# Chemoenzymic Synthesis of *N*-(Phosphonomethyl)glycine

John E. Gavagan, Susan K. Fager, John E. Seip, Dawn S. Clark, Mark S. Payne, David L. Anton, and Robert DiCosimo*

*Central Research and Development Department, E. I. du Pont de Nemours & Co., Experimental Station, P.O. Box 80328, Wilmington, Delaware 19880-0328*

*Received March 12, 1997*[⊗]

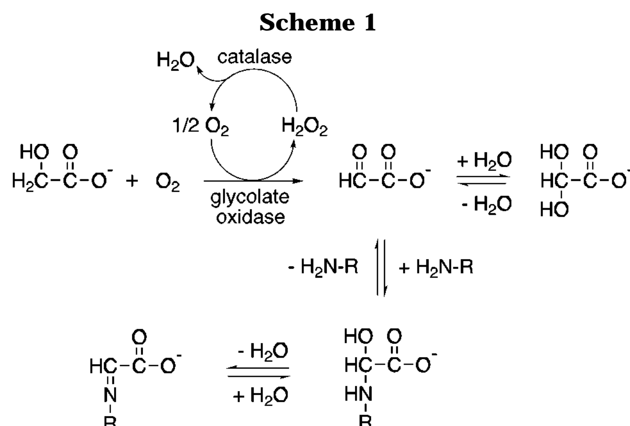
Permeabilized, metabolically-inactive transformants of the methylotrophic yeasts *Hansenula polymorpha* and *Pichia pastoris* which contain significant quantities of the enzymes spinach glycolate oxidase ((*S*)-2-hydroxyacid oxidase, EC 1.1.3.15), *Saccharomyces cerevisiae* catalase T (EC 1.11.1.6), and endogenous catalase have been used as catalysts for the oxidation of glycolic acid by oxygen to produce glyoxylic acid in aqueous mixtures containing (aminomethyl)phosphonic acid. After separation and recovery of the microbial catalyst from the oxidation product mixture for reuse, the resulting solution of glyoxylic acid and (aminomethyl)phosphonic acid was subsequently hydrogenated with a palladium/carbon catalyst to produce *N*-(phosphonomethyl)glycine (glyphosate), a broad-spectrum, postemergent herbicide. Complete conversion of (aminomethyl)phosphonic acid in the hydrogenation allowed the use of a simple acid precipitation for isolation of the *N*-(phosphonomethyl)glycine from the hydrogenation product mixture in high purity and yield.

## Introduction

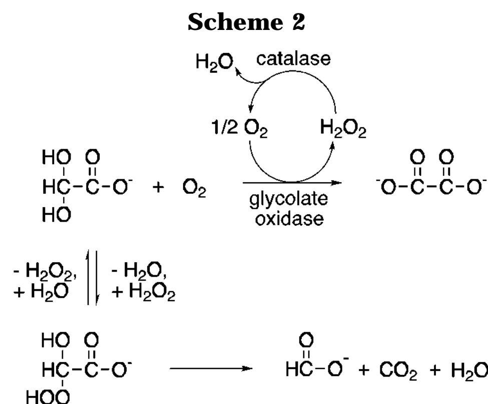
We have recently reported the oxidation of glycolic acid by oxygen in aqueous solution to produce glyoxylic acid in >98% yield by using the enzymes spinach glycolate oxidase[1] ((*S*)-2-hydroxyacid oxidase, EC 1.1.3.15) and catalase[2] (EC 1.11.1.6) as cocatalysts (Scheme 1). The oxidation was initially performed using soluble enzymes,[3] then improvements in reaction rates and catalyst productivity were achieved by co-immobilizing the soluble enzymes on an insoluble support,[4] and finally by using a metabolically-inactive transformant of the methylotrophic yeast *Pichia pastoris* or *Hansenula polymorpha* which expressed both spinach glycolate oxidase and an endogenous catalase as catalyst.[5] An unexpected increase in glyoxylic acid yield was produced by the synergistic effect of using catalase (to destroy byproduct hydrogen peroxide) with an aliphatic amine buffer capable of reacting with the glyoxylic acid to produce a hemiaminal or imine. Rapid decomposition of hydrogen peroxide limited the nonenzymatic oxidation of glyoxylate to formate and carbonate, and the trapping of glyoxylate with an amine buffer significantly reduced both product inhibition of glycolate oxidase and the further enzymatic oxidation of glyoxylate to oxalate by glycolate oxidase (Scheme 2).

The improvement in yield of glyoxylate which resulted from the formation of an oxidation-resistant N-substituted hemiaminal and/or imine of glyoxylate and an amine buffer was found to be dependent on the p$K_a$ of the protonated amine.[3] Of the amines examined, those with a p$K_a$ approximately equal to or lower than the pH of the reaction mixture (i.e., ethylenediamine and, less preferably, tris(hydroxymethyl)aminomethane) produced

**Scheme 1**




**Scheme 2**




much higher yields of glyoxylate (and correspondingly lower yields of formate and oxalate) than amine buffers whose p$K_a$s were higher than the pH at which the reaction was performed. These results were consistent with the expectation that an unprotonated amine was necessary to form an oxidation-resistant N-substituted hemiaminal and/or imine complex with glyoxylate; an amine buffer whose p$K_a$ was much higher than the pH of the reaction mixture would be present predominantly as the protonated ammonium ion and therefore be less likely to form such complexes with glyoxylate.

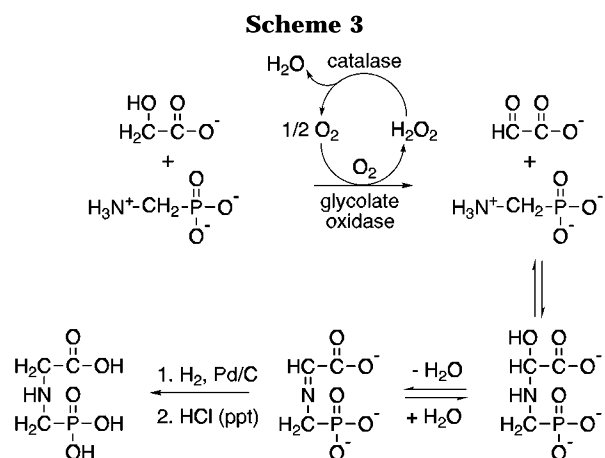
While examining the affect of the addition of various amines on the yield of glyoxylate produced by the

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1997.

(1) (a) Zelitch, I.; Ochoa, S. *J. Biol. Chem.* **1953**, *201*, 707−718. (b) Richardson, K. E.; Tolbert, N. E. *J. Biol. Chem.* **1961**, *236*, 1280−1284.
(2) Schonbaum, G. R.; Chance, B. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1976; 3rd ed., Vol. 13, part C, pp 363−408.
(3) Seip, J. E.; Fager, S. K.; Gavagan, J. E.; Gosser, L. W.; Anton, D. L.; DiCosimo, R. *J. Org. Chem.* **1993**, *58*, 2253−2259.
(4) Seip, J. E.; Fager, S. K.; Gavagan, J. E.; Anton, D. L.; DiCosimo, R. *Bioorg. Med. Chem.* **1994**, *2*, 371−378.
(5) Gavagan, J. E.; Fager, S. K.; Seip, J. E.; Payne, M. S.; Anton, D. L.; DiCosimo, R. *J. Org. Chem.* **1995**, *60*, 3957−3963.

S0022-3263(97)00455-6 CCC: $14.00 © 1997 American Chemical Society

**Scheme 3**




enzymatic oxidation of glycolate, it was found that the addition of (aminomethyl)phosphonic acid (AMPA) resulted in unexpectedly high yields. The enzymatic oxidation was routinely run at an initial pH of 8.5–9.0, and within this pH range AMPA (p$K_a$ 10.08)[6] would be present predominately as the protonated ammonium salt and not as the corresponding free amine which could react with glyoxylate to produce an oxidation-resistant hemiaminal or imine. The use of AMPA also resulted in an improvement in recovery of glycolate oxidase and catalase activity when compared to reactions run in the absence of added AMPA. The subsequent hydrogenation of the resulting product mixture containing the imine of glyoxylate and AMPA resulted in the direct production of *N*-(phosphonomethyl)glycine[7] (NPMG, also known as glyphosate), which is a broad-spectrum, postemergent herbicide widely used in agriculture. We now report the optimization of a chemoenzymic synthesis of NPMG (Scheme 3) which starts with glycolic acid and (aminomethyl)phosphonic acid.

## Results

**Oxidation of Glycolic Acid/AMPA Mixtures Using Soluble and Immobilized Enzymes.** A series of oxidations of glycolate by oxygen in an aqueous solution containing soluble glycolate oxidase and *Aspergillus niger*[8] catalase, and a molar equivalent of AMPA, were initially performed to determine the dependence of glyoxylate yield on catalase concentration (Table 1). For comparison purposes, reactions at the lowest catalase concentration were also run using phosphate or bicine buffer in place of AMPA to determine the effect of AMPA on glyoxylate yield. At the lowest catalase concentration, the yield of glyoxylate in the presence of 1 equiv of AMPA was significantly greater than when phosphate or bicine was used, and the yield of glyoxylate obtained with AMPA increased with increasing catalase concentration up to ca. 14 000 IU/mL. Figure 1 depicts a time course for the oxidation of glycolate with AMPA and only 1 400 IU/mL of *A. niger* catalase; when the concentration of catalase is insufficient to rapidly decompose byproduct hydrogen peroxide, significant amounts of formate are produced by the reaction of hydrogen peroxide with glyoxylate. Figure 1 also illustrates an increase in the rate of conversion of glyoxylate to oxalate when greater than 95% of the glycolate has been converted; this increase in rate of oxalate production was independent of catalase concentration.

Reactions were next run using 14 000 IU/mL of *A. niger* catalase and increasing concentrations of glycolate and AMPA to determine the optimal glycolate concentration for yield and reaction time (Table 2). In these reactions, the concentration of AMPA was limited to 80% of glycolate concentration in order to produce an excess of glyoxylate relative to the amount of AMPA necessary for

**Table 1. Dependence of Glyoxylate, Formate, and Oxalate Yields on Soluble Catalase Concentration in Oxidations of Glycolate by Soluble Glycolate Oxidase[a]**

| [catalase] (IU/mL) | buffer | glyoxylate (%) | formate (%) | oxalate (%) | glycolate (%) |
|---|---|---|---|---|---|
| 1400 | phosphate | 39 | 45 | 15 | 0 |
| 1400 | bicine | 43 | 50 | 10 | 0.2 |
| 1400 | AMPA | 70 | 20 | 2.2 | 5.3 |
| 5600 | AMPA | 86 | 7.6 | 3.3 | 2.5 |
| 14000 | AMPA | 88 | 3.3 | 3.0 | 3.4 |
| 56000 | AMPA | 84 | 0.4 | 2.5 | 8.4 |

[a] Aqueous solutions containing *A. niger* catalase, spinach glycolate oxidase (1.0 IU/mL), flavin mononucleotide (0.01 mM), glycolic acid (0.25 M), and AMPA (0.26 M) or phosphate (0.33 M) or bicine (0.26 M) buffers at pH 8.5 were stirred at 15 °C under oxygen at 70 psig.

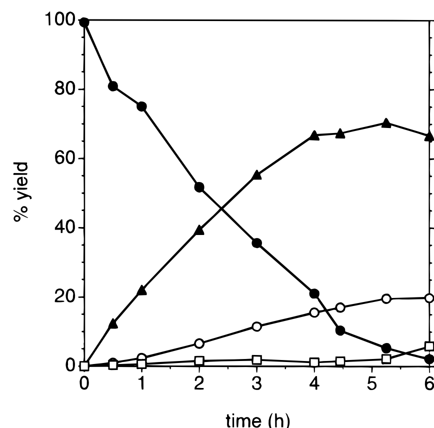



**Figure 1.** Time course for the oxidation of glycolic acid (0.25 M) in an aqueous solution containing AMPA (0.26 M), flavin mononucleotide (0.01 mM), spinach glycolate oxidase (1.0 IU/mL), and *A. niger* catalase (1400 IU/mL) at pH 8.5, 15 °C, and 70 psig of oxygen: glycolate (●), glyoxylate (▲), formate (○), oxalate (□).

**Table 2. Dependence of Glyoxylate, Formate, and Oxalate Yields and Reaction Time on Glycolate Concentration[a]**

| [glycolate] (M) | [AMPA] (M) | reactn time (h) | glyoxylate (%) | formate (%) | oxalate (%) | glycolate (%) |
|---|---|---|---|---|---|---|
| 0.25 | 0.20 | 6 | 92 | 4.4 | 5.5 | 0 |
| 0.50 | 0.40 | 17.5 | 91 | 2.9 | 2.9 | 4.1 |
| 0.75 | 0.60 | 40 | 83 | 2.3 | 7.5 | 0 |
| 1.00 | 0.80 | 66 | 79 | 2.2 | 12 | 2.0 |

[a] Aqueous solutions containing soluble *A. niger* catalase (14 000 IU/mL) and spinach glycolate oxidase (1.0 IU/mL), flavin mononucleotide (0.01 mM), glycolic acid, and AMPA at pH 8.5 were stirred at 5 °C under oxygen at 70 psig.

(6) Song, B.; Chen, D.; Bastian, M.; Bruce, M. R.; Sigel, H. *Helv. Chim. Acta* **1994**, *77*, 1738–1756.

(7) Franz, J. E. *Adv. Pestic. Sci., Plenary Lect. Symp. Pap. Int. Congr. Pestic. Chem., 4th (1979)*, meeting date 1978; Geissbuehler, H., Ed.; Pergamon: Oxford, England; Vol. 2, pp 139–47.

(8) (a) Mosavi-Movahedi, A. A.; Wilkinson, A. E., Jones, M. N. *Int. J. Biol. Macromol.* **1987**, *9*, 327–332. (b) Kikuchi-Torii, K.; Hayashi, S.; Nakamoto, H., Nakamura, H. *J. Biochem.* **1982**, *92*, 1449–1456. (c) Wasserman, B. P.; Hultin, H. O. *Arch. Biochem. Biophys.* **1981**, *212*, 385–392. (d) Gruft, H.; Ruck, R.; Traynor, J. *Can. J. Biochem.* **1978**, *56*, 916–919. (e) Scott, D.; Hammer, F. *Enzymologia* **1960**, *22*, 229–237.

**Table 3. Glyoxylate, Formate, and Oxalate Yields for Glycolate Oxidations using *H. polymorpha* and *P. pastoris* Transformant Catalysts with EDA, AMPA, or DEAMPA**

| catalyst | catalase T (% total catalase) | amine | glyoxylate (%) | formate (%) | oxalate (%) | glycolate (%) |
|---|---|---|---|---|---|---|
| *P. p.* MSP10 | 0 | EDA | 99 | 0 | 0.3 | 0 |
| *P. p.* MSP10 | 0 | AMPA | 78 | 12 | 7.2 | 2.7 |
| *P. p.* MSP10 | 0 | DEAMPA | 93 | 3.6 | 3.9 | 0 |
| *P. p.* MSP8.6 | 25 | AMPA | 90 | 4.4 | 4.9 | 3.6 |
| *H. p.* GO1 | 0 | EDA | 98 | 0.1 | 1.0 | 0.9 |
| *H. p.* GO1 | 0 | AMPA | 59 | 34 | 2.9 | 4.9 |
| *H. p.* GO1 | 0 | DEAMPA | 84 | 16 | 5.6 | 0 |
| *H. p.* 13.168 | 70 | AMPA | 90 | 1.8 | 3.1 | 5.0 |

[a] Aqueous reaction mixtures containing glycolic acid (0.50 M), AMPA (0.375 M), or DEAMPA (0.375 M) and flavin mononucleotide (0.01 mM) at pH 8.3 were stirred with 5% (wet weight cell weight/volume) of permeabilized *P. pastoris* transformant MSP10 (spinach glycolate oxidase and endogenous catalase) or MSP8.6 (spinach glycolate oxidase, endogenous catalase, and *S. cerevisiae* catalase T), or permeabilized *H. polymorpha* transformant GO1 (spinach glycolate oxidase and endogenous catalase) or 13.168 (spinach glycolate oxidase, endogenous catalase, and *S. cerevisiae* catalase T), at 5 °C under oxygen (70 psig) with oxygen sparging. Reactions containing EDA (0.788 M) and glycolate (0.75 M) were run under similar conditions.

the subsequent hydrogenation of the product mixture to produce NPMG (see below). At concentrations greater than 0.50 M glycolate, the decrease in glyoxylate yield was due to an increase in the enzymatic oxidation of the hydrate of glyoxylate by glycolate oxidase to produce oxalate. The reaction times required to reach >95% conversion of glycolate increased markedly with increasing glycolate concentration and were most likely due to product inhibition of the glycolate oxidase activity.

When soluble enzymes were used as catalysts for the preparation of glyoxylic acid/AMPA mixtures, the sparging of oxygen into the reaction mixture could not be used to increase the rate of oxygen dissolution because of the resulting rapid inactivation of glycolate oxidase, and reaction rates were primarily limited by the rate at which oxygen could dissolve into the reaction mixture.[3] Reaction rates were significantly increased when a catalyst consisting of spinach glycolate oxidase and *A. niger* catalase co-immobilized on oxirane acrylic beads was substituted for the soluble enzymes.[4] Sparging oxygen into the reaction mixture containing the co-immobilized enzymes at 5 °C resulted in complete oxidation of 0.5 M glycolate in 3.5 h, compared to a reaction time of 18 h when the reaction was repeated using the same concentrations of soluble enzymes without oxygen sparging. Because the co-immobilized enzyme catalyst could be readily recovered for reuse, ten consecutive oxidations of 0.50 M glycolate in 0.375 M AMPA were run with catalyst recycle. By the third reaction with catalyst recycle, there was a loss of ca. 50% of glycolate oxidase activity, with a corresponding increase in reaction time from 3.5 h to ca. 16 h. No further loss of glycolate activity occurred after the third consecutive reaction, and no significant loss of catalase activity was measured from the initial available activity; the yield of glyoxylate in each of these consecutive batch reactions was between 87 and 91%.

An added advantage of using a co-immobilized enzyme catalyst instead of soluble enzymes was the much higher yields of glyoxylate and much lower yields of byproduct formate produced when low concentrations of immobilized catalase were used when compared to reactions run with the same concentration of soluble catalase. For the oxidation of 0.50 M glycolate in 0.375 M AMPA at 5 °C, a concentration of co-immobilized catalase of only 2400 IU/mL resulted in yields of glyoxylic acid (88%) and formate (3.3%) similar to that obtained when 14 000 IU/mL of soluble catalase was used (Table 2).

**Methylotrophic Yeast Transformant Catalysts Which Express Spinach Glycolate Oxidase.** When permeabilized, genetically-engineered transformants of the methylotrophic yeasts *P. pastoris* MSP10[9] or *H. polymorpha* GO1[10] that contain significant quantities of both spinach glycolate oxidase and an endogenous catalase were used to catalyze the oxidation of glycolic acid to glyoxylic acid in aqueous reaction mixtures containing ethylenediamine, almost quantitative yields of glyoxylic acid were obtained.[5] Permeabilization of the cells with a cationic detergent (e.g., benzylcetyldimethylammonium chloride[11]) was required prior to their use as catalyst to access the enzyme activities contained within the cells. When these same catalysts were employed for the oxidation of glycolic acid in aqueous reaction mixtures containing AMPA, the yield of glyoxylate was markedly lower, and a corresponding increase in the amount of the formate was observed (Table 3).

*P. pastoris* endogenous catalase[11] and *H. polymorpha* endogenous catalase[12] were each isolated and examined for their ability to decompose hydrogen peroxide generated during the oxidation of glycolic acid in aqueous mixtures containing AMPA. The catalase enzymes from *A. niger*,[8] *A. nidulans*,[13] *Saccharomyces cerevisiae* (catalase T and catalase A),[14] and bovine liver[15] were also examined. The activity of catalase from *A. niger* and *A. nidulans* was unaffected by AMPA, as was catalase T from *S. cerevisiae*. The catalase from *H. polymorpha*, *P. pastoris*, and bovine liver and catalase A from *S. cerevisiae* were each found to be reversibly inhibited by AMPA. This inhibition occurred regardless of whether a soluble

(9) Payne, M. S.; Petrillo, K. L.; Gavagan, J. E.; Wagner, L. W.; DiCosimo, R.; Anton, D. L. *Gene* **1995**, *167*, 215–219.

(10) (a) Gellissen, G.; Melber, K.; Janowicz, Z. A.; Dahlems, U. M.; Weydemann, U.; Piontek, M.; Strasser, A. W. M.; Hollenberg, C. P. *Antonie van Leeuwenhoek* **1992**, *62*, 79–93. (b) Gellissen, G.; Weydemann, U.; Strasser, A. W. M.; Piontek, M.; Janowicz, Z. A.; Hollenberg, C. P. *TIBTECH* **1992**, *10*, 413–417. (c) Sudbery, P. E.; Gleeson, M. A.; Veale, R. A.; Ledeboer, A. M.; Zoetmulder, M. C. M. *Biochem. Soc. Trans.* **1988**, *16*, 1081–1083.

(11) Zhang, M.; Wang, H. Y. *Enzyme Microb. Technol.* **1994**, *16*, 10–17.

(12) (a) Van Dijken, J. P.; Otto, R.; Harder, W. *Arch. Microbiol.* **1975**, *106*, 221–226. (b) Van Dijken, J. P.; Veenhuis, M.; Vermeulen, C. A.; Harder, W. *Arch. Microbiol.* **1975**, *105*, 261–267.

(13) Navarro, R. E.; Stringer, M. A.; Hansberg, W.; Timberlake, W. E.; Aguirre, J. *Curr. Genet.* **1996**, *29*, 352–359. (b) Devchand, M.; Skipper, N.; Anton, D. L.; DiCosimo, R.; Gavagan, J. E. *Biotechnol. Bioeng.* **1996**, *50*, 341–346.

(14) (a) Hoertner, H.; Ammerer, G.; Hartter, E.; Hamilton, B.; Rytka, J.; Bilinski, T.; Ruis, H. *Eur. J. Biochem.* **1982**, *128*, 179–184. (b) Sels, A. A.; Brygier, J. *Eur. J. Biochem.* **1980**, *112*, 283–291. (d) Seah, T. C. M.; Bhatti, A. R.; Kaplan, J. G. *Can. J. Biochem.* **1973**, *51*, 1551–1555.

(15) (a) Vasudevan, P. T.; Weiland, R. H. *Biotechnol. Bioeng.* **1990**, *36*, 783–789. (b) Altomare, R. E.; Kohler, J.; Greenfield, P. F.; Kittrell, J. R. *Biotechnol. Bioeng.* **1974**, *16*, 1659–1673.

**Table 4. Comparison of AMPA and DEAMPA in Glycolate Oxidations Using *A. niger* catalase or *H. polymorpha* Catalase**[a]

| catalase | [catalase] (IU/mL) | [glycolate] (M) | amine | [amine] (M) | glyoxylate (%) | formate (%) |
|---|---|---|---|---|---|---|
| *A. niger* | 1400 | 0.25 | AMPA | 0.26 | 70 | 20 |
| *A. niger* | 14000 | 0.50 | AMPA | 0.375 | 92 | 2 |
| *A. niger* | 14000 | 0.50 | AMPA | 0.50 | 89 | 2 |
| *A. niger* | 1400 | 0.50 | DEAMPA | 0.525 | 95 | 4 |
| *A. niger* | 14000 | 0.50 | DEAMPA | 0.40 | 97 | 1 |
| *H. poly.* | 5600 | 0.50 | AMPA | 0.375 | 43 | 45 |
| *H. poly.* | 14000 | 0.50 | AMPA | 0.375 | 64 | 24 |
| *H. poly.* | 56000 | 0.50 | AMPA | 0.375 | 68 | 6 |
| *H. poly.* | 1400 | 0.50 | DEAMPA | 0.525 | 84 | 12 |
| *H. poly.* | 14000 | 0.50 | DEAMPA | 0.525 | 98 | 1 |

[a] Aqueous solutions containing soluble catalase, spinach glycolate oxidase (1.0 IU/mL), flavin mononucleotide (0.01 mM), glycolic acid, and either AMPA or DEAMPA at pH 8.5 were stirred at 5 °C under oxygen at 70 psig.

catalase, immobilized catalase, or whole cell catalyst was employed. The addition of soluble catalase from *A. niger* or *S. cerevisiae* (catalase T), or permeabilized whole cells of *S. cerevisiae*, to reaction mixtures as a supplemental source of catalase resulted in marked improvements in glyoxylic acid production in reactions using either the *H. polymorpha* GO1 or *P. pastoris* MSP10 transformant as catalyst. Addition of 14 000 IU/mL of soluble *A. niger* catalase to an oxidation of glycolate (0.50 M) catalyzed by *H. polymorpha* GO1 in aqueous AMPA (0.375 M) resulted in an increase in glyoxylate yield from 59 to 90% and a decrease in formate yield from 34 to only 1.3%.

As an alternative to adding an additional source of AMPA inhibition-resistant catalase to reactions using either the *P. pastoris* MSP10 or *H. polymorpha* GO1 transformant catalyst, the substitution of a dialkyl (aminomethyl)phosphonate for AMPA in these same reactions resulted in a significant reduction in the inhibition of the endogenous catalase activities of these transformants. AMPA and diethyl (aminomethyl)phosphonate[16] (DEAMPA) were first compared in oxidations of glycolic acid which used soluble glycolate oxidase and either soluble *A. niger* catalase (which was not inhibited by AMPA) or soluble *H. polymorpha* catalase (reversibly inhibited by AMPA); the yields of glyoxylic acid and formic acid obtained under reaction conditions optimal for glyoxylic acid production are listed in Table 4. The yields of glyoxylic acid produced with DEAMPA were greater than those obtained with AMPA when either *A. niger* or *H. polymorpha* soluble catalase was used. The improvement in glyoxylate yield at lower concentrations of the uninhibited *A. niger* catalase may be due to the lower p$K_a$ of the DEAMPA-protonated amine (p$K_a$ 6.4) when compared to AMPA (p$K_a$ 10.08);[6] the lower p$K_a$ of DEAMPA favors the formation of oxidation-resistant hemiaminal or imine complexes of unprotonated DEAMPA with glyoxylic acid at the pH at which the reactions were performed (pH 8.5–9.0).

Comparison of glyoxylate and formate production in reactions which employed soluble *H. polymorpha* catalase and either DEAMPA or AMPA illustrates the unexpected, marked increase in glyoxylate yield and decrease in formate production when DEAMPA was used as amine additive. Significantly lower concentrations of *H. polymorpha* soluble catalase could be employed to obtain high yields of glyoxylate with DEAMPA; increasing the concentration of this same catalase when AMPA was used did not produce yields of glyoxylate comparable to those obtained with DEAMPA. Similar improvements in glyoxylate yield and decreases in formate production were obtained when DEAMPA was substituted for AMPA in reactions using either *H. polymorpha* GO1 or *P. pastoris* MSP10 transformants as catalyst (Table 3; 0.50 M glycolic acid, 0.375 M DEAMPA). When the concentration of DEAMPA in reactions using *H. polymorpha* GO1 or *P. pastoris* MSP10 transformant catalysts was increased to 0.525 M, glyoxylate yields increased to 98 and 95%, respectively; no significant improvement in glyoxylate yield was obtained with a corresponding increase in AMPA concentration.

**Methylotrophic Yeast Transformant Catalysts Which Coexpress Spinach Glycolate Oxidase and *S. cerevisiae* Catalase T.** As a preferable alternative to either substituting DEAMPA for AMPA or adding an additional source of inhibition-resistant catalase to glycolic acid/AMPA reaction mixtures, the *H. polymorpha* GO1 transformant and *P. pastoris* MSP10 transformant were re-engineered to produce new transformants, *H. polymorpha* 13.168[10] and *P. pastoris* MSP8.6,[17] which coexpress both spinach glycolate oxidase and *S. cerevisiae* catalase T. Using these microbial cell "double" transformants for the oxidation of glycolate to glyoxylate in reaction mixtures containing AMPA resulted in a significant increase in glyoxylate yield, and reduction in formate production, when compared to that obtained with the single transformants whose endogenous catalase was inhibited by AMPA (Table 3). Although the yield of glyoxylate was 90% in the initial reaction when either *H. polymorpha* 13.168 or *P. pastoris* MSP8.6 was used, recovery and reuse of the catalyst in subsequent batch reactions typically resulted in lower yields of glyoxylate. The yield of glyoxylate decreased to ca. 67% in subsequent reactions when *P. pastoris* MSP8.6 was recycled as catalyst, while a decrease in yield of from 90 to ca. 84% glyoxylate was obtained in consecutive batch reactions when *H. polymorpha* 13.168 was used; the higher relative concentration of *S. cerevisiae* catalase T in *H. polymorpha* 13.168 is most likely responsible for the higher yields obtained with recycle of this catalyst (Table 3).

Figure 2 illustrates the results of 30 consecutive batch reactions with catalyst recycle using *H. polymorpha* 13.168 for the oxidation of 0.50 M glycolic acid to glyoxylic acid in reaction mixtures containing 0.375 M AMPA. After a slight decrease in glyoxylate yield to 84% after the first several reactions, no further decrease in glyoxylate yield was observed. No significant loss of either total catalase activity or glycolate oxidase activity of the microbial catalyst was observed over the course of 30 reactions, and increases in the measured levels of either enzyme activity relative to the initial activities was attributed to an increase in the permeabilization of the cells. The reaction time increased from 1.0 to 1.5 h over the course of this series of recycle reactions. Flavin mononucleotide (FMN, enzyme cofactor of glycolate oxidase)[1,18] was included in the reaction mixtures at a concentration of 0.01 mM (4.56 mg/L); this concentration of FMN was sufficient to eliminate the gradual loss of glycolate oxidase activity otherwise observed for reactions

(16) Yamauchi, K.; Mitsuda, Y.; Kinoshita, M. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 3285–3286.

(17) Payne, M. S.; Petrillo, K. L.; Gavagan, J. E.; DiCosimo, R.; Wagner, L. W.; Anton, D. L. *Gene* **1997**, *194*, 179–182.

(18) Frigerio, N. A.; Harbury, H. A. *J. Biol. Chem.* **1958**, *231*, 135–157.

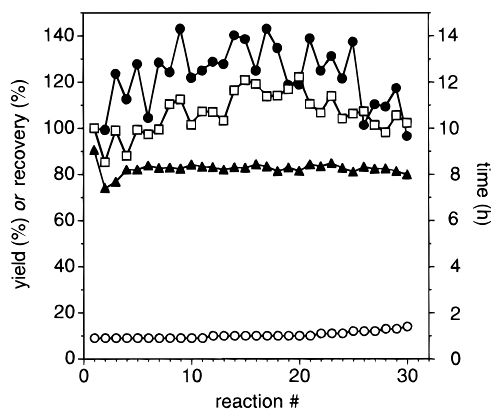



**Figure 2.** Glyoxylate yield (▲), recovered glycolate oxidase (●) and total catalase (□) activities of recycled *H. polymorpha* 13.168 double-transformant catalyst and reaction time (○) for 30 consecutive batch oxidations of glycolic acid. Reaction conditions: glycolic acid (0.50 M), AMPA (0.375 M), flavin mononucleotide (0.01 mM), isobutyric acid (0.100 M, HPLC internal standard), 5% (wet cell weight/volume) permeabilized *H. polymorpha* 13.168 cells (6.55 DCIP IU glycolate oxidase/mL, 5900 IU total catalase/mL), pH 8.3, 40 psig $O_2$ (with sparging), 5 °C, stirring at 700 rpm.

run in the absence of added FMN and extended the number of times the whole cell catalysts could be recycled.

**Hydrogenation of Glyoxylic Acid/AMPA Mixtures.** The 0.375 M concentration of AMPA in oxidations of 0.50 M glycolate was selected on the basis of a determination of the minimum excess concentration of glyoxylate in a hydrogenation reaction which gave complete conversion of AMPA to NPMG. Because free glyoxylate was in equilibrium with the imine produced by reaction of glyoxylate with AMPA (Scheme 3), some glyoxylate was hydrogenated to glycolate over the course of the reaction; an excess of glyoxylate was therefore necessary to ensure complete conversion of AMPA to NPMG, which simplified the subsequent isolation of NPMG from the resulting product mixture. *N,N*-Diacetic acid (aminomethyl)phosphonic acid (the product from hydrogenation of an imine formed by the reaction of NPMG and glyoxylate) was not detected in any of the hydrogenation product mixtures.

A series of hydrogenations of enzymatically-produced glyoxylate/AMPA mixtures were initially performed at 25 °C and 50 psig hydrogen using a 5% Pd/carbon catalyst,[19] where the ratio of glyoxylate concentration to AMPA concentration was varied from 1.28 to 1.04 (Figure 3). At glyoxylate/AMPA concentration ratios of greater than 1.12, the conversion of AMPA to glyoxylate was greater than 98%. In oxidation reactions of 0.50 M glycolate, the concentration of glyoxylate produced during consecutive batch reactions with catalyst recycle was typically at least 0.42 M; therefore employing an AMPA concentration of 0.375 M resulted in the desired excess concentration of glyoxylate (12% molar excess of glyoxylate relative to AMPA) prior to hydrogenation.

The dependence of the yield of NPMG on changes in temperature (5−50 °C), hydrogen pressure (50−500 psig), catalyst concentration (0.1−2% dry weight 5% Pd/C per volume of reaction mixture), and pH (6.0−8.0) in reactions containing 0.435 M glyoxylic acid and 0.375 M AMPA (produced by the enzymatic oxidation of glycolate)

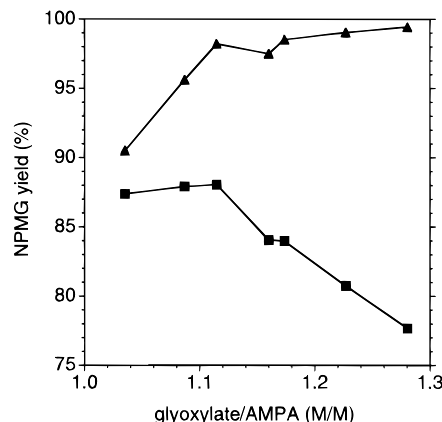



**Figure 3.** Dependence of NPMG yield (based on glyoxylate, ■; based on AMPA, ▲) on the ratio of glyoxylate concentration to AMPA concentration in hydrogenations of glyoxylate/AMPA mixtures. A solution containing glyoxylate (0.435 M) and AMPA (0.375 M, glyoxylate/AMPA = 1.16), prepared by enzymatic oxidation of glycolate (0.50 M) in aqueous AMPA (0.375 M), was adjusted to the indicated ratios of glyoxylate/AMPA concentrations by the addition of either glyoxylate or AMPA and then the resulting solutions were adjusted to pH 7.0 and hydrogenated for 19 h at 25 °C and 50 psig hydrogen using 1% (wt/v) of 5% Pd/carbon as catalyst.

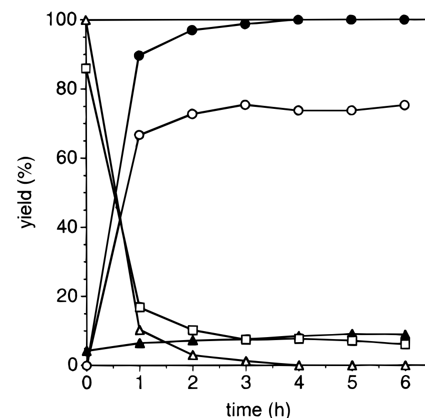



**Figure 4.** Time course for the hydrogenation of a filtered solution (prepared by the oxidation of glycolate (0.500 M) in aqueous AMPA (0.375 M) using permeabilized *H. polymorpha* 7.13.3 cells) of glyoxylate (0.435 M) and AMPA (0.375 M) at 25 °C, pH 7.0, and 250 psig of hydrogen and with 1% (wt/v) of 5% Pd/carbon as catalayst. The yields of glyoxylate (□), glycolate (▲), and NPMG (○) are based on initial glycolate concentration (0.500 M), and the yields of AMPA (△) and NPMG (●) are based on initial AMPA concentration (0.375 M). The yields of formate (5.6%) and oxalate (4.2%) (produced during the enzymatic oxidation and based on initial glycolate concentration) remained unchanged over the course of the hydrogenation.

was examined. Yields of NPMG under these reaction conditions were only dependent on changes in catalyst concentration and hydrogen pressure. Optimal reaction conditions for hydrogenation of enzymatically-produced 0.435 M glyoxylic acid/0.375 M AMPA reaction mixtures employed 500 psig of hydrogen and 1% dry weight/weight of solution of 5% Pd/carbon at pH 7.0 and 27.5 °C, affording 100% NPMG on the basis of AMPA (Figure 4). No detectable AMPA remained in hydrogenation product mixtures produced under these optimal reaction conditions.

Enzymatically-produced reaction mixtures of glyoxylic acid/AMPA were subjected to different levels of filtration

(19) Rogers, T. E.; Smith, L. R. US Patent 5,578,190, 1996.

prior to hydrogenation under the optimal reaction conditions described above, and reaction mixtures which were filtered only through 0.20 μm, 30 kDa, or 10 kDa molecular weight cutoff filters showed slower reaction rates and left a small but detectable amount of AMPA unreacted when compared to reaction mixtures which were additionally filtered through 1 kDa filters. When enzymatically-produced glyoxylate/AMPA mixtures were filtered through a 1 kDa filter before hydrogenation, identical product yields and reaction rates were obtained when compared to the hydrogenation of mixtures prepared from glyoxylic acid and AMPA.

Filtration also produced enzymatically-produced glyoxylic acid/AMPA mixtures which were stable for storage at 5 °C prior to hydrogenation. Reaction mixtures which were prepared with commercially available glyoxylic acid were stable for at least 2 weeks at room temperature with no change in composition, rate of hydrogenation, or yield of NPMG, while the concentration of glyoxylic acid in enzymatically-produced mixtures left in contact with the permeabilized cell catalyst for 16 h at 5 °C decreased approximately 6%, with a concomitant 3% increase in both glycolate and oxalate. Presumably, a Cannizzaro-type reaction occurs in the presence of the microbial catalyst, where 2 equiv of aldehyde disproportionate to a carboxylic acid and the corresponding alcohol. Immediate removal of the cells by centrifugation and subsequent filtration of the filtrate through 0.22 μm cellulose acetate and 10 kDa Diaflo-Amicon filters eliminated this undesirable side reaction.

Recycle of the 5% Pd/C catalyst in consecutive hydrogenations of 0.435 M glyoxylic acid/0.375 M AMPA mixtures at 275 psig of hydrogen, 27 °C (pH 7), resulted in significant decreases in reaction rate in subsequent reactions when the catalyst was recovered from the first hydrogenation reaction and reused without further treatment. Increasing the hydrogen pressure from 275 to 500 psig in subsequent reactions did not result in an increase in reaction rate. When, after six consecutive reactions, the catalyst was washed with a small quantity of water prior to recycle in a seventh reaction, a significant amount of the catalyst activity was recovered. A series of hydrogenation reactions with catalyst recycle was then repeated at an initial hydrogen pressure of 500 psig, except that the recovered catalyst was briefly washed with water prior to each reuse; seven consecutive hydrogenations with catalyst recycle were run with no change in reaction rate, and each reaction produced a quantitative yield of NPMG based on AMPA.

**Isolation of NPMG from Hydrogenation Product Mixtures.** After the hydrogenation of an enzymatically-produced glyoxylate/AMPA mixture and subsequent filtration to remove the 5% Pd/C catalyst, the resulting aqueous product mixture was concentrated 2-fold by distillation under reduced pressure to produce a concentrated solution typically containing formic (0.062 M), oxalic (0.038 M), glycolic (0.084 M), and glyoxylic (0.076 M) acids and NPMG (0.750 M, 127 g/L). The pH of the concentrate was next adjusted to 2.1 with 12 N HCl at 25 °C (NPMG $pK_{a1}$ = 2.229).[20] At pH 2.1, the final concentrations of formic, oxalic, glycolic, and glyoxylic acids were all below their reported solubility limits and these organic acids remained in solution, as did the sodium chloride produced during the pH adjustment, while ca. 92% of the NPMG immediately precipitated (NPMG solubility = 12 g/L at 25 °C).[21] This precipitate was typically only ca. 93% pure (molar basis), with ca. 3% oxalic acid as organic impurity. It was found that a simple water wash of the precipitate would decrease the concentration of oxalic acid impurity to less than 0.1% (molar basis) with a 94% recovery of ≥98% pure NPMG, indicating that the oxalic acid was only adsorbed on the surface of the NPMG precipitate. Recrystallization of the impure NPMG precipitate from water also readily removed the oxalate impurity.

## Discussion

For any chemical process, the steps necessary to isolate and purify the final product, and also treat and properly dispose of the waste products generated, can add significantly to the cost of manufacture. In designing a chemoenzymic synthesis of NPMG from glycolic acid (which is considerably less expensive than glyoxylic acid) and (aminomethyl)phosphonic acid, minimization of both waste generation and the number of process steps were important factors in determining the reaction conditions for the enzyme oxidation step and the subsequent hydrogenation so as to produce a final product mixture from which NPMG could be easily recovered in high yield and purity. It was first determined that NPMG could be readily precipitated from aqueous mixtures containing glycolic, glyoxylic, formic, and oxalic acids in high yield and purity but that any unreacted AMPA present would coprecipitate with the NPMG. This required that AMPA be completely converted to NPMG in the hydrogenation step, which in turn required the presence of an excess of glyoxylic acid in the hydrogenation step. Because the yield of glyoxylic acid produced in the initial enzymatic oxidation of glycolic acid was typically about 84% in consecutive batch reactions with catalyst recycle, the concentration of AMPA in the enzymatic oxidation was therefore set at 75% of the initial glycolic acid concentration (0.375 M when using 0.050 M glycolic acid) to produce a sufficient excess of glyoxylic acid to obtain complete conversion of AMPA to NPMG in the subsequent hydrogenation. The significantly higher cost of AMPA relative to glycolic acid was an additional reason to optimize the individual process steps to obtain a quantitative yield of NPMG based on AMPA.

The present chemoenzymic synthesis of NPMG is simple to perform and requires very few processing steps. The *H. polymorpha* and *P. pastoris* transformant catalysts were prepared using inexpensive media and standard fermentation techniques, much the same as used in the commercial preparation of bakers' yeast or brewers' yeast. The initial aqueous reaction mixture containing 0.50 M glycolic acid and 0.375 M AMPA at pH 8.0−9.0 was cooled to 5 °C, then a 5 wt % charge of permeabilized microbial catalyst was added, and the resulting mixture was sparged with oxygen under pressure at 5 °C. At the conclusion of the oxidation reaction, the microbial catalyst was recovered from the mixture by membrane filtration for catalyst recycle, and the filtrate was hydrogenated directly without isolation or purification of the reaction intermediates to produce NPMG, which was recovered from the hydrogenation product mixture in

(20) Motekaitis, R. J.; Martell, A. E. *J. Coord. Chem.* **1985,** *14*, 139−149.

(21) *The Pesticide Manual: Incorporating the Agrochemicals Handbook*, 10th ed.; Tomlin, C., Ed.; British Crop Protection Council, Royal Society of Chemistry (Great Britain), The Bath Press: Bath, 1994; p 543.

high yield and purity by 2-fold concentration and acid precipitation. Aside from the small amounts of organic acid byproducts produced, the major waste product of this process is the sodium chloride generated by the use of sodium hydroxide to adjust the pH of the initial oxidation reaction mixture and the hydrochloric acid used to precipitate NPMG in the final step. By comparison, the current multistep chemical process for NPMG produces aqueous waste streams containing a large number of byproducts, including *N*-(phosphonomethyl)iminodiacetic acid, AMPA, iminodiacetic acid, *N*-formyl-*N*-(phosphonomethyl)glycine, phosphoric acid, phosphorous acid, formaldehyde, formic acid, and carbon dioxide.[22] Many of these byproducts are produced in the final step, where PMIDA undergoes an oxidative dealkylation of one of its two acetate substituents.[23]

The permeabilized single transformants *H. polymorpha* GO1 and *P. pastoris* MSP10, which had been previously used to oxidize glycolic acid to glyoxylic acid in >98% yield when ethylenediamine was used as an amine additive, produced significantly lower yields of glyoxylic acid when (aminomethyl)phosphonic acid was substituted for ethylenediamine. The endogenous catalase activities of these two methylotrophic yeast transformants were reversibly inhibited by AMPA and were less effective at decomposing byproduct hydrogen peroxide to water and oxygen, resulting in the production of significant quantities of formate (a product of the oxidation of glyoxylate by hydrogen peroxide). The mechanism of this previously-unreported inhibition of certain catalase enzymes by AMPA is unknown, but of the catalase sources examined, cytosolic catalases (*A. niger* and *A. nidulans* catalase, *S. cerevisiae* catalase T) were not inhibited, while catalases which are normally membrane-associated (peroxisomal *H. polymorpha* and *P. pastoris* catalase, bovine liver catalase, and *S. cerevisiae* catalase A) were reversibly inhibited by AMPA.

As a preferable alternative to the addition of a second inhibition-resistant catalase to the reaction mixture, or the substitution of DEAMPA for AMPA (which would require an additional step to hydrolyze the resulting phosphonate diester to NPMG), increased yields of glyoxylic acid were obtained from the enzymatic oxidation of glycolate/AMPA mixtures by employing double transformants of *H. polymorpha* and *P. pastoris* which coexpressed both spinach glycolate oxidase and an additional exogenous catalase, *S. cerevisiae* catalase T, which was not inhibited by AMPA. Extracts of the *P. pastoris* MSP8.6 double transformant initially had only 25% of total catalase as catalase T,[17] and both the percentage of catalase T and the yields of glyoxylate decreased significantly when this catalyst was recycled in consecutive batch reactions. Extracts of the double-transformant *H. polymorpha* 13.168 initially contained ca. 70% of total catalase as *S. cerevisiae* catalase T[10] and were successfully recycled in 30 consecutive batch oxidations of glycolic acid/AMPA mixtures, with an average glyoxylate yield of 84%.

The initial yield of glyoxylate in the first oxidation reaction which used freshly prepared, permeabilized *H. polymorpha* 13.168 double transformant as catalyst was typically 89−91%, and the yield dropped to ca. 84% over the course of the next several consecutive batch reactions run with catalyst recycle. The percentage of catalase T relative to total catalase (the sum of catalase T and endogenous catalase) which could be measured by assay of the permeabilized microbial cells after several catalyst recycles dropped from an initial value of ca. 50% to ca. 20% of total catalase, and this loss of catalase T activity was most likely responsible for the drop in glyoxylate yield. Leakage of some fraction of the cytosolic catalase T from the permeabilized cells over the course of the first several catalyst recycles may have caused some of this loss in activity, in which case the peroxisomal targeting of catalase T, as is the case for the coexpressed glycolate oxidase, might lead to an improved microbial transformant catalyst.

Additional improvements in the yield of NPMG based on glycolic acid in the present process (currently at 75%) might be obtained by further optimization of the ratio of glyoxylate to AMPA required in the hydrogenation step. Initial hydrogenation studies performed using only 50 psig of hydrogen indicated that a ratio of glyoxylate/AMPA of at least 1.12 was required to obtain complete conversion of AMPA to NPMG, but optimization of the hydrogenation reaction conditions using this glyoxylate/AMPA ratio indicated that at higher hydrogen pressures (250−500 psig of hydrogen) significant concentrations of glyoxylate remained (ca. 0.038 M, up to 9% of the initial glyoxylate concentration) at the reaction times required to obtain complete conversion of AMPA. At these higher hydrogen pressures, a ratio of glyoxylate/AMPA of as low as 1.04 might still produce complete conversion of AMPA, which could potentially increase NPMG yield based on glycolate from 75 to ca. 83%.

## Experimental Section

**Materials and Methods.** All chemicals were obtained from commercial sources and used as received unless otherwise noted. Soluble spinach glycolate oxidase, *A. niger* catalase, and bovine liver catalase were obtained from Sigma. DEAMPA was prepared according to a literature procedure,[16] and the p$K_a$ of the DEAMPA ammonium ion (p$K_a$ 6.40) was measured by titration at 25 °C. Oxidations of glycolic acid were performed in a 300 mL EZE-Seal (Autoclave Engineers) stirred autoclave reactor equipped with Dispersimax impeller, which sparges the reaction mixture as the mixture is stirred, or in a 3 oz. Fischer−Porter tube equipped with a magnetic stir bar and no oxygen sparging. Hydrogenations were performed in the same 300 mL autoclave reactor. Samples from oxidation reactions using enzyme catalysts were prepared for HPLC analysis by centrifugation, followed by filtration of the supernatant using a Millipore Ultrafree MC (10 000 MWCO) filter unit. Yields of glyoxylate, formate, and oxalate, and the recovered yield of glycolate, were calculated as percentages based on the total amount of glycolic acid present at the beginning of the reaction. Analyses were performed using high-pressure liquid chromatography (HPLC): organic acid analyses were performed using a Bio-Rad HPX-87H column and AMPA and *N*-(phosphonomethyl)glycine were analyzed using a Bio-Rad Aminex Glyphosate Analysis column, using HPLC conditions recommended by the column manufacturer.

Co-immobilized glycolate oxidase and catalase on oxirane acrylic beads were prepared as previously described.[4] Single transformants of *P. pastoris* MSP10[9] and *H. polymorpha* GO1[10] (expressing spinach glycolate oxidase and an endogenous catalase) and double transformants of *P. pastoris* MSP8.6[17] and *H. polymorpha* 13.168[10] (expressing spinach glycolate oxidase and catalase T from *S. cerevisiae*) were prepared as previously described. Glycolate oxidase, total catalase (*S.*

(22) Smith, L. R. Eur. Pat. Appl. EP 413,672, 1991.
(23) (a) Riley, D. P.; Fields, D. L.; Rivers, W. *J. Am. Chem. Soc.* **1991**, *113*, 3371−3378. (b) Riley, D. P.; Fields, D. L.; Rivers, W. *Inorg. Chem.* **1991**, *30*, 4191−4197. (c) Riley, D. P.; Fields, D. L. *J. Am. Chem. Soc.* **1992**, *114*, 1881−1882. (d) Bakthazor, T. M.; Pulwer, M. J. US Patent 4,654,429, 1987.

*cerevisiae* catalase T plus endogenous *H. polymorpha* catalase), and catalase T activities present in extracts of *H. polymorpha* double transformants ranged from 95 to 160 DCIP IU/g of wet cells for glycolate oxidase, 165000–315000 IU/g of wet cells for total catalase, and 90000–140000 IU/g of wet cells for catalase T.

Wet cell weights of permeabilized or whole cell microbial transformants used as catalysts or in assays were determined by blotting a known weight (ca. 0.250 g) of cell paste (obtained by centrifugation of fermentation broth or from cell suspensions in buffer) on filter paper to remove excess moisture, then reweighing the blotted cell paste to determine wet cell weight; this procedure provided a reproducible method for determining the fraction of water in fresh or frozen cell paste. G.O. activities are reported in DCIP units unless otherwise noted.

**Permeabilization of *P. pastoris* and *H. polymorpha* Microbial Transformants.** A suspension of 10 wt % wet cells in 50 mM phosphate buffer (pH 7.0) containing 0.1% (w/v) of either benzalkonium chloride or Barquat MB-50 (Lonza) was mixed for 60 min at 25 °C.[3,6] The mixture was then centrifuged, the supernatant was decanted, and the cells were washed three times (10% w/v) in 50 mM phosphate buffer (pH 7.0) at 5 °C. The resulting cell paste could be used directly in oxidation reactions, or frozen at −20 or −80 °C and stored at these temperatures until needed. The amount of permeabilized cell catalyst added to a reaction mixture was chosen so as to provide the desired concentrations of glycolate oxidase and catalase activities. Recoveries of permeabilized cell catalyst from reactions with glycolate oxidase and catalase activities of greater than 100% of their initial values were due to increased permeabilization of the whole-cell catalyst during the course of the reaction.

**Glycolate Oxidase and Catalase Assays of Whole Cell Transformants.** Whole cell transformant catalysts were assayed for glycolate oxidase activity by first blotting ca. 0.25 g of permeabilized or unpermeabilized wet cells on filter paper to remove excess water and then accurately weighing ca. 5–10 mg of the blotted wet cells into a 3 mL quartz cuvette containing a magnetic stirring bar and 2.0 mL of a solution which contained 2,6-dichlorophenol–indophenol (DCIP, 0.12 mM) and TRIS buffer (80 mM, pH 8.3). The cuvette was capped with a rubber septum and the solution deoxygenated by bubbling with nitrogen for 5 min. To the cuvette was then added by syringe 0.040 mL of an aqueous solution of glycolic acid (1.0 M) and TRIS buffer (1.0 M, pH 8.3), and the mixture was stirred while the change in absorption with time at 606 nm was measured ($\epsilon$ = 22 000 L mol$^{-1}$ cm$^{-1}$).[24]

Catalase activity of permeabilized microbial single transformants, and total catalase activity (*S. cerevisiae* catalase T plus endogenous catalase) of permeabilized microbial double transformants, was assayed by accurately weighing ca. 2–5 mg of the blotted wet cells into a 3 mL quartz cuvette containing a magnetic stirring bar and 2.0 mL of 16.7 mM phosphate buffer (pH 7.0), then adding 1.0 mL of 59 mM hydrogen peroxide in 16.7 mM phosphate buffer (pH 7.0), and measuring the change in absorption with time at 240 nm ($\epsilon$ = 39.4 L mol$^{-1}$ cm$^{-1}$).[25] The catalase T and endogenous catalase activities of microbial double transformants were separately determined by preparing extracts of the double transformants and assaying the extracts as described above at pH 7.0 and at pH 4.0; at pH 4.0, the endogenous *H. polymorpha* catalase retains 7% of its activity at pH 7.0, while the *S. cerevisiae* catalase T retained 60% of its activity at pH 7.0.

**Oxidation of Glycolic Acid/AMPA Mixtures Using Soluble Glycolate Oxidase and Catalase.** Into a 3 oz. Fischer–Porter glass aerosol reaction vessel were placed a magnetic stirring bar and 10 mL of an aqueous solution containing glycolic acid (0.25 M), (aminomethyl)phosphonic acid (AMPA, 0.20 M), FMN (0.01 mM), butyric acid (HPLC internal standard, 0.10 M), glycolate oxidase (from spinach; 1.0 IU/mL), and catalase (from *A. niger*, 14 000 IU/mL) at pH 8.5. The reaction vessel was sealed, the reaction mixture was cooled to 5 °C and then the vessel was flushed with oxygen by pressurizing to 70 psig and venting to atmospheric pressure five times with stirring. The vessel was then pressurized to 70 psig of oxygen and the mixture stirred at 5 °C. Aliquots (0.10 mL) were removed by syringe through a sampling port (without loss of pressure in the vessel) at regular intervals for analysis by HPLC to monitor the progress of the reaction. After 6 h, the HPLC yields of glyoxylate, formate, and oxalate were 92.3, 4.36, and 5.5%, respectively, and no glycolate remained. The remaining activity of glycolate oxidase and catalase were 87 and 88%, respectively, of their initial values.

**Oxidation of Glycolic Acid/AMPA Mixtures Using Co-Immobilized Glycolate Oxidase and Catalase.** In a typical procedure, a 300 mL stirred autoclave was charged with 100 mL of a solution containing glycolic acid (0.50 M), (aminomethyl)phosphonic acid (0.375 M), isobutyric acid (0.10 M, HPLC internal standard), and flavin mononucleotide (FMN, 0.01 mM), and the solution was cooled to 5 °C. The pH of the solution was adjusted to 8.5 with 50% sodium hydroxide, and then 80 IU of spinach glycolate oxidase and 240 000 IU of *A. niger* catalase co-immobilized[4] on 24 g of oxirane acrylic beads (Eupergit C, Accurate Chemical and Scientific Corp.) was added (0.80 IU/mL glycolate oxidase, 2400 IU/mL catalase). The resulting mixture was stirred at 400 rpm and 5 °C under 70 psig of oxygen, while oxygen was sparged through the mixture at 50 mL/min. The reaction was monitored by removing a 0.40 mL aliquot of the reaction mixture at regular intervals, filtering the aliquot using a Millipore Ultrafree-MC 10000-MW-cutoff filter unit (to remove the enzyme catalyst), and analyzing by HPLC. After 3.5 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 87.8, 2.5, and 3.3%, respectively, with 9.2% glycolic acid remaining. The final activities of glycolate oxidase and catalase were 78 and 94% of their initial values, respectively. The immobilized enzyme catalyst was recycled by filtering the product mixture through a 2.5 cm i.d. × 20 cm glass column equipped with a 20 $\mu$m polyethylene bed support. The remaining liquid adsorbed on the catalyst was removed by briefly passing a stream of nitrogen through the column, then the catalyst was resuspended in 100 mL of a fresh 5 °C solution containing glycolic acid (0.50 M), (aminomethyl)phosphonic acid (0.375 M), isobutyric acid (0.10 M, HPLC internal standard), and flavin mononucleotide (0.01 mM) at pH 8.5. The 300 mL autoclave reactor was again charged with this reaction mixture and the reaction repeated.

**Oxidation of Glycolic Acid/AMPA Mixtures Using Permeabilized Microbial Transformant Catalysts.** In a typical procedure, a 300 mL stirred autoclave reactor was charged with 100 mL of a solution containing glycolic acid (0.500 M), AMPA (0.375 M), isobutyric acid (0.100 M, HPLC internal standard), and FMN (0.01 mM) at pH 8.3 (adjusted with 50% NaOH), and the solution was cooled to 5 °C. To the reactor were then added 5.0 g of permeabilized *H. polymorpha* double transformant 13.168 (357 IU glycolate oxidase and 600 000 IU total catalase (50% *S. cerevisiae* catalase T, 50% *H. polymorpha* endogenous catalase)). The pH of the resulting mixture was readjusted to 8.3 with 5% NaOH. This mixture was stirred at 750 rpm, which sparged oxygen through the mixture via the action of the turbine impeller, and at 5 °C under 40 psig of oxygen. The reaction was monitored by taking a 0.20 mL aliquot of the reaction mixture at regular intervals, filtering the aliquot using a Millipore Ultrafree-MC 10000 NMWL filter unit, and analyzing the filtrate by HPLC. After 1.5 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 90.0, 3.1, and 1.8%, respectively, with 5.0% recovery of glycolic acid. The final activities of permeabilized-cell glycolate oxidase and total catalase were 146 and 113%, respectively, of their initial values after permeabilization.

The microbial cell catalyst was recovered from the reaction mixture described above by centrifugation. Without further treatment the cell pellet was mixed with 100 mL of fresh reaction mixture and the reaction repeated. After 1.5 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 84.3, 4.9, and 9.9%, respectively, with 1.7% recovery of glycolic acid. The final activities of permeabilized-cell glycolate oxidase and

(24) Armstrong, J. McD. *Biochim. Biophys. Acta* **1964**, *86*, 194–197.
(25) Aebi, H. E. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Verlag Chemie: Deerfield Beach, FL 1983; 3rd ed.; Vol. III, pp 273–286.

total catalase were 143 and 104% of their initial values after permeabilization. The percentage of total catalase for catalase T and *H. polymorpha* catalase in extracts of the recovered cells after two uses were 46 and 54%, respectively.

**Hydrogenation of Glyoxylate/AMPA Mixtures To Produce NPMG.** A 100 mL aqueous solution containing glycolic acid (3.84 g, 99% purity, 0.500 M) and AMPA (4.24 g, 98.3% purity, 0.375 M) at pH 8.3 (adjusted with 50% NaOH) and at 5 °C and under 120 psig of oxygen was oxidized in a 300 mL autoclave reactor as described above using 7.1 g of permeabilized *H. polymorpha* double-transformant catalyst. After 1.25 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 86.8, 3.8, and 6.0%, respectively, with a 3.5% recovery of glycolic acid. The product mixture was centrifuged at 5 °C and the supernatant decanted from the cell pellet. The supernatant, containing glyoxylic acid (0.434 M) and AMPA (0.375 M), was immediately filtered through a 0.2 $\mu$m cellulose acetate filter and then consecutively through an Amicon YM30, YM10, and YM1 membrane filter disk. The resulting filtrate (pH 7.0) was placed in a 300 mL stirred autoclave reactor, along with 1.0 g (dry weight) of 5% Pd/C (Grace). The reactor was purged with nitrogen and then pressurized to 275 psig of hydrogen with stirring at 1000 rpm and at 27 °C. The reaction was monitored by taking a 0.20 mL aliquot of the reaction mixture at regular intervals. The sample was centrifuged, 0.10 mL of the supernatant was added to 0.10 mL of 0.100 M isobutyric acid (HPLC internal standard), and the solution was filtered using a Millipore Ultrafree-MC 10,000 NMWL filter unit and then analyzed the by HPLC. After 4 h, the unisolated yield of *N*-(phosphonomethyl)glycine was 100% based on AMPA.

**Isolation of *N*-(Phosphonomethyl)glycine from Hydrogenation Product Mixtures.** A typical hydrogenation product mixture, containing formic (0.031 M), oxalic (0.019 M), glycolic (0.042 M), and glyoxylic (0.038 M) acids, and NPMG (0.375 M, 63.5 g/L) was concentrated to half its initial volume by evaporation at reduced pressure. Concentrated hydrochloric acid was added dropwise to the concentrate until the mixture reached a pH of 2.1. A solid white precipitate formed with a simultaneous increase in pH. The pH was continually readjusted to 2.1 until precipitation stopped and the pH no longer increased upon standing. The mixture was filtered, and the precipitate was washed with 2 $\times$ 10 mL of cold water and dried under vacuum to a constant weight. Analysis by HPLC indicated the white solid was primarily *N*-(phosphonomethyl)glycine (93% purity, 92% yield), with 3% oxalic acid. A 1.00 g portion of this mixture was recrystallized from water to afford 0.94 g (94% yield, 98% purity) of *N*-(phosphonomethyl)glycine which now contained 0.08% oxalic acid.

**Acknowledgment.** Winnie Wagner and co-workers of the DuPont Fermentation Research Facility provided supplies of microbial catalysts used in this work. Fermentation optimizations of *P. pastoris* and *H. polymorpha* transformants and some multiple-batch glycolate oxidations were performed by Ken Schneider, Ross Jacobson, Mark Schendel, and co-workers at Bio-Technical Resources (Manitowoc, WI). Expression of spinach glycolate oxidase and *S. cerevisiae* catalase T in *H. polymorpha* was performed by Gerd Gellisen, Michael Piontek, Ulrike Dahlems, Volker Jenzelewski, Zbigniew Janowicz, and co-workers at Rhein Biotech, Düsseldorf, Germany). Kelly Petrillo (DuPont) assisted Mark Payne in the preparation of the *P. pastoris* transformants. Charles E. Nakamura (DuPont) prepared the sample of DEAMPA and determined the p$K_a$ of the DEAMPA ammonium salt.

JO970455F