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# Chemoenzymatic Synthesis of Glycolic Acid

Anna Panova,[a] Lawrence J. Mersinger,[a] Qiang Liu,[a] Thomas Foo,[a] D. Christopher Roe,[a] William L. Spillan,[a] Amy E. Sigmund,[a] Arie Ben-Bassat,[a] L. Winona Wagner,[a] Daniel P. O'Keefe,[a] Shijun Wu,[a] Kelly L. Petrillo,[a] Mark S. Payne,[a] Stephen T. Breske ,[a] F. Glenn Gallagher,[a] and Robert DiCosimo[a,*]

[a] DuPont Central Research and Development, Experimental Station, P.O. Box 80328, Wilmington, DE 19880-0328, USA
Fax: (+1)-302-695-8114; e-mail: Robert.DiCosimo@usa.dupont.com

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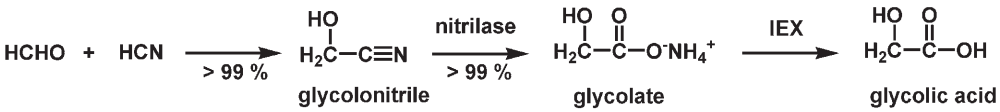
**Abstract:** A chemoenzymatic process for the production of high-purity glycolic acid has been demonstrated, starting with the reaction of formaldehyde and hydrogen cyanide to produce glycolonitrile in >99% yield and purity. The resulting aqueous glycolonitrile was used without further purification in a subsequent biocatalytic conversion of glycolonitrile to ammonium glycolate. A high-activity biocatalyst based on an *Acidovorax facilis* 72W nitrilase was developed, where protein engineering and optimized protein expression in an *E. coli* transformant host were used to improve microbial nitrilase specific activity by 33-fold compared to the wild-type strain. A biocatalyst productivity of >1000 g glycolic acid/g dry cell weight was achieved using a glutaraldehyde/polyethylenimine cross-linked carrageenan-immobilized *E. coli* MG1655 transformant expressing the *A. facilis* 72W nitrilase mutant, where 3.2 M ammonium glycolate was produced in consecutive batch reactions with biocatalyst recycle, or in a continuous stirred-tank reactor. Direct conversion of the unpurified ammonium glycolate product solution to high-purity aqueous glycolic acid was accomplished by fixed-bed ion exchange over a strong acid cation resin.

**Keywords:** enzyme catalysis; glycolic acid; glycolonitrile; nitrilases

## Introduction

Glycolic acid (GLA) is used in the preparation of a large variety of products, including consumer and industrial cleaners,[1] personal care products,[2] and as a monomer in the preparation of polyglycolic acid (PGA) for dissolvable sutures,[3] drug-delivery materials[4] and gas barrier packaging materials.[5] The majority of glycolic acid currently produced commercially is prepared by the high-pressure, high-temperature reaction of formaldehyde and carbon monoxide by acid catalysis.[6] Alternative processes produce glycolic acid by the chemical hydrolysis of chloroacetic acid[7] or by the chemical hydrolysis of glycolonitrile to produce the ammonium salt of glycolic acid, which is subsequently converted to methyl glycolate, distilled and hydrolyzed.[8] The use of *Rhodococcus rhodochrous*,[9] *Acidovorax facilis* 72W,[10] *Brevibacterium casei*,[11] or *Acinetobacter* sp. AK226[12] microbial nitrilase for hydrolysis of glycolonitrile to ammonium glycolate, followed by conversion of the salt to GLA, has been reported. The oxidation of ethylene glycol to glycolic acid has been demonstrated using an *E. coli* recombinant having enhanced lactoaldehyde reductase and lactoaldehyde dehydrogenase activities,[5] *Candida xestobii* GA28,[13] and *Aureobasidium pullulans* GA37or *Williopsis saturnus* GA101.[14]

Many current chemical or enzymatic methods for the synthesis of GLA produce significant amounts of impurities that must be removed prior to its use in some applications, such as production of PGA. We now describe a chemoenzymatic process for the production of GLA (Scheme 1), where formaldehyde (HCHO) and hydrogen cyanide (HCN) are first react-




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

ed to prepare an aqueous solution of glycolonitrile (GLN) in high yield and purity, and without further purification, the aqueous GLN is converted to ammonium glycolate ($NH_4GLA$) in high yield and purity using an immobilized microbial nitrilase, and the product mixture containing $NH_4GLA$ is directly converted to GLA using ion exchange chromatography. The present process offers an advantage over most alternative existing processes, where a high-purity GLA is produced without the need for expensive distillation or crystallization steps.

## Results and Discussion

### Preparation of Glycolonitrile

The present chemoenzymatic process for production of GLA required high-purity GLN. Methods to synthesize GLN by reacting aqueous solutions of formaldehyde and hydrogen cyanide have previously been reported,[15] but these methods typically result in an aqueous reaction product that requires significant purification (e.g., distillation). Impurities and/or by-products of the reaction (including excess reactive formaldehyde) can interfere with the biocatalytic hydrolysis of GLN to GLA, including enzyme inactivation and/or inhibition that decreases the overall productivity of the catalyst (i.e., total grams of glycolic acid produced per gram of catalyst). A GLN synthesis was required that 1) increased overall GLN yield, 2) minimized unwanted impurities and/or by-products, especially HCHO, and 3) employed a relatively simple process to reduce manufacturing costs.

The reaction of HCN and HCHO has been measured to be first order in both cyanide anion and unhydrated monomeric formaldehyde.[16] Pure, dry monomeric HCHO is reactive and readily polymerizes as a gas or liquid at ordinary temperatures; therefore it is produced commercially as an aqueous solution, where HCHO is present in an equilibrium mixture composed predominately of the monomeric hydrate and a series of low molecular weight polymeric hydrates.[17] The equilibrium concentration of unhydrated monomeric HCHO is well under 0.1% even in highly concentrated solutions.[18] Consequently, the reaction of HCN and HCHO under published conditions, where decomposition of GLN is minimized, generally results in product mixtures in which significant fractions of the HCHO remain unreacted.[19] Residual HCHO is a problematic inhibitor of nitrilase (see below); therefore, to increase the conversion of HCHO to GLN while minimizing GLN degradation,[15a,20] new ways to access more reactive lower molecular weight distributions of aqueous HCHO were needed.

Consistent with entropic considerations, lower molecular weight distributions of HCHO are favored at higher temperatures and lower total HCHO concentrations.[17] Such thermodynamic considerations suggested heating a diluted formaldehyde feed stream and mixing the pre-heated solution directly into a reaction mixture containing an excess of HCN. The use of NaOH as catalyst increased the rate of equilibration of the HCHO oligomer distribution and the rate of reaction of HCN with HCHO to make GLN. The amount of NaOH needed to effectively activate aqueous formaldehyde at 100–120 °C over a residence time of about 2–5 min was as low as 0.1 mol% of the HCHO, but depending on the acidity of the HCHO source, addition of NaOH up to 0.5 mol% of the HCHO may be desirable to adjust the HCHO feed solution to pH 9–10 prior to heating.

The use of formaldehyde feed pre-heating to produce high-purity GLN solutions was demonstrated in semi-batch reactions at a scale of 2.5 mol of glycolonitrile per batch (~500 mL scale) and subsequently scaled up to 25-mol batches. To ensure that the reaction was conducted in the presence of excess HCN throughout, the reaction vessel was initially charged with an aqueous solution containing an amount of HCN equivalent to 5–10 mol% of the total HCHO to be fed. The HCN and pre-heated HCHO feed solutions were then fed at equimolar rates to the vessel over a 2 h period. Pre-heating the formaldehyde feed allowed the reaction with HCN to be conducted in a temperature range of 20–30 °C, which minimized the degradation of glycolonitrile. The beneficial effect of formaldehyde feed pre-heating has also been demonstrated across a range of methanol concentrations in the formaldehyde source (from $<1$% in 52% HCHO to 15% in 37% HCHO, added during manufacture as a stabilizer). Upon completing the reactant feeds to the semi-batch reaction, the GLN product solution was stabilized at an acidic pH ($<$pH 4) by the addition of GLA (0.5–1.5 mol% of the GLN produced), then both the volatile excess HCN and any methanol contained in the formaldehyde feed was removed by concentration of the solution by distillation.

Concentrated aqueous GLN product solutions were analyzed using quantitative $^{13}C$ NMR and/or $^{1}H$ NMR spectroscopy (see Experimental Section); Figure 1 depicts the $^{13}C$ NMR spectrum of one such GLN product solution. Using this analytical method, it was demonstrated that the GLN synthesis process employing the pre-heating of the formaldehyde feed solution reproducibly yielded a high-purity product, with organic impurities totaling less than 1 mol% of the GLN produced, with no single impurity appearing to exceed 0.25 mol%. The aqueous GLN solution prepared by this optimized reaction was sufficiently pure to allow its use directly in the subsequent biocatalytic hydrolysis step, without any significant purification to elimi-

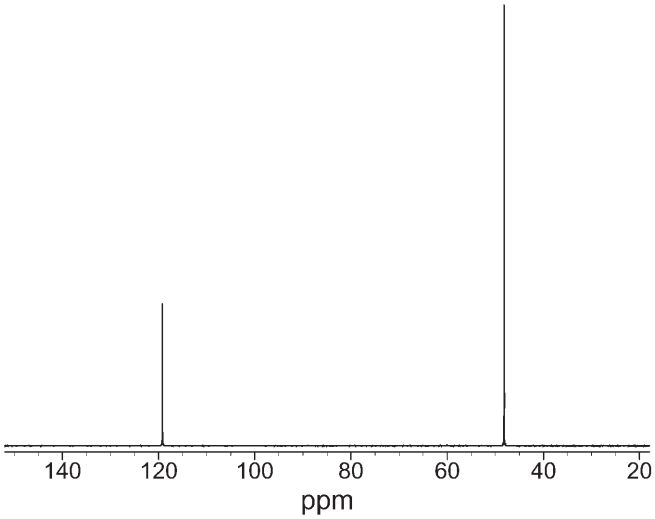



**Figure 1.** $^{13}C$ NMR spectrum of concentrated glycolonitrile product solution.

nate or limit the presence of unreacted starting materials, by-products or impurities that have a negative impact on nitrilase stability and productivity, or on GLA product purity.

## Optimization of Biocatalyst Specific Activity

The gene encoding *Acidovorax facilis* 72W (ATCC 55746) nitrilase has been previously cloned and expressed in *E. coli*.[21] This nitrilase converts a wide variety of nitriles to the corresponding carboxylic acids, including GLN to GLA, but an initial evaluation of this nitrilase in the present application determined that a nitrilase biocatalyst with increased glycolonitrile specific activity was required to achieve a volumetric productivity target of 17 g GLA/L/h. A combination of protein engineering, high throughput screening, and nucleotide sequence analysis was used to identify *A. facilis* 72W nitrilase mutants having improved GLN specific activity.[22] Recombinant strains of *E. coli* that expressed the native 72W nitrilase or nitrilase mutants were evaluated at the 10 L scale for growth ($OD_{550}$), nitrilase specific activity [IU/g dry cell weight (dcw)] and nitrilase volumetric productivity (IU/L/h). Table 1 summarizes the specific activity and improvement in enzyme and biocatalyst specific activity for several *E. coli* transformants that express nitrilase mutants with a single amino acid substitution at position 168, relative to the specific activity of the *A. facilis* 72W strain from which the nitrilase gene was isolated. A 4.1-fold increase in GLN specific activity was obtained for the Phe168Val mutant nitrilase in *E. coli* MG1655 when comparing native and mutant nitrilases at the same protein expression level. An overall increase in microbial cell specific activity of 33-fold was obtained compared to *A. facilis* 72W by improvement in both nitrilase specific activity and protein expression level in *E. coli* MG1655.

## Immobilization of *E. coli* Nitrilase Transformants

Immobilization of the microbial cell nitrilase provided several advantages when compared to the use of unimmobilized cells in batch or continuous reactions to produce glycolic acid; the immobilized cells were readily recovered and recycled in consecutive batch reactions, and immobilization provided improved nitrilase stability under reaction conditions and higher

**Table 1.** Specific activity and relative enzyme and microbial specific activity for GLN hydrolysis by microbial nitrilase catalysts.

| Microbial nitrilase | Microbial specific activity[a] (GLN IU/g dcw) | Relative enzyme specific activity[b] | Relative microbial specific activity[c] |
|---|---|---|---|
| *A. facilis* 72W | 60 | | 1.0 |
| *E. coli* BL21(DE3)/pnitex2 | 125 | – | 2.1 |
| *E. coli* FM5/pNM18 | 387 | | 6.5 |
| *E. coli* MG1655/pSW138 | 490 | 1.0 | 8.2 |
| *E. coli* MG1655/pSW138-Phe168Met | 1460 | 3.0 | 24.3 |
| *E. coli* MG1655/pSW138-Phe168Lys | 1542 | 3.1 | 25.7 |
| *E. coli* MG1655/pSW138-Phe168Val | 1992 | 4.1 | 33.2 |

[a] Microbial specific activities (GLN IU/g dcw) measured at 25 °C using 5–25 mg dcw/mL in 0.10 M potassium phosphate buffer (pH 7.0) containing 0.50 M GLN.
[b] Comparison of mutant nitrilase specific activities to *A. facilis* 72W nitrilase specific activity at the same protein expression level in *E. coli* MG1655.
[c] Comparison of *E. coli* transformant specific activities to *A. facilis* 72W specific activity.

product purity, eliminating the need for product purification prior to ion exchange chromatography. In previous evaluations of methods for immobilization of nitrile-hydrolyzing microbial catalysts, encapsulation in either alginate or carrageenan beads was simple to perform, inexpensive, and produced a robust catalyst.[23] Microbial nitrilase catalysts immobilized in calcium-cross-linked alginate beads could not be used for production of glycolic acid, as both the acid and its salts were highly effective for sequestration of calcium ion, leading to the dissolution of the alginate gel during the reaction.

The immobilization protocol started with heating a 12.5% dry cell weight suspension in 0.35 M phosphate buffer (pH 7) to 50 °C prior to mixing with a 5 wt% carrageenan solution at 50 °C, and the resulting 50 °C cell/carrageenan suspension was immediately dripped into a potassium bicarbonate buffer to produce the catalyst beads (*ca.* 2.5 mm diameter). The immobilized cells (5% dcw in 3% carrageenan beads) were subsequently chemically cross-linked with glutaraldehyde (GA) and polyethylenimine (PEI), producing a physically-robust immobilized-cell catalyst with acceptable gel strength for use in both batch and continuous biocatalytic reactions. Batch immobilization of microbial nitrilase in carrageenan required that the nitrilase activity be stable at 50 °C for *ca.* 30 min. Temperature stabilities of cell suspensions in 0.35 M phosphate buffer (pH 7) were measured at 50 °C, and *E. coli* transformants expressing the native 72W nitrilase lost 40-50% of initial activity after 6 h and 87% of initial activity after 24 h, whereas transformants expressing the Phe168Val mutant nitrilase lost only *ca.* 6–13% of initial activity after 24 h under identical conditions. Comparisons of the specific activities of uncross-linked versus GA/PEI cross-linked immobilized cell catalysts indicated that there was no measurable loss of enzyme activity due to the GA/PEI cross-linking step.

### Temperature Dependence of *E. coli* MG1655/pSW138 and *E. coli* MG1655/pSW138-Phe168Val Nitrilase Specific Activity

The dependence of specific activity on temperature was determined for both unimmobilized *E. coli* MG1655/pSW138 and *E. coli* MG1655/pSW138-Phe168Val (Figure 2). 3-Hydroxybutyronitrile was substituted for GLN in the assays, as GLN was not chemically stable under the assay conditions over the temperature range employed. The Phe168Val mutant nitrilase was slightly less stable at higher temperatures than the native nitrilase, where the specific activity of the Phe168Val mutant nitrilase began to decrease at a temperature greater than 60 °C, whereas a decrease in specific activity of the native nitrilase did not occur until the temperature exceeded 65 °C; both activities sharply declined above 65 °C.

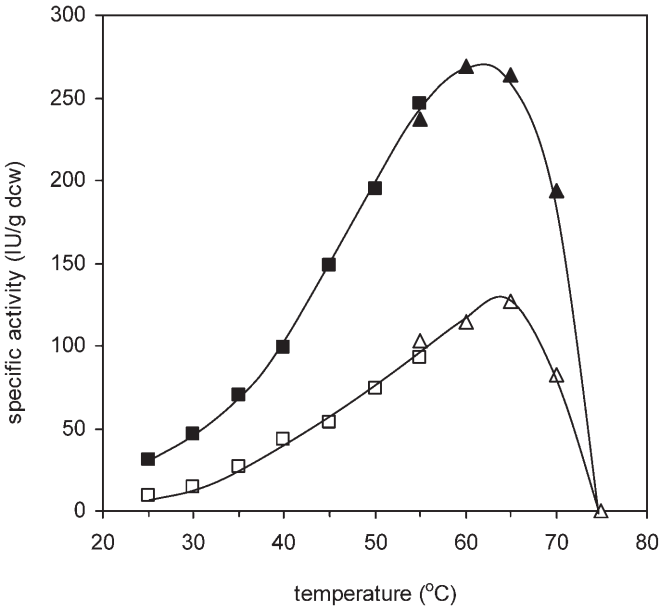



**Figure 2.** Temperature dependence of unimmobilized *E. coli* MG1655/pSW138 (□, △) and *E. coli* MG1655/pSW138-Phe168 Val (■, ▲) nitrilase activity (2.5–25 mg dcw/mL in 0.10 M $KH_2PO_4$ buffer (pH 7.0)) assayed using 3-hydroxybutyronitrile (0.50 M) as substrate. At < 55 °C, cell suspensions (1 mL) were heated to assay temperature for 5 min prior to mixing with heated assay solution (3 mL); at > 55 °C, unheated cell suspensions (0.1 mL) were mixed with heated assay solution (3.9 mL) to start the assay.

The specific activity of the immobilized Phe168Val mutant nitrilase did not increase significantly with increasing temperature above 25 °C, which may be due to diffusional limitations when using 2.5 mm diameter catalyst beads containing 5% dcw microbial nitrilase having high specific activity; above a certain reaction temperature, diffusion of GLN into the catalyst bead may become rate-limiting. The recovered catalyst activity in consecutive batch reactions with catalyst recycle decreased more rapidly when using the Phe168Val mutant nitrilase at 35 °C when compared to reactions run at 30 °C or 25 °C. A reaction temperature of 25 °C was optimal for GLN stability, volumetric productivity and biocatalyst productivity.

### Optimal GLN and GLA Concentrations

In aqueous solution, GLN is in equilibrium with HCN and HCHO, although the reported equilibrium constant strongly favors the formation of the α-hydroxynitrile.[16] GLN readily polymerizes at neutral to basic pH to form a complex mixture of products,[24] and is normally sold as a 55–70 wt% aqueous solution that is stabilized with 0.5 wt% sulfuric acid or phosphoric

acid at a pH of $<2$. The affect of GLN concentration on biocatalyst activity and productivity was evaluated, where optimal reaction conditions were chosen to balance the dependence of nitrilase activity and productivity on GLN concentration, the pH of the reaction, and on the chemical stability of GLN under reaction conditions, which was also pH dependent.

An initial study of the affect of GLN concentration on catalyst activity and stability in consecutive batch reactions with catalyst recycle was performed using GA/PEI cross-linked, carrageenan-immobilized *E.coli* BL21(DE3)/pnitex2 (7.5% dcw) as catalyst at pH 7.5 and 25°C. Three modes of GLN addition to the reaction were compared: a single addition of GLN to produce a final concentration of 0.90 M or 2.70 M, and a sequential addition of three equal GLN aliquots to produce a final concentration of 2.30 M, with each aliquot added after complete conversion of the previous aliquot. Sequential addition of three equal GLN aliquots to each batch reaction to produce 2.3 M GLA resulted in a biocatalyst stability over consecutive batch reactions *ca.* equivalent to that obtained with a single GLN addition to produce 0.9 M GLA; biocatalyst productivity (g GLA/g dcw) was *ca.* 2.5-fold greater per batch reaction that produced the higher GLA concentration, with no decrease in yield or product purity. An initial GLN concentration of 2.7 M resulted in rapid GLN oligomerization and significant color formation, along with significant loss of nitrilase activity, over the course of a single batch reaction.

In three separate series of consecutive batch reactions using GA/PEI cross-linked, carrageenan-immobilized *E. coli* MG1655/pSW138 to produce 2.4–2.5 M ammonium glycolate at pH 7.5, sequential additions to produce initial GLN concentrations of 0.3 M, 0.4 M or 1.0 M resulted in similar biocatalyst stabilities and productivities over consecutive batch reactions, therefore initial GLN concentrations of $\leq 1$ M were employed in all subsequent batch recycle reactions. There was no significant difference in GLN hydrolysis rates measured using GA/PEI cross-linked immobilized *E. coli* MG1655/pSW138-Phe168Val in batch reactions containing 1.0 M GLN and 0.10 M, 0.60 M or 3.0 M NH4GLA (pH 7.3), indicating no measurable product inhibition of nitrilase within this product concentration range.

## Dependence of Biocatalyst Specific Activity and Productivity on pH

The dependence of initial specific activity of GA/PEI cross-linked immobilized *E. coli* MG1655/pSW138 and/pSW138-Phe168Val biocatalysts on pH was measured (Figure 3). The activity of the immobilized native 72W nitrilase was constant over the pH range of 7.5 to 5.5, and then decreased rapidly from pH 5.5 to 4.0. The specific activity of the immobilized Phe168Val nitrilase mutant at pH 6.5 was more than 2.5-fold higher than that of the immobilized native microbial nitrilase, but decreased by *ca.* 86% as the pH decreased from 6.5 to 5.8. Recovered biocatalyst activity (U/g dcw) of the immobilized Phe168Val microbial nitrilase was measured over the course of fifteen consecutive batch reactions with catalyst recycle at pH 5.8, 6.5, and 7.5, where for each reaction eight GLN aliquots (producing an initial GLN concentration of 0.40 M) were added sequentially to convert a total of 2.5 M GLN to GLA (20 wt% biocatalyst charge in the final reaction volume, 0.8 M initial $NH_4$GLA concentration at the start of the first batch reaction run at 25°C under nitrogen). The recovered nitrilase activity (relative to the initial catalyst activity in the first batch reaction) after each batch reaction was similar at each reaction pH, but the reaction rates at pH 5.8 and 6.5 were *ca.* 33% and 80%, respectively, of the rate at pH 7.5. At a reaction pH $>8.0$, significant GLN oligomerization and color formation was observed, therefore, a reaction pH of *ca.* 7.5 was employed in subsequent batch and continuous biocatalytic reactions to achieve optimal reaction rate, catalyst and volumetric productivity, and product purity.

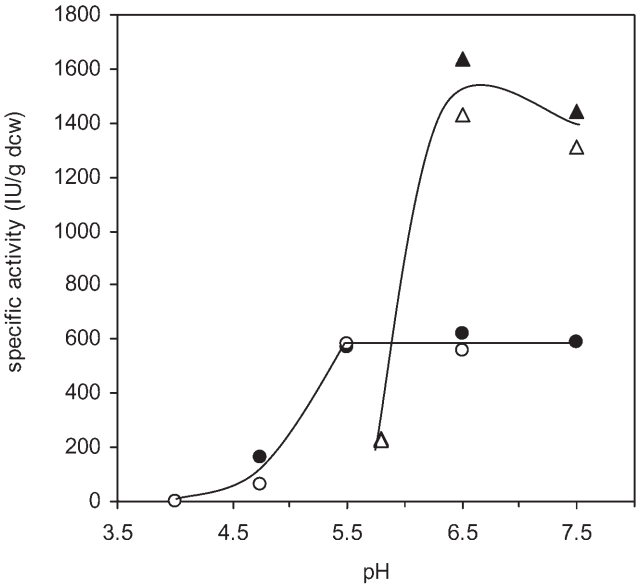



**Figure 3.** Dependence of nitrilase activity of GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138 (20 wt% biocatalyst, 1.0 M GLN; ●, ○) and *E. coli* MG1655/pSW138-Phe168Val (27 wt% biocatalyst, 0.4 M GLN; △, ▲) on pH at 25°C.

### Dependence of Biocatalyst Activity and Productivity on HCHO and HCN Concentrations

Low concentrations of HCHO and/or HCN may be present in the biocatalytic reaction mixture, either from incomplete conversion during GLN synthesis or from dissociation of glycolonitrile (although the equilibrium constant[16] strongly favors GLN formation). The concentration of HCHO in GLN prepared in the first process step was typically 0.2–0.4 mol% of GLN, equivalent to *ca.* 5–10 mM HCHO in a single batch reaction during conversion of 2.4 M GLN. The effect of low concentrations of HCHO and HCN on biocatalyst activity and productivity was initially evaluated in consecutive batch reactions with catalyst recycle using immobilized *E. coli* FM5/pNM18, where either 5 mol% HCN (relative to 2.4 M final GLN concentration) or 5 mol% HCHO were added to each reaction. There was no significant decrease (<4%) in reaction rate or catalyst productivity over the course of seven consecutive batch reactions containing added HCN, and no difference in recovered catalyst activity when compared to a set of control reactions run with no added HCHO or HCN.

The dependence of nitrilase specific activity on HCHO concentration was examined in a series of consecutive batch reactions with catalyst recycle of GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val using a GLN solution containing 0.4, 0.8, 1.0, 1.3, or 2.6 mol% HCHO (relative to GLN) (Figure 4). Eleven consecutive batch reactions were run using GLN containing 0.4, 0.8, and 1.0 mol% HCHO, and seven consecutive reactions were run with GLN containing 1.3 or 2.6 mol% HCHO prior to switching to GLN containing 0.4 mol% added HCHO in subsequent recycle reactions in these series. Decreasing catalyst specific activity was observed with increasing concentration of HCHO over the course of the first seven consecutive batch reactions in these series; substituting 0.4 mol% HCHO for 1.3 or 2.6 mol% HCHO after the seventh batch reaction of a series resulted in an increase in catalyst activity in subsequent reactions to finally equal the specific activity observed when only 0.4 mol% HCHO was added to consecutive batch reactions, again demonstrating the reversible nature of nitrilase inhibition by HCHO.

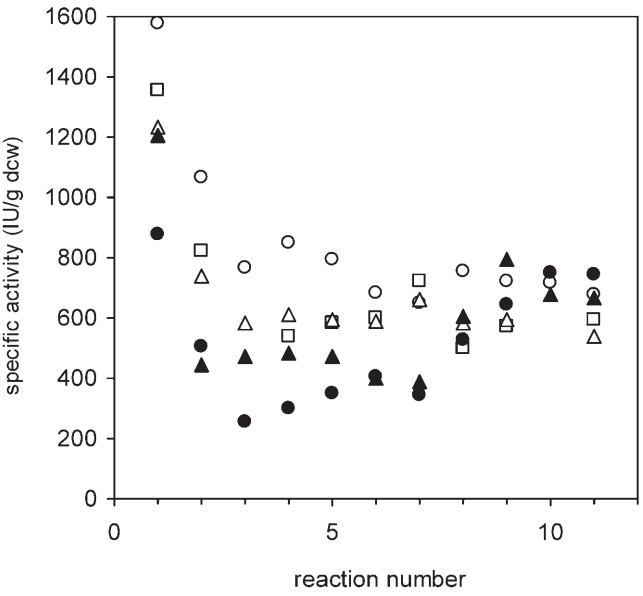



**Figure 4.** Effect of HCHO concentration on recovered biocatalyst activity of GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val in consecutive batch reactions with biocatalyst recycle (20 wt% biocatalyst charge) at pH 7.5 and 25 °C. Eight equivalent GLN aliquots were added sequentially (at complete GLN conversion) to produce $NH_4GLA$ (2.5 M) in the final reaction volume. Eleven consecutive batch reactions were run using GLN containing 0.4 (○), 0.8 (□), and 1.0 mol% HCHO (△), and seven consecutive reactions were run with GLN containing 1.3 (▲) or 2.6 mol% HCHO (●) prior to switching to GLN containing 0.4 mol% added HCHO in subsequent recycle reactions in these two series.

### Volumetric and Catalyst Productivity of Immobilized Microbial Nitrilase Mutants

Consecutive batch reactions with catalyst recycle were run under nitrogen at 25 °C using three GA/PEI cross-linked carrageenan-immobilized cell catalysts: *E. coli* MG1655/pSW138-Phe168Val, Phe168Met and Phe168Lys (Figure 5). The total concentration of GLN (commercial grade, purified prior to use by vacuum distillation of the GLN) converted in each reaction was 2.4 M (added in eight equivalent aliquots). The decrease in biocatalyst specific activity after the first batch reaction was 43% for Phe168Val, 51% for Phe168Lys, and 26% for Phe168Met, but the remaining nitrilase activity was relatively stable in the following nineteen batch reactions. Nitrilase mutants Phe168Met and Phe168Lys each lost substantial activity after *ca.* 22 recycles, and were discontinued after 27 reactions (biocatalyst productivity = 494 g GLA/g dcw). For recycle reactions using the Phe168Val biocatalyst, 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 the series). The initial and final volumetric productivities were 46 g GLA/L/h and 36 g GLA/L/h, respectively.

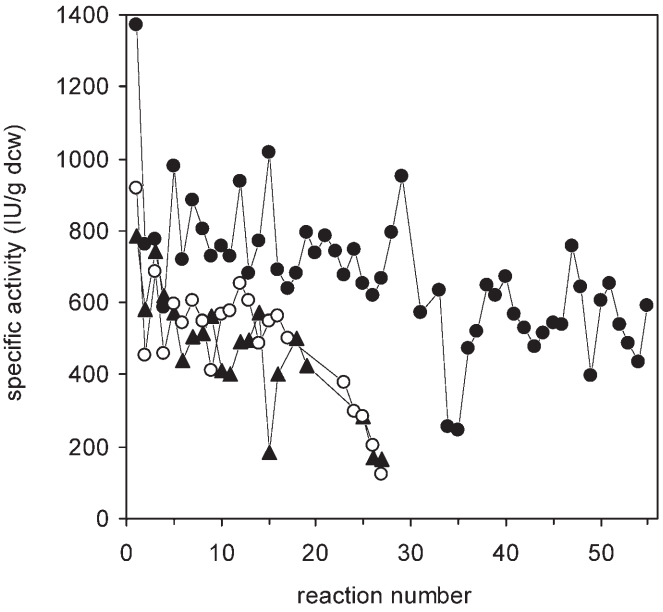



**Figure 5.** Consecutive batch reactions with biocatalyst recycle of GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val (●), *E. coli* MG1655/pSW138-Phe168Lys (○) and *E. coli* MG1655/pSW138-Phe168Met (▲) (20 wt% biocatalyst charge) at pH 7.5 and 25°C. Eight equivalent GLN aliquots were added sequentially (at complete GLN conversion) to produce 2.4 M $NH_4$GLA in the final reaction volume; carryover of reaction "heel" from previous the batch reaction results in a total final concentration of 3.2 M $NH_4$GLA in each batch reaction.

## Continuous Stirred-Tank Reactor and Packed-Bed Column Reactor for Ammonium Glycolate Production

A continuous stirred-tank reactor (CSTR)[25] was run to demonstrate that GLN produced by the reaction of HCHO and HCN could be used without further purification in the biocatalytic hydrolysis reaction; the GLN product solution was simply concentrated by distillation to remove water, methanol (a stabilizer in some grades of commercial formaldehyde) and HCN (used in excess during the reaction to produce GLN), and subsequently treated with activated carbon. The CSTR was started as a batch reaction containing 35 wt% (60 vol%) GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val catalyst beads, and then converted to continuous operation by separately feeding aqueous GLN (3.45 M, stabilized with 1.2 mol% GLA) and aqueous ammonium hydroxide (1 wt%, to provide pH control) to the reactor. Product mixture was continuously removed to maintain a constant reaction volume. The feed rate was adjusted over the course of the CSTR to maintain a 98–99% GLN conversion, producing a steady-state concentration of *ca.* 3.2 M $NH_4$GLA.

Table 2 lists the feed rates, average GLN conversion, residence time (reaction volume/total feed rate), volumetric productivity, and biocatalyst productivity for each time period at constant feed rate over 644 h of CSTR operation. The initial volumetric productivity was 29.7 g GLA/L/h at 99.0% GLN conversion (7.6 h residence time). After 644 h (26.8 days) of continuous operation, the residence time increased to 8.9 h, and the volumetric productivity and catalyst productivity were 25.1 g GLA/L/h and 1027 g GLA/g dcw, respectively. The final biocatalyst specific activity was *ca.* 82% of initial activity, and there was no observable attrition of the catalyst beads over the course of CSTR operation. A second CSTR run using vacuum-distilled GLN as feed produced almost identical results for all CSTR parameters, demonstrating that GLN produced in the first chemoenzymatic process step could be used without affecting volumetric or catalyst productivity.

The dependence of GLN conversion on residence time was measured in a third CSTR (Figure 6). An initial GLN conversion of 97.5% to produce *ca.* 3.3 M GLA was obtained using an 8.2 h residence time. Feed rates were subsequently decreased over the

**Table 2.** GLN conversion, residence time, volumetric productivity and total biocatalyst productivity in a CSTR using unpurified GLN to 3.2 M $NH_4$GLA.[a]

| Time [h] | GLN feed [mL/h] | $NH_4OH$ feed [mL/h] | Residence time [h] | GLN conversion [%] | Volumetric productivity [g GLA/L/h] | Total biocatalyst productivity [g GLA/g dcw] |
|---|---|---|---|---|---|---|
| 21 | 45.7 | 6.9 | 7.6 | 99.0 | 29.7 | 36 |
| 141 | 45.2 | 6.8 | 7.7 | 98.5 | 29.2 | 236 |
| 168 | 44.6 | 6.6 | 7.8 | 98.7 | 28.9 | 280 |
| 313 | 44.3 | 6.8 | 7.8 | 98.5 | 28.6 | 517 |
| 477 | 42.4 | 6.4 | 8.2 | 98.5 | 27.4 | 774 |
| 573 | 42.7 | 6.4 | 8.1 | 98.3 | 27.6 | 925 |
| 644 | 39.0 | 5.8 | 8.9 | 98.2 | 25.1 | 1027 |

[a] The CSTR was run at 25°C with pH control at pH 7.0–7.5 using GA/PEI cross-linked carrageenan immobilized *E. coli* MG1655/pSW138-Phe168Val biocatalyst beads (35 wt% catalyst charge). Aqueous GLN (3.45 M, stabilized with 1.2 mol% GLA at pH 3) and aqueous ammonium hydroxide (1:38 dilution of concentrated $NH_4OH$) were fed at the indicated rates to maintain GLN conversion at 98–99%.

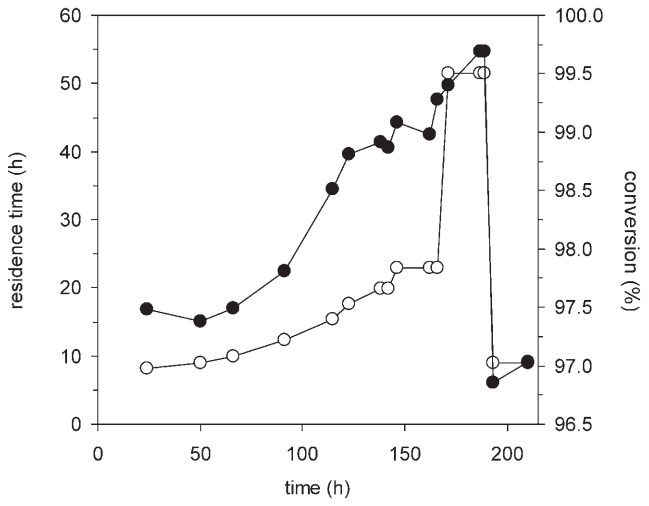



**Figure 6.** Dependence of GLN conversion (●) on residence time (○) in a CSTR using GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val (35 wt% catalyst charge) for production of $NH_4GLA$ (3.2 M) at 25 °C and pH 7.3–7.5.

course of CSTR operation to increase GLN conversion to *ca.* 99% (22.9 h residence time), and then to 99.7% (51.6 h residence time), where the concentration of unconverted GLN was *ca.* 34 mM and 10 mM, respectively. After 189 h of continuous operation, feed rates were reset to those initially used for an 8.2 h residence time, and the conversion decreased to 97.0%, indicating that there was no significant ($<0.5\%$) loss of catalyst activity.

To measure the change in rate of a reaction as the concentration of GLN approached zero, a batch reaction was run using a 20 wt% catalyst charge of GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val catalyst beads to convert three equivalent GLN additions to ammonium glycolate (3.3 M final product concentration), where the change in concentration of GLN with time was measured continuously after the final GLN addition. A $K_M$ for GLN of 180 mM was determined for the carrageenan-immobilized microbial nitrilase catalyst beads using kinetic analysis of the resulting progress curve.[26] The relatively-high $K_M$ for GLN limits the conversion that can be achieved in an acceptable residence time using a single CSTR, but the use of two CSTRs in series, or a CSTR followed by a packed-bed column reactor (see below), can produce $>99.95\%$ GLN conversion using residence times acceptable for commercial manufacture.

A packed-bed column reactor containing GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val catalyst beads was run using a CSTR product mixture containing 3.19 M $NH_4GLA$ and 152 mM unpurified GLN (95% GLN conversion in the CSTR). A column residence time of 90 min resulted in a 99.97% total conversion of the GLN (conversion based on total of GLA and GLN in column product mixture; the conversion in the column itself was 99.30–99.35% based on 152 mM GLN concentration in column feed). The feed rate to the column reactor at a residence time of *ca.* 90 min was *ca.* equivalent to the CSTR product solution production rate, indicating that the 400 mL CSTR containing a 35 wt% catalyst charge (140 g catalyst beads) and the 75 mL packed-bed column reactor containing 57.4 g catalyst beads, were appropriately matched so that a continuous feed of the CSTR product solution directly to the column reactor could achieve the desired GLN conversion.

The recovered biocatalyst activity at the conclusion of the packed-bed column reactor (344 h continuous operation) was 88% of initial activity, demonstrating that a constant feed of 150 mM GLN to the column did not rapidly inactivate the nitrilase catalyst. Constant exposure of the nitrilase in beads at the column inlet to 152 mM GLN could have resulted in a greater rate of loss of activity than was observed when the biocatalyst was constantly mixed in a CSTR during GLN addition, where in a CSTR there was an immediate dilution of the feed to a steady-state GLN concentration between 50 and 60 mM, and where all of the catalyst beads were equally exposed to this steady-state concentration.

## Chemoenzymatic GLA Process Demonstration

Four batch reactions of HCN with heat-treated aqueous HCHO (15–28 mol GLN/batch) were run to produce a total of 82 mol GLN ($>99\%$ yield and purity based on added HCHO). Except for concentration of the aqueous product mixtures to 40 wt% GLN by distillation of water, followed by treatment of the resulting concentrates with 0.5–1.0 wt% decolorizing carbon, the aqueous GLN was used without further purification in consecutive 4-L batch reactions with recycle of GA/PEI cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-Phe168Val biocatalyst. The resulting $NH_4GLA$ product solutions (3.0 M, $>99\%$ yield and purity) from six batch reactions were combined, and without dilution the aqueous $NH_4GLA$ (48.5 mol) was converted to GLA using fixed-bed cation exchange over a strong acid cation resin. The column fractions containing low ammonia (typically $<7$ ppm) were combined (25 mol GLA) and concentrated to produce 2.6 kg of high-purity 72 wt% GLA in water.

The extremely high ionic load in the feed to the cation exchange column limited the efficiency of the column for converting $NH_4GLA$ to GLA, which in turn limited the number of column fractions that could be collected prior to breakthrough of ammonia.

A typical strong acid cation resin possesses 1.8–2.0 equivalents of exchange sites per liter of resin, and the NH4GLA feed contained 3 equivalents per liter of exchangeable cations; *ca.* 32% of the theoretical column capacity was utilized prior to ammonium ion breakthrough under these operating conditions. Dilution of the feed would have increased column efficiency and yield of high purity glycolic acid, but would add significant cost for the subsequent removal of the additional water in a commercial process. Traditional fixed-bed ion exchange would be unsuitable for continuous processing due to the extremely rapid exhaustion of the beds, whereas a simulated moving-bed (SMB) type of continuous ion exchange system could be used at commercial scale; SMB units, while allowing for continuous operation, provide better resin utilization and more efficient regeneration as a result of the effective counter-current contacting that is achieved in such a system. Yields of high purity GLA of >98% (based on $NH_4$GLA) would be expected using an SMB ion exchange unit.

The concentration of organic impurities in the product of each step of the chemoenzymatic process were determined by quantitative $^1$H NMR, and indicated that total organic impurities did not increase during the biocatalytic hydrolysis step. The concentration of total organic impurities detected in the final product (72 wt% high purity GLA in water) was 0.2 mol% (relative to GLA). The chemoenzymatic product had no significant (<1 ppm) concentrations of either diglycolic acid or methoxyacetic acid, unlike the technical grade GLA produced by the chemical reaction of HCHO and CO; applications requiring high purity GLA require a crystallization of this commercial technical grade GLA to significantly lower the concentration of these two impurities, adding an additional cost that is avoided in the present process.

## Conclusions

High purity GLA produced by crystallization of technical grade material is currently available commercially as either a 70 wt% aqueous solution, or as a crystalline solid. A chemoenzymatic process has been demonstrated that can produce high purity GLA (comparable purity to existing commercially available high purity GLA) without isolation and/or purification of the GLN or $NH_4$GLA intermediates, and without purification of the final aqueous product, such that the column effluent from the final ion exchange step requires only concentration to 70 wt% GLA prior to use.

The preparation of GLN required a heat treatment of commercial grade HCHO prior to reaction with HCN. This heat treatment could be run continuously in a semi-batch process, and re-equilibrated the less reactive oligomeric forms of HCHO present in commercial product to more reactive oligomers, producing a HCHO conversion in excess of 99% when the reaction was run with a 5% molar excess of HCN. The excess HCN and methanol (present as stabilizer added to many commercial HCHO formulations) was readily stripped from the reaction product with the water removed by distillation during concentration. Aqueous GLN was only stable at acidic pH (pH of <3–4), and substitution of GLA for the sulfuric acid or phosphoric acid used as stabilizer in commercial GLN preparations provided the necessary product stability, even during concentration under reduced pressure at 65 °C, without adding a contaminant that would need to be removed from the final product (the acid stabilizer in this instance is also the final product).

Increases in both the specific activity of the *Acidovorax facilis* 72W nitrilase through protein engineering (with a change at position 168 from phenylalanine to valine) and the level of nitrilase expression in a microbial transformant (by expression in *E. coli* MG1655/pSW138-Phe168Val) were required to achieve volumetric productivity and catalyst productivity targets (1000 g GLA/g dcw and 17 g GLA/L/h, respectively) in consecutive batch reactions with catalyst recycle and in a CSTR. The nitrilase total turnover number in the CSTR was estimated to be *ca.* $4.4\times10^6$ mol GLA/mol nitrilase, with *ca.* 82% of the initial enzyme activity remaining when the catalyst productivity target was met. The use of nitrilase as catalyst for the production of pharmaceutical and agrochemical intermediates, monomers for polymers, and specialty chemicals is now well-documented,[27] and the chemoenzymatic production of high purity GLA reported herein is an additional example of how nitrilase can be employed as catalyst to produce a product in higher yield and purity, and with fewer process steps than alternative chemical processes.

## Experimental Section

### Materials and Methods

Chemicals were obtained from commercial sources unless otherwise noted, and used as received. The calculated % recovery of GLN and % yield of GLA were based on initial GLN 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.

The isolation and growth of microbial cultures described herein has been reported.[17–21] Cell paste isolated from fermentation was frozen at −80 °C without pre-treatment with glycerol or DMSO, and thawed prior to use in reactions or for immobilization in carrageenan. Wet cell weights of microbial catalysts employed in reactions or assays were ob-

tained from cell pellets prepared by centrifugation of fermentation broth or cell suspensions in buffer. Dry cell weights were determined by microwave drying of wet cells. Microbial cell enzyme activity was measured by stirring a suspension of 8.5–12.5 mg dry cell weight/mL in 100 mM phosphate buffer (pH 7.0) and 0.5 M substrate at 25 °C, and analyzing aliquots removed at 1, 5, 10 and 15 min for the rate of substrate disappearance. A unit of enzyme activity (IU) was equivalent to 1 micromol/min of GLN conversion.

Initial identification and quantitative analysis of impurities in GLN was performed by $^{13}C$ NMR spectroscopy using a Varian Unity Inova spectrometer (Varian, Inc., Palo Alto, CA) operating at 400 MHz. Samples were prepared by mixing 3.0 mL of the reaction product and 0.5 mL of $D_2O$ in a 10-mm NMR tube. $^{13}C$ NMR spectra were typically acquired using a spectral width of 26 KHz with the transmitter located at 100 ppm, 128 K points, and a 90-degree pulse (pw90 = 10.7 μs at a transmitter power of 56 db). The longest $^{13}C$ $T_1$ (23 s) was associated with the GLN nitrile carbon, and the total recycle time was set greater than ten times this value (recycle delay d1 = 240 sec, acquisition time at = 2.52 s). Signal averaging of 360 scans gave a total experiment time of 26.3 h. The nuclear Overhauser enhancement (NOE) was suppressed by gating on the Waltz-modulated $^1H$ decoupling only during the acquisition time.

Quantitative $^1H$ NMR spectra were obtained using Varian Unity Inova spectrometers operating either at 400 MHz or at 500 MHz. The majority of data was acquired on the latter system using a high sensitivity cryoprobe, and typical sample preparation involved adding to a 5-mm NMR tube 20 μL of the reaction product along with 530 μL of a $D_2O$ solution containing 0.1 mM DSS as internal reference. The sample temperature was maintained at 15 °C in order to resolve the residual HOD solvent resonance (4.890 ppm) from nearby signals observed for formaldehyde hydrate (4.812 ppm) and related impurities (4.971 ppm). Solvent suppression was obtained by a combination of on-resonance low power presaturation and the use of the first slice of a NOESY pulse sequence in order to suppress B1 inhomogeneity effects. Spectra were acquired using a spectral width of 6000 Hz, a 90-degree pulse (5.9 μs at a transmitter power of 48 db), and an acquisition time of 4 s. The longest $^1H$ $T_1$ (8 s) was associated with the methanol $CH_3$ protons, and the total delay time prior to acquisition was set to 50 s. Signal averaging of 16 scans was preceded by 2 steady-state scans to give a total experiment time of approximately 16 min.

## Semi-Batch Glycolonitrile Synthesis (2.5-mol Scale)

Aqueous sodium hydroxide (16.7 wt%, 0.56 mL) was added to 37 wt% aqueous formaldehyde [218.0 g, stabilized with 7–8% methanol (Aldrich)], and the resulting slightly hazy solution used for the continuous formaldehyde feed. The reaction vessel, equipped with a magnetic stir bar, was initially charged with a mixture of 3.3 g HCN in 35.3 g water and placed within a water bath maintained at *ca.* 20 °C, on top of a stir plate and lab jack assembly in a lowered position. The approximately 36-inch section of the formaldehyde feed line (1/8-in OD × 0.085-in ID) directly preceding the inlet to the reaction flask was heated to 120 °C after filling the formaldehyde feed line, and the flow of heated formaldehyde feed was first established by observing two-phase flow from the outlet of the formaldehyde feed line. After establishing two-phase flow out of the formaldehyde feed line, the reaction vessel was raised to introduce the formaldehyde feed directly into the liquid reaction mixture. The stir plate, water bath, and lab jack assembly was then raised to provide mixing and to maintain the reaction temperature at *ca.* 20–25 °C by periodically adding ice and/or dry ice to the water bath. The reactants were each continuously pumped into the reaction vessel over a period of 2.0 h as follows: 82.4 mL/h of 50 wt% aqueous HCN solution ($d = 0.86$ g/mL), 92.7 mL/h of 37 wt% aqueous formaldehyde feed (described above, $d = 1.09$ g/mL). After 2.0 h, the feeds were stopped, and the reaction vessel, water bath, stir plate, and lab jack assembly was lowered to remove the formaldehyde feed line from the reaction product to avoid any potential draining or siphoning of additional formaldehyde from the feed line. The reaction mixture was removed from the reaction vessel and then quenched by adding of 1.3 mL of 70 wt% glycolic acid (Glypure®, E.I. DuPont de Nemours, Wilmington, DE), resulting in a glycolonitrile product solution at about pH 3. The glycolonitrile solution was concentrated to remove excess unreacted HCN and methanol under vacuum at 60–70 °C. A typical preparation yielded 150 g of a 16.7 M solution of aqueous glycolonitrile (> 99% yield based on HCHO by quantitative $^{13}C$ NMR, 95% isolated yield after concentration).

## Fermentation of *E. coli* MG1655/pSW138-Phe168Val

A fermentor seed culture was prepared by charging a 2-L shake flask with 0.5 L seed medium containing yeast extract (Ambrex 695, 5.0 g/L), $K_2HPO_4$ (10.0 g/L), $KH_2PO_4$ (7.0 g/L), sodium citrate dihydrate (1.0 g/L), $(NH_4)_2SO_4$ (4.0 g/L), $MgSO_4$ heptahydrate (1.0 g/L) and ferric ammonium citrate (0.10 g/L). The pH of the medium was adjusted to 6.8 and sterilized in the flask. Post sterilization additions include glucose (50 wt% in water, 10.0 mL) and ampicillin (25 mg/mL, 1 mL). The seed medium was inoculated with a 1 mL suspension of *E. coli* MG1655/pSW138-Phe168Val[23] in 20% glycerol, and cultivated at 35 °C and 300 rpm. The seed culture was transferred at *ca.* 1.5 $OD_{550}$ to a 14-L fermentor (Braun) with 8 L of medium at 35 °C containing $KH_2PO_4$ (3.50 g/L), $FeSO_4$ heptahydrate (0.05 g/L), $MgSO_4$ heptahydrate (2.0 g/L), sodium citrate dihydrate (1.90 g/L), yeast extract (Ambrex 695, 5.0 g/L), Biospumex153 K antifoam (0.25 mL/L, Cognis Corporation), NaCl (1.0 g/L), $CaCl_2$ dihydrate (10 g/L), and NIT trace elements solution (10 mL/L). The trace elements solution contained citric acid monohydrate (10 g/L), $MnSO_4$ hydrate (2 g/L), NaCl (2 g/L), $FeSO_4$ heptahydrate (0.5 g/L), $ZnSO_4$ heptahydrate (0.2 g/L), $CuSO_4$ pentahydrate (0.02 g/L) and $NaMoO_4$ dihydrate (0.02 g/L). Post-sterilization additions included glucose (50 wt% in water, 80.00 g) and ampicillin (25 mg/mL, 16.00 mL). Glucose feed to the fermentor was initiated when the glucose concentration decreased below 0.5 g/L (50 wt% aqueous glucose, 0.35 g min$^{-1}$ for 3 h, then increased to 0.5 g min$^{-1}$). At an $OD_{550}$ of 25 the feed was changed to aqueous lactose (25 wt%, 0.6 g min$^{-1}$) to induce nitrilase expression. The lactose feed was adjusted to 1.3 g min$^{-1}$, 0.89 g min$^{-1}$, and 1.0 g min$^{-1}$ at 2 h, 20 h and 30 h, respectively, after lactose feed was first initiated, and the run ended 40 h after lactose feed was initiated. The dissolved oxygen

(DO) concentration was controlled at 25% of air saturation at the preinduction phase and at 10% of air saturation post induction phase. The DO was controlled first by impeller agitation rate (400 to 1000 rpm) and later by aeration rate (2 to 10 sLpm). The pH was controlled at 6.8 pre-induction and 7.2 post-induction using $NH_4OH$ (29% w/w) and $H_2SO_4$ (20% w/v). The head pressure was 0.5 bar.

## Preparation of GA/PEI Cross-Linked Carrageenan-Immobilized *E. coli* MG1655/pSW-Phe168Val

With rapid stirring, 12 g of carrageenan (Gelcarin® GP911, FMC) were slowly added to 228 g deionized distilled water at 50 °C, the resulting mixture heated to 80 °C until the carrageenan was completely dissolved, and the resulting solution cooled with stirring to 52 °C. In a separate beaker equipped with stir bar, 74.9 g of frozen *E. coli* MG1655/pSW138-Phe168Val cells (26.7% dcw) was added to 85.1 g of 0.35 M $Na_2HPO_4$ (pH 7.3) at *ca.* 25 °C and mixed until the cells were suspended, then a deoxyribonuclease I solution [10 μL of 12,500 U/mL DNase (Sigma)/100 mL of cell suspension] was added. The cell suspension was filtered consecutively through a 230 micron and a 140 micron Nupro TF strainer element filter, and heated with stirring to 50 °C immediately before addition to carrageenan solution. With stirring, 160.0 g of the cell suspension at 50 °C were added to the carrageenan solution at 52 °C, and the cell/carrageenan suspension immediately transferred to an electrically-heated ISCO syringe pump (47 °C). The suspension was pumped at 50 mL $min^{-1}$ through two 230-micron filters and a transfer line (all heated to 47 °C) to an electrically-heated 19-hole die (47 °C) equipped with 18-gauge blunt-tip needles and dripped into 1000 mL of 0.25 M $KHCO_3$ (pH 7.3, adjusted with phosphoric acid) at 38 °C. The resulting beads were allowed to harden for 1 h with stirring, and stored in 0.25 M $KHCO_3$ (pH 7.3). Chemical cross-linking of the beads was performed by addition of 0.5 g of 25% glutaraldehyde (GA) in water (Sigma) to 20 g beads suspended in 48 mL of 0.25 M $KHCO_3$ (pH 7.3), and stirring for 1 h at room temperature. To the suspension of beads was then added 2.0 g of 12.5 wt% polyethylenimine [PEI, Lupasol® PS (BASF)] in water, and mixing for an additional 18 h at room temperature. For long-term storage (from one month to more than one year), The GA/PEI cross-linked beads were suspended in 1.5 volumes of aqueous $NH_4HCO_3$ (1.0 M, pH 7.3) at 5 °C.

## GLN Hydrolysis in Batch Reactions with Catalyst Recycle

In a typical procedure, 8.0 g of GA/PEI cross-linked immobilized *E. coli* MG1655/pSW138-Phe168Val catalyst beads [5.0% dcw, 3.0% carrageenan; washed twice for 15 min with 9 volumes of 0.1 M ammonium glycolate (pH 7.0)], 14.52 mL of deionized water and 6.0 mL of 4.0 M ammonium glycolate (pH 7.0) were placed in a 50-mL reaction vessel (jacketed double sidearm Celstir®, Wheaton) with overhead stirring and temperature control at 25 °C. The vessel was flushed with nitrogen, then 1.08 mL of glycolonitrile (59.93 wt% in water containing 1.2 mol% GLA) and 0.375 mL of 1:16 ammonium hydroxide (to maintain the reaction pH between 7.0 and 7.5) were each individually added simultaneously while stirring by programmable syringe pump (Model 200P programmable dual syringe pump, KD Scientific) every 2 h (30 mL initial reaction volume after first addition of GLN and $NH_4OH$). A total of eight additions of each solution were added over 14 h (40 mL final reaction volume), and the reaction was stirred an additional 2 h after the final addition to achieve complete GLN conversion. The reaction rate was measured immediately after the first addition of GLN (< 0.400 M GLN) by sampling the reaction at predetermined times, then mixing 50 μL of sample with 200 μL of 0.25 M *n*-propanol (HPLC external standard) in water and 10 μL of 6 N HCl, and analyzing the resulting solution by HPLC. At the end of the first reaction (and for each subsequent reaction), *ca.* 30 g of product mixture were decanted from the reaction vessel, leaving a "heel" containing 10.3 g of product mixture and biocatalyst. To the reactor were added 18.2 mL of deionized water, then the next batch reaction was initiated by addition of the GLN and ammonium hydroxide solutions as before.

## GLN Hydrolysis in a CSTR

The CSTR was a 500-mL jacketed resin kettle equipped with four internal baffles and a side arm through which an extra-course gas dispersion tube was placed in the reaction mixture (subsurface and flush with the side wall of the reactor) to allow for continuous product removal over the course of the reaction. Mixing was accomplished using a four-blade impeller, where each blade was at a 45° angle to the stirring shaft. The CSTR was started as a batch reaction, where 140 g of GA/PEI cross-linked carrageenan-immobilized MG1655/pSW138-Phe168Val beads [washed twice for 15 min with 1.40 L of 0.1 M $NH_4GLA$ (pH 7.0)] and 118 mL of 2.03 M $NH_4GLA$ (pH 7.0) were added and the mixture stirred at 200 rpm while flushing the reactor with nitrogen for 15 min. While stirring, 41.4 mL of GLN (41.8 wt%, 0.31 mol) were added to the flask, followed immediately by 5.93 mL of 1:16 dilution of conc. $NH_4OH$ (final reaction mixture pH 7.5). The reaction was sampled and analyzed by HPLC every 2 min for 20 min to determine the initial reaction rate. After 2 h, additional 41.4 mL of GLN solution and 3.0 mL of 1:16 $NH_4OH$ were added (final pH 7.5), and after additional 2 h, 41.4 mL of GLN solution and 4.0 mL of 1:16 $NH_4OH$ were again added and the mixture stirred overnight. The final reaction volume, including 140 g of catalyst beads (35 wt% catalyst charge, *ca.* 60 volume% of reaction volume), was 400 mL.

At the conclusion of the batch reaction, the reactor was switched to continuous operation, where an Accu FM10 pump equipped with RH00STY-LF piston pump head (SciLog) was used to add 0.762 mL $min^{-1}$ of GLN (3.45 M in deionized water, stabilized with 1.2 mol% GLA), and a ChemTec CP-8 pump with Tandem 1081 peristaltic pump head (SciLog) was used to simultaneously add 0.115 mL $min^{-1}$ of aqueous ammonium hydroxide (1:38 dilution of concentrated ammonium hydroxide) to the reactor. A second peristaltic pump was employed to maintain the reaction volume at 400 mL by continuously removing product mixture through the submerged gas dispersion tube at a rate *ca.* equivalent to the combined addition rate of the two feeds. The reaction mixture was analyzed for GLA by adding 60 μL of sample to 960 μL of 0.214 M *n*-propanol (HPLC external standard) in water and 36 μL of 6 N HCl,

and analyzing the resulting solution by HPLC (5 μL injection volume). The reaction mixture was analyzed for GLN by adding 50 μL of 1.00 M *n*-propanol (HPLC external standard) in water to 950 μL of the same sample and analyzing the resulting solution by HPLC (25 μL injection volume). Volumetric and biocatalyst productivity for a CSTR run using unpurified GLN prepared from HCHO and HCN are reported in Table 2.

### GLN Hydrolysis in a Packed-bed Column

A Hoke sample cylinder (304 stainless steel, 75 mL internal volume, 121 mm length × 38 mm OD) equipped with a 120 in coil of 1/16-in OD × 0.04-in ID 316 stainless steel tubing (heat exchanger for column feed) was charged with 57.43 g of GA/PEI cross-linked *E. coli* MG1655/pSW138-Phe168Val beads (1764 GLN IU/g dcw) in 3.22 M ammonium glycolate (pH 7.3). The packed-bed column and heat exchanger were placed in a jacketed water bath maintained at 25 °C using a recirculating temperature bath. An Accu FM10 pump equipped with RH00STY-LF piston pump head (SciLog) was used to pump a column feed solution containing $NH_4GLA$ (3.19 M, product mixture collected from a CSTR run using unpurified GLN prepared from HCHO and HCN) and GLN (152 mM, unpurified, from HCHO and HCN) at 7 °C through the heat exchanger (24.7 °C exit temperature to the column inlet). The concentrations of $NH_4GLA$ and GLN in the column product solution were analyzed by HPLC using the sample analysis methods described for the CSTR. A column feed rate of 49.9 mL/h (1.5 h residence time) produced a total GLN conversion [mol GLN/(mol GLN+mol GLA)] of 99.97%. The recovered biocatalyst activity after 344 h of continuous operation was 1559 GLN IU/g dcw (88% of initial activity).

### Preparation of Chemoenzymatic Glycolic Acid

A 4-L jacketed glass resin kettle equipped with overhead stirring (maintained at 25 °C with a recirculating temperature bath) was charged with 843 g of GA/PEI cross-linked *E. coli* MG1655/pSW138-Phe168Val beads that had been washed four times with 9 volumes of aqueous $NH_4GLA$ [0.1 M, pH 7.0, prepared using high-purity glycolic acid (Glypure®, DuPont)] to remove the aqueous $NH_4HCO_3$ (1.0 M, pH 7.3) catalyst storage buffer. To the reactor were added 1975 mL deionized water, and the resulting biocatalyst suspension stirred under nitrogen as aqueous GLN (450 mL, 40 wt%, unpurified product from the reaction of HCHO and HCN) was added in one portion. The pH of the reaction mixture was immediately adjusted to 7.0 with aqueous $NH_4OH$ (1:8 dilution of concentrated $NH_4OH$, *ca.* 20–25 mL). Samples were periodically withdrawn by syringe and analyzed by HPLC to determine the rate of conversion of GLN to GLA, and after complete GLN conversion was observed (*ca.* 3 h), a second aliquot of GLN and $NH_4OH$ (equal to first aliquot) was added, and after 3 h a third addition of GLN and $NH_4OH$ was made. At complete conversion of GLN, the product mixture (*ca.* 3.0 M $NH_4GLA$) was withdrawn by polyethylene cannula from the reactor, leaving a reaction heel comprised of biocatalyst and a small amount of reaction product. The next batch reaction was initiated by the addition of deionized water, GLN and $NH_4OH$ as described above. A total of eight consecutive batch reactions were performed, where each reaction produced *ca.* 3.3 L of aqueous $NH_4GLA$ (3.0 M, >99% yield and purity). Product solutions from the final six batch reactions of this series were combined and used without dilution as feed for conversion of $NH_4GLA$ to GLA by fixed-bed cation exchange over a strong acid cation resin. A 10 cm ID × 114 cm fixed-bed column (8.95 L) containing Dowex G-26 $H^+$ cation exchange resin (*ca.* 2 equivalents/L bed volume) was flushed with ultra-pure water to >5 MOhm resistivity prior to use. A total of 48.5 mol $NH_4GLA$ in two equal *ca.* 8-L portions was converted to GLA in two separate runs. The ion exchange was performed using an 80 mL $min^{-1}$ upflow, and effluent fractions were collected at 400 mL intervals. The fractions containing low ammonia (typically <7 ppm) were combined (25 mol GLA total from two runs) and concentrated by distillation of water to produce 2.64 kg of high purity 72 wt% GLA in water [>99 pure based on wt% of organic impurities (*ca.* 0.23 mol% total organic impurities relative to GLA)].

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