Production of Acrylamide using Alginate-Immobilized *E. coli* Expressing *Comamonas testosteroni* 5-MGAM-4D Nitrile Hydratase

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Abstract: A thermally-stable nitrile hydratase produced by *Comamonas testosteroni* 5-MGAM-4D has been expressed in *Escherichia coli*, and the alginate-immobilized transformant evaluated as catalyst for the conversion of acrylonitrile to acrylamide. In batch reactions with catalyst recycle, the catalyst productivity decreased with increasing acrylonitrile concentration or reaction temperature, but was relatively insensitive to acrylamide concentration. A total of 206 consecutive batch reactions with catalyst recycle were run at 5 °C and produced 1035 g acrylamide/g dry cell weight and 95 g acrylamide/L; the initial and final volumetric productivities of this series of recycle reactions were 197 and 96 g acrylamide/L/h, respectively. A packed column reactor yielded lower catalyst productivity than consecutive batch recycle reactions, presumably due to contact of the fixed-bed catalyst with a constant high concentration of acrylonitrile.

Keywords: acrylamide; acrylonitrile; catalyst productivity; nitrile hydratase

Introduction

The development of a lab-scale biocatalytic reaction into a commercial process requires the determination of the contribution of volumetric productivity (g product/L/h) and catalyst productivity (g product/g biocatalyst) to the overall cost of manufacture. For the commercial process employing biocatalytic conversion of acrylonitrile to acrylamide, successive generations of nitrile hydratase biocatalysts have continuously improved upon volumetric and catalyst productivities. A variety of bacterial genera are known to possess a diverse spectrum of nitrile hydratase (NHase, EC 4.2.1.84) activities.[1] Many mesophilic NHases are remarkably unstable, having very short enzyme activity half-lives in the growth temperature range of 20–35 °C.[1a,2] Wild-type cells with NHase activity such as *Rhodococcus* sp. N-774,[3] *Pseudomonas chlororaphis* B23,[4] and *Rhodococcus rhodochrous* J1[5] have each been used commercially to convert acrylonitrile to acrylamide. The NHase activities of *Rhodococcus* sp. N-774 and *P. chlororaphis* B23 were not stable above 10 °C, and although the NHase activity of *R. rhodochrous* J1 showed comparatively greater thermal stability, the reaction temperature for commercial production of acrylamide using this biocatalyst was maintained at 10 °C to improve enzyme stability and catalyst productivity [g product/g dry cell weight (dcw)].[5]

In addition to temperature instability, both mesophilic and thermally stable microbial NHase catalysts are susceptible to inactivation by high concentrations of acrylonitrile. An acrylonitrile concentration of 1.5–2 wt % was employed when using *Rhodococcus* sp. N-774 and *P. chlororaphis* B23 catalysts for commercial acrylamide production, while up to 7 wt % acrylonitrile has been employed with *R. rhodochrous* J1.[5] *Bacillus* sp. BR449 expresses a thermostable NHase that undergoes rapid inactivation at an acrylonitrile concentration of only 2 wt % at 22–50 °C.[6] The thermophilic NHase of *Bacillus* spp. RAPc8 lost activity after a short time when used for the continuous conversion of 100 mM acrylonitrile in a column reactor at 40 °C, where low catalyst productivity was attributed to exposure of NHase to acrylonitrile.[7] A comparison of two *Rhodococcus* isolates, one with only a nitrilase activity and one with only a combination of NHase and amidase activities, as catalysts for ammonium acrylate production concluded that the combination of NHase and amidase activities was less preferred due to the susceptibility of both enzymes to deactivation by acrylonitrile, as well as inhibition of the two enzymes by their respective products.[8]

Recombinant organisms expressing exogenous NHase have also been demonstrated to be effective catalysts for the hydration of nitriles. Expression of nitrile hydratase genes isolated from *C. testosteroni* NI1,[9]

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Rhodococcus sp. N-771,[10] *Rhodococcus* sp. N-774,[11] *R. rhodochrous* J1[12] and *P. putida* 5B[13] in *E. coli* has been reported. The NHase of the moderate thermophile *Pseudonocardia thermophila* JCM 3095 has been cloned and expressed in *E. coli* for use in the commercial production of acrylamide.[14] *Comamonas testosteroni* 5-MGAM-4D expresses a thermally stable NHase and amidase, and has been used for conversion of a variety of nitriles to their corresponding carboxylic acids;[15] the NHase has recently been cloned and sequenced, and active NHase has been over-produced in *Escherichia coli* SW132.[16] We now report an examination of this transformant catalyst for the conversion of acrylonitrile to acrylamide, where the dependence of catalyst productivity on reaction temperature and concentration of acrylonitrile and acrylamide has been evaluated.

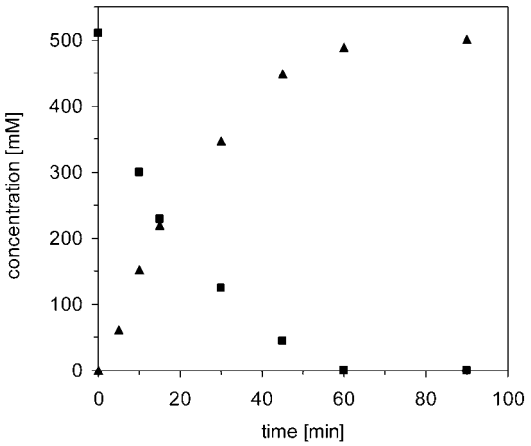

Figure 2. Time course for hydration of 0.51 M acrylonitrile using unimmobilized *E. coli* SW132 cells (0.441 mg dcw/mL) in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C: acrylonitrile (■), acrylamide (▲).

Results and Discussion

The K_m (2.0 mM) and specific activity [31.1 μmol/min/mg dry cell weight (dcw)] were determined for the NHase activity of *E. coli* SW132 cells (4.412 μg dcw/mL) by measuring the rate of acrylamide production over a range of acrylonitrile concentrations of 0.40–80 mM (Figure 1). The microbial K_m was similar to that reported for the NHase of *R. rhodochrous* J1 (1.89 mM), and considerably lower than that of *P. putida* B23 (34.6 mM) and *Brevibacterium* sp. R312 (16.7 mM).[5] Complete conversion of 0.51 M acrylonitrile using unimmobilized cells (0.44 mg dcw/mL) produced a quantitative yield of acrylamide, and no acrylic acid was detected (Figure 2). *E. coli* SW132 cells (7.5% dcw) were immobilized in 2.75 wt % alginate beads (*ca.* 3 mm diameter), which were subsequently cross-linked with glutaraldehyde and polyethyleneimine.[17] Batch reactions for hydration of 0.53, 1.06, 2.05 and 3.04 M acrylonitrile to acrylamide were initially run at 25 °C using a catalyst charge of 20 wt % alginate-immobilized cells in the reaction mixture, and quantitative conversion to acrylamide was observed at each concentration; all reactions additionally contained 2 mM calcium chloride to maintain the integrity of the calcium-cross-linked alginate gel beads. Measurement of reaction rates for hydration of 2–3 M acrylonitrile was initially problematic; the solubility of acrylonitrile in water has been reported to be 74 g/L (1.4 M) at 25 °C,[18] and reactions containing 2–3 M acrylonitrile were initially two-phase mixtures of acrylonitrile and the aqueous catalyst suspension. At these higher concentrations of acrylonitrile, it was also noted that a decrease in total volume of the initially two-phase mixture occurred over the course of the reaction; when using 3 M acrylonitrile, the final volume of the reaction mixture was *ca.* 90% of the initial volume. Dilution of the final reaction mixture to exactly one-tenth of the initial reaction volume allowed for the accurate measurement of the concentration of acrylamide present.

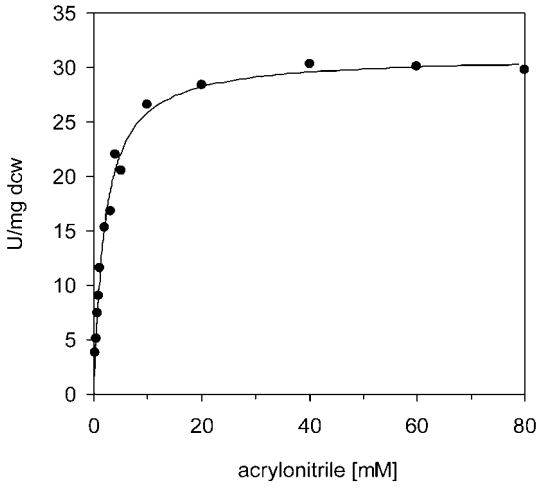

Figure 1. Dependence of specific activity on acrylonitrile concentration for unimmobilized *E. coli* SW132 NHase at 25 °C (4.412 μg dcw/mL).

The thermal stability of *E. coli* SW132 NHase has been previously determined, and the enzyme has a half-life of 3.0 h at 50 °C.[16] Consecutive batch reactions with catalyst recycle were initially performed at 35 °C to determine the effect of acrylonitrile concentration on NHase stability at a higher temperature than is typically employed for commercial production of acrylamide (5–10 °C).[5] In consecutive batch reactions with catalyst recycle, the product mixture was decanted from the catalyst beads, leaving a reaction "heel" containing the catalyst beads and residual product mixture, and the reactor was recharged with acrylonitrile and 2 mM calcium chloride to produce the desired initial concentration of reactant. The catalyst beads were *ca.* 90% water by weight and contained an acrylamide concentration

equivalent to that of the final concentration of acrylamide in the product mixture of the previous batch reaction, so the acrylamide remaining in the reaction heel was carried over into subsequent reactions in a series. For reactions employing a 20 wt % immobilized cell catalyst charge and acrylonitrile concentrations of 1.06 M, 2.05 M and 3.04 M, the final concentrations of acrylamide produced after *ca.* five consecutive batch reactions with catalyst recycle remained constant at *ca.* 1.33 M, 2.56 M and 3.80 M, respectively. For 1.06 M acrylonitrile recycle reactions at 35 °C, the initial volumetric productivity was 737 g acrylamide/L/h, and a total catalyst productivity of 126 g acrylamide/g (dcw) catalyst was obtained over the course of 25 catalyst recycles, with 43% of the initial catalyst activity remaining (Table 1). The recovered catalyst activity in recycle reactions decreased with increasing acrylonitrile concentration (Figure 3), where at 3.04 M acrylonitrile only 5% of the initial catalyst activity remained after four catalyst recycles.

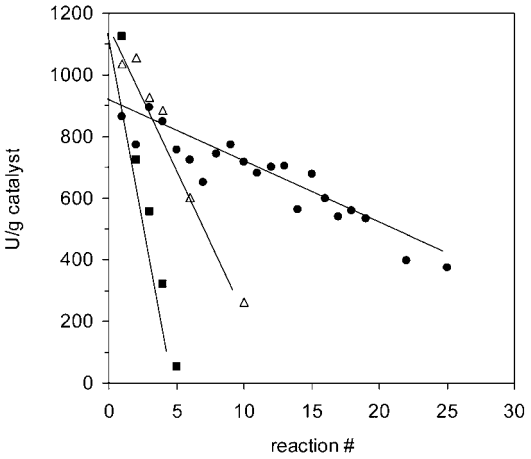

Figure 3. Effect of acrylonitrile concentration on recovered NHase activity of alginate-immobilized *E. coli* SW132 in consecutive batch recycle reactions at 35 °C; acrylonitrile: 1.06 M (●), 2.05 M (△), 3.04 M (■).

A significant improvement in catalyst productivity was achieved by decreasing the reaction temperature. The immobilized-cell NHase activity was measured in consecutive batch reactions with 1 M acrylonitrile at 5 °C and 10 °C using 20 wt % immobilized-cell catalyst (Figure 4). At 5 °C, a total of 206 consecutive batch reactions with catalyst recycle produced 1035 g acrylamide/g (dcw) catalyst and 95 g acrylamide/L. The catalyst activity in the final reaction was 49% of the initial activity, and the initial and final volumetric productivities of this series of reactions were 231 and 96 g acrylamide/L/h, respectively. Although decreasing the reaction temperature from 35 °C to 5 °C resulted in a decrease in reaction rate (and volumetric productivity), the initial and final reaction times in this set of recycle reactions were only 40 minutes and 80 minutes, respectively. When the reaction temperature was increased from 5 °C to 10 °C in a parallel series of reactions, there was a decrease in catalyst productivity (Table 1); after 149 consecutive batch reactions, the recovered NHase activities at 5 °C and 10 °C were 67% and 31% of their respective initial activities.

The immobilized-cell recycle reactions performed at 5 °C (described above) were run over a period of four months, where the catalyst was stored in either product mixture (containing *ca.* 1.33 M acrylamide) or 2 mM calcium chloride (containing *ca.* 0.28 M acrylamide) for periods of up to seven days at 5 °C between reactions; no significant loss of catalyst activity was observed during these storage periods, indicating that concentrations of acrylamide of up to 1.33 M did not result in significant loss of NHase activity over the course of these reactions. The effect of acrylonitrile concentration on recovered

Table 1. Catalyst specific activity, catalyst productivity and volumetric productivity for the conversion of acrylonitrile to acrylamide using alginate-immobilized *E. coli* SW132 in consecutive batch reactions with catalyst recycle.[a]

Temp. [°C]	Acrylonitrile [M]	Initial catalyst specific activity [U/g][b]	Initial volumetric productivity [g/L/h]	Catalyst recycles	Final volumetric productivity [g/L/h]	Remaining catalyst activity [% initial activity]	Total catalyst productivity [g acrylamide/g dry cell wt]
35	1.06	864	737	25	319	43	126
35	2.05	1037	884	10	225	25	97
35	3.04	1124	958	4	275	29	58
25	0.53	447	381				
25	1.06	559	476				
10	1.06	252	215	163	50	32	819
5	1.06	231	197	206	96	49	1035
5	3.04	524	447	19	88	20	274

[a] Reaction conditions: 20-mL reaction volume containing 4.0 g alginate-immobilized *E. coli* SW132 [3 mm diameter beads, 7.5% dry cell weight (dcw), cell lot A] in 2.0 mM calcium chloride.

[b] Specific activity was determined by measuring the rate (μmol/min) of acrylamide production per g dcw *E. coli* SW132 in the reaction mixture.

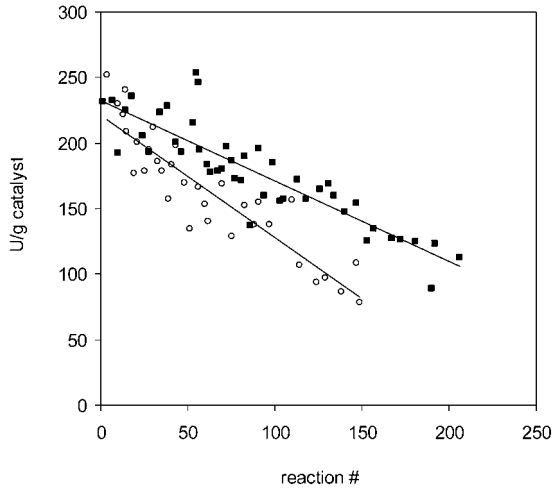

Figure 4. Specific activity of alginate-immobilized *E. coli* SW132 in consecutive 1.06 M acrylonitrile batch reactions with catalyst recycle: 5 °C (■), 10 °C (○).

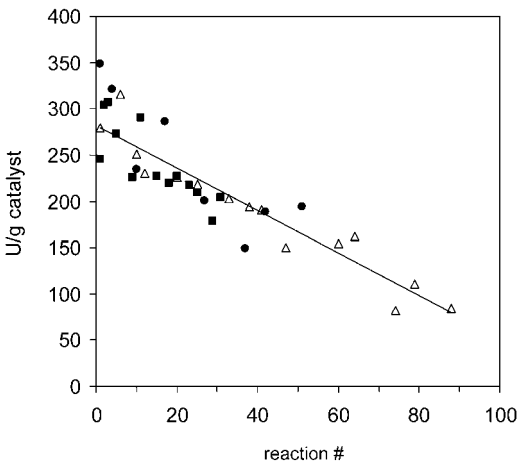

Figure 5. Specific activity of alginate-immobilized *E. coli* SW132 (1-mm diameter catalyst beads) in consecutive 1.06 M acrylonitrile batch reactions with catalyst recycle at 5 °C: no added acrylamide (■), 1.4 M added acrylamide (△, trend line), 2.9 M acrylamide (●).

NHase activity in consecutive batch reactions was also examined at 5 °C where, for reactions employing 3.04 M acrylonitrile, an 80% decrease in catalyst activity occurred after only 19 recycle reactions; increasing the concentration of acrylonitrile from 1.06 M to 3.04 M was deleterious to *E. coli* SW132 NHase activity at either 35 °C or 5 °C.

When using alginate-immobilized microbial cells, volumetric productivity can often be increased by decreasing the diameter of the catalyst bead. The reaction rate may be limited by the rate of diffusion of reactant into the catalyst bead and, if there is product inhibition, by the rate of diffusion of product out of the bead.[19] If the reaction rate is significantly lower than the rate of diffusion of reactant into the bead, no effect on reaction rate would be expected; conversely, where the reaction rate is significantly greater than the rate of diffusion, a dependence of rate on diameter is expected. The reaction rate and bead diffusion rate could also be independently affected by reaction temperature. The 3.0-mm diameter catalyst beads employed in the reactions described above were produced by dripping the cell/alginate suspension from a 20 gauge needle into an aqueous solution containing calcium chloride; the diameter of the resulting beads size is typically a function of the cell/alginate suspension viscosity and surface tension, not the diameter of the needle. Modifying this technique by placing the needle within the lumen of a second tube through which air can be metered produced beads having a smaller diameter.[20] *E. coli* SW132 cells were encapsulated in 1-mm diameter alginate beads using this annular air-flow technique, and at 5 °C the specific activity of the 1-mm diameter beads was *ca.* 1.4-fold greater than that of the corresponding 3-mm diameter beads.

The dependence of catalyst productivity on acrylamide concentration was examined (Figure 5). Catalyst beads having a 1-mm diameter were employed to improve volumetric productivity at the same catalyst charge as was previously employed using 3-mm catalyst beads. Consecutive 1.06 M acrylonitrile batch reactions with catalyst recycle were performed at 5 °C in the presence of either 1.4 M or 2.9 M added acrylamide; recycle reactions with no added acrylamide were run as a control. No significant difference in the rate of loss of catalyst activity was observed over the course of thirty consecutive recycle reactions that had final concentrations of acrylamide of 1.33 M (control), 2.73 M (1.4 M added acrylamide) and 4.23 M (2.9 M added acrylamide), corresponding 95, 194 and 301 g acrylamide/L, respectively. Based on these results, it should be possible to further increase catalyst productivity by continuously adding acrylonitrile to a reaction to maintain a constant concentration significantly lower than 1 M [but at a concentration of at least five times the K_m (2.0 mM) to maintain maximum reaction rate] and produce at least 30 wt % acrylamide in water.

The catalyst used to measure catalyst productivity at 5 °C (Figure 4) was 3-mm diameter beads prepared using a first lot (lot A) of *E. coli* SW132 cells, whereas the recycle reactions depicted in Figure 5 were performed with 1-mm diameter catalyst beads prepared using a second lot (lot B) of *E. coli* SW132 cells. The specific activity of lot B cells was only *ca.* 83% of that of lot A cells. Comparison of the catalyst stability of the two catalyst bead preparations for reactions run with 1.06 M acrylonitrile at 5 °C (no added acrylamide) indicated that the rate of loss of catalyst activity with the 1-mm diameter lot B cell catalyst beads was significantly greater than for the 3-mm lot A cell catalyst beads. The dependence of catalyst stability and productivity on bead diameter and cell lot was therefore evaluated

using three different catalyst bead preparations: (1) 3-mm diameter beads, lot A cells, (2) 3-mm diameter beads, lot B cells, and (3) 1 mm diameter beads, lot B cells (Figure 6). There was no significant dependence of catalyst stability on bead diameter, but there was a difference in catalyst stability between the two lots of immobilized cells; beads produced with lot B cells lost their activity at a greater rate than beads produced with lot A cells. Also, there was an initial increase in the specific activity of lot B catalyst beads after the first recycle reaction that was not observed for lot A catalyst beads; the reason for this initial increase in lot B catalyst specific activity has not been determined, but could be due to partial lysis of the immobilized cells under the reaction conditions. These results indicate that the fermentation of *E. coli* SW132 requires further optimization, and that multiple lots of cells produced by fermentation should routinely be compared to determine the variability in NHase specific activity and productivity of microbial cell catalysts.

Immobilized cell catalyst productivity in a column reactor at 5 °C (Figure 7) was evaluated using the same catalyst bead preparation employed for the determination of catalyst productivity at 5 °C in batch reactions with catalyst recycle (3-mm diameter beads, lot A cells; Figure 3). After an initial adjustment in the feed rate of 1.13 M acrylonitrile/2 mM calcium chloride to maintain 90–95% conversion of acrylonitrile to acrylamide (running at less than 100% conversion allows one to immediately detect a loss of catalyst activity), the column was run continuously for a total of 399 h. At 263 h, the conversion of acrylonitrile began to decrease markedly at constant feed rate; the catalyst productivity at this point was *ca.* 155 g acrylamide/g (dcw) catalyst. A lower catalyst productivity was observed for the catalyst in a column reactor when compared to consecutive batch reactions, and this lower productivity may be due at least in part to the constant exposure of the catalyst beads at the column inlet to a high concentration of acrylonitrile, whereas in batch operation the entire catalyst charge is initially exposed to this same acrylonitrile concentration, but the concentration subsequently decreases over the course of the reaction.

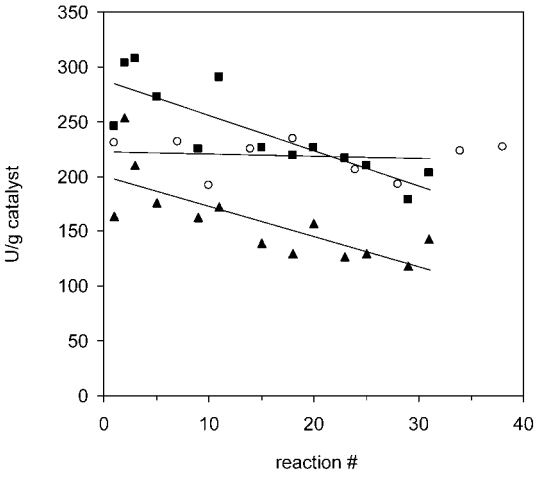

Figure 6. Comparison of recovered activity of 1-mm and 3-mm bead diameter immobilized cell catalysts in consecutive 1.06 M acrylonitrile batch reactions with catalyst recycle at 5 °C: 1 mm diameter, cell lot B (■), 3 mm diameter, cell lot B (▲), 3 mm diameter, cell lot A (○).

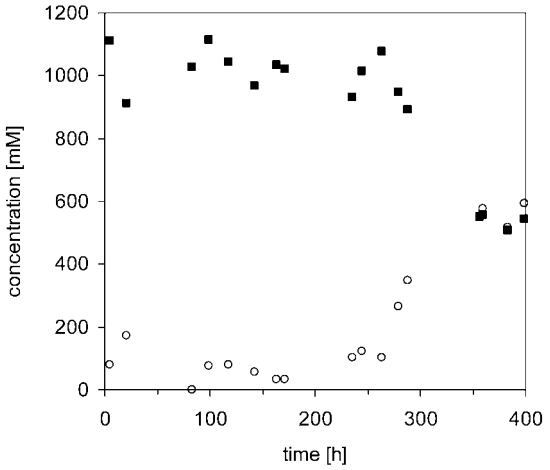

Figure 7. Time course for conversion of 1.13 M acrylonitrile in a column reactor containing 3 mm diameter catalyst beads (cell lot A) at 5 °C: acrylamide (■), acrylonitrile (○). Flow rate adjusted to maintain > 90% conversion: 0–21 h at 0.5 mL/min, 21–118 h at 0.4 mL/min, 118–143 h at 0.35 mL/min, 143–399 h at 0.3 mL/min. Catalyst productivity from 0–263 h was 155 g acrylamide/g dcw.

Conclusion

A catalyst productivity of 1035 g acrylamide/g dcw for alginate-immobilized *E. coli* SW132 is comparable to the commercial catalyst productivities for polyacrylamide-immobilized *Rhodococcus* sp. 774 and *P. putida* B23 (500 and 850 g acrylamide/g dcw, respectively),[5] but less than that of the polyacrylamide-immobilized *R. rhodochrous* J1 currently employed for commercial production of acrylamide (> 7000 g acrylamide/g dcw).[5] Although the catalyst productivity of thermophilic NHase catalysts *Bacillus* sp. BR449[6] or alginate-immobilized *Bacillus* spp. RAPc8[7] was not reported for reactions run at 5–10 °C, comparison of the catalyst productivity of unimmobilized *E. coli* SW132 at 35 °C with that of *Bacillus* sp. BR449 at 30–40 °C indicates a significantly greater catalyst productivity for *E. coli* SW132. It is possible that additional improvement in *E. coli* SW132 catalyst productivity might be achieved by further optimization of the immobilization matrix; for example, a significant increase in the operational stability of immobilized *Brevibacterium* sp. CH1 NHase for hydration of acrylonitrile was observed when cells were

immobilized in polyacrylamide instead of calcium alginate.[21]

Despite the fact that *E. coli* SW132 NHase is relatively thermally stable compared to the NHase of most mesophilic bacteria, the catalyst productivity at 35 °C was significantly less than that at 5 °C. The major determinant of NHase catalyst productivity at either 5 °C or 35 °C was the concentration of acrylonitrile in the reaction mixture. This result is in agreement with those of previous studies with thermally stable microbial NHases, where the nucleophilic reaction of protein functional groups with acrylonitrile has been proposed as a mechanism for enzyme inactivation.[6,7] Performing the reaction at 5–10 °C decreased this rate of enzyme inactivation, while at the same time the specific activity of the microbial catalyst was sufficiently high in this temperature range to produce acceptable reaction rates (and volumetric productivities) for biocatalytic conversion of acrylonitrile to acrylamide.

Experimental Section

General Remarks

Chemicals were obtained from commercial sources and used as received. Cell paste was stored frozen at −80 °C. Wet cell weight (wcw) of microbial catalysts utilized in reactions or assays was obtained from cell pellets prepared by centrifugation of fermentation broth, dry cell weight (dcw) of cells was determined by microwave drying of wet cells. Analysis for acrylonitrile and acrylamide were performed by HPLC using a refractive index detector and a Bio-Rad HPX-87H column (30 cm × 7.8 mm diameter) at 50 °C; the solvent was 0.001 N sulfuric acid. The calculated yields of recovered acrylonitrile and acrylamide were based on initial acrylonitrile concentrations.

Fermentation of *Escherichia coli* SW132 Cells

E. coli strain SW132[16] was grown in a 500-mL seed culture flask at 36 °C, 300 rpm for 10 h to an $OD_{\lambda=550}$ of > 2.0 prior to inoculation of the fermentor. The fermentation medium was prepared in an initial batch of 7.5 L, and contained 32 g KH_2PO_4, 8.0 g $MgSO_4 \cdot 7\ H_2O$, 8.0 g $(NH_4)_2SO_4$, 50 g yeast extract, and 10 mL Mazu DF204 antifoam (BASF). Following sterilization, 369 g glucose solution (60% w/w), 160 mL trace element solution (citric acid, 10 g/L; $CaCl_2 \cdot 2\ H_2O$, 1.5 g/L; $FeSO_4 \cdot 7\ H_2O$, 5.0 g/L; $ZnSO_4 \cdot 7\ H_2O$, 0.39 g/L; $CuSO_4 \cdot 5\ H_2O$, 0.38 g/L; $CoCl_2 \cdot 6\ H_2O$, 0.20 g/L; $MnCl_2 \cdot 4\ H_2O$, 0.30 g/L), and 100 mg/L ampicillin were added. NH_4OH (40% w/v) and 20% w/v H_2SO_4 were used for pH control. The initial set points were: agitation, 400 rpm; aeration, 2 L/min; pH, 6.8; pressure, 0.5 psig; dissolved oxygen concentration (DO), 25%; temperature, 36 °C. At culture densities of 20–30 OD additional AMP was added to 100 mg/L. IPTG was added to 1 mM at culture densities of 30–35 OD. Glucose feed was started at < 5 g/L and feed rate was reduced if glucose accumulated above 2 g/L. IPTG (1 mM) was added to the fermentor at 30–35 $OD_{\lambda=550}$ and cells were harvested 5 h after IPTG addition; final $OD_{\lambda=550}$ was 57.6. The cells were chilled to 5–10 °C and recovered by centrifugation; 490 g (wet cells) were isolated. Based on SDS-PAGE analysis, NHase protein constituted approximately 30% of total soluble protein in induced *E. coli* SW132.

Hydration of Acrylonitrile to Acrylamide using Unimmobilized *E. coli* SW132 cells

A 20-mL reaction vessel equipped with magnetic stirring was charged with 0.336 mL (0.271 g, 5.10 mmol) of acrylonitrile and 9.46 mL of 0.1 M potassium phosphate buffer (pH 7.0). To the reaction vessel was next added 0.200 mL of an aqueous suspension of 22.06 mg dcw/mL of *E. coli* SW132 cells in 0.10 M potassium phosphate buffer (pH 7.0), and the resulting mixture stirred at 25 °C in a constant temperature bath. Samples (0.100 mL) of the reaction mixture were mixed with 0.100 mL of water, 0.020 mL of 6 N HCl and 0.200 mL of aqueous 0.200 M sodium butyrate (HPLC standard). The resulting mixture was centrifuged, and the supernatant analyzed by HPLC. All reactions produced only the amide as the hydration product at 100% conversion of nitrile, with no hydrolysis of the nitrile to the corresponding carboxylic acid.

Immobilization of *E. coli* SW132 Cells in Calcium Cross-Linked Alginate

A 250-mL media bottle (equipped with magnetic stir bar containing 59.7 g of distilled, deionized water at 50 °C) was slowly charged with 3.30 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. To the alginate suspension was added 40.8 g of *E. coli* SW132 wet cell paste (22% dry cell weight) and 16.2 mL of distilled water with stirring. The cell/alginate mixture was added dropwise by syringe 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 and the resulting beads (82 g, 3-mm diameter) were resuspended in 200 mL of 0.20 M calcium acetate buffer (pH 7.0) at 25 °C. With stirring, 4.10 g of 25 wt % glutaraldehyde (GA) in water was added and the beads were mixed for 1.0 h at 25 °C. To the bead suspension was then added 16.4 g of 12.5 wt % polyethyleneimine (PEI, BASF Lupasol® PR971L, average molecular weight *ca.* 750,000) in water, and the beads were mixed for an additional 18 h at 25 °C. The GA/PEI-cross-linked beads were then washed twice with 250 mL of 0.05 M calcium acetate buffer (pH 7.0) at 25 °C, and stored in this same buffer at 5 °C.

Hydration of Acrylonitrile to Acrylamide using Alginate-Immobilized *E. coli* SW132 Cells in Consecutive Batch Reactions with Biocatalyst Recycle

A 50-mL jacketed reaction vessel (equipped with an overhead stirrer and temperature-controlled at 5, 10 or 35 °C with a recirculating temperature bath) was charged with 4.0 g of GA/PEI-cross-linked *E. coli* SW132 cell/alginate beads. To the reaction vessel was added 0.2 mL of 0.20 M calcium acetate buffer (pH 7.0, 2.0 mM final calcium ion concentration), 1.40 mL (1.13 g, 21.3 mmol) of acrylonitrile, and the final volume of

the reaction mixture adjusted to 20 mL by the addition of distilled, deionized water. The mixture was stirred at 5, 10 or 35 °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 were mixed with 0.200 mL of 0.200 M sodium butyrate (HPLC external standard); the resulting mixture was centrifuged and the supernatant analyzed by HPLC. At the completion of the reaction (100% conversion of acrylonitrile), the product mixture was decanted from the biocatalyst beads, and additional distilled, deionized water, 0.2 mL of 0.20 M calcium acetate buffer and 1.40 mL (1.13 g, 21.3 mmol) of acrylonitrile mixed with the reaction heel (immobilized-cell catalyst and remaining product mixture from the previous reaction) at 5, 10 or 35 °C. At the completion of the second reaction, the product mixture was decanted and subsequent reactions performed as before.

Hydration of Acrylonitrile to Acrylamide using Alginate-Immobilized *E. coli* SW132 Cells in a Packed Column Reactor

A 12 cm × 2.5 cm diameter stainless steel column was charged with 34 g of GA/PEI-cross-linked *E. coli* SW132 cell/alginate beads (3-mm diameter beads, lot A cells) suspended in 0.05 M calcium acetate buffer (pH 7.0). The column was placed in a controlled-temperature bath at 5 °C, and an aqueous solution of 1.13 M acrylonitrile and 2 mM calcium chloride was fed to the column at an initial flow rate of 0.5 mL/min using a peristaltic pump. Samples (0.100 mL) of the column effluent were mixed with 0.400 mL of water, and then 0.200 mL of the diluted sample were mixed with 0.200 mL of 0.200 M sodium butyrate (HPLC external standard) and analyzed by HPLC. The column flow rate was adjusted during initial operation to achieve 90–95% conversion of acrylonitrile to acrylamide: 0–21 h at 0.50 mL/min, 21–118 h at 0.40 mL/min, 118–143 h at 0.35 mL/min, 143–399 h at 0.3 mL/min. After continuous operation for 263 h (120 h at 0.30 mL/min), the catalyst productivity was *ca.* 155 g acrylamide/g dcw catalyst.

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References

[1] a) D. A. Cowan, R. A. Cameron, T. L. Tsekoa, *Adv. Appl. Microbiol.* **2003**, *52*, 123–158; b) L. Martinkova, V. Kren, *Biocatal. Biotransform.* **2002**, *20*, 73–93.
[2] D. Cowan, R. Cramp, R. Pereira, D. Graham, Q. Almatawah, *Extremophiles* **1998**, *2*, 207–216.
[3] I. Watanabe, Y. Satoh, K. Enomoto, *Agric. Biol. Chem.* **1987**, *51*, 3193–3199; I. Watanabe, Y. Satoh, K. Enomoto, S. Seki, K. Sakashita, *Agric. Biol. Chem.* **1987**, *51*, 3201–3206.
[4] T. Nagasawa, H. Nanba, K. Ryuno, K. Takeuchi, H. Yamada, *Eur. J. Biochem.* **1987**, *162*, 691–698.
[5] T. Nagaswa, H. Shimizu, H. Yamada, *Appl. Microbiol. Biotechnol.* **1993**, 40, 189–195.
[6] R. Padmakumar, P. Oriel, *Appl. Biochem. Biotechnol.* **1999**, *77–79*, 671–679.
[7] D. Graham, R. Pereira, D. Barfield, D. Cowan, *Enz. Microb. Technol.* **2000**, *26*, 368–373.
[8] N. A. Webster, D. K. Ramsden, J. Hughes, *Biotechnology Lett.* **2001**, *23*, 95–101.
[9] J. M. Stevens, N. Rao Saroja, M. Jaouen, M. Belghazi, J.-M. Schmitter, D. Mansuy, I. Artaud, M.-A. Sari, *Protein Expression and Purification* **2003**, *29*, 70–76.
[10] M. Nojiri, M. Yohda, M. Odaka, Y. Matsushita, M. Tsujimura, T. Yoshida, N. Dohmae, K. Takio, I. Endo, *J. Biochem. (Tokyo)* **1999**, *125*, 696–704.
[11] Y. Hashimoto, M. Nishiyama, S. Horinouchi, T. Beppu, *Biosci. Biotechnol. Biochem*. **1994**, *58*, 1859–1865.
[12] M. Kobayashi, M. Nishiyama, T. Nagasawa, S. Horinouchi, T. Beppu, H. Yamada, *Biochim. Biophys. Acta* **1991**, *1129*, 23–33.
[13] S. Wu, R. D. Fallon, M. S. Payne, *Appl. Microbiol. Biotechnol.* **1997**, *48*, 704–708.
[14] T. Yamaki, T. Oikawa, K. Ito, T. Nakamura, *J. Ferment. Bioeng.* **1997**, *83*, 474–477; A. Miyanaga, S. Fushinobu, K. Ito, T. Wakagi, *Biochem. Biophys. Res. Commun*. **2001**, *288*, 1169–1174; A. Miyanaga, S. Fushinobu, K. Ito, H. Shoun, T. Wakagi, *Eur. J. Biochem.* **2004**, *271*, 429–438.
[15] a) J. E. Gavagan, S. Fager, R. D. Fallon, P. W. Folsom, F. E. Herkes, A. E. Eisenberg, E. C. Hann, R. DiCosimo, *J. Org. Chem.* **1998**, *63*, 4792–4801; b) E. C. Hann, A. E. Sigmund, S. K. Fager, F. B. Cooling, J. E. Gavagan, A. Ben-Bassat, S. Chauhan, M. S. Payne, S. M. Hennessey, R. DiCosimo, *Adv. Syn. Catal.* **2003**, *345*, 775–782; c) DuPont, *US Patent* 6,670,158 B2, **2003**; *Chem. Abstr.* **2003**, *139*, 148573.
[16] K. L. Petrillo, S. Wu, E. C. Hann, F. B. Cooling, A. Ben-Bassat, J. E. Gavagan, R. DiCosimo, M. S. Payne. *Appl. Microbiol. Biotechnol*. **2005**, in press.
[17] E. C. Hann, A. E. Sigmund, S. M. Hennessey, J. E. Gavagan, D. R. Short, A. Ben-Bassat, S. Chauhan, R. D. Fallon, M. S. Payne, R. DiCosimo, *Org. Proc. Res. Dev.* **2002**, *6*, 492–496.
[18] H. S. Davis, O. F. Wiedeman, *Ind. Eng. Chem*. **1945**, *37*, 482–485.
[19] B. Atkinson, F. Mavituna, *Biochemical Engineering and Biotechnology Handbook*, 2nd edn., Macmillan Publishers, Stockton Press, New York, **1991**, pp. 582, 764–765.
[20] J. Klein, J. Stock, K.-D. Vorlop, *Eur. J. Appl. Microbiol. Biotechnol.* **1983**, *18*, 86–91; T. Rehg, C. Dorger, P. C. Chau, *Biotechnol. Lett.* **1986**, *8*, 111–114; D. B. Seifert, J. A. Phillips, *Biotechnol. Prog.* **1997**, *13*, 562–568.
[21] J. S. Hwang, H. N. Chang, *Biotechnol. Bioeng.* **1989**, *34*, 380–386.

