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5-Cyanovaleramide Production using Immobilized *Pseudomonas chlororaphis* B23

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Abstract—A biocatalytic process for the hydration of adiponitrile to 5-cyanovaleramide has been developed which can be run to higher conversion, produces more product per weight of catalyst, and generates significantly less waste products than alternate chemical processes. The biocatalyst consists of *Pseudomonas chlororaphis* B23 microbial cells immobilized in calcium alginate beads. The cells contain a nitrile hydratase (EC 4.2.1.84) which catalyzes the hydration of adiponitrile to 5-cyanovaleramide with high regioselectivity, and with less than 5% selectivity to byproduct adipamide. Fifty-eight consecutive batch reactions with biocatalyst recycle were run to convert a total of 12.7 metric tons of adiponitrile to 5-cyanovaleramide. At 97% adiponitrile conversion, the yield of 5-cyanovaleramide was 13.6 metric tons (93% yield, 96% selectivity), and the total weight of 5-cyanovaleramide produced per weight of catalyst was 3150 kg/kg (dry cell weight).

Introduction

The first step in the manufacture of a new herbicide required the conversion of adiponitrile (ADN) to 5-cyanovaleramide (5CVAM). Chemical catalysts for the hydration of nitriles to amides,[1–3] such as Raney copper[4] or manganese dioxide,[5] produced significant quantities of adipamide (ADAM) in addition to 5CVAM at high conversions of ADN (Scheme 1). The amount of catalyst waste produced per kilogram of product was also significant when employing many of these chemical catalysts. As an alternative to chemical catalytic methods, the use of enzymatic catalysis for the production of 5CVAM from ADN was examined.

Aliphatic nitriles are readily hydrated to the corresponding amides by the nitrile hydratase (EC 4.2.1.84) of a variety of bacteria and fungi.[6–14] For the preparation of an ω-cyanocarboxylic acid amide from an aliphatic dinitrile, a regioselective nitrile hydratase was required to prevent significant production of the corresponding diamide. When employing a bacterial cell as catalyst, the absence of amidase activity that could result in further hydrolysis of the amide to carboxylic acid was also important. Bacterial strains of *Bacillus*,[15,16] *Bacteridium*,[15,16] *Brevibacterium*,[15,16] *Micrococcus*,[15,16] *Pseudomonas*,[17] and *Acinetobacter*[18] have each been reported to produce a regioselective aliphatic nitrile hydratase, and a variety of these strains were evaluated as a catalyst for hydration of ADN to 5CVAM.

Whole microbial cells of *Pseudomonas chlororaphis* B23, first isolated and characterized by H. Yamada and coworkers,[19,20,21] and subsequently employed by the Nitto Chemical Industry Co. (now merged with Mitsubishi Rayon Co.) for the commercial production of acrylamide from acrylonitrile,[22–25] were found to be a highly regioselective catalyst for the production of 5CVAM. This biocatalyst was superior in both enzyme stability and productivity of 5CVAM (kg 5CVAM/kg biocatalyst/h) to all other whole-cell microbial catalysts which were examined. The demonstration of a commercial-scale process for the production of 5CVAM using immobilized *P. chlororaphis* B23 cells is now described.

Results and Discussion

Storage stability of *P. chlororaphis* B23 nitrile hydratase activity

The nitrile hydratase activity of *P. chlororaphis* B23 cells suspended in aqueous buffer was found to be most

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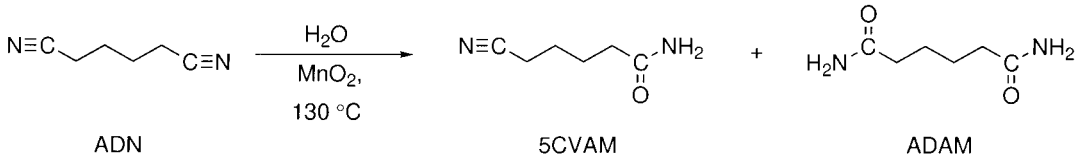

Scheme 1.

stable at ca. 5°C, and was increasingly less stable with increasing temperature from 5 to 25°C.[19] Because the B23 cell nitrile hydratase rapidly and irreversibly lost activity at a pH below pH 6, a buffer was required to maintain the pH of the cell suspension between 6.5 and 7.5, which was the optimum pH range for stabilization of the enzyme activity.[19] Phosphate buffer could not be employed, as it reacted with calcium ions used to cross-link sodium alginate during cell immobilization, producing insoluble calcium phosphate and causing dissolution of the alginate beads. Millimolar concentrations of organic acids (e.g. 20 mM *n*-butyric acid) had previously been shown to markedly improve the stability of B23 nitrile hydratase activity.[19] *P. chlororaphis* B23 cell suspensions (50% wet cell weight (wcw) suspensions) containing 0.5% sodium propionate (w/v) at pH 6.2 lost over 50% of initial nitrile hydratase specific activity after three weeks at 5°C. Cell suspensions at 10 mg wcw/mL were stable at 5°C for as long as 6 weeks in the presence of 23 mM sodium butyrate at pH 7.0. Cell suspensions at 200 mg wcw/mL in 23 mM sodium butyrate were much less stable, suggesting that higher butyrate or propionate concentrations were required for suspensions containing cells at or above 200 mg wcw/mL.

The nitrile hydratase activity of B23 cell paste which was isolated by centrifugation and subsequently frozen at −20°C or −80°C was stable for at least 12 months. Similar stabilities were observed for frozen 38 and 54 wt% cell suspensions (wcw) in 0.5% sodium propionate (pH 6.2). Cell breakage or lysis after one freeze–thaw cycle was minimal, with only 1–5% of the total nitrile hydratase activity present measured in assays as cell-free enzyme activity. A second and third freeze–thaw of frozen B23 cells resulted in extracellular nitrile hydratase activity of 9 and 13% of total activity, respectively, indicating an increase in permeabilization of the B23 cell wall and membrane with increasing number of freeze–thaw cycles. B23 cells subjected to one freeze–thaw cycle and subsequently used in the preparation of B23/alginate beads (see below) produced a catalyst with superior selectivity to 5CVAM in batch recycle reactions than B23/alginate beads made with cells which had not undergone at least one freeze–thaw cycle.

Optimization of reaction conditions using unimmobilized and immobilized B23 cells

ADN hydration reactions were run at 5°C with unimmobilized B23 cells to determine the stability of the microbial nitrile hydratase activity. The concentration of 5CVAM produced in these reactions was varied from 0.40 to 1.50 M. The measured solubility of ADN at 5°C in either the initial 20 mM sodium butyrate/5 mM calcium chloride buffer (pH 7) or in 0.38 M 5CVAM product mixture was only 0.45 and 0.52 M, respectively, so batch reactions which were run to produce greater than 0.45 M 5CVAM started as two-phase mixtures of aqueous cell suspension and ADN. At 5°C, the solubility of byproduct ADAM was only 37–42 mM in 1 to 1.5 M 5CVAM. The nitrile hydratase activity of B23 cells in these reactions was inhibited by sodium butyrate; for the production of 0.75 M 5CVAM at 5°C, the reaction rate decreased 41% as the concentration of sodium butyrate was increased from 10 to 40 mM.

Six consecutive reactions were run at 5°C to convert 0.40 M ADN (in a single-phase aqueous solution) to 5CVAM with a recycle of the unimmobilized cells; the time required for complete conversion of ADN increased three-fold over the course of the six reactions as the cells lost activity. At complete conversion of ADN, the ratio of 5CVAM to ADAM was $\geq$100:1 in each of the six reactions. If a reaction was allowed to continue after complete conversion of ADN, 5CVAM was further hydrolyzed to ADAM at ca. 1–2% of the initial rate of 5CVAM production. Consecutive batch reactions were next performed at 5°C to produce a final concentration of 1.5 M 5CVAM by starting with a two-phase mixture of aqueous cell suspension (containing the solubility limit of ADN) and ADN; only the first reaction gave complete conversion of ADN, and an attempt to reuse the unimmobilized cells in a second reaction resulted in incomplete conversion of ADN at extended reaction times. Only cell debris (no intact cells) was recovered from this second reaction, indicating that the unimmobilized cells were readily lysed in the two-phase mixture of ADN and aqueous buffer, with concomitant loss of nitrile hydratase activity. Addition of ADN over the course of a reaction to produce 1.5 M 5CVAM resulted in only a modest improvement in cell stability relative to complete addition of ADN at the start of a reaction.

B23 cells must be rapidly separated from the product mixture after complete conversion of ADN to prevent over-conversion of 5CVAM to ADAM, and filtration or centrifugation for catalyst separation and recovery could not be performed on a commercial scale in a sufficiently short time. Several methods of cell immobilization were examined to produce a catalyst which could be readily separated from the product mixture, and which would also improve the stability of the whole cells against lysis in a two-phase mixture of ADN and aqueous buffer. An immobilization method was required that was simple to perform on a large scale, and produced an immobilized catalyst that had a high specific activity (IU/g catalyst), and was stable to the reaction conditions employed. Immobilization by entrapment in agarose[26] or carrageenan[27] could not be utilized, since

the nitrile hydratase activity of B23 cells was rapidly deactivated at the temperatures required for immobilization in these matrices (35–50°C). Immobilization on the surface of porous inorganic supports (such as Celite or porous silica) would produce a catalyst with a low specific activity relative to gel entrapment, and in stirred batch reactions, rapid loss of activity due to attrition of the particle surface during mechanical mixing of the suspended catalyst could occur.[28] Immobilization in either polyacrylamide gel (PAG)[29] or calcium alginate[30] produced a catalyst with high specific activity, but under identical reaction conditions the B23/alginate catalyst was more stable, and produced more product per weight of catalyst, than B23/PAG catalyst. B23 cells were immobilized in calcium alginate by first preparing a suspension of 20% wcw (5% dry cell weight (dcw)) cells in an aqueous mixture containing sodium alginate (Pronova Protanal® LF 10/60, 2.75% w/w final concentration) and sodium butyrate (23 mM final concentration, pH 7) at 5°C, then the resulting cell suspension was filtered through consecutive 230 and 140 micron steel mesh filters prior to adding the suspension dropwise with stirring to a solution of 0.20 M calcium chloride/23 mM sodium butyrate (pH 7) at 5°C. After the resulting beads were allowed to harden in this solution for 18 h, the beads were washed twice with 23 mM sodium butyrate/5 mM calcium chloride buffer and stored at 5°C in this same buffer for up to 135 days with no significant loss of activity. The inclusion of 5 mM calcium ion in both storage and reaction mixture buffer maintained the calcium-crosslinked form of the alginate gel.

The dependence of specific activity (ADN IU/gram catalyst) of the B23/alginate beads on pH at 5°C was determined (Fig. 1). The specific activity decreased markedly below pH 7, and was fairly constant from pH 7.5–9. As the optimum pH for B23 nitrile hydratase stability was between pH 6.5 and 7.5, reactions were typically run in a pH range of 6.8–7.2. The dependence of B23/alginate bead specific activity on temperature was also measured (Fig. 2); an increase in reaction temperature from 5 to 10°C resulted in an increase in catalyst specific activity (and for a given catalyst loading, reaction rate) of ca. 60%. Specific activity also increased with decreasing diameter of the B23/alginate beads (measured over a range of 3.5 mm–2.0 mm diameter), indicating that the reaction rate was limited at least in part by the rate of diffusion of ADN into the alginate bead.

B23/alginate beads (ca. 2.5 mm dia.) were used as catalyst for the production of either 0.75 or 1.5 M 5CVAM in up to 60 consecutive batch reactions with catalyst recycle at 5°C and in 23 mM butyrate/5 mM calcium chloride (pH 7). Using a 7.5 wt % catalyst charge of B23/alginate beads to produce 0.75 M 5CVAM, the ratio of 5CVAM/ADAM averaged ca. 28:1 at complete conversion of ADN. When a similar set of recycles was run at 10°C, the initial reaction time decreased from 3 to 2 h, but the ratio of 5CVAM/ADAM decreased to ca. 14:1. Repeating the hydration reactions at 5 and 10°C with unimmobilized cells showed no difference in selectivity to 5CVAM at the two temperatures (5CVAM/ADAM of > 100:1). The decrease in 5CVAM selectivity with increasing temperature when using B23/alginate beads was most likely due to a larger increase in the cellular nitrile hydratase activity relative to the increase in the rate of diffusion of 5CVAM out of the alginate beads, which resulted in the production of more ADAM. Recycle reactions to produce 0.75 M 5CVAM were also run at 5°C in the absence of 23 mM sodium butyrate (substituting 20 mM Tris buffer), and the activity of the beads was observed to decrease over time much more rapidly than for recycle reactions run within the presence of butyrate, demonstrating the stabilizing effect of butyrate on enzyme activity.

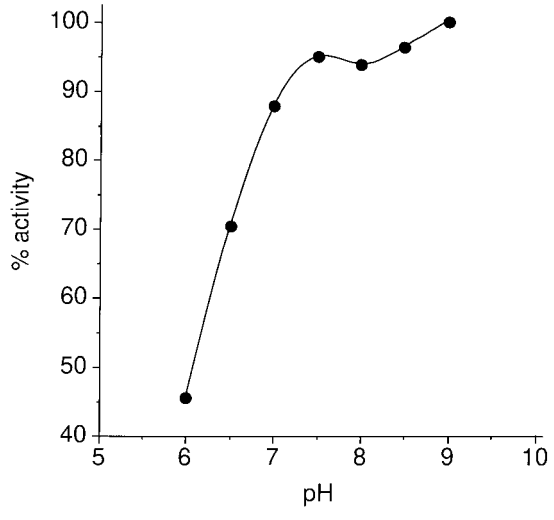

Figure 1. Activity of B23/alginate beads as a function of pH (catalyst activity at pH 9 = 100%). Reactions were run with pH monitoring to produce 0.75 M 5CVAM in two-phase reaction mixtures containing ADN and 7.5% (w/w) B23/alginate beads in 23 mM sodium butyrate/5 mM calcium chloride (pH 6–9) at 5°C.

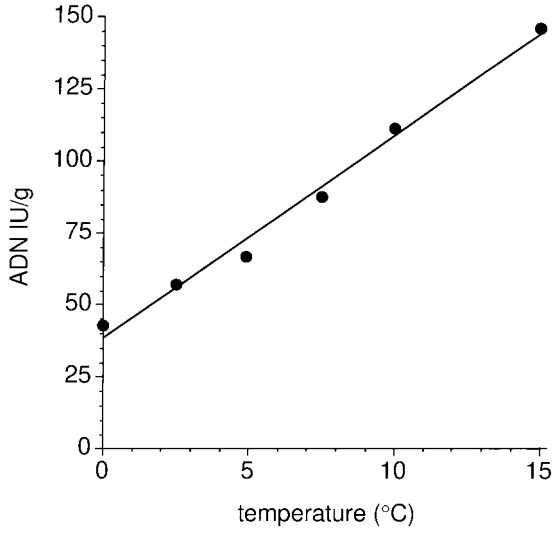

Fig. 2. Dependence of B23/alginate bead specific activity (ADN IU/g) on temperature. Reactions were run to produce 0.75 M 5CVAM in two-phase reaction mixtures containing ADN and 7.5% (w/w) B23/alginate beads in 23 mM sodium butyrate/5 mM calcium chloride (pH 7).

Fifty consecutive batch reactions to produce 1.5 M 5CVAM at 5°C were run using 7.5 wt% B23/alginate catalyst beads; after 50 reactions, the catalyst productivity was 1910 g 5CVAM/g dcw B23 cells. After the first reaction, the final concentration of 5CVAM in subsequent batch reactions exceeded 1.7 M, as consecutive reactions were run by decanting the product mixture from the previous reaction, and adding fresh buffer and ADN to the catalyst and remaining product mixture from the previous batch reaction (ca. 16 wt% of the initial reaction mixture). The ratio of 5CVAM to ADAM with alginate beads made using frozen/thawed B23 cells decreased from 36:1 to ca. 24:1 after the first several catalyst recycles (Fig. 3), while alginate beads prepared using B23 cells that had not undergone a freeze/thaw cycle produced a final ratio of 5CVAM to ADAM of ca. 15:1 under these same conditions. This difference in selectivity was most likely due to an increase in permeability of the cells to 5CVAM, caused by the freezing and thawing of the cells prior to immobilization.[31,32] This significant improvement in 5CVAM selectivity was an additional benefit of storing B23 cell suspensions frozen at −80°C prior to immobilization in alginate beads.

A time course for the production of 1.5 M 5CVAM using B23/alginate beads at 5°C is illustrated in Figure 4; at the reaction endpoint, the yields of 5CVAM and ADAM were 95 and 3.5% respectively, with 1.2% ADN remaining. This reaction was repeated, but the reaction was allowed to continue past the point of complete conversion of ADN until ADAM precipitated from the product mixture; under these reaction conditions, complete conversion of ADN occurred within 4 h, and ADAM was first observed to precipitate in the reaction mixture between 7 and 8 h. The concentration of ADAM reached ca. 110–120 mM before precipitation, which was more than twice the measured solubility limit for ADAM, and may indicate that the product mixture was supersaturated with ADAM. When reactions were run to produce ≥ 1.5 M 5CVAM, no precipitation of ADAM was observed in these reaction mixtures if the catalyst was immediately separated from the product mixture at the end of the reaction. Some B23/alginate beads recovered after use in 60 consecutive batch reactions to produce ≥ 1.5 M 5CVAM had ADAM precipitated within the beads, but a significant decrease in catalyst activity for these catalyst beads was not observed.

As an alternative to running consecutive batch reactions, the use of a fixed-bed packed column containing B23/alginate beads was also examined. Packed-column reactors containing B23/alginate beads were run at 5 and 10°C while feeding 0.40 M ADN (ca. the solubility limit of ADN in reaction buffer). At 5°C, the average yields of 5CVAM and ADAM produced over the first 800 h of continuous operation were 85 and 10%, respectively, with a 95% conversion of ADN (5CVAM: ADAM = 8.5:1); after 800 h, the catalyst began to slowly lose activity. At 10°C, the column began to lose activity after only 430 h, and produced up to 14% ADAM. Because of the lower selectivity to 5CVAM in the fixed-bed column reaction, and the possibility of ADAM precipitation and plugging of the column, the batch reaction was chosen for scale-up.

Commercial-scale preparation of 5CVAM

Scale-up of production of B23/alginate catalyst beads was accomplished using a 7-inch diameter stainless steel "nozzle" equipped with 288 20-gauge hollow stainless steel tubes, 3.5 cm in length. The B23 cell/alginate suspension was pumped at 3 to 7°C through consecutive 230 and 140 μm steel screens to remove particulates, then through the nozzle, and the resulting droplets collected and hardened in 0.20 M calcium chloride/23 mM sodium butyrate buffer for 18 h at 5°C. The catalyst

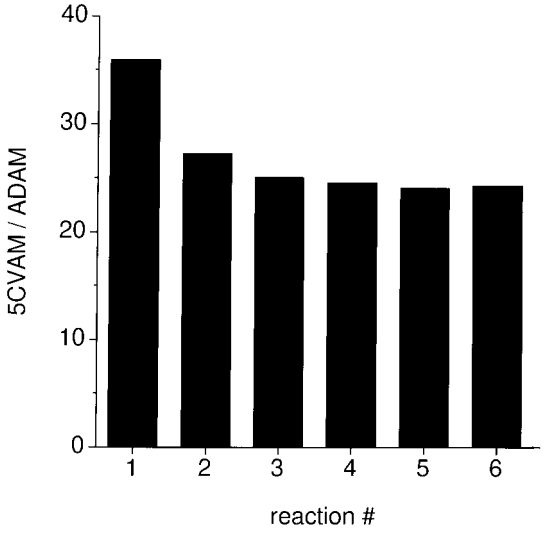

Figure 3. Ratio of 5CVAM/ADAM produced in consecutive batch reactions with recycle of B23/alginate beads. Reactions were run to produce 1.5 M 5CVAM in two-phase reaction mixtures containing ADN and 7.5% (w/w) B23/alginate beads in 23 mM sodium butyrate/5 mM calcium chloride (pH 7.0) at 5°C.

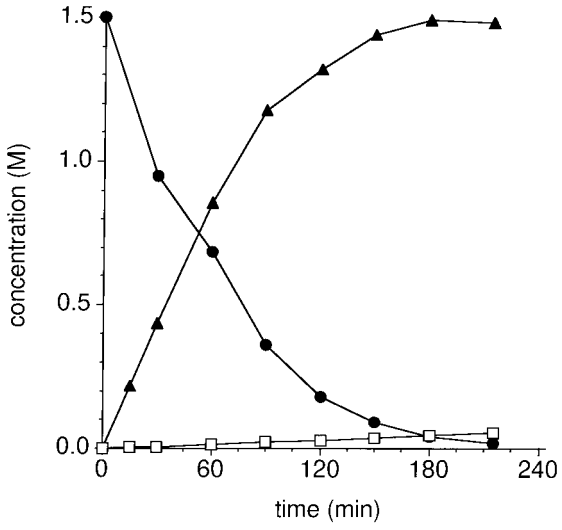

Figure 4. Time course for the production of 1.5 M 5CVAM in a two-phase reaction mixture containing ADN and 7.5% (w/w) B23/alginate beads in 23 mM sodium butyrate/5 mM calcium chloride (pH 7.0) at 5°C: ADN (●), 5CVAM (▲), ADAM (□).

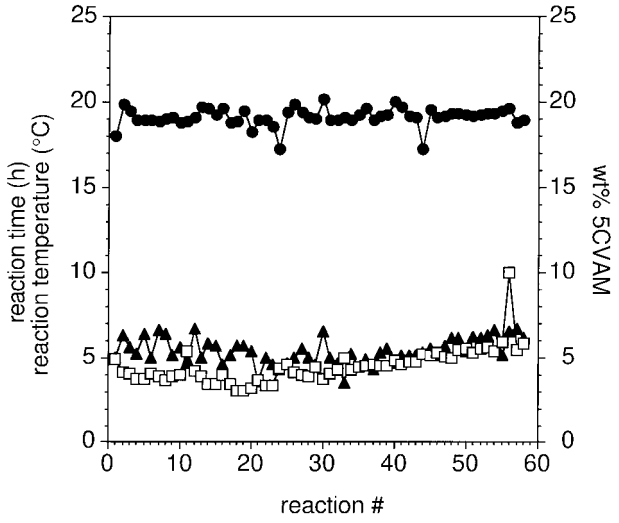

Figure 5. Reaction time (□), average reaction temperature (▲), and weight % 5CVAM (●) for 58 consecutive batch reactions with catalyst recycle to produce at total of 13.6 metric tons of 5CVAM. Reactions were run in two-phase reaction mixtures containing ADN and 5.8% (final weight catalyst/weight reaction mixture) B23/alginate beads in 23 mM sodium butyrate/5 mM calcium chloride (pH 7) at 5°C.

beads were washed twice with 23 mM butyrate/5 mM calcium chloride buffer and stored in this same buffer at 5°C until needed.

The first commercial-scale production of 5CVAM using B23/alginate beads was performed by converting a total of 12.7 metric tons of ADN in 58 consecutive 400-gallon batch reactions with catalyst recycle. The reaction time, average reaction temperature, and wt%5CVAM produced in the reactions are illustrated in Figure 5. At 97% overall conversion of ADN, the combined yield of recovered 5CVAM was 13.6 metric tons (93% yield). The catalyst productivity was 3150 kg of 5CVAM produced per kg of B23 cells (dcw). The total weight of product mixture produced was 70.7 metric tons, which contained 19.2 wt% 5CVAM, 0.99 wt% ADAM, and 0.52 wt% ADN. The product was isolated from the reaction mixture by removal of water by distillation, then dissolution of the resulting oil at >65°C in methanol, which precipitated the byproduct ADAM as well as calcium and butyrate salts. The methanolic 5CVAM solution was used directly in the next process step for herbicide synthesis. The reaction time for reaction number 56 (Fig. 5) was allowed to extend for 4 h past the endpoint of the reaction, then two more reactions were run to determine if the increased production and precipitation of ADAM in reaction number 56 produced a loss of catalyst activity in subsequent reactions; no increase in reaction time for the final two batch reactions was observed.

Conclusions

A biocatalytic process for the highly regioselective hydration of ADN to 5CVAM has been demonstrated using *P. chlororaphis* B23 cells immobilized in calcium alginate beads. The reaction was run to higher conversion, produced more product per weight of catalyst, and generated significantly smaller amounts of byproducts and waste products than alternate chemical processes which were examined. In particular, the ratio of catalyst waste to 5CVAM produced in the process was very low; only 0.006 kg of catalyst waste was produced per kg 5CVAM, and the catalyst waste was 93% by weight water. The reaction produced ca. 190 g 5CVAM per liter of reaction volume, and purification and recovery of the product for use in the next process step was readily performed.

Experimental

Materials and methods

All materials were obtained from commercial sources and used as received unless otherwise noted. Water used in catalyst preparation and reactions was distilled and deionized. Sodium alginate (Protonal® LF 10/60) was obtained from Pronova Biopolymers. *Pseudomonas chlororaphis* B23 cell suspensions obtained from the Nitto Chemical Industry Co. were stored frozen at −80°C. Wet cell weights of whole cell 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 lyophilization of wet cells, and the ratio of dry cell weight to wet cell weight for all cells was typically 0.25:1. Reaction temperatures were thermostated using recirculating temperature baths. The percent recovery of ADN and the percent yields of ADAM and 5CVAM were based on the initial concentration of ADN present in the reaction mixture, and was determined by HPLC using refractive index detection and a Supelcosil LC-18 DB column (25 cm×4.6 mm dia.) with 20 mM Tris buffer at pH 7.5 in 2.5% methanol/water as mobile phase.

Nitrile hydratase assay of B23 cells. Into a 20 mL glass vial equipped with magnetic stir bar was added 10 mL of an aqueous solution containing 0.20 M adiponitrile, 23 mM sodium butyrate, and 50 mM potassium phosphate (pH 7) at 25°C. With stirring, 0.100 mL of a B23 cell suspension (10.0 mg wet cell wt/mL) in an aqueous buffer containing 23 mM sodium butyrate and 50 mM potassium phosphate (pH 7) at 5°C was added to the vial. After 15 min, a 0.200 mL aliquot of the reaction mixture was added to 20 µl of 1 N HCl (to stop the reaction) and 0.200 mL of 20 mM *N*-methylpropionamide in water (HPLC standard) in a 1.5 mL microcentrifuge tube. The contents of the tube were mixed and then centrifuged to pellet the cells, then the supernatant was analyzed for 5CVAM by HPLC. The specific activity (IU/g) of the B23 cells was then calculated from the reaction rate in micromoles 5CVAM per minute and the weight of B23 cells employed in the assay.

Preparation of *P. chlororaphis* B23/calcium alginate beads. To a 250 mL media bottle was added 90.2 mL of 38 mM sodium butyrate (pH 7), and the bottle placed in water bath at 25°C. Using an overhead stirrer, 4.125 g

of sodium alginate was slowly added to the bottle with stirring, and the resulting mixture heated briefly to 80°C with stirring to dissolve the alginate. The resulting solution was cooled to 5°C, then 55.7 g of frozen B23 cell suspension (53.8% wet cell wt) was slowly added with stirring to the alginate solution at 5°C. After the cells were fully dispersed and suspended in the mixture at 5°C, the cell suspension was degassed under partial vacuum for ca. 1 h at 5°C. The mixture was then pumped at 2 mL/min through consecutive 230 and 140 μm steel mesh filters to remove particulates, then through a 20 gauge needle (5 mm length, blunt tip) while maintaining the temperature of the mixture at 3–7°C. The resulting droplets of B23 cell/alginate suspension produced by the needle were collected in a 1 L beaker containing 488 mL of a stirred aqueous hardening solution containing 0.20 M calcium chloride and 23 mM sodium butyrate (pH 7) at 5°C. A distance of at least 20 cm between the needle tip and the hardening solution was required for the formation of spherical gel beads. After completion of the addition, the resulting B23/alginate beads were stirred in the hardening solution at 5°C for 2 h, then the hardening solution was replaced with an additional 488 mL of hardening solution at 5°C and the bead suspension mixed an additional 16 h at 5°C. The catalyst beads were washed three times for 15 min with 5 mM calcium chloride, 23 mM sodium butyrate at 5°C, and stored in this same buffer at 5°C until ready for use (final bead diameter: 2.5–3.0 mm).

Assay of B23/alginate beads. Into a 50 mL Erlenmeyer flask equipped with magnetic stir bar was weighed 1.88 g of B23/alginate beads and 20.98 mL of aqueous buffer containing 23 mM sodium butyrate and 5 mM calcium chloride (pH 7) at 5°C. To the flask was then added 2.15 mL (2.048 g, 18.9 mmol) of adiponitrile, and the resulting mixture stirred at 5°C in a thermostated temperature bath. Aliquots (0.100 mL) of the reaction were removed at 1, 5, 10 and 15 min and immediately mixed with 5 μL of 2 N HCl (to stop the reaction) and 0.100 mL of 0.20 M *N*-methylpropionamide (HPLC standard), and the resulting solution analyzed by HPLC for 5-cyanovaleramide. The specific activity (IU/g) of the B23/alginate beads was then calculated from the reaction rate (micromoles 5CVAM per min) and the weight of catalyst beads employed in the assay.

Recycle reactions using B23/alginate beads. Into a 125 mL Erlenmeyer flask equipped with magnetic stir bar was placed 7.5 g of B23/alginate beads, 75.3 mL of aqueous buffer containing 23 mM sodium butyrate and 5 mM calcium chloride (pH 7) at 5°C, and 17.2 mL (16.49 g, 0.152 mol) of adiponitrile. The resulting mixture was stirred at 5°C in a thermostated temperature bath. The progress of the reaction was monitored by removal of 0.100 mL aliquots of reaction mixture, which were mixed with 5 μL of 1.0 N HCl (to stop the rxn) and 0.100 mL of 0.20 M *N*-methylpropionamide (HPLC standard), and then analyzed by HPLC. When the conversion of adiponitrile was at least 97%, 83.25 mL of the product mixture was decanted and 66.05 mL of reaction buffer at 5°C and 17.2 mL (16.49 g) of adiponitrile was added and the reaction repeated. After the desired number of recycle reactions were completed, the final product mixture was separated from the catalyst beads by filtration, and the catalyst beads washed twice by stirring with reaction buffer for 15 min at 5°C. The catalyst was then stored in reaction buffer at 5°C until the next set of recycle reactions were started.

Scale-up of B23/alginate bead preparation and 5-cyanovaleramide production. Into a 100-gallon reactor was charged 140 L of 23 mM sodium butyrate buffer (pH 6.8–7.2), then 6.42 kg of sodium alginate was added and the resulting mixture heated to 80°C for 1 h with stirring. The resulting solution was cooled to 10°C, then 87.2 kg of frozen *P. chlororaphis* B23 cell slurry (54% wcw/slurry weight) was added with stirring while maintaining the temperature between 3 and 7°C. After the final addition of frozen cell slurry, the suspension was stirred until all the cells were suspended, then the cell suspension was degassed under partial vacuum for an additional 2 h. The resulting cell slurry was maintained at 3–7°C while pumping through a 230 and 140 μm filter, then through a 7-inch diameter stainless steel nozzle equipped with 288 20-gauge hollow stainless steel tubes (3.5 cm in length) attached to the top of a 300-gallon reactor containing 757 L of 0.20 M calcium chloride/23 mM sodium butyrate hardening buffer at 5°C. After the dropwise addition of the cell slurry was complete, the resulting B23 alginate beads were stirred for an additional 2 h, then the stirring was stopped and the hardening buffer decanted. An equivalent volume of fresh hardening buffer at 5°C was added to the catalyst beads and the mixture stirred an additional 16 h. The hardening buffer was again decanted, and the catalyst beads washed twice with ca. 400 L of 23 mM sodium butyrate/5 mM calcium chloride buffer (pH 7.0) at 5°C to yield 201 kg of B23/alginate beads. The beads were stored in the wash buffer at 5°C until needed.

Into a 500-gallon reactor was charged 1007 L of 23 mM sodium butyrate/5 mM calcium chloride buffer (pH 7) at 5°C and 55.4 kg of B23/alginate beads, followed by 218 kg (2015 mol) of adiponitrile. The resulting mixture was stirred at 5°C under nitrogen, and samples were withdrawn at regular intervals and analyzed by HPLC to determine the progress of the reaction. When the conversion of adiponitrile was $\geq$97% (ca. 4 h initial reaction time), the stirring was stopped and ca. 90% of the product mixture was decanted. The reactor was immediately charged with 1007 L of reaction buffer and 218 kg (2015 mol) of adiponitrile, and the reaction repeated. After running 13 consecutive batch reactions to establish a reaction baseline, a final additional 20.4 kg of B23/alginate beads was added to the fourteenth consecutive batch reaction (5.8 wt % final catalyst weight/weight reaction mixture). Each decanted product mixture was briefly heated to 40°C to deactivate any catalyst activity that was present. Fifty-eight consecutive batch reactions produced 13.6 metric tons of 5CVAM, and the yields of 5-cyanovaleramide and adipamide from the combined product mixtures were 93 and 4%, respectively, with 3% recovery of unconverted adiponitrile.

Isolation and purification of 5-cyanovaleramide. A 2 L sample of product mixture from a commercial-scale reaction (190 g 5-cyanovaleramide/L) was continuously extracted with 1.5 L of ethyl acetate. The ethyl acetate fraction was then concentrated by rotary evaporation to produce ca. 696 g of a wet white solid, which was not dried further to remove remaining ethyl acetate, but was divided into two equal portions by weight. Each portion was dissolved in a solution of 200 mL of methanol and 800 mL of toluene and heated to 60°C in a water bath with stirring. Undissolved solids were removed from the resulting warm solution by filtration, and the filtrate was then cooled to 5°C. Addition of 1.4 L of ethyl ether to the cold filtrate with stirring immediately precipitated a white solid, which was collected by vacuum filtration, and washed with two 200 mL portions of cold (5°C) ethyl ether. The combined ether precipitates were combined and dried to constant weight under vacuum to yield 376 g (99% yield) of 99% pure 5CVAM (0.7% ADAM and 0.3% ADN by HPLC). A portion of this 5CVAM was further purified by continuous extraction at 100°C with toluene, followed by separation of the toluene fraction and concentration by rotary evaporation. The resulting white solid was recrystallized from ethyl acetate at −20°C to yield purified 5CVAM: mp 66.0–66.9°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.24 (bs, 1H), 5.99 (bs, 1H), 2.40 (t, $J = 6.7$ Hz, 2H), 2.28 (t, $J = 6.9$ Hz, 2H),1.85–1.67 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.8, 119.6, 34.6, 24.9, 24.4, 17.0; IR 3414, 3217, 2959, 2885, 2245, 1677, 1621 cm^{-1}; MS (EI) m/z 127 (MH^+, 7), 110 (4), 86 (9), 82 (11), 59 (100), 54 (31); HRMS calcd for $C_6H_{10}N_2O$ (MH^+) 127.0871, found 127.0871.

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