Glyoxylic Acid Production Using Microbial Transformant Catalysts

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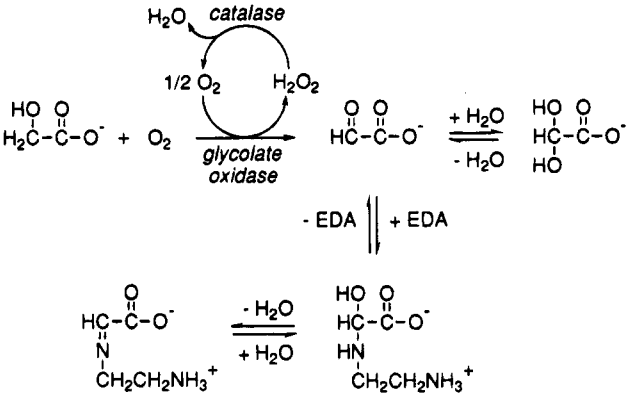
A practical method for the biocatalytic oxidation of glycolic acid to glyoxylic acid which uses a genetically engineered microbial cell as catalyst has been developed. Oxidations of glycolic acid at concentrations ranging from 0.75 to 1.5 M were run in aqueous solution in the presence of oxygen, ethylenediamine, and a metabolically inactive transformant of *Aspergillus nidulans*, *Hansenula polymorpha*, *Pichia pastoris*, or *Escherichia coli*. Each of the transformant catalysts contained significant quantities of both spinach glycolate oxidase ((*S*)-2-hydroxy acid oxidase, EC 1.1.3.15) and an endogenous catalase (EC 1.11.1.6). The *H. polymorpha* and *P. pastoris* transformant catalysts were recycled in up to 30 consecutive batch reactions, where the selectivity to glyoxylic acid was typically >98% at 100% conversion of glycolic acid. Under optimum reaction conditions and with endpoint monitoring, selectivities to glyoxylic acid of at least 99.7% were obtained.

Introduction

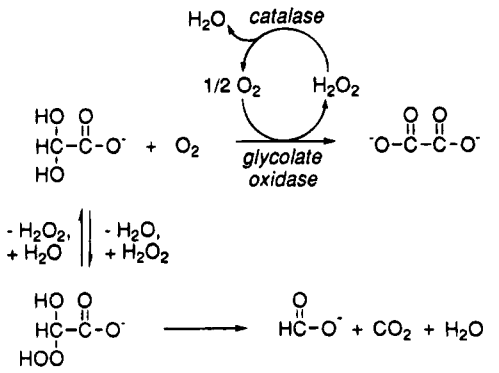
Glyoxylic acid is currently produced either by the nitric acid oxidation of glyoxal or by a three-step process which starts with the ozonolysis of dimethyl maleate, followed by hydrogenation of the hydroperoxide intermediate and hydrolysis of the resulting methyl glyoxylate hemiacetal.[1] As an alternative to these methods of production, we have been examining the biocatalytic oxidation of glycolic acid (hydroxyacetic acid) to glyoxylic acid (Scheme 1). Oxidations of glycolic acid were previously run in aqueous solution in the presence of oxygen and ethylenediamine (EDA) using either the soluble enzyme catalysts glycolate oxidase ((*S*)-2-hydroxy acid oxidase, EC 1.1.3.15) and catalase (EC 1.11.1.6)[2] or these same enzymes coimmobilized on a solid support.[3] These biocatalytic oxidations produced a higher yield of glyoxylic acid and fewer undesirable byproducts than current methods of production.

Including both catalase and EDA in oxidations of glycolic acid resulted in selectivities to glyoxylic acid of >99% at 100% conversion of glycolic acid. Catalase was added to decompose the stoichiometric amount of byproduct hydrogen peroxide produced during the oxidation, and EDA was added to react with glyoxylate, producing an equilibrium mixture of the corresponding imine and hemiaminal. Rapid decomposition of hydrogen peroxide limited the non-enzymatic oxidation of glyoxylate to formate and carbonate, and the trapping of glyoxylate with EDA significantly reduced both the product inhibition of glycolate oxidase and the further enzymatic oxidation of glyoxylate to oxalate (Scheme 2). Neither the separate addition of catalase nor EDA produced the high selectivity to glyoxylic acid observed when both were present, and the almost quantitative yields obtained were more than expected from a simple additive effect of using catalase or EDA alone.[2]

Scheme 1



Scheme 2



The technical feasibility of a biocatalytic oxidation of glycolate to glyoxylate was demonstrated using soluble enzymes as catalysts, but these soluble enzyme catalysts were neither practical nor cost-effective. Glycolate oxidase was not commercially available in significant quantities and had to be isolated from spinach leaves. The soluble enzyme was not very stable in aqueous reaction mixtures and was rapidly inactivated by the sparging of reaction mixtures with oxygen; reactions could only be run under pressure without sparging, resulting in a significant limitation of reaction rates. Finally, attempts to recover the remaining glycolate oxidase and catalase from product mixtures at the end of a reaction resulted

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in significant losses of both enzyme activities. Many of these deficiencies were overcome by coimmobilization of glycolate oxidase and catalase on an oxirane–acrylic bead solid support, but immobilization still required prior isolation of soluble glycolate oxidase, and the yield of enzyme activity upon immobilization of glycolate oxidase on the support was less than 20% of the initial activity.[3]

As an alternative to using soluble or immobilized enzyme catalysts for the biocatalytic production of glyoxylic acid, we have now demonstrated that genetically engineered microbial cell transformants of *Aspergillus nidulans*, *Hansenula polymorpha*, *Pichia pastoris*, and *Escherichia coli*, which express spinach glycolate oxidase and an endogenous catalase, can be used as catalysts for this reaction. The expression of foreign proteins in *A. nidulans*,[4] *H. polymorpha*,[5] and *P. pastoris*[6] has been well-documented. Active spinach glycolate oxidase has been previously expressed in both *E. coli*[7] and *Saccharomyces cerevisiae*,[8] but the use of such transformants as whole cell catalysts for the oxidation of glycolate to glyoxylate under nonfermentative reaction conditions has not been previously demonstrated.

Results

Catalyst Preparation, Permeabilization, and Stability. A brief description of the preparation and growth of transformants of *A. nidulans*, *H. polymorpha*, and *P. pastoris* is given in the Experimental Section; a more detailed description of the preparation of these transformants will be published separately. The preparation and growth of an *E. coli* transformant which expresses spinach glycolate oxidase has been previously described.[7] Representative levels of glycolate oxidase and catalase activities found in extracts of the *A. nidulans*, *H. polymorpha*, *P. pastoris*, and *E. coli* transformants and the corresponding activities observed by enzymatic assay of the whole cells are listed in Table 1. Enzyme catalyst activities are reported in international units (IU); an IU is the amount of enzyme which converts 1 μmol of substrate per minute under standard assay conditions. A significant portion of the total available enzyme activities of the *A. nidulans* and *E. coli* transformants was observed when assaying the whole cells; therefore, permeabilization of these transformants to increase accessibility of glycolate and oxygen to the intracellular enzymes was not required. These cells were frozen in liquid nitrogen and stored at −80 or −20 °C until needed, and some permeabilization of these cells prior to assay or use as catalyst may have taken place by the action of freezing and thawing.

Table 1. Glycolate Oxidase and Catalase Specific Activities of Cell Extracts or Whole Cells of Microbial Transformants[a]

	cell extract		whole cells	
microbial transformant	G.O. (oAB IU/g)[b]	catalase (IU/g)	G.O. (DCIP IU/g)[c]	catalase (IU/g)
A. nidulans	12	8200	7.0	1210
H. polymorpha	267	74000	1.4	5189
P. pastoris	432	200000	1.1	2714
E. coli	5	700	2.4	1330

[a] Measured as international units (IU) per gram of blotted wet cells; an IU is the quantity of enzyme which converts 1 μmol of substrate per minute under standard assay conditions. [b] Activity measured using the *o*-aminobenzaldehyde (oAB) standard assay. [c] Activity measured using the dichlorophenolindophenol (DCIP) standard assay; 0.55 DCIP IU ≅ 1 oAB IU.

Less than 1% of the total available enzyme activities of *P. pastoris* or *H. polymorpha* transformants was accessible by assay of the whole cells, even after freezing and thawing (Table 1). Permeabilization[9] of these cells was required prior to use as catalyst, and several methods of permeabilization were examined for preparing cells with sufficient glycolate oxidase activity. Initially, permeabilization was performed by mixing a 10 wt % suspension of cells in phosphate buffer with 0.1% (v/v) Triton X-100 for 15 min and then spraying the resulting suspension into liquid nitrogen (rapid freezing was imperative for optimal permeabilization), followed by thawing and centrifugation to recover the permeabilized cells;[10] at least 25% of the total available glycolate oxidase activity was accessed by this method. Additional freeze–thaws (up to six cycles) of these permeabilized cells in liquid nitrogen typically doubled the permeabilized cell enzyme activity. These permeabilized cells performed well as catalyst in subsequent oxidations, but the amount of liquid nitrogen used in their preparation would be impractical in a large-scale process.

Yields of glycolate oxidase activity obtained by the permeabilization of *P. pastoris* and *H. polymorpha* transformants with 0.1% (w/v) solutions of cetyltrimethylammonium bromide[11] (CTAB), benzylcetyldimethylammonium chloride[12] (BCDAC), *N*,*N*′,*N*′-poly(oxyethylene)(10)-*N*-tallow-1,3-diaminopropane[13] (EDT-20), sodium dodecylsulfate[14] (SDS), and Triton X-100[10,12b] are listed in Table 2. The cationic detergents CTAB, BCDAC, and EDT-20 each produced permeabilized cells with significantly increased glycolate oxidase and catalase activities, while the anionic detergent SDS and nonionic Triton X-100 did not produce a measurable increase in either enzyme activity compared to the activities of unpermeabilized cells. Of the three cationic detergents tested, BCDAC produced the highest levels of glycolate oxidase and catalase for permeabilized cells; a mixture of C_{12}-, C_{14}-, and C_{16}-alkyldimethybenzylammonium chlorides

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Table 2. Permeabilization of *P. pastoris* and *H. polymorpha* Transformants[a]

	P. pastoris		*H. polymorpha*	
detergent	G.O. (DCIP IU/g)[b]	catalase (IU/g)	G.O. (DCIP IU/g)[b]	catalase (IU/g)
CTAB	55	68 000	64	30 900
BCDAC	87	94 000	128	42 000
EDT-20	76	96 000	56	25 000
SDS	1.7	2 700	1.2	3 600
Triton X-100	1.4	3 400	1.0	1 700

[a] Ten percent (weight of wet cells/volume) suspensions of *P. pastoris* or *H. polymorpha* transformants in phosphate buffer (50 mM, pH 7.0, 25 °C) were treated with 0.1% (w/v) of detergent for 1 h: CTAB, cetyltrimethylammonium bromide; BCDAC, benzylcetyldimethylammonium chloride; EDT-20, *N, N', N'*-poly(oxyethylene)(10)-*N*-tallow-1,3-diaminopropane; SDS, sodium dodecylsulfate. [b] Activity measured using the DCIP standard assay.

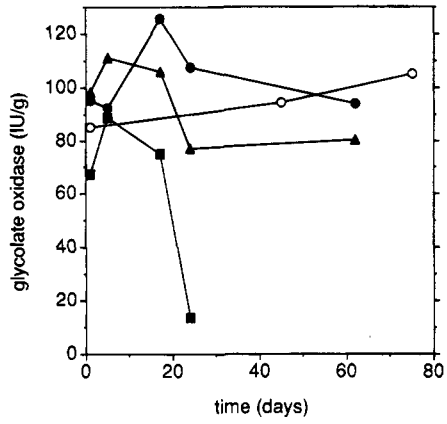

Figure 1. Stability of glycolate oxidase activity of permeabilized *P. pastoris* transformant cells at 5 °C (■), −20 °C (▲), and −80 °C (●) and of unpermeabilized cells stored at 5 °C (○). Cells were permeabilized with 0.2% (w/v) Barquat MB-50 and stored at 5 °C as a 10% (w/v) suspension in 50 mM phosphate buffer (pH 7.0), or the suspension was centrifuged and the resulting cell pellet frozen at −20 or −80 °C.

(Lonza Barquat OJ-50 or MB-50) was subsequently found to perform as well as the more expensive BCDAC.

The stability of both glycolate oxidase and catalase activities of permeabilized *P. pastoris* cells stored at −80, −20, and 5 °C was observed to decrease with increasing storage temperature; the dependence of recovered glycolate oxidase activity in permeabilized cells upon storage time at these three temperatures is depicted in Figure 1. At 5 °C, the remaining glycolate oxidase and catalase activities in permeabilized cells were ca. 10 and 50%, respectively, of their initial levels after 24 days, while storage at −80 °C for the same time resulted in little or no loss of either enzyme activity. In contrast, unpermeabilized cells showed little loss in either enzyme activity when stored at 5 °C for more than two months (Figure 1). The enzyme activities of permeabilized *H. polymorpha* cells were less stable than those of *P. pastoris*, and they required storage at −80 °C to prevent significant losses in glycolate oxidase and catalase activities.

Oxidation Reactions. Reactions were run at 5 °C in a 300 mL stainless steel batch reactor with oxygen sparging under pressure and with stirring provided by an overhead impeller. Aqueous reaction mixtures were typically comprised of glycolic acid (0.75 M), EDA (0.788 M), and an *A. nidulans*, *E. coli*, *H. polymorpha*, or *P. pastoris* transformant as catalyst; the initial pH of this mixture was ca. 8.9–9.1 and required no adjustment. The concentration of EDA was increased to 0.863 M in reaction mixtures where either propionic or isobutyric acid (0.075 M) was added as a HPLC internal standard. The glycolate oxidase cofactor flavin mononucleotide (FMN, 0.10–0.01 mM) was also initially added to reaction mixtures, as the presence of added FMN had been previously shown to enhance the stability of soluble glycolate oxidase under similar reaction conditions. The amount of wet cell paste added as catalyst was chosen so as to result in glycolate oxidase and catalase activities of at least 0.5 and 350 IU/mL, respectively. Table 3 lists representative yields of glyoxylate, formate, and oxalate produced in oxidations of 0.75 M glycolate when using each of the four transformants and the number of reactions run by recycling the same catalyst in consecutive batch reactions. Incomplete conversion of glycolate was obtained for single batch reactions when using the *E. coli* catalyst; these cells lysed during the reaction, releasing the contents of the cells into the reaction mixture with significant loss of catalyst activity. Individual batch reactions using the *A. nidulans* catalyst could be run to complete conversion of glycolate and near quantitative yields of glyoxylate, but the low specific activity of these cells (ca. 3–5 IU of glycolate oxidase/gram wet cell weight) required the use of an excessive amount of catalyst per volume of reaction mixture (ca. 20% w/v). Recycling the *A. nidulans* catalyst to a second batch reaction resulted in significant loss of catalyst activity and incomplete conversion of glycolate to glyoxylate.

The addition of FMN (glycolate oxidase cofactor) was omitted from a series of batch reactions using an *H. polymorpha* transformant catalyst in order to determine the stability of the intracellular glycolate oxidase in the absence of added FMN. A 99% yield of glyoxylic acid was obtained when a solution of glycolic acid (0.75 M) and EDA (0.788 M) was stirred at 5 °C with 4.7% (weight of wet cells/volume) of a permeabilized (1% Triton X-100/1 freeze–thaw) *H. polymorpha* transformant under 70 psig of oxygen with sparging. This catalyst was recycled in 20 consecutive 0.75 M batch reactions; the reaction times during this series increased from 1.0 to 1.5 h, and the final glycolate oxidase and catalase activities of the catalyst were 61 and 86%, respectively, of their initial values when the series was discontinued. No difference in glycolate oxidase recovery was observed when the series of reactions run in the absence of added FMN was compared to a similar series of batch reactions run with catalyst recycle and in the presence of 0.01 mM FMN (Table 3); this result was in marked contrast to those of reactions run using soluble glycolate oxidase, which exhibit a rapid loss of glycolate oxidase activity in the absence of added FMN.

P. pastoris transformants also produced high yields of glyoxylic acid under the same reaction conditions described for *H. polymorpha* transformants. When using either transformant, stopping the reaction as soon as the concentration of remaining glycolate dropped to less than 0.1% resulted in glyoxylate yields in excess of 99%, with less than 0.1% formate and 0.3% oxalate produced as unwanted byproducts. Figure 3 depicts the yields of glyoxylate and oxalate for 14 consecutive recycle batch reactions using a *P. pastoris* transformant catalyst and reaction endpoint monitoring by HPLC. The progress of the reaction was easily controlled by starting and stopping the oxygen sparging through the reaction mixture. When careful control of the reaction endpoint was not employed, and the reaction was simply terminated with

Table 3. Comparison of Microbial Transformants as Catalyst for the Oxidation of Glycolic Acid[a]

microbial transformant	cells (g/100 mL)	glyoxylate (%)	formate (%)	oxalate (%)	glycolate (%)	recycles
E. coli	30	74.4	5.6	1.1	6.3	0
A. nidulans	26	98.0	0	0	0	0
H. polymorpha	5	98.2	0.1	1.0	0.9	26
P. pastoris	5	99.7	0	0.3	0	30

[a] The indicated amount of wet cells was added to 100 mL of an aqueous solution containing glycolic acid (0.75 M), EDA (0.86 M), and either propionic acid (0.075 M) or isobutyric acid (0.100 M) as HPLC internal standard at 5 °C and pH 9.0–9.3; reactions were performed under oxygen pressure while sparging oxygen through the reaction mixture.

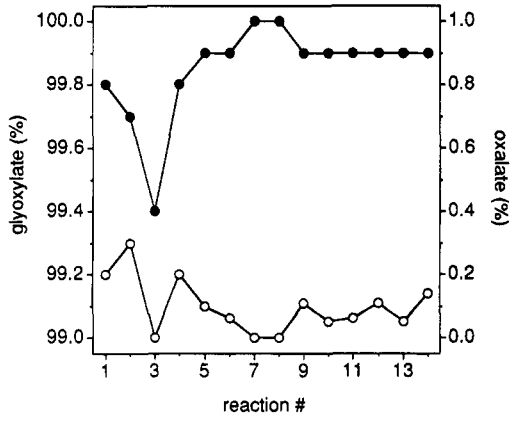

Figure 2. Yields of glyoxylate (●) and oxalate (○) obtained for consecutive batch oxidations of glycolic acid with catalyst recycle and endpoint monitoring. Reactions were terminated immediately upon complete conversion (>99.9%) of glycolate; formate yields were <0.1%. Reaction conditions: glycolic acid (0.750 M), EDA (0.863 M), FMN (0.01 mM), isobutyric acid (0.100 M, HPLC internal standard), 5% (w/v) permeabilized *P. pastoris* transformant cells (3.07 DCIP IU of glycolate oxidase/mL, 3360 IU of catalase/mL), pH 9.1, 120 psig O_2 (with sparging), 5 °C, and stirring at 1000 rpm.

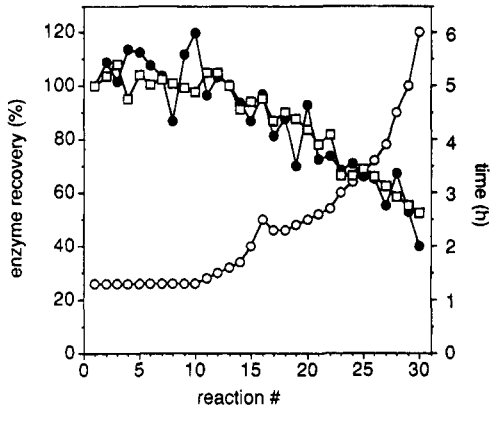

Figure 3. Reaction time (○) and recovered glycolate oxidase (●) and catalase (□) activities of a recycled *P. pastoris* transformant catalyst for 30 consecutive batch oxidations of glycolic acid. Reaction conditions: glycolic acid (0.750 M), EDA (0.863 M), isobutyric acid (0.100 M, HPLC internal standard), 5% (w/v) permeabilized *P. pastoris* transformant cells (5.55 DCIP IU of glycolate oxidase/mL, 5900 IU of catalase/mL), pH 8.9, 120 psig O_2 (with sparging), 5 °C, and stirring at 1000 rpm.

ca. 1% or less glycolate remaining, the yield of glyoxylic acid was typically greater than 98%, with less than 1% oxalate and 0.1% formate produced. If the reaction was allowed to proceed past the point at which less than 0.1% glycolate remained, the product glyoxylate (as its hydrate, Scheme 2) was subsequently oxidized to oxalic acid by glycolate oxidase. Because the reactions were run with a 5% molar excess of EDA present, the concentration of free glyoxylate was very low, and its further oxidation

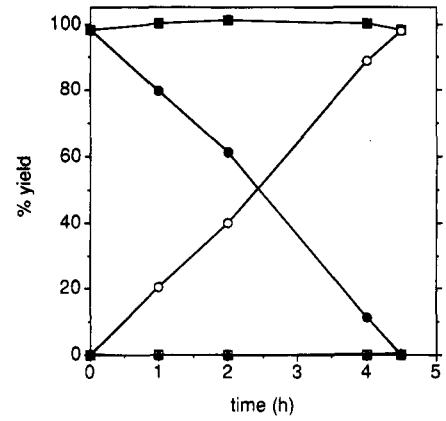

Figure 4. Time course for the oxidation of 1.50 M glycolic acid in aqueous EDA (1.575 M) using 2% (w/v) permeabilized *P. pastoris* transformant cells (2.32 DCIP IU of glycolate oxidase/mL, 3036 IU of catalase/mL) at pH 9.3, 120 psig O_2 (with sparging), 5 °C, and stirring at 1000 rpm: glycolate (●), glyoxylate (○), formate (□), oxalate (▲), and mass balance (■).

to oxalate was slow compared to the rate of oxidation of glycolate (K_m (glycolate) = 0.38 mM, K_m (glyoxylate) = 5.4 mM).[15] Also, the production of oxalate inhibits glycolate oxidase (K_i (oxalate) = 3.1 mM),[15] so that yields of glyoxylate of at least 98% could be easily obtained without careful monitoring of the reaction endpoint.

The recovered glycolate oxidase and catalase activities of a recycled *P. pastoris* transformant catalyst and the corresponding reaction times for 30 consecutive batch oxidations of 0.75 M glycolate are illustrated in Figure 3. As was the case for the *H. polymorpha* transformant, the omission of added FMN to this series of reactions resulted in no change in the rate of loss of glycolate oxidase activity when compared to the rate of loss in a similar series performed with added FMN. Catalyst recycle was simply performed by removal of the product mixture from the reactor, centrifugation, decantation of the product mixture, and resuspension of the catalyst pellet in fresh reaction mixture. The catalyst could be stored at 5 °C in either the product mixture or the freshly prepared reaction mixture for 24 h with no measurable loss of enzyme activities. When the concentration of glycolic acid was increased from 0.75 to 1.50 M (114 g/L), the reaction time increased from 1.5 to 4.5 h, with a 98.0% yield of glyoxylic acid (Figure 4). Thirteen consecutive batch oxidations with catalyst recycle of aqueous solutions containing 1.5 M glycolic acid, 1.575 M EDA, and 5% (w/v) *P. pastoris* transformant catalyst were performed before this series was discontinued; the first ten reactions were completed in less than 6 h, and an additional three reactions were run until the reaction time of the final reaction increased to 13 h. The total turnover number (moles of glyoxylate/moles of enzyme)

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for the glycolate oxidase catalyst in this series of reactions was ca. 2×10^7.

Optimum glyoxylate yields and enzyme recoveries were obtained when the concentration of glycolate oxidase employed in the oxidations (added as permeabilized whole cells of *H. polymorpha* or *P. pastoris*) was between 1.0 and 10.0 IU/mL. The amount of wet cell weight required to provide these concentrations of glycolate oxidase was typically between 2.0 and 5.0% (w/v) of the reaction volume, depending on the total glycolate oxidase concentration within the cell and the extent of permeabilization of the cells prior to use. The corresponding concentration of catalase in these reaction mixtures was no less than 350 IU/mL and usually greater than 5000 IU/mL. A ratio of concentrations of catalase to glycolate oxidase (measured in IU for each) of at least 250:1 was also required for optimum yields and enzyme recoveries. It was also demonstrated that having both glycolate oxidase and catalase enzymes present within the same permeabilized cell produced higher yields of glyoxylate and lower yields of formate than when using two separate whole cell catalysts, where one catalyst contained glycolate oxidase and the other catalase.

The initial pH of reaction mixtures was adjusted to between 8.9 and 9.5 by employing a slight molar excess of EDA (relative to glycolate), since the maximum activity of spinach glycolate oxidase occurs at ca. pH 8.3, and the pH of the mixture decreased by ca. 0.5 pH units over the course of the reaction. No pH control of the reaction was required. At least a 5% molar excess of the added amine was necessary to ensure optimal selectivity to glyoxylate. The reaction temperature was typically adjusted to 5 °C, but temperatures of up to 15 °C could be employed with little deleterious effect on enzyme recoveries or selectivity to glyoxylic acid. The reaction rate was at least partially controlled by the rate at which oxygen could be dissolved into the aqueous reaction mixture, and sparging of oxygen through the mixture was necessary to maintain a high oxygen dissolution rate. Oxygen was sparged through the mixture at a rate of at least 0.2 volumes of oxygen (measured at atmospheric pressure) per volume of reaction mixture per minute (volume/volume/minute). The reactions were also run under pressure to provide a higher concentration of dissolved oxygen; although no upper limit of oxygen pressure was determined, pressures from 0.584 to 1.76 MPa (70–240 psig) oxygen were typically employed.

Discussion

For a biocatalytic process to be practical, the catalyst must be inexpensive and easy to prepare and must exhibit good stability and productivity under reaction conditions. 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 baker's yeast or brewer's yeast. The cell densities produced by fermentation of either methylotrophic yeast transformant were as high as 80 g dry cell weight (ca. 270 g wet cell paste) per liter of fermentation broth; cell densities for the parent methylotrophic yeast strains of up to 130 g dry cell weight (430 g wet cell paste) per liter have been reported.[16] After isolation from the fermentation broth by centrifugation, the catalyst was stored as frozen cell paste for as long as 18 months at either −80 or −20 °C without significant loss of activity. Prior to use as catalyst, the cells were treated with an inexpensive quarternary ammonium compound (a biocide) which permeabilized the cell wall and membrane; this treatment rendered the cells metabolically inactive and allowed access to the required enzyme activities contained within the dead cells. These permeabilized cells were either used immediately or stored for several months at −80 or −20 °C prior to use.

The oxidation of glycolate with either the *H. polymorpha* or *P. pastoris* transformant catalysts typically produced near quantitative selectivities to glyoxylate (as a mixture of imine and hemiaminal with EDA). Running a reaction was simply performed by dissolving the desired amount of glycolic acid in water, adding a 5% molar excess of EDA, cooling the resulting solution to 5 °C, adding the desired amount of catalyst, and stirring under oxygen pressure with sparging. Reaction times were from 1 to 2 h for oxidations of 0.75 M glycolate (57 g/L) and from 4 to 5 h for 1.50 M glycolate (114 g/L). Catalyst recovery was performed by centrifugation of the product mixture, and glyoxylic acid could be readily separated from EDA by using a strong acid ion-exchange resin (as has been previously demonstrated when using soluble enzymes as catalysts).[2] The recovered EDA would be recycled to a subsequent batch reaction. Unlike reactions run with soluble or immobilized glycolate oxidase, the addition of flavin mononucleotide (FMN, an enzyme cofactor) was not required to maintain enzyme activity, thus eliminating an additional step for removal of the added FMN from the product mixture.

Of the two methylotrophic yeast catalysts, the *P. pastoris* transformant produced higher levels of glycolate oxidase and catalase and performed marginally better in recycle reactions than the *H. polymorpha* transformant. For recycle reactions which utilized the *P. pastoris* transformant catalyst, the catalyst productivity was ca. 112 g of glyoxylic acid per gram dry cell weight for 30 recycles at 0.75 M glycolate and ca. 96 g of glyoxylic acid per gram dry cell weight for 13 recycles at 1.5 M glycolate. These levels of catalyst productivity are within acceptable limits for a commercial-scale biocatalytic process for the production of glyoxylic acid which uses a permeabilized yeast cell transformant as catalyst.

Experimental Section

Materials and Methods. All chemicals were obtained from commercial sources and used as received unless otherwise noted. Soluble spinach glycolate oxidase (for comparative enzyme assays) was obtained from Sigma. Oxidations of glycolic acid were performed in a 300 mL EZE-Seal Autoclave Engineers stirred autoclave reactor equipped with a Dispersimax impeller, which sparges the reaction mixture as the mixture is stirred. Samples were prepared for HPLC analysis by centrifugation, followed by filtration of the supernatant using a Millipore Ultrafree MC (10 000 NMWL) filter unit. Analyses for glycolic acid, glyoxylic acid, oxalic acid, and formic acid were performed by HPLC on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) at 50 °C, using as solvent an aqueous solution of H_2SO_4 (0.01 N) and 1-hydroxyethane-1,1-diphosphonic acid (0.1 mM) at 1.0 mL/min. Analysis was performed by UV at 210 nm and by refractive index detection.

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

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by centrifugation of fermentation broth or from cell suspensions in buffer) on filter paper to remove excess moisture and then reweighing the blotted cell paste to determine the wet cell weight; this procedure provided a reproducible method for determining the fraction of water in fresh or frozen cell paste. Glycolate oxidase activities are reported in DCIP units unless otherwise noted.

Catalyst Preparation. *A. nidulans* transformants were prepared by first cloning the spinach gene which codes for glycolate oxidase and then introducing this gene into a strain of *A. nidulans* which already produced acceptable levels of the endogenous catalase. The resulting transformants were cultured in various media (minimal or SYG rich media) in shaker flasks or fermenters, and different agents such as oleic acid, hydroxyacetic acid, or corn steep liquor were added to the media to increase levels of expression of glycolate oxidase and/or catalase. The transformants were then screened by assaying cell extracts for glycolate oxidase and catalase activity. A transformant harboring multiple copies of the spinach glycolate oxidase-encoding DNA (under expression control of the *A. nidulans* alcA promoter) and multiple copies of the *A. nidulans* alcR gene (the product of which regulates function of the alcA promoter) was designated *A. nidulans* strain FT17.[17] Spores of the *A. nidulans* transformant were inoculated into 10 L of rich (SYG) media composed of 0.5% yeast extract, 100 mM NH_4NO_3, 33 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, trace metals, and 2% glucose. The fermentation was performed at 30 °C with 150–800 rpm agitation and aeration at 2–10 slpm to maintain 10–20% dissolved oxygen. When glucose was depleted, the culture was induced for glycolate oxidase expression by the addition of methyl ethyl ketone (100 mM) and fructose (0.1% w/v). At optimum expression of glycolate oxidase activity, poly(ethylene glycol) 8000 (PEG 8000) was added to the fermenter to a final concentration of 1.0–1.5%, and the mycelia was harvested by centrifugation, frozen in liquid nitrogen, and stored at −80 °C.

H. polymorpha transformants which express the glycolate oxidase enzyme from spinach as well as an endogenous catalase have been prepared by inserting the DNA for glycolate oxidase into an expression vector under the control of the formate dehydrogenase (FMD) promoter. *H. polymorpha* was transformed with this vector, and a strain producing high levels of glycolate oxidase was selected and designated *H. polymorpha* strain GO1.[18] *H. polymorpha* transformant catalysts were typically prepared by first growing an inoculum in 500 mL of 0.28% Yeast Nitrogen Base (YNB (Difco), without ammonium sulfate or amino acids), 1.0% ammonium sulfate (w/v), and 2.0% glycerol at pH 5.0 and 30 °C. This culture was then inoculated into a fermenter containing 10 L of the same media and grown for 17–20 h at 30 °C. Induction was initiated by the addition of 100 g of methanol. Peak glycolate oxidase activity occurred 36–45 h after induction. At the conclusion of the fermentation, 1.0 kg of glycerol was added and the cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80 °C.

P. pastoris transformants which express the glycolate oxidase enzyme from spinach and an endogenous catalase were prepared by inserting a DNA fragment containing the spinach glycolate oxidase gene into a *P. pastoris* expression vector (pHIL-D4) such as to be under control of the methanol-inducible alcohol oxidase I promoter, generating the plasmid pMP1. *P. pastoris* strain GTS115 (NRRL Y-15851) was transformed by plasmid pMP1, and a selection was done to allow for integration of the linearized plasmid pMP1 into the chromosomal alcohol oxidase I locus and replacement of the alcohol oxidase gene with the glycolate oxidase gene. A pool of such transformants was next selected for the maximal number of integrated copies of the expression cassette, and a high copy number transformant was isolated and designated *P. pastoris* strain MSP10.[19] *P. pastoris* cells were typically prepared by first growing an inoculum in 500 mL of MGY media which is composed of YNB without amino acids (13.4 g/L), glycerol (10 g/L), and biotin (0.4 mg/L) at pH 5.0 and 30 °C. After growing for 48 h at 30 °C, the inoculum was transferred to a fermenter containing 10 L of MGY media. The fermentation was operated at pH 5.0 (controlled with NH_4OH) at 30 °C with an agitation rate of 200–800 rpm and an aeration rate of 2–8 slpm of air at 5 psig and while the dissolved oxygen was maintained at 25–40% saturation. When glycerol was depleted, the cells were induced to express glycolate oxidase by the addition of methanol (50 g). Glycolate oxidase activity during induction was followed by enzyme assay. After 24 h of induction, glycerol (1 kg) was added to the fermenter, and the cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −80 °C.

The preparation of the *E. coli* WT-GAO transformant which expresses the glycolate oxidase enzyme from spinach and an endogenous catalase has been previously described.[7]

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 was 0.12 mM in 2,6-dichlorophenolindophenol (DCIP) and 80 mM in Tris buffer (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 1.0 M glycolic acid/1.0 M Tris (pH 8.3), and the mixture was stirred while the change in absorption with time at 606 nm (ϵ = 22 000 L mol^{-1} cm^{-1}) was measured.[20]

Catalase activity 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 (ϵ = 39.4 L mol^{-1} cm^{-1}).[21]

Glycolate Oxidase and Catalase Assays of Transformant Extracts. Extracts of whole cell transformants of *A. nidulans*, *H. polymorpha*, and *P. pastoris* were prepared by first blotting ca. 0.25 g of wet cells on filter paper to remove excess water and then accurately weighing 50–100 mg of blotted cells into a 17 × 100 mm polypropylene test tube. To the tube was then added 1 mL of an aqueous buffer containing 20 mM potassium phosphate (pH 8.0), 1 mM EDTA, 0.1 mM FMN, and 0.1 mM PMSF at 25 °C. To the tube was then added 1 mL of 0.5 mm glass beads, and the resulting mixture was vortexed at high speed for 2 min while the cells were ground with a glass rod. The resulting mixture was centrifuged and the supernatant assayed for glycolate oxidase or catalase activity. Extracts of *E. coli* were prepared according to a published procedure.[7]

Extracts were assayed for glycolate oxidase activity using a modification of an enzyme assay which utilizes *o*-aminobenzaldehyde and glycine.[22] A stock solution of assay reagents was prepared by mixing 8.97 mL of 1 M glycine/1 M KCl buffer (pH 8.0), 0.6 mL of distilled deionized water, 0.03 mL of a solution of *o*-aminobenzaldehyde (100 mg) in acetone (0.30 mL), 0.1 mL of 1 mM FMN, and 0.2 mL of 0.20 M sodium glycolate (pH 8.0). Into a 1.5 mL cuvette was placed 0.99 mL of the assay solution, and then 10 μL of extract was added and the change in absorption with time measured at 440 nm (ϵ = 2036 L mol^{-1} cm^{-1}).[22] Extracts were assayed for catalase activity using the same procedure described above for whole cells.

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Permeabilization of *P. pastoris* and *H. polymorpha* transformants. Method A. A suspension of 10 wt % wet cells in 20 mM phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100 was mixed for 15 min at 25 °C and then added dropwise to liquid nitrogen. The frozen suspension was collected, allowed to thaw at room temperature, and then washed with 20 mM phosphate/0.1 mM FMN buffer (pH 7.0).

Method B. 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. The mixture was then centrifuged and the supernatant decanted, and the cells were washed three times (10% w/v) in 50 mM phosphate buffer (pH 7.0) at 5 °C.

Oxidation of Glycolic Acid Using *A. nidulans* FT17 as Catalyst. In a typical procedure, a 300 mL stirred autoclave reactor was charged with 100 mL of a solution containing glycolic acid (0.75 M), EDA (0.86 M, pH 9.0), propionic acid (0.075 M, HPLC internal standard), and flavin mononucleotide (0.01 mM), and the solution was cooled to 5 °C. To the reactor was then added 26 g (wet cell weight) of frozen (−80 °C) *A. nidulans* FT17 (24 IU of glycolate oxidase and 192 000 IU of catalase), and the cells were allowed to thaw at 5 °C. The resulting mixture was stirred at 400 rpm and 5 °C under 120 psig of oxygen while oxygen was bubbled through the mixture at 50 mL/min. The reaction was monitored by taking a 0.100 mL aliquot of the reaction mixture at regular intervals, filtering the aliquot using a Millipore Ultrafree MC 10 000 NMWL filter unit, and analyzing by HPLC. After 11.5 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 98, 0, and 0%, respectively, with complete conversion of glycolic acid. The final activities of glycolate oxidase and catalase were 100 and 62% of their initial values, respectively.

At the completion of the reaction, the reaction mixture was centrifuged at 5 °C and the supernatant decanted. The resulting pellet of *A. nidulans* cells was resuspended in 100 mL of fresh reaction mixture at 5 °C, and the reaction was repeated under conditions identical to those described above. After 16 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 47, 0, and 0 %, respectively, with a 54% recovery of glycolic acid.

Oxidation of Glycolic Acid Using *P. pastoris* MSP10 as Catalyst. In a typical procedure, a 300 mL stirred autoclave reactor was charged with 100 mL of an aqueous solution containing glycolic acid (0.750 M), EDA (0.863 M), isobutyric acid (0.100 M, HPLC internal standard), and flavin mononucleotide (0.01 mM) at pH 9.25, and the solution was cooled to 5 °C. To the reactor was then added 5.0 g (wet cell weight) of *P. pastoris* MSP10 (423 IU of glycolate oxidase and 869 000 IU of catalase) which had been permeabilized by treatment with 0.1% benzalkonium chloride, and the reactor was purged with oxygen. The mixture was then stirred at 1000 rpm, which caused oxygen to bubble through the mixture via the action of the turbine impeller, and at 5 °C under 120 psig of oxygen. The reaction was monitored by taking a 0.40 mL aliquot of the reaction mixture at regular intervals, filtering the aliquot using a Millipore Ultrafree MC 10 000 NMWL filter unit, and analyzing the filtrate by HPLC. After 1.0 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 98.7, 1.3, and 0%, respectively, with no remaining glycolic acid. The recovered activities of permeabilized cell glycolate oxidase and catalase were 87 and 84% of their initial values, respectively. The microbial cell catalyst was recovered from the reaction mixture by centrifugation at 5 °C and was recycled to subsequent reactions without further treatment.

Oxidation of Glycolic Acid Using *H. polymorpha* GO1 as Catalyst. In a typical procedure, a 300 mL stirred autoclave reactor was charged with 100 mL of an aqueous solution containing glycolic acid (0.750 M), EDA (0.863 M), and isobutyric acid (0.100 M, HPLC internal standard), at pH 9.3, and the solution was cooled to 5 °C. To the reactor was then added 5.0 g of *H. polymorpha* transformant GO1 (880 IU of glycolate oxidase and 453 000 IU of catalase) which had been permeabilized by treatment with 0.1% Triton X-100/1 freeze–thaw, and the reactor was purged with oxygen. The mixture was then stirred at 1000 rpm, which caused oxygen to bubble through the mixture via the action of the turbine impeller, and at 5 °C under 250 psig of oxygen. The reaction was monitored by taking a 0.40 mL aliquot of the reaction mixture at regular intervals, filtering the aliquot using a Millipore Ultrafree MC 10 000 NMWL filter unit, and analyzing the filtrate by HPLC. After 1.0 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 98.0, 1.0, and 0.1%, respectively, with 0.9% glycolic acid remaining. The recovered activities of permeabilized cell glycolate oxidase and catalase were 107 and 103% of their initial values, respectively. The microbial cell catalyst was recovered from the reaction mixture by centrifugation at 5 °C and was recycled to subsequent reactions without further treatment.

Oxidation of Glycolic Acid Using *E. coli* WT-GAO as Catalyst. A 300 mL stirred autoclave reactor was charged with 100 mL of a solution containing glycolic acid (0.750 M), EDA (0.863 M), isobutyric acid (0.100 M, HPLC internal standard), and flavin mononucleotide (0.01 mM) at pH 9.2, and the solution was cooled to 5 °C. To the reactor was then added 30 g of *E. coli* transformant WT-GAO (72 IU of glycolate oxidase and 29 600 IU of catalase), and the mixture was stirred at 1000 rpm, which caused oxygen to bubble through the mixture via the action of the turbine impeller, and at 5 °C under 120 psig of oxygen. The reaction was monitored by taking a 0.40 mL aliquot of the reaction mixture at regular intervals, filtering the aliquot using a Millipore Ultrafree MC 10 000 NMWL filter unit, and analyzing the filtrate by HPLC. After 23 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 74.4, 1.1, and 5.6%, respectively, with 6.3% glycolic acid remaining. The recovered activities of microbial glycolate oxidase and catalase were 30 and 199% of their initial values, respectively.

Oxidation of 1.5 M Glycolic Acid Using *P. pastoris* MSP10 as Catalyst. The procedure described above for the oxidation of 0.75 M glycolic acid was repeated using 100 mL of an aqueous solution containing glycolic acid (1.500 M), EDA (1.575 M), isobutyric acid (0.300 M, HPLC internal standard), and flavin mononucleotide (0.01 mM) at pH 9.25, to which was added 2.0 g of *P. pastoris* MSP10 (114 IU of glycolate oxidase and 148 000 IU of catalase) which had been permeabilized by treatment with 0.1% Triton X-100/1 freeze–thaw. After 4.5 h, the yields of glyoxylic acid, oxalic acid, and formic acid were 98.0, 0.4, and 0%, respectively, with no glycolic acid remaining. The final activities of permeabilized cell glycolate oxidase and catalase were 136 and 113% of their initial values, respectively.

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