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# Optimization of Biocatalyst Specific Activity for Glycolic Acid Production

Arie Ben-Bassat,[a] Alison M. Walls,[a] Matthew A. Plummer,[a] Amy E. Sigmund,[a] William L. Spillan,[a] and Robert DiCosimo[a,*]

[a] DuPont Central Research and Development, Experimental Station, P.O. Box 80328, Wilmington, DE 19880-0328, USA
Fax: (+1)-302-355-3839; e-mail: Robert.DiCosimo@usa.dupont.com

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This manuscript is dedicated to Professor Chi-Huey Wong on the occasion of his 60th birthday.

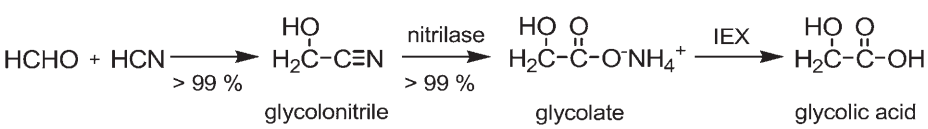
**Abstract:** A chemoenzymatic process has been developed that employs an immobilized microbial nitrilase biocatalyst for the conversion of glycolonitrile to high-purity glycolic acid. The specific activity of this immobilized cell biocatalyst decreased significantly during initial use in either consecutive batch reactions with catalyst recycle, or in a continuous stirred-tank reactor, but the nitrilase activity remaining after this initial decrease was stable under the reactions conditions. The initial stability of this immobilized cell nitrilase catalyst has been improved by treatment of the microbial cells with glutaraldehyde prior to immobilization. Conditions for glutaraldehyde treatment were defined that completely inactivated the culture without significantly affecting nitrilase activity. A method for dehydration, storage and rehydration of the carrageenan-immobilized cells has also been demonstrated that further improves the specific activity of this biocatalyst.

**Keywords:** enzyme catalysis; glutaraldehyde; glycolic acid; microbial sterilization; nitrilase

## Introduction

We have previously reported the development of a chemoenzymatic process for the production of high-purity glycolic acid (Scheme 1), where formaldehyde and hydrogen cyanide were first reacted to produce glycolonitrile in >99% yield and purity, the resulting aqueous glycolonitrile was used without further purification in a subsequent biocatalytic conversion of glycolonitrile to ammonium glycolate, and the ammonium glycolate was directly converted to glycolic acid using ion exchange over a strong acid cation resin (>99% yield and purity after three process steps).[1] A glutaraldehyde/polyethylenimine cross-linked carrageenan-immobilized *E. coli* MG1655 transformant expressing the *Acidovorax facilis* 72W nitrilase[2] having a Phe168Val mutation[3] was employed as biocatalyst, and a biocatalyst productivity of >1000 g glycolic acid/g dry cell weight was achieved during the production of 3.2 M ammonium glycolate in either consecutive batch reactions with biocatalyst recycle or in a continuous stirred-tank reactor.

The specific activity of the biocatalyst (measured as units of nitrilase activity per gram dry cell weight in the immobilized cell biocatalyst) decreased significantly during its initial use in either consecutive batch reactions with catalyst recycle, or in a continuous stirred-tank reactor, where 40–50% of the initial specific activity was lost in a first batch reaction, but the nitrilase activity remaining after this initial decrease was stable and robust under the reaction conditions such that targets for both biocatalyst productivity (grams glycolic acid/gram biocatalyst) and volumetric productivity (grams glycolic acid/L/h) were achieved. The economics of this process have now been further improved by treatment of the microbial cell nitrilase with glutaraldehyde (GA) at the end of the fermentation, resulting in a significant stabilization of the nitri-




**Scheme 1.** Three-step chemoenzymatic glycolic acid process.

lase specific activity of the immobilized cell biocatalyst when used for ammonium glycolate production.

## Results and Discussion

### Glutaraldehyde Treatment of the Microbial Nitrilase Catalyst

Inactivation of a microbial catalyst culture [reduction in colony-forming units/mL (CFU/mL) by $\geq$ 6-log] at the conclusion of fermentation avoids containment and safety issues for handling, storage and transportation associated with live recombinant cultures.[4] Any inactivation method for commercial operation should be cost effective and practical, and provide complete inactivation of the microbial cells without undue risk to the operators or the environment; this method should also not affect the activity of the enzyme of interest, or interfere with the downstream processing of the microbial catalyst. Inactivation of the culture by heat treatment, extreme pH (e.g., below 3 or above 10) or with chemical reagents are common practices to inactivate microbial cultures.[5] Conditions for heat treatment of a fermentation broth containing *E. coli* MG1655/pSW138-Phe168Val were not found that provided complete inactivation of high-density cultures without inactivation of nitrilase. Treatment using an extreme pH was not considered because nitrilase was rapidly inactivated at a pH below 5 and above 10. A practical and cost effective method has been developed that utilizes treatment with GA, or GA followed by bisulfite, for complete inactivation of high-density *E. coli* cultures, where nitrilase activity is preserved in cells in suspension or in an immobilized form.

GA is widely used as a biocide and disinfectant for surfaces, medical instruments and cooling towers.[6] Biocidal activity is mainly due to reaction of the GA aldehyde functional groups with primary amines on cell walls of living organisms (from lysine and arginine), and by complex chemical cross-linking reactions where essential metabolic functions are disrupted and the cells die.[7] The biocidal action of GA is generally enhanced with increasing concentration and temperature, and at an optimal pH of 8–9; components of a fermentation broth that can interfere with the biocidal activity of GA include amino acids, peptides and ammonia.[6c]

Cultures were prepared by 10-L fermentations of *E. coli* MG1655/pSW138-Phe168Val. The culture density at the end of these runs was 120–140 $OD_{550}$ and cell viability ranged from $3\times10^{10}$ to $5\times10^{10}$ CFU/mL. A mineral medium with glucose, ammonia and 5 g/L yeast extract was used for cell culture, where the pH was maintained with ammonia titration, glucose was supplied first in batch and fed batch, and later in the run lactose was added as substrate and inducer.[1] Washing the cells removed ammonia (30–50 mM) from the culture and rendered the washed cells significantly more sensitive to GA treatment than cells treated directly with GA in a broth, but for practical reasons an inactivation method was developed using unwashed cultures that can be used directly in the fermentation vessel.

To produce a high level of inactivation, GA (5–10 wt% in water) was added with efficient mixing to high-density fermentation culture (100–150 $OD_{550}$) at 50 mg to 100 mg GA/L min$^{-1}$, to a final concentration of 3.5–5.0 g GA/L (about 0.030 g to about 0.042 g GA per $OD_{550}$). The residual GA concentration is typically low, in the range of 10–200 ppm, and can be further reduced by the addition of *ca.* 1 g/L sodium bisulfite (as a 10 wt% aqueous solution).[8] The GA-treated culture or GA/bisulfite-treated culture was chilled to 5–10 °C prior to immobilization, and optionally washed (by concentration and re-dilution of the cell suspension or fermentation broth) with water or an appropriate storage buffer to remove residual bisulfite and unreacted GA.

The effect of pH on culture inactivation by GA is depicted in Figure 1, where optimal inactivation was observed at pH 5.0–5.5, and at a higher pH the rate of inactivation declined significantly; at the optimal inactivation pH ammonia is predominately present as ammonium ion, and is mostly unreactive with GA. The measured concentration of GA in the culture over the course of GA addition was significantly lower than the cumulative concentration of GA added (Figure 2), where GA concentration in the culture peaked at 0.34 g/L at the end of GA addition

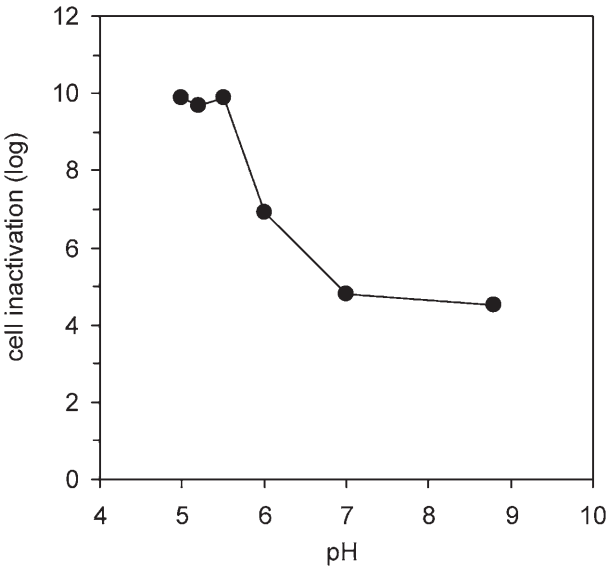



**Figure 1.** Dependence of *E. coli* inactivation by GA on pH. GA was added to a final concentration of 5 g/L at 50 mg GA/L min$^{-1}$, and cell inactivation [log (initial CFU/mL)/(final CFU)/mL] measured after 5 h total treatment time.

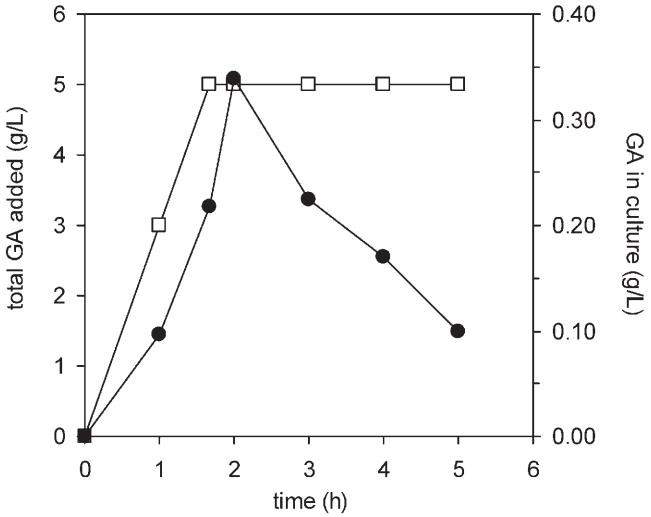



**Figure 2.** Total GA added to cell culture (□ g/L), and GA concentration measured in cell culture (●, g/L) over 5 h total treatment time.

(*ca.* 100 min) and declined to 0.1 g/L at 5 h; GA concentration continued to decrease with longer incubation times at ambient temperature or 5 °C. The dependence of culture inactivation on the ratio of GA concentration (g/L) to culture density ($OD_{550}$) was evaluated at pH 5.2 and 35 °C (Figure 3); to insure complete inactivation of the culture, a ratio of GA concentration (g/L) to $OD_{550}$ of $\geq 0.035$ was utilized.

A large-scale demonstration of culture inactivation was performed at pH 5.2 and 35 °C using 180 L of fermentation broth having a culture density of 139 $OD_{550}$ ($3.5 \times 10^{10}$ CFU/mL); GA (10% w/w) was added to 5 g/L at rate of 50 mg GA/L min$^{-1}$, and after 5 h sodium bisulfite (10 wt% aqueous solution) was

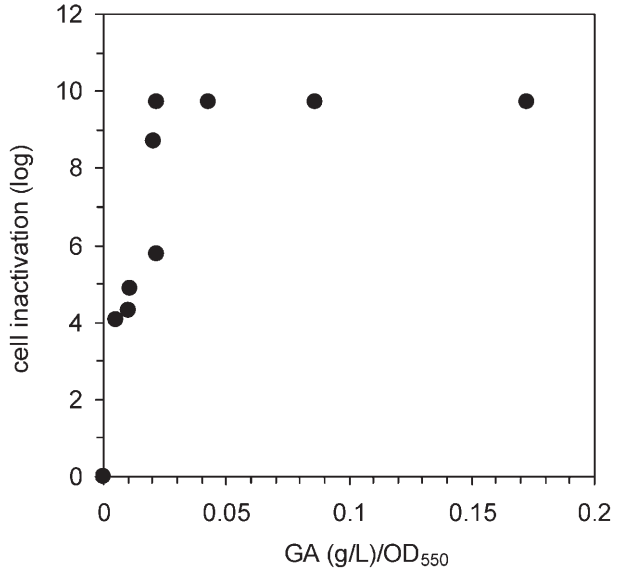



**Figure 3.** Dependence of cell inactivation [log (initial CFU)/mL/(final CFU)/mL] on GA (g/L)/$OD_{550nm}$.

added to a final concentration of 1 g/L. The culture inactivation exceeded 9.6 logs (< 10 CFU/mL remaining) and the microbial nitrilase specific activities before treatment, after treatment with GA, and after treatment with bisulfite were 2819, 3300 and 2493 benzonitrile U/g dcw, respectively. Cells recovered from this run after treatment with GA and bisulfite were immobilized in carrageenan and subsequently cross-linked with GA and polyethylenimine (PEI),[2b] and the resulting immobilized microbial nitrilase catalyst retained significantly greater activity in batch reactions with catalyst recycle when compared to immobilized microbial nitrilase catalysts that were prepared from cells that were not treated with GA (see below).

### Effect of GA Treatment of Cells on Specific Activity and Stability of Immobilized *E. coli* MG1655/pSW138-Phe168Val Biocatalyst

The use of GA/PEI cross-linked carrageenan-immobilized cells (*E. coli* MG1655/pSW138-Phe168Val) in consecutive batch reactions with biocatalyst recycle has been previously reported,[1] where in each batch reaction a total of 2.4 M glycolonitrile was completely (> 99%) converted to ammonium glycolate. The decrease in biocatalyst specific activity after a first batch reaction was typically 40–50%, but the remaining nitrilase activity was relatively stable; the biocatalyst productivity after 55 consecutive batch reactions was 1010 g GLA/g dcw, with a final catalyst specific activity of *ca.* 78% of the initial activity (measured at the second batch reaction of a series). To determine the effect of glutaraldehyde treatment on the nitrilase activity of *E. coli* MG1655/pSW138-Phe168Val, cells from the 180 L GA-treated fermentation (described above) were immobilized using the previously described immobilization protocol.[1] The resulting GA/PEI cross-linked carrageenan-immobilized cell biocatalyst was evaluated in duplicate sets of four consecutive batch reactions, and compared to a duplicate set of batch reactions run using a similar biocatalyst prepared without GA treatment prior to cell immobilization (Figure 4); all batch reactions reported herein produced > 99% conversion of GLN to GLA prior to recycle of the biocatalyst in a subsequent batch reaction. The initial specific activity of the GA-treated immobilized cells was significantly higher than that of the immobilized cells that were not GA treated, indicating a possible stabilization of the microbial nitrilase activity to the immobilization procedure by prior GA treatment (batch immobilization of the microbial nitrilase in carrageenan was performed at 50 °C).[1] The loss in specific activity after the first batch reaction in duplicate sets of reactions was 11% and 17% for the GA-treated immobilized cells, compared to 45% and 47% for the immobilized cells that were not

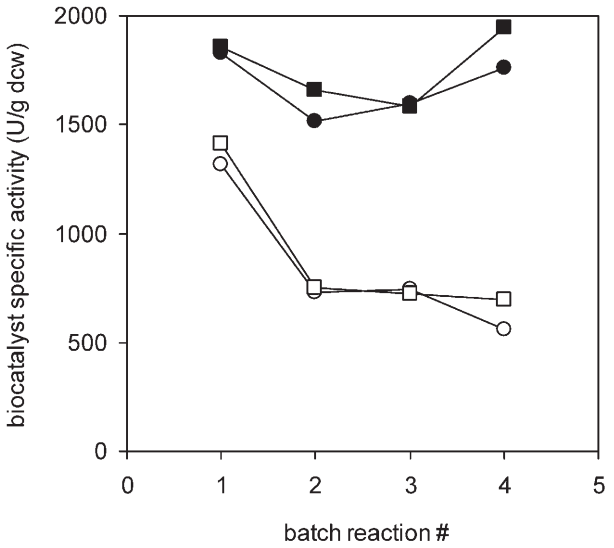



**Figure 4.** Consecutive batch reactions with biocatalyst recycle of either GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val (○, □) or GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val prepared with GA-treated cells (●, ■) at pH 7.5 and 25 °C (duplicate runs with each biocatalyst using an equivalent dry cell weight biocatalyst charge). Eight equivalent GLN aliquots were added sequentially (at complete GLN conversion) to produce a total of 2.4 M glycolonitrile in the final reaction volume; carry-over of reaction "heel" from previous batch reaction resulted in a total final concentration of 3.5 M ammonium glycolate in each consecutive batch reaction.

GA treated. The reason for the observed increase in biocatalyst activity of the GA-treated immobilized cells over the course of four consecutive batch reactions after an initial decrease has not been determined, but may be due to increased permeability of the immobilized cells. Immobilization of cells that were not GA treated in carrageenan, and subsequent chemical cross-linking of the immobilized cell biocatalyst first with GA and then with PEI, did not prevent a significant loss of nitrilase activity in a first batch reaction to convert glycolonitrile to ammonium glycolate. The recovered biocatalyst's specific activity of the GA-treated immobilized cells in consecutive batch reactions was *ca.* twice that of the untreated immobilized cells, resulting in a two-fold improvement in volumetric productivity (grams GLA/L/h).

### Specific Activity and Stability of Dehydrated/Rehydrated Immobilized *E. coli* MG1655/pSW138-F168 V Biocatalyst Prepared without GA Treatment of Cells

Dehydration and subsequent rehydration of GA/PEI cross-linked carrageenan-immobilized cells provided several advantages in the preparation and use of an immobilized cell biocatalyst for glycolic acid production. Prior to final chemical cross-linking with GA and PEI, the carrageenan-immobilized microbial biocatalyst was *ca.* 92% (w/w) water, 5% dcw and 3% carrageenan. Dehydration of the GA/PEI cross-linked carrageenan-immobilized cell beads (*ca.* 2.5 mm dia.) resulted in a significant decrease in biocatalyst mass and bead diameter. The dehydrated biocatalyst occupied a significantly smaller volume than the original catalyst beads, and was more economically stored and shipped; the original biocatalyst beads were typically stored in an equal weight of aqueous buffer, where the dcw concentration was only *ca.* 2.5% of the weight of immobilized cell biocatalyst plus buffer. When GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val biocatalyst beads were dehydrated to a constant weight in a vacuum oven (176 mm Hg vacuum at 35 °C with nitrogen purge for 24 h), the resulting dehydrated beads were only 9.1% of their original weight (*ca.* 56% dcw). A second advantage of dehydration/rehydration was that the dehydrated beads did not absorb an equivalent weight of water upon rehydration as was lost during dehydration, resulting in a higher specific activity of the rehydrated immobilized cell biocatalyst; a higher specific activity allows one to operate a batch reactor or CSTR with a higher volumetric productivity at an equivalent biocatalyst loading (weight biocatalyst/reaction mass).

Dehydrated biocatalyst (stored at ambient temperature for 7 days) was rehydrated in 0.10 M $NH_4GLA$ (pH 7.3) for 24 h at either 25 °C or 5 °C, and the resulting rehydrated biocatalyst weighed only 21% of the original hydrated biocatalyst weight. The rehydrated biocatalyst was assayed for activity (based on dry cell weight content), and the measured specific activities compared to that of the original immobilized cell biocatalyst prior to dehydration/rehydration. There was no difference in recovered activity based on rehydration temperature, but the specific activity of the dehydrated/rehydrated immobilized cell biocatalysts decreased by 41–42% compared to their initial activity (Table 1). A significant decrease in initial activity of fresh beads after a first batch reaction was also observed (see above), and it is possible that a fraction of the total nitrilase present in the microbial catalyst was unstable to dehydration/rehydration, and to reaction conditions that generated high concentrations of ammonium glycolate.

The catalyst productivity of GA/PEI cross-linked, carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val prepared without GA treatment of cells and stored in dehydrated form for 32 days at 25 °C prior to rehydration was evaluated in consecutive batch reactions (Figure 5). Although this biocatalyst lost *ca.* 59% of its initial specific activity (U/g dcw)

**Table 1.** Specific activity of glutaraldehyde/polyethylenimine cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-F168V transformant before and after dehydration/rehydration.

| Glutaraldehyde treatment of cells prior to immobilization | Dehydration/rehydration of immobilized cells | Dehydration temperature [°C] | Storage at 5 °C [days] | Rehydration temperature [°C] | Initial specific activity [U/g dcw] | Activity loss after rehydration [%] |
|---|---|---|---|---|---|---|
| no | no | - | 7 | - | 1787[a] | - |
| no | yes | 35 | 7 | 5 | 1049[a] | 41 |
| no | yes | 35 | 7 | 25 | 1032[a] | 42 |
| no | yes | 35 | 32 | 25 | 734[a] | 59 |
| yes | no | - | 3 | - | 1841[b,c] | - |
| yes | yes | 35 | 3 | 5 | 1622[b,c] | 12 |
| yes | no | - | 28 | - | 1949[b,c] | - |
| yes | yes | 35 | 28 | 25 | 1484[b,c] | 19 |

[a] Conversion of 1.0 M GLN.
[b] Average of two experiments.
[c] Conversion of 2.4 M GLN.

during dehydration, storage and rehydration (Table 1), the remaining biocatalyst activity was relatively stable over twenty-four consecutive batch reactions (performed over 31 days). The batch-to-batch recovery of this dehydrated/rehydrated biocatalyst (measured as biocatalyst specific activity) was similar to that of immobilized microbial biocatalyst that had not been dehydrated/rehydrated (Figure 5, reactions performed over 35 days), and the dehydration/rehydration of the biocatalyst did not adversely affect the robustness of the biocatalyst beads under the reaction conditions over an extended period of use (no biocatalyst attrition or disintegration was observed).

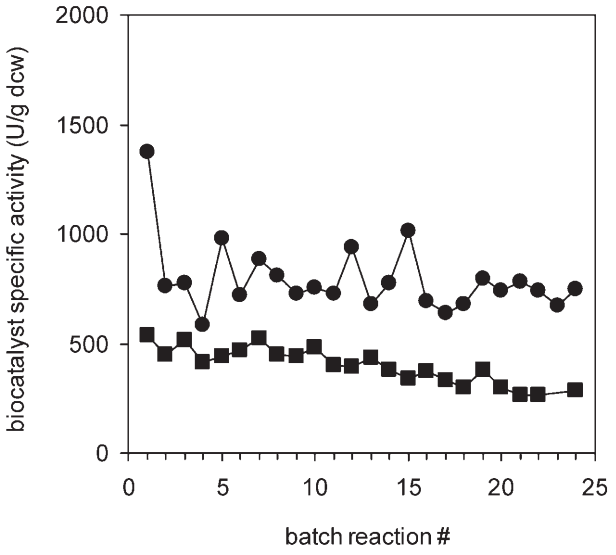



**Figure 5.** Consecutive batch reactions with biocatalyst recycle of either GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val (●) or dehydrated/rehydrated GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val (■) (equivalent dry cell weight biocatalyst charge) at pH 7.5 and 25 °C. Eight equivalent GLN aliquots were added sequentially (at complete GLN conversion) to produce a total of 2.4 M $NH_4GLA$ in the final reaction volume; carry-over of reaction "heel" from previous batch reaction results in a total final concentration of 3.5 M $NH_4GLA$ in each batch reaction.

## Specific Activity and Stability of Dehydrated/Rehydrated Immobilized *E. coli* MG1655/pSW138-F168 V Biocatalyst Prepared with GA Treatment of Cells

Dehydration of immobilized cell biocatalyst prepared using GA-treated cells was performed as described above for non-GA treated immobilized cells; the resulting dehydrated beads were 22% of their original weight. A portion of the dehydrated beads was immediately rehydrated in 0.10 M $NH_4GLA$ (pH 7.3) for three days at 5 °C, and the fully rehydrated beads increased to 60% of their original hydrated weight. The specific activity (U/g dcw) of the GA-treated immobilized cell biocatalyst decreased only 12% after dehydration/rehydration, whereas the immobilized cell biocatalyst prepared without GA treatment of cells prior to immobilization decreased 42% after dehydration/rehydration (Table 1).

Four consecutive batch reactions with biocatalyst recycle were run with dehydrated/rehydrated immobilized cell biocatalyst prepared with GA-treated cells, and compared to the same biocatalyst without dehydration/rehydration (using a catalyst charge containing an equivalent dry cell weight). The initial activity of these two biocatalysts is reported in Table 1, and the dependence of recovered specific activity on batch reaction with biocatalyst recycle is depicted in Figure 6 (reported data are an average of two separate sets of batch reactions). The biocatalyst specific activity for each of these two biocatalysts decreased by only 14–15% after the first batch reaction. The recovered specific activity in consecutive batch reac-

tions of either GA-treated catalyst (with or without dehydration/rehydration) was *ca.* two-fold greater than that of the biocatalyst produced without GA treatment of cells prior to immobilization and without dehydration/rehydration (Figure 5).

An aliquot of the freshly prepared and dehydrated GA-treated immobilized cell biocatalyst was stored for 28 days at 5 °C, then rehydrated as described above and evaluated in consecutive batch reactions with catalyst recycle. A separate set of batch reactions was run with the same lot of catalyst beads that were not dehydrated/rehydrated, but were stored for 28 days at 5 °C in 1.0 M ammonium bicarbonate buffer (pH 7.3). The initial activity of these two biocatalysts after 28 days is reported in Table 1; the dehydrated/rehydrated biocatalyst lost 8% activity during storage, whereas the biocatalyst that was not dehydrated/rehydrated increased in specific activity by 6%. The dependence of recovered specific activity of these two biocatalysts on batch reaction with biocatalyst recycle is depicted in Figure 6 (reported data are an average of two separate sets of batch reactions), where the biocatalyst specific activity of the dehydrated/rehydrated biocatalyst decreased by only 3% after the first batch reaction, and the biocatalyst specific activity of the corresponding biocatalyst that was not dehydrated/rehydrated decreased by 26% after the first batch reaction. Although there was a significantly greater decrease in specific activity after a first batch reaction when using the biocatalyst that was not dehydrated prior to storage for 28 days at 5 °C, the biocatalyst specific activity for each of these two biocatalysts was approximately the same after the first batch reaction.

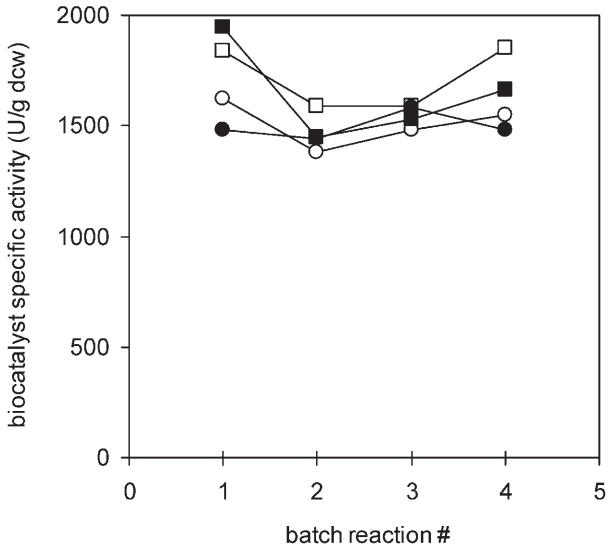



**Figure 6.** Consecutive batch reactions with biocatalyst recycle at pH 7.5 and 25 °C using either GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val prepared with GA-treated cells [stored for 1 day (□) or 28 days (■) at 5 °C], or dehydrated/rehydrated GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val prepared with GA-treated cells [stored for 1 day (○) or 28 days (●) at 5 °C] (equivalent dry cell weight biocatalyst charge, average of two sets of reactions with each biocatalyst). Eight equivalent GLN aliquots were added sequentially (at complete GLN conversion) to produce a total of 2.4 M $NH_4GLA$ in the final reaction volume of each batch reaction; carry-over of reaction "heel" from a previous batch reaction results in a total final concentration of 3.5 M $NH_4GLA$ in each batch reaction.

## Conclusions

A method for complete inactivation of a microbial nitrilase transformant with GA has been developed that provides >9-log reduction in viable cells without a significant decrease in nitrilase activity, thus avoiding containment and safety issues for handling, storage and transportation of live recombinant cultures. Although GA has been successfully employed to stabilize microbial glucose isomerase[9] and penicillin acylase,[10] it has also been reported that enzymes with a thiol functional group in or very near the active site of the enzyme (e.g., nitrilase) are inactivated by thiol-reactive agents such as GA,[11] and use of GA to inactivate live cells while retaining microbial nitrilase activity was potentially problematic.[12] Preservation of nitrilase activity during GA treatment of the microbial nitrilase biocatalyst was dependent on reaction time, temperature, GA concentration, pH and the concentration of ammonia and other amines (e.g., amino acids and peptides) in the culture media that react with GA. A preferred GA treatment method reacted cells from high density fermentation (100–150 $OD_{550}$) with 5–10 wt% aqueous GA that was added with efficient mixing at 50 mg to 100 mg GA/L $min^{-1}$, resulting in a final concentration of about 3.5 g to 5 g GA/L (*ca.* 0.030 g to 0.042 g GA per $OD_{550}$). A 10 wt% solution of sodium bisulfite in water was added at 1 g/L to inactivate residual unreacted GA. This method of culture inactivation may have utility in other processes, where optimization of GA concentration and addition rate (dependent on microbial strain, culture density and media) may result in little or no inactivation of a desired intracellular enzyme.

GA treatment of cells prior to immobilization additionally resulted in several improvements in the resulting biocatalyst when used for conversion of glycolonitrile to ammonium glycolate. Without GA treatment, freshly prepared immobilized cell biocatalyst typically lost between 35% and 50% of initial activity after a single batch reaction. GA treatment of cells followed by immobilization resulted in significantly less activity loss in a first batch reaction with catalyst recycle, where the specific activity recovered in subse-

quent batch reactions was *ca.* twice that of the biocatalyst produced without GA treatment of cells. Biocatalyst specific activity has a direct impact on process economics, where increasing the specific activity at a constant catalyst loading results in an increase in volumetric productivity; decreasing batch reaction time, or residence time in a CSTR, is one way to reduce overall manufacturing cost. Alternatively, the catalyst charge can be reduced to maintain volumetric productivity when substituting a biocatalyst with higher specific activity, thereby allowing a greater percentage of the working volume of a batch reactor to be recovered and processed prior to start of the next batch reaction.

GA treatment of cells prior to immobilization also makes possible the production and storage of a dehydrated form of the biocatalyst that retains significantly more nitrilase activity when rehydrated than dehydrated/rehydrated biocatalyst produced without GA treatment of cells prior to immobilization. Storage of a dehydrated immobilized cell biocatalyst, and more importantly, transport of said biocatalyst between manufacturing sites, is considerably more cost-effective than storing or shipping an immobilized cell biocatalyst in aqueous buffer, particularly where storage and shipment at 5 °C is preferred to ambient temperature. Rehydration can be performed directly in a batch reactor or CSTR prior to commencement of ammonium glycolate production. There was no significant loss of nitrilase specific activity in GA-treated immobilized cell catalysts that had been dehydrated and then rehydrated immediately after preparation, or rehydrated after storage for 28 days at 5 °C, when compared to biocatalyst that had not been dehydrated/rehydrated. After dehydration/rehydration, the immobilized cell catalyst produced using GA-treated cells weighed only 60% as much as the biocatalyst prior to dehydration/rehydration, resulting in an increase in the biocatalyst dcw content from 5.0% dcw to 8.3% dcw; this increase in dcw content provided an additional increase in biocatalyst specific activity when measured as units/g immobilized cell biocatalyst instead of U/g dcw in biocatalyst, making possible a further reduction in catalyst charge to deliver a specified volumetric productivity.

## Experimental Section

### Materials and Methods

Chemicals were obtained from commercial sources unless otherwise noted, and used as received. The calculated % recovery of glycolonitrile and % yield of ammonium glycolate were based on initial glycolonitrile concentration, and determined by HPLC using a refractive index detector and a Bio-Rad HPX-87H column (30 cm × 7.8 mm dia.) and 0.001 N sulfuric acid as mobile phase at 50 °C. Fermentation of *E. coli* MG1655/pSW138-Phe168Val has been previously reported.[1] Nitrilase specific activity (U/g dcw) of whole cells recovered from fermentation (before or after GA treatment) was determined by mixing a cell suspension (8.5–12.5 mg dcw/mL) with 9.5 mM benzonitrile in 0.10 M potassium phosphate buffer (pH 7.0) and measuring conversion to ammonium benzoate at 245 nm, where a unit of enzyme activity (U) was equivalent to 1 micromole/min of benzonitrile conversion. Wet cell weights of microbial catalysts employed in immobilizations or assays were obtained from cell pellets prepared by centrifugation of fermentation broth or cell suspensions in buffer. Dry cell weights were determined using a Mettler-Toledo HR83-P halogen moisture analyzer. Specific activity of immobilized cell biocatalysts was based on dcw content and measured by determining the rate of conversion of 0.4 M glycolonitrile to ammonium glycolate by HPLC.

### Treatment of *E. coli* MG1655/pSW138-Phe168Val with GA and Sodium Bisulfite Prior to Immobilization

Aliquots (500 mL) were taken from 10-L fermentations of *E. coli* MG1655/pSW138-Phe168Val and placed in 2-L polyethylene flasks; the culture density was typically 120–140 $OD_{550}$ and cell viability ranged from $3 \times 10^{10}$ to $5 \times 10^{10}$ CFU/mL. The flasks were incubated in a shaking water bath at 35 °C and 175 rpm, and samples were taken and tested for cell viability and GA concentration immediately on or after GA inactivation with 1 g/L sodium bisulfite. The fermentation broth was adjusted to pH 5.2 and maintained at this pH with $H_2SO_4$ (2 N) and NaOH (2 N) while aqueous GA (5% w/w) was added at a rate of 50 mg GA/L fermentation broth/min; the final concentration of GA added was *ca.* 5.0 g GA/L (0.035 g GA/$OD_{550}$). At 5 h after initiation of GA addition, the pH was adjusted to 7.0 and aqueous sodium bisulfite (10% w/w, pH 7) was added to 1.0 g sodium bisulfite/L to neutralize residual GA, and the broth incubated for an additional 15 min. The resulting cell suspension was stored at 5 °C until the cells were recovered by centrifugation and immobilized.

Cell viability (in CFU/mL) was determined in samples after appropriate serial dilution with sterile saline, spreading on Luria Broth plates, incubation at 36 °C for 20 h and counting discernible colonies. Each sample was tested with two independent dilutions and each dilution plated on two LB plates. In instances where no cell growth was observed on any of the four plates with samples taken without dilution, it was assumed that there was one colony count in the four plates and results were expressed as equal or below the resulting calculated CFU/mL. Log reduction of viable cells was determined by subtraction of culture density in log (CFU/mL) before treatment from the culture density in log (CFU/mL) after treatment. GA concentration was determined after derivatization with dinitrophenylhydrazine and analysis by HPLC.[8]

### Immobilization of GA/Sodium Bisulfite-Treated *E. coli* MG1655/pNM18-Phe168Val in GA/PEI Cross-Linked Carrageenan Beads

The final cell suspension recovered from the GA/sodium bisulfite-treated fermentation broth described above was centrifuged at 5 °C. The resulting cell pellet was resuspended at 20 wt% (wcw) in 0.35 M potassium phosphate buffer (pH 7.2), and centrifugation of the resulting cell suspension at 5 °C produced a washed wet cell paste that was immobilized and then chemically cross-linked with GA and PEI as previously described.[1]

### Storage of GA/PEI Cross-Lnked Carrageenan-Immobilized *E. coli* MG1655/pSW138-Phe168Val

Freshly-prepared GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val prepared using cells either treated or not treated with GA/sodium bisulfite prior to immobilization were stored in 1.0 M ammonium bicarbonate (pH 7.3) at 5 °C. The biocatalyst beads were subsequently washed twice for 15 min with a 20-fold excess (w/w) of 0.1 M ammonium glycolate (pH 7.3) at room temperature, then evaluated in duplicate sets of consecutive batch reactions with biocatalyst recycle as described above.

### Dehydration/Rehydration of GA/PEI Cross-Linked Carrageenan-Immobilized *E. coli* MG1655/pSW138-Phe168Val Transformant

GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-F168V biocatalyst beads were dehydrated in a vacuum oven (176 mm Hg) at 35 °C with nitrogen purge for 24 h. The ratio of dehydrated bead weight to original (not dehydrated) bead weight was 0.0914. Dehydrated immobilized cell biocatalyst was stored under nitrogen at 5 °C. The dehydrated beads were subsequently rehydrated by placing the dehydrated beads in a 20-fold excess (w/w) of 0.10 M ammonium glycolate (pH 7.3) at either 5 °C or 25 °C for 18 h. The resulting rehydrated beads were washed twice with a 9-fold excess (w/w) of 0.10 M ammonium glycolate (pH 7.3), then weighed; the ratio of rehydrated bead weight to original (not dehydrated) bead weight was 0.210 for beads rehydrated at 5 °C, and the ratio of rehydrated bead weight to original bead weight was 0.212 for beads rehydrated at 25 °C. Dehydration of immobilized cell biocatalyst beads prepared with GA/sodium bisulfite-treated cells was performed as described above; the ratio of final dehydrated bead weight to original (not dehydrated) bead weight was 0.217, and the ratio of rehydrated bead weight to original bead weight was 0.578 for beads rehydrated at 5 °C.

### Consecutive Batch Reactions with Catalyst Recycle using GA/PEI Cross-Linked Carrageenan-Immobilized *E. coli* MG1655/pSW138-Phe168Val Transformant

In a typical procedure, duplicate sets of batch reactions for the conversion of glycolonitrile to glycolic acid were run in 50-mL jacketed reaction vessels equipped with overhead stirring and temperature control. Each reactor was charged with 8 g of GA/PEI-cross-linked *E. coli* MG1655/pSW138-Phe168Val/carrageenan beads containing 5% (dcw) cells that were either treated or not treated with GA/sodium bisulfite prior to immobilization. To the vessel was then added 14.78 mL of distilled water and 6.0 mL of aqueous ammonium glycolate (4.0 M, pH 7.0), and the reaction vessel flushed with nitrogen. The mixture was stirred at 25 °C while programmable syringe pumps were used to simultaneously add 1.07 mL of 60 wt% glycolonitrile in water (12.0 mmol glycolonitrile, 0.084 mmol formaldehyde (Fluka; redistilled, stabilized with 0.5 wt% glycolic acid)) and 0.150 mL of aqueous ammonium hydroxide (1.875 wt%); one equivalent volume of glycolonitrile and ammonium hydroxide solutions was added simultaneously every 2 h (for a total of eight equivalent additions each of glycolonitrile solution and aqueous ammonium hydroxide) to maintain the concentration of glycolonitrile at $\leq$400 mM and the pH within a range of 6.5–7.5. Four 0.050-mL reaction samples were removed at predetermined times after the first glycolonitrile addition and analyzed by HPLC to determine the initial reaction rate and the biocatalyst specific activity (μmol glycolic acid/min/g dcw biocatalyst). At completion of the reaction, there was 100% conversion of glycolonitrile to produce glycolic acid (as the ammonium salt) in >99% yield. At the end of the first reaction, the aqueous product mixture was decanted from the catalyst (under nitrogen), leaving *ca.* 10.3 g of a mixture of immobilized cell catalyst (8.0 g) and remaining product mixture (*ca.* 2.3 g). To the reaction vessel were then added 20.78 mL of distilled, deionized water, and a second reaction was performed at 25 °C by the addition of aliquots of aqueous GLN and ammonium hydroxide as described immediately above.

Reactions run to compare immobilized cell biocatalysts before and after dehydration/rehydration followed a similar procedure, where a catalyst loading was chosen to provide an equivalent concentration of immobilized dcw per reaction volume.

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