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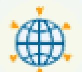

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Applied Cellular Physiology and Metabolic Engineering

Enantioselective oxidation of 2-hydroxy carboxylic acids by glycolate oxidase and catalase coexpressed in methylotrophic *Pichia pastoris*

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KEYWORDS

glycolate oxidase • biocatalysis • 2-hydroxy carboxylic acid • resolution • Pichia pastoris

ABSTRACT

Glycolate oxidase (GO; (*S*)-2-hydroxyacid oxidase, EC 1.1.3.15) is a flavin mononucleotide (FMN)-dependent enzyme, which catalyzes the oxidation of 2-hydroxy carboxylic acids to the corresponding 2-keto acids. Catalase has been used as cocatalyst to decompose hydrogen peroxide produced in the reaction, thus limiting peroxide-based side reactions and GO deactivation. GO from spinach and catalase T from Saccharomyces cerevisiae previously coexpressed in Pichia pastoris strain NRRL Y-21001, was permeabilized and used for the oxidation of 3-phenyllactic acid, 3-indolelactic acid, 3-chlorolactic acid, 2-hydroxybutanoic acid, and 2-hydroxydecanoic acid to demonstrate high degree of selectivity to the (S)-enantiomers, leaving (R)-isomers intact. The rates of oxidation ranged from 1.3 to 120.0%, relative to the oxidation of lactic acid to pyruvic acid. The best substrates were 3-chlorolactic acid (110%) and 2-hydroxybutanoic acid (120%). Oxidation was carried out with (R)-, (S)-, and (RS)-3-phenyllactic acid, (RS)-lactic acid, and (RS)-2-hydroxybutanoic acid in 500 mL scale to characterize the products and stoichiometry of the reaction. All (RS)- and (S)-2-hydroxy acids produced 2-keto acids at close to the theoretical yield in 1-9 h. (R)-3-Phenyllactic acid was not oxidized over a period of 9 h. Addition of exogenous FMN and catalase were not required for this oxidation using double recombinant Pichia pastoris whole cells. As GO is absolutely specific to (S)-enantiomers, it can be used for resolution of racemic 2-hydroxy acids to (R)-2-hydroxy acids as well as for production of 2-keto acids. This is the first report on the selectivity of a broad range of 2-hydroxy acids by GO. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 2010

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ARTICLE TEXT

Introduction

Optically active 2-hydroxy acids are important building blocks for the asymmetric synthesis of wide variety of bioactive molecules, such as virus protease and angiotensin converting enzyme (ACE) inhibitors, beta-blockers, A2 antagonists, and Ca-channel blockers.[1] Optically pure 2-hydroxy acids are used for making chiral synthons like glycols,[2] halo esters,[3] and epoxides.[4] Derivatives of optically active 2-hydroxy acids such as 3-cyclohexyl-2-hydroxy acids are used for making essential component of sialyl Lewis analogs, which are currently in test for treatment of inflammatory disorders.[5]

Several chemical[6] and enzymatic methods[7][8] have been reported previously on the synthesis of optically active 2-hydroxy acids. Most reports involve reduction of 2-keto acids with Baker's yeast and lactate dehydrogenase, the addition of prussic acid to aldehydes by oxynitrilase, enantioselective oxidation of 1,2-diols with dehydrogenase, stereoselective esterification of 2-hydroxy acids with lipase and resolution of racemic 2-hydroxy acids using optically active amine.[9][10] The disadvantages of these methods include lack of substrate selectivity,[7][8] requirement of cosubstrates[7] or coenzymes,[9] requirement of organic solvents,[9] or non-availability of enzyme on a large scale.[8]

Resolution of racemic 2-hydroxy acids (1) can be achieved by selective oxidation of one of the enantiomers by an enzyme such as glycolate oxidase (GO). Adam et al.[10][11] tried the synthesis of (*R*)-2-hydroxy acids by enantioselective oxidation of racemic 2-hydroxy acids by using spinach-GO. They used soluble enzyme, expensive cofactor, FMN (since GO is not active in solution in the absence of FMN) and exogenously added catalase to decompose the byproduct hydrogen peroxide. An alternate, more economical way for the efficient synthesis of optically active 2-hydroxy acids is desirable.

GO (2-hydroxyacid oxidase, EC 1.1.3.15) is a flavin mononucleotide (FMN)-dependent peroxisomal enzyme, which catalyzes oxidation of 2-hydroxy carboxylic acids such as lactate or glycolate, to the corresponding 2-keto acids (Figure 1). GO is found in many green plants and animals including the leaves of spinach,[12] pea,[13] sugar beet,[12] lettuce,[14] tobacco,[15] pumpkin,[16] cucumber cotyledons,[17] and the liver of pigs,[18] rats,[19] and humans.[20] In green plants, GO is one of the enzymes involved in photorespiration, a pathway that results in reduced net photosynthesis. In animals, the enzyme participates in the production of oxalate through metabolic pathways from serine and carbohydrates.

Figure 1. GO catalyzed oxidation of (*RS*)-2-hydroxy acids (1). The product, 2-keto acids formed from (*S*)-enantiomers of 1, is designated as 2.
[Normal View 17K | Magnified View 41K]

The X-ray crystal structure of spinach-GO has been published.[21] GO was reported to be catalytically active only as tetramers or octamers of identical subunits, which have a molecular weight of 43,000, with one FMN per subunit.[22] The enzyme activity was also found to be relatively unstable in solution; the protein tended to irreversibly aggregate into an inactive form, especially in the absence of added FMN. Undesirable reactions could take place when the product, 2-keto acids react directly with coproduct of GO reaction, hydrogen peroxide. Addition of catalase[23] to the reaction mixture was reported to improve the yield of 2-keto acids by suppressing or deactivating the hydrogen peroxide-mediated reactions including GO deactivation.

To economically produce chiral 2-hydroxy acids using GO, the enzyme needs to be cloned in a suitable host. Eukaryotic yeasts, such as methylotrophic *Pichia pastoris* (*P. pastoris*) offers several advantages to commercial applications of GO. This organism can generally be grown to higher densities than bacteria and are readily adaptable to continuous fermentation. It has been reported that *P. pastoris* can be grown to cell densities in excess of 100 g/L (U.S. Patent No. 4,414,329). Additional advantages of the yeast host include the fact that many critical functions of the organism, such as oxidative phosphorylations, are located within organelles and thus are not exposed to the possible deleterious effects of the overexpression of foreign enzymatic products. In contrast, *Escherichia coli* (*E. coli*) has the tendency to express recombinant (r) proteins as inclusion bodies. Also, yield of biomass of *E. coli* is not as high.[24]

In this study, GO from spinach and catalase T from *Saccharomyces cerevisiae* previously coexpressed in *Pichia pastoris* strain NRRL Y-21001 was used as biocatalyst.[25][26] Permeabilized r*P. pastoris* exhibited activity with a broad range of substrates including glycolic acid, lactic acid, 3-chlorolactic acid, 3,3,3-trifluorolactic acid, mandelic acid, 3-phenyllactic acid, 3-(*p*-hydroxyphenyl)-lactic acid, 3-indolelactic acid, 2-hydroxybutanoic acid, 2-hydroxy-3-methylbutanoic acid, and 2-hydroxydecanoic acid (structures shown in Figure 2). We demonstrate for the first time, high yield of 2-keto acids and absolute enantioselectivity for (*S*)-2-hydroxy acids using lactic acid, 2-hydroxybutanoic acid, and 3-phenyl lactic acid in 500 mL scale by r*P. pastoris* whole cells expressing GO. Exogenous addition of FMN and catalase are not necessary for high yield of 2-keto acids.

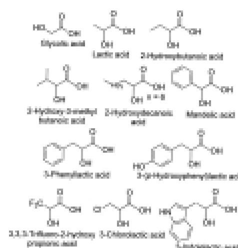


Figure 2. Chemical structures of 2-hydroxy acids used for GO catalyzed oxidations.
[Normal View 14K | Magnified View 36K]

Materials and Methods

Materials

Benzalkonium chloride (BC) and 2,6-dichlorophenol-indophenol (DCIP) were purchased from Sigma Chemical Co (St. Louis, MO). Hydrogen peroxide (30%) and sulfuric acid (36 N) were obtained from Fisher Scientific (Pittsburgh, PA). All 2-hydroxy acids were purchased either from Fluka or Sigma-Aldrich. DL-2-hydroxybutanoic acid and DL-3-phenyllactic acid were purchased from TCI America (Portland, OR) and Bachem Bioscience (King of Prussia, PA), respectively, for the use in bioreactors. All other chemicals were analytical grade reagents, commercially available, and were used without further purification. Water used throughout this work was purified using a Barnstead (Dubuque, IA) Nanopure Ultrapure Water System (D 4751).

Instrumentation

High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-20AT dual pumping system connected to a Shimadzu SPD-M20A diode array and RID-10A refractive index detectors and a Shimadzu CBM-20 system controller (Kyoto, Japan). Separations were carried out over an Aminex HPX-87H column (300 × 7.8 mm^2 i.d., 9 μm particle size, Biorad, Hercules, CA). Samples were eluted with a mobile phase consisting of 5 mM sulfuric acid in water at a flow rate of 0.6 mL/min, while eluting peaks were detected by both diode array (at 209 nm) and refractive index detectors. Liquid chromatography-mass spectrometry (LC-MS) was performed using a Shimadzu 2010 EV (Shimadzu Scientific Instruments, USA) system. GO and catalase assays were done with a Shimadzu UV-2450 UV-visible scanning spectrophotometer (Shimadzu Corporation, Kyoto, Japan). r*Pichia pastoris* was produced in 30 L Sartorius Biostat C-DCU (Sartorius BBI Systems). Large scale GO catalyzed oxidation reactions were performed in 1 L Dasgip Bioreactors (DASGIP BioTools, LLC; Shrewsbury, MA).

Double recombinant Pichia pastoris used in this study

Recombinant *P. pastoris* MSP8.6[25][26] was a gift from Dupont Central Research and Development, Wilmington, DE to The University of Iowa. This strain was constructed as follows. A 1110-bp DNA fragment encoding spinach GO gene was cloned into the *P. pastoris* expression plasmid pMP1,[25] in which GO expression is under control of the *Pichia pastoris* strong, methanol-inducible alcohol oxidase I (*AOX1*) promoter. Plasmid pMP1 was linearized and used to transform the *Pichia pastoris* host strain GTS115 (*his4*) (Phillips petroleum). This clone was designated as MSP 10. A 1850-bp DNA fragment encoding *S. cerevisiae* catalase T gene was cloned in an expression vector pMP8[26] under the methanol-inducible *AOX1* promoter. Plasmid pMP8 was linearized and used to retransform MSP10. Both genes were integrated into *Pichia pastoris* chromosome. This sequentially transformed *Pichia pastoris* MSP 10 (designated as MSP8.6) was used as biocatalyst for the oxidation of 2-hydroxy acids.

Fermentation of recombinant Pichia pastoris

Fermentation was done at 30 L scale to obtain biomass containing GO and catalase. Inoculum was grown to an optical density of 1.5 in shake flasks with medium consisting of 15.5 g/L glycerol, 5 g/L ammonium sulfate, 1.5 g/L yeast nitrogen base (YNB), and 0.16 mg/L biotin. Fermentation medium contained 10 g/L glycerol, 3.5 g/L ammonium sulfate, 4.7 g/L spray-dried corn steep, and P2000 antifoam. Fermentation conditions were: 30°C, pH of 5 controlled with 4 M sodium hydroxide and 25 LPM airflow with agitation increasing from 300 to 800 rpm to maintain 30% dissolved oxygen. The glycerol concentration was monitored, and glycerol (50.0 g/L) and spray-dried corn steep (18.3 g/L) feed was initiated when glycerol levels fell below 2 g/L (at 18 h). When OD at 595 nm reached 121 at 34 h, 200 mL (1% v/v) methanol was added to the fermentor to start induction of GO and catalase. The glycerol and corn steep feed was stopped 30 min after methanol addition. Methanol concentration was monitored, and methanol feed was initiated to maintain concentrations between 2 and 10 g/L. At 7 h postinduction, YNB and biotin feed was started, with a total addition of 27 g YNB and 8 mg biotin over 10 h. Samples were withdrawn at different time intervals and OD, concentration of glycerol and methanol, and GO activity were measured. Cells were harvested at stationary phase when GO activity reached maximum level, and stored at -80°C until further use.

Permeabilization of rP. pastoris whole cells

Frozen cells kept at -80°C did not lose any activity over the one year period before or after permeabilization. Hence, cells were taken from the -80°C freezer and thawed at room temperature for permeabilization by BC. Stock of 100 mg/mL of BC was prepared in 50 mM of phosphate buffer, pH 7.0 to make different concentration of BC. Blotted cells (40 mg) were taken in four 2 mL natural microcentrifuge tubes for permeabilization by 0, 0.02, 0.1, and 0.5% (w/v) of BC. Cells were resuspended in 400 μL of 50 mM phosphate buffer, pH 7.0 and then desired amount of BC stock-solution was added and incubated at room temperature at 150 rpm for 1 h. After incubation, cells were centrifuged at 9900*g* for 10 min and washed four times with 800 μL of 50 mM phosphate buffer, pH 7.0. Every washing involved resuspension of cells in buffer followed by centrifugation at 9900*g* for 5 min. After final wash, cells were stored at -80°C freezer overnight or used immediately for GO and catalase activities. For checking substrate specificity and other studies, 100-300 g of frozen cells were permeabilized by 0.1% of BC by using the aforementioned method and stored at -80°C and used as needed.

Enzyme assays

GO activity was measured using a modification of a reported procedure.[27] Approximately, 0.5 g of frozen permeabilized cells were taken from the -80°C freezer and thawed at room temperature for the enzyme assay. After thawing, cells were blotted on a filter paper and about 40 mg of blotted cells were weighed into a 15 mL sterile polypropylene centrifuge tube (Fisherbrand, Cat. No. 05-539-12). DCIP assay solution (8 mL, 0.12 mM of DCIP in 80 mM of Tris-buffer, pH 8.3) was added to the centrifuge tube and

cells were suspended. Cell suspension (50 µL, ca. 0.25 mg of blotted cells) was removed from the centrifuge tube and placed in a 3.0 mL of quartz cuvette with a flea stirrer. DCIP solution (2 mL) was added to the cuvette. The cuvette was capped with a rubber septum and bubbled with nitrogen for 3 min to remove oxygen from the contents of the cuvette. Lactic acid (40 µL, 1.0 M) dissolved in 1.0 M Tris-buffer at pH 8.3 was added to the cuvette by a syringe. Immediately, change in absorbance at 606 nm for 30 s with stirring was measured (molar extinction coefficient, ϵ = 22,000 L mol^{-1} cm^{-1}).

Catalase activity was measured using a modified method of Eisenberg.[27] Like the GO assay, about 40 mg of blotted cells was weighed into a 15 mL sterile polypropylene centrifuge tube. Catalase assay buffer (8 mL, 0.0167 mM of phosphate buffer, pH 7) was added to the centrifuge tube and cells were suspended. Then, 50 µL of cell suspension (ca. 0.25 mg of blotted cells) was removed from the centrifuge tube and placed in a 3.0 mL of quartz cuvette with a flea stirrer. Catalase assay buffer (2.0 mL) was added into the cuvette. Hydrogen peroxide solution (1 mL, 67 µL of 30% peroxide in 10 mL of assay buffer, pH 7.0) was added to the cuvette. Immediately, change in absorbance at 240 nm was measured for 30 s while stirring (ϵ = 39.4 L mol^{-1} cm^{-1}).

GO activity of 2-hydroxy acids

Different 2-hydroxy acids (Figure 2) were chosen to examine GO activity. All 2-hydroxy acids were dissolved in 1.0 M of Tris-buffer, pH 8.3 to make 1 M stock solution for DCIP assay. To compare GO activity on different 2-hydroxy acids, activity on lactic acid was designated as 100%. Individual 2-hydroxy acids mentioned earlier were substituted for lactic acid in the assay mixture.

Enantioselectivity of GO

For checking stereoselectivity of GO, lactic acid, 3-phenyllacticacid, and 2-hydroxybutanoic acid were chosen. Both (*R*)- and (*S*)-isomers of these acids were separately assayed by DCIP. (*R*)- and (*S*)-isomers of the aforementioned acids were dissolved in 1.0 M Tris-buffer, pH 8.3 to make 1 M stock solutions.

Kinetics of 2-hydroxy acids

Lactic acid, 3-phenyllacticacid, and 2-hydroxybutanoic acid were chosen to study kinetic properties of GO. (*S*)-Enantiomers of aforementioned acids were separately dissolved in 1.0 M Tris-buffer, pH 8.3 to make a stock solution of 1 M. GO activity was measured at concentrations of 0.1, 0.4, 1, 4, 10, and 20 mM (final concentration) for the aforementioned substrates. Lineweaver-Burk plots were made by plotting the inverse values of activity and substrate concentration. K_m and V_{max} of lactic acid, 3-phenyllactic acid, and 2-hydroxybutanoic acid were determined from these plots.

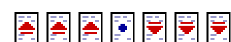
Reactions in 1.0 L bioreactors

Lactic acid, 3-phenyllactic acid, and 2-hydroxybutanoic acid were chosen for the oxidation reaction in bioreactors. Frozen permeabilized cells (60 g/L) were suspended in water. The final volume of the reaction was 500 mL. Substrate concentration used was 300 mM for (*RS*)-lactic acid and (*RS*)-2-hydroxybutanoic acid, 500 mM for (*RS*)-3-phenyllactic acid, and 250 mM for (*R*)-3-phenyllactic acid and (*S*)-3-phenyllactic acid. The pH and temperature were maintained at 7.0 and 18°C, respectively. The rate of agitation was 800 rpm. Air-flow was set at 0.4 L/min. At 0 h and indicated time intervals, samples were taken out and subjected to HPLC for the quantification of substrates and products.

Characterization of oxidized products by LC-MS

The oxidized product was isolated using Biorad HPX-87H Aminex column, which was also used for quantification of substrate and product by HPLC. Same solvent system, flow rate, and other parameters used for quantification, were used for isolation of the products except injection-volume, which was 10 µL. Appropriate fractions were concentrated and injected into LC-MS without passing through any column. Mobile phase used was 85% (v/v) MeOH, 15% (v/v) H_2O, and 0.1% (v/v) HCOOH. Flow rate was 0.2 mL/min. ESI probe was used at negative mode.

Results and Discussion



Recombinant *Pichia pastoris* containing GO and catalase T was grown in a 30 L fermentor. After a lag phase of 14 h, the culture growth reached stationary phase in 42 h (Figure 3). As both GO and catalase genes were integrated into the *P. pastoris* chromosome under control of the methanol-inducible *AOX1* promoter, methanol was added to start induction of GO and catalase. Methanol concentration was maintained at 2-10 g/L for optimum induction of GO and catalase. GO activity was checked at 6, 12, 15, 25, 28.5, and 30.5 h of postmethanol induction. Peak enzyme activity of GO and catalase (data not shown) was achieved at 28.5 h, at ~62.5 h total run time. Biomass (3.1 kg of wet weight) was harvested by centrifugation at OD_{595} of 150.

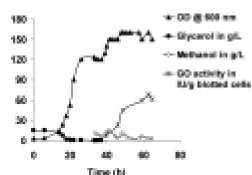


Figure 3. Fermentation profile of recombinant *Pichia pastoris* at 30 L scale.
[Normal View 20K | Magnified View 46K]

The biomass produced in the fermentor was permeabilized with the cationic surfactant, BC. Different concentrations of BC were used to determine optimum GO and catalase activity. For checking maximum GO activity at different concentrations of BC, lactic acid, 3-phenyl lactic acid, and 2-hydroxybutanoic acid were used as substrates. GO and catalase activity of frozen cells without BC-treatment and with 0.02, 0.1, and 0.5% (w/v) BC-treatment are summarized in Figures 4A,B, respectively. Frozen cells without

the treatment of BC showed poor activity for both enzymes. Thus, permeabilization with BC was necessary for maximum catalytic activities of intracellular enzymes, GO and catalase. The poor catalytic activities of GO and catalase without permeabilization is probably due to the impermeability of the cells to substrates.[28] The optimum concentration of BC to achieve maximum GO and catalase activities was 0.1%. Lower activity of 0.5% BC-treated cells could be due to the inactivation of the enzymes.

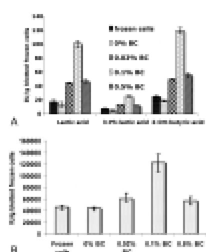


Figure 4. GO (A) and catalase (B) activity of permeabilized cells at different concentrations of benzalkonium chloride.
[Normal View 36K | Magnified View 107K]

BC (0.1%, w/v)-permeabilized whole cells of recombinant *Pichia pastoris* were used as biocatalyst to examine the oxidation of various 2-hydroxy acids shown in Figure 2. Glycolic acid, lactic acid, 2-hydroxybutanoic acid, 2-hydroxy-3-methylbutanoic acid, 3-chlorolactic acid, and 3,3,3-trifluorolactic acid were chosen as representatives of substrates with short chain alkyl groups. 2-Hydroxydecanoic acid was chosen as a long chain aliphatic substrate. Mandelic acid, 3-phenyllactic acid, and 3-(*p*-hydroxyphenyl)-lactic acid represented substrates with aromatic substitution. 3-Indolelactic acid was selected as the lone heterocyclic substrate. GO oxidized most of these acids to the corresponding 2-keto acids with the exception of 2-hydroxy-3-methylbutanoic acid and mandelic acid. High activity was observed with glycolic acid, lactic acid, 3-chlorolactic acid, and 2-hydroxybutanoic acid, whereas 3,3,3-trifluorolactic acid, 3-phenyllactic acid, 3-(*p*-hydroxyphenyl)-lactic acid, 3-indolelactic acid, and 2-hydroxydecanoic acid showed moderate activity. The rates of oxidation ranged from 1.3 to 120% (Table 1), relative to the oxidation of lactic acid to pyruvic acid. All the high and moderately active substrates (except glycolic acid and 3,3,3-trifluorolactic acid) have primary carbon atom at 3-position containing two hydrogen atoms. Carbon atom at 3-position of 2-hydroxy-3-methylbutanoic acid, which showed very poor activity, has methyl substituent. The other poor substrate, mandelic acid, has C-3 atom as part of phenyl ring. Judging from the substrate specificity of GO, steric demand in close proximity to the α-hydroxy functionality with substituent at 3-position, may determine the activity and specificity of GO. Similar results were observed by Adam et al. where mandelic acid and 2-hydroxy-3-methylbutanoic acid were not oxidized by GO.[10][11]

Table 1. Relative GO Activity of 2-Hydroxy Acids Compared with Lactic Acid (100%)

Racemic 2-Hydroxy Acids	Relative Activity (%)*
3-Indolelactic acid	17.7 ± 1.4
3-Phenyllactic acid	25.2 ± 0.4
3-(*p*-Hydroxyphenyl)-lactic acid	25.7 ± 2.1
3-Chlorolactic acid	110 ± 2.2
3,3,3-Trifluorolactic acid	11.4 ± 1.0
2-Hydroxybutanoic acid	120 ± 3.1
2-Hydroxy-3-methylbutyric acid	1.3 ± 0.1
Glycolic acid	89.6 ± 1.9
Mandelic acid	2.8 ± 0.3
2-Hydroxydecanoic acid	40.2 ± 1.3

* **Activity of lactic acid is considered as 100%.**

The oxidation of 2-hydroxy acids to 2-keto acids is believed to proceed via a mechanism similar to that observed in other flavoprotein oxidases.[29][30] This involves binding of 2-hydroxy acids to the enzyme active site, two electron reduction of FMN, and reoxidation of the reduced FMN by oxygen to produce hydrogen peroxide. Permeabilized *Pichia* cells did not require exogenous addition of FMN. Externally added FMN did not increase GO activity above the level without the cofactor addition. At higher than 100 μM, FMN inhibited the reaction. Also, addition of riboflavin during *Pichia pastoris* fermentation did not augment growth or GO activity yields. Both these studies indicated that the GO expressed *Pichia pastoris* had sufficient cofactor for maximum activity. Catalase coexpressed in r*Pichia* suppressed the destruction of the 2-oxo acids with hydrogen peroxide by the reduction of the latter to water (Figure 1).[10][23]

To check the enantioselectivity of GO, lactic acid, 3-phenyllactic acid, and 2-hydroxybutanoic acid were chosen as substrates. These acids were selected due to the following reasons. (*R*)-Isomer of lactic acid is very expensive compared with its racemic mixture and (*S*)-isomer. (*R*)-Isomer of lactic acid is used as a standard in the laboratory and starting material for the synthesis of chiral compounds.[31] Moreover, the oxidized product, pyruvic acid is extensively used in chemical, cosmetic, food and drug

industry. 3-Phenyllactic acid was chosen because the compound and its derivatives are frequently used in nonracemic form as components of pharmaceuticals and natural antibiotic agents.[32] They represent an integral part of bioactive peptides, such as aeruginosins[33] and microcin,[34] which are potent protease inhibitors. *p*-Fluoro-substituted 3-phenyllactic acid is a key building block for the synthesis of AG7088 (Rupintrivir), a potent rhinovirus inhibitor used for the treatment of common cold.[35][36] 2-Hydroxybutanoic acid was chosen as an aliphatic compound because (*R*)-isomer of 2-hydroxybutanoic acid derivatives are important building block for the production of a large variety of ACE inhibitors, which have in common, the (*S*)-homophenylalanine moiety as the central pharmacophore unit.[37]

(*R*)- and (*S*)-isomers of these three important 2-hydroxy acids were tested individually by incubating with GO. GO oxidized all of these (*S*)-acids, whereas (*R*)-isomers were totally inactive (Figure 5). Thus, GO can be utilized for the resolution of racemic 2-hydroxy acids to (*R*)-hydroxy acids. Nonselective reduction of the product (2) will create a dynamic process for resolution of α -hydroxy acids (1) with high yield of the (*R*)-enantiomers (Figure 1). A similar dynamic process to enrich (*R*)-enantiomers was reported by Oikawa et al.[31] They used racemic mixture of lactate with purified (*S*)-lactate oxidase (from *Aerococcus viridans*) to obtain (*R*)-lactate with 99% yield. In their one-pot system, (*S*)-lactic acid was enantiospecifically oxidized to pyruvic acid, which was chemically reduced by sodium borohydride to (*RS*)-lactic acid. Thus, (*RS*)-lactic acid was fully converted to the (*R*)-enantiomer. In a similar manner, a dynamic resolution process can be conceived for the production of (*R*)-2-hydroxy acids with high yield from racemic 2-hydroxyacids, using the r*Pichia pastoris* expressing GO. GO has several advantages over lactate oxidase[31]: (a) Oikawa et al. have not examined other hydroxyacids compared with GO which works not only with lactate but also with numerous 2-hydroxy acids (listed in Table 1). (b) The substrate loading for lactate oxidase is 5 mM racemic lactic acid whereas for GO, it is 300-500 mM racemic 2-hydroxy acids, which is 60-100 times higher. (c) Lactate oxidase was used at 1.0 mL scale vs. GO at 500.0 mL scale. (d) Use of purified L-lactate oxidase supplemented with FMN, is not economical, given the cost of enzyme purification and FMN. In addition, catalase (commercially purchased) and BSA (commercially purchased, as a stabilizing agent) were added to the reaction mixture. In contrast, permeabilized double recombinant *Pichia pastoris* obtained from high-cell density fermentation (Figure 3) requires no exogenous addition of cofactor, stabilizing agent or catalase. (e) Last but not least, lactate oxidase reaction was conducted in 500 mM phosphate buffer as opposed to water for r*Pichia* expressing GO.

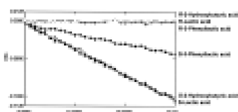


Figure 5. Enantioselective oxidation of (*S*)-hydroxy acids by DCIP assay. [Normal View 17K | Magnified View 41K]

To study the kinetic properties of GO, 2-hydroxybutanoic acid, 3-phenyl lactic acid, and lactic acid were chosen as substrates. K_m and V_{max} of these acids were calculated from Lineweaver-Burk plots and summarized in Table 2. (*S*)-2-Hydroxybutanoic acid is the best substrate. V_{max}/K_m for (*S*)-2-hydroxybutanoic acid, (*S*)-lactic acid, and (*S*)-3-phenyllactic acid are 60.5, 11.9, and 34.29, respectively.

Table 2. K_m and V_{max} of 2-Hydroxy Acids

Substrates	K_m (mM)	V_{max} (μmol^{-1} min^{-1} g^{-1})
(*S*)-2-Hydroxybutanoic acid	1.90 ± 0.07	114.9 ± 3.1
(*S*)-3-Phenyllactic acid	1.96 ± 0.08	23.36 ± 0.09
(*S*)-Lactic acid	2.43 ± 0.06	83.33 ± 2.5

GO-catalyzed reactions with select substrates were carried out in 1 L bioreactors to characterize the products and stoichiometry of the reaction. Reactions were carried out with 300 mM of (*RS*)-lactic acid and (*RS*)-2-hydroxybutanoic acid, 500 mM of (*RS*)-3-phenyllactic acid, and 250 mM of (*R*)-3-phenyllactic acid and (*S*)-3-phenyllactic acid. HPLC profiles of reaction mixtures of these acids at different time points are shown in the Figures 6 and 7. The composition of lactic acid (300 mM) used in the bioreactor was (*S*)-isomer, 93% and (*R*)-isomer, 7%. At 0 h sample of lactic acid reaction, 4.7 mM of pyruvic acid was produced. This is due to the time consumed between sampling and HPLC analysis. At 1 h 15 min of the reaction, most of the lactic acid was converted to pyruvic acid (77.6%). At 1 h 45 min, 2 h 25 min, and 2 h 40 min of reaction, the amount of pyruvic acid produced was 89, 93, and 93%, respectively. At 2 h 25 min, the reaction was complete. Rate of formation of pyruvic acid and disappearance of lactic acid with time in the Figure 6B shows nearly total conversion. Few reports are available on the production of pyruvic acid from (*S*)-lactic acid.[38] Most of the reports showed use of lactate oxidase as catalyst. Ma et al.[38] published space-time yield of pyruvate from (*S*)-lactate as 1.33 gl^{-1} h^{-1}. They used lactate oxidase as catalyst. The space-time yield of pyruvate from this study using (*RS*)-lactate (93% *S*) was 10.14 gl^{-1} h^{-1}, which is 7.6 times higher.

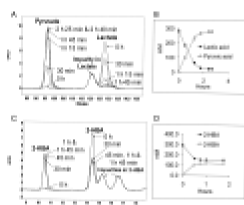


Figure 6. HPLC profiles of (*RS*)-lactic acid (A) and (*RS*)-2-hydroxybutanoic acid (C) reaction mixtures at different time points in 1 L bioreactor. (B) Rate of formation of pyruvic acid and disappearance of lactic acid. (D) Rate of formation of 2-ketobutanoic acid (2-KBA) and disappearance of 2-hydroxybutanoic acid (2-HBA).
[Normal View 52K | Magnified View 142K]

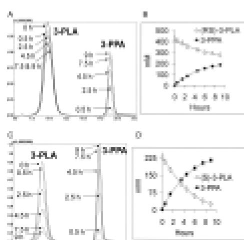


Figure 7. HPLC profiles of (*RS*)-3-phenyllactic acid (A) and (*S*)-3-phenyllactic acid (C) reaction mixtures at different time points in 1 L bioreactor. (B, D) Rate of formation of 3-phenylpyruvic acid (3-PPA) and disappearance of (*RS*)-3-phenyllactic acid (B) and (*S*)-3-phenyllactic acid (D). Phenyllactic acid is designated as PLA.
[Normal View 59K | Magnified View 165K]

The (*RS*)-2-hydroxybutanoic acid used from the commercially supplied bottle had impurities (Figure 6C). Quantification by HPLC determined the exact concentration of (*RS*)-2-hydroxybutanoic acid in the bioreactor, which was 300 mM. Similar to lactic acid reaction, at 0 h sample of 2-hydroxybutanoic acid reaction, 5.8 mM of 2-ketobutanoic acid was produced. At 30 min, 45 min, 60 min, and 1 h 45 min of reaction, the amount of product, 2-ketobutanoic acid was 102.6 mM (34.2%), 109.3 mM (36.4%), 116.2 mM (38.7%), and 113.2 mM (37.8%), respectively. Maximum theoretical yield of 2-ketobutanoic acid was 77.46% (assuming 50% *S*-enantiomers present in the racemic mixture, according to the manufacturer). Rate of formation of 2-ketobutanoic acid and disappearance of 2-hydroxybutanoic acid with time in the Figure 6D shows very rapid conversion of 2-hydroxybutanoic acid to its keto-form.

GO-catalyzed reaction in the bioreactor could also be tracked online by monitoring dissolved oxygen in the reaction mixture, since oxygen is stoichiometrically consumed during the reaction. The DO tracing at the beginning of the reaction and at the end of the reaction matched HPLC analysis. Without HPLC measurement of reactants and products, just observing DO plot would give an excellent idea about the progress of this oxidation reaction. Based on DO tracings, the rate of conversion of 2-hydroxybutanoic acid and lactic acid also matched the rates shown in Table 1. When reaction started, percent dissolved oxygen (DO) dropped down and reached steady state of 25-40%. At the end of reaction, DO returned to 100%. DO plots of (*RS*)-lactic acid and (*RS*)-2-hydroxybutanoic acid reactions are shown in the Figure 8. From the DO plot of 2-hydroxybutanoic acid, it can be concluded that the reaction terminated at 45 min, which matched the total reaction time determined by HPLC (Figures 6C,D). Similarly, DO plot of lactic acid showed a total reaction time of 2 h 25 min, matching the HPLC data (Figures 6A,B and 8). Thus monitoring of the DO during the bioreactor studies was extremely useful to note the end of the reaction for other analytical measurements and product analysis.

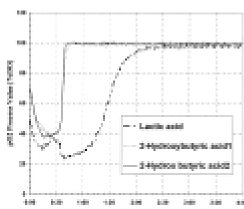


Figure 8. Dissolved oxygen plots of (*RS*)-lactic acid and (*RS*)-2-hydroxybutanoic acid reactions in 1 L bioreactor.
[Normal View 29K | Magnified View 77K]

The (*S*)-enantioselectivity of GO determined by initial velocity studies was confirmed in the bioreactor studies with (*RS*)-3-phenyllactic acid (500 mM), (*R*)-3-phenyllactic acid (250 mM), and (*S*)-3-phenyllactic acid (250 mM). Like lactic acid and 2-hydroxybutanoic acid reactions, at 0 h sample of (*RS*)-3-phenyllactic acid reaction, 2.24 mM of 3-phenylpyruvic acid was produced. At 30 min, 2 h 30 min, 6 h, 7 h 30 min, and 9 h, the amount of 3-phenylpyruvic acid produced was 26.6 mM (5.3%), 86.9 mM (17.3%), 156.8 mM (31.4%), 176.6 mM (35.3%), and 188.6 mM (37.7%), respectively. With (*S*)-3-phenyllactic acid, the amount of product formed at 0 min, 30 min, 3 h 30 min, 6 h, 7 h 30 min, and 9 h, were 2.4 mM (1.0%), 26.8 mM (10.7%), 129.8 mM (51.9%), 181.0 mM (72.4%), 203.2 mM (81.3%), and 214.9 mM (85.9%), respectively. Nearly theoretical yield of product was obtained from both of (*RS*)-3-phenyllactic acid and (*S*)-3-phenyllactic acid (Figures 7B,D). When (*R*)-3-phenyllactic acid was used as a substrate in the reactor, no product (3-phenylpyruvic acid) was detected by HPLC after 9 h of incubation. This data further supported absolute selectivity of GO toward (*S*)-enantiomers.

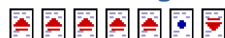
The oxidized products were confirmed by HPLC, based on the retention times of standards and by LC-MS. Lactic acid, 2-hydroxybutanoic acid, and 3-phenyllactic acid oxidized products showed very sharp *m/z* peak (100%) at 87, 101, and 163, respectively, at ESI negative mode, which corresponded to the molecular masses of pyruvic acid, 2-ketobutanoic acid, and 3-phenylpyruvic acid, respectively.

Conclusions

Permeabilized double recombinant *P. pastoris* containing GO and catalase catalyzed oxidation of several 2-hydroxy acids such as glycolic acid, lactic acid, 3-chlorolactic acid, 3,3,3-trifluorolactic acid, 2-hydroxybutanoic acid, 2-hydroxydecanoic acid, 3-phenyllactic acid, 3-(*p*-hydroxyphenyl)-lactic acid, and 3-indolelactic acid. Conversion of three substrates, (*S*)- and (*RS*)-3-phenyllactic acid,

(*RS*)-lactic acid, and (*RS*)-2-hydroxybutanoic acid to the corresponding 2-keto acids was achieved in near theoretical yields, in 500 mL scale. External addition of FMN and catalase was not required for maximum conversion. GO was highly specific for the (*S*)-enantiomers.

Acknowledgements



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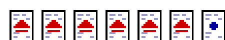

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