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Optimization of an Immobilized-Cell Biocatalyst for Production of 4-Cyanopentanoic Acid

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Abstract:

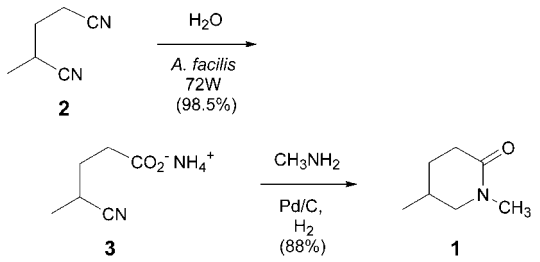
Optimization of microbial cell immobilization, catalyst specific activity, and volumetric productivity were required for scale-up of the nitrilase-catalyzed hydrolysis of 2-methylglutaronitrile to 4-cyanopentanoic acid, an intermediate in the preparation of 1,5-dimethyl-2-piperidone. As an alternative to the immobilization of *Acidovorax facilis* 72W cells in carrageenan, immobilization in alginate, followed by cross-linking with glutaraldehyde and polyethylenimine, produced a catalyst which was stable in reaction mixtures containing high concentrations of 4-cyanopentanoic acid ammonium salt. Immobilization in alginate produced catalysts with a higher nitrilase specific activity than was achieved in carrageenan, and volumetric productivity of 4-cyanopentanoic acid was increased from 19 to 49 g/L/h. Substituting alginate for carrageenan also eliminated one process step in the immobilization. A further increase in volumetric productivity to 79 g/L/h was achieved by using an immobilized *Escherichia coli* transformant which expresses *A. facilis* 72W nitrilase.

Introduction

1,5-Dimethyl-2-piperidone (**1**, Xolvone) is a precision cleaning solvent currently in commercial development by DuPont for use in a variety of industrial applications, including electronics cleaning, photoresist stripping, industrial degreasing and metal cleaning, and resin cleanup; it can also be used in the formulation of inks and industrial adhesives and as a reaction solvent for the production of polymers and chemicals. Lactam **1** is not flammable, is completely miscible with water, has a good toxicological profile, and is readily biodegradable.

The first commercial production of Xolvone employed a direct hydrogenation of 2-methylglutaronitrile (**2**) in the presence of methylamine and produced a mixture of 1,3- and 1,5-dimethyl-2-piperidones.[1] A chemoenzymatic process for production of **1** has now been developed[2] and is scheduled to replace the current chemical process. In a first step, the nitrilase activity of immobilized *Acidovorax facilis* 72W cells

Scheme 1



is used to convert **2** to 4-cyanopentanoic acid (**3**) (as the ammonium salt) with greater than 98% regioselectivity at 100% conversion. Without further purification, the aqueous solution of **3** is subsequently concentrated by distillation, methylamine is added, and **3** is hydrogenated to produce **1** in 88% yield (Scheme 1). The chemoenzymatic process produces a single geometric isomer of dimethyl-2-piperidone with a higher boiling point than the mixture of geometric isomers produced in the chemical process, and in higher yield with less byproduct formation.

The initial piloting of the chemoenzymatic process for commercial production of **1** revealed the need for several process improvements, including optimization of microbial cell immobilization and increasing volumetric productivity for the production of **3**.

Results and Discussion

Scale-Up of Cell Immobilization using Carrageenan. Immobilization of *A. facilis* 72W protects the cells from lysis, stabilizes the nitrilase activity under reaction conditions, and allows for facile separation of the catalyst from the product mixture for recycling in subsequent reactions.[2] After several methods of cell immobilization were screened, carrageenan[3] was initially chosen as an immobilization matrix. Operational stability of the immobilized cells was further increased by subsequent treatment of the catalyst beads with the cross-linking agents glutaraldehyde (GA) and polyethylenimine (PEI).[4] Nitrilase enzymes function by the nucleophilic attack

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(1) (a) Kosak, J. R. U.S. Patent 5,449,780, 1995. (b) Herkes, F. E. U.S. Patent 5,808,078, 1998.

(2) Cooling, F. B.; Fager, S. K.; Fallon, R. D.; Folsom, P. W.; Gallagher, F. G.; Gavagan, J. E.; Hann, E. C.; Herkes, F. E.; Phillips, R. L.; Sigmund, A.; Wagner, L. W.; Wu, W.; DiCosimo, R. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 295.

(3) (a) Chibata, I.; Tosa, T.; Sato, T.; Takata, I. *Methods Enzymol.* **1987**, *135*, 189. (b) Scott, C. D. *Ann. N.Y. Acad. Sci.* **1987**, *501* (Enzyme Eng. 8), 487.

(4) (a) Calik, G.; Savasci, H.; Calik, P.; Ozdamar, T. H. *Enzyme Microb. Technol.* **1999**, *24*, 67. (b) Park, Y.-M.; Rhee, S.-K.; Choi, E.-S.; Chung, I. S. *J. Microbiol. Biotechnol.* **1998**, *8*, 696. (c) Bahulekar, R.; Ayyangar, N. R.; Ponrathnam, S. *Enzyme Microb. Technol.* **1991**, *13*, 858. (d) Chao, K. C.; Haugen, M. M.; Royer, G. P. *Biotechnol. Bioeng.* **1986**, *28*, 1289. (e) Takata, I.; Kayashima, K.; Tosa, T.; Chibata, I. *J. Ferment. Technol.* **1982**, *60*, 431. (f) Tosa, T.; Sato, T.; Mori, T.; Yamamoto, K.; Takata, I.; Nishida, Y.; Chibata, I. *Biotechnol. Bioeng.* **1979**, *21*, 1697.

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on the nitrile carbon by an activated thiol residue,[5] and it has been reported that thiol-containing enzymes are inactivated by glutaraldehyde.[6] There was no significant loss of nitrilase activity due to the GA/PEI cross-linking of the immobilized cells, despite the fact that glutaraldehyde was shown to inactivate the isolated *A. facilis* 72W nitrilase enzyme. The resulting cross-linked catalyst was robust and maintained excellent physical integrity, as well as nitrilase activity, under reaction conditions which produced up to 240 g/L of **3**.

Piloting of cell immobilization in carrageenan to produce several hundred kilograms of catalyst revealed this process could be problematic on a large scale.[7] Impurities in cell suspensions produced by dewatering fermentation broth, a more economical method of cell isolation than batch centrifugation to produce cell paste, often resulted in catalyst beads having lower-than-expected gel strength. Carrageenans which were selected for high gel strength typically had gelling temperatures above 45 °C, which required that a short residence time (4−5 h) at elevated temperature be employed during immobilization to maintain nitrilase activity of the cells. In-line heating and mixing of carrageenan solution and cell suspension could be employed, but required special equipment and control of process temperature between 45 and 50 °C so as not to prematurely gel the cell/carrageenan suspension or inactivate the nitrilase activity. The cell/carrageenan suspension was also sensitive to shear during gelation, which was induced by a decrease in temperature of the suspension, and cross-linking of the carrageenan by potassium ion. The formation of catalyst beads by extrusion of the heated cell/carrageenan suspension from a multiorifice die into an aqueous solution of potassium chloride or bicarbonate at 25 °C required low feed rates (<0.5 lb per orifice per hour), a short drop height into the gelling solution, and low-shear mixing of the resulting bead suspension until gelation of the outermost layer of the beads was complete. The requirements for strict temperature control, short processing times, and the low volumetric productivity of the immobilization process led us to substitute alginate for carrageenan in the immobilization process.

Immobilization of *A. facilis* 72W in Alginate. Alginate (FMC Protanal LF 10/60) is used by DuPont for the immobilization of a microbial nitrile hydratase, which is employed in a commercial process for the hydration of adiponitrile to 5-cyanovaleramide.[8] Immobilization in alginate[9] can typically be performed between 5 and 35 °C, which is beneficial for maintaining the nitrilase activity of *A. facilis* 72W, and premature gelation of the mixture due to an unexpected drop in temperature can be avoided. Alginate/cell suspensions are not as shear-sensitive as carrageenan/cell suspensions, and much higher production rates of catalyst beads can be achieved; the reaction of alginate with calcium ion in the gelling solution rapidly cross-links the alginate/cell suspension to produce a gel, unlike carrageenan, where gelation rates are much slower.

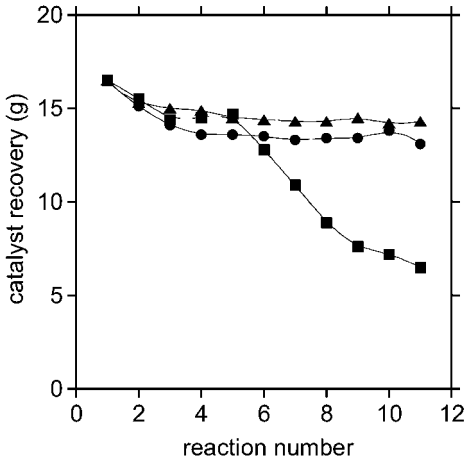

Figure 1. **Effect of calcium ion concentration on recovery of GA/PEI-cross-linked *A. facilis* 72W (5% dcw)/alginate catalyst. Initial catalyst charge of 16.5 g of catalyst in 100 g of total reaction mass to convert 1.25 M 2 to 3 at 30 °C. Calcium acetate concentration: (▲) 5 mM, (●) 2 mM, (■) 0 mM.**

Alginate was initially not chosen for the present process because calcium-cross-linked alginate gels are unstable in the presence of high concentrations of ammonium ion; it has been recommended not to exceed a ratio of ammonium/calcium ion of 20:1 or 25:1,[10] whereas the concentration of ammonium ion can range from 1.5 to 2.0 M during the production of **3**.[2] After cross-linking with GA and PEI,[4,11] the alginate gel enzyme catalyst was found to retain its physical integrity when recycled in consecutive batch reactions to produce high concentrations of **3**, but only when calcium ion was added to batch recycle reactions (Figure 1). Recycle reactions were run at calcium acetate concentrations of 5, 3, 2, 1, 0.5, or 0.1 mM, and the catalyst beads at calcium ion concentration less than 2 mM began to disintegrate after several recycle reactions. When using a minimum calcium ion concentration of 2 mM, the ratio of ammonium ion (produced by the hydrolysis of **2**) to calcium ion is typically at least 750:1. Cross-linking 72W/alginate beads with GA/PEI also stabilizes nitrilase activity in

(5) Cowan, D.; Cramp, R.; Pereira, R.; Graham, D.; Almatawah, Q. *Extremophiles* **1998**, *2*, 207.
(6) (a) Gestrelius, S. M. U.S. Patent 4,288,552, 1981. (b) Lantero, O. J., Jr. U.S. Patent 4,355,105, 1982.
(7) Hennessey, S. M.; DiCosimo, R. Manuscript in preparation.
(8) (a) DiCosimo, R.; Hann, E. C.; Eisenberg, A.; Fager, S. K.; Perkins, N. E.; Gallagher, F. G.; Cooper, S. M.; Gavagan, J. E.; Stieglitz, B.; Hennessey, S. M. *Green Chemical Syntheses and Processes*; ACS Symposium Series 767; American Chemical Society: Washington: DC, 2000; p 114. (b) Hann, E. C.; Eisenberg, A.; Fager, S. K.; Perkins, N. E.; Gallagher, F. G.; Cooper, S. M.; Gavagan, J. E.; Stieglitz, B.; Hennessey, S. M.; DiCosimo, R. *Bioorg. Med. Chem.* **1999**, *7*, 2239.
(9) (a) Blandino, A.; Macias, M.; Cantero, D. J. *Biosci. Bioeng.* **1999**, *88*, 686. (b) Ogbonna, J. C.; Amano, Y.; Nakamura, K. *J. Ferment. Bioeng.* **1989**, *67*, 92. (c) Martinsen, A.; Skjaak-Braek, G.; Smidsroed, O. *Biotechnol. Bioeng.* **1989**, *33*, 79. (d) Bucke, C. *Methods Enzymol.* **1987**, *135*, 175. (e) Klein, J.; Stock, J.; Vorlop, K. D. *Eur. J. Appl. Microbiol. Biotechnol.* **1983**, *18*, 86. (f) Cheetham, P. S. J.; Blunt, K. W.; Bucke, C. *Biotechnol. Bioeng.* **1979**, *21*, 2155. (g) Cheetham, P. S. J. *Enzyme Microb. Technol.* **1979**, *13*, 183.
(10) (a) Klein, J.; Vorlop, K. D. In *Biotechnology Focus I*; Finn, R. F., Ed.; Oxford University Press: New York, 1998; pp 325−336. (b) Smidsroed, O.; Skjaak-Braek, G. *Trends Biotechnol.* **1990**, *8*, 71.
(11) Birnbaum, S.; Pendleton, R.; Larsson, P.-O.; Mosbach, K. *Biotechnol. Lett.* **1981**, *3*, 393.

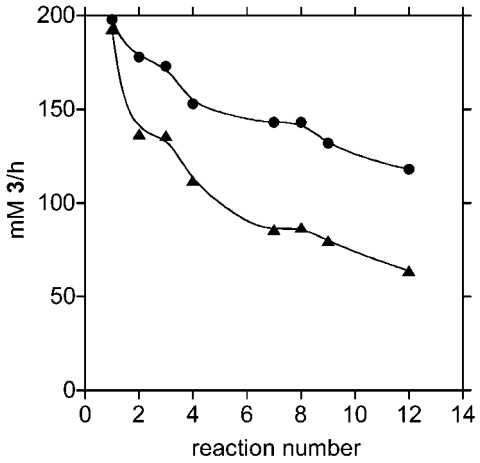

Figure 2. **Effect of GA/PEI-cross-linking on stability of *A. facilis* 72W (5% dcw)/alginate catalyst nitrilase activity. Initial catalyst charge of 16.5 g of catalyst in 100 g of total reaction mass to convert 1.25 M 2 to 3 at 30 °C (5 mM calcium acetate): (▲) no GA/PEI cross-linking, (●) GA/PEI cross-linking.**

consecutive batch reactions (Figure 2); these reactions were run with 5 mM calcium ion added to each reaction, and the loss in nitrilase activity was not due to a loss of catalyst in consecutive reactions due to catalyst dissolution.

Inactivation of *A. facilis* 72W Nitrile Hydratase Activity. Dinitrile **2** is converted with greater than 98% regioselectivity to the ω-cyanocarboxylic acid **3** (as the ammonium salt) by the aliphatic nitrilase (EC 3.5.5.7) activity of *A. facilis* 72W.[12] This biocatalyst also contains an undesirable, nonregioselective nitrile hydratase (EC 4.2.1.84) and an amidase (EC 3.5.1.4) which together can convert **2** to 2-methylglutaric acid (**4**). Prior to immobilization, the nitrile hydratase and amidase were inactivated by heating an aqueous suspension of the cells at 50 °C for 20−60 min without measurable inactivation of the thermostable nitrilase.[2,13] When immobilizing the cells in carrageenan, this heating of cell suspensions to 50 °C was also required to mix the cell suspension with a carrageenan solution at a temperature above the gelling temperature of the carrageenan (45 °C).

When *A. facilis* 72W cells were immobilized in alginate at 25 °C and the resulting immobilized cell catalyst cross-linked with GA and PEI, the nitrile hydratase activity was unexpectedly found to be selectively and completely inactivated without producing a measurable loss of nitrilase activity (Table 1). The heat-treatment step normally required to inactivate the nitrile hydratase activity prior to immobilization was no longer required.

Improving Immobilized-Cell Specific Activity and Volumetric Productivity Using *A. facilis* 72W. By immobilizing *A. facilis* 72W cells in alginate instead of carrageenan, a significant increase in both catalyst specific activity and reactor productivity was obtained. Increasing the % dry cell weight (dcw) in carrageenan/cell beads from

Table 1. **Remaining nitrile hydratase activity of *A. facilis* 72W after immobilization in alginate and GA/PEI cross-linking**

catalyst	cells heated at 50 °C[a]	GA/PEI cross-linking	% nitrile hydratase activity[b]
A. facilis 72W cells	no	no	34.0
A. facilis 72W cells	yes	no	1.5
72W/alginate beads	no	no	3.6
72W/alginate beads	no	GA only	1.7
72W/alginate beads	no	GA and PEI	1.5

[a] Cell suspension (11.5% dcw) in 0.15 M aqueous sodium acetate heated for 15 min. [b] Nitrile hydratase activity as a percentage of nitrilase activity, measured as the rate of production of **4** relative to **3**; **4** is produced at 1.5% the rate of **3** by *A. facilis* 72W nitrilase.

Table 2. **Dependence of immobilized *A. facilis* 72W specific activity on % dry cell weight in alginate and carrageenan catalyst gel beads**

rxn temp (°C)	gel bead	% gel[a]	% dcw in bead	wt % cat.[b]	rate[b] (mM **3**/h)	specific activity (mmol **3**/h/g cat.)
30	alginate	2.75	5.0	16.5	213	1.29
30	alginate	2.75	7.5	16.5	301	1.82
30	alginate	2.75	7.5	22.0	377	1.71
35	alginate	2.75	5.0	16.5	272	1.64
35	alginate	2.75	7.5	22.0	663	3.01
30	carrageenan	3.00	5.0	16.5	200	1.21
30	carrageenan	3.00	7.5	16.5	213	1.29
35	carrageenan	2.25	5.0	16.5	359	2.17
35	carrageenan	2.25	7.5	16.5	283	1.72

[a] Dry wt % alginate or carrageenan in catalyst gel bead. [b] Catalyst bead (wt %) in total reaction mass to convert 1.25 M **2** to **3**; reactions using 72W/alginate catalyst included 2 mM calcium acetate in reaction mixture.

5 to 7.5% (a 50% increase) resulted in only a 10% increase in reaction rate to produce **3** from **2** at 30 °C, but the same increase in dcw in alginate/cell beads produced a 41% increase in rate; similar results were obtained at 35 °C (Table 2). To increase the volumetric productivity for **3** from an average value of 19 g/L/h over the course of 55 recycle reactions, the % dcw in catalyst beads, catalyst loading, and reaction temperature were each optimized. Increasing % dcw from 5 to 7.5% at 30 °C produced an average volumetric productivity of 37 g/L/h over 40 recycle reactions. Additionally increasing the catalyst loading (from 165 to 220 g/L) and reaction temperature (from 30 to 35 °C) further increased the average volumetric productivity for **3** to 49 g/L/h over 78 recycle reactions. The gradual loss of catalyst activity over the course of recycle reactions at the higher % dcw and catalyst loadings mirrors the rate of loss in the initial conditions.

Alginate/cell beads prepared using dewatered fermentation broth produced an immobilized cell catalyst which had stability of activity and physical integrity in recycle reactions comparable to that of catalyst produced with cell paste isolated by batch centrifugation; this was not the case for immobilization in carrageenan.

Immobilized *Escherichia coli* Transformant Which Expresses *A. facilis* 72W Nitrilase. Further improvement in catalyst specific activity and volumetric productivity was achieved by successfully expressing the *A. facilis* 72W

(12) Gavagan, J. E.; Fager, S. K.; Fallon, R. D.; Folsom, P. W.; Herkes, F. E.; Eisenberg, A.; Hann, E. C.; DiCosimo, R. *J. Org. Chem.* **1998**, *63*, 4792.
(13) (a) Gavagan, J. E.; DiCosimo, R.; Eisenberg, A.; Fager, S. K.; Folsom, P. W.; Hann, E. C.; Schneider, K. J.; Fallon, R. D. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 654. (b) DiCosimo, R.; Fallon, R. D.; Gavagan, J. E.; Herkes, F. E. U.S. Patent 5,814,508, 1998.

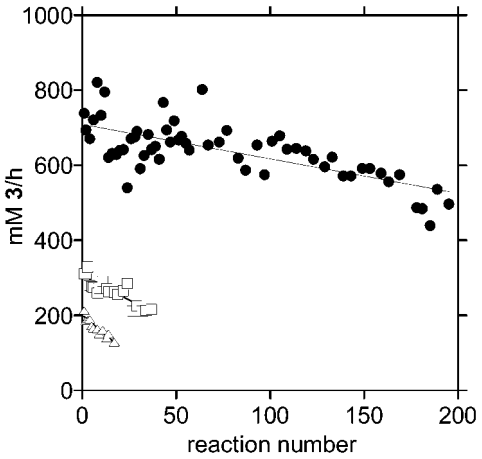

Figure 3. **Relative reaction rates for conversion of 1.25 M 2 to 3 using alginate-immobilized, GA/PEI-cross-linked *A. facilis* 72W or *E. coli* SW91 (30 °C; calcium acetate, 2 mM): (△) *A. facilis* 72W(5% dcw)/alginate (16.5 wt % catalyst); (□) *A. facilis* 72W(7.5% dcw)/alginate (22 wt % catalyst); (●) *E. coli* SW91-(5% dcw)/alginate (16.5 wt % catalyst).**

nitrilase in an *E. coli* transformant[14] and immobilizing the transformant in alginate. Prior to immobilization, cells of *E. coli* transformant strain SW91 were assayed to have a specific activity for hydrolysis of **2** at 25 °C of ca. 662 IU/g dcw, compared to a typical specific activity of 200−225 IU/g dcw for *A. facilis* 72W. Recycle reactions to produce **3** (1.5 M, 216 g/L as the ammonium salt) were run at 30 °C with *E. coli* SW91 cells immobilized at 5% dcw in FMC Protanal LF 10/60 alginate (16.5 wt % catalyst loading); 2 mM calcium acetate was added to each batch reaction to maintain alginate bead integrity. The reaction rate for the first batch reaction was 739 mM **3**/h, and the reaction was complete in less than 2.5 h; the initial volumetric productivity for **3** using this catalyst was 93 g/L/h.

Figure 3 charts the reaction rate for consecutive batch reactions at 30 °C using the 5% dcw *E. coli* SW91/alginate catalyst, relative to reactions using *A. facilis* 72W cells immobilized in alginate at 5 or 7.5% dcw. Two reactions a day were run consecutively with catalyst recycle, and then the catalyst was stored in the product mixture of the second reaction overnight at 5 °C before reuse in additional batch reactions. A total of 195 consecutive batch reactions with catalyst recycle were run with the immobilized *E. coli* SW91 transformant catalyst, producing 3500 g of **3**/g dcw (compared to ca. 1000 g of **3**/g dcw *A. facilis* 72W), and at the end of the series of reactions complete conversion of 162 g of **2**/L was obtained in less than 3 h. The recovered catalyst activity after 195 recycles was 67% of initial activity. The volumetric productivity of the final reaction in this series was 63 g **3**/L/h (497 mM **3**/h), and the average volumetric productivity of the series of reactions was 78 g **3**/L/h, a 4-fold improvement in average volumetric productivity relative to 19 g **3**/L/h obtained using 5% dcw *A. facilis* immobilized in alginate. The use of immobilized *E. coli* transformant cells resulted in a very significant improvement in the overall process to produce **3**, both in immobilization cost and in processing costs, where the reaction can now be run in ca. one-third the time required when using the immobilized *A. facilis* 72W catalyst.

(14) Fallon, R. D.; Payne, M. S.; Chauhan, S.; DiCosimo, R. PCT Int. Appl. WO 0175077A2, 2001.

Conclusions

The development of a commercial biocatalytic process which will initially utilize contract manufacturing often requires that the process be designed to run in existing equipment, and it is highly desirable to avoid new capital expenditures for individual processes. Piloting of the first-generation biocatalytic process to produce intermediate **3** revealed, upon scale-up, several problems related to immobilization, and volumetric productivity for production of **3**, that adversely affected the overall economics of the process. Immobilization in carrageenan required that the process be run with stringent temperature control to avoid loss of catalyst activity and to prevent premature gelation of the catalyst during processing. The shear sensitivity of cell/carrageenan suspensions also limited the volumetric productivity of catalyst production. The carrageenan-immobilized cells had a relatively low specific activity, which limited volumetric productivity for production of **3**.

Significant improvement in overall process economics was first achieved by switching from carrageenan to alginate for cell immobilization. Immobilization of *A. facilis* 72W cells in alginate eliminated the problems of catalyst inactivation, premature gelation, and shear sensitivity during immobilization. Immobilized *A. facilis* 72W catalysts with increased specific activity could be prepared when using alginate, and further optimization of reaction conditions (catalyst loading, temperature) produced a marked increase in volumetric productivity of **3**. Finally, the specific nitrilase activity of the bacterial cells was improved by expressing the *A. facilis* 72W nitrilase in *E. coli* and then immobilizing the resulting transformant and using it as biocatalyst for the production of **3**. The immobilized *E. coli* SW91 transformant proved to be an exceedingly robust catalyst with a significantly improved specific activity; not only was the volumetric productivity of **3** improved by a factor of 4, but the productivity of the transformant catalyst was at least 3500 g of **3**/g dcw, which reduces catalyst cost as a percentage of the total cost of manufacture of **1**. None of the changes to the catalyst or reaction conditions affected the hydrogenation of **3** to **1** in the subsequent process step, where the yield of **1** is typically as high as 88%.

Experimental Section

General. All chemicals were obtained from commercial sources and used as received unless otherwise noted. The isolation and characterization of **3** (as the carboxylic acid) produced by enzymatic hydrolysis of **2** using *A. facilis* 72W has been reported.[12] Isagel RG 300 carrageenan and Protanal LF 10/60 alginate were obtained from FMC. Dinitrile **2** was purified by filtration through a column of basic, activity 1 alumina (5 parts **2**:1 part alumina), or by stirring **2** with 5 wt % basic, activity 1 alumina at 25 °C for 18 h. Water was distilled and deionized. The percent recovery of **2** and the percent yields of **3** and **4** were based on the initial amount

of **2** present in the reaction mixture, and were determined by HPLC using a Supelcosil LC-18 DB column (15 cm × 4.6 mm diameter), 10 mM acetic acid/10 mM sodium acetate in 7.5% methanol/water as mobile phase, and a refractive index detector; *N*-methylpropionamide was employed as external standard. *A. facilis* 72W[12,13] (ATCC 55746) and *E. coli* SW91[14] (ATCC PTA-1175) were prepared as previously described and stored frozen at −80 °C. Wet cell weights of *A. facilis* 72W cells employed in reactions, immobilizations, or assays were obtained from cell pellets prepared by centrifugation of either fermentation broth or cell suspensions in buffer. Dry cell weight (dcw) was determined by lyophilization of wet cells, and the ratio of dcw to wet cell weight (wcw) was typically 0.25. Nitrilase activity of microbial biocatalyst was performed by measuring the rate of conversion of a 0.30 M solution of **2** in 25 mM potassium phosphate buffer containing 12.5 mg dcw biocatalyst/mL at 25 °C.[2] Immobilization of cells in carrageenan to produce 10−100 g quantities of catalyst was performed as previously described.[2]

Immobilization of *A. facilis* 72W or *E. coli* SW91 Cells in Calcium Alginate. Into a 100-mL media bottle equipped with magnetic stir bar and containing 22.9 g of distilled, deionized water at 50 °C was slowly added 1.10 g of FMC BioPolymer Protanal LF 10/60 alginate with rapid stirring. The mixture was heated to 75−80 °C with rapid stirring until the alginate was completely dissolved, and the resulting solution cooled to 25 °C in a water bath. A suspension of 1.84 g (dry cell weight) *A. facilis* 72W in 0.15 M sodium acetate buffer (16 g total weight, pH 7.0) was heated to 50 °C for 15 min, cooled to 25 °C, and then added to the alginate solution at 25 °C with stirring. The cell/alginate mixture was added dropwise by syringe to 213 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 84 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C. With stirring, 0.88 g of 25 wt % glutaraldehyde (GA) in water was added, and the beads were mixed for 1.0 h at 25 °C. To the suspension was then added 3.5 g of 12.5 wt % polyethylenimine (PEI, BASF Lupasol PR971L, average molecular weight ca. 750 000) in water, and the beads were mixed for an additional hour at 25 °C. The cross-linked beads were then washed twice with 84 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C, and stored in this same buffer at 5 °C. Uncross-linked beads were prepared as above except that GA and PEI were not added to the suspension of beads in 0.20 M calcium acetate buffer.

For immobilization of *E. coli* SW91, the above procedure was employed except the cell suspension was not heated to 50 °C prior to immobilization.

Biocatalyst Recycle Reactions for Production of 3. In a typical procedure, a 125-mL jacketed reaction vessel (temperature-controlled at 30 °C with a recirculating temperature bath) was charged with 16.5 g of either uncross-linked or GA/PEI-cross-linked *A. facilis* 72W/alginate beads, or GA/PEI-cross-linked *E. coli* SW91b/alginate beads, 68.25 mL of distilled, deionized water, 1.0 mL of 0.50 M calcium acetate buffer (pH 7.0, 5.0 mM final calcium ion concentration in reaction mixture), and 14.25 mL (13.54 g, 1.25 M) of **2**, and the mixture was stirred at 30 °C. Samples were analyzed by HPLC and the rate of conversion of **2** and rate of production of **3** and **4** measured over time. At complete conversion of **2**, the yields of **3** and **4** were typically 98.5 and 1.5%, respectively. The product mixture was decanted from the catalyst beads, the catalyst weight was measured, and then 1.0 mL of 0.50 M calcium acetate buffer and enough water were added to the catalyst to give a final total weight of 85.75 g. An additional 14.25 mL (13.54 g, 1.25 M) of **2** was added to the catalyst suspension and the reaction repeated. The aqueous product mixtures were combined and concentrated by distillation to 78 wt % **3** (as the ammonium salt), and this concentrate was used directly in the hydrogenation of **3** to **1** without isolation or further purification.[2]

Acknowledgment

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