.

Keywords: amidase; enzyme catalysis; 3-hydroxyalkanenitrile; 3-hydroxyalkanoic acid; nitrilase; nitrile hydratase

Introduction

3-Hydroxyalkanoic acids can be utilized to prepare linear or branched copolyesters for fiber, film, molding, and coating applications,[1] and a variety of biological and chemical methods for the production of 3-hydroxyalkanoic acids have been reported.[2] 3-Hydroxyvaleric acid (3-HVA) has been prepared by the hydroxylation of valeric acid in fermentation using *Candida rugosa*,[3] and a single enantiomer of 3-HVA was similarly prepared using *Pseudomonas putida*, *Pseudomonas fluorescens*, *Arthrobacter oxydans* and *Arthrobacter crystallopietes*.[4] (*R*)-(−)-3-HVA and (*R*)-(−)-3-hydroxybutyric acid (3-HBA) have been prepared by chemical degradation[5] or fermentative autodegradation[6] of poly(3-hydroxybutyrate/3-hydroxyvalerate). (*R*)-3-HVA has also been prepared by the asymmetric hydrogenation of methyl 3-oxovalerate, followed by saponification.[7] Several (*R*)- and (*S*)-3-hydroxyalkanoic acids have been prepared by the enzymatic reduction of the corresponding 3-oxoalkanoic acid.[8] 3-Hydroxypropionic acid (3-HPA) has been produced by fermentative conversion of carbohydrates.[9]

3-Hydroxyalkanoic acids can also be prepared by the chemical or enzymatic hydrolysis of the corresponding nitriles, the nitriles being readily prepared by a variety of chemical routes.[10] Chemical hydrolysis of nitriles to the corresponding acids typically employs strongly acidic or basic reaction conditions and high reaction temperatures,[11] and usually produces unwanted by-products and/or large amounts of inorganic salts. Reaction conditions for the chemical hydrolysis of alkanenitriles which additionally have a hydroxy substituent can also result in the undesirable elimination of primary, secondary, or tertiary hydroxy groups to produce carbon-carbon double bonds, reducing yield and requiring separation of the resulting alkenylnitrile by-products from the 3-hydroxyalkanoic acid to avoid detrimental affects in the end use of the product (e.g., color formation in polymerization reactions).

Enzyme-catalyzed hydrolysis of nitriles can be performed at neutral pH, and with extremely high selectivity to the corresponding acid.[12] A wide variety of bacterial genera are known to possess a diverse spectrum of nitrilases, nitrile hydratases and amidases.[13] The immobilized nitrile hydratase and amidase from *Rhodococcus* sp. (SP409 from Novo Industri) was used

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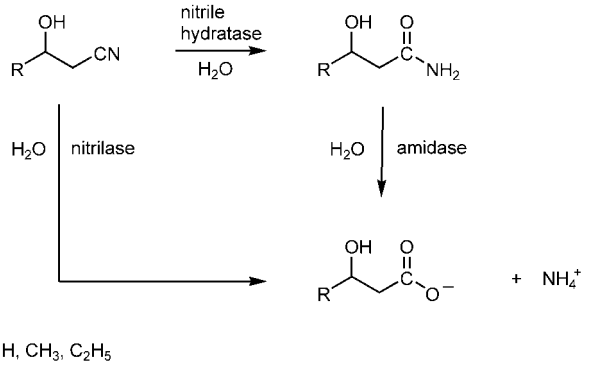
to hydrolyze 3-hydroxypropionitrile (3-HPN), 3-hydroxyheptanenitrile, and 3-hydroxynonanenitrile to the corresponding 3-hydroxycarboxylic acids in 63%, 62% and 83% yields, respectively;[14] in contrast, the purified nitrile hydratase of *Bacillus pallidus* Dac521 hydrolyzed a variety of aliphatic nitriles, but did not hydrolyze 3-HPN.[15] The efficacy of nitrilases for the enantioselective hydrolysis of 2- and 3-hydroxynitriles has recently been described.[16] Scale-up of the enzymatic hydrolysis for production of kilogram quantities of 3-hydroxyalkanoic acids will require a biocatalyst with good enzyme stability during cell immobilization and under reaction conditions, and a variety of microbial nitrilase and nitrile hydratase/amidase biocatalysts have now been examined for these properties.



Scheme 1. 3-Hydroxyalkanenitrile hydrolysis by nitrilase or nitrile hydratase/amidase.

Results and Discussion

Biocatalyst Screening for 3-Hydroxyalkanenitrile Hydrolysis

More than 500 microbial strains having nitrilase or nitrile hydratase/amidase activities (Scheme 1) were isolated from environmental samples, or obtained from internal or public culture collections, and screened as catalysts for hydrolysis of 3-hydroxyalkanenitriles. No microbial nitrilases were isolated by the culturing of environmental samples with 3-hydroxyvaleronitrile (3-HVN) as sole nitrogen source. The nitrilase catalysts *E. coli* SS1001 (ATCC PTA-1177),[17] *Rhodococcus rhodochrous* NCIMB 11216,[18] and *Rhodococcus* sp. (ATCC 39484)[19] were each examined for hydrolysis of 3-HVN. For *E. coli* SS1001, which expresses the nitrilase of *Acidovorax facilis* 72W (ATCC 55746),[20] the specific activity for 3-HVN [5 International Units (IU)/g dry cell weight (dcw)] was only 1.7% that of 2-methylglutaronitrile, and this catalyst had low volumetric productivity in the present application when compared to nitrile hydratase/amidase microbial catalysts (*vide supra*). The nitrilase of *R. rhodochrous* NCIMB 11216 and *Rhodococcus* sp ATCC 39484 also had low 3-HVN specific activity. The specific activities of both *E. coli* SS1001 and *A. facilis* 72W microbial nitrilase catalysts were also measured using 3-hydroxybutyronitrile (3-HBN) as substrate at 25 °C, and low specific activities similar to those for 3-HVN were obtained (11 IU/g dcw and 6 IU/g dcw for *E. coli* SS1001 and *A. facilis* 72W, respectively).

Screening of culture collections and environmental samples for microbial growth on either 3-HVN or 3-hydroxyvaleramide (3-HVAm) as sole nitrogen source produced a significant number of biocatalysts having a combination of nitrile hydratase and amidase activities, and three of the isolates with high specific activity for hydrolysis of 3-HVN were selected for further evaluation. The specific activities for *Comamonas testosteroni* 22–1 (ATCC PTA-1853),[21] *Dietzia* sp. ADL1 (ATCC PTA-1854)[21] and *Comamonas testosteroni* 5-MGAM-4D (ATCC 55744),[21,22] initially produced in unoptimized 10-L fermentations are listed in Table 1. Nitrile hydratase and amidase specific activities were determined by measuring the change in concentration of 3-HVN and 3-HVA, respectively, over time using data collected at 3-HVN or 3-HVAm concentrations greater than *ca.* five times K_m (to ensure substrate saturation of enzyme). The K_m for *C. testosteroni* 22–1 and *C. testosteroni* 5-MGAM-4D nitrile hydratase were 10 mM and 75 mM, respectively, and for amidase, 34 mM and 78 mM, respectively. The 3-HVAm K_m for *Dietzia* sp. ADL1 amidase was 20.5 mM; the nitrile hydratase K_m was not determined, but rates were linear above a 3-HVN concentration of 0.1 M.

Each microbial strain was immobilized in alginate beads, which were subsequently cross-linked with glutaraldehyde (GA) and polyethyleneimine (PEI) to stabilize the calcium-cross-linked alginate beads in product mixtures containing high concentrations of ammonium ion.[23] The chemical cross-linking of algi-

Table 1. Initial specific activities of *C. testosteroni* 22–1, *Dietzia* sp ALD-1, and *C. testosteroni* 5-MGAM-4D produced in 10-L fermentation.

Catalyst	Nitrile hydratase [IU/g dcw]	Amidase [IU/g dcw]
C. testosteroni 22–1[a]	1523	1046
Dietzia sp. ALD-1[a]	4261	251
C. testosteroni 5-MGAM-4D[b]	2811	869

[a] Specific activities for 10.5 mg dcw/mL suspension in 0.10 M potassium phosphate (pH 7.0) at 25 °C, using 0.3 M 3-HVN (nitrile hydratase) or 0.3 M 3-HVAm (amidase).

[b] Specific activities for 8.5 mg dcw/mL suspension in 0.10 M potassium phosphate (pH 7.0) at 25 °C, using 0.75 M 3-HVN (nitrile hydratase) or 0.75 M 3-HVAm (amidase).

Table 2. Immobilized-cell biocatalyst specific activities for hydrolysis of 1.0 M 3-HVN in consecutive batch reactions with biocatalyst recycle.

Rxn #	*C. testosteroni* 22 – 1[a]		*C. testosteroni* 5-MGAM-4D[a]		*Dietzia* sp. ALD-1[b]	
	nitrile hydratase [IU/g][c]	amidase [IU/g][d]	nitrile hydratase [IU/g][c]	amidase [IU/g][d]	nitrile hydratase [IU/g][c]	amidase [IU/g][d]
1	34	8.5	93	28	96	6.8
2	20	5.1	65	23	122	3.8
3	3.4	1.0	56	14	101	3.4

[a] Reactions run unbuffered at 15 °C with 20 wt % biocatalyst beads in reaction mixture.
[b] Reactions run unbuffered at 25 °C with 20 wt % biocatalyst beads in reaction mixture.
[c] Specific activity for conversion of 3-HVN.
[d] Specific activity for conversion of 3-HVAm.

nate-immobilized *C. testosteroni* 22 – 1 cells with GA and PEI resulted in a significant loss of nitrile hydratase and amidase activity, 26% and 42%, respectively, when compared to biocatalyst beads which were not cross-linked with GA and PEI. No significant loss of either enzyme activity was observed for GA/PEI-cross-linking of alginate-immobilized *C. testosteroni* 5-MGAM-4D; the effect of chemical cross-linking on immobilized *Dietzia* sp ALD-1 was not determined. The specific activity of the resulting biocatalyst beads was measured in three consecutive reactions with biocatalyst recycle for conversion of 1.0 M 3-HVN to 3-HVA (Table 2). Immobilized *C. testosteroni* 22 – 1 readily lost activity at 25 °C, and only one batch reaction could be completed at extended reaction times using a 20 wt % biocatalyst bead loading. At 15 °C, there was some improvement of enzyme stability with this same biocatalyst, but there was a 90% loss of both enzyme activities in three consecutive reactions. The recovered activities using either immobilized *Dietzia* sp ALD-1 or *C. testosteroni* 5-MGAM-4D in consecutive reactions were significantly greater than *C. testosteroni* 22 – 1, and *C. testosteroni* 5-MGAM-4D was chosen for further optimization due to its higher amidase specific activity relative to *Dietzia* sp. ALD-1.

Characterization of Unimmobilized and Immobilized *C. testosteroni* 5-MGAM-4D Nitrile Hydratase and Amidase Activity

The stability of nitrile hydratase and amidase activities of a 20% wet cell weight (wcw) suspension of *C. testosteroni* 5-MGAM-4D in 0.35 M potassium phosphate (pH 7.0) at 50 °C was checked over 1 – 3 h to determine the relative thermal stabilities of the two enzyme activities. There was a rapid initial loss of nitrile hydratase activity after 1 h, with a subsequent slower rate of inactivation at extended heating times (Figure 1); the amidase activity was relatively stable at this same temperature. *C. testosteroni* 5-MGAM-4D has previously been reported to have two nitrile hydratase activities, where one nitrile hydratase could be completely inactivated by heating a suspension of the microbial cells at 50 °C for 1 h.[22] The initial rapid loss of nitrile hydratase activity observed in the first hour of heating at 50 °C was most likely due to the inactivation of the less-thermally stable nitrile hydratase activity, and the rate of nitrile hydratase inactivation after 1 h was representative of the thermal stability of the remaining nitrile hydratase.

The pH dependence of nitrile hydratase and amidase activities of heat-treated *C. testosteroni* 5-MGAM-4D [20% wcw suspension in 0.35 M phosphate buffer (pH 7.0) heated 0.5 h at 50 °C] was determined at 25 °C for 20% wcw suspensions in 0.1 M pyrophosphate at pH 4 – 10 (Figure 2). The optimal pH for nitrile hydratase was between pH 7 – 8, while the amidase showed an optimum at pH 7. The recovered nitrile hydratase and amidase activities after 24 h were optimal at pH 8 (79% and 90%, respectively). Suspensions of cells that were not heat-treated rapidly lost activity over

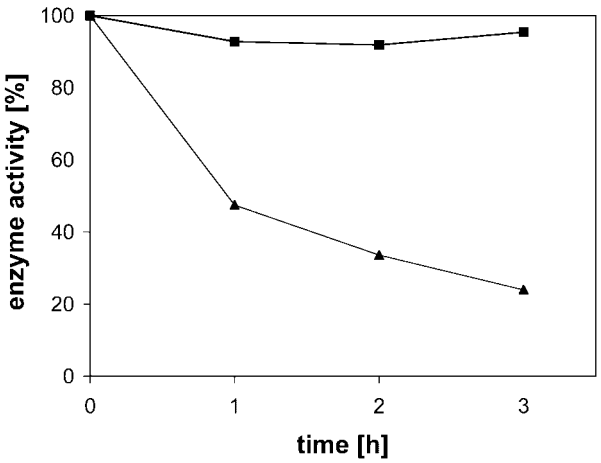


Figure 1. Stability of *C. testosteroni* 5-MGAM-4D nitrile hydratase and amidase activity at 50 °C; nitrile hydratase (▲), amidase (■).

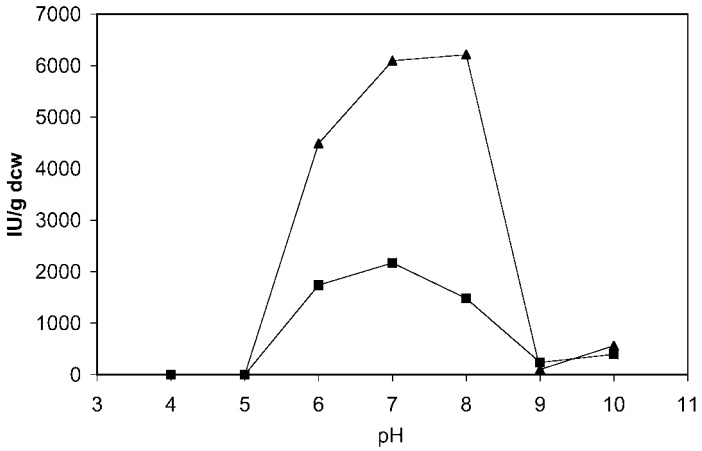


Figure 2. Dependence of *C. testosteroni* 5-MGAM-4D nitrile hydratase and amidase specific activity on pH; nitrile hydratase (▲), amidase (■).

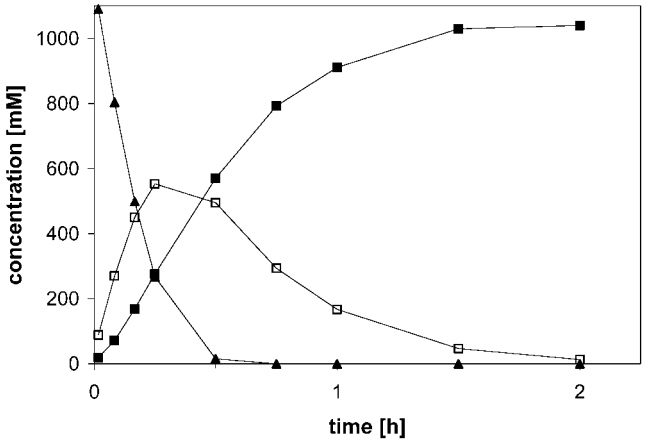


Figure 3. Time course for hydrolysis of 1.08 M 3-HVN using 20 wt % GA/PEI-cross-linked *C. testosteroni* 5-MGAM-4D/ alginate beads as catalyst at 25 °C: 3-HVN (▲); 3-HVAm (□); 3-HVA (■).

the entire pH range in 24 h. Hydrolysis of 3-hydroxyalkanenitriles using either unimmobilized or alginate-immobilized cells in the absence of added buffer produced the ammonium salt of the corresponding carboxylic acid, which conveniently buffered the reaction mixture at pH 7.3 to 7.4.

To test the effect of 3-HVA concentration (as the ammonium salt) on the stability of *C. testosteroni* 5-MGAM-4D nitrile hydratase and amidase, a 50 mg wcw/mL cell suspension in 0.10 M phosphate buffer (pH 7.0) containing 0.50 M 3-HVA ammonium salt was mixed for 24 h at 25 °C, then the cell suspension was centrifuged, and the cell pellet washed twice with 0.10 M phosphate buffer (pH 7.0) and assayed using either 0.5 M 3-HVN or 3-HVAm. There was no measurable loss of nitrile hydratase or amidase activity versus a control maintained for 24 h in the absence of added 3-HVA. Assays for the effect of 3-HVA on *C. testosteroni* 5-MGAM-4D amidase activity were also run using 3-HVAm as substrate; the rate of hydrolysis of 0.5 M 3-HVAm in the presence of 0.5 M 3-HVA ammonium salt was 77% of the amidase activity with no added 3-HVA, indicating partial inhibition of amidase activity at high concentrations of 3-HVA.

Performance of Immobilized *C. testosteroni* 5-MGAM-4D for Production of 3-Hydroxyalkanoic Acids

Optimization of fermentation conditions and scale-up of fermentations from 10 L to 200 L increased the *C. testosteroni* 5-MGAM-4D cell specific nitrile hydratase and amidase activities to 8785 IU/g dcw and 1505 IU/g/dcw, respectively (measured without prior heat-treatment of cell suspensions); these cells were immobilized as described above and used at a 20 wt % biocatalyst bead loading to convert 1.0 M 3-HVN to 3-HVA (Figure 3, 118 g/L final product concentration). All reactions which employed alginate-immobilized cells additionally contained either 2.0 mM or 5.0 mM calcium acetate in the reaction mixture to maintain the physical integrity of the calcium alginate beads in the presence of up to 1.0 M ammonium ion (generated by hydrolysis of the nitrile).[20a] In consecutive batch reactions, a portion of the reaction mixture (*ca.* 20 wt % biocatalyst beads and 2.5 wt % of the product solution) was carried over into each subsequent reaction, such that the final product concentration of 3-HVA in the fifth (and subsequent) recycle reactions was *ca.* 1.30 M (153 g 3-HVA/L). Nitrile hydratase and amidase specific activities were determined by measuring the change in concentration of 3-HVN and 3-HVA, respectively, over time using data collected at 3-HVN or 3-HVAm concentrations greater than ca. five times K_m.

The ratio of nitrile hydratase/amidase specific activity of unimmobilized *C. testosteroni* 5-MGAM-4D cells did not change significantly over the temperature range of 5 – 25 °C, whereas the ratio decreased with increasing temperature when using 7.5% dcw cells immobilized in GA/PEI-cross-linked alginate beads (Table 3). After calculation of the specific activity of the beads based on dcw content (e.g., 1493 nitrile hydratase IU/g dcw and 244 amidase U/g/dcw for beads at 5 °C), the rates of either enzymatic reaction were relatively unaffected by immobilization at 5 °C, but with increasing temperature the specific activity of the immobilized-cell nitrile hydratase did not increase proportionately to the amidase activity. This could be the result of the nitrile hydratase-catalyzed reaction becoming rate-limited by diffusion of 3-HVN into the biocatalyst beads, relative to the increase in reaction rate as the temperature increases. There was less of an effect of 3-HVAm diffusion on the amidase reaction rate with increasing

Table 3. Dependence of specific activity of unimmobilized or immobilized *C. testosteroni* 5-MGAM-4D cells on temperature.

Temperature [°C]	Catalyst[a]	Nitrile hydratase [IU/g][b]	Amidase (IU/g)[c]	Nitrile hydratase/amidase
25	cells	8785	1505	5.8
25	beads	303	87.8	3.5
15	cells	3968	676	5.9
15	beads	220	50.6	4.3
5	cells	1560	239	6.5
5	beads	112	18.3	6.1

[a] Specific activity for cells based on IU/g dcw, for beads based on IU/g beads.
[b] Nitrile hydratase specific activity measured for conversion of 1.0 M 3-HVN.
[c] Amidase specific activity measured for conversion of 1.0 M 3-HVAm.

temperature, possibly due to the significantly lower amidase specific activity. Decreasing the biocatalyst bead diameter from its current value of 2 – 3 mm might decrease diffusion-related limitations on nitrile hydratase-catalyzed reaction rates.[24]

There was no significant difference in the recovered nitrile hydratase or amidase activities over the course of five recycle reactions at 5, 10, or 25 °C using GA/PEI-cross-linked *C. testosteroni* 5-MGAM-4D/alginate beads for hydrolysis of 1.0 M 3-HVN. In each set of recycle reactions, there was a *ca.* 50% loss of both nitrile hydratase and amidase activities by the fifth reaction. At 5 °C, the intermediate 3-HVAm was not completely converted to 3-HVA in 24 h. At 35 °C, there was an increase in the rate of loss of recovered catalyst activity with consecutive batch reactions relative to reactions at 15 – 25 °C. A set of recycle reactions were run at 15 °C with 1.5 M 3-HVN, and these reactions did not completely convert 3-HVN to 3-HVA; there was a significantly greater loss of amidase activity after five recycle reactions at this higher 3-HVN concentration.

To test the effect of initial 3-HVN concentration and final 3-HVA concentration on enzyme stability, recycle reactions were run using the immobilized cell biocatalyst at 25 °C to produce 1.0 M 3-HVA by slowly feeding 3-HVN by syringe pump (over the course of 2 h), and these reactions were compared to reactions which produced 1.0 M (12 wt %) 3-HVA, or 0.5 M 3-HVA, with a single addition of 3-HVN at the start of the reaction. For the slow-feed reaction, the instantaneous concentration of either 3-HVN or 3-HVAm was maintained below 200 mM (less than $5 \times K_m$), so the reaction rate was partially limited by substrate concentration. There was no significant difference in loss of nitrile hydratase or amidase activity over five consecutive reactions for these three sets of reactions. As a control, the same catalyst beads were maintained at 25 °C in 0.2 M calcium acetate for 66 h, and 92% recoveries of both nitrile hydratase and amidase activity were obtained over this same period of time. The initial significant loss of both enzyme activities in these reactions remains unexplained.

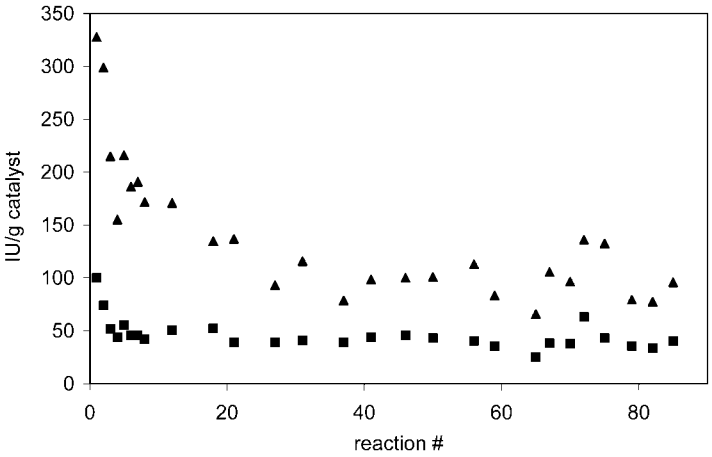


Figure 4. Nitrile hydratase and amidase specific activity of GA/PEI-cross-linked *C. testosteroni* 5-MGAM-4D/alginate beads in consecutive 1.0 M 3-HVN batch reactions at 25 °C with catalyst recycle: nitrile hydratase (▲), amidase (■).

The recovery of nitrile hydratase and amidase activities of the immobilized-cell biocatalyst (20 wt %) in reactions run at 25 °C to produce 1.0 M 3-HVA was measured over a total of 85 consecutive reactions with biocatalyst recycle (Figure 4). The recoveries of nitrile hydratase and amidase from the final reaction were 29% and 40%, of initial values, respectively, with complete conversion of 3-HVN and less than 2% amide remaining after 7 h in the final reaction of the series. The catalyst productivity for this set of reactions was 670 g 3-HVA/g dcw (50 g 3-HVA/g biocatalyst bead), and the volumetric productivity of the initial and final reactions in the series were 44 g 3-HVA/L/h and 17 g 3-HVA/L/h, respectively. Despite the initial significant loss of biocatalyst activity in the first several recycle reactions, the 3-HVA volumetric productivity obtained over the course of the entire series of reactions was acceptable for further development work.

The nitrile hydratase specific activity of immobilized *C. testosteroni* 5-MGAM-4D was measured for 3-HVN, 3-HBN, and 3-HPN, as was the amidase specific activity for the corresponding amides; the nitrile hydratase

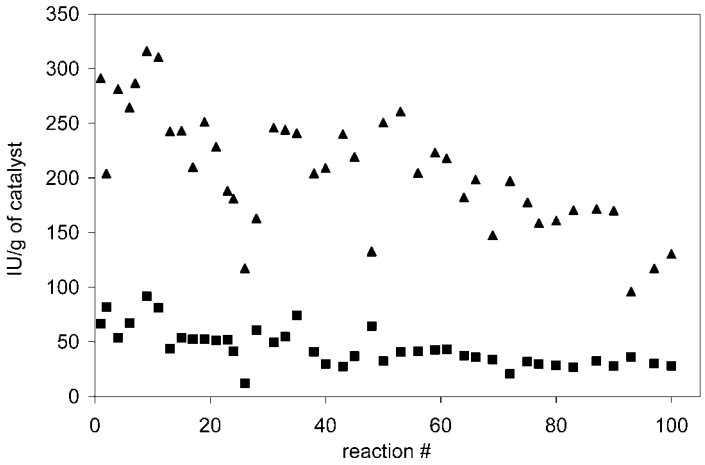


Figure 5. Nitrile hydratase and amidase specific activity of GA/PEI-cross-linked *C. testosteroni* 5-MGAM-4D/alginate beads in consecutive 1.0 M 3-HPN batch reactions at 15 °C with catalyst recycle: nitrile hydratase (▲), amidase (■).

specific activity for 3-HVN and 3-HBN were 75% and 78%, respectively, that of 3-HPN, and the amidase specific activity for 3-HVAm and 3-hydroxybutyramide (3-HBAm) were 45% and 53%, respectively, that of 3-hydroxypropionamide (3-HPAm). A second series of 100 recycle reactions was run using immobilized *C. testosteroni* 5-MGAM-4D to convert 1.0 M 3-HPN to 3-HPA at 15 °C (Figure 5). The final recoveries of nitrile hydratase and amidase were 45% and 42%, respectively, of their initial activities. The catalyst productivity for this series of reactions was 693 g 3-HPA/g dcw (52 g 3-HPA/g catalyst bead), and the volumetric productivity of the initial and final reactions in the series were 23 g 3-HPA/L/h and 7 g 3-HPA/L/h, respectively. The initial significant decrease in recovered nitrile hydratase activity during the first several 3-HVN recycle reactions at 25 °C (Figure 4) or 15 °C was not observed with 3-HPN, indicating that 3-HVN, or an unidentified impurity in 3-HVN, may be responsible for enzyme inactivation.

Conclusions

Scale-up and commercialization of the biocatalytic hydrolysis of 3-hydroxyalkanenitriles will require a robust biocatalyst with high specific activity, and good stability under reaction conditions which produce high concentrations of an ammonium carboxylate salt. An exhaustive screening of environmental samples using 3-HVN as substrate did not result in the identification of a nitrilase-based microbial catalyst, and low nitrilase specific activity for 3-hydroxyalkanenitrile hydrolysis was obtained with a small number of known microbial nitrilases. A large number of microbial nitrile hydratase/amidase catalysts were isolated from the same environmental samples, but not all cultures had the desired combination of stability and specific activity for the desired biocatalytic conversions.

Immobilized *C. testosteroni* 5-MGAM-4D was physically robust over the course of up to 100 recycle reactions, with little or no decrease in the mass of recovered biocatalyst, and no significant attrition of the catalyst beads. The storage stability of the immobilized cell enzyme activities was also excellent, with no measurable loss of nitrile hydratase or amidase activity of the GA/PEI-cross-linked *C. testosteroni* 5-MGAM-4D/alginate bead biocatalyst after 106 days at 5 °C in 0.2 M calcium acetate (pH 7.0). Acceptable catalyst and volumetric productivities were obtained for hydrolysis of several 3-hydroxyalkanenitriles, and this biocatalyst is currently being utilized for the production of 100-kilogram quantities of 3-HVA.

Experimental Section

Materials and Methods

Chemicals were obtained from commercial sources unless otherwise noted, and used as received. 3-HVN was prepared according to a literature procedure.[10c] 3-HVAm[25] and 3-HVA[26] were prepared as described below; isolated yields are unoptimized and melting points uncorrected. The calculated recovery of 3-hydroxyalkanenitriles and yields of the hydrolysis products were based on initial 3-hydroxyalkanenitrile concentration, and determined by HPLC using a refractive index detector and either a Supelcosil LC-18 DB column (15 cm × 4.6 mm dia.) and 10 mM acetic acid/10 mM sodium acetate in 7.5% methanol/water as mobile phase (for hydrolysis of 3-HVN), or a Bio-Rad HPX-87H column (30 cm × 7.8 mm dia.) and 0.001 N sulfuric acid as mobile phase at 50 °C (for hydrolysis of 3-HBN and 3-HPN). Chemical shifts for ^{1}H and ^{13}C NMR spectra are expressed in parts per million positive values downfield from internal TMS. Identification of hydrolysis products of 3-HBN and 3-HPN was made by comparison of HPLC retention times to commercially available samples.

Microbial cultures having nitrilase or nitrile hydratase/amidase activities were initially identified by using an assay for ammonia[27] released during hydrolysis of 3-HVN or 3-HVAm. The isolation and growth of microbial cultures described herein has been reported.[17–21] Cell paste isolated from fermentation was frozen at −80 °C without pre-treatment with glycerol or DMSO, and thawed prior to use in reactions or for immobilization in alginate. Wet cell weights of microbial catalysts employed in reactions or assays were obtained from cell pellets prepared by centrifugation of fermentation broth or cell suspensions in buffer. Dry cell weights were determined by microwave drying of wet cells. Microbial cell enzyme activity was measured by stirring a suspension of 8.5 – 12.5 mg dry cell weight/mL in 25 mM phosphate buffer (pH 7.0) and 0.30 – 0.75 M substrate at 25 °C, and analyzing aliquots removed at 1, 5, 10 and 15 min for the rate of substrate disappearance. A unit of enzyme activity (IU) was equivalent to 1 micromole/min of substrate conversion. K_m values were determined using kinetic analysis of progress curves.[28]

Fermentation of *C. testosteroni* 5-MGAM-4D

The production train included a first seed produced in 500 mL of medium, a second seed produced by 10-L fermentation, and a 200-L production fermentation. The medium was based on mineral medium with 5 g/L yeast extract, 5 g/L ammonium sulfate and 6 g/L sodium lactate. The fermentations were run at all stages at 32 °C, the pH was controlled at 6.8 with 85% lactic acid and 40% sodium hydroxide, and the dissolved oxygen concentration controlled at 25% of air saturation by adjustment of aeration and agitation rates. *n*-Butyramide was added twice during the 200-L production run as an inducer and additional nitrogen source, with additions of 2 g/L at 23 h and 29 h in response to cessation of growth. The fermentation was stopped at 32.5 h, the culture chilled and *ca.* 8.2 kg of wet cells paste were isolated by centrifugation. During the production run the microbial nitrile hydratase and amidase specific activities increased two-fold and 1.6-fold, respectively, after induction, with final specific activities of 8785 3-HVN IU/g dcw and 1505 3-HVAm IU/g/dcw, respectively.

Preparation of 3-Hydroxyvaleramide (3-HVAm)

To a suspension of 10 g wcw of *Rhodococcus* sp. A4[29] (formerly *Brevibacterium* sp. R312 strain A4,[30] Technische Universiteit Delft, LMD#79.2) in 890 mL of deionized water at 5 °C were added 100 g (0.99 mol) of 3-HVN with stirring. After 3 h, further 9.0 g wcw of A4 were added, and the mixture stirred at 5 °C for 19 h to give quantitative conversion of 3-HVN to 3-HVAm. The reaction mixture was centrifuged, and the supernatant decanted, filtered (30,000 molecular weight cut-off), and the filtrate concentrated by rotary evaporation. The resulting oil was mixed with consecutive 1.5 L aliquots of dichloromethane until all the oil had dissolved, then the dichloromethane solutions were dried over magnesium sulfate, filtered, and cooled to − 18 °C. The resulting white crystalline solid was collected from each dichloromethane fraction by vacuum filtration and dried under vacuum to afford 3-HVAm; yield: 73 g (62%); mp 53.0 – 53.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 6.39$ (bs, 1H), 6.21 (bs, 1H), 3.91 – 3.78 (m, 2H), 2.41 – 2.27 (m, 2H), 1.56 – 1.48 (m, 2 H), 0.95 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 175.5$, 69.2, 42.0, 29.9, 9.9; MS (CI): $m/z = 118$ (MH^+, 21), 100 (100), 82 (36), 59 (22), 57 (25); HRMS: calcd. for $C_5H_{12}NO_2$ (MH^+): 118.0868; found: 118.0869.

Preparation of 3-Hydroxyvaleric Acid (3-HVA) using Unimmobilized *C. testosteroni* 5-MGAM-4D Cells

A 3-L reaction mixture containing 300 g (1.0 M) of 3-HVN and 134 g wcw (23 g dcw) of *C. testosteroni* 5-MGAM-4D cells in 20 mM potassium phosphate buffer (pH 7.0) was mixed at 25 °C. After 46.5 h, the conversion of 3-HVN was 100%, and the HPLC yield of 3-HVA was 100%. The reaction mixture was centrifuged and the supernatant decanted. The supernatant was filtered (30,000 molecular weight cut-off), then adjusted to pH 1.9 with concentrated HCl. The resulting solution was saturated with sodium chloride, then extracted with four 1.5-L portions of ethyl ether. The combined organic extracts were dried over magnesium sulfate, filtered, and the ether solution concentrated to 2.4 L by rotary evaporation. Hexanes (1.6 L) were added to the concentrate, and the resulting mixture cooled in dry ice/acetone. The resulting crystalline precipitate was collected by vacuum filtration, washed with cold hexanes, and dried under vacuum to afford 3-hydroxyvaleric acid; yield: 274 g (77%); mp 43.0 – 44.5 °C (reported 43 – 44 °C);[26] ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.10$ (bs, 1H), 4.02 – 3.97 (m, 1H), 2.56 – 2.46 (m, 2H), 1.60 – 1.51 (m, 2H), 0.95 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): = 175.8, 69.2, 40.4, 28.7, 9.1; IR (melt): $\nu = 3700 – 2300$ (br), 2965, 2939, 2882, 2663 (br), 2573 (br), 1702, 1443, 1306, 1184 cm^{-1}; MS (EI): $m/z = 119$ (MH^+, 1.5), 101 (8.4), 100 (8.4), 89 (34), 71 (100), 59 (36) 43 (60); HRMS: calcd. for $C_5H_{11}O_3$ (MH^+): 119.0708; found: 119.0712.

Preparation of GA/PEI-Cross-linked *C. testosteroni* 5-MGAM-4D/Alginate Beads

Into a 250-mL media bottle equipped with magnetic stir bar and containing 68.7 g of distilled, deionized water at 50 °C were slowly added 3.30 g of FMC BioPolymer Protanal® LF 10/60 alginate with rapid stirring. The mixture was heated to 75 – 80 °C until the alginate was completely dissolved, and the resulting solution cooled to 25 °C in a water bath. A suspension of *C. testosteroni* 5-MGAM-4D cells (28.6 g wcw) in 0.185 M sodium acetate buffer (19.4 mL, pH 7.0) was prepared at 25 °C and added to the alginate solution at 25 °C with stirring. The cell/alginate mixture was added dropwise at 2-mL/min by syringe pump to 640 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C with stirring. After stirring for 2 h, the buffer was decanted from the resulting beads, which were resuspended in 293 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C. With stirring, 6.0 g of 25 wt % glutaraldehyde in water was added and the beads mixed for 1.0 h at 25 °C. To the resulting suspension was then added 24.0 g of 12.5 wt % polyethyleneimine (BASF Lupasol® PR971L, average molecular weight *ca.* 750,000) in water, and the beads mixed for an additional 18 h at 25 °C. The cross-linked beads were then washed twice with 300 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C, and stored in this same buffer at 5 °C.

Hydrolysis of 3-Hydroxyalkanenitriles using GA/PEI-Cross-Linked *C. testosteroni* 5-MGAM-4D/Alginate Beads

In a typical procedure, 4.0 g of GA/PEI-cross-linked *C. testosteroni* 5-MGAM-4D/alginate beads, 13.45 mL of distilled, deionized water, 0.5 mL of 0.20 M calcium acetate (pH 7.0, 5.0 mM final calcium ion concentration in reaction mixture) and 2.05 mL (1.98 g, 1.0 M) of 3-HVN were added to a 50-mL jacketed reaction vessel (temperature-controlled at 25 °C with a recirculating temperature bath), and the mixture stirred at 25 °C. Samples (0.100 mL) of the reaction mixture were mixed with 0.400 mL of water, and then 0.200 mL of the diluted sample was mixed with 0.200 mL of 0.200 M sodium butyrate (HPLC external standard) in water. The resulting sample was centrifuged, and the supernatant analyzed by HPLC. After 7 h, the conversion of 3-HVN was 100%, and the HPLC yield of 3-HVA was 100%.

At the end of the reaction the product mixture was decanted from the catalyst beads. The catalyst was reused in a further

three consecutive batch reactions under the conditions as described above; reactions 2, 3, and 4 produced HPLC yields of 3-HVA of 99.7%, 99.7%, and 99.6% at 100% conversion of 3-HVN. At the completion of four recycle reactions, the final concentration of 3-hydroxyvaleric acid in the final product mixture was 1.29 M.

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