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Journal of Molecular Catalysis B: Enzymatic 2 (1997) 223–232

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Pyruvic acid production using methylotrophic yeast transformants as catalyst

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Received 15 August 1996; accepted 16 September 1996

Abstract

Permeabilized transformants of the methylotrophic yeasts *Hansenula polymorpha* and *Pichia pastoris* which express both the glycolate oxidase ((*S*)-2-hydroxyacid oxidase, EC 1.1.3.15) from spinach and an endogenous catalase (EC 1.11.1.6) have been used as catalysts for the oxidation of L-lactic acid to pyruvic acid. Oxidations of the sodium or ammonium salts of L-lactate at concentrations of up to 1.06 M were run in unbuffered aqueous solution without pH control and with oxygen sparging. The permeabilized transformant catalysts were recovered and recycled in up to 12 consecutive oxidations of the sodium or ammonium salts of 0.50 M L-lactate, where the initial selectivity to pyruvic acid was typically > 98% at 98% conversion of L-lactate. The pyruvic acid salt was readily recovered from unbuffered reaction mixtures in high yield and purity by separation of the catalyst from the reaction mixture, followed by removal of water by evaporation or freeze-drying.

Keywords: L-Lactic acid oxidation; Pyruvic acid production; Glycolate oxidase; Catalase; *Hansenula polymorpha*; *Pichia pastoris*; Microbial transformants

1. Introduction

Pyruvic acid has been prepared by both fermentation and by enzyme-catalyzed reactions. Fermentation of glucose has produced pyruvic acid in concentrations as high as 648 mM (57.0 g/l as pyruvic acid) [1], but the yield based on added carbon source was relatively low (57%), and isolation of purified pyruvic acid from such complex fermentation broths are generally difficult and expensive to perform. L-Lactate oxidase (L-lactate:oxygen oxidoreductase, non-decarboxylating, EC 1.1.3.2) from *Pediococcus* was used to catalyze the oxidation of L-lactate by oxygen to produce pyruvate and hydrogen peroxide [2]. The L-lactate oxidase was coimmobilized with catalase to limit further oxidation of pyruvate by byproduct hydrogen peroxide, which produces acetate, carbon dioxide and water. Oxidation of 0.049 M L-lactate by L-lactate oxidase produced yields of pyruvic acid (isolated as the 2,4-dinitrophenylhydrazone derivative) of up to 47%; recycle of the co-immobilized lactate oxidase/catalase catalyst in a subsequent oxidation reaction resulted in significantly lower yields. Glycolate oxidase ((S)-2-hydroxy-acid oxidase, EC 1.1.3.15), an enzyme commonly found in leafy green plants and

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PII S1381-1177(96)00021-5

mammalian cells, catalyzes the oxidation of glycolic acid (hydroxyacetic acid) to glyoxylic acid, or L-lactic acid to pyruvic acid, with the concomitant production of hydrogen peroxide [3–5]. This soluble enzyme, together with an excess of added soluble catalase, has been used to oxidize 3.3 mM solutions of L-lactate in 50 mM phosphate buffer (pH 8.0) to produce 3.2 mM pyruvic acid [5].

We have previously reported the use of permeabilized transformants of either *Hansenula polymorpha* or *Pichia pastoris* which express spinach glycolate oxidase and an endogenous catalase as catalysts for the oxidation of glycolic acid to glyoxylic acid [6]. Oxidations of glycolic acid at concentrations of up to 1.5 M were run in aqueous solution in the presence of oxygen and ethylenediamine (EDA), and selectivities to glyoxylic acid of $> 99\%$ at 100% conversion of glycolic acid were obtained. Catalase decomposed the stoichiometric amount of byproduct hydrogen peroxide produced during the oxidation, limiting the non-enzymatic oxidation of glyoxylate to formate and carbon dioxide. EDA was added to produce an equilibrium mixture of the corresponding imine and hemiaminal with glyoxylate, which significantly reduced both product inhibition of glycolate oxidase and the further enzymatic oxidation of glyoxylate to oxalate. Neither the separate addition of catalase or 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. We now describe the use of these same methylotrophic yeast transformants as catalysts for the production of pyruvate from L-lactate.

2. Experimental section

2.1. Materials and methods

All chemicals were obtained from commercial sources and used as received unless otherwise noted. Soluble spinach glycolate oxidase was obtained from Sigma. Oxidations of L-lactic acid using permeabilized cell transformants as catalyst 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 70 ml Fischer Porter glass aerosol reaction vessel under oxygen pressure without sparging. Soluble enzyme reactions were run without oxygen sparging to prevent rapid loss of glycolate oxidase activity. Samples were prepared for HPLC analysis by centrifugation, followed by filtration of the supernatant using a Millipore Ultrafree® MC (10000 MWCO) filter unit. Analyses for L-lactic acid, pyruvic acid, and acetic acid were performed by HPLC on a Bio-Rad Aminex® HPX-87H column (300 × 7.8 mm) at 50°C, using 0.01 N H_2SO_4 as solvent at 1.0 ml/min. Analysis was performed at 210 nm (UV), or by refractive index detection.

2.2. Catalyst preparation

Hansenula 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 [7].

Fermentations for the production of *H. polymorpha* transformant catalysts were performed 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. When glycerol was depleted, induction of glycolate oxidase production was initiated by the

addition of 100 g of methanol. Peak glycolate oxidase activity occurred 36–45 h post induction. At the conclusion of the fermentation, 1.0 kg of glycerol was added and the cells harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C.

Pichia 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 as to allow integration of the linearized plasmid pMP1 into the chromosomal alcohol oxidase **I** locus and replacement of alcohol oxidase gene with glycolate oxidase gene. A pool of such transformants were next selected for maximal number of integrated copies of the expression cassette, and a high copy number transformant was isolated and designated *P. pastoris* strain MSP10 [8].

Fermentations for the production of *Pichia pastoris* transformants were performed by first growing an inoculum in 500 ml of MGY media which is composed of yeast nitrogen base (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 48 h growth 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), 30°C, agitation rate of 200–800 rpm, aeration of 2–8 slpm of air at 5 psig, and dissolved oxygen 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 collected by centrifugation, frozen in liquid nitrogen and stored at −80°C.

After isolation from the fermentation broth by centrifugation, the cell paste was frozen at −80°C and stored for as long as 18 months at either −80°C or −20°C without significant loss of activity. Prior to use as catalyst, the cells were treated with a quaternary 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 [6]. Permeabilization of *P. pastoris* and *H. polymorpha* transformants was performed by mixing 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) for 60 min at 25°C. The mixture was then centrifuged, the supernatant decanted, and the cells washed three times (10% w/v) in 50 mM phosphate buffer (pH 7.0) at 5°C. These permeabilized cells were either used immediately, or stored for over one year at −80°C or −20°C prior to use.

Once permeabilized, the amount of whole cell catalyst added to a reaction mixture was chosen so as to provide the desired concentrations of glycolate oxidase and catalase activities (assayed as described below). Recoveries of glycolate oxidase and catalase activities of whole cell catalysts were measured at the end of a reaction, and recoveries of greater than 100% of initial values were attributed to increased permeabilization of the cells during the course of the reaction. Wet cell weights of whole cell or permeabilized microbial transformants used in reactions 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 re-weighing 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. Lyophilization of blotted wet cell paste prepared in this manner routinely resulted in a ratio of dry cell weight/blotted wet cell weight of 0.30.

2.3. Enzyme assays of permeabilized cell transformants

Permeabilized cell transformant catalysts were assayed for glycolate oxidase activity by first blotting ca. 0.25 g of permeabilized wet cells on filter paper to remove excess water, 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 an aqueous solution containing 0.12 mM 2,6-dichlorophenol-indophenol (DCIP) and 80 mM TRIS buffer (pH 8.3). The cuvette was capped with a rubber septum and the contents 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 resulting mixture stirred while measuring the change in absorption with time at 606 nm ($\varepsilon = 22000$ l mol^{-1} cm^{-1}) [9]. For assays of permeabilized cell transformants, no difference in activity was measured when substituting L-lactic acid for glycolic acid in this assay procedure.

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 ($\varepsilon = 39.4$ l mol^{-1} cm^{-1}) [10].

2.4. L-Lactate oxidation using soluble enzyme catalysts

Into a 3 oz. Fischer–Porter glass aerosol reaction vessel was placed a magnetic stirring bar and 10 ml of an aqueous solution containing sodium L-lactate (0.500 M), isobutyric acid (HPLC internal standard, 0.100 M), soluble spinach glycolate oxidase (6.0 IU/ml), and soluble *Aspergillus niger* catalase (10,000 IU/ml) at pH 9.0 (adjusted with 50% NaOH) and at 15°C; no buffer was added. The reaction vessel was sealed and the reaction mixture was cooled to 15°C, 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 15°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 5 h, the HPLC yields of pyruvate and acetate were 95.3% and 0.9%, respectively, and 4.5% lactate remained. The remaining activities of glycolate oxidase and catalase were 68% and 100%, respectively, of their initial values.

2.5. L-Lactate oxidation using permeabilized cell catalysts

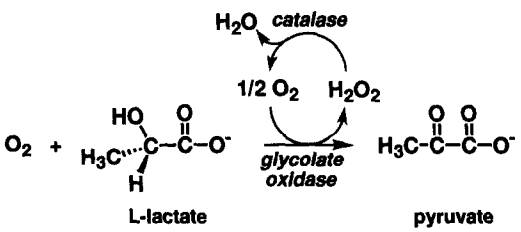
A 300 ml EZE-Seal stirred autoclave reactor equipped with a Dispersimax impeller (Autoclave Engineers) was charged with 100 ml of a solution containing sodium L-lactate (5.50 g, 0.50 M). To the reactor was then added 6.70 g of *Pichia pastoris* transformant strain MSP10 (670 IU glycolate oxidase and 1,177,000 IU catalase) which had been permeabilized by treatment with 0.1% (w/w) Barquat™ MB-50, and the mixture adjusted to pH 9.0 with 50% NaOH and cooled to 5°C. The reactor was purged with oxygen, then the 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 0.40 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 using 0.10 M isobutyric acid added to the sample as internal standard. After 3.0 h, the HPLC yields of pyruvate and acetate were 99.2% and 1.4%, respectively, and 0.6% lactate remained. The recovered activities of permeabilized-cell glycolate oxidase and catalase were 107 and 106% of their initial values, respectively.

The reaction mixture was centrifuged to remove the permeabilized-cell catalyst, and the resulting supernatant filtered through a 0.2 μm nylon filter. The pH of the resulting filtrate was adjusted to 4.6 with 1.0 N HCl, then the water was removed by freeze-drying to produce 5.20 g of sodium pyruvate (96% isolated yield, 98% sodium pyruvate as determined by HPLC analysis).

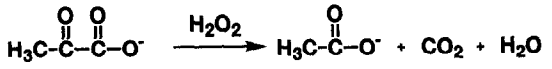
3. Results and discussion

3.1. Effect of buffer on pyruvate yield

When glycolate oxidase (GO) and catalase were employed as catalysts for the oxidation of L-lactate to pyruvate (Scheme 1), there was no byproduct formation due to the further enzymatic oxidation of pyruvate, but the hydrogen peroxide which was also produced could react with pyruvate to produce acetate and carbon dioxide (Scheme 2). For the previously reported oxidation of glycolic acid to glyoxylic acid using either soluble glycolate oxidase and catalase [11] or microbial transformants which coexpress these enzymes [6] as catalyst, including a stoichiometric amount of a primary amine buffer such as ethylenediamine (EDA) or tris(hydroxymethyl)aminomethane (TRIS) in the reaction mixture resulted in a higher yield of glyoxylate compared to that obtained when either phosphate or bicine buffer were employed. The oxidation of L-lactate to pyruvate when using either soluble enzymes or whole cell microbial transformants as catalyst was therefore initially run



Scheme 1. Oxidation of L-lactate to pyruvate using glycolate oxidase and catalase.

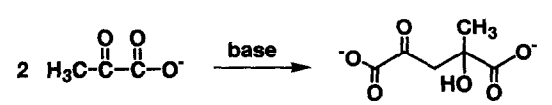


Scheme 2. Oxidation of pyruvate by hydrogen peroxide.

in the presence or absence of several amine buffers to determine the effect of added primary amine buffer on pyruvate yield (Table 1).

When using soluble glycolate oxidase and catalase as catalysts, a low concentration (1,400 IU/ml) of soluble catalase resulted in the production of significant amounts of acetate when using bicine as buffer (bicine does not complex with pyruvate). Substitution of EDA for bicine eliminated the production of acetate, but a low mass balance was also observed, presumably due to the base-catalyzed self-aldol condensation of pyruvate to produce 4-methyl-4-hydroxy-2-oxoglutarate [12] (Scheme 3). A tenfold increase in the soluble catalase concentration markedly decreased the amount of acetate produced when using phosphate buffer, where under these same conditions the use of primary amine buffers such as TRIS or ethanolamine produced much lower yields of pyruvate and low mass balances. When *P. pastoris* transformant MSP10 was employed as catalyst, excellent yields of pyruvate were obtained when using either bicine or phosphate buffer, or when using no added buffer and allowing the pH of the reaction to drift over the course of the oxidation; substitution of EDA for phosphate or bicine again resulted in no acetate production, but much lower pyruvate yields. A time course for the oxidation of 0.47 M sodium lactate in the absence of added buffer and without pH control when using the *P. pastoris* transformant catalyst is illustrated in Fig. 1.

The ability to oxidize L-lactate to pyruvate with high selectivity and in high yield in the absence of an added buffer and without pH



Scheme 3. Base-catalyzed self aldol condensation of pyruvate.

Table 1
Effect of buffer on pyruvate yield [a]

catalyst	buffer	lactate (M)	pH	GO (IU/ml)	catalase (IU/ml)	pyruvate (%)	acetate (%)	lactate (%)
sol. enzymes	bicine	0.75	8.9	1.0	1400	48	46	6.6
	EDA	0.75	8.9	1.0	1400	50	0	31
	phosphate	0.75	8.1	1.0	14000	80	5.2	16
	TRIS	0.75	8.3	1.0	14000	55	6.3	2.7
	ethanolamine	0.75	8.3	1.0	14000	46	0	2.8
P. pastoris	bicine	0.50	9.0	6.5	10100	93	6.3	0.4
	phosphate	0.50	9.0	6.5	10100	98	2.5	0.3
	no buffer	0.50	9.0	6.5	10100	99	0.7	0.4
	no buffer	0.95	9.0	3.8	11800	91	6.7	0.8
	EDA	1.01	9.1	5.3	26000	76	0	0

[a] Reactions were run at 5°C under oxygen.

control simplifies the isolation and purification steps required to separate pyruvate from the reaction mixture. A process for the production of pyruvate can be run by simply diluting a purified lactic acid solution produced be fermentation to the appropriate concentration, adjusting the pH with caustic or ammonia, adding the catalyst and sparging the resulting mixture with oxygen under pressure. The sodium or ammonium salt of pyruvic acid is then readily isolated by first separating the catalyst from the product mixture, then removing the remaining water from the pyruvate salt by evaporation, distillation, or freeze-drying.

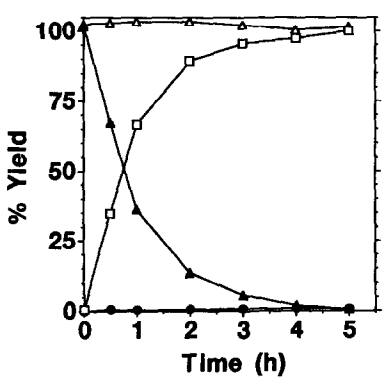


Fig. 1. Time course for the oxidation of 0.47 M sodium L-lactate in aqueous solution with no added buffer, using 5% (w/v) permeabilized *P. pastoris* transformant cells (4.12 DCIP IU glycolate oxidase/ml, 12700 IU catalase/ml) as catalyst at an initial pH 9.0, 10 psig O_2 (with sparging), 5°C, and stirring at 500 rpm: lactate (▲), pyruvate (□), acetate (●), and mass balance (△).

3.2. Determination of optimum pH for pyruvate production

To determine the optimum initial pH for an unbuffered reaction run without pH control, the dependence of the lactate oxidase activity of soluble spinach glycolate oxidase on pH was first determined (Fig. 2). The optimum lactate oxidase activity of glycolate oxidase occurred at pH 8.0, and this activity decreased by ca. 40% at 1.5 pH units above or below this optimum pH. The pK_a of L-lactic acid and pyruvic acid are 3.79 and 2.49, respectively, therefore, it was expected that the oxidation of a 1.0 M solution of L-lactate to produce ca. 1.0 M pyruvate would result in a decrease in the pH of an unbuffered reaction mixture by ca. 1.3 pH units when the

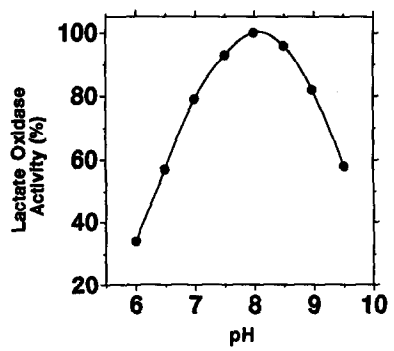


Fig. 2. Dependence of lactate oxidase activity of soluble spinach glycolate oxidase on pH. The measured activity at pH 8.0 and 25°C (standard assay conditions) was taken as 100%.

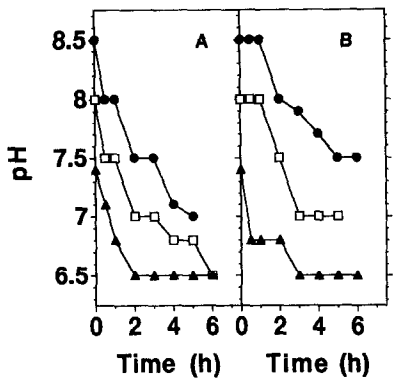


Fig. 3. pH of reaction mixture during oxidation of sodium lactate (A) or ammonium lactate (B) by *P. pastoris* transformant in unbuffered aqueous solution. Initial pH: (A, sodium lactate) pH 8.5 (●), pH 8.0 (△), pH 7.4 (▲); (B, ammonium lactate) pH 8.5 (●), pH 8.0 (△), pH 7.4 (▲) Reaction conditions: sodium or ammonium lactate (0.9–1.0 M), 5% (w/v) permeabilized *P. pastoris* transformant cells, 70 psig O_2 (with sparging), 5°C, stirring at 750 rpm.

initial pH of the reaction mixture was adjusted to between 8.5 and 7.5. Oxidations of 1.0 M sodium lactate or ammonium lactate were run using the *P. pastoris* transformant as catalyst and in the absence of added buffer and without pH control, starting from an initial pH of 8.5, 8.0, or 7.4, and the pH of the reaction mixtures were monitored over the course of the reaction (Fig. 3). The decrease in pH over the course of oxidations having an initial pH of 8.5 or 8.0 typically ranged between 1.5 and 2.0 pH units, while a pH decrease of only 1.0 pH unit was observed when starting at a pH of 7.5. Both the mass balance and yield of pyruvate decreased for reactions run at an initial pH greater than 8.0 when compared to the same reactions run at an initial pH of 8.0 or less, which was attributed to the chemical instability of pyruvate under basic reaction conditions [12].

It was not possible to continuously adjust the pH of the reaction mixture over the course of a reaction when using the autoclave reactor under oxygen pressure with sparging. An oxidation of 0.50 M sodium lactate was performed in this reactor with periodic readjustment of the pH of the reaction mixture to pH 7.4 by the addition of aliquots of 0.5% sodium hydroxide, and an increase in the reaction rate of ca. 10% was obtained when compared to the same reaction run without pH control; no change in the yield or selectivity to pyruvate was observed. With appropriate equipment it should be possible to continuously adjust the pH, which would allow the reaction to be run at close to the optimum reaction rate.

3.3. Dependence of pyruvate yield on L-lactate and catalase concentrations

Table 2 provides a comparison of the results obtained for the oxidation of L-lactate solutions using soluble glycolate oxidase (GO) and soluble catalase, *H. polymorpha* permeabilized-cell catalyst, and *P. pastoris* permeabilized-cell catalyst at several L-lactate and catalase concentrations. At 0.5 M L-lactate, the yield of pyruvate is higher, and the production of byproduct ac-

Table 2
Dependence of pyruvate yield on initial lactate [a] concentration using soluble enzymes or microbial transformants as catalyst

lactate (M)	catalyst	buffer	pH	GO (IU/ml)	catalase (IU/ml)	pyruvate (%)	acetate (%)	lactate (%)
0.50	sol. enzymes	none	9.0	6.0	10000	95	4.5	0.9
0.50	*H. polymorpha*	none	9.0	6.5	5000	97	2.5	0.4
0.50	*P. pastoris*	none	9.0	1.1	2500	93	5.0	3.3
0.50	*P. pastoris*	none	9.0	2.3	5000	97	2.3	0.4
0.50	*P. pastoris*	none	9.0	6.5	10100	99	0.7	0.4
0.75	sol. enzymes	phosphate	8.1	1.0	14000	80	3.8	20
0.71	*P. pastoris*	none	7.5	3.3	10000	98	1.3	2.7
1.00	*H. polymorpha*	none	7.5	4.1	9700	94	1.5	4.9
1.01	*P. pastoris*	none	7.5	6.3	17000	93	3.3	4.2

[a] Oxidation of 0.50 M sodium lactate in water with no added buffer at an initial pH of 9.0, 5°C and 70 psig oxygen.

etate is lower, when using the *P. pastoris* permeabilized whole-cell catalyst in place of soluble enzymes at similar enzyme concentrations (ca. 6 IU/ml GO, 10000 IU/ml catalase). When either the *H. polymorpha* or *P. pastoris* permeabilized-cell catalyst was employed at a concentration of catalase activity half that of the soluble catalase reaction (5000 IU/ml vs. 10000 IU/ml for soluble catalase), more pyruvate and less acetate was formed; when the *P. pastoris* permeabilized cell catalyst was used at a catalase concentration of only 25% of soluble catalase activity, comparable yields of pyruvate and acetate were obtained. Lowering the concentration of catalase in the soluble enzyme reactions resulted in significantly lower yields of pyruvate and an increase in acetate production (see Table 1). These results demonstrate the advantage of having the catalase located in close physical proximity to the glycolate oxidase (the site of hydrogen peroxide production) within the microbial cell transformant catalyst, compared to when the two soluble enzymes are each uniformly dispersed within a reaction mixture.

The improvement in pyruvate yields when using the permeabilized whole cell catalysts compared to using soluble enzymes increased as the initial concentration of L-lactate was increased above 0.50 M (Table 2). Even though the concentration of catalase in the soluble enzyme reaction run at 0.75 M L-lactate was ca. 40% greater than that of the *P. pastoris* permeabilized whole-cell catalyst reaction run at 0.71 M L-lactate, the permeabilized whole-cell catalyst produced 18% more pyruvate, and the reaction was complete in ca. one-tenth the time. This soluble enzyme reaction did not proceed to complete conversion because of the total loss of the soluble glycolate oxidase activity under these reaction conditions, while the glycolate oxidase activity of the permeabilized whole cell transformants was almost completely recovered. When the L-lactate concentration was increased to 1.0 M, the yield of pyruvate obtained when using the permeabilized whole-cell catalyst was typically as high as 94%; these yields could not be achieved with the soluble enzymes as catalyst at this concentration of L-lactate, nor could the soluble enzymes be easily recovered for reuse in a subsequent reaction.

Table 3
Effect of reaction temperature on pyruvate yield [a]

temperature (°C)	pyruvate (%)	acetate (%)	lactate (%)
5	97	2.3	0.4
15	97	2.5	0.4
30	90	6.5	0.6

[a] Oxidation of 0.50 M sodium lactate with 5000 IU catalase/ml and no added buffer, at an initial pH of 9.0 and 70 psig oxygen. Catalyst: 5°C, *P. pastoris*; 15°C and 30°C, *H. polymorpha*.

When using the permeabilized microbial transformants as catalyst, increasing the reaction temperature from 5°C to 30°C resulted in a decrease in pyruvate yield and an increase in acetate production at temperatures above 15°C (Table 3); this result is consistent with previous studies of catalase stability and temperature dependence, where lower temperatures favor catalase stability [13,14].

3.4. Recycle of permeabilized cell catalyst

The recovered glycolate oxidase and catalase activities of a recycled *P. pastoris* transformant catalyst, and the corresponding reaction times and pyruvate yields for 12 consecutive batch oxidations of 0.50 M L-lactate are illustrated in Fig. 4. As was also the case for the *H. polymorpha* transformant, the omission of added flavin mononucleotide (FMN) to this series of reactions resulted in no change in the rate of loss of glycolate oxidase activity when compared to a similar series performed with added FMN; reactions using soluble glycolate oxidase required the addition of at least 0.01 mM FMN to stabilize glycolate oxidase activity [6]. Catalyst recycle was simply performed by removal of the product mixture from the reactor, centrifugation and decantation of the supernatant from the catalyst pellet, and resuspension of the catalyst in fresh reaction mixture. The recovered catalyst could be stored at 5°C in freshly-prepared L-

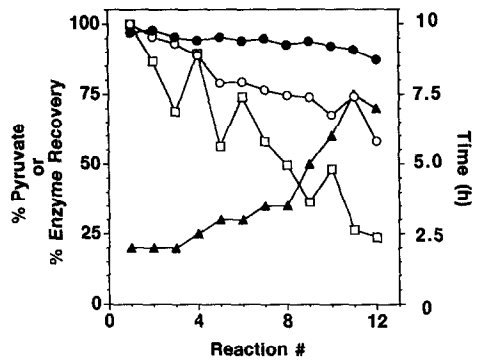


Fig. 4. Pyruvate yield (●), reaction time (▲) and recovered glycolate oxidase (○) and catalase (□) activities of a recycled *P. pastoris* transformant catalyst for twelve consecutive batch oxidations of sodium L-lactate. Reaction conditions: sodium lactate (0.50 M), 5% (w/v) permeabilized *P. pastoris* transformant cells (8.84 DCIP IU glycolate oxidase/ml, 18,000 IU catalase/ml), initial pH 7.5 (no buffer), 70 psig O_2 (with sparging), 5°C, stirring at 750 rpm.

lactate reaction mixture for 24 h under nitrogen with no measurable loss of either enzyme activities. Storage of the recovered catalyst in product mixture (ca. 0.5 M pyruvate) resulted in a gradual loss of catalase activity, while no comparable loss of glycolate oxidase activity was observed; a comparison of the stability of the catalase activity of *P. pastoris* when stored in 50 mM phosphate buffer (pH 7.5) or 0.43 M ammonium pyruvate (pH 7.4) is illustrated in Fig. 5.

When the concentration of L-lactate in recycle reactions was increased from 0.50 M to 1.00 M (ca. 90 g/l as lactic acid), the reaction time increased from 2 h to 5 h, with a 96% yield of pyruvate in the first reaction (Table 4). Measurement of recovered glycolate oxidase activities of greater than 100% were most likely due to an initial increase in permeability of the whole-cell catalyst during the course of the first reaction. Five consecutive batch oxidations with catalyst recycle of aqueous solutions containing 1.0 M L-lactate and 5% (w/v) *P. pastoris* transformant catalyst were performed before the series was discontinued; the catalase activity of the recovered catalyst dropped markedly after the fourth use of the catalyst, and the pyruvate yield dropped below 90%. The stability study of glycolate oxidase and catalase activities of the microbial transformants in pyruvate product mixtures indicated that the rapid loss of catalase activity in recycle reactions is most likely due to the presence of high concentrations of pyruvate which are produced, and the much slower rate of loss of glycolate oxidase activity can be attributed to this loss of catalase activity, as the catalase activity protects the glycolate oxidase enzyme from deactivation due to reaction with hydrogen peroxide [11].

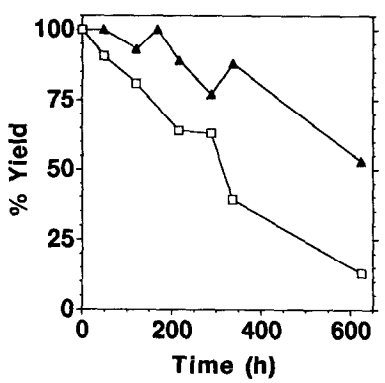


Fig. 5. Stability of catalase activity of permeabilized *P. pastoris* transformant cells stored at 5°C as a 10% (w/v) suspension in 50 mM phosphate buffer (pH 7.5) (▲) or in a product mixture containing 0.43 M ammonium pyruvate (pH 7.4) (□).

Table 4
Oxidation of sodium lactate (1.06 M) in water with no added buffer at pH 7.5 and at 5°C with catalyst recycle

run no.	time (h)	GO (%)	catalase (%)	pyruvate (%)	acetate (%)	lactate (%)
1	5	116	107	95.7	2.5	1.9
2	6	110	88	96.0	4.5	0.8
3	7	108	71	92.3	5.4	2.4
4	8	125	89	90.7	7.2	2.1
5	10	112	41	86.1	10.2	3.7

4. Conclusions

Pyruvic acid has been prepared via the enzyme-catalyzed oxidation of L-lactic acid with selectivities as high as 99%, and isolated in 96% yield (98% purity, sodium salt). The high

initial concentration of L-lactic acid employed did not result in substrate inhibition of the glycolate oxidase, which would have limited the reaction rate and/or the final concentration of product. Similarly, a high concentration of pyruvic acid might have resulted in product inhibition of the enzyme, again limiting the concentration of product obtained. The yields of pyruvate obtained in the absence of buffer and without pH control during reaction equaled or exceeded those obtained in the presence of added buffer. The preparation of pyruvate in the absence of added buffer allowed for a simple isolation of product from a reaction mixture; the catalyst was removed by filtration or centrifugation, leaving an aqueous solution of a relatively pure pyruvic acid salt that was easily recovered by removal of water.

The use of a permeabilized microbial cell transformant catalyst for the oxidation of L-lactate to pyruvate has been demonstrated for the first time, and these catalysts offer several advantages over the use of the corresponding soluble enzymes. Reaction rates are typically limited by mass transport of oxygen into the reaction mixture, and mixtures containing permeabilized microbial transformant catalysts can be sparged with oxygen, while sparging a reaction mixture containing soluble enzymes results in the rapid, irreversible denaturation of glycolate oxidase. At the conclusion of the reaction, the whole-cell catalyst is easily recovered for reuse by filtration or centrifugation, while the soluble enzymes cannot be recovered by centrifugation, and filtration results in the loss of much of the soluble glycolate oxidase activity. The recovery of reusable enzyme activities for the microbial cell catalysts was significantly greater than that of the respective soluble enzymes after a single reaction. For recycle reactions using either the permeabilized *P. pastoris* or *H. polymorpha* transformant catalysts, the catalyst productivity for oxidations of 0.50 M sodium L-lactate was ca. 33 g pyruvic acid/gram dry cell weight after 12 reactions with catalyst recycle, and for oxidation of 1.06 M sodium L-lactate, ca. 29 g pyruvic acid/g dry cell weight after 5 reactions with catalyst recycle. These levels of catalyst productivity are within acceptable limits for a commercial-scale biocatalytic process for the production of pyruvic acid which uses a permeabilized microbial transformant as catalyst.

Acknowledgements

Expression of glycolate oxidase in *H. polymorpha* was performed by Gerd Gellisen, Michael Piontek, Zbigniew Janowicz and coworkers at Rhein Biotech, Düsseldorf, Germany. Fermentation optimizations of *P. pastoris*, and *H. polymorpha* transformants were performed by Ken Schneider, Ross Jacobson, and co-workers at Bio-Technical Resources (Manitowoc, WI). L. Winnie Wagner and coworkers of the DuPont Fermentation Research Facility provided supplies of catalyst used in this work.

References

[1] T. Yonehara and R. Miyata, J. Ferment. Bioeng., 78 (1994) 155.
[2] B.A. Burdick and J.R. Schaeffer, Biotech. Lett., 9 (1987) 253.
[3] C.O. Clagett, N.E. Tolbert and R.H. Burris, J. Biol. Chem., 178 (1949) 977.
[4] N.E. Tolbert, C.O. Clagett, R.H. Burris, J. Biol. Chem., 181 (1949) 905.
[5] I. Zelitch and S. Ochoa, J. Biol. Chem., 201 (1953) 707.
[6] J.E. Gavagan, S.K. Fager, J.E. Seip, M.S. Payne, D.L. Anton, and R. DiCosimo, J. Org. Chem., 60 (1995) 3957.
[7] G. Gellissen, M. Piontek, U. Dahlems, V. Jenzelewski,; J.E. Gavagan, R. DiCosimo, D.L. Anton and Z.A. Janowicz, Appl. Microbiol. Biotechnol., 46 (1996) 46.
[8] M.S. Payne, K.L. Petrillo, J.E. Gavagan, L.W. Wagner, R. DiCosimo and D.L. Anton, Gene, 167 (1995) 215.
[9] J. McD. Armstrong, Biochim. Biophys. Acta, 86 (1964) 194.
[10] H.E. Aebi, In H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis (3rd Edition), Vol. III, pp. 273–286, Verlag Chemie, Deerfield Beach, FL, 1983.
[11] J.E. Seip, S.K. Fager, J.E. Gavagan, L.W. Gosser, D.L. Anton and R. DiCosimo, J. Org. Chem., 58 (1993) 2253.
[12] R.W. Von Korff, Methods Enzymol., 13 (1969) 519.
[13] R.E. Altomare, P.F. Greenfield and J.R. Kittrell, Biotechnol. Bioeng., 16 (1974) 1675.
[14] D. Scott and F. Hammer, Enzymologia, 22 (1960) 229.