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A NOVEL SPRAY-DRYING PROCESS TO STABILIZE GLYCOLATE OXIDASE
AND CATALASE IN *PICHLA PASTORIS* AND OPTIMIZATION OF PYRUVATE
PRODUCTION FROM LACTATE USING THE SPRAY-DRIED BIOCATALYST

by

James Huston Glenn IV

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Chemical and Biochemical Engineering
in the Graduate College of
The University of Iowa

December 2009

Thesis Supervisor: Professor Mani Subramanian

ABSTRACT

Pyruvate is a valuable chemical intermediate in the production of fine chemicals used by agrochemical, pharmaceutical, and food industries. Current technology for production of pyruvic acid is based on conversion from tartaric acid and results in environmentally incompatible byproducts. An enzymatic approach to making pyruvate was developed by cloning the glycolate oxidase (GO) gene from spinach into *Pichia pastoris* (Payne, et al., (1995). High-level production of spinach glycolate oxidase in the methylotrophic yeast *Pichia pastoris*: Engineering a biocatalyst. *Gene*, 167(1-2), 215-219). GO is a flavoprotein (FMN dependent) which catalyzes the conversion of lactate to pyruvate with the equimolar production of hydrogen peroxide. Hydrogen peroxide can lower GO activity and make non-catalytic byproducts, so catalase was also cloned into *P. pastoris* to create a double transformant.

Process development work was completed at the University of Iowa's Center for Biocatalysis and Bioprocessing. High-density *P. pastoris* fermentation (7.2 kg cells/L) was completed at the 100 L scale. Critical fermentation set-points were confirmed at 14 h glycerol feeding followed by methanol induction at 2 – 10 g/L for 30 h. After fermentation, these cells were permeabilized with benzalkonium chloride (BAC) to enable whole-cell biocatalysis and increase enzyme activity, yielding 100 U/g for GO. In 30 L enzyme reactions, permeabilized cells were recycled three times for over 92% conversion of 0.5 M lactate with an “enzyme to product” ratio of approximately 1:2 (Gough, et al., (2005). Production of pyruvate from lactate using recombinant *Pichia pastoris* cells as catalyst. *Process Biochemistry*, 40(8), 2597-2601). Though effective, the post-fermentation process for GO recovery involved several unit-operations, including multiple washing steps to remove residual BAC.

The present work has focused on minimizing unit-operations by spray-drying the fermentation product to create a powdered biocatalyst. Optimal spray-drying conditions

for the Buchi B-190 instrument were 150°C drying air, 15 mL/min liquid feed rate, and 600 mg cells/mL liquid feed. These conditions resulted in *P. pastoris* biocatalyst with activities of 80 – 100 U/g for GO and 180,000 – 220,000 U/g for catalase. The spray-dried cells retained nearly 100% of the enzyme activity compared to BAC treated cells as reported by Gough et al. Additionally, the spray-dried biocatalyst was stable at room temperature for 30 days, and no measurable enzyme leaching was observed. Then, *P. pastoris* was spray-dried under optimal conditions and tested for conversion of lactate to pyruvate for an improved “enzyme to product” ratio.

Enzyme reaction optimization was done at the one-liter scale in DASGIP reactors. The DASGIP system contained four parallel reactors with control of temperature, pH, and dissolved oxygen. Other key variables included substrate loading, conducting the reaction in buffer or water, minimizing enzyme concentration, and maximizing the number of enzyme recycles. Optimal performance was achieved in water at pH 7.0 with an operating temperature of 25°C and 1.0 M substrate loading. Enzyme loading was at 12 g/L for the first two cycles, and subsequently, 2 – 3 g/L of fresh cells were added every alternate cycle to reach 15 cycles. Under these conditions, 75 – 95% conversion of lactate to pyruvate was accomplished for every 12 – 16 h reaction cycle. Based on these parameters, an “enzyme to product” ratio of 1:41 was achieved.

Abstract Approved: _____
 Thesis Supervisor

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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee
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To my wife, Jackie, who truly has a knack for finding the beauty in any situation.

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Dr. Gopishetty and Dr. Shuvendu Das helped to extend the application of my thesis work by investigating other important substrates. Dr. Das spent a few sleepless nights watching over fermentations with me. Jody Ehler was also committed to the success of our fermentations regardless of the time of day. Kevin Erpelding assisted with experimental design and equipment configuration to streamline the project and maximize success. Russell Hamilton pitched-in with fermentation sampling, as well as Wensheng Liu, Deanna Lettington, Alyssa Langerud, Mary Garner, Shelly Railsback, Larry Dostal and others. I’m sure everyone at the CBB has taken a sample or helped in some way.

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TABLE OF CONTENTS

LIST OF TABLES	viii
LIST OF FIGURES	ix
CHAPTER 1: INTRODUCTION AND BACKGROUND	1
Pyruvate Usage	1
Pyruvate Market Analysis.....	1
Current Production Methods for Pyruvate.....	2
Green Chemistry: Use of Enzymes for Biocatalysis	3
Thrust of Current Work: Enzymatic Process for Conversion of Lactate to Pyruvate.....	7
Cloning of Spinach GO: Construction of GO Expression Vector Used in this Work	9
Incorporation of Catalase T from <i>S. cerevisiae</i> into GO Expression Vector	11
Advantages of Whole-Cell Biocatalysis for Lactate to Pyruvate Conversion.....	12
Enzymatic Process for Conversion of Lactate to Pyruvate with Unit Operations Based on Detergent Permeabilization of <i>P. pastoris</i>	13
CHAPTER 2: MATERIALS AND METHODS	16
Detergent Cell Permeabilization for Biocatalysis.....	16
Materials for Detergent Cell Permeabilization.....	16
Method for Detergent Cell Permeabilization	16
Buchi B-190 Spray-Dryer.....	16
Spray-Drying Materials.....	17
Spray-Drying Method.....	18
GO and Catalase Activity Calculation.....	18
Materials for GO and Catalase Activity Assays.....	19
DCIP Assay for GO Activity.....	20
H ₂ O ₂ Assay for Catalase Activity.....	20
GO and Catalase Stability Testing.....	21
Detergent Permeabilization of Spray-Dried Cells	21
GO and Catalase Leaching Tests.....	22
Microscopy of Spray-Dried <i>P. pastoris</i>	22
Expression of GO in <i>E. coli</i>	22
Materials for Growth of <i>E. coli</i>	23
Growth of <i>E. coli</i> Expressing GO.....	23
Spray-Drying <i>E. coli</i> for Active GO.....	23
Spray-Drying <i>S. cerevisiae</i> for Active Catalase	24
DASGIP Fed-Batch Pro System.....	24
Materials for GO-Based Production of Pyruvate	25
GO-Based Production of Pyruvate in DASGIP.....	26
Analox GM8 Metabolite Analyzer	29
Materials for Analox GM8 Measurement	30
L-lactate Measurement by Analox GM8.....	31
Pyruvate Measurement by Analox GM8.....	31
Glycerol Measurement by Analox GM8	32

Methanol Measurement by Analox GM8.....	32
HPLC Analysis of Lactate and Pyruvate.....	32
30 L Fermentation of <i>P. pastoris</i>	33
Materials for <i>P. pastoris</i> Fermentation.....	33
Method for <i>P. pastoris</i> Fermentation.....	33
SDS-PAGE Analysis of Enzyme Expression.....	36
Materials for SDS-PAGE Analysis.....	36
Method for SDS-PAGE Analysis.....	37
Preparation of <i>P. pastoris</i> Seed Bank.....	38
CHAPTER 3: DETERMINATION OF SPRAY-DRYING AS A ROBUST PROCESS FOR <i>PICHIA PASTORIS</i> EXPRESSING GLYCOLATE OXIDASE AND CALALASE FOR BIOCATALYTIC CONVERSION OF LACTATE TO PYRUVATE.....	39
Traditional Biotransformation Processes.....	39
Advantages of Spray-drying.....	39
Specific Aim 1.....	40
Initial Screening of Spray-Drying Parameters to Determine GO and Catalase Activity.....	42
GO Activity Results for Initial Screening of Spray-Drying Parameters.....	44
Catalase Activity Results for Initial Screening of Spray-Drying Parameters.....	46
First-Level Optimization of Spray-Drying Parameters with Respect to GO and Catalase Activity.....	48
GO Activity Results for First-Level Optimization of Spray-Drying Parameters.....	49
Catalase Activity Results for First-Level Optimization of Spray- Drying Parameters.....	51
Final Optimization of Spray-Drying Parameters to Create a Robust Process.....	53
GO and Catalase Activity Results for Final Optimization of Spray- Drying Parameters.....	54
Stability of GO and Catalase to Create a Robust Process.....	56
Comparison of GO and Catalase Activities of Spray-Dried Cells vs. BAC Treated Cells.....	57
Determination of GO and Catalase Leaching from Spray-Dried Cells to Ensure a Robust Process.....	59
Discussion of Optimal Spray-Drying Parameters with Respect to Enzyme Activity and Process Robustness.....	61
Conclusions for Specific Aim 1.....	64
Microscopic Characterization of Spray-Dried <i>P. pastoris</i>	66
Spray-Drying as a Broad Technology for Biocatalysis.....	68
Spray-drying Greatly Reduces <i>P. pastoris</i> Viability.....	70
CHAPTER 4: OPTIMIZATION OF THE REACTION PROCESS FOR CONVERSION OF LACTATE TO PYRUVATE USING SPRAY- DRIED <i>PICHIA PASTORIS</i> EXPRESSING GLYCOLATE OXIDASE AND CALALASE.....	72
Previous Enzyme Reaction Studies.....	72
Specific Aim 2.....	77
pH Optimization Study.....	77

Pyruvate Formation at Various Lactate Concentrations.....	79
Operating Temperature and Initial Cell Loading Study	81
Medium Composition and Decreased Cell Loading Study	83
Product Inhibition Testing and Cell Loading Optimization	86
Determination of Enzyme Recycling to Improve “Enzyme to Product” Ratio.....	89
Conclusions for Specific Aim 2.....	92
CHAPTER 5: DEMONSTRATION OF NEW PROCESS FOR PYRUVATE PRODUCTION “END TO END” USING SPRAY-DRIED <i>PICHIA</i> <i>PASTORIS</i> AND PREPARATION OF NEW SEED BANK.....	95
Early Process Development Work at the University of Iowa’s Center for Biocatalysis and Bioprocessing	95
Specific Aim 3	98
Verification of <i>P. pastoris</i> Fermentation Conditions for Maximum GO and Catalase Yield.....	99
“End to End” Demonstration of the New Process for Production of Pyruvate from Lactate by Spray-Dried <i>P. pastoris</i> to Confirm Process Inputs and Outputs.....	103
Conclusions for Specific Aim 3.....	111
CHAPTER 6: SUMMARY OF COMPLETED RESEARCH AND SUGGESTED FUTURE WORK.....	113
Second-Generation Process Summary.....	113
Suggested Future Work	115
APPENDIX A: ENANTIOSELECTIVITY OF GLYCOLATE OXIDASE FROM SPRAY-DRIED <i>PICHIA PASTORIS</i>	117
Importance of Chiral 2-Hydroxy Acids.....	117
Enantioselectivity of GO from Spray-Dried <i>P. pastoris</i>	117
APPENDIX B: PUBLICATONS AND PRESENTATIONS.....	120
REFERENCES	121

LIST OF TABLES

Table 1.	Pyruvate production and byproduct formation using soluble GO and catalase compared to using microbial transformants expressing recombinant GO from spinach and catalase T from <i>S. cerevisiae</i>	13
Table 2.	Factorial design parameters for screening spray-dryer cell concentration, feed rate, and operating temperature to determine GO and catalase activity.....	43
Table 3.	Factorial design parameters for first-level optimization of spray-dryer cell concentration, feed rate, and operating temperature with respect to GO and catalase activity.	49
Table 4.	Final optimization of spray-drying cell concentration.....	54
Table 5.	Spray-drying parameter optimization and determination of process robustness.....	61
Table 6.	Conditions tested for pH optimization study.	78
Table 7.	Conditions tested for lactate concentration effect.....	80
Table 8.	Conditions tested for temperature and initial cell loading in the enzyme reaction.....	82
Table 9.	Conditions tested for medium composition and decreased cell loading in the enzyme reaction.	84
Table 10.	Conditions for substrate feeding experiment to test product inhibition.....	86
Table 11.	Conditions tested for enzyme recycling experiment.....	89
Table 12.	GO process changes and their impact.....	94
Table 13.	Best <i>P. pastoris</i> fermentations with respect to overall GO and catalase yield from early process development studies at the University of Iowa's Center for Biocatalysis and Bioprocessing.	96
Table 14.	Best <i>P. pastoris</i> fermentation conditions from early process development studies at the University of Iowa's Center for Biocatalysis and Bioprocessing.....	97
Table 15.	Summary of <i>P. pastoris</i> fermentation conditions for maximum biomass production and GO and catalase yield.	102
Table 16.	15 Reaction cycles using spray-dried biocatalyst from new seed bank to verify reaction conditions and determine upper limit for "enzyme to product" ratio	107
Table A1.	GO activity for racemic 2-hydroxy carboxylic acids where the optically active enantiomer is industrially important.....	118

LIST OF FIGURES

Figure 1.	End-use breakdown for pyruvate.	2
Figure 2.	Cumulative number of biotransformation processes that have been started on an industrial scale.	4
Figure 3.	Enzyme types used in industrial biotransformations based on 134 processes.....	5
Figure 4.	Use of enzymes or whole-cells in industrial biotransformations based on 134 processes.....	6
Figure 5.	Enzymatic conversion of lactic acid to pyruvic acid by glycolate oxidase with catalase employed to break-down the hydrogen peroxide byproduct and minimize further oxidation of pyruvic acid to byproducts such as acetic acid.....	8
Figure 6.	Glycolate oxidase yield and specific activity from various plant sources. Open bars denote total glycolate oxidase yield in U/g wet plant weight, while filled bars show specific activity in U/mg protein. Glycolate oxidase activities from plant extracts were measured by o-aminobenzaldehyde assay	9
Figure 7.	pMP1 expression plasmid contained the 110 base pair fragment encoding for GO from spinach downstream of the strong <i>AOX1</i> promoter. Other plasmid functions include Km^R for resistance to kanamycin, neomycin and G418, Ap^R for resistance to ampicillin, <i>HIS4</i> for chromosomal integration in <i>his4</i> hosts, and the <i>ori</i> replication origin for plasmid manipulations in <i>E. coli</i>	10
Figure 8.	pMP8 expression plasmid contained the 1850 base pair <i>CTT1</i> fragment obtained from PCR amplification of <i>S. cerevisiae</i> DNA. Other functions in the vector include Ap^R for resistance to ampicillin, <i>HIS4</i> for chromosomal integration in <i>his4</i> hosts, the <i>ori</i> replication origin for plasmid manipulations in <i>E. coli</i> , and <i>SUC2</i> for selection in hosts previously transformed to His^+	11
Figure 9.	Enzymatic process for conversion of lactate to pyruvate with unit operations based on benzalkonium chloride (BAC) permeabilization of <i>P. pastoris</i>	15
Figure 10.	Buchi B-190 spray-dryer. Specific to the Buchi B-190 apparatus, the feed (1) is transferred by peristaltic pump (2, not visible) to the spray nozzle (3). Pressurized air drives the feed through the nozzle, creating tiny droplets that enter the drying chamber (4). Simultaneously, ambient air is drawn from the intake (5), through an electric heater (6, not visible) and into the drying chamber. The carrier liquid evaporates in the drying chamber and the dry powder is drawn by vacuum into the cyclone (7). The dry powder settles in the cyclone, and the product is gathered in the collection vessel (8).	17

Figure 11. Reduction of 2, 6-dichloroindophenol.....	19
Figure 12. DASGIP Fed-Batch Pro system of four parallel reactors.....	25
Figure 13. Analox GM8 Metabolite Analyzer for measurement of L-lactate, pyruvate, glycerol, and methanol by enzymatic assay.	30
Figure 14. Spray-drying flowchart with benchmarks to determine process robustness.	41
Figure 15. Effect of temperature (120 – 150°C) on spray-dried GO activity. Outer red lines represent 95% confidence interval.....	44
Figure 16. Effect of feed rate (5 – 15 mL/min) on spray-dried GO activity. Outer red lines represent 95% confidence interval.....	45
Figure 17. Effect of cell concentration (30 – 100 mg/mL) on spray-dried GO activity. Outer red lines represent 95% confidence interval.	45
Figure 18. Effect of temperature (120 – 150°C) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.....	46
Figure 19. Effect of feed rate (5 – 15 mL/min) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.....	47
Figure 20. Effect of cell concentration (30 – 100 mg/mL) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.	47
Figure 21. Effect of temperature (120 – 195°C) on spray-dried GO activity. Outer red lines represent 95% confidence interval.....	50
Figure 22. Effect of cell concentration (100 – 400 mg/mL) on spray-dried GO activity. Outer red lines represent 95% confidence interval.	50
Figure 23. Effect of temperature*cell concentration interaction on spray-dried GO activity. Outer red lines represent 95% confidence interval.	51
Figure 24. Effect of temperature (120 – 195°C) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.....	52
Figure 25. Effect of cell concentration (100 – 400 mg/mL) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.	52
Figure 26. Effect of temperature*cell concentration interaction on spray-dried catalase. Outer red lines represent 95% confidence interval.....	53
Figure 27. GO activity for spray-dried cells at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL.	55
Figure 28. Catalase activity for spray-dried cells at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL.	55

Figure 29. Average GO activity of spray-dried cells stored at room temperature. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL. Error bars signify one standard deviation (n=6).....	56
Figure 30. Average catalase activity of spray-dried cells stored at room temperature. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL. Error bars signify one standard deviation (n=6).....	57
Figure 31. GO activity of BAC treated and spray-dried cells. Blue Bars represent no BAC treatment and red bars indicate BAC treatment. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL. Error bars represent one standard deviation (n=6).....	58
Figure 32. Catalase activity of BAC treated and spray-dried cells. Blue Bars represent no BAC treatment and red bars indicate BAC treatment. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200 mg/mL, 400, and 600 mg/mL. Error bars represent one standard deviation (n=6). Cells not spray dried and not treated with BAC had no catalase activity.	59
Figure 33. Test for GO leaching from spray-dried cells. Spray-drying conditions were 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration.	60
Figure 34. Test for catalase leaching from spray-dried cells. Spray-drying conditions were 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration.	60
Figure 35. GO activity Pareto chart for spray-drying Trials 1 – 20 in Table 5.	62
Figure 36. Catalase activity Pareto chart for spray-drying Trials 1 – 20 in Table 5.....	62
Figure 37. Achieved benchmarks for the new spray-drying process to prepare <i>P. pastoris</i> for conversion of lactate to pyruvate.....	65
Figure 38. New and simplified spray-drying process for GO-based conversion of lactate to pyruvate.	66
Figure 39. Scanning electron micrograph of spray-dried <i>P. pastoris</i> cell cluster at the 3.0 µm scale.....	67
Figure 40. Scanning electron micrograph of the surface of spray-dried <i>P. pastoris</i> at the 1.0 µm scale to investigate porosity.	67
Figure 41. GO activity of spray-dried <i>E. coli</i> and control cells (not spray-dried). Spray-drying was done at 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration. Error bars represent one standard deviation (n=3).....	68
Figure 42. Catalase activity of spray-dried <i>S. cerevisiae</i> and control cells (not spray-dried). Spray-drying was done at 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration. Error bars represent one standard deviation (n=3).	69

Figure 43.	Viability comparison between spray-dried <i>P. pastoris</i> (left) and <i>P. pastoris</i> seed vial (right). Seed bank preparation, fermentation, and spray-drying were completed as described in Materials and Methods. Both conditions were streaked on YPD agar plates and incubated at 30°C for 48 h.	71
Figure 44.	Percent conversion of L-lactic acid vs. initial L-lactic acid concentration by permeabilized <i>P. pastoris</i> in 500 mL Belco spinner vessels. Reaction conditions were 500 rpm stirring, 5°C, pH 7.0, and 1 L/min air flow.	73
Figure 45.	Percent conversion of 0.5 M L-lactic acid vs. initial pyruvic acid concentration by permeabilized <i>P. pastoris</i> in 500 mL Belco spinner vessels. Reaction conditions were 500 rpm stirring, 5°C, pH 7.0, and 1 L/min air flow.	74
Figure 46.	Effect of temperature on percent conversion of 0.5 M L-lactic acid by permeabilized <i>P. pastoris</i> in 500 mL Belco spinner vessels. Reaction conditions were 500 rpm stirring, temperatures of 5, 10, 15, and 22°C, pH 7.0, and air flow at 1 L/min.	75
Figure 47.	Effect of pH on percent conversion of 1.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in 80 mM Tris, 60 g/L spray-dried <i>P. pastoris</i> , 15°C, 1 L/min air flow, and 1250 rpm.	78
Figure 48.	Effect of pH on pyruvate formed from 1.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in 80 mM Tris, 60 g/L spray-dried <i>P. pastoris</i> , 15°C, and 1 L/min air flow.	79
Figure 49.	Effect of lactate concentration on percent conversion of lactate. Reaction conditions were L-lactic acid in 80 mM Tris, pH 6.0, 60 g/L spray-dried <i>P. pastoris</i> , 15°C, 1 L/min air flow, and 1250 rpm agitation.	81
Figure 50.	Effect of cell concentration and temperature on percent conversion of 1.0 M lactate. Reaction conditions were L-lactic acid in 80 mM Tris, pH 7.0, 25°C, 1 L/min air flow, and 1250 rpm.	83
Figure 51.	Effect of enzyme concentration and medium composition on pyruvate formed from 1.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in 80 mM Tris or water, pH 7.0, 25°C, 1 L/min air flow, and 1250 rpm.	85
Figure 52.	Effect of product inhibition on GO reaction by feeding 4.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in water supplemented by pyruvate feeding, pH 7.0, 25°C, 1 L/min air flow, and 1250 rpm.	88
Figure 53.	Percent conversion of 1.0 M lactate at the end of each 10 – 12 h reaction cycle. Reaction conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 10 reaction cycles. Initial 15 g/L cell concentrations were supplemented with either (a) 2 g/L fresh cells at cycles 4, 6, and 8, or (b) 5 g/L fresh cells at cycle 4 only.	90

Figure 54.	HPLC chromatograms of pyruvic acid product from reactor 1 at completed cycles 1, 5, and 10 compared to 98% pyruvic acid standard from Sigma. Reactor conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 10 reaction cycles. Initial 15 g/L cell concentration was supplemented with 2 g/L of fresh cells at cycles 4, 6, and 8. HPLC conditions were BioRad Aminex column at 30°C with 0.008 N sulfuric acid solvent at 0.6 mL/min and UV detection at 215 nm.	92
Figure 55.	Process development fermentation 99999-13: <i>P. pastoris</i> MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 10 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h. Optical density (OD ₆₀₀) was tracked by UV-VIS spectroscopy, glycerol and methanol concentrations were measured by HPLC, and GO activity was followed by oAB assay.....	98
Figure 56.	GO and catalase activities of spray-dried samples from fermentation 27016-1e plotted as a function of induction time. Fermentation conditions were <i>P. pastoris</i> MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 35 h.	100
Figure 57.	GO and catalase activities of spray-dried samples from fermentation 27016-1f plotted as a function of induction time. Fermentation conditions were <i>P. pastoris</i> MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 10 – 20 g/L methanol induction for 30 h.	101
Figure 58.	Fermentation 27016-1h: <i>P. pastoris</i> MSP 8.6 new seed bank, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h. Optical density was tracked by UV-VIS spectroscopy, glycerol and methanol concentrations were measured by Analox instrument, and GO activity was followed by DCIP assay of spray-dried samples.	104
Figure 59.	SDS-PAGE analysis of fermentation 27016-1h to test the new seed bank. Fermentation conditions were <i>P. pastoris</i> MSP 8.6 new seed bank, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h.	105
Figure 60.	GO and catalase activities of spray-dried samples from fermentation 27016-1h plotted as a function of induction time. Fermentation conditions were <i>P. pastoris</i> MSP 8.6 new seed bank, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 10 – 20 g/L methanol induction for 30 h.	106
Figure 61.	Percent conversion of 1.0 M lactate at the end of each 12 – 16 h reaction cycle. Reaction conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 15 reaction cycles. Initial cell concentrations were supplemented with either 2 g/L fresh cells or 3 g/L fresh cells at the start of the indicated reaction cycle.....	108

Figure 62.	HPLC chromatograms of pyruvic acid product from reactor 1 at completed cycles 1, 4, 8, 12, and 15 compared to 98% pyruvic acid standard from Sigma. Reactor conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 15 reaction cycles. Initial 15 g/L cell concentration was supplemented with 2 g/L of fresh cells at cycles 3, 5, 7 and 9, and fresh cell additions of 3 g/L at cycles 11 and 15. HPLC conditions were BioRad Aminex column at 30°C with 0.008 N sulfuric acid solvent at 0.6 mL/min and refractive index detection at 215 nm.	110
Figure 63.	Spray-drying as a platform technology to yield <i>P. pastoris</i> biocatalyst for production of several keto-acids, resolution of chiral hydroxy-acids, and enzyme activity tests such as the diagnosis of glycolate.	114
Figure A1.	UV-VIS spectrometer trace of Δ absorbance/time for optically pure 2-hydroxy acids. <i>P. pastoris</i> biocatalyst was prepared by spray-drying and GO activity was measured by DCIP enzyme assay as described in Materials and Methods.	119

CHAPTER 1: INTRODUCTION AND BACKGROUND

Pyruvate Usage

Pyruvate is an important chemical intermediate in the production of pharmaceuticals, agrochemicals, and fine chemicals. Pyruvic acid can be found in polymers and food additives, while pyruvate esters are used in the cosmetic industry and as “green” solvents. Common pharmaceutical elements synthesized from pyruvate are L-tryptophan, L-tyrosine, alanine, and the Parkinson’s drug, L-DOPA (Li, et al., 2001). Pyruvate is used in the making of agrochemicals, including indoxacarb based pesticides (McCann, et al., 2001), and indol-3-pyruvic acid for plant growth regulation (Arshad & Frankenberger, 1991). Although the mechanism is unclear, calcium pyruvate has recently been found to accelerate human fatty acid metabolism (Kalman, et al., 1999), suggesting pyruvate for weight loss applications.

Pyruvate Market Analysis

In 2005, the global pyruvate market was estimated at 1,490 tons (pyruvic acid equivalent) with ethyl and methyl pyruvate in highest demand (Avalon Consulting, 2005). Bulk pyruvate cost was found to be 11 USD/kg based on interviews with consumers, suppliers, and agents. This report and cost analysis was used to arrive at a global market value of 15 million USD for pyruvate. The end-use breakdown of pyruvate is shown in Figure 1. For the market analysis, health supplements or “nutraceuticals” derived from pyruvate have been categorized with other food additives. These food additives, along with agrochemicals, comprise almost one-half of pyruvate usage, while the remaining demand is entirely from the pharmaceutical industry. In pharmaceuticals, pyruvate is largely used to synthesize amino acids and building-blocks of complex drug molecules.

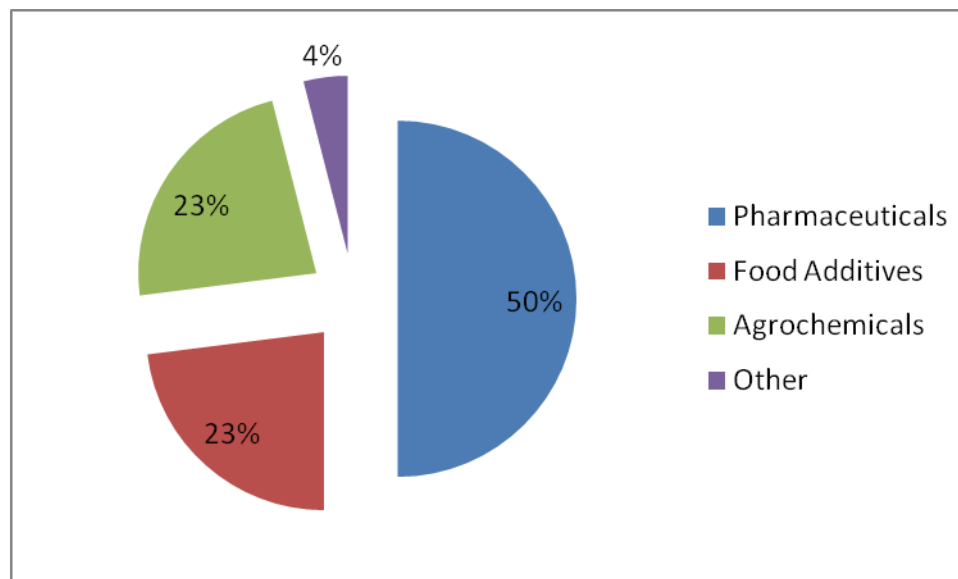


Figure 1. End-use breakdown for pyruvate.

Source: Avalon Consulting (2005). Pyruvate Market Analysis. In University of Iowa (Ed.).

Current Production Methods for Pyruvate

Today, there are three main routes for industrial production of pyruvate: (1) synthesis from tartaric acid, (2) direct fermentation, and (3) air oxidation of lactic acid.

- Synthesis from tartaric acid is the oldest method for pyruvate manufacture and is currently being used by at least two commercial plants. This route yields pyruvic acid by dehydration of tartaric acid in the presence of potassium hydrogen sulfate, followed by decarboxylation and multiple distillations (Howard & Fraser, 1932). The process is simple to achieve but requires extensive energy input and generates environmentally incompatible byproducts.

- Direct fermentation is an environmentally-friendly alternative to the tartaric acid process. In direct fermentation, an organism, such as the yeast *Torulopsis glabrata*, is modified to overproduce pyruvic acid during cell metabolism (Miyata & Yonehara, 1996; Yonehara & Miyata, 1994). Disadvantages of direct fermentation include low pyruvic acid yield based on glucose input and difficulties with product separation from complex fermentation broths.
- Comparatively, air oxidation of lactic acid is a superior process. This method offers 100% lactic acid conversion with single-pass pyruvic acid yields of 50 mol% (Ai & Ohdan, 1997). Air oxidation is based on vapor-phase oxidative dehydrogenation of lactic acid over an iron phosphate catalyst. This technique is now being adapted for large-scale production at Fudan University, China. However, it is not clear what percent of the world's pyruvate demand will be met by the air oxidation process. The company sponsoring this project (biocatalysis-based production of pyruvate) will perform a detailed economic analysis for comparison to other manufacturing routes.

Green Chemistry: Use of Enzymes for Biocatalysis

Biocatalysis can be defined as the use of enzymes, including whole-cells, to catalyze chemical reactions. Enzymes are capable of accepting a variety of substrates with extremely high chiral and positional selectivity (Schmid, et al., 2001). This high selectivity makes enzymes ideal for complex transformations by eliminating blocking groups required in the chemical synthesis of some molecules. One example is the production of L-DOPA (L-3,4-dihydroxyphenylalanine) using tyrosine phenol lyase by Ajinomoto (Ishige, et al., 2005). Some complex reactions, such as the stereo-specific hydroxylation of steroids, can be done in a single step (Das, et al., 2002). Additionally, high selectivity from renewable biocatalysts provide efficient reactions, typically occurring at near ambient conditions, with few byproducts (Schmid, et al., 2001). This

results in environmentally friendly “green” chemistry that is often safer than chemical synthesis by limiting exposure to harsh reaction conditions, including extreme pH, temperature, and pressure, as well as hazardous solvents. These specific advantages have led to an increase in the start-up of industrial-scale biotransformation processes in recent history (Figure 2).

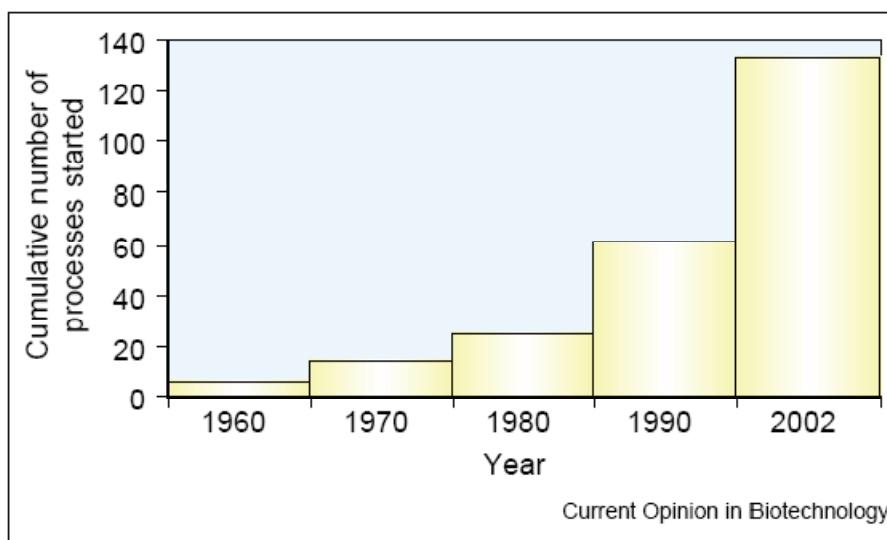


Figure 2. Cumulative number of biotransformation processes that have been started on an industrial scale.

Source: Straathof, et al. (2002). The production of fine chemicals by biotransformations. *Current opinion in biotechnology*, 13(6), 548-556.

According to Straahof, et al., the cumulative number of biotransformation processes that have been initiated on an industrial scale has increased from ≈ 60 in 1990 to ≈ 135 in 2002, and this trend is expected to continue. Criteria for inclusion as an industrial biotransformation process involved reactions in which pre-formed precursor molecules were converted, use of enzymes or whole cells either free or immobilized, production of a fine chemical or commodity that was later recovered, and successful

scale-up to over 100 kg product per year (Straathof, et al., 2002). While the largest enzyme-based processes were for production of carbohydrates, pharmaceuticals registered the greatest number of enzymatic processes. Of the nearly 135 enzyme-based processes analyzed, slightly over 50% were in the pharmaceutical sector, with other sectors (in order of decreasing process number) being food, agricultural, cosmetic, and polymer sectors (Straathof, et al., 2002). The large number of pharmaceutical processes underscores the importance of biocatalysis to generate chiral molecules often required for biological activity. Given the high substrate specificity and selectivity of enzymes, it is not surprising to find pharmaceutical applications that require chiral products or the resolution of racemic mixtures to pure enantiomers.

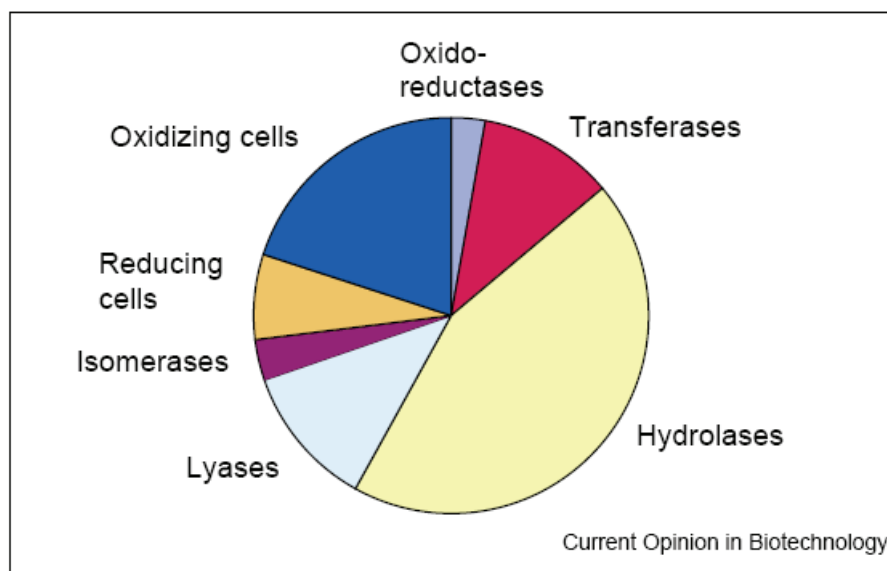


Figure 3. Enzyme types used in industrial biotransformations based on 134 processes.

Source: Straathof, et al. (2002). The production of fine chemicals by biotransformations. *Current opinion in biotechnology*, 13(6), 548-556.

Hydrolases are the most frequently used class of enzymes for commercial biotransformations (Figure 3). Other industrially important enzyme classes include transferases and lyases, followed in importance by isomerases and oxido-reductases. Oxido-reductases have the fewest industrial applications because of the requirement for expensive cofactors and their regeneration (Wildeman, et al., 2007). Hence, redox reactions are conducted using metabolically active cells or via another enzyme for regeneration of the cofactor (Zhao & van der Donk, 2003). One recent example is the use of metabolically active *P. pastoris* for improved NADH regeneration through an engineered methanol oxidation pathway (Schroer, et al., 2009).

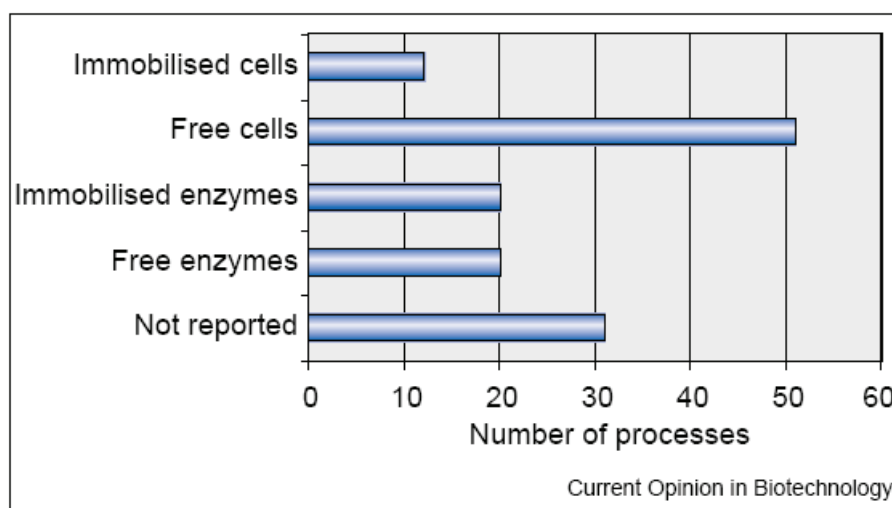


Figure 4. Use of enzymes or whole-cells in industrial biotransformations based on 134 processes.

Source: Straathof, et al. (2002). The production of fine chemicals by biotransformations. *Current opinion in biotechnology*, 13(6), 548-556.

Whole-cells are also used in many other types of biotransformations in addition to “redox” reactions. When using whole-cells for other reactions, the cells need not be

metabolically active other than one or two enzymes required for biocatalysis. At the industrial scale, whole-cells are the preferred method for biotransformation compared to purified enzymes (Figure 4). Whole-cells alleviate the need for expensive enzyme purification and generally do not require immobilization to preserve enzyme activity or aid recovery, such as in the industrial production of acrylamide from acrylonitrile (Yamada & Kobayashi, 1996).

Thrust of Current Work: Enzymatic Process for Conversion of Lactate to Pyruvate

Complex syntheses, high separation costs, generation of undesirable side-products and low yields have made enzymatic methods for producing pyruvate more attractive (Anton, et al., 1996; Gough, et al., 2005). For example, pyruvate can be produced via use of lactate oxidase (Ma, et al., 2004); however, the yield is low. Glycolate oxidase (GO) is a peroxisomal enzyme commonly found in the leaves of green plants, such as spinach and tobacco (N. Tolbert, et al., 1968; N. E. Tolbert, et al., 1949), as well as in animals (Duley & Holmes, 1976; Fry & Richardson, 1979; Schuman & Massey, 1971). In green plants, GO is involved in photorespiration (Zelitch, 1973), while in animals the enzyme is used for production of oxalate through metabolic pathways from serine and carbohydrates (Holmes & Assimos, 1998).

GO is a member of the oxidoreductase enzyme class; specifically, GO is a flavoprotein oxidase (Cederlund, et al., 1988; Lindqvist, 1989; Lindqvist & Branden, 1989). Flavoprotein oxidases catalyze redox reactions in which electrons are transferred from an electron donor to a flavin ring and, ultimately, to molecular oxygen (Ghisla & Massey, 1989). GO catalyzes the oxidation of several α -hydroxy acids to their corresponding α -ketoacids with the stoichiometric production of hydrogen peroxide (Clagett, et al., 1949; N. E. Tolbert, et al., 1949). This hydrogen peroxide byproduct can deactivate GO or further react with pyruvate to yield unwanted products. As a result,

catalase is introduced as a second enzyme to break-down the hydrogen peroxide byproduct (J. E. Seip, et al., 1993). Figure 5 shows the synergistic roles of GO and catalase in the production of pyruvic acid where GO catalyzes the oxidation of lactate acid and molecular oxygen is reduced.

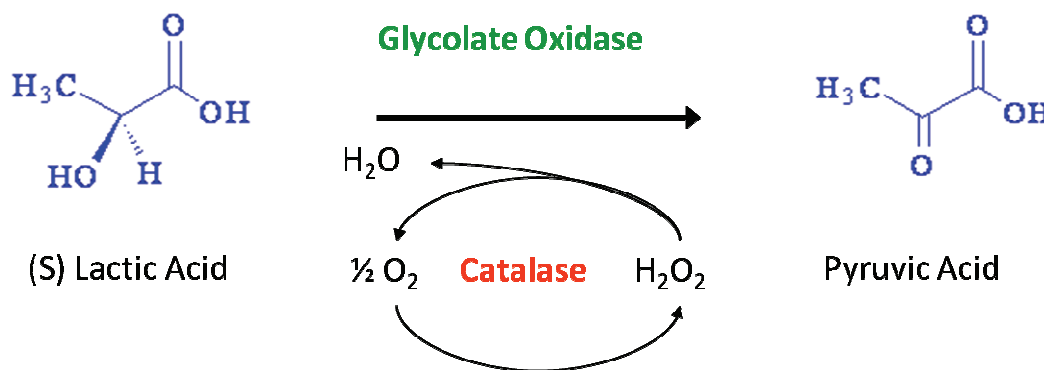


Figure 5. Enzymatic conversion of lactic acid to pyruvic acid by glycolate oxidase with catalase employed to break-down the hydrogen peroxide byproduct and minimize further oxidation of pyruvic acid to byproducts such as acetic acid.

As shown in Figure 6, GO from spinach was found to have the highest yield per gram of plant material and highest enzyme activity (J. E. Seip, et al., 1993). Spinach yielded 1.0 U of GO activity per mg plant material and 0.5 U per mg protein where 1.0 U of activity is defined as the amount of enzyme needed to catalyze 1 μ mol of substrate per minute (Karlson, et al., 1979). Soluble GO from spinach was combined with catalase T from *S. cerevisiae* to demonstrate feasibility of an enzymatic process for the production of pyruvate. Co-immobilization of the enzymes on oxirane acrylic beads increased enzyme stability and recovery (J. Seip, et al., 1994). However, the immobilization process did not alleviate problems associated with initially extracting GO from spinach. Also, significant loss of enzyme activity was observed after immobilization.

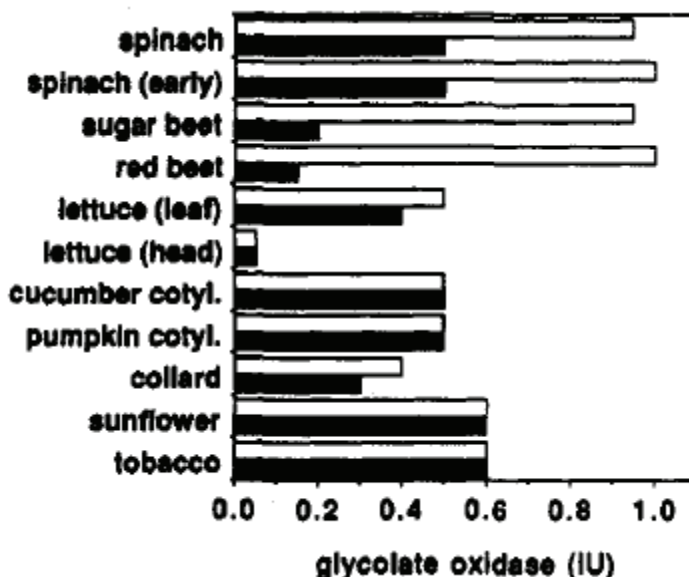


Figure 6. Glycolate oxidase yield and specific activity from various plant sources. Open bars denote total glycolate oxidase yield in U/g wet plant weight, while filled bars show specific activity in U/mg protein. Glycolate oxidase activities from plant extracts were measured by o-aminobenzaldehyde assay

Source: Seip, J. E., et al. (1993). Biocatalytic production of glyoxylic acid. *The Journal of Organic Chemistry*, 58(8), 2253-2259.

Cloning of Spinach GO: Construction of GO Expression

Vector Used in this Work

The pHIL-D4 expression plasmid (Phillips Petroleum) was used for cloning GO into *P. pastoris* (M. S. Payne, et al., 1995). The pHIL-D4 plasmid contained a strong methanol-inducible promoter, alcohol oxidase I, (*AOX1*) for expression of the GO gene. A 110 base-pair DNA fragment encoding for spinach GO (Volkita & Somerville, 1987) was subcloned into the *EcoRI* site of the pMP1 expression plasmid (Figure 7). The pMP1 plasmid was cut with restriction enzyme *ScaI*, and the linearized plasmid was used to transform *P. pastoris* GTS115, a his4 host (Phillips Petroleum). Approximately 100

His⁺ hosts were screened for reduced growth on methanol (Mut^S) due to disruption of the *AOX1* gene by integration of the GO expression cassette (*AOX1* transplacement). Slow growth on methanol was observed for 17 clones, and these clones were transferred to YPD medium. The YPD was supplemented with increasing concentrations of G418 to screen for high plasmid copy numbers based on Km^R resistance to G418. Two clones were resistant to over 1000 µg G418/mL, so these clones were selected as having the highest plasmid copy numbers and designated MSP10 and MSP12.

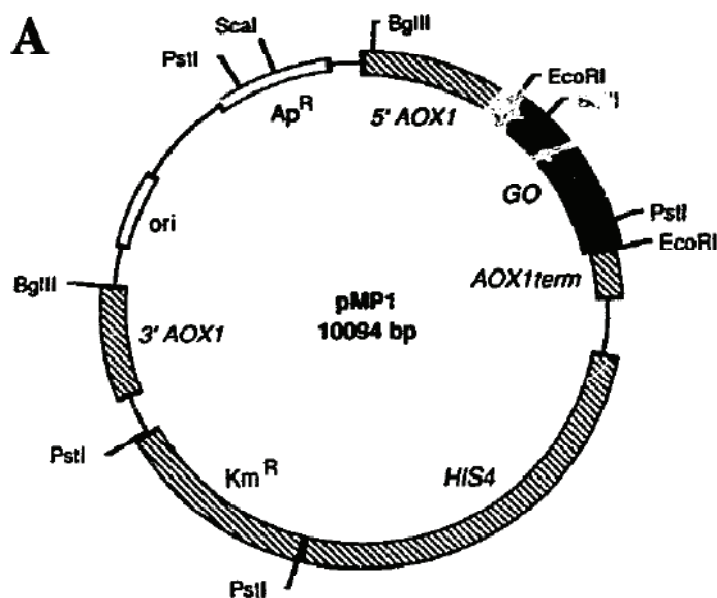


Figure 7. pMP1 expression plasmid contained the 110 base pair fragment encoding for GO from spinach downstream of the strong *AOX1* promoter. Other plasmid functions include Km^R for resistance to kanamycin, neomycin and G418, Ap^R for resistance to ampicillin, *HIS4* for chromosomal integration in *his4* hosts, and the *ori* replication origin for plasmid manipulations in *E. coli*.

Source: Payne, M. S., et al. (1995). High-level production of spinach glycolate oxidase in the methylotrophic yeast *Pichia pastoris*: Engineering a biocatalyst. *Gene*, 167(1-2), 215-219.

Incorporation of Catalase T from *S. cerevisiae* into GO

Expression Vector

The 1850 base pair *CTT1* gene encoding for catalase T was obtained by PCR amplification from *S. cerevisiae* DNA. An *EcoRI* site within the *CTT1* gene and lack of a plasmid that was suitable for multiple transformations made construction of several intermediate plasmids necessary. The resulting plasmid (pMP8) contained the *CTT1* expression cassette following the *AOX1* promoter, *HIS4*, Ap^R , *ori*, and also *SUC2* for selection in hosts previously transformed to His⁺ (Figure 8).

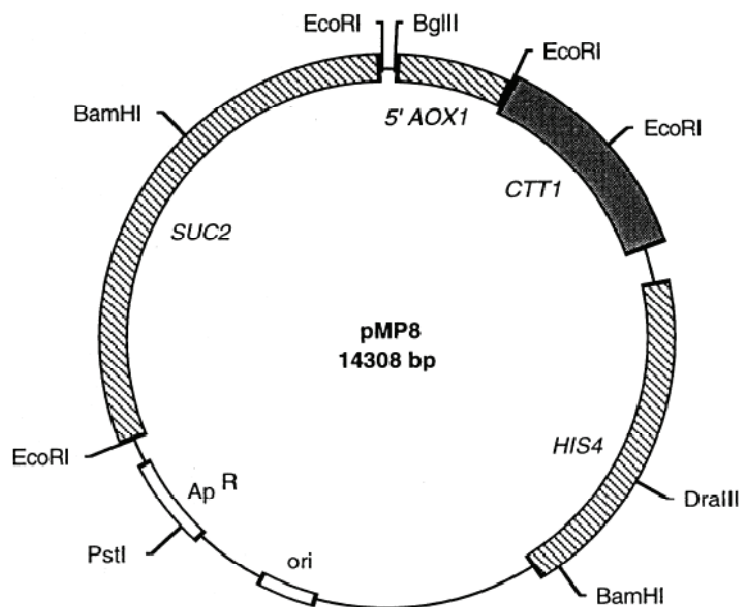


Figure 8. pMP8 expression plasmid contained the 1850 base pair *CTT1* fragment obtained from PCR amplification of *S. cerevisiae* DNA. Other functions in the vector include Ap^R for resistance to ampicillin, *HIS4* for chromosomal integration in *his4* hosts, the *ori* replication origin for plasmid manipulations in *E. coli*, and *SUC2* for selection in hosts previously transformed to His⁺.

Source: Payne, M. S., et al. (1995). High-level production of spinach glycolate oxidase in the methylotrophic yeast *Pichia pastoris*: Engineering a biocatalyst. *Gene*, 167(1-2), 215-219.

Plasmid pMP8 was cut with restriction enzyme *DraIII* and used to re-transform *P. pastoris* MSP10 that was previously developed for GO production. Plasmid pMP8 was cut at *HIS4* to minimize loss of the GO cassette by adding the *CTTI* cassette at an alternate location in the genome. Transformants were selected by re-plating on sucrose for selection against clones expressing invertase encoded by *SUC2*. PCR examination of the selected transformants showed 41% retained the GO expression cassette, and 89% of those clones had incorporated the *CTTI* cassette to yield double-transformants expressing both GO and catalase T (Mark S. Payne, et al., 1997).

Advantages of Whole-Cell Biocatalysis for Lactate to Pyruvate Conversion

Pyruvic acid production using a double-recombinant yeast for biocatalysis (Eisenberg, et al., 1997), has the advantage of expressing both GO and catalase together in one organism for maximum product yield (compared to adding partially purified soluble GO and catalase enzymes, which are expensive). Also, in this whole-cell method, exogenous FMN addition is not required. *P. pastoris* and *H. polymorpha* were each double-transformed to express recombinant GO from spinach and recombinant catalase T from *S. cerevisiae*. After fermentation, these organisms were treated with detergent to permeabilize cells, allowing substrate access to the internal enzymes for biocatalysis (Gavagan, et al., 1995). Results for the oxidation of 0.5 M L-lactic acid (Table 1) showed higher pyruvate yields and less acetate formation when using either permeabilized *P. pastoris* or permeabilized *H. polymorpha* compared to using similar concentrations of soluble GO and catalase (Eisenberg, et al., 1997).

Soluble GO (6.0 U/mL) and catalase (10000 U/mL) yielded 95% pyruvate and 4.5% acetate from 0.5 M L-lactate solutions. In contrast, permeabilized *P. pastoris* expressing recombinant GO (6.5 U/mL) and recombinant catalase (10100 U/mL) yielded 99% pyruvate and only 0.7% acetate. These results show the importance of co-locating

catalase and GO for rapid destruction of the hydrogen peroxide byproduct. Also, the effectiveness of whole-cell biocatalysis was more evident at higher starting lactate concentrations. At 0.75 M initial L-lactate concentrations, permeabilized *P. pastoris* generated 18% more pyruvate in one-tenth of the time compared to soluble GO and catalase (Eisenberg, et al., 1997). This was partially due to activity loss of soluble enzymes from high aeration and agitation conditions required for the oxidation process. In addition to higher stability, the permeabilized whole-cell biocatalysts offered simple enzyme recovery by centrifugation for reuse.

Table 1. Pyruvate production and byproduct formation using soluble GO and catalase compared to using microbial transformants expressing recombinant GO from spinach and catalase T from *S. cerevisiae*.

Lactate [M]	Catalyst	GO [U/mL]	Catalase [U/mL]	Pyruvate [%]	Acetate [%]	Lactate [%]
0.5	Soluble Enzymes	6.0	10000	95	4.5	0.9
0.5	<i>H. polymorpha</i>	6.5	5000	97	2.5	0.4
0.5	<i>P. pastoris</i>	1.1	2500	93	5.0	3.3
0.5	<i>P. pastoris</i>	2.3	5000	97	2.3	0.4
0.5	<i>P. pastoris</i>	6.5	10100	99	0.7	0.4

Source: Eisenberg, A., et al. (1997). Pyruvic acid production using methylotrophic yeast transformants as catalyst. *Journal of Molecular Catalysis B-Enzymatic*, 2(4-5), 223-232.

Enzymatic Process for Conversion of Lactate to Pyruvate
with Unit Operations Based on Detergent Permeabilization
of *P. pastoris*

Patents related to the cloning and expression of GO in *P. pastoris* were donated to the University of Iowa Research Foundation by DuPont de Nemours and Company

(Anton & DiCosimo, 1998; Anton, et al., 1995; Anton, et al., 1997; Anton, et al., 1993a, 1993b; Anton, et al., 1996). Early process evaluations were completed at the University of Iowa's Center for Biocatalysis and Bioprocessing using *P. pastoris* to yield pyruvate (Gough, et al., 2001; Gough, et al., 2005). Key results have been summarized:

- Production of GO at high yields (100 U/g) via high cell density *P. pastoris* fermentation at 100 L scale (7.2 Kg cells/100 L).
- Permeabilization of cells was achieved by simple detergent treatment using the cationic surfactant, benzalkonium chloride (BAC), to enable efficient whole-cell biocatalysis. However, prolonged exposure to BAC decreased enzyme activity, so permeabilized cells were washed extensively to remove the residual detergent.
- Several parameters for the enzyme reaction were established at the 30 L scale, including temperature, substrate loading, and enzyme recycle. Permeabilized whole-cells containing 6.7 U/mL GO and 12,500 U/mL catalase were recycled three times, with each cycle yielding around 97% conversion of 0.5 M lactate to pyruvate. This resulted in a modest “enzyme to product” ratio of about 1:2 based on calculations from reported data. Enzyme recycles were limited due to 50% GO activity loss in 80 h at 15°C and 50% catalase activity loss in 30 h. As a result of enzyme instability at 15°C, reaction temperatures were lowered to 5°C to extend useable biocatalyst life.
- In addition, recovery of pyruvate as ethyl ester was also demonstrated.

Though effective, the post-fermentation process for GO recovery and preparation for biocatalysis required several unit operations (a) solid/liquid separation to recover *P. pastoris*, (b) washing with phosphate buffer, (c) recovery of cells, (d) permeabilization of cells with BAC, (e) several cycles of washing with buffer to remove residual BAC, (f) recovery of cells, and (g) re-suspending cells in buffer for biocatalysis (Figure 9).

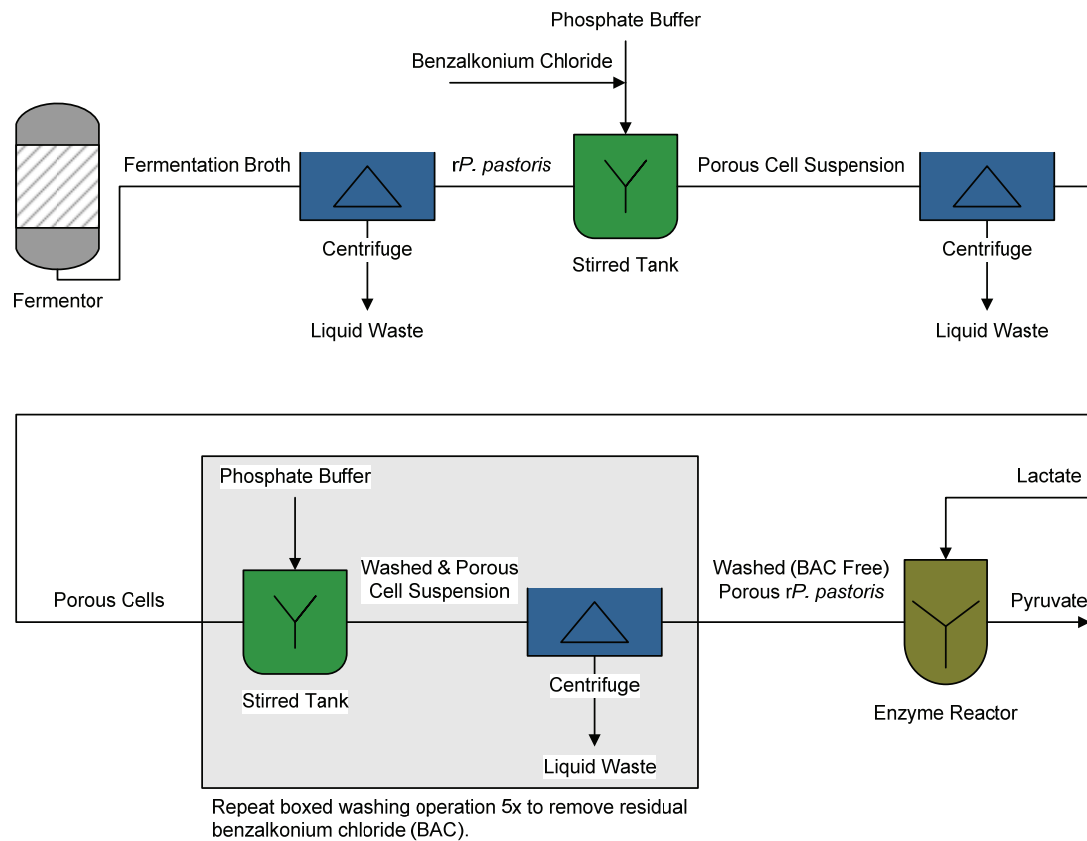


Figure 9. Enzymatic process for conversion of lactate to pyruvate with unit operations based on benzalkonium chloride (BAC) permeabilization of *P. pastoris*.

While the above process is an improvement over use of soluble GO and catalase, the calculated “enzyme to product” ratio is still too low to yield a commercially viable technology. Hence, the specific aims for the completed research were:

- To minimize unit-operations required for stable biocatalyst preparation,
- To optimize the enzyme reaction for best “enzyme to product” ratio,
- And to complete an “end to end” demonstration of the entire process to confirm “enzyme to product” ratio and pyruvate yield.

CHAPTER 2: MATERIALS AND METHODS

Detergent Cell Permeabilization for Biocatalysis

GO and catalase are expressed inside *P. pastoris* cells, therefore cells must be perforated to enable efficient biocatalysis. Previous work that was completed at the University of Iowa's Center for Biocatalysis and Bioprocessing used benzalkonium chloride detergent to permeabilize cell membranes (Gough, et al., 2001).

Materials for Detergent Cell Permeabilization

Potassium phosphate monobasic USP and potassium phosphate dibasic USP were obtained from Fisher, Pittsburgh, PA. Benzalkonium chloride detergent (BAC) was ordered from Sigma, St. Louis, MO.

Method for Detergent Cell Permeabilization

1. Prepared mixture of 10% w/v cells in 50 mM potassium phosphate buffer, pH 7.0.
2. Added 0.1% w/v BAC.
3. Placed solution on rotary shaker at 200 rpm for 60 min at room temperature.
4. Recovered cells by centrifugation (9950 x g for 10 min at 5°C).
5. Discarded supernatant and washed cells by re-suspension (10% w/v) in 50 mM phosphate buffer, pH 7.0.
6. Repeated washing (step 5) three times, and recovered cells by centrifugation.

Permeabilized cells were used immediately or stored at -80°C until use.

Buchi B-190 Spray-Dryer

Spray-drying was investigated as an alternative to detergent treatment to prepare *P. pastoris* cells for biocatalysis. A Buchi B-190 spray-dryer, New Castle, DE, (Figure 10) equipped with pneumatic nozzle cleaner was used for all spray-drying procedures. In the spray-drying process, a liquid feed is pressurized through a spray nozzle to create fine droplets in the presence of a hot gas. The feed can be a solution or suspension, and the

gas is typically air. The fine droplets and elevated temperatures cause evaporation of the carrier liquid, leaving the solute as dry powder.

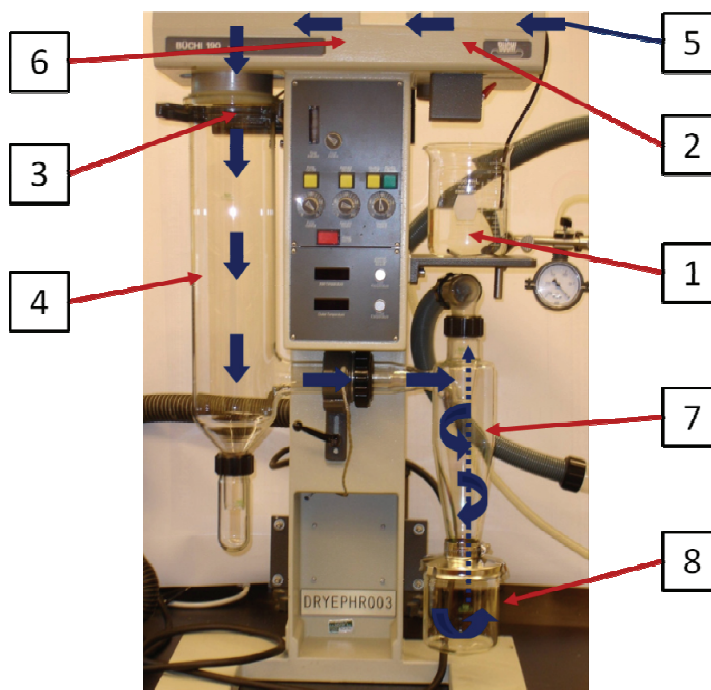


Figure 10. Buchi B-190 spray-dryer. Specific to the Buchi B-190 apparatus, the feed (1) is transferred by peristaltic pump (2, not visible) to the spray nozzle (3). Pressurized air drives the feed through the nozzle, creating tiny droplets that enter the drying chamber (4). Simultaneously, ambient air is drawn from the intake (5), through an electric heater (6, not visible) and into the drying chamber. The carrier liquid evaporates in the drying chamber and the dry powder is drawn by vacuum into the cyclone (7). The dry powder settles in the cyclone, and the product is gathered in the collection vessel (8).

Spray-Drying Materials

P. pastoris was obtained by the fermentation process described in Chapter 5. These cells were either spray-dried directly after fermentation or kept frozen at -80°C until needed. When frozen cells were used, they were thawed at 4°C . Excess water was removed by blotting the cells on filter paper. The blotted cells were diluted in deionized water to appropriate cell concentrations for spray-drying.

Spray-Drying Method

A Buchi B-190 spray-dryer with a pneumatic nozzle cleaner was used for all spray-drying procedures. Controlled operating parameters included cell concentration, feed rate, airflow, temperature, and vacuum level. For all experiments, the airflow was set to 700 L/h and the vacuum level was constant at -50 mbar. Experimental cell concentrations ranged from 30 to 600 mg/mL, feed rates spanned from 5 to 15 mL/min, and operating temperatures were tested from 120 to 195°C.

Blotted cells were weighed into separate containers to yield the appropriate cell concentrations after dilution to 100 mL total volume with deionized water. Then, the containers were mixed to form cell suspensions of 30 to 600 mg/mL.

Air flow was initiated to the spray-dryer at 700 L/h, the aspirator was set to -50 mbar, and the heater was powered on. Deionized water was pumped through the spray nozzle. The feed rate and operating temperature were set to desired experimental values. After reaching temperature equilibrium, the deionized water feed was replaced by the cell suspension. The spray-drying process lasted between 6 and 20 min based on the feed rate used. When the 100 mL cell suspension had been processed, the feed pump and heater were stopped, and air was circulated to reduce the spray-dryer outlet temperature to 70°C. Then, the aspirator and air supply were shut down. The collection vessel was removed, and the spray-dried product was transferred to glass vials. These vials were stored under dry conditions at room temperature. The feed line was flushed with water and the glassware rinsed before each cycle of spray-drying.

GO and Catalase Activity Calculation

GO and catalase activities were the two responses used for screening spray-dryer operating parameters. GO enzyme activity was measured by coupling the conversion of lactate to the anaerobic reduction of 2, 6-dichloroindophenol (DCIP). The bound FMN cofactor is reduced by lactate. In the absence of oxygen, DCIP accepts electrons from

reduced FMN, which alters double-bond conjugation to yield a color change (Figure 11). This change in color can be monitored by UV-VIS spectroscopy at 606 nm to determine GO activity (Armstrong, 1964).

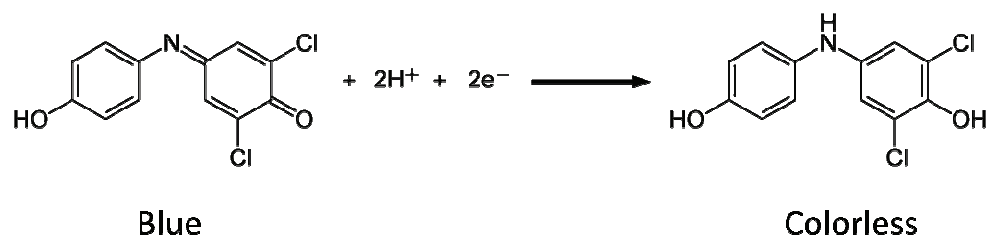


Figure 11. Reduction of 2, 6-dichloroindophenol.

UV-VIS spectroscopy was also used to measure catalase activity. Catalase breaks-down hydrogen peroxide to oxygen and water. This reaction was measured by tracking the disappearance of hydrogen peroxide at 215 nm (Aebi, 1983).

All enzyme activity measurements were performed using a Shimadzu UV-2450 spectrometer, Columbia, MA, equipped with Shimadzu magnet stir plates. The change in light absorbance per minute ($\Delta\text{Abs}/\text{min}$) at 606 nm or 215 nm for GO and catalase, respectively, was used in Equation 1 for enzyme activity. Other variables included the cuvette volume (V), molar extinction coefficient (ϵ), light path distance (L), and cell mass (M). Extinction coefficients were $\epsilon_{\text{DCIP}} = 22000 \text{ L mol}^{-1} \text{ cm}^{-1}$ (Armstrong, 1964) and $\epsilon_{\text{H}_2\text{O}_2} = 39.4 \text{ L mol}^{-1} \text{ cm}^{-1}$ (Aebi, 1983).

$$\text{Enzyme Activity [U/g]} = (\Delta \text{ Abs}/\text{min} * V) / (\epsilon * L * M) \quad (1)$$

Materials for GO and Catalase Activity Assays

P. pastoris cells were obtained by fermentation as described in Chapter 5 and prepared for biocatalysis either by detergent cell permeabilization or by spray-drying.

Molecular biology grade Tris base, potassium phosphate monobasic USP, potassium phosphate dibasic USP, and certified 30% hydrogen peroxide were acquired from Fisher, Pittsburgh, PA. 2, 6-Dichloroindophenol sodium salt and DL-lactic acid containing approximately equal amounts of D/L isomers were purchased from Sigma, St. Louis, MO. 3.0 mL quartz cuvettes with screw-top septum caps and 6 mm x 3 mm magnetic stir bars were obtained from Starna Cells, Atascadero, CA.

DCIP Assay for GO Activity

1. Transferred approximately 40 mg *P. pastoris* cells to a 15 mL centrifuge tube.
2. Added 8.0 mL of DCIP assay solution (0.12 mM 2, 6-dichloroindophenol in 80 mM Tris, pH 8.3) to the centrifuge tube.
3. Mixed to suspend cells and then removed 50 μ L of suspension (\approx 0.25 mg cells) and placed in 3.0 mL quartz cuvette with magnetic stir bar.
4. Added 2.0 mL of DCIP assay solution to the cuvette. Capped with septum and bubbled with nitrogen for 3 min.
5. To initiate the reaction, 40 μ L of 1.0 M L-lactic acid in 1.0 M Tris (pH 8.3) was added to the cuvette via syringe. Final concentrations were 0.12 mg/mL cells, 0.12 mM DCIP, 19.1 mM L-lactic acid, and 97.6 mM Tris.
6. Measured change in absorbance at 606 nm for 30 s with stirring.
7. Calculated GO activity using Equation 1.

H₂O₂ Assay for Catalase Activity

1. Transferred approximately 40 mg *P. pastoris* cells to a 15 mL centrifuge tube.
2. Added 8.0 mL of catalase assay buffer (16.7 mM phosphate buffer, pH 7) to the centrifuge tube.
3. Mixed to suspend cells and then removed 50 μ L of suspension (\approx 0.25 mg cells) and placed in a 3.0 mL quartz cuvette with magnetic stir bar.
4. Added 2.0 mL of catalase assay buffer to the cuvette.

5. To initiate the reaction, 1.0 mL of hydrogen peroxide solution (67 μ L of 30% hydrogen peroxide solution in 10 mL assay buffer, pH 7.0) was added. Final concentrations were 0.08 mg/mL cells, 0.07% hydrogen peroxide, and 16.7 mM phosphate buffer.
6. Measured change in absorbance at 240 nm for 30 s with stirring.
7. Calculated catalase activity using Equation 1.

GO and Catalase Stability Testing

Detergent permeabilized cells must be stored frozen at or below -20°C to preserve GO and catalase enzyme activity. However, stable room-temperature storage of the spray-dried biocatalyst would reduce process cost by (a) removing the need for frozen storage, and (b) allowing for spray-dried biomass from larger fermentations to be used on an “as needed” basis. The spray-dried cells were stored at room temperature in stoppered vials to prevent hydration. GO stability was evaluated by measuring GO activity over 19 days after spray-drying. Catalase activity was evaluated by measuring catalase activity over 20 days after spray-drying.

Detergent Permeabilization of Spray-Dried Cells

It was hypothesized that sharp temperature shifts and shear forces associated with the spray-drying process would perforate cells without the need for detergent treatment. To verify the hypothesis, spray-dried cells were treated with BAC detergent to determine if added detergent would further permeabilize cells for increased enzyme activity. The spray-dried cells were permeabilized as described in the Detergent Cell Permeabilization for Biocatalysis section. GO and catalase activities of spray-dried cells were compared to (a) cells that were treated with BAC after the spray-drying process, (b) cells that were treated with BAC only, and (c) cells that were neither spray-dried or treated with BAC.

GO and Catalase Leaching Tests

It is important to minimize leaching of GO and catalase from spray-dried cells to maintain enzyme activity and facilitate enzyme recovery. The GO leaching test was done by following a modified version of the DCIP Assay for GO Activity. After initial activity calculation (step 7 from DCIP assay), the cells were recovered by centrifugation, and the activity assay was repeated on the supernatant (step 8 listed below). The cell pellet was then re-suspended in fresh DCIP assay buffer for another activity measurement (step 9 listed below). This process of activity measurement and re-suspension in fresh assay buffer was repeated four times to determine enzyme leaching from cells.

8. Centrifuged cell suspension at 9950 x g for 10 min. Measured enzyme activity of supernatant according to steps 4 – 7 using supernatant instead of buffer in step 4.
9. Mixed to suspend cell pellet in 8.0 mL fresh DCIP assay buffer and repeated assay on cell suspension and supernatant.

The leaching test for catalase was done by measuring catalase activity as modified above.

Microscopy of Spray-Dried *P. pastoris*

Scanning electron microscopy of spray-dried cells was performed at the University of Iowa's Central Microscopy Research Facility. The spray-dried samples were mounted on aluminum Cambridge stubs using carbon tape, and the samples were sputter-coated with gold in the presence of argon. After sample preparation, analysis was completed using a Hitachi S-4800 scanning electron microscope, Pleasanton, CA, with an extraction voltage of 3.0 kV. Electron accelerating voltage was set to 18.0 kV and 30.0 kV to yield resolutions of 3.0 μm and 1.0 μm , respectively.

Expression of GO in *E. coli*

Dr. Michael Louie cloned the GO gene into *E. coli* expression plasmid pET-32a (Novagen), EMD Biosciences, San Diego, CA, from plasmid pPM1 (M. S. Payne, et al., 1995). The resulting plasmid was named pET-GO#11 and was transformed into *E. coli*

Rosetta-gami B(DE3) cells (Novagen) for protein expression. This recombinant strain was used for spray-drying studies.

Materials for Growth of *E. coli*

LB broth was obtained from Difco, Becton Dickinson, Franklin Lakes, NJ, and ampicillin sodium salt was purchased from Research Products International, Mt. Prospect, IL. Dioxane-free isopropyl- β -D-thiogalactopyranoside (IPTG) and chloramphenicol were received from Calbiochem, EMD Biosciences, San Diego, CA.

Growth of *E. coli* Expressing GO

A 100-mL seed culture of *E. coli* was grown in LB medium supplemented with ampicillin (100 μ g/mL) and chloramphenicol (34 μ g/mL) at 37°C for 16 h. The seed culture was used to inoculate 500 mL of the same LB medium to an initial cell density of 0.1 OD₆₀₀. The culture was incubated at 29°C with shaking at 180 rpm, until the OD₆₀₀ reached 0.3. The incubation was continued at 25°C with shaking at 230 rpm until the OD₆₀₀ reached 0.5. IPTG was then added to the culture (final concentration of 0.2 mM) to induce GO production. The induced culture was incubated at 25°C with shaking at 230 rpm for 16 h. The cells were harvested by centrifugation at 10,000 \times g and 4 °C for 10 min. Cells were stored frozen at -80°C, and the above growth procedure was repeated to generate a total of 11 g wet cell weight for spray-drying studies.

Spray-Drying *E. coli* for Active GO

E. coli cells were thawed at 4°C. The 11 g cell pellet was divided into two parts: 10 g were spray-dried, and the remaining 1 g was kept as control. The 10 g portion was diluted with deionized water to 40 mL final volume. This yielded a cell concentration of 250 mg/mL for the spray-dryer feed. The cells were spray-dried as described in the Spray-Drying Method section using the optimal conditions determined for *P. pastoris*. The resulting biocatalyst powder was collected for GO activity measurement. The

remaining 1 g *E. coli* cells (not spray-dried) were blotted on filter paper to remove moisture. The spray-dried cells and blotted (control) cells were assayed for GO activity.

Spray-Drying *S. cerevisiae* for Active Catalase

SAF-Instant *S. cerevisiae* was obtained from Lesaffre Yeast Corporation, Milwaukee, WI, as granular free-flowing yeast for use in baking dough. *S. cerevisiae* (10 g) was diluted with deionized water to a total volume of 100 mL. This yielded a cell concentration of 100 mg/mL for the spray-dryer feed. The cells were spray-dried using the optimal conditions determined for *P. pastoris*. The resulting biocatalyst powder was collected for catalase activity measurement. Another 40 mg of *S. cerevisiae* (not spray-dried) was used as control. The spray-dried cells and control cells were both assayed for catalase activity.

DASGIP Fed-Batch Pro System

The DASGIP Fed-Batch Pro system, Shrewsbury, MA, commissioned at the University of Iowa's Center for Biocatalysis and Bioprocessing had four parallel reactors, each with individual pH, dissolved oxygen, and temperature control (Figure 12). The reactors had a total volume of 1.5 L, with a working volume from 500 mL to approximately 1.2 L. Dissolved oxygen (DO) and pH levels were measured by Broadley-James K9 FermProbes, Irvine, CA, and Broadley-James 12 mm x 220 mm DO sensors, respectively. The dissolved oxygen was maintained by air supplied to the reactors at one VVM (volume of air per volume of medium per min) through sintered stones. These sintered stones yielded small bubbles with high surface area per volume of air to increase oxygen transfer. Additionally, oxygen levels were controlled by variable agitation using flat-blade Rushton impellers. After maximum agitation was reached, the sparge gas was enriched with pure oxygen as needed to meet oxygen requirements. The pH was controlled by acid and base addition using a DASGIP MP8 pump. The MP8 module contained eight pump heads, so each reactor had its own means of acid and base addition

(for two-sided pH control). As an alternative, one pump for each reactor could be used for one-sided pH control (either acid or base), and the second pump could be used to supply feed or substrate. Temperatures were measured with platinum electrodes, and all four reactors were contained within a BioBlock. Chilled glycol at 4°C was circulated through the BioBlock to maintain temperature set-points. Chilled glycol was also pumped through exhaust condensers to minimize water loss through exhaust vent filters. All pH, dissolved oxygen, temperature, and other control parameters were monitored by the DASGIP computer system and outputted to Microsoft Excel.



Figure 12. DASGIP Fed-Batch Pro system of four parallel reactors.

Materials for GO-Based Production of Pyruvate

P. pastoris cells were obtained by the fermentation process as described in Chapter 5. These cells were kept frozen at -80°C and were thawed at 4°C prior to use.

Excess water was removed by blotting the cells on filter paper. The blotted cells were diluted in deionized water for spray-drying at the optimized conditions determined in Chapter 3. For reaction studies, 88% biologically produced lactic acid, with 95% L isometric purity, was purchased from ADM, Decatur, IL. This stock lactic acid was pH adjusted using 10.0 M NaOH from Fisher, Pittsburgh, PA, and then diluted to appropriate concentrations with Tris base from Fisher or with deionized water. The reaction pH was maintained with addition of 5.0 M HCl and 5.0 M NaOH, both supplied by Fisher. Prior to analysis, samples were removed using 3.0 mL syringes, centrifuged in 1.5 mL micro centrifuge tubes, and supernatant was filtered through 0.2 μm Millex-GN syringe filter units purchased from Millipore, Billerica, MA.

GO-Based Production of Pyruvate in DASGIP

The pH optimization study utilized 1.0 M L-lactic acid in 80 mM Tris buffer, 60 g/L spray-dried cells, 15°C, and pH values of 5.0, 6.0, 7.0, and 8.0. This procedure was consistent for all enzyme reaction experiments unless otherwise noted. Approximately 108 g of stock lactic acid solution was combined with 80 mL of 1.0 M Tris, and pH was adjusted to 5.0 using 10.0 M sodium hydroxide. The substrate solution was diluted to 1.0 L, yielding a solution of 1.0 M L-lactic acid in 80 mM Tris at pH 5.0. This process was repeated to generate three additional 1.0 L solutions of 1.0 M L-lactic acid in 80 mM Tris at pH values of 6.0, 7.0, and 8.0 for the pH experiment.

A new workflow was created in the DASGIP software with one-sided pH control using acid, temperature control, and dissolved oxygen control using variable agitation. The option for pH and pO_2 probe calibration was chosen, and the workflow was labeled “pH optimization” to easily identify the experiment. The reactor type was selected as SR1000 ODSS, which is the DASGIP stirrer pro 1000 mL culture vessel with overhead motor-driven agitation. The reactor state was then changed to “free” to allow for further configuration of operating set-points. At this point, the individual reactors were labeled

pH 5.0, pH 6.0, pH 7.0 and pH 8.0 to indicate values for the experimental variable. The temperature was set to 15°C for all four reactors, and the pH was set to 5.0, 6.0, 7.0, and 8.0 for reactors 1, 2, 3, and 4, respectively. The pH was controlled by the addition of 5.0 M HCl, and default pH control parameters were adjusted for a more aggressive response. This resulted in an increased pH control proportional response from 15 to 40 and lowered reset time from 2400 to 1200 s.

For all reactors, the dissolved oxygen concentration was set to 90% (with air saturation calibrated as 100%). However, oxygen control was limited to air addition and agitation only. This decision was made based on studies showing that full conversion of lactic acid to pyruvic acid did not need oxygen enrichment (Gough, et al., 2005). Air was bubbled through the reaction solutions at 0.9 L/min (about 0.9 VVM) using submersed stone sparging elements. Minimum agitation was set to 500 rpm (46 mm OD, 6-blade Rushton impellers), and the agitation was increased to 1250 rpm based on oxygen demand. Operating the reactors with this oxygen control strategy resulted in an immediate increase to maximum agitation upon initiation of the reaction. During the oxidation reaction, the agitation remained at the maximum 1250 rpm, and the dissolved oxygen readings decreased to between 5% and 30% based on amount of spray-dried cells used for biocatalysis. The dissolved oxygen level was used to gauge the progress of the reaction because the oxygen reading would return to the controlled level of 90% after the oxidation of lactic acid was complete.

After the workflow was created and all operating set-points were specified, the pH probes were calibrated using pH 4.0 and pH 7.0 buffers. Concerning the dissolved oxygen probes, the integrity of the pO₂ membranes was checked and the electrolyte was replaced. The 1.0 M L-lactic acid solutions in 80 mM Tris at pH 5.0, 6.0, 7.0, and 8.0 were added to the appropriate reactors. The addition lines for pH control were primed with 5.0 M HCL. Each reactor was fitted with pO₂, pH, and temperature probes, and the probes were connected to their corresponding control modules. The pO₂ probe must be

connected to the dissolved oxygen control module for at least 6 h to polarize the membrane prior to calibration. Then, the agitator motors, acid addition lines, inlet air lines, and exhaust condensers were connected. The chilled glycol bath was set to 4°C and glycol was circulated to the BioBlock and exhaust condensers. The reactors were chilled to the 15°C operating temperature and held overnight while the pO₂ membranes were allowed to polarize. The next morning, airflow was started to the reactors at 0.9 L/min and agitation was initiated at 1250 rpm. The pO₂ readings stabilized, and the dissolved oxygen probes were calibrated to 100% air saturation at the given conditions.

The experimental control procedure was started, and probe readings were checked for accuracy using real-time DASGIP chart displays. Spray-dried cells (60 g) were each added to beakers labeled 1 – 4. A 60 mL syringe was used to remove 100 mL of substrate solution (multiple transfers) from reactor 1 through the sample port and this solution was used to re-suspend the biocatalyst in beaker 1. The procedure was repeated with separate syringes to re-suspend the biocatalyst for each reactor. After all biocatalyst had been re-suspended, the syringes were used to return the 100 mL substrate/biocatalyst mixtures to each reactor. Then, the inoculation timer was started and time zero samples were taken for lactate and pyruvate analysis.

Beginning with time zero, hourly samples were removed until completion of the oxidation reaction, as signified by depressed pO₂ levels returning to their 90% set-points. A 5 mL volume of reaction mixture was pulled (by syringe) from each reactor to flush sample lines, followed by removal of 1.5 mL samples for analysis. The 1.5 mL samples were then transferred to micro-centrifuge tubes and spun for 2 min at 16,000 x g. The supernatant was passed through 0.2 µm syringe filters to remove any remaining biocatalyst and stop reaction progress. All reaction optimization samples were quickly analyzed by enzymatic assay for L-lactic acid and pyruvic acid concentrations as described in L-lactate Measurement by Analox GM8 and Pyruvate Measurement by

Analox GM8, respectively. L-lactic acid disappearance and pyruvate formation, as well as product purity, was confirmed by HPLC.

After completion of the pH experiment, the run was “finished” in the DASGIP control procedure. Chilled glycol circulation was stopped, the inlet airflow was stopped, and all probes, addition lines, air lines, and exhaust condensers were disconnected. Reactors were removed from the BioBlock and the reaction mixture from each vessel was divided equally between two 800 mL centrifuge bottles. Separation of biocatalyst from pyruvic acid was done by centrifugation at 9950 x g for 10 min at 4°C. The product was stored at 4°C, while the biocatalyst was discarded or immediately reused (during enzyme recycling experiments). Water was used to flush HCl from the acid addition lines, the reaction vessels were washed with detergent, and the vessels and probes were rinsed with deionized water. Dissolved oxygen probes were stored in air and pH probes were stored in potassium chloride solution. Again, all enzyme reaction procedures were consistent with the pH experiment outlined in this section. Changes in set-points, cell concentrations, or substrate composition for the purpose of testing other experimental variables have been noted alongside presentation of those results.

Analox GM8 Metabolite Analyzer

The Analox GM8 Metabolite Analyzer, Lunenburg, MA, was used to measure L-lactate, pyruvate, glycerol, and methanol concentrations by enzymatic assay (Figure 13). Separate buffer and enzyme kits were required for each metabolite. In the presence of the substrate, the supplied enzymes catalyzed an oxidation reaction, which lowered the dissolved oxygen concentration in the reaction buffer. The oxidation could be direct, such as conversion of L-lactate to pyruvate and hydrogen peroxide by L-lactate oxidase. The oxidation could also be indirect, such as the conversion of glycerol and ATP to glycerol-3-phosphate by glycerol kinase, followed by the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate by glycerol-3-phosphate oxidase. In either

scenario, the decrease in dissolved oxygen was proportional to the initial substrate concentration, and this was compared to the oxygen consumption of a standard solution with known substrate concentration.

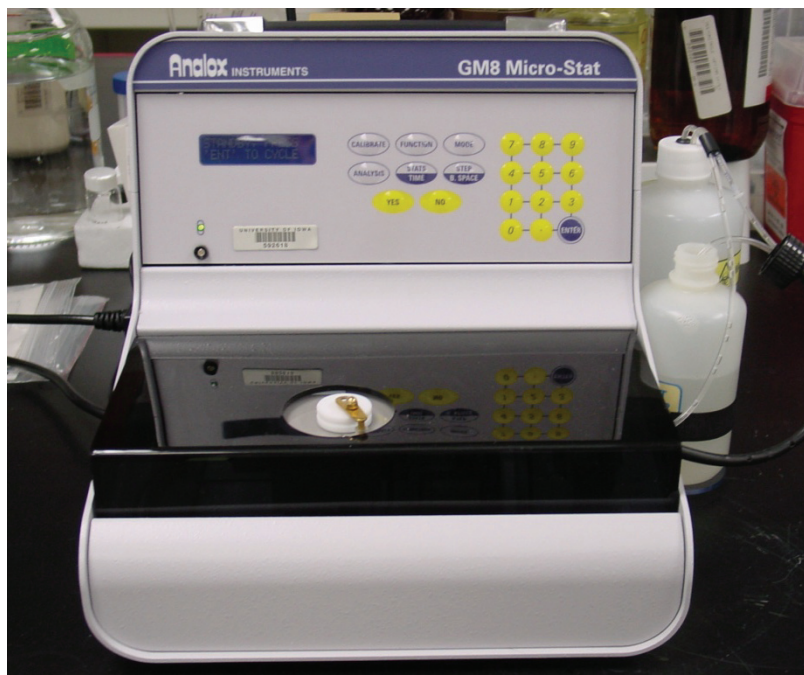


Figure 13. Analox GM8 Metabolite Analyzer for measurement of L-lactate, pyruvate, glycerol, and methanol by enzymatic assay.

Materials for Analox GM8 Measurement

For each metabolite assay, a separate kit containing lyophilized enzyme, buffer, additional reagents, and substrate standard was purchased from Analox, Lunenburg, MA. The L-lactate kit had L-lactate oxidoreductase, buffer solution at pH 6.5, and 8.0 mM L-lactate standard. The pyruvate kit had lactate dehydrogenase, peroxidase, NADH, Tris-succinate buffer, and 0.45 mM pyruvate standard. The glycerol kit provided glycerol kinase, glycerol-3-phosphate oxidase, ATP, PIPES buffer, and 20 g/L glycerol standard.

The methanol kit contained alcohol oxidase, and buffer solution at pH 7.4. A separate 20 g/L methanol standard was purchased from Analox. All reagents were stored at 4°C.

Prior to analysis, reagents were brought to room temperature and the lyophilized enzymes were reconstituted in the provided buffers. The buffer solution was connected to the instrument supply line, and the appropriate method (lactate, pyruvate, glycerol, or methanol) was selected. Buffer was cycled through the machine four times to prime the reaction chamber before calibration. Samples were prepared for analysis as described in Chapter 2 and Chapter 5.

L-lactate Measurement by Analox GM8

The Analox instrument was calibrated with 7.0 μ L of provided 8.0 mM L-lactate standard for measurements of 0.05 – 10.0 mM L-lactate. Samples were typically diluted 1:100 with deionized water, and 7.0 μ L of the diluted sample was used for analysis. Samples with initial L-lactate concentrations above 1.0 M were diluted accordingly.

Pyruvate Measurement by Analox GM8

The pyruvate analysis was indirect, so additional enzymes and co-factors were involved. A reagent blank was prepared with 25 μ L of NADH solution, 3.0 μ L of lactate dehydrogenase (LDH) suspension, and 100 μ L of deionized water. The standard was prepared in the same manner with 25 μ L of NADH, 3.0 μ L of LDH, and 100 μ L of 0.45 mM pyruvate. The blank and standard were incubated at room temperature for at least 5 min prior to calibration. The two-point calibration was performed by injecting 25 μ L of the blank followed by 25 μ L of the standard. This calibration gave linear reading for measurements of 0.02 mM – 1.5 mM pyruvate. Samples were typically diluted 1:1000, and 100 μ L of diluted sample was incubated with 25 μ L of NADH and 3.0 μ L of LDH for 5 min. Then, 25 μ L of incubated sample was injected for analysis. Samples with initial pyruvate concentrations above 1.0 M were diluted accordingly before incubation.

Glycerol Measurement by Analox GM8

For glycerol measurement, 7.0 μL of the 20 g/L standard was diluted in 250 μL deionized water, and the instrument was calibrated to 20 g/L by injecting 7.0 μL of diluted glycerol standard. To be consistent with the calibration, samples were also diluted 7:257 in deionized water and 7.0 μL of the dilution was used for analysis. This method provided linear readings from 4.0 – 30 g/L concentrations. For samples below 4.0 g/L, the 20 g/L standard was diluted 1:10. Then, the Analox was recalibrated with the new 2.0 g/L standard for linear measurements from 1.0 to 4.0 g/L.

Methanol Measurement by Analox GM8

For the methanol analysis, 5.0 μL of the 20 g/L standard was diluted in 500 μL of deionized water, and the instrument was calibrated to 20 g/L by injecting 5.0 μL of the diluted standard. For consistency with the calibration, samples were diluted 5:505 in deionized water and 5.0 μL of the dilution was used for analysis. This method gave linear readings from 5.0 – 40 g/L concentrations. Samples below 5.0 g/L were reanalyzed by injecting 10 μL of the diluted sample and multiplying the result by 0.5.

HPLC Analysis of Lactate and Pyruvate

The Shimadzu LC-20 Series HPLC, Columbia, MD, was used to confirm the conversion of lactate to pyruvate in the enzyme reaction. Samples for analysis were prepared as described in the GO-Based Production of Pyruvate in DASGIP section. Samples were analyzed with an Aminex HPX-87H 300 mm x 7.8 mm column purchased from BioRad, Hercules, CA, and 0.008 N sulfuric acid solvent. The column temperature was maintained at 30°C, and the solvent flow rate was held constant at 0.6 mL/min. Samples were diluted and loaded onto the column in 2 μL injections. Samples were analyzed for lactate and pyruvate content by UV detection at 215 nm, peak integration by Shimadzu software, and comparison to standard curves. At the specified conditions, approximate retention times were 8.9 and 11.8 min for pyruvate and lactate, respectively.

30 L Fermentation of *P. pastoris*

Fermentation of *P. pastoris* was completed at the 30 L scale for optimization of glycerol feeding, methanol induction, and vitamin addition for maximum biomass yield and GO and catalase activities. During the growth phase, total glycerol additions of 1,500 g and 2,000 g were tested for increased biomass yield. For induction, methanol concentration ranges of 2 – 10 g/L and 10 – 20 g/L were studied to determine the best levels for GO and catalase expression and final enzyme activities. Induction length from 24 to 35 h was studied for recovery of *P. pastoris* at peak enzyme activities. Also, riboflavin was added as an economical precursor to the flavin mononucleotide cofactor (FMN) needed for active GO (Lindqvist & Branden, 1985, 1989).

Materials for *P. pastoris* Fermentation

The following materials were purchased from Fisher, Pittsburgh, PA: USP grade glycerol, Optima methanol, enzyme grade ammonium sulfate, 28% certified ACS ammonium hydroxide, and FCC grade hydrochloric acid. YNB without amino acids and ammonium sulfate was obtained from Difco, Becton, Dickinson, Franklin Lakes, NJ. Spray-dried corn steep was purchased from Roquette, Geneva, IL. Reagent grade D-biotin was ordered from Amresco, Solon, OH, and poly-(propylene glycol) antifoam was ordered from Sigma, St. Louis, MO.

Method for *P. pastoris* Fermentation

Inoculum for the 30 L fermentor was grown in shake flasks. Shake flask medium (2.5 L) at pH 5.0 was prepared with 15.2 g/L glycerol, 5.0 g/L ammonium sulfate, 2.8 g/L YNB, and 320 µg/L biotin; deionized water was used to reach the final volume. 38 g glycerol and 12.5 g ammonium sulfate were dissolved in deionized water to a volume of 2.4 L. The glycerol and ammonium sulfate solution was equally distributed into four 1.0 L baffled Fernbach flasks and autoclaved for 20 min. 12.5 mL of post-sterile addition (7.0 g YNB and 800 µg biotin in 100 mL deionized water, filtered at 0.2 µm) was added

to each flask. The flasks were each inoculated with 0.5 mL *P. pastoris* Y-21187 seed culture at ≈ 0.5 OD₆₀₀ and incubated at 30°C and 200 rpm for 48 h.

The 30 L fermentor was prepared by replacing the DO probe electrolyte, checking the integrity of the membrane, and connecting the probe to polarize. The pH probe was connected and calibrated with pH 7.0 and pH 4.0 buffers. Both probes were installed in the vessel, and a new fermentation batch was initiated in the computer control software. The fermentor was sterilized for 30 min with 21.0 L deionized water containing 3.5 g/L ammonium sulfate, 10 g/L glycerol, and 5 mL poly-(propylene glycol) antifoam. The first post-sterile addition (141 g spray-dried corn steep in 1.0 L deionized water) was autoclaved for 60 min. The second addition of 11 mg biotin in 55 mL deionized water (0.2 mg/mL biotin) was sterile-filtered, and both solutions were aseptically added to the fermentor. The fermentor was held overnight, and sterility was confirmed the next day by microscopic examination.

The fermentation set-points were 30°C, 40% dissolved oxygen, and pH 5.0. The dissolved oxygen cascade was specified for air flow at 25 L/min and variable agitation from 200 to 1000 rpm. The pH control strategy was set for addition of 28% ammonium hydroxide and 5 M hydrochloric acid. The polarized DO probe was calibrated to 100% at the above operating conditions and maximum agitation. A sample was taken from the fermentor and the OD₆₀₀ was measured to establish a baseline.

After 48 h incubation, samples were removed from each flask and examined microscopically. The OD₆₀₀ was determined and expected to be in the range of 3 – 4. With confirmation of culture purity and cell density, two flasks were used to inoculate the fermentor, and the control system was synchronized to initiate the batch. A zero-hour sample was taken from the fermentor for cell density measurement and glycerol/methanol metabolite analysis. The metabolite sample was placed in a 1.5 mL micro-centrifuge tube and spun for 2 min at 16,000 x g. Then, the supernatant was filtered and analyzed for glycerol (pre-induction) or methanol (post induction) as described in Glycerol

Measurement by Analox GM8 or Methanol Measurement by Analox GM8, respectively. Hourly samples were removed to track cell density and maintain specified glycerol/methanol concentrations by adjusting the feed rates of later glycerol and methanol additions.

To increase biomass, glycerol feeding was initiated when batch concentrations fell below 2.0 g/L. Glycerol (10 L) feed solution was prepared with 150 g/L or 200 g/L glycerol, 18.8 g/L spray-dried corn steep, 1.0 mg/L biotin, and deionized water to obtain the final volume. 1,500 g or 2,000 g glycerol was dissolved in deionized water to a volume of 8.9 L. Separately, 188 g spray-dried corn steep was added to 1.0 L deionized water in a flask. Both feed solutions were autoclaved for 60 min, and the spray-dried corn steep solution was added to the glycerol solution. The post-sterile addition was prepared by dissolving 10 mg biotin in 100 mL deionized water, followed by filtration at 0.2 μm . The biotin solution was aseptically combined with the glycerol and spray-dried corn steep in an addition vessel for glycerol feeding by peristaltic pump. When fermentor glycerol levels fell below 2 g/L, the glycerol feed solution was started at 10.0 mL/min. Samples were taken for glycerol analysis, and the feed rate was adjusted to maintain the specified 2 – 10 g/L concentration.

When approximately 300 mL of glycerol feed remained, a 500 mL pre-induction sample was removed from the fermentor. The sample was centrifuged at 7,000 x g for 10 min and stored at 4°C for later spray-drying and GO and catalase activity assays. The culture was induced with addition of 10% or 20% v/v methanol. It was critical for resulting enzyme activity that methanol was added before complete glycerol exhaustion. Therefore, the glycerol feed was continued until the remaining 300 mL was depleted (another 30 min at 10 mL/min). When fermentor methanol levels fell below 4 g/L in the low concentration experiment or 10 g/L in the high concentration experiment, the methanol feed was started at 2 mL/min. Samples were removed for methanol analysis, and the feed rate was adjusted to maintain the specified 2 – 10 g/L or 10 – 20 g/L

concentrations. Additionally, 500 mL samples were taken at 15, 24, 30, and 35 h post-induction for spray-drying, along with the pre-induction sample, to track GO and catalase activities during the fermentation.

At 7 h post-induction a continuous YNB (containing riboflavin) and biotin feed was initiated. The YNB and biotin feed with YNB concentrations of 48.5 g/L or 97.0 g/L was tested for GO activity. The post-induction feed was prepared by adding 48.5 g or 97.0 g YNB and 28.6 mg biotin to 1.0 L deionized water. The feed was sterile-filtered and aseptically added to the fermentor at 0.6 mL/min by peristaltic pump. Enzyme activities were monitored throughout fermentation, and *P. pastoris* cells were harvested at peak GO and catalase activities ($\approx 30 - 35$ h post-induction) by centrifugation at 7,000 x g for 10 min. Total wet cell weight was recorded, and the biomass was divided into 1.0 kg aliquots and stored in freezer bags at -80°C . The fermentor was cleaned following standard operating procedures, and plots of operating parameters (DO, pH, temperature, and agitation) were generated. Cell densities and enzyme activities were plotted vs. run time, and enzyme expression data was obtained by SDS-PAGE.

SDS-PAGE Analysis of Enzyme Expression

In addition to GO and catalase enzyme assays, protein expression was confirmed by SDS-PAGE. Samples were taken right before induction and at 15, 24, 30, and 35 h post-induction for cell disruption, total protein measurement, and gel analysis. Samples were compared to molecular weight standards and *E. coli* transformed for GO expression. *P. pastoris* was obtained by fermentation and *E. coli* was grown in shake flasks as described previously.

Materials for SDS-PAGE Analysis

Potassium phosphate monobasic USP and potassium phosphate dibasic USP were obtained from Fisher, Pittsburgh, PA. Acid-washed glass beads (425 – 600 μm), and DL-dithiothreitol (DTT) were purchased from Sigma, St. Louis, MO. Yeast protein extract

reagent (Y-PER) and 2 mg/mL bovine serum albumin (BSA) were received from Pierce, Thermo Fisher Scientific, Rockford, IL. Bradford protein assay reagent was obtained from Bio-Rad, Hercules, CA. The following materials were purchased from Invitrogen, Carlsbad, CA: 12% Bis-Tris gel (10 well, 30 μ L comb), NuPage 4x lithium dodecyl sulfate (LDS) sample buffer, SeeBlue Plus2 pre-stained standard, NuPage MOPS SDS running buffer, and SimplyBlue safe stain.

Method for SDS-PAGE Analysis

Prior to gel analysis, cell disruption was accomplished by rapid mixing in the presence of Y-PER and glass beads. Approximately 200 mg of *P. pastoris* or *E. coli* samples were placed in 1.5 mL micro-centrifuge tubes and washed with 500 μ L of 50 mM potassium phosphate buffer at pH 7.5. Samples were centrifuged at 16,000 x g for 1 min, and the supernatant was discarded. An equal volume of glass beads (with respect to the cell pellet) was added to each sample along with 200 μ L of Y-PER. The samples were mixed rapidly for 30 s and cooled for 30 s, and the procedure was repeated to reach 4 total min of mixing. After cell disruption, samples were centrifuged at 16,000 x g for 2 min. Soluble fractions were retained for *P. pastoris* samples, and both soluble and insoluble fractions were saved for *E. coli* samples.

The soluble fractions were diluted 1:2000 with deionized water, and total protein concentrations were determined by Bradford assay using BSA standard. For preparation of the gel load, the original soluble fractions were pre-diluted to approximately 1.5 μ g/ μ L protein concentration based on Bradford results. 7.0 μ L of each pre-diluted sample was combined with 2.5 μ L of 4x LDS and 0.5 μ L of 1.0 M DTT. 1.0 μ L insoluble *E. coli* fraction was diluted with 6.0 μ L deionized water and combined with LDS and DTT as described for soluble fractions, and all samples were heated to 99°C for 6 min. The 12% Bis-Tris gel was loaded with 10 μ L SeeBlue Plus2 pre-stained standard and 10 μ L of each sample preparation (approximately 10 μ g protein loads). The gel was run using

NuPage MOPS SDS running buffer at 200 V for 50 min and stained with SimplyBlue safe stain following the Invitrogen microwave protocol.

Preparation of *P. pastoris* Seed Bank

Dehydrated yeast-tryptone-dextrose (YPD) was obtained from Difco, Becton, Dickinson, Franklin Lakes, NJ, and USP grade glycerol was ordered from Fisher, Pittsburgh, PA. 1.0 L of YPD medium at pH 6.5 was prepared by dissolving 50 g YPD in 1.0 L deionized water. The medium was equally divided (5 x 200 mL) among five 500 mL baffled Fernbach flasks. 50 mL of glycerol was added to one flask, and the flask was labeled YPD containing 20% glycerol. All flasks were autoclaved at 121°C for 20 min along with 115 cryovials, plastic trays to hold the cryovials, pipette tips, and an empty 250 mL flask.

Two sterile YPD flasks were each inoculated with 0.5 mL *P. pastoris* Y-21187 seed culture and incubated at 30°C and 200 rpm for 6 to 8 h. Incubation was stopped when cell densities reached 0.8 – 1.2 OD₆₀₀, and culture purity was confirmed by microscopic examination. One flask was selected, and 60 mL of the culture was mixed with 60 mL of the YPD containing 20% glycerol in the previously empty 250 mL flask. 1.0 mL cell suspension was dispensed into each cryovial, and the seed vials were capped, numbered, and labeled “*P. pastoris*, (MSP 8.6), Project 99999, Y-21187.” The new seed bank was stored in a cryovial freezer box at -80°C. The following day, two seed vials were randomly selected for inoculation of the two remaining YPD flasks. The flasks were incubated at 30°C and 200 rpm for 24 h, at which time a sample was taken to confirm cell growth (OD₆₀₀ > 6.0) and culture purity by microscopic inspection.

CHAPTER 3: DETERMINATION OF SPRAY-DRYING AS A
ROBUST PROCESS FOR *PICHIA PASTORIS* EXPRESSING
GLYCOLATE OXIDASE AND CALALASE FOR BIOCATALYTIC
CONVERSION OF LACTATE TO PYRUVATE

Traditional Biotransformation Processes

Most industrial biotransformation processes involve whole-cells, immobilized enzymes, or immobilized cells (Figure 4). However, there some disadvantages to using these systems. For example, the product could be metabolized by free or immobilized cells, especially a product like pyruvate. Processes with immobilized enzymes or cells must also account for the cost of immobilization and the cost of the inert support. Furthermore, immobilized biocatalysis may be compromised in terms of rate of product formation due to activity loss associated with immobilization (J. Seip, et al., 1994).

Incidentally, very few biotransformations involve spray-dried cells; i.e., there are limited references (Hanson, et al., 2000; Hutter, et al., 1995). One possible reason for this could be the elevated temperatures involved in spray-drying cells or enzymes. Typically, enzymes and cells are sensitive to higher temperatures. However, the spray-drying process involves only short residence time at higher temperatures. In addition, there is an added advantage in preparation of a dehydrated biocatalyst with the potential of offering stability for transport and storage. Spray-drying may also help to permeabilize cells by fracturing the cell wall, although cell fracture could result in leaching of enzymes from the cells. Nevertheless, in the present work it was decided to try spray-drying to (a) reduce unit operations shown in Figure 9 and (b) to stabilize and permeabilize the whole-cells, while retaining as much enzyme activity as possible.

Advantages of Spray-drying

Spray-drying is a well-established industrial process, most notably, in the dairy industry to yield dry powders of milk, cheese, and creamers (Henning, et al., 2006). In

the pharmaceutical industry, spray-drying of therapeutic enzymes is less common, but studies have shown that stabilizing agents, such as polyols, can be introduced to decrease activity losses (Mumenthaler, et al., 1994). Additionally, there has been success in spray-drying whole-cells with retained enzyme activity, including brewer's yeast (Luna-Solano, et al., 2005) and yogurt to preserve starter cultures (Bielecka & Majkowska, 2000). While full characterization of the spray-drying process can be challenging, scale-up from laboratory to production spray-dryers is readily accomplished from average particle residence time and spray-dryer evaporation capacity (Mazza, et al., 2003).

Specific Aim 1

Minimize unit-operations required for stable biocatalyst preparation. Spray-drying was investigated for replacement of BAC permeabilization and washing steps. Initial studies examined the feasibility of spray-drying *P. pastoris* for GO and catalase activity. It is important that both enzymes retain activity in one spray-drying process. The expectations and decision points for spray-drying *P. pastoris* to preserve GO and catalase activity are provided in Figure 14.

- For spray-drying to be an effective process model, spray-dried cells must yield dry powder that retains > 80% GO and catalase activity relative to BAC treated cells. Also, the dry powder should have a shelf-life of at least 30 days at room temperature, and enzyme leaching from cells should be < 10% of the original activity, based on activity loss after each reaction cycle. If these criteria are met, GO enantioselectivity will also be tested for retention of absolute selectivity to S hydroxyacids. This selective oxidation is not critical for the conversion of lactate to pyruvate. However, it will be ideal to use the spray-drying process for other GO-based applications, including production of chiral hydroxyacids, several of which are important pharmaceutical intermediates (Larissegger-Schnell, et al., 2006b; Storz, et al., 2003) and Appendix A.

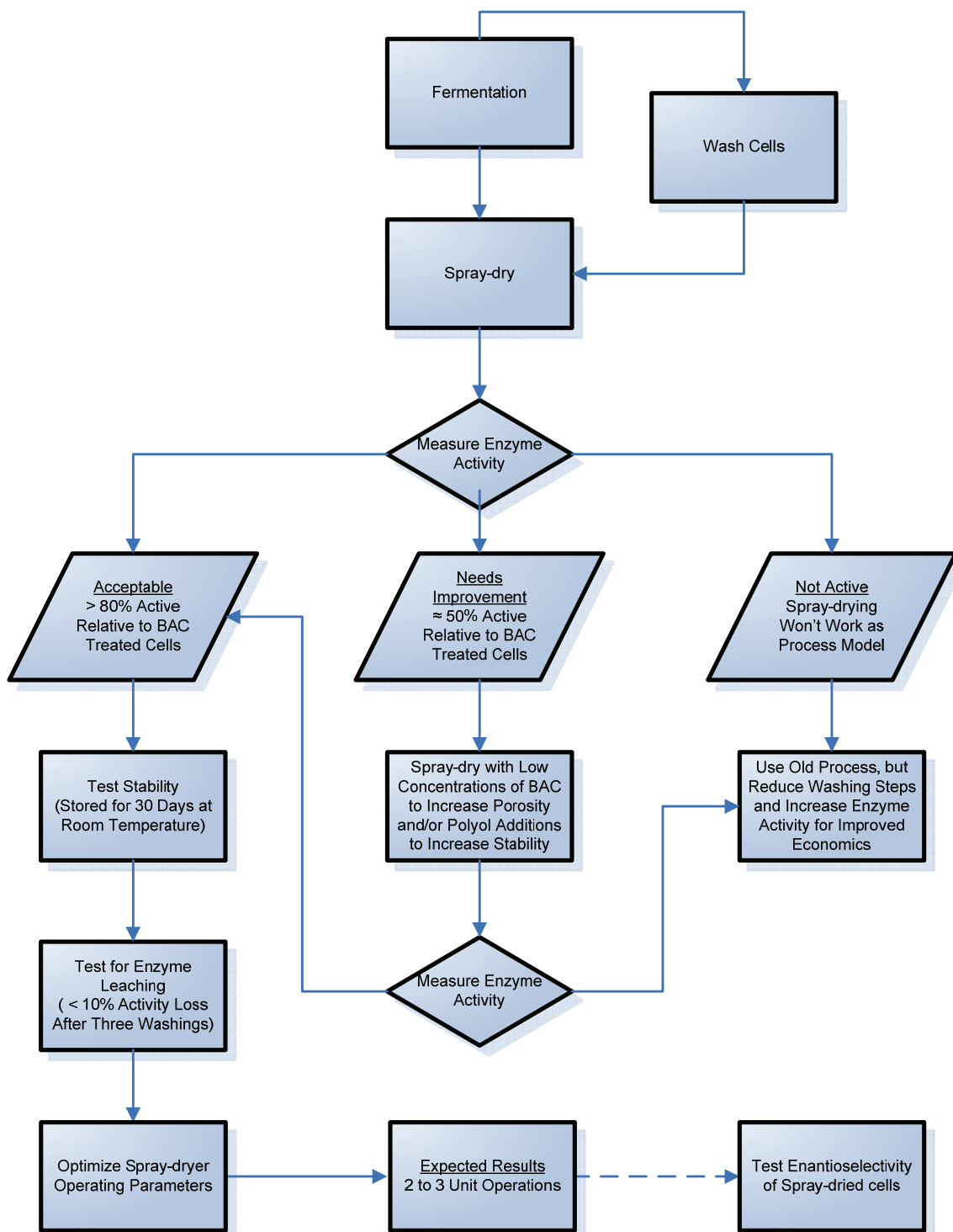


Figure 14. Spray-drying flowchart with benchmarks to determine process robustness.

If spray-drying is proven successful, the process will be optimized, and the unit-operations are expected to be reduced from over 7 to 2 – 3. Additionally, BAC will be completely removed from the process, thus omitting the need for an additional chemical treatment step and the disposal of this undesirable material.

- If the spray-dried cells retain $\approx 50\%$ activity observed with the BAC treated cells, it could be caused by inadequate cell permeabilization and/or activity loss due to high temperatures associated with the spray-drying process. In order to achieve $> 80\%$ enzyme activity, attempts would be made to spray-dry *P. pastoris* in the presence of low concentrations of BAC to increase cell porosity or in the presence of polyols to promote enzyme stability. Elevated spray-drying temperatures could lower BAC concentrations needed for permeabilization, and possibly, reduce washing steps necessary for residual BAC removal. Polyols would be added to the spray-dryer feed based on their known ability to stabilize some spray-dried enzymes (Liao, et al., 2002). Spray-dryer operating temperature can be lowered, and the residence time decreased, to reduce high temperature exposure and help to preserve GO and catalase enzyme activity.
- If the spray-dried cells achieve $< 25\%$ activity observed with BAC treated cells, it will be concluded that spray-drying will not be an effective process model. This could be due to inactivation of the enzymes by high temperatures and/or BAC exposure during spray-drying. If spray-drying does not work, attempts will be made to reduce the washing steps in the old process (Figure 14) in order to minimize unit operations and improve overall process economics.

Initial Screening of Spray-Drying Parameters to Determine GO and Catalase Activity

The initial screening investigated the feasibility of spray-drying *P. pastoris* to preserve GO and catalase activity. It was hypothesized that temperature shifts associated

with the spray-drying process would break-open the cells, and rapid moisture loss would stabilize the enzymes. The initial spray-drying study was designed to screen cell concentration, feed rate, and operating temperature for their relative effects on GO and catalase activity. Choices for these parameters were based on a 2002 paper by Liao, et al. Determination of spray-drying parameters with the greatest impact on enzyme activity would help in planning additional experiments to optimize these parameters.

A full-factorial experiment was designed with three factors: cell concentration, feed rate, and temperature. GO and catalase activities were the two responses used to screen the spray-dryer operating parameters. Each sample was spray-dried and assayed for GO and catalase activity. The appropriate cell concentrations were prepared by dilution of blotted cells in deionized water to yield a cell suspension for feed to the spray-dryer (mg blotted cells/mL liquid). The feed rate was the volume of cell suspension processed per minute (mL/min), and temperature was specified as inlet air temperature (°C). A high and low value was chosen for each of the three factors, resulting in eight experimental runs shown in Table 2.

Table 2. Factorial design parameters for screening spray-dryer cell concentration, feed rate, and operating temperature to determine GO and catalase activity.

Trial	Pattern	Temperature [C]	Feed Rate [mL/min]	Cell Concentration [mg/mL]
1	---	120	5	30
2	--+	120	5	100
3	-+-	120	15	30
4	-++	120	15	100
5	+--	150	5	30
6	+-+	150	5	100
7	++-	150	15	30
8	+++	150	15	100

JMP statistical software, SAS, Cary, NC, was used to design the screening experiment and analyze data. The constructed model incorporated cell concentration, feed rate, temperature, and all three interaction parameters (cell concentration*feed rate, cell concentration*temperature, and feed rate*temperature). Leverage plots are shown to gauge relative impact of the spray-drying parameters on GO and catalase activity.

GO Activity Results for Initial Screening of Spray-Drying Parameters

All tested spray-drying parameters were significant for GO activity as shown by the 95% confidence interval crossing the horizontal axes. Increasing temperature from 120 to 150°C had a slight negative effect on GO activity (Figure 15).

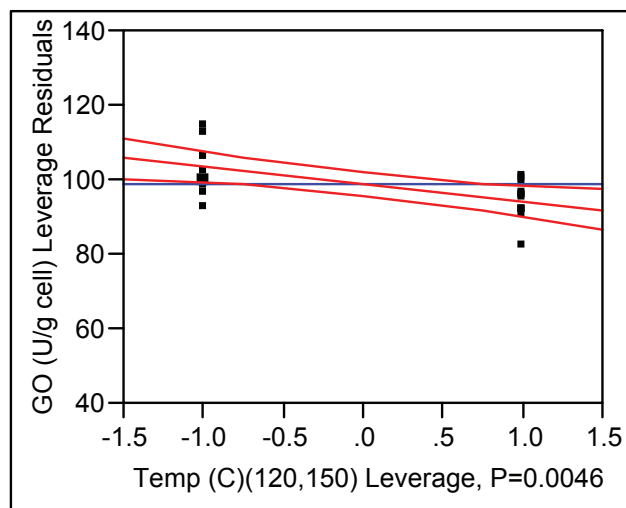


Figure 15. Effect of temperature (120 – 150°C) on spray-dried GO activity. Outer red lines represent 95% confidence interval.

Increasing the feed rate from 5 to 15 mL/min had the steepest leverage slope and the most significant effect on GO activity (Figure 16).

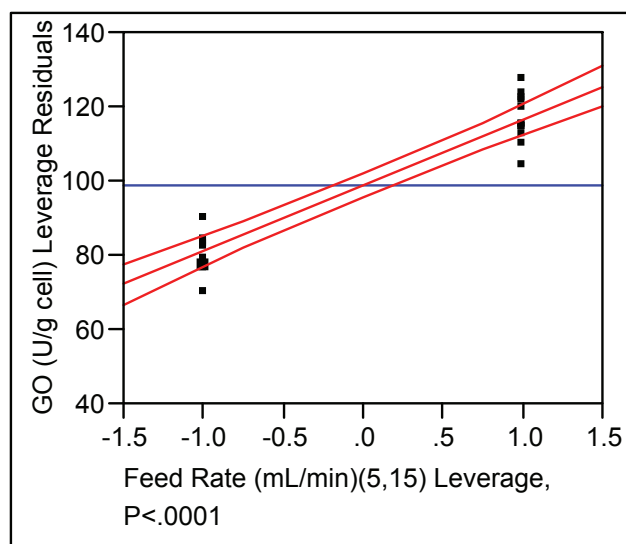


Figure 16. Effect of feed rate (5 – 15 mL/min) on spray-dried GO activity. Outer red lines represent 95% confidence interval.

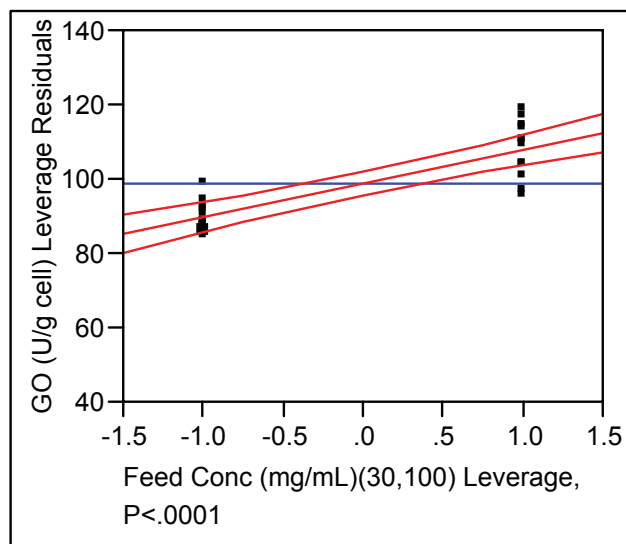


Figure 17. Effect of cell concentration (30 – 100 mg/mL) on spray-dried GO activity. Outer red lines represent 95% confidence interval.

While, increasing cell concentration from 30 to 100 mg/mL also increased GO activity (Figure 17). The temperature*feed rate interaction plot (not shown) suggests increasing the feed rate would minimize GO activity loss incurred when spray-drying at higher temperatures.

Catalase Activity Results for Initial Screening of Spray-Drying Parameters

Spray-drying temperature, feed rate, and cell concentration were also significant for catalase activity. Increasing the temperature from 120 to 150°C had a more drastic decrease on catalase activity compared to GO activity (Figure 18). Consistent with GO results, increasing the feed rate from 5 to 15 mL/min had the greatest positive impact on catalase activity (Figure 19). In addition, elevating the cell concentration from 30 to 100 mg/mL increased catalase activity (Figure 20).

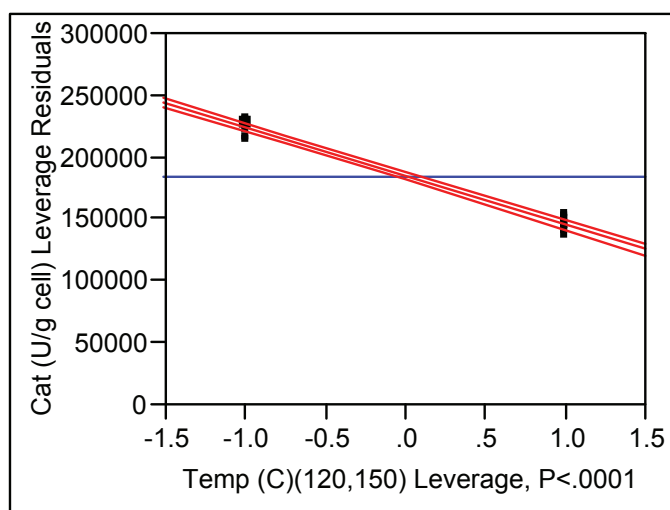


Figure 18. Effect of temperature (120 – 150°C) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.

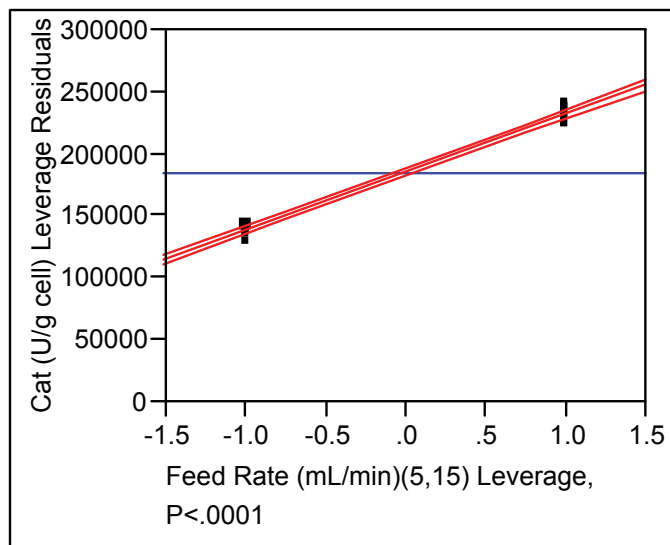


Figure 19. Effect of feed rate (5 – 15 mL/min) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.

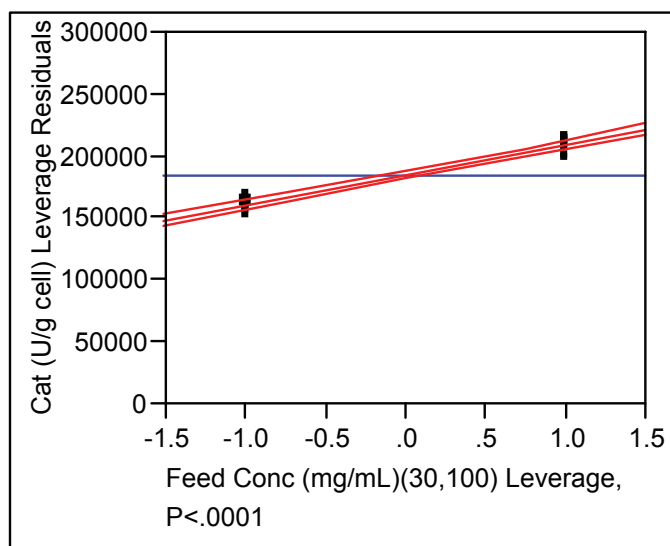


Figure 20. Effect of cell concentration (30 – 100 mg/mL) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.

Interaction plots for temperature*feed rate and temperature*cell concentration suggest that increasing the feed rate and the cell concentration would minimize catalase activity loss caused by spray-drying at higher temperatures (not shown).

First-Level Optimization of Spray-Drying Parameters with Respect to GO and Catalase Activity

After demonstrating that spray-drying *P. pastoris* preserved the activity of both GO and catalase, spray-drying conditions were then optimized to achieve maximum enzyme activity. When considering results from initial screening experiments for both enzymes (a) the higher feed rate resulted in better enzyme activity, (b) increased temperature lowered enzyme activity, and (c) increased cell concentration further helped to minimize activity losses when spray-drying at temperatures above 120°C.

Based on positive results, the spray-dryer feed rate was set to the instrument maximum of 15 mL/min. However, the more beneficial temperature of 120°C produced a “caked” powder (due to residual moisture) when operating at these higher feed rates. As an alternative, higher cell concentrations were investigated to compensate for the enzyme activity loss at temperatures above 120°C. A full factorial experiment was designed with two factors that were both important for enzyme activity (temperature and cell concentration). Three temperatures and three cell concentrations were tested, resulting in a total of nine combinatorial runs as shown in Table 3.

GO and catalase activities were responses used for screening the spray-dryer operating parameters. Each spray-dried sample was assayed for GO and catalase activity. JMP statistical software was used to develop a model based on temperature, cell concentration, and one interaction parameter (temperature*cell concentration). From Table 3, trial 9 (120°C, 100 mg/mL) and trial 12 (150°C, 100 mg/mL) were previous conditions that were repeated for consistency. In addition, higher feed concentrations and higher temperatures were investigated. The maximum controlled temperature (at the

specified feed rate) was 195°C. This upper temperature limit was studied to enhance results from increasing the feed concentration and to help determine a temperature optimum. Leverage plots are shown for temperature, cell concentration, and the temperature*cell concentration interaction.

Table 3. Factorial design parameters for first-level optimization of spray-dryer cell concentration, feed rate, and operating temperature with respect to GO and catalase activity.

Trial	Pattern	Temperature [C]	Cell Concentration [mg/mL]
9	1-1	120	100
10	1-2	120	200
11	1-3	120	400
12	2-1	150	100
13	2-2	150	200
14	2-3	150	400
15	3-1	195	100
16	3-2	195	200
17	3-3	195	400

GO Activity Results for First-Level Optimization of Spray-Drying Parameters

For trials 15, 16, and 17 (Table 3), spray-drying at 195°C resulted in significantly lower GO activities compared to the 120 and 150°C conditions (Figure 21). The benefit of spray-drying at higher cell concentrations is not evident from Figure 22 directly. The 95% confidence interval does not cross the horizontal axis, and increasing the cell concentration does not show a statistically significant change in GO activity.

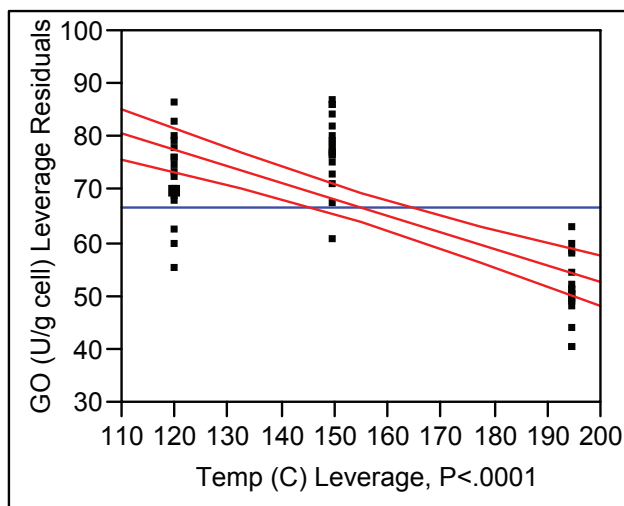


Figure 21. Effect of temperature (120 – 195°C) on spray-dried GO activity. Outer red lines represent 95% confidence interval.

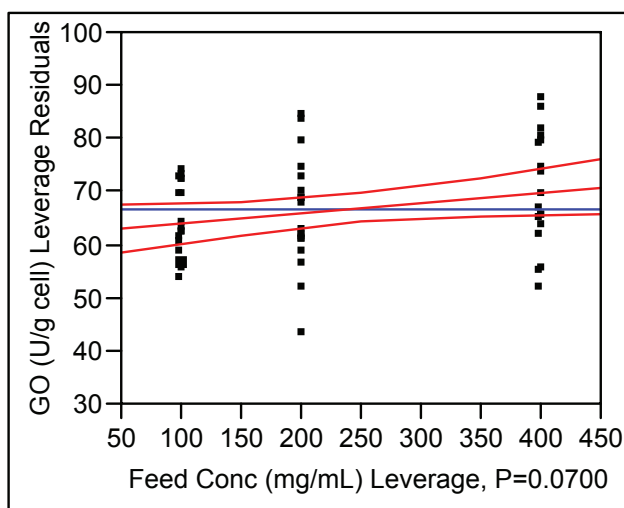


Figure 22. Effect of cell concentration (100 – 400 mg/mL) on spray-dried GO activity. Outer red lines represent 95% confidence interval.

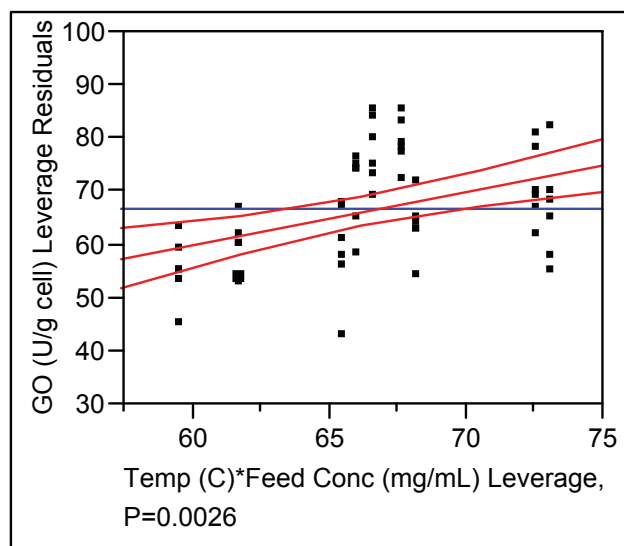


Figure 23. Effect of temperature*cell concentration interaction on spray-dried GO activity. Outer red lines represent 95% confidence interval.

However, there are two benefits for increased cell concentrations (a) more *P. pastoris* can be processed per unit volume, and (b) the temperature*cell concentration interaction plot (Figure 23) shows that higher cell concentrations helped achieve comparable GO activity between the 120 and 150°C runs. These results are important because spray-drying at 150°C yields a more uniform biocatalyst powder with less residual moisture than operating at 120°C.

Catalase Activity Results for First-Level Optimization of Spray-Drying Parameters

Consistent with GO, spray-drying at 195°C significantly decreased catalase activity compared to lower operating temperatures (Figure 24). Spray-drying at 120 and 150°C yielded comparable catalase activity.

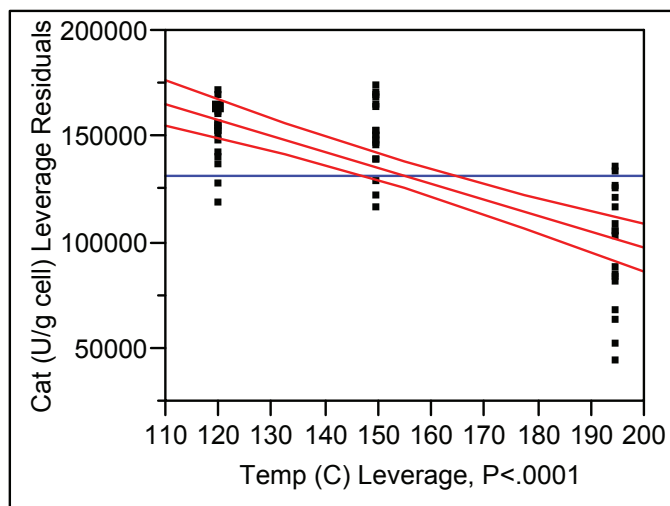


Figure 24. Effect of temperature (120 – 195°C) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.

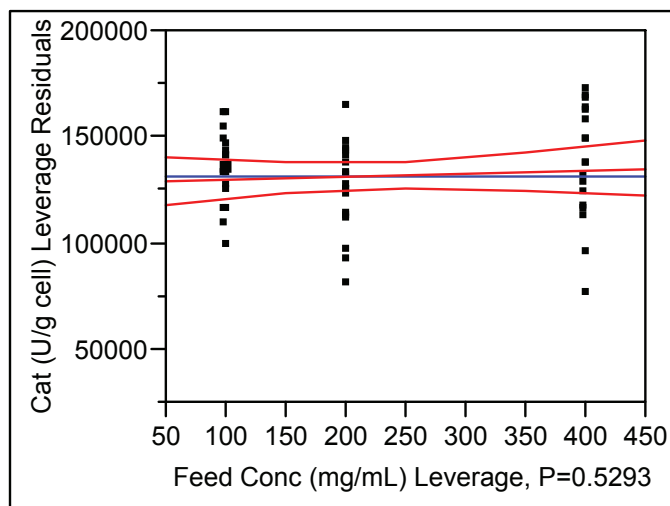


Figure 25. Effect of cell concentration (100 – 400 mg/mL) on spray-dried catalase activity. Outer red lines represent 95% confidence interval.

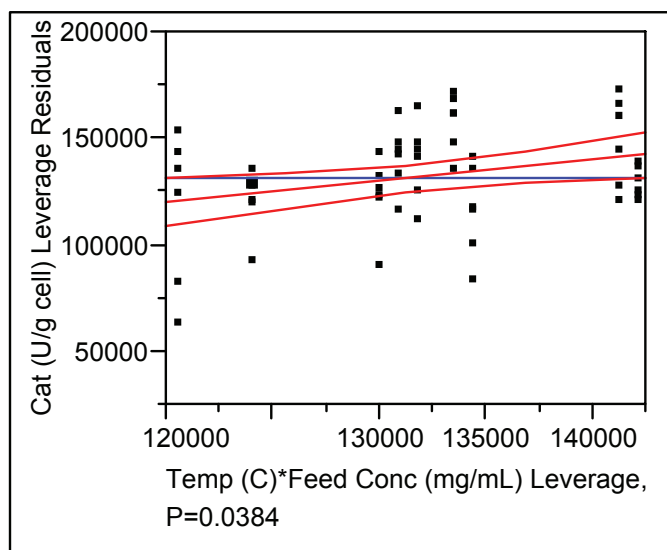


Figure 26. Effect of temperature*cell concentration interaction on spray-dried catalase. Outer red lines represent 95% confidence interval.

Increasing the feed concentration provided no statistical change in catalase activity, demonstrated by the feed concentration plot (Figure 25) and the temperature*feed concentration interaction plot (Figure 26). Catalase did not receive the same benefit as GO when spray-dried at higher feed concentrations. Catalase could be more resilient to temperature increases from 120 to 150°C, and therefore, not require higher cell concentrations to offset activity loss from spray-drying at elevated temperatures. To summarize, spray-drying *P. pastoris* cell suspensions up to 400 mg cells/mL can be accomplished without loss in GO or catalase activity.

Final Optimization of Spray-Drying Parameters to Create a Robust Process

Previous results demonstrated that increasing feed rate was the best way to retain maximum GO and catalase activity in spray-dried cells. Thus, the feed rate was set to the instrument maximum of 15 mL/min where higher temperatures and cell concentrations

were tested. Temperatures of 120 and 150°C yielded similar enzyme activities, but the 150°C condition provided a more uniform and dry biocatalyst powder. The maximum operating temperature of 195°C was studied, and clearly this temperature resulted in inactivation of both enzymes. Also, cell concentrations up to 400 mg/mL were tested without any statistical change in enzyme activity.

For further optimization of parameters, the best feed rate (15 mL/min) and best temperature (150°C) from previous studies were fixed. Increasing cell concentrations could still prove beneficial by increasing processing volumes and helping to maintain enzyme activity when spray-drying at higher temperatures. Cell concentrations of 200 and 400 mg /mL were repeated, along with one new parameter (600 mg/mL), to find an upper limit on cell concentration. The viscosity of the 600 mg/mL condition decreased the feed rate to 13 mL/min. Concentrations above 600 mg/mL were not tested because viscosity issues and pump limitations significantly decreased feed rates below the 15 mL/min optimum. Tested conditions are summarized in Table 4.

Table 4. Final optimization of spray-drying cell concentration.

Trial	Cell Concentration [mg/mL]
18	200
19	400
20	600

GO and Catalase Activity Results for Final Optimization of Spray-Drying Parameters

For final optimization, the feed rate was constant at 15 mL/min, temperature was set to 150°C, and cell concentrations of 200, 400 and 600 mg/mL were tested.

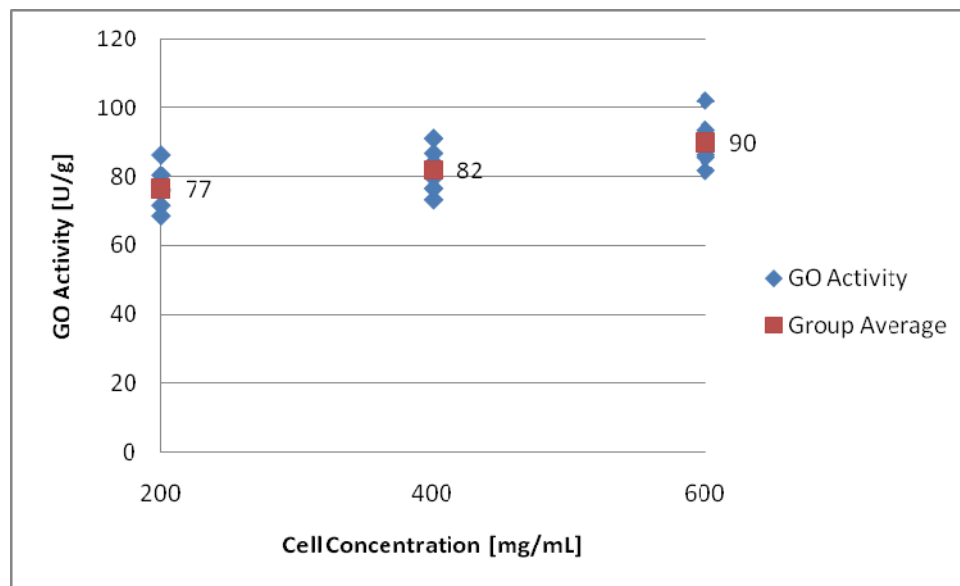


Figure 27. GO activity for spray-dried cells at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL.

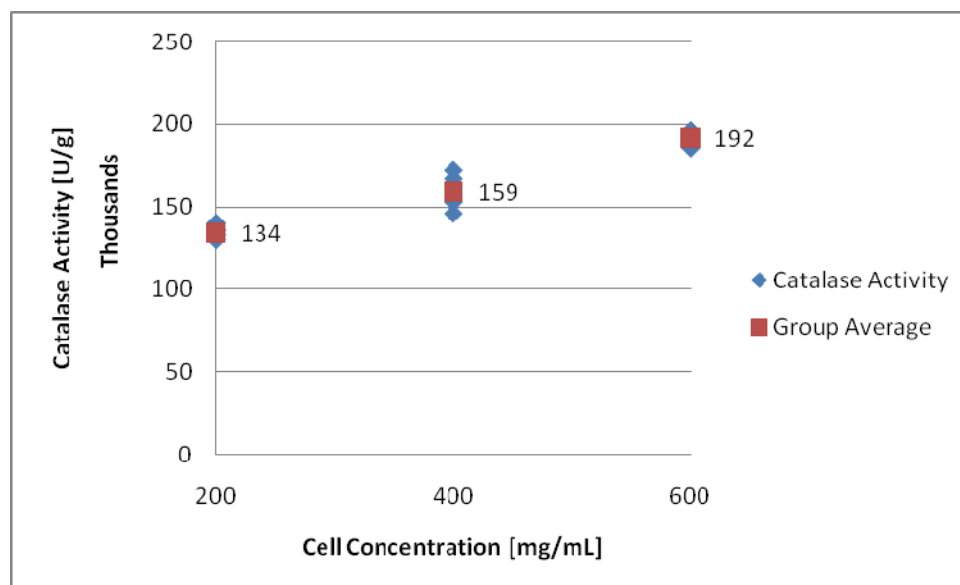


Figure 28. Catalase activity for spray-dried cells at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL.

Six assays were completed for each condition and used to calculate enzyme activities. These enzyme activities were plotted along with their average values. All three conditions tested had overlapping GO activities, but the average values increased slightly with each successive feed concentration (Figure 27). The catalase assay showed less variability, and the 600 mg/mL condition clearly had the highest activity (Figure 28).

Stability of GO and Catalase to Create a Robust Process

Earlier work used BAC detergent to permeabilize cells in order to maximize GO and catalase activity of *P. pastoris* (Gough, et al., 2001). The BAC treated cells required storage at -80°C to maintain enzyme activity (Gough, et al., 2001). In contrast, Spray-dried cells (present work) were stored at room temperature. The spray-dried cells were sampled for enzyme activity three times over 19 days to determine the stability of GO and catalase at room temperature.

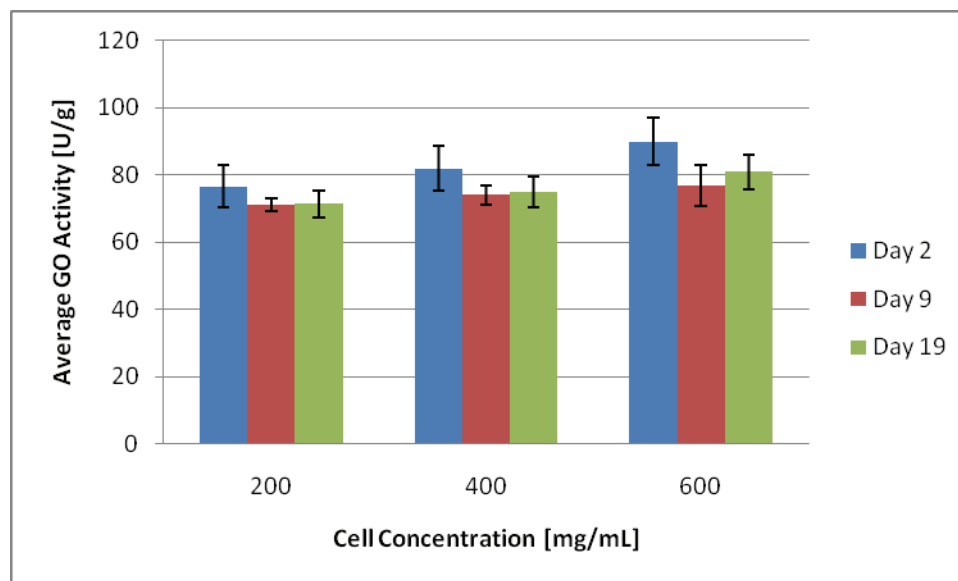


Figure 29. Average GO activity of spray-dried cells stored at room temperature. Spray-drying was done at 150°C , 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL. Error bars signify one standard deviation ($n=6$).

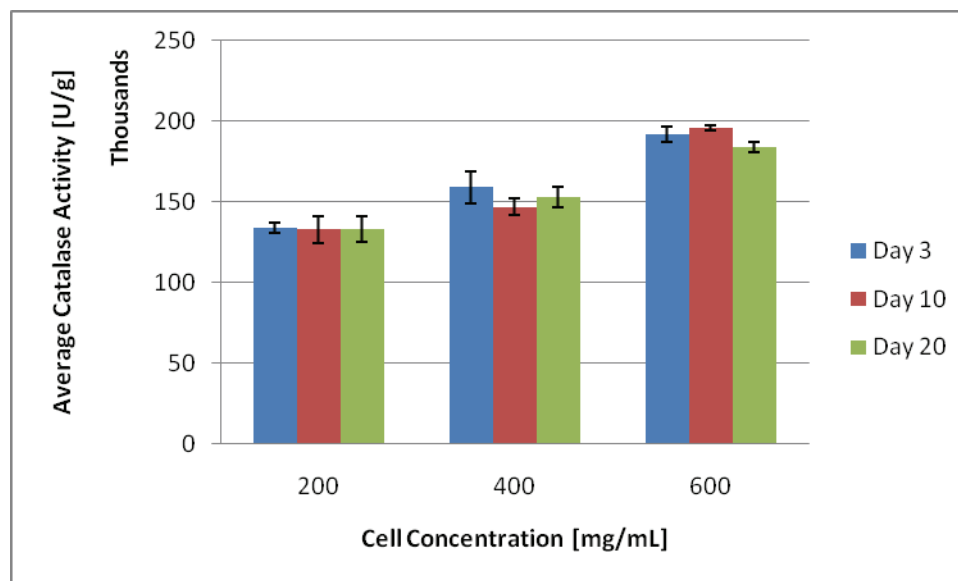


Figure 30. Average catalase activity of spray-dried cells stored at room temperature. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL. Error bars signify one standard deviation (n=6).

For each condition, six absorbance measurements were taken. The average value was plotted with error bars signifying one standard deviation. As is evident from Figures 29 and 30, no statistical loss in activity was observed for GO or catalase under any spray-drying conditions tested in Table 4. While GO and catalase stability was confirmed up to 20 days for the given batch of cells, another batch maintained 90% of initial GO and catalase activity at the benchmark of 30 days.

Comparison of GO and Catalase Activities of Spray-Dried

Cells vs. BAC Treated Cells

The current procedure of spray-drying *P. pastoris* to obtain maximum enzyme activity was compared to previously established methods for permeabilization via BAC treatment (Gough, et al., 2001). BAC treated cells were permeabilized and washed following procedures outlined in Materials and Methods. GO and catalase activities of

spray-dried cells were compared to (a) cells that were treated with BAC after the spray-drying process, (b) cells that were treated with BAC only, and (c) cells that were neither spray-dried or treated with BAC. As shown by Figures 31 and 32, cells that were not spray-dried required BAC treatment to reach maximum levels of GO and catalase activity, but spray-dried cells already had high levels of activity without adding the detergent. Furthermore, cells that were treated with BAC detergent after spray-drying had lower GO activity compared to non-BAC treated and spray-dried cells (Figure 31) but gave comparable or slightly higher catalase activity (Figure 32). This demonstrates that spray-drying had already permeabilized *P. pastoris* for maximum activity and additional treatment with BAC detergent compromised GO activity. Thus, additional permeabilization of spray-dried cells was not necessary.

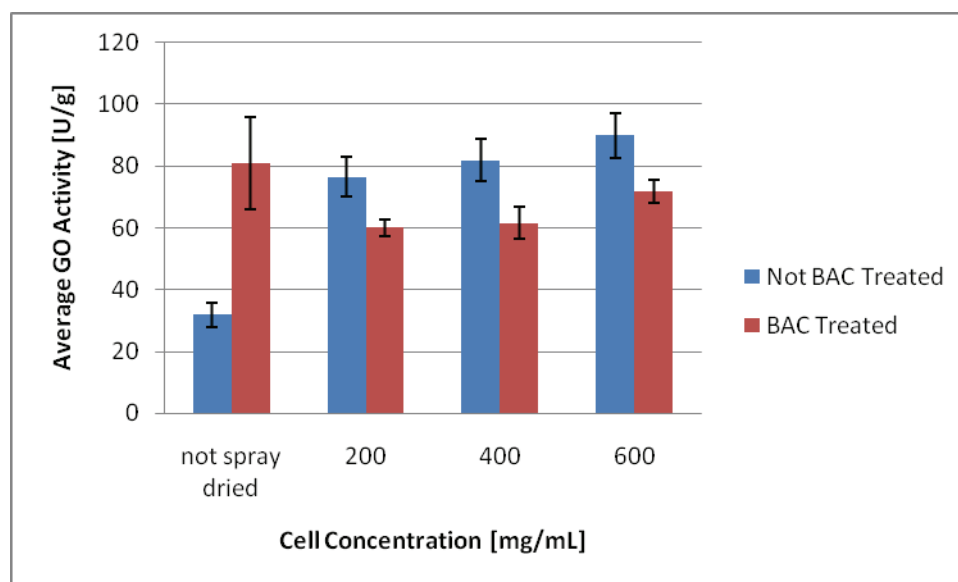


Figure 31. GO activity of BAC treated and spray-dried cells. Blue Bars represent no BAC treatment and red bars indicate BAC treatment. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200, 400, and 600 mg/mL. Error bars represent one standard deviation (n=6).

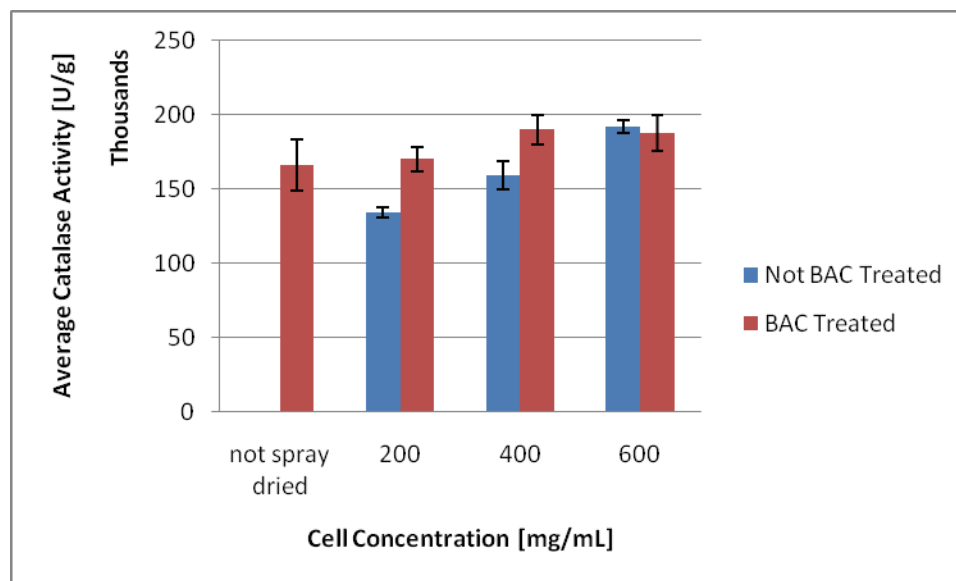


Figure 32. Catalase activity of BAC treated and spray-dried cells. Blue Bars represent no BAC treatment and red bars indicate BAC treatment. Spray-drying was done at 150°C, 15 mL/min feed rate, and cell concentrations of 200 mg/mL, 400, and 600 mg/mL. Error bars represent one standard deviation (n=6). Cells not spray dried and not treated with BAC had no catalase activity.

Determination of GO and Catalase Leaching from Spray-Dried Cells to Ensure a Robust Process

P. pastoris was spray-dried at optimal conditions obtained from factorial design studies (150°C, 15 mL/min feed rate and 600 mg/mL cell concentration) to test for leaching of GO and catalase from spray-dried cells. Enzyme activities were measured, cells were washed with fresh assay buffer, and activity measurements were repeated as described previously. No statistical change in GO or catalase activity was observed after each of three washings (Figures 33 and 34). Furthermore, no measurable enzyme activity was detected in the wash supernatant (not shown). This is a strong indication that spray-dried cells did not leach GO and catalase at detectable levels and is promising for industrial biocatalytic applications.

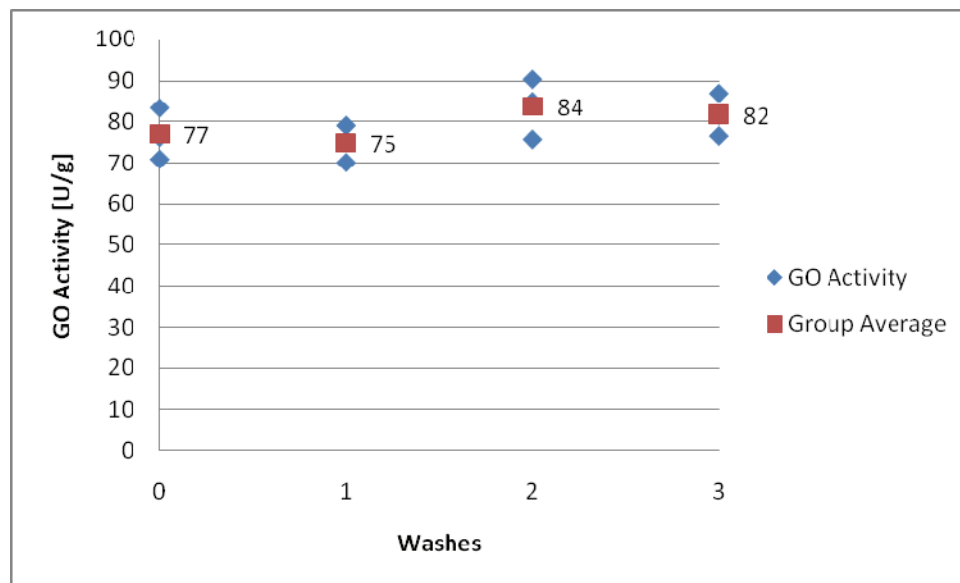


Figure 33. Test for GO leaching from spray-dried cells. Spray-drying conditions were 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration.

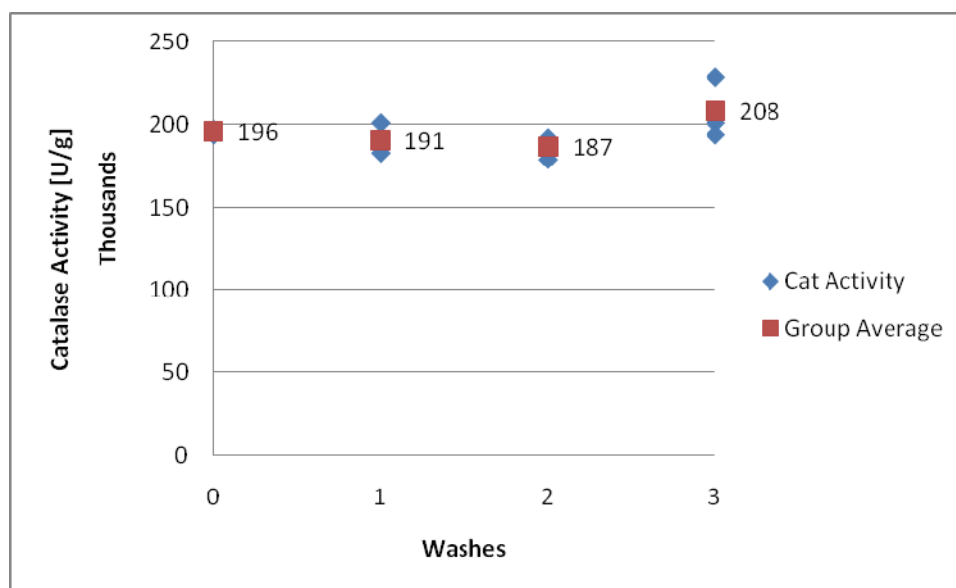


Figure 34. Test for catalase leaching from spray-dried cells. Spray-drying conditions were 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration.

Discussion of Optimal Spray-Drying Parameters with
Respect to Enzyme Activity and Process Robustness

The above investigations suggested that spray-drying was a viable alternative to the permeabilization of *P. pastoris* using BAC. Key parameters for retaining maximum GO and catalase activities while spray-drying were temperature, cell concentration, and feed rate. Results for all spray-drying studies are summarized in Table 5.

Table 5. Spray-drying parameter optimization and determination of process robustness.

Trial	Temperature	Feed Rate	Cell Concentration	Cell Batch	GO Activity	Catalase Activity
	[C]	[mL/min]	[mg/mL]		[U/g]	[U/g]
1	120	5	30	A	86	183000
2	120	5	100	A	105	224000
3	120	15	30	A	104	237000
4	120	15	100	A	119	257000
5	150	5	30	A	56	33000
6	150	5	100	A	78	115000
7	150	15	30	A	113	191000
8	150	15	100	A	130	245000
9	120	15	100	B	67	148000
10	120	15	200	B	71	151000
11	120	15	400	B	79	158000
12	150	15	100	B	71	143000
13	150	15	200	B	79	143000
14	150	15	400	B	84	166000
15	195	15	100	B	56	117000
16	195	15	200	B	51	86000
17	195	15	400	B	46	93000
18	150	15	200	B	77	134000
19	150	15	400	B	82	159000
20	150	13	600	B	90	192000

JMP software was used to analyze all spray-drying experiments and determine the best conditions for GO and catalase activity. *P. pastoris* was obtained by fermentation process as described in Chapter 5 and frozen in batches until needed for spray-drying. The cell batch was entered into JMP as a categorical factor (A or B). This was completed so each factor, cell batch, spray-drying temperature, spray-drying feed rate, and spray-drying cell concentration, could be considered independently. Figures 35 and 36 show Pareto charts for the cell batch, all spray-drying parameters, and relevant interaction parameters for GO and catalase, respectively.

Term	Range Tested		t Ratio	Prob> t
Cell Batch	A or B		6.64	<.0001
Feed Rate	5 to 15 [mL/min]		4.43	0.00
Cell Concentration	30 to 600 [mg/mL]		3.39	0.00
Temperature	120 to 195 [C]		-3.18	0.01
Temperature*Feed Rate			1.79	0.09

Figure 35. GO activity Pareto chart for spray-drying Trials 1 – 20 in Table 5.

Term	Range Tested		t Ratio	Prob> t
Feed Rate	5 to 15 [mL/min]		5.94	<.0001
Cell Batch	A or B		5.44	<.0001
Temperature	120 to 195 [C]		-5.29	<.0001
Cell Concentration	30 to 600 [mg/mL]		4.07	0.00
Temperature*Feed Rate			3.64	0.00

Figure 36. Catalase activity Pareto chart for spray-drying Trials 1 – 20 in Table 5.

Figures 35 and 36 show the relative contributions of each factor to the spray-dried enzyme activity. Factors (cell batch, spray-drying temperature, spray-drying feed rate,

spray-drying cell concentration, and the temperature*feed rate interaction parameter) with positive t-ratios contribute to increased enzyme activity, while factors with negative t-ratios contribute to decreased enzyme activity. The cell batch was highly important to enzyme activity with batch “A” giving the best GO and catalase activities. Focusing on spray-drying parameters, high feed rates, high feed concentrations, and low temperatures gave the best GO and catalase activities.

High feed rate was the most significant spray-drying factor for increased enzyme activity, so the instrument maximum of 15 mL/min was chosen. High cell concentration was also important for increased enzyme activity with the 600 mg/mL concentration being optimal. Cell concentrations above 600 mg/mL were too viscous and reduced the feed rate below the 15 mL/min optimum. Because the feed rate was more significant than cell concentration with respect to GO (Figure 35) and catalase activity (Figure 36), cell concentrations above 600 mg/mL were not tested.

Low operating temperatures provided the best enzyme activities, but the 120°C condition did not yield a dry powder. As shown by the temperature*feed rate interaction parameters (Figures 35 and 36), the negative effects of operating at higher temperatures can be offset to some extent by spray-drying at higher feed concentrations. Both the 150°C and 195°C conditions yielded dry powder, but the lower temperature of 150°C was optimal. At 195°C, there was clearly enzyme inactivation, and the effect of higher feed rates combined with higher feed concentrations could not be tested due to instrument limitations. Operating conditions that resulted in the highest GO and catalase activities with the Buchi B-190 spray dryer were 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration. These optimal spray-drying conditions yielded enzyme activities of 90 U/g for GO and 192,000 U/g for catalase, which were better than results from the BAC-treated permeabilization of *P. pastoris* (Gough, et al., 2001).

Conclusions for Specific Aim 1

The purpose of Specific Aim 1 was to minimize unit-operations needed for stable biocatalyst preparation by testing spray-drying as an alternate process model. Initial tests demonstrated comparable or better enzyme activities in spray-dried cells compared to detergent permeabilized cells (> 80 U/g for GO and $> 150,000$ U/g for catalase). The clear advantage of the spray-drying process, besides reducing unit operations required for BAC treatment (Figure 9), is the stability of the biocatalyst. Both GO and catalase activities were stable up to 20 days when stored at room temperature (Figures 29 and 30), and over 90% activity remained after 30 days when testing a separate batch of spray-dried cells. In addition, results showed that there was no significant loss of enzyme activity due to leaching (Figures 33 and 34).

The robustness of spray-drying was further confirmed by repeating the process under optimized conditions (150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration). Consistently, the spray-dried powder yielded enzyme activities of 80 – 100 U/g for GO and 160,000 – 220,000 U/g for catalase. Thus, it was concluded that spray-drying *P. pastoris* for use as biocatalyst for the production of pyruvate from lactate provided large advantages compared to the previous process (Gough, et al., 2005). Based on the process evaluation (Figure 37), spray-drying was determined to be an effective process model for the conversion of lactate to pyruvate. With spray-drying proven as robust, the number of unit operations in the old process (Figure 9) has been reduced in the new process (Figure 38).

In the new, simplified process, BAC treatment and several washing steps have been replaced by spray-drying. The liquid broth is taken directly from the fermentor and concentrated to the optimal spray-dryer feed concentration of 600 mg/mL, while extra liquid is removed as waste. The concentrated fermentation broth is then spray-dried at a feed rate of 15 mL/min and temperature of 150°C. The spray-dried product is a porous and stable powder with GO activity > 80 U/g and catalase activity $> 160,000$ U/g.

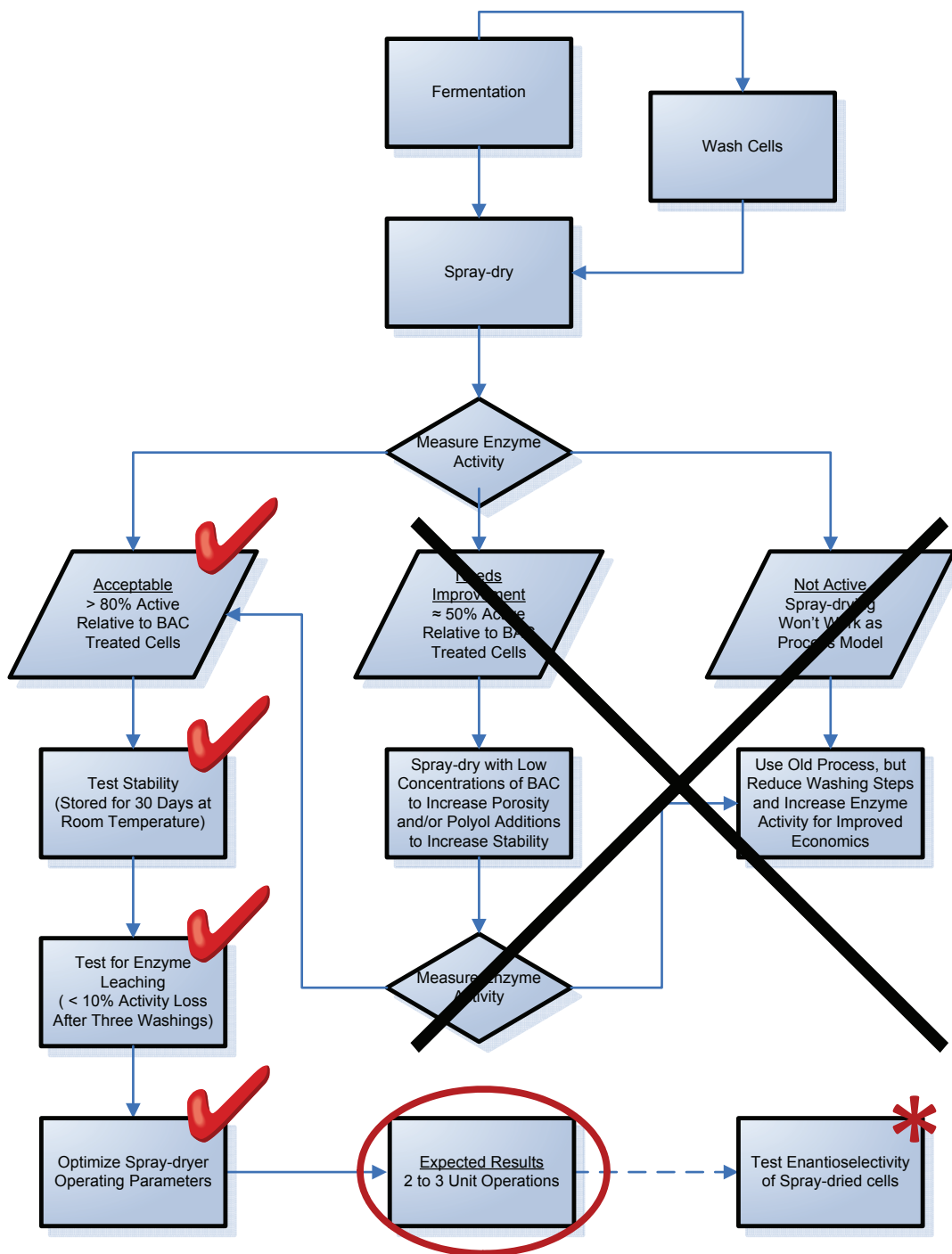


Figure 37. Achieved benchmarks for the new spray-drying process to prepare *P. pastoris* for conversion of lactate to pyruvate.

This spray-dried biocatalyst can be stored at room temperature for 30 days and used as needed for conversion of lactate to pyruvate in the enzyme reactor. Additionally, centrifugation can be used to recover the cells for enzyme reuse. Recycling the enzymes will increase “enzyme to product” ratio and improve overall process economics.

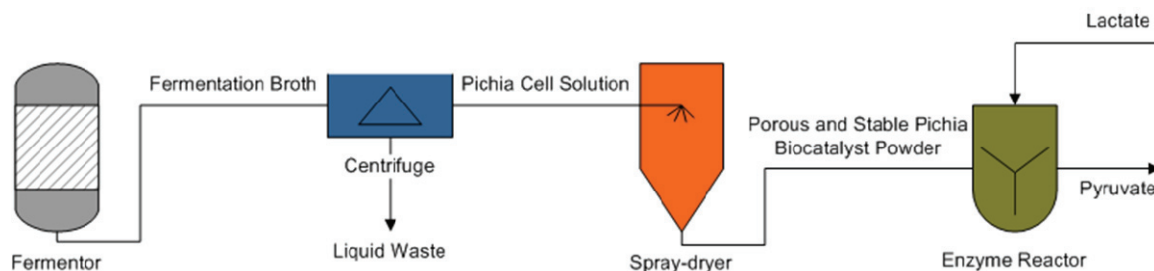


Figure 38. New and simplified spray-drying process for GO-based conversion of lactate to pyruvate.

Microscopic Characterization of Spray-Dried *P. pastoris*

Scanning electron microscopy was used to investigate the surface characteristics of spray-dried cells (Figures 39 and 40). Cells were prepared by mounting to Cambridge stubs using carbon tape and sputter coating with gold in the presence of argon as described previously. Individual spray-dried cells were oval in shape with approximate dimensions of 1.0 μm by 2.5 μm . During the spray-drying process, these cells formed spherical aggregates that were approximately 5.0 μm in diameter and contained about 30 cells. The spray drying process did not cause visible damage to the cells. On the contrary, cell surfaces appeared to be uniformly smooth at the 3.0 μm scale (Figure 39) and the 1.0 μm scale (Figure 40). The thickness of cell aggregates complicated attempts to increase resolution beyond the 1.0 μm level and compare cell surface porosity between spray-dried cells and BAC-treated cells.

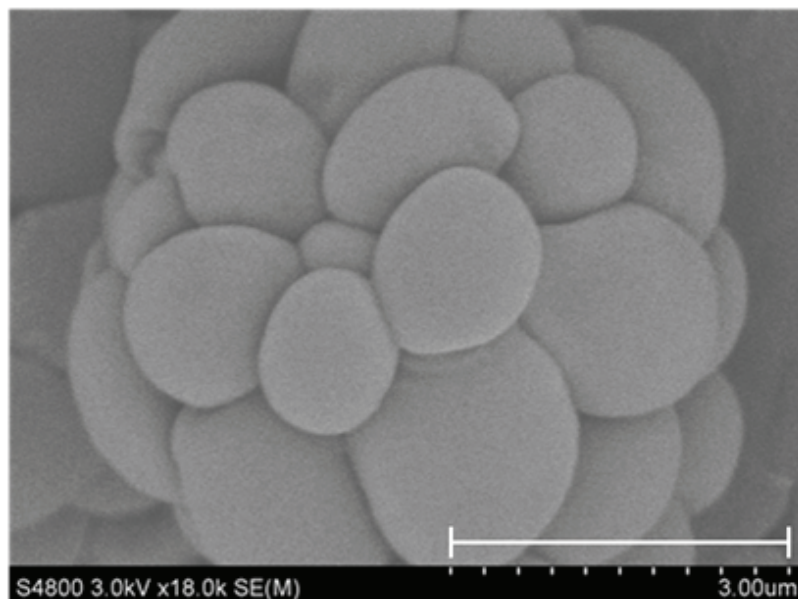


Figure 39. Scanning electron micrograph of spray-dried *P. pastoris* cell cluster at the 3.0 μm scale.

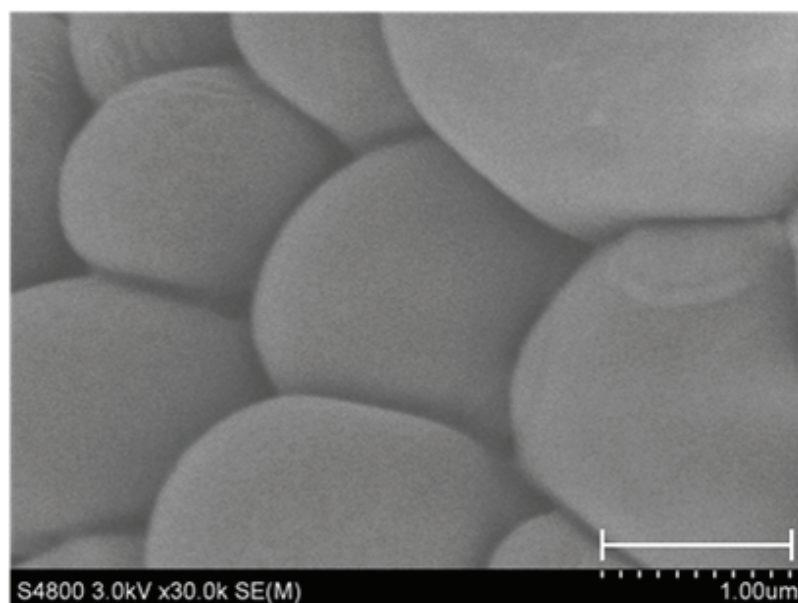


Figure 40. Scanning electron micrograph of the surface of spray-dried *P. pastoris* at the 1.0 μm scale to investigate porosity.

Spray-Drying as a Broad Technology for Biocatalysis

Given the success of spray-dried *P. pastoris* with respect to GO and catalase for the production of pyruvate (Figure 38), the broad application of this process to other organisms and reactions was also examined. Specifically, *E. coli* and *S. cerevisiae* expressing GO catalase were evaluated. The cloning of GO into *E. coli* was completed by Dr. Michael Louie. Growth and spray-drying procedures related to *E. coli* are described in Materials and Methods. For *S. cerevisiae*, yeast expressing native catalase was obtained from Lesaffre Yeast Corporation, Milwaukee, WI, and spray-dried for active catalase. Spray-drying conditions that were optimized for *P. pastoris* (150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration) were used for both organisms. GO activities for spray-dried *E. coli* were compared to non spray-dried cells (Figure 41).

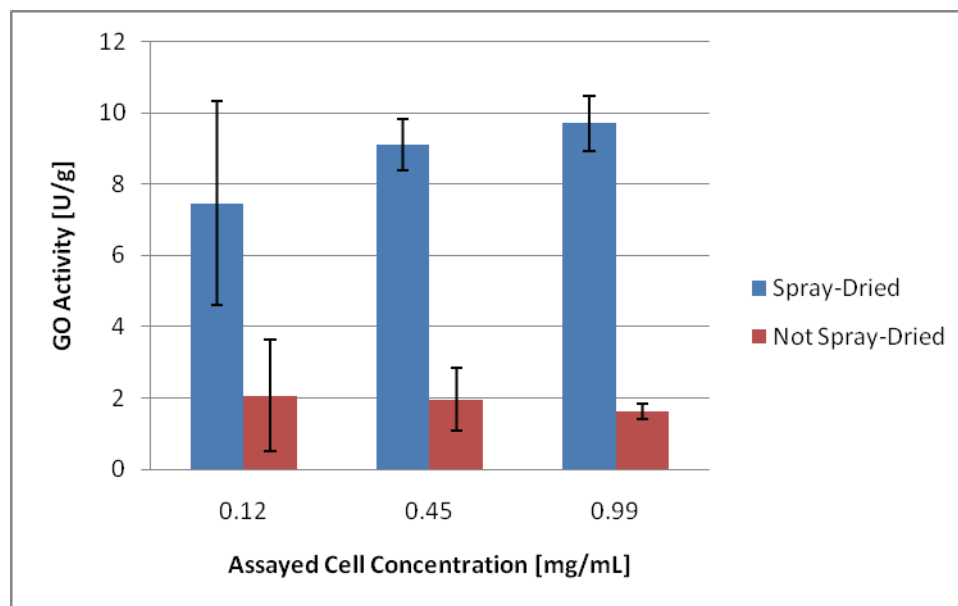


Figure 41. GO activity of spray-dried *E. coli* and control cells (not spray-dried). Spray-drying was done at 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration. Error bars represent one standard deviation (n=3).

Three cell concentrations were tested, and GO activity was calculated on a per gram basis. The lowest cell concentration (0.12 mg/mL) was near the sensitivity limit of the spectrometer, resulting in higher error for the first data point. The spray-dried *E. coli* retained ≈ 10 U/g of GO activity. Based on protein gel analysis detailed in Chapter 5, most recombinant GO was expressed as inactive inclusion bodies (Figure 59); however, spray-dried *E. coli* yielded a measurable increase in GO activity that was much higher than the controls.

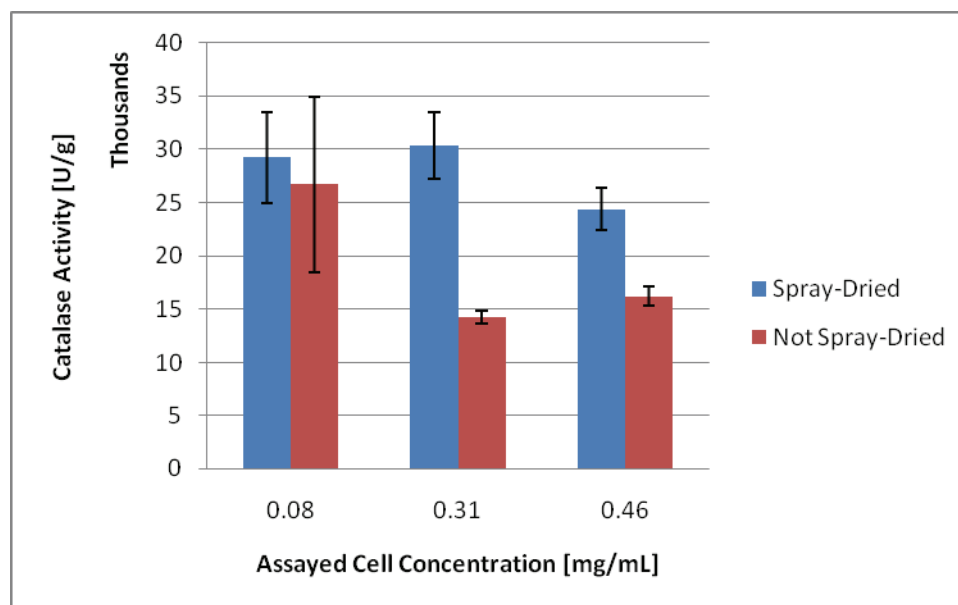


Figure 42. Catalase activity of spray-dried *S. cerevisiae* and control cells (not spray-dried). Spray-drying was done at 150°C, 15 mL/min feed rate, and 600 mg/mL cell concentration. Error bars represent one standard deviation (n=3).

S. cerevisiae containing native catalase was spray-dried to examine the activity. Catalase activity results for spray-dried *S. cerevisiae* were compared to control cells that were not spray-dried (Figure 42). Three cell concentrations were tested, and catalase activity was calculated on a per gram basis. The lowest cell concentration (0.08 mg/mL)

was near the sensitivity limit of the spectrometer, resulting in higher error for the first data point. The spray-dried *S. cerevisiae* retained 25,000 – 30,000 U/g of catalase activity. In comparison, the control cells (*S. cerevisiae* that was not spray-dried) had approximately 15,000 U/g of catalase activity. The control cells may have been subjected to processing conditions by the manufacturer to create the granular yeast, and those conditions could have partially permeabilized the cells and increased catalase activity in the non spray-dried samples. Nevertheless, spray-dried preparations yielded higher catalase activity.

These results indicate that spray-drying has the potential for larger application in preparing cells for biocatalysis. Several microorganisms can be spray-dried in order to create stable preparations of biocatalysts with minimum unit operations to retain maximum enzyme activity. Stability of the biocatalyst and lack of leaching add distinct advantages to spray-dried preparations in terms of minimum enzyme usage in the reaction of interest and maximum cell recycle.

Spray-drying Greatly Reduces *P. pastoris* Viability

Pre and post spray-drying viability of *P. pastoris* was investigated to determine if spray-dried cells were metabolically active. Metabolic activity is significant, especially with pyruvate, as active cells can consume the biocatalytic product. For this comparison, one seed vial was taken from the *P. pastoris* seed bank that was aseptically prepared as described in Materials and Methods. An inoculation loop was used to streak a portion of the seed vial on an YPD agar plate for the control. A second YPD agar plate was streaked with spray-dried product after the fermentation process described previously. Both plates were incubated at 30°C for 48 h with results shown in Figure 43. Some growth was observed on the plate containing spray-dried cells but this could be due to storage for over 30 days in a non-sterile environment. Nonetheless, viability of spray-dried *P. pastoris* is extremely low compared to cells before spray-drying.

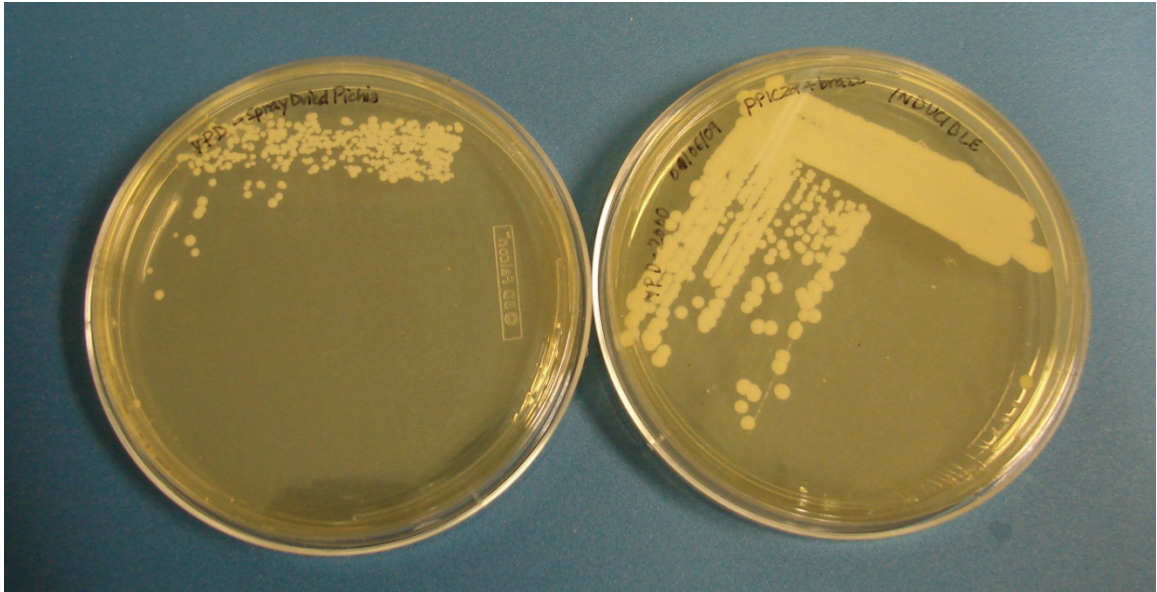


Figure 43. Viability comparison between spray-dried *P. pastoris* (left) and *P. pastoris* seed vial (right). Seed bank preparation, fermentation, and spray-drying were completed as described in Materials and Methods. Both conditions were streaked on YPD agar plates and incubated at 30°C for 48 h.

CHAPTER 4: OPTIMIZATION OF THE REACTION PROCESS FOR
CONVERSION OF LACTATE TO PYRUVATE USING SPRAY-
DRIED *PICHLIA PASTORIS* EXPRESSING GLYCOLATE OXIDASE
AND CATALASE

Previous Enzyme Reaction Studies

The *P. pastoris* double-transformant, MSP8.6, (expressing recombinant GO from spinach and catalase T from *S. cerevisiae*) developed at DuPont (Mark S. Payne, et al., 1997) was studied for enzymatic pyruvate production at the University of Iowa's Center for Biocatalysis and Bioprocessing. Initially, *P. pastoris* was grown on glycerol medium and induced with methanol as described in Materials and Methods. Peak GO activity was reached at 30 h post-induction, at which time the culture was harvested by centrifugation and cells were permeabilized with detergent (Gough, et al., 2001).

Permeabilized *P. pastoris* was used to study the effects of substrate inhibition, product inhibition, and reaction temperature on L-lactic acid conversion to pyruvic acid. For substrate inhibition experiments, L-lactic acid solutions ranging from 0.1 M to 1.5 M were prepared in 50 mM phosphate buffer with final pH adjusted to 7.0 using NaOH. Lactic acid solutions were divided into 100 mL aliquots and added to 500 mL Belco spinner vessels. Stirring was controlled at 500 rpm by magnetic stir bar, and air was bubbled through the mixture at 1 L/min. The reaction was initiated by adding permeabilized cells with the equivalent of 6.7 U/mL GO, 11,700 U/mL of endogenous catalase, and 806 U/mL of catalase T.

As shown by Gough, et al. in Figure 44, the best lactic acid concentration for the given conditions was 0.5 M, where 100% conversion was achieved in 8 h. Lactate concentrations above 0.5 M did not reach full conversion in 8 h, indicating the possibility of substrate or product inhibition. For this reason, product inhibition was investigated by repeating the 0.5 M lactate condition as described above. One modification was the

addition of pyruvate to the initial lactate solution, yielding a final lactate concentration of 0.5 M and final pyruvate concentrations ranging from 0% to 80% (v/v) (Figure 45).

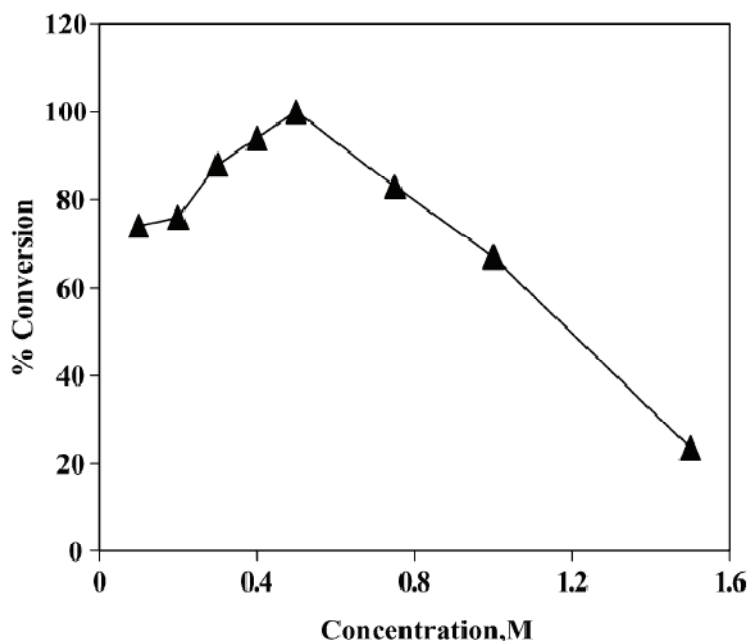


Figure 44. Percent conversion of L-lactic acid vs. initial L-lactic acid concentration by permeabilized *P. pastoris* in 500 mL Belco spinner vessels. Reaction conditions were 500 rpm stirring, 5°C, pH 7.0, and 1 L/min air flow.

Source: Gough, S., et al. (2005). Production of pyruvate from lactate using recombinant *Pichia pastoris* cells as catalyst. *Process Biochemistry*, 40(8), 2597-2601.

Approximately 88% conversion of 0.5 M lactate was observed until pyruvate concentrations exceeded 40%. At 60% initial pyruvate concentration, only 40% lactate conversion was observed, and the reaction was completely inhibited at 80% starting pyruvate concentrations. While previous substrate and product inhibition experiments demonstrated the usefulness of lactate to pyruvate conversion by whole-cell biocatalyst, several questions remained concerning the best enzyme reaction conditions with respect

to production cost. Substrate is an important cost component, as well as recovery of product from aqueous solution. For this reason, reaction conditions must be specified such that (1) most lactate is converted to pyruvate and (2) this pyruvate is concentrated to simplify recovery. Early work achieved conversion of 0.5 M lactate in 8 h, but it might be more cost effective to attempt conversion of over 1.0 M lactate solutions (to reach higher product concentrations) even if more time is required. Furthermore, some lactate disappearance was observed in the presence of 20% and 40% initial pyruvate solutions, however, mass balances were not performed to confirm the direct conversion of lactate to pyruvate with minimal byproducts.

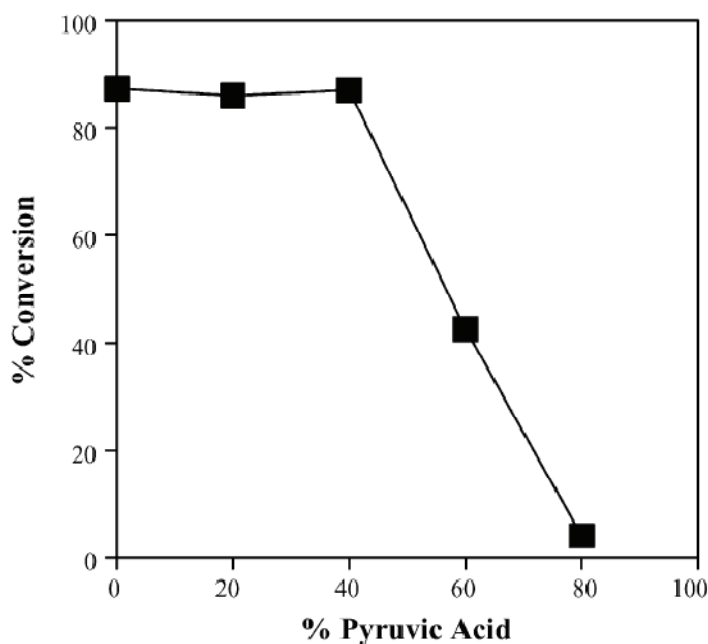


Figure 45. Percent conversion of 0.5 M L-lactic acid vs. initial pyruvic acid concentration by permeabilized *P. pastoris* in 500 mL Belco spinner vessels. Reaction conditions were 500 rpm stirring, 5°C, pH 7.0, and 1 L/min air flow.

Source: Gough, S., et al. (2005). Production of pyruvate from lactate using recombinant *Pichia pastoris* cells as catalyst. *Process Biochemistry*, 40(8), 2597-2601.

Additionally, Gough et al. determined enzyme reaction temperatures below 15°C were best for lactate conversion, providing additional data to show that higher temperatures resulted in decreased activity (Figure 46). Temperatures from 5°C to 22°C were tested using conditions similar to those of substrate and product inhibition experiments (100 mL of 0.5 M lactic acid solution in 50 mM phosphate buffer, pH 7.0, permeabilized *P. pastoris*, 500 mL Belco spinner vessels with 500 rpm agitation, and air supplied at 1 L/min). Temperatures of 5°C, 10°C, and 15°C achieved roughly 100% lactate conversion in 8 to 9 h while the 22°C trial only reached 30% conversion.

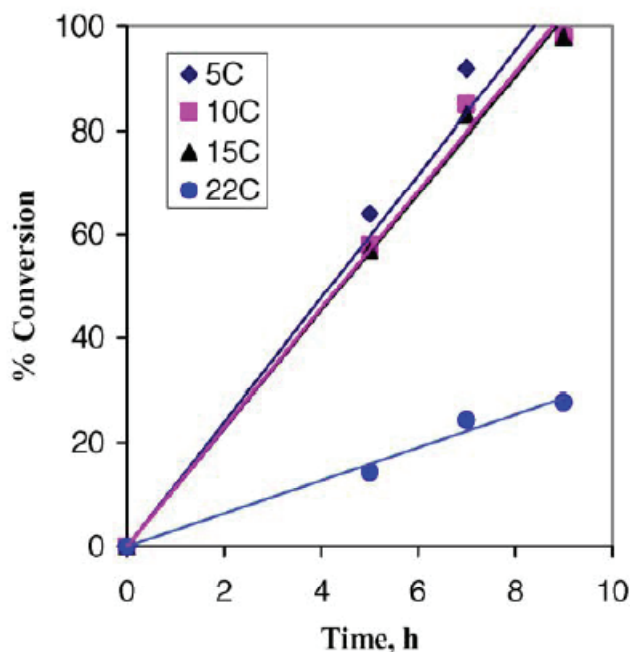


Figure 46. Effect of temperature on percent conversion of 0.5 M L-lactic acid by permeabilized *P. pastoris* in 500 mL Belco spinner vessels. Reaction conditions were 500 rpm stirring, temperatures of 5, 10, 15, and 22°C, pH 7.0, and air flow at 1 L/min.

Source: Gough, S., et al. (2005). Production of pyruvate from lactate using recombinant *Pichia pastoris* cells as catalyst. *Process Biochemistry*, 40(8), 2597-2601.

Further work by Gough et al. investigated using air or pure oxygen to supply the reducing power required for conversion of lactate to pyruvate (Gough, et al., 2005). Again, similar reaction conditions were used (100 mL of 0.5 M lactic acid solution in 50 mM phosphate buffer, pH 9.0, permeabilized *P. pastoris*, 500 mL Belco spinner vessels with 500 rpm agitation, and air or oxygen supplied at 1 L/min). In oxygen concentration experiments, lactate conversion was measured at 5 h when air (21% oxygen) or pure oxygen was bubbled through the reaction solution at 1 L/min. After the 5 h reaction, cells were recovered by centrifugation and re-used in three separate reactions. Results indicate $\approx 60\%$ conversion for all three cycles with air, and over 97% conversion for the three cycles when using pure oxygen.

Past temperature and oxygen concentration studies underscore the important relationship between reaction conditions and the time needed to achieve full conversion. While a faster reaction is generally preferred, it might not be the economic optimum. Therefore, it could be more cost effective to operate near ambient temperatures with added biocatalyst to compensate for enzyme activity loss. Also, pure oxygen is highly expensive compared to compressed air. Previous substrate inhibition experiments confirmed approximately 100% conversion of 0.5 M lactate when the reaction time was extended to 8 h. Using pure oxygen under the same conditions can reduce conversion time to 5 h, but this will add significant cost. Since the reaction does reach full conversion with air (albeit over a longer period of time), increasing oxygen transfer rates in the reactor could be a more economical method for reducing the time needed to achieve full conversion using air. Gough et al. demonstrated the ability to recover the biocatalyst for use in subsequent reactions. For the air condition, lactate conversion decreased from around 65% conversion to 60% conversion over the three cycles. However, the biocatalyst could potentially be used beyond three cycles with addition of small amounts of fresh biocatalyst to boost enzyme activity.

Specific Aim 2

Optimize enzyme reaction for best “enzyme to product” ratio and pyruvate yield. The following reaction variables will be addressed to achieve maximum “enzyme to product” ratio and pyruvate yield:

- Optimize lactate concentration for maximum conversion.
- Determine allowable pyruvate levels before significant product inhibition.
- Minimize enzyme required to achieve conversion.
- Test operating conditions for cell destruction and enzyme leaching.
- Optimize reaction parameters, including temperature, pH, dissolved oxygen concentration, and buffer composition.
- Maximize enzyme recycles for best “enzyme to product” ratio.

pH Optimization Study

The pH optimization experiment was conducted as described in Materials and Methods. Reactor set-points are summarized in Table 6. From work by Gough et al., reaction temperatures of 5°C, 10°C, and 15°C offered similar lactate vs. time conversions. 15°C was chosen for the current pH study because it was nearest to ambient temperature (for reduced cooling costs) without affecting conversion. Gough et al. performed all enzyme reactions using L-lactic acid in 80 mM Tris buffer; thus, 80 mM Tris buffer was used for current pH testing. Also, the group used GO concentrations of 6.7 U/mL for enzyme reactions. The spray-dried biocatalyst yielded GO activity of roughly 100 U/g. As a result, 60 g/L of spray-dried cells were used for comparable GO activity (6.0 U/mL) to studies performed by Gough et al. While the group observed substrate inhibition above 0.5 M lactate, economic reasons (cost of separating dilute product solutions) prompted choosing a higher 1.0 M lactate concentration for current work. Hourly samples were analyzed by Analox enzymatic assay for lactic acid conversion and pyruvic acid formation as described previously.

Table 6. Conditions tested for pH optimization study.

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Medium	Temperature [°C]
1	5.0	1.0	60	80 mM Tris	15
2	6.0	1.0	60	80 mM Tris	15
3	7.0	1.0	60	80 mM Tris	15
4	8.0	1.0	60	80 mM Tris	15

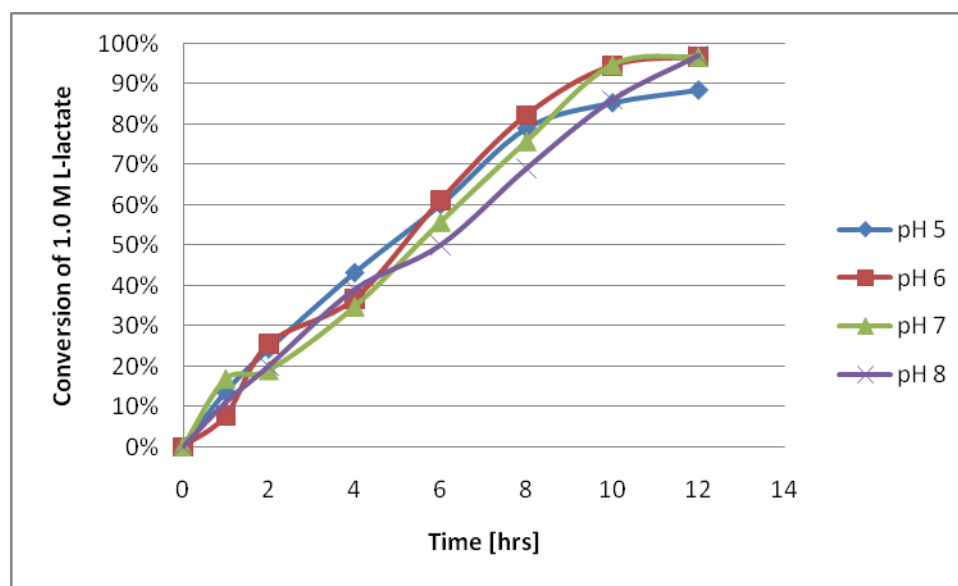


Figure 47. Effect of pH on percent conversion of 1.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in 80 mM Tris, 60 g/L spray-dried *P. pastoris*, 15°C, 1 L/min air flow, and 1250 rpm.

While pH 6.0, 7.0 and 8.0 conditions achieved full consumption of 1.0 M lactate in 12 h (Figure 47), only pH 6.0 and 7.0 set-points yielded 1.0 M pyruvate (Figure 48). The lower pyruvate yield at pH 8.0 could be caused by the base-catalyzed self-aldol condensation of pyruvate to produce 4-methyl-4-hydroxy-2-oxoglytarate (Von Korff,

1969). Another possibility for decreased pyruvate yield at elevated pH could be formation of hydrogen peroxide caused by depressed catalase activity. In the presence of hydrogen peroxide, pyruvate can be further oxidized to unwanted byproducts, such as acetate (N. E. Tolbert, et al., 1949). As a result, a pH operating window was specified from 6.0 to 7.0 for optimal pH control and pyruvate yield.

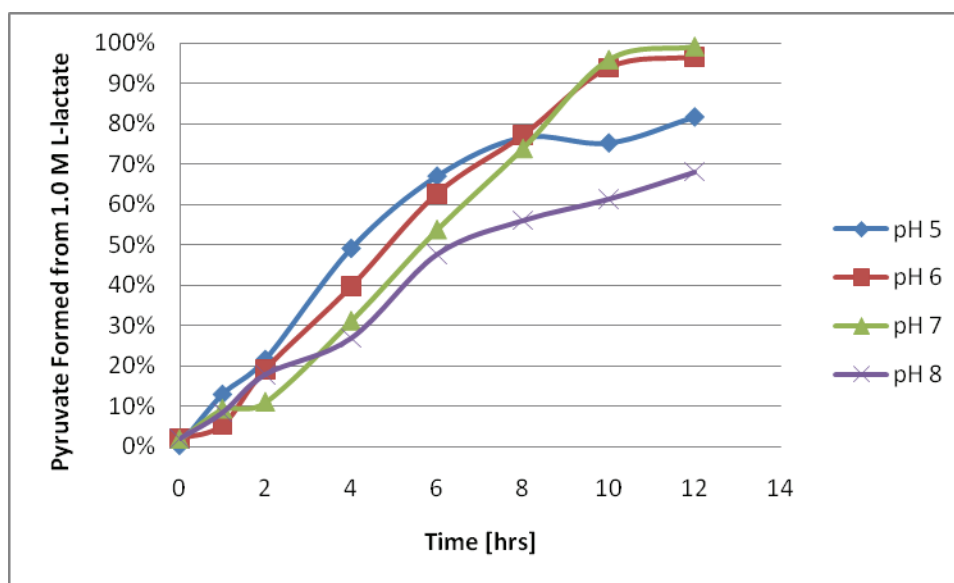


Figure 48. Effect of pH on pyruvate formed from 1.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in 80 mM Tris, 60 g/L spray-dried *P. pastoris*, 15°C, and 1 L/min air flow.

Pyruvate Formation at Various Lactate Concentrations

For the substrate concentration effect, pH 6.0 was chosen to verify the lower limit of the pH operating window. Other reactor set-points are summarized in Table 7. Deviations from the procedure for pyruvate production in DASGIP were the pH was set to 6.0 and four different substrate solutions were prepared for lactate inhibition testing. Gough et al. had observed full conversion of 0.5 M lactate with decreased reaction rates

above this concentration. However, higher substrate concentrations (resulting in more concentrated product) could reduce separation costs and yield a more economical process even if reaction rates are depressed by elevated substrate levels. Moreover, 1.0 M lactate was successfully converted to pyruvate in 12 h during the pH experiment (Figure 48). For these reasons, four substrate solutions were prepared ranging from 1.0 M to 4.0 M L-lactic acid in 80 mM Tris at pH 6.0. Samples were taken every 4 h and evaluated by Analox enzymatic assay.

Table 7. Conditions tested for lactate concentration effect.

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Medium	Temperature [°C]
1	6.0	1.0	60	80 mM Tris	15
2	6.0	2.0	60	80 mM Tris	15
3	6.0	3.0	60	80 mM Tris	15
4	6.0	4.0	60	80 mM Tris	15

In Figure 49, the 1.0 M and 2.0 M lactate conditions achieved > 95% conversion based on initial concentrations, while significant substrate inhibition was observed over 2.0 M lactate. The 1.0 M trial reached 95% conversion in approximately 10 h, which is consistent with the earlier pH experiment. In contrast, the 2.0 M trial required nearly three times as long (36 h) to reach over 95% conversion. It is important to note the value of a concentrated product solution, but the substrate is also costly. At 10 h, the 1.0 M lactate condition yielded almost 0.95 M pyruvate (95% yield based on initial lactate concentration). At 36 h, all of the 2.0 M lactate was consumed, but only 1.1 M pyruvate had been formed (55% yield from initial lactate). Also, for the 1.0 M lactate trial, the pyruvate yield decreased from 95% at 10 h to 76% at 36 h.

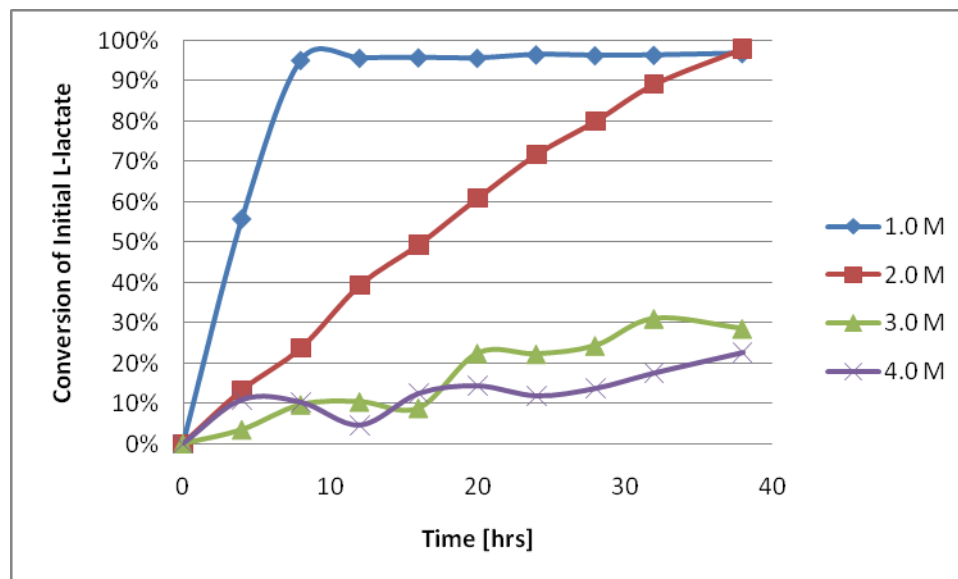


Figure 49. Effect of lactate concentration on percent conversion of lactate. Reaction conditions were L-lactic acid in 80 mM Tris, pH 6.0, 60 g/L spray-dried *P. pastoris*, 15°C, 1 L/min air flow, and 1250 rpm agitation.

This added time could have increased pyruvic acid exposure to hydrogen peroxide causing the formation of acetic acid. Additionally, native *P. pastoris* enzymes could have been preserved in the spray-drying process. These native enzymes are found in smaller quantities compared to recombinant GO and catalase, but increased time may have provided an opportunity for conversion of pyruvate to unwanted products. As a result, the optimal lactic acid concentration was determined to be 1.0 M, and the pyruvic acid should be recovered directly after complete substrate conversion.

Operating Temperature and Initial Cell Loading Study

To this point, the pH and substrate inhibition experiments used spray-dried cell concentrations of 60 g/L in the enzyme reaction to generate pyruvic acid. This particular cell concentration was chosen to replicate GO activity levels from studies published by

Gough et al. and may not be optimum for the new process. For conversion of 1.0 M lactate to pyruvate, 60 g/L spray-dried cells gave an approximate “enzyme to product” ratio of 2:3. However, reducing the enzyme required to maintain comparable pyruvate productivity could improve this “enzyme to product” ratio for a more economical process. Another economical benefit would be operation of the enzyme reaction near ambient temperatures for reduced cooling costs. An experiment (Table 8) was designed with 15 to 30 g/L cell concentrations at 25°C reaction temperature to determine pyruvate yields at these more economical conditions and compare to previous results. 1.0 M L-lactic acid in 80 mM Tris was chosen from substrate inhibition results, while the pH was set to 7.0 to verify the upper limit specified in the pH experiment. Samples were taken every 2 h, until 10 h; then, samples were withdrawn hourly. Lactate and pyruvate concentrations were analyzed by Analox.

Table 8. Conditions tested for temperature and initial cell loading in the enzyme reaction.

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Medium	Temperature [°C]
1	7.0	1.0	15	80 mM Tris	25
2	7.0	1.0	15	80 mM Tris	25
3	7.0	1.0	30	80 mM Tris	25
4	7.0	1.0	30	80 mM Tris	25

As noted in Figure 50, the 15 g/L and 30 g/L conditions achieved average lactate conversions of 95.9% and 96.8%, respectively. In addition, these conditions reached over 95% pyruvate yields in 12 h, which is equivalent to previous studies at 60 g/L spray-dried cells. The “enzyme to product” ratio was improved from 2:3 (at 60 g/L) to a more economical 1:6 (at 15 g/L). However, the 15 g/L trial offered similar results to 30 g/L, and 60 g/L trials, so an optimal cell concentration had not been determined. Later

experiments were completed to test cell loading below 15 g/L. No significant changes were observed by increasing the temperature from 15°C to 25°C (Figure 47 compared to Figure 50). All four conditions tested at 25°C yielded > 95% lactate conversions which are comparable to results at 15°C. Therefore, the enzyme reaction can be operated at 25°C and lower cell concentrations without a reduction in pyruvate productivity.

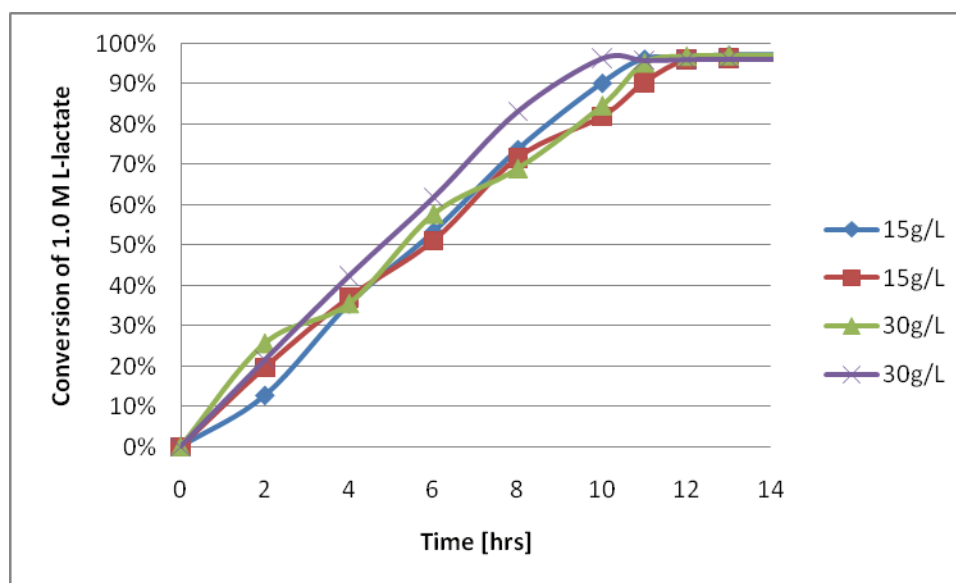


Figure 50. Effect of cell concentration and temperature on percent conversion of 1.0 M lactate. Reaction conditions were L-lactic acid in 80 mM Tris, pH 7.0, 25°C, 1 L/min air flow, and 1250 rpm.

Medium Composition and Decreased Cell Loading Study

The newly defined parameters of 1.0 M lactate and 25°C operating temperature were used for an experiment to explore cell concentrations below 15 g/L and investigate simplified reaction medium. Cell concentrations of 8 g/L and 15 g/L were studied along with removal of Tris buffer, an expensive material, from the reaction medium (Table 9). Conversion rates at 8 g/L and 15 g/L spray-dried cells were investigated to increase “enzyme to product” ratio without sacrificing productivity. Also, the conversion of

lactate to pyruvate was found to be highly dependent on pH. However, a large pH operating window was specified (pH 6.0 to 7.0), and the DASGIP system offered very good pH control (± 0.04 pH units) with the given control parameters. For these reasons, the target pH might be maintained without the need for Tris buffer. Removing Tris buffer in systems with good pH control would alleviate a major cost and could simplify pyruvate recovery. Samples were taken every 2 h, and lactate and pyruvate concentrations were measured by Analox.

Table 9. Conditions tested for medium composition and decreased cell loading in the enzyme reaction.

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Medium	Temperature [°C]
1	7.0	1.0	8	80 mM Tris	25
2	7.0	1.0	15	80 mM Tris	25
3	7.0	1.0	8	water	25
4	7.0	1.0	15	water	25

As shown in Figure 51, the 15 g/L cells in water achieved 96.9% pyruvate yield from 1.0 M lactate in 12 h. At 12 h, the condition of 15 g/L cells in Tris had slightly over 100% pyruvate yield. This value is most likely closer to 98% given the 2% error of the Analox instrument for pyruvate analysis. A better representation for the 15 g/L condition in Tris would be the 13 h time point where 97.0% of the initial 1.0 M lactate had been converted to pyruvate. Considering the 2% measurement error, both 15 g/L conditions (Tris and water) had reached over 97% pyruvate yield in 12 h. Also, the conditions with 8 g/L cells in Tris and the 8 g/L cells in water had similar pyruvate yields of 81.9% and 77.7%, respectively.

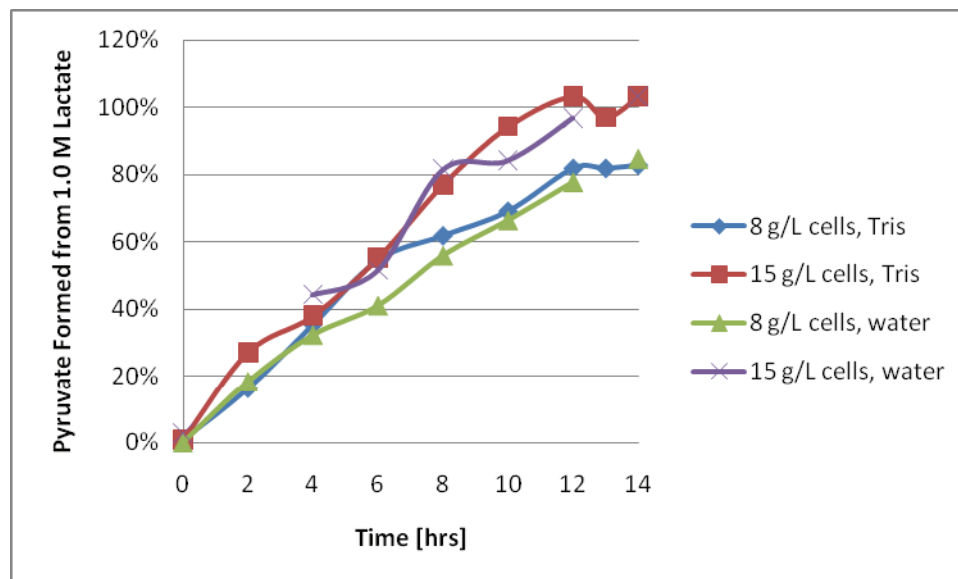


Figure 51. Effect of enzyme concentration and medium composition on pyruvate formed from 1.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in 80 mM Tris or water, pH 7.0, 25°C, 1 L/min air flow, and 1250 rpm.

Since results were consistent for the Tris and water diluents, Tris buffer is not needed for systems with the ability to control pH in the 6.0 to 7.0 operating window. This is significant because a costly component can be removed from the medium and will not need to be separated from the pyruvate product. Concerning optimization of the “enzyme to product” ratio, the 8 g/L cell concentrations achieved approximately 80% pyruvate yield in 12 h compared to the 15 g/L trials at > 97% pyruvate yield. The 8 g/L conditions technically gave better “enzyme to product” ratios than trials at 15 g/L, but it is important to note that lactate is the most expensive component of the enzyme reaction. As shown in earlier work, significantly increasing reaction time also increases pyruvate exposure to further oxidation by hydrogen peroxide or conversion to unwanted byproducts by native *P. pastoris* enzymes. Thus, the reaction should not be extended to conserve enzyme at the risk of losing the more expensive product. Cell concentrations

between 8 g/L and 15 g/L were tested to determine the optimal enzyme concentration for full conversion of lactate to pyruvate without significantly increasing reaction time.

Product Inhibition Testing and Cell Loading Optimization

Results of the substrate inhibition experiment show consumption of 1.0 M initial lactate in approximately 10 h, and disappearance of 2.0 M lactate in 40 h (Figure 49). While a more concentrated product solution is desired, the 2.0 M condition only offered 55% pyruvate yield from 2.0 M lactate, resulting in a 1.1 M pyruvate solution. However, the 1.0 M initial lactate condition gave a 95% pyruvate yield. This resulted in a 0.95 M solution of pyruvate. Because lactate is the most expensive component of the enzyme reaction, 1.0 M lactate was chosen as the optimal starting lactate concentration. Gough et al. saw 88% disappearance of 0.5 M lactate in the presence of 40% v/v pyruvate, or roughly 4.5 M pyruvate (Gough, et al., 2005). While the group saw consumption of lactate in the presence of 40% added pyruvate, it was unclear if feeding low levels of lactate (to overcome substrate inhibition), would yield solutions with high product concentrations from the GO-based conversion of lactate. A substrate feeding experiment (Table 10) was designed to determine the maximum pyruvate concentration that could be directly achieved by GO-based conversion of lactate to pyruvate.

Table 10. Conditions for substrate feeding experiment to test product inhibition.

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Medium	Temperature [°C]
1	7.0	1.0 M + 4.0 M Feed	10	water	25
2	7.0	1.0 M + 4.0 M Feed	10	water	25
3	7.0	1.0 M + 4.0 M Feed	15	water	25
4	7.0	1.0 M + 4.0 M Feed	15	water	25

Targeted final working volume was 1.0 L, so vessels were charged with 500 mL of 1.0 M L-lactic acid in water to allow for an additional 500 mL of 4.0 M feed addition. As noted, the conversion of lactate to pyruvate is an oxidation reaction where reaction progress was monitored by dissolved oxygen level. Dissolved oxygen probes were calibrated to 100% air saturation at 0.9 VVM and 1250 rpm. Dissolved oxygen levels fell below 20% saturation during active lactate conversion and returned to around 90% saturation at reaction completion. Based on this trend, a dissolved oxygen “trigger feature” was used to add the concentrated lactate feed. The feed pumps were turned off when dissolved oxygen levels dropped below 20% during active lactate conversion. Then, feed pumps were turned on when the dissolved oxygen concentrations climbed above 30% as lactate was consumed. Feed pumps cycled off and on between dissolved oxygen concentrations of 20% and 30% saturation, respectively. This feed strategy helped maintain 200 to 400 mM lactate concentrations at all times during the reaction. Because reaction volume varied from 0.5 to 1.0 L, aeration was adjusted for 0.9 VVM, and agitation was changed for constant power input per unit volume. Consistent with other experiments, pH was set to 7.0 and temperature was controlled at 25°C. 15 g/L cell concentrations were repeated, and 10 g/L concentrations were tested to find the optimal “enzyme to product” ratio. Lactic acid and pyruvic acid concentrations were determined by Analox assay as described in Materials and Methods.

For all conditions, the initial 1.0 M lactate concentration decreased to between 200 and 400 mM in 8 h. This decrease in lactate concentration caused oxygen levels to rise above 30%, which triggered the feed pumps to add 4.0 M lactate. The feed was stopped when oxygen levels returned below 20%, and the cycle repeated approximately every 70 min for the next 40 h. Each cycle introduced around 15 mL of 4.0 M lactate feed, resulting in approximately 1.0 L final volume after 40 h. The addition of 500 mL of 4.0 M lactate feed to the initial 500 mL of 1.0 M lactate batch medium would yield a theoretical pyruvate concentration of 2.5 M. The entire lactate feed volume was added to

the batch medium over time to maintain 200 to 400 mM batch lactate concentrations. However, only 1.2 M pyruvate was achieved for all conditions (Figure 52), thus indicating significant product inhibition above 1.2 M pyruvate. This result is consistent with the 2.0 M lactate condition of the substrate inhibition experiment where 1.1 M pyruvate was formed.

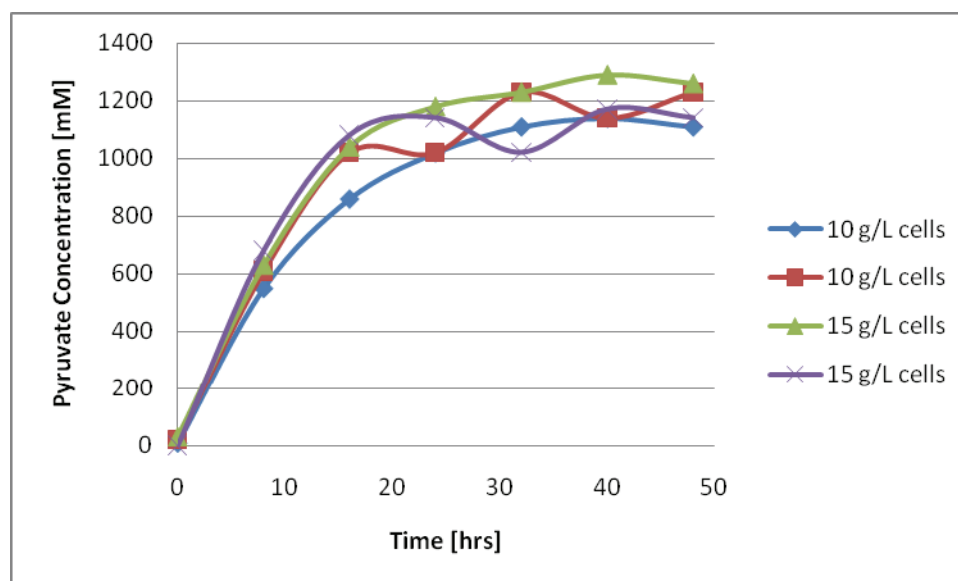


Figure 52. Effect of product inhibition on GO reaction by feeding 4.0 M lactate. Reaction conditions were 1.0 M L-lactic acid in water supplemented by pyruvate feeding, pH 7.0, 25°C, 1 L/min air flow, and 1250 rpm.

Another similarity between the substrate and product inhibition experiments is the reduced reaction rate leading to much longer reaction time for full lactate consumption. This added reaction time could expose the pyruvate product to further oxidation by hydrogen peroxide or degradation by native *P. pastoris* enzymes. Therefore, the 1.0 M initial lactate concentration is optimal, and no substrate feeding is advised as very little added lactate is converted to pyruvate. Substrate feeding complicated the determination

of pyruvate productivity at 10 g/L and 15 g/L conditions, so similar cell concentrations were repeated in later enzyme recycling experiments.

Determination of Enzyme Recycling to Improve “Enzyme
to Product” Ratio

Reuse of the enzyme after each reaction cycle has the potential to maximize productivity in the form of “enzyme to product” ratio. Based on the previous reaction studies, the conditions given in Table 11 were chosen for enzyme recycling. Due to strong substrate and product inhibition, 1.0 M initial L-lactate in water was chosen and the reaction was set as a batch-wise operation. The reaction was controlled at pH 7.0 and the near-ambient temperature of 25°C for reduced cooling costs. The 15 g/L cell concentration achieved comparable pyruvate productivity to the 30 g/L, 60 g/L, and 90 g/L conditions, (but with better “enzyme to product” ratios) while the 8 g/L cell concentration did not reach the same pyruvate yields. For this reason, reactions were initiated with 15 g/L spray-dried cells.

Table 11. Conditions tested for enzyme recycling experiment.

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Cycles	Medium	Temperature [°C]
1	7.0	1.0	15 + 2	10	water	25
2	7.0	1.0	15 + 2	10	water	25
3	7.0	1.0	15 + 5	10	water	25
4	7.0	1.0	15 + 5	10	water	25

After the 10 – 12 h reaction cycles, cells were removed from the product solution by centrifugation at 7,000 x g and 4°C for 10 min. A large batch of substrate was prepared for the entire experiment to keep consistency among cycles and stored at 4°C

until needed. The recovered cells were then re-suspended in fresh substrate solution, and the process was repeated for 10 cycles. Fresh cells (unused spray-dried cells) were added throughout the experiment to maintain pyruvate productivity and compensate for minor enzyme activity losses that were previously observed with increasing reaction cycles (Gavagan, et al., 1995). For reactors 1 and 2, 2 g/L of fresh cells were added at the start of cycles 4, 6, and 8, while reactors 3 and 4 received one addition of 5 g/L fresh cells at the beginning of cycle 4 only. Initial and final samples were taken for each reaction cycle and analyzed for lactate and pyruvate concentrations by Analox (Figure 53).

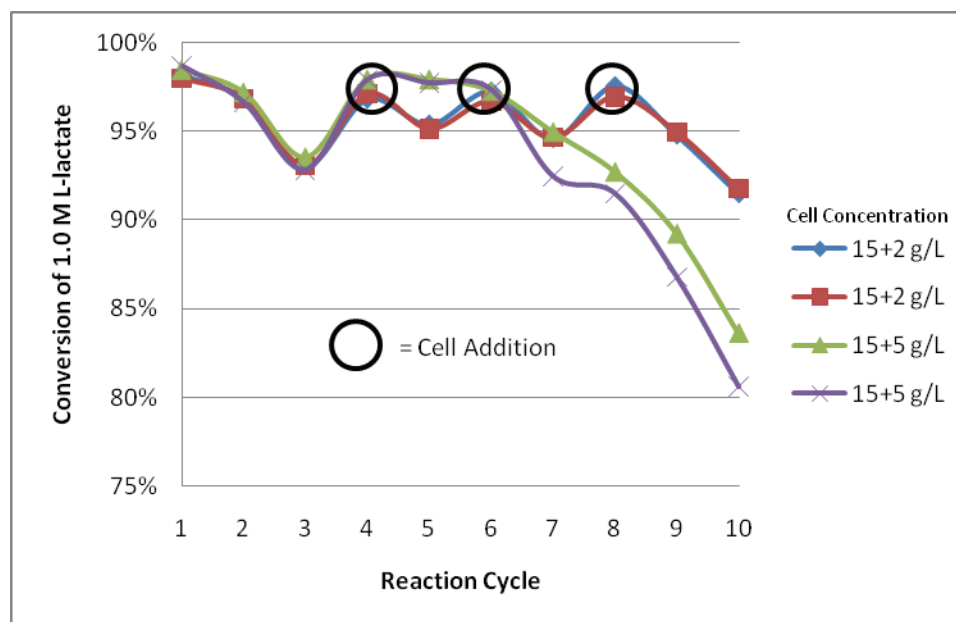


Figure 53. Percent conversion of 1.0 M lactate at the end of each 10 – 12 h reaction cycle. Reaction conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 10 reaction cycles. Initial 15 g/L cell concentrations were supplemented with either (a) 2 g/L fresh cells at cycles 4, 6, and 8, or (b) 5 g/L fresh cells at cycle 4 only.

All four reactors gave consistent results for the first three cycles, with cycle 1 yielding about 98% conversion of 1.0 M lactate, cycle 2 offering 97% conversion, and

cycle 3 conversion dropping to 93%. At the beginning of cycle 4, 2 g/L of fresh cells were added to reactors 1 and 2, while 5 g/L fresh cells were added to reactors 3 and 4. This fresh cell addition boosted the productivity by compensating for activity loss during the first three cycles. These cell additions also maintained lactate conversions of 97 – 98% for cycle 4. The single 5 g/L cell addition for reactors 3 and 4 helped keep lactate conversions at 97 – 98% for cycles 4 through 6; however, conversion rates quickly dropped after cycle 6 without further fresh cell supplementation. In contrast, reactors 1 and 2 were able to maintain lactate conversions between 95% and 97% from fresh cell additions at cycle 4, 6, and 8 before decreasing to 92% conversion at cycle 10. These results indicate that supplementation of spray-dried cells at 1 – 2 g/L of fresh cells are preferred, possibly beginning at cycle 3 and continuing every other cycle through cycle 10 to maintain conversion above 95% for each cycle.

Pyruvate product formation was confirmed by HPLC analysis following HPLC Analysis of Lactate and Pyruvate (Figure 54). HPLC chromatograms are shown for the 98% pyruvic acid standard obtained from Sigma as well as the pyruvic acid product from completed cycles 1, 5, and 10 for reactor 1. The first reactor had an initial cell concentration of 15 g/L and received fresh cell additions at the beginning of cycles 4, 6, and 8. For HPLC analysis at the given conditions, approximate retention times were 8.9 min for pyruvate and 11.8 min for lactate. Therefore, it can be seen that lactic acid was the main contaminant in the 98% Sigma pyruvic acid. However, this lactic acid peak is not visible in the pyruvic acid product from cycles 1, 5, and 10, for reactor 1. While Analox results indicate the highest lactate conversions achieved were 98% for a roughly equivalent purity to the Sigma pyruvic acid, HPLC results suggest higher purity based on undetectable levels of lactate in the reactor 1 pyruvate product. Furthermore, these chromatograms are consistent throughout all 10 cycles showing strong pyruvic acid peaks with no other measureable peaks that would indicate byproducts. The purity of the biocatalytically derived pyruvate is equal, if not better than the commercial standard.

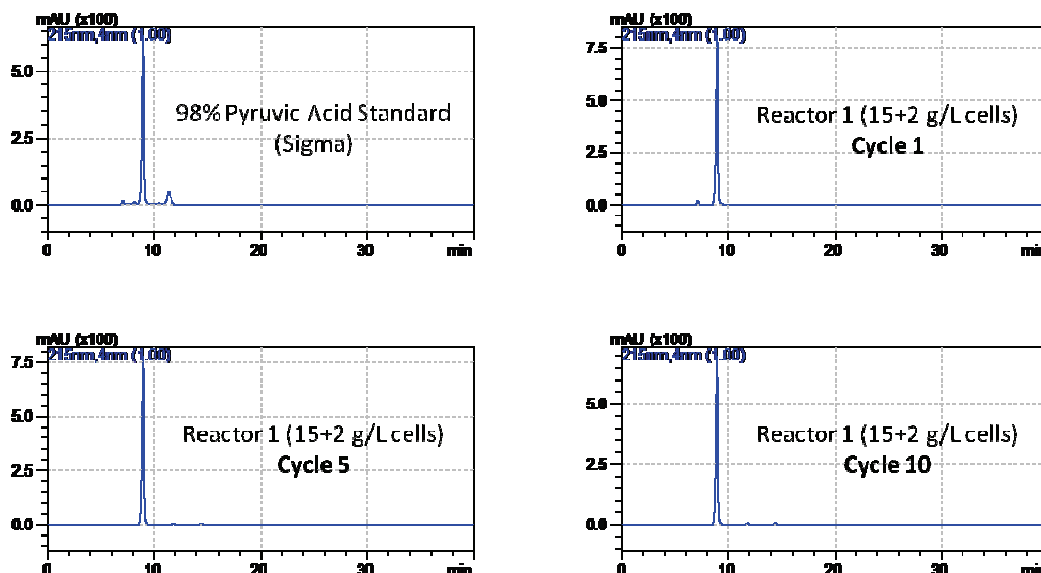


Figure 54. HPLC chromatograms of pyruvic acid product from reactor 1 at completed cycles 1, 5, and 10 compared to 98% pyruvic acid standard from Sigma. Reactor conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 10 reaction cycles. Initial 15 g/L cell concentration was supplemented with 2 g/L of fresh cells at cycles 4, 6, and 8. HPLC conditions were BioRad Aminex column at 30°C with 0.008 N sulfuric acid solvent at 0.6 mL/min and UV detection at 215 nm.

Conclusions for Specific Aim 2

Initial enzyme reaction set-points were determined from published results: 0.5 M L-lactic acid in 80 mM Tris, pH 7.0, 6.7 U/mL GO (equivalent to approximately 60 g/L spray-dried *P. pastoris*), 15°C, and 1 VVM air flow (Gough, et al., 2005). Gough et al. achieved a total of three reaction cycles using 0.5 M lactate to yield an “enzyme to product” ratio slightly above 1:2 calculated from published results.

Based on these parameters, experiments were performed in DASGIP reactors to improve “enzyme to product” ratio and pyruvate yield. pH values between 6.0 and 7.0 were determined to yield almost 100% conversion of lactate to pyruvate, so a pH operating window from 6.0 to 7.0 was specified for studies to optimize other parameters.

The high cost of product separation from dilute solutions prompted testing lactate concentrations above 0.5 M to generate more concentrated product solutions. Strong substrate inhibition was observed above 1.0 M lactate, which significantly decreased reaction rates and allowed time for byproduct formation. The increased lactate time exposure to hydrogen peroxide or native *P. pastoris* enzymes may have resulted in this formation of unwanted products. Substrate feeding was investigated to maintain low levels of lactate in the reaction medium, thus avoiding substrate inhibition, and to find an upper limit on product concentration. Significant product inhibition was discovered above 1.2 M pyruvate levels, so batch medium containing 1.0 M initial lactate without substrate feeding was determined optimal.

Further optimization studies were performed using the 6.0 to 7.0 pH operating range and 1.0 M lactate solutions without substrate feeding. Previous work involved preparation of reaction medium by dilution of lactate in 80 mM Tris buffer to maintain pH control. However, Tris buffer is costly and is an additional component that must be removed during the pyruvate purification process. With a large pH operating window (from 6.0 to 7.0 pH units) and good pH control in the DASGIP reactors, the effects of lactate dilution in water were tested. Comparable pyruvate yields were observed when using either Tris or water diluents, so the costly Tris buffer was eliminated in systems with the ability to maintain the specified 6.0 to 7.0 pH range. For another economic improvement, the reaction temperature was increased from 15 to 25°C and pyruvate production was verified to be consistent with that obtained at lower temperatures. Therefore, Tris buffer was removed from the process and cooling costs were reduced, both without reduction in pyruvate yields.

With the reaction pH, medium composition, and operating temperature defined, enzyme usage was optimized for the best “enzyme to product” ratio. Published work used the equivalent of 60 g/L spray-dried cells (Gough, et al., 2005), so this concentration was used for initial studies. 60 g/L cell loading resulted in $\approx 100\%$ conversion of 1.0 M

lactate to pyruvate. Other cell concentrations from 8 g/L to 90 g/L were also tested to determine optimal pyruvate yield and “enzyme to product” ratio. The 15 g/L condition gave comparable pyruvate productivity to the 30 g/L, 60 g/L, and 90 g/L concentrations, while the 8 g/L trial did not reach the same pyruvate yields. For this reason, 15 g/L cell concentrations were chosen for enzyme recycling experiments to further improve “enzyme to product” ratios. The 15 g/L spray-dried cells were recycled 10 times for conversion of 1.0 M lactate in 10 – 14 h. Then, 2 g/L of fresh cells were added at the beginning of cycles 4, 6, and 8, to maintain catalytic power. This resulted in > 95% lactate conversion for cycles 1 through 9 before decreasing to 92% conversion at cycle 10. As a result, minimal cell loading of 15 g/L coupled with small additions of fresh cells over 10 reaction cycles improved “enzyme to product” ratios from \approx 1:2 to over 1:38. These process improvements are summarized in Table 12.

Table 12. GO process changes and their impact.

Old Process	New Process	Impact
Solid/liquid separation	Stacked-disc centrifugation	Speed, high cell density paste ready for spray-drying
Cell re-suspension in buffer	Spray-drying	Reduced unit operations, stable powder for catalysis in water
BAC treatment	Eliminated toxic chemical	Reduced unit operation, reduced waste
Several washing steps	Eliminated multiple washing steps	Reduced unit operations
Wet paste for catalysis in buffer	Spray-dried powder for catalysis in water	> Enzyme activity, > operating temp., no buffer for simple product recovery

CHAPTER 5: DEMONSTRATION OF NEW PROCESS FOR
PYRUVATE PRODUCTION “END TO END” USING SPRAY-DRIED
PICHIA PASTORIS AND PREPARATION OF NEW SEED BANK

Early Process Development Work at the University of
Iowa’s Center for Biocatalysis and Bioprocessing

The choice of host and cloning procedures for GO and catalase are briefly described in Chapter 1 (Gavagan, et al., 1995; Mark S. Payne, et al., 1997). Thirteen fermentations were previously completed at the University of Iowa’s Center for Biocatalysis and Bioprocessing. This early process development work was consistent with *P. pastoris* fermentation procedures as described in Materials and Methods. During these fermentations, GO activities were tracked by o-aminobenzaldehyde assay (oAB) (Gavagan, et al., 1995) and recorded in International Units (U). One U is defined as the amount of enzyme needed to convert one micromole of substrate per minute under standard conditions (Aebi, 1983). Recent work involved a simplified dichloroindophenol assay (DCIP) to track GO activities during fermentations. It was reported that 0.55 DCIP units were approximately equal to 1 oAB unit (Gavagan, et al., 1995). Thus, early oAB results can be multiplied by 0.55 for translation to DCIP results. Catalase measurements were consistent for all work, so no additional conversions were required.

At DuPont, several organisms were genetically engineered and patented for GO production, including *Aspergillus nidulans*, *Hansenula polymorpha*, *Pichia pastoris*, and *Escherichia coli* (Anton & DiCosimo, 1998; Anton, et al., 1995; Anton, et al., 1997; Anton, et al., 1993a, 1993b; Anton, et al., 1996). *P. pastoris* was determined to have the best GO activity from oAB assay of cell extracts (Gavagan, et al., 1995). Based on these results, two strains of *P. pastoris* were tested for GO and catalase expression at the University of Iowa’s Center for Biocatalysis and Bioprocessing. Single-recombinant *P. pastoris* MSP 10 expressed recombinant GO and native catalase, while double-

recombinant *P. pastoris* MSP 8.6 expressed recombinant GO and recombinant catalase T from *S. cerevisiae*. Results of these six best fermentations are summarized in Table 13.

Table 13. Best *P. pastoris* fermentations with respect to overall GO and catalase yield from early process development studies at the University of Iowa's Center for Biocatalysis and Bioprocessing.

Batch	Strain	GO Activity*	Catalase Activity	Catalase T Activity	Wet Cell Weight
		[U/g]	[U/g]	[U/g]	[g/L]
99999-5	MSP 10	95	148,000	-----	30
99999-9	MSP 10	71	152,000	-----	82
99999-10	MSP 10	116	372,000	-----	100
99999-11	MSP 10	150	353,000	-----	100
99999-12	MSP 8.6	160	224,000	16,000	75
99999-13	MSP 8.6	160	229,000	20,000	110

Note: GO activity was measured with oAB assay of cell extracts, where 1 oAB U \approx 0.55 DCIP U.

P. pastoris MSP 8.6 resulted in the highest GO activity with 160 U/g measured by oAB assay or an equivalent 88 U/g by DCIP assay. While the double-recombinant MSP 8.6 strain had lower total catalase activity, only a base level of catalase is needed to break-down the hydrogen peroxide byproduct. The MSP 8.6 strain meets the required base level of catalase as demonstrated by the superior GO activity. Batch 99999-13 had the best combined GO activity and wet cell weight, resulting in an overall GO yield of 17,600 U/L based on the oAB assay or 9,680 U/L by DCIP assay. Process development work resulted in fermentation conditions shown in Table 14 for batch 99999-13.

For batch 99999-13, double-recombinant *P. pastoris* MSP 8.6 was grown according to documented procedures with the set-points in Table 14. The fermentor was controlled at 30°C, pH 5.0, and 40% dissolved oxygen. Dissolved oxygen levels were

maintained by airflow at 0.8 VVM and increasing agitation from 200 to 800 rpm. After maximum agitation, airflow was supplemented with oxygen to maintain the 40% dissolved oxygen setting. Optical density (OD₆₀₀), glycerol concentrations, methanol concentrations, and GO activities were tracked during the fermentation (Figure 55).

Table 14. Best *P. pastoris* fermentation conditions from early process development studies at the University of Iowa's Center for Biocatalysis and Bioprocessing

Operating Parameter	Set-Point
Organism	Double-recombinant <i>P. pastoris</i> MSP 8.6
Temperature	30°C
pH	5.0, controlled with NH ₄ OH
Dissolved Oxygen	40% (calibrated to 100% in air-saturated medium)
Growth	22 h batch growth followed by 10 h fed-batch
Induction	2 – 10 g/L MeOH induction for 30 h

After the initial lag phase of batch growth, cell densities increased to approximately 20 OD₆₀₀ at 22 h. Prior fermentations of *P. pastoris* strain MSP 8.6 have demonstrated low glycerol levels in the batch medium (4 – 10 g/L) at 22 h, so glycerol feed was started to maintain exponential growth. This glycerol feed was continued until 32 h, at which time the feed was stopped, and glycerol levels fell to between 4 and 6 g/L. When the glycerol concentrations reached the 4 – 6 g/L target, the fermentation was induced with 10% v/v methanol. Note the residual glycerol at the time of induction, because the smooth transition from glycerol to methanol consumption was essential for adequate protein expression. When methanol concentrations decreased below 4 g/L, methanol feed was initiated to maintain 2 – 10 g/L levels. GO activity of cell extracts was monitored by oAB assay (Gavagan, et al., 1995). Then, the culture was harvested at peak GO activity, which typically occurred 30 h post-induction.

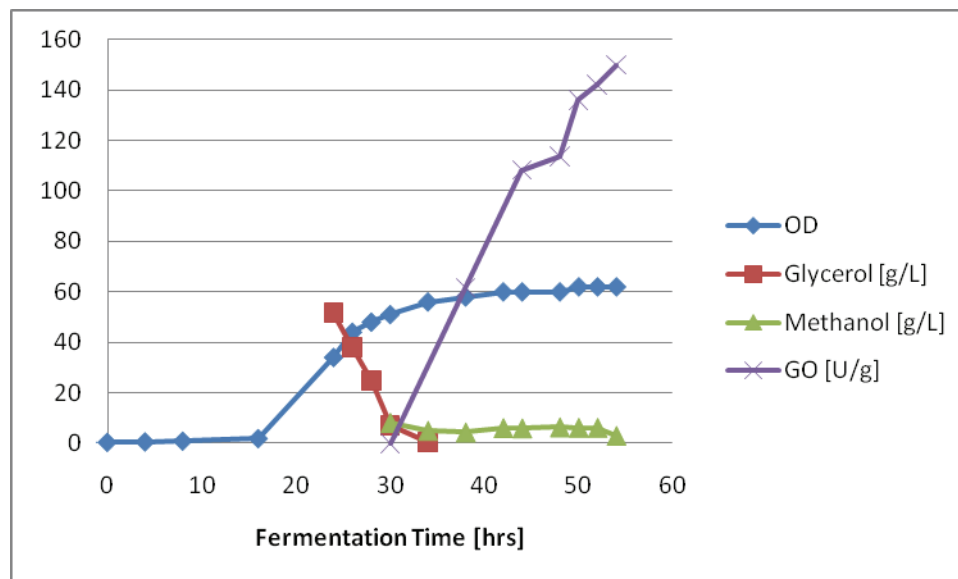


Figure 55. Process development fermentation 99999-13: *P. pastoris* MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 10 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h. Optical density (OD₆₀₀) was tracked by UV-VIS spectroscopy, glycerol and methanol concentrations were measured by HPLC, and GO activity was followed by oAB assay

Specific Aim 3

Verification of *P. pastoris* fermentation conditions, preparation of a new seed bank, and “end to end” demonstration of the entire process determine overall “enzyme to product” ratio and confirm pyruvate yield. Glycerol feeding and methanol induction parameters were tested to verify established fermentation conditions and to check for improvements. During the growth phase, glycerol feeding was extended beyond 10 h to investigate greater biomass production. For induction, an elevated methanol concentration range of 10 – 20 g/L was compared to the established 2 – 10 g/L range to define the best conditions for GO and catalase expression at higher cell densities. Also, the length of induction was studied from 24 to 35 h to recover the biomass at peak activity levels.

In addition to verifying *P. pastoris* fermentation conditions, the entire process was demonstrated from seed bank through fermentation, spray-drying, and the enzyme reaction to determine overall “enzyme to product” ratio and confirm pyruvate yield. The established process used benzalkonium chloride (BAC) to permeabilize *P. pastoris* cells for biocatalysis (Gough, et al., 2005). These BAC treated cells were recycled three times for conversion of 0.5 M lactate solutions, yielding an “enzyme to product” ratio of approximately 1:2 from published results (Gough, et al., 2005). Major process changes were implemented by replacing BAC treatment with spray-drying and optimizing the enzyme reaction, including biocatalyst recycling, to improve “enzyme to product” ratio. These significant changes required an “end to end” demonstration of the entire process to test the new seed bank, define overall inputs and outputs, and confirm the final product for preparation of a technology transfer package and marketing to industry.

Verification of *P. pastoris* Fermentation Conditions for Maximum GO and Catalase Yield

The fermentation conditions for batch 99999-13 (Table 14) resulted in peak cell densities of ≈ 60 OD₆₀₀ (Figure 55) and a biomass yield of 110 g/L wet cell weight (Internal Communications). It has been reported that *P. pastoris* fermentations have achieved biomass yields on the order of 100’s of grams per liter with cell densities up to 500 OD₆₀₀ (Cregg, et al., 2000). For this reason, the glycerol fed-batch was extended to increase biomass levels and test protein expression at higher cell densities. Fermentation 27016-1e was completed as described in Materials and Methods with the glycerol fed-batch extended beyond 10 h. After 14 h of glycerol feeding, the cell densities plateaued around 200 OD₆₀₀, so the feed was transitioned to methanol for induction. Methanol feeding continued for another 35 h with samples removed at 0, 15, 24, 30, and 35 h post-induction. The fermentation was harvested 35 h after induction with a final biomass yield of 136 g/L wet cell weight.

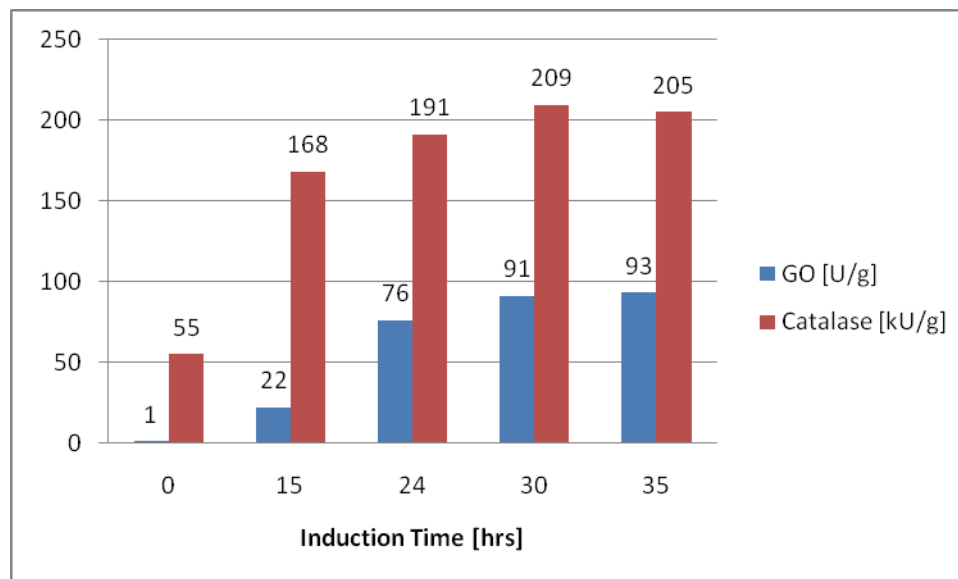


Figure 56. GO and catalase activities of spray-dried samples from fermentation 27016-1e plotted as a function of induction time. Fermentation conditions were *P. pastoris* MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 35 h.

While batch 27016-1e had an improved biomass yield compared to 99999-13 (136 g/L vs. 110 g/L wet cell weight), enzyme activities were measured to determine GO and catalase yield. Post-induction samples were spray-dried and analyzed for GO and catalase activities as described in Materials and Methods. Enzyme activities for the spray-dried samples were plotted as a function of induction time in Figure 56. Final enzyme activities for fermentation 27016-1e were 93 U/g for GO by DCIP assay and 205,000 U/g for catalase after 35 h induction. However, no appreciable change in enzyme activity was observed from 30 h to the final harvest at 35 h. Therefore, the 30 h methanol induction that was established during early process development was confirmed as optimal. At the suggested 30 h post-induction harvest, batch 27016-1e had enzyme activities of 91 U/g for GO and 209,000 U/g for catalase. The combined GO activity and

136 g/L wet biomass weight resulted in an overall GO yield of 12,380 U/L by DCIP assay. This was compared to fermentation 99999-13 with 110 g/L wet cell weight, resulting in the lower GO yield of 9,680 U/L by DCIP assay. Therefore, the glycerol fed-batch was extended from 10 h to 14 h to build biomass up to 200 OD₆₀₀ before methanol induction. The longer glycerol feeding time achieved greater biomass concentrations without sacrificing enzyme activity; thus, resulting in higher total GO and catalase yields.

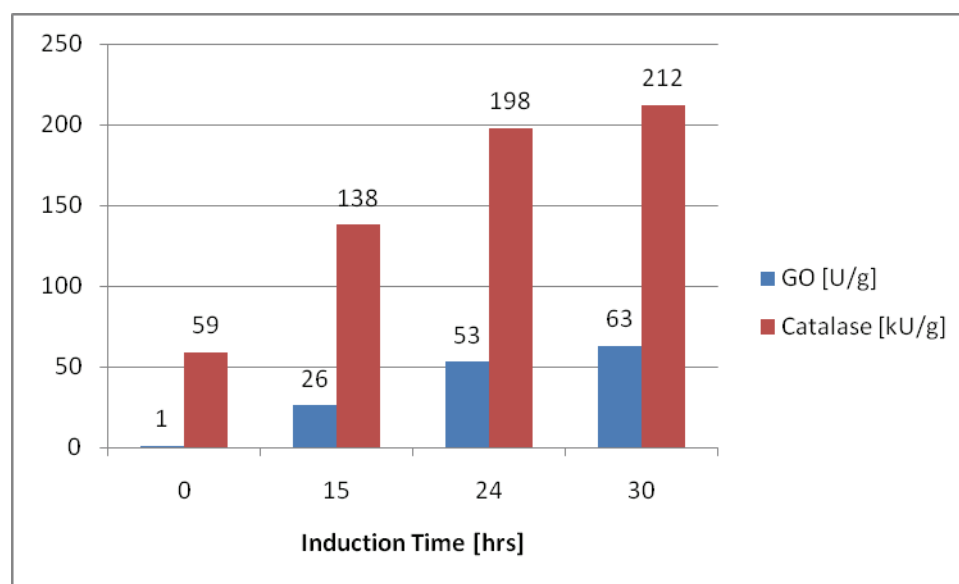


Figure 57. GO and catalase activities of spray-dried samples from fermentation 27016-1f plotted as a function of induction time. Fermentation conditions were *P. pastoris* MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 10 – 20 g/L methanol induction for 30 h.

In addition to the optimal 30 h induction time, methanol concentration during induction is another significant parameter for recombinant enzyme expression. The defined fermentation conditions for batch 99999-13 indicated a methanol concentration range of 2 – 10 g/L. Methanol concentration was controlled offline by Analox enzymatic assay and adjusting the methanol feed rate as appropriate. With the success of glycerol

feeding to increase biomass yield, it was hypothesized that higher cell densities might require elevated methanol levels for maximum GO and catalase induction.

Fermentation 27016-1f was completed as described previously with set-points modified from Table 14. Glycerol feeding was set to 14 h for increased biomass production, and an elevated methanol concentration of 10 – 20 g/L was investigated for greater GO and catalase expression at higher cell densities. Methanol feeding continued for 30 h with samples removed at 0, 15, 24, and 30 h post-induction. Samples were spray-dried and GO and catalase activities were measured. Figure 57 shows GO and catalase activities of the spray-dried samples as a function of induction time for fermentation 27016-1f. Enzyme activities for fermentation 27016-1f were 63 U/g by DCIP assay for GO and 212,000 U/g for catalase at 30 h post-induction. Compared to the same time points for batch 27016-1e (91 U/g for GO and 209,000 U/g for catalase), the 10 – 20 g/L methanol concentration did not promote additional enzyme expression in the higher biomass culture. In contrast, the elevated methanol levels hindered induction for GO. Furthermore, cell densities decreased from pre-induction measurements of ≈ 200 OD₆₀₀ to ≈ 160 OD₆₀₀ after induction, suggesting methanol toxicity above 10 g/L.

Table 15. Summary of *P. pastoris* fermentation conditions for maximum biomass production and GO and catalase yield.

Operating Parameter	Set-Point
Organism	Double-recombinant <i>P. pastoris</i> MSP 8.6
Temperature	30°C
pH	5.0, controlled with NH ₄ OH
Dissolved Oxygen	40% (calibrated to 100% in air-saturated medium)
Growth	22 h batch growth followed by 14 h fed-batch
Induction	2 – 10 g/L MeOH induction for 30 h

Established parameters of 30 h induction time and 2 – 10 g/L methanol concentration were verified to be optimal for resulting GO and catalase activities. Additionally, the glycerol fed-batch was modified from 10 to 14 h to build biomass up to 200 OD₆₀₀ without sacrificing enzyme activity. Table 15 summarizes the verified conditions for maximum biomass production and enzyme expression, resulting in optimal GO and catalase yield. Low cell densities of ≈ 60 OD₆₀₀ were observed in previous fermentations, so glycerol feeding was modified to increase biomass to ≈ 200 OD₆₀₀. The induction parameters of 2 – 10 g/L methanol, maintained for 30 h, were verified as optimal for GO and catalase yield at these increased cell densities. *P. pastoris* fermentation inputs were confirmed to generate outputs of 80 – 100 U/g for GO, 180,000 – 220,000 U/g for catalase, and 120 – 140 g/L wet biomass weight or GO and catalase yields of approximately 11,700 U/L and 26,000,000 U/L, respectively.

“End to End” Demonstration of the New Process for
Production of Pyruvate from Lactate by Spray-Dried *P.*
pastoris to Confirm Process Inputs and Outputs

A new seed bank was prepared as described in Materials and Methods for the proposed technology transfer package. The integrity of the seed bank was confirmed by 30 L fermentation to analyze biomass production and GO and catalase activities for consistency with defined process outputs (80 – 100 U/g for GO, 180,000 – 220,000 U/g for catalase, and 120 – 140 g/L wet biomass weight). The new seed bank fermentation 27016-1h was completed using the optimized conditions verified in Table 15. Optical density, glycerol concentrations, methanol concentrations, and GO activities were tracked during the fermentation (Figure 58).

After 20 h of batch growth, the new seed bank reached an approximate cell density of 40 OD₆₀₀, depleting glycerol levels to 0.9 g/L. Glycerol feed was started at this time to maintain exponential growth and increase biomass to the ≈ 200 OD₆₀₀ target.

Several samples were taken throughout glycerol feeding to carefully control levels below 5 g/L for an immediate switch to methanol induction. Glycerol feeding was continued until 34 h, at which time the cell density reached 188 OD₆₀₀ and the culture was induced with 10% v/v methanol. When methanol concentrations decreased below 4 g/L, methanol feed was initiated to maintain 2 – 10 g/L levels. For direct comparison of the seed banks, fermentation 27016-1h was harvested at 30 h after induction. Samples were taken at 0, 15, 24, and 30 h post-induction for analysis of enzyme expression by SDS-PAGE as described in Materials and Methods. In addition to the molecular weight standard, 27016-1h was compared to batch 27016-1e grown using the old seed bank at the same fermentation conditions. *E. coli* expressing GO was also used as a reference, and results of 27016-1e, 27016-1h, and markers are shown in Figure 59.

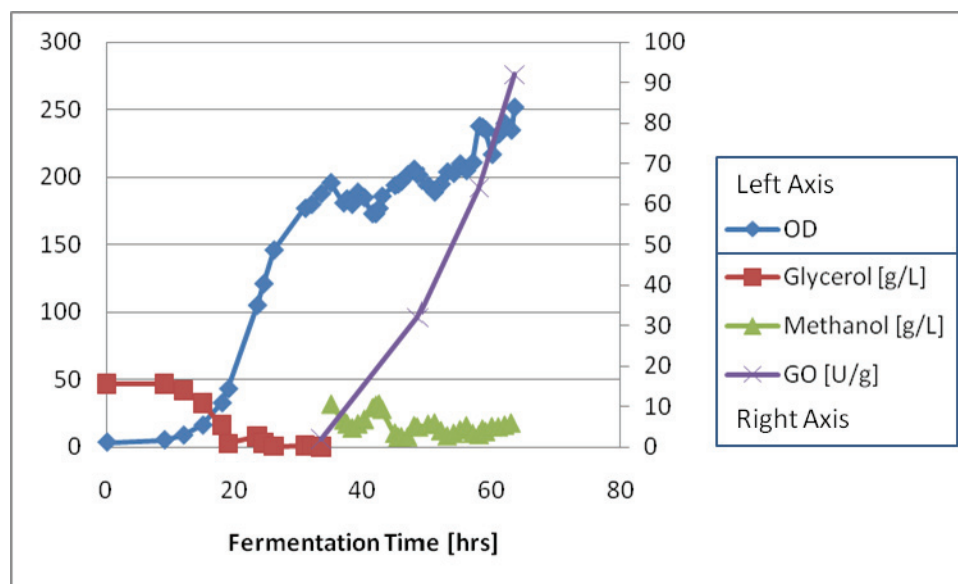


Figure 58. Fermentation 27016-1h: *P. pastoris* MSP 8.6 new seed bank, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h. Optical density was tracked by UV-VIS spectroscopy, glycerol and methanol concentrations were measured by Analox instrument, and GO activity was followed by DCIP assay of spray-dried samples.

GO is a tetramer, composed of four subunits, each with a molecular weight of approximately 43,000 Da (Lindqvist & Branden, 1985). *E. coli* expressing GO was also used as a marker. Shown by the heavy insoluble fraction, most GO from *E. coli* formed inclusion bodies with subunits confirmed at 43,000 Da. For the 27016-1h sample taken before induction, only a slight band was visible at the specified molecular weight. However, the 43,000 Da band became increasingly darker as the length of induction progressed from 15 to 24, and 30 h.

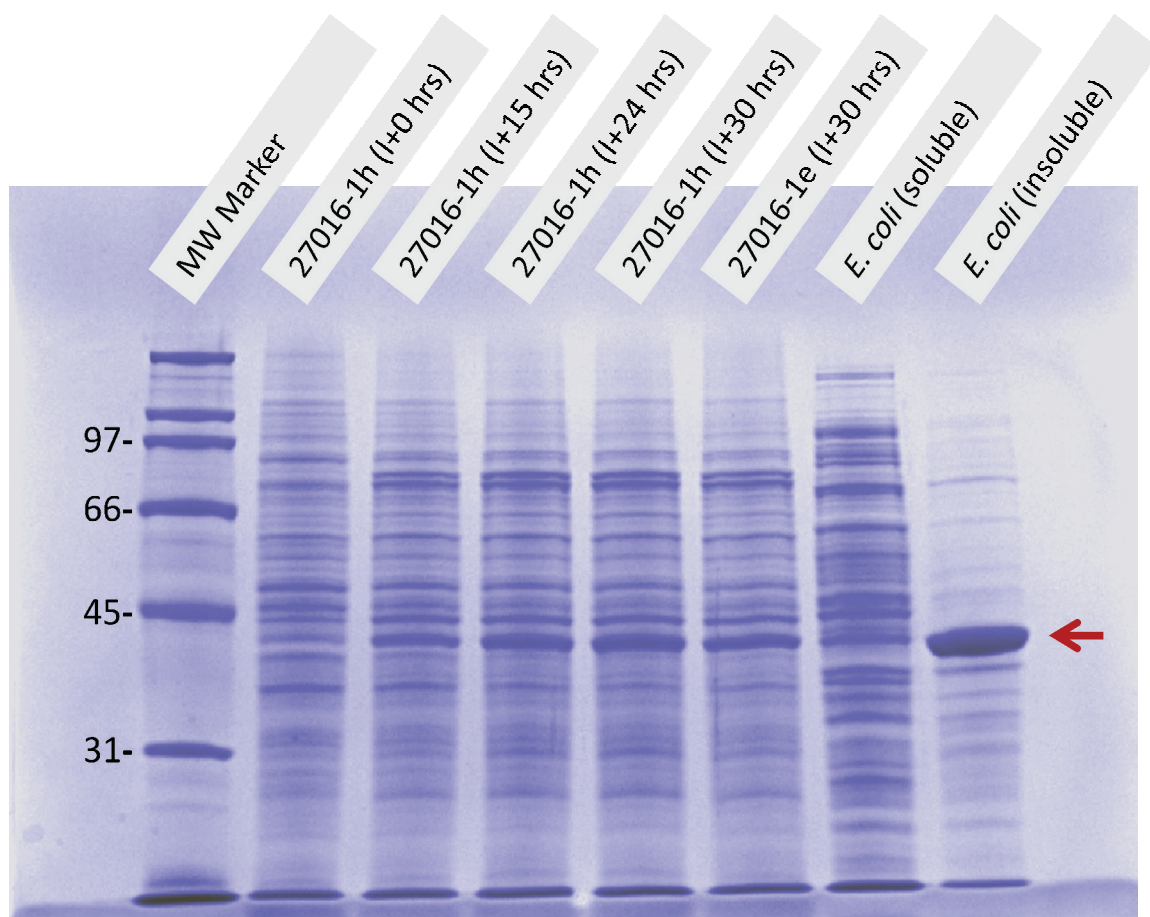


Figure 59. SDS-PAGE analysis of fermentation 27016-1h to test the new seed bank. Fermentation conditions were *P. pastoris* MSP 8.6 new seed bank, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h.

Also, final samples were compared between batch 27016-1h using the new seed bank and batch 27016-1e using the old seed bank at the same conditions (*P. pastoris* MSP 8.6, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 2 – 10 g/L methanol induction for 30 h). The 30 h sample for batch 27016-1h yielded a slightly darker band compared to the corresponding time point for batch 27016-1e, thus suggesting an equal or greater level of GO expression for the new seed bank. Catalase is also a tetramer with the molecular weight of each subunit around 60,000 Da (Klei, et al., 1990). Catalase was present in two forms: a native catalase, which was constitutively expressed, and catalase T linked to a methanol-inducible promoter. Due to the constant expression of the native catalase, a band at 60,000 Da was visible in all samples except those for *E. coli*.

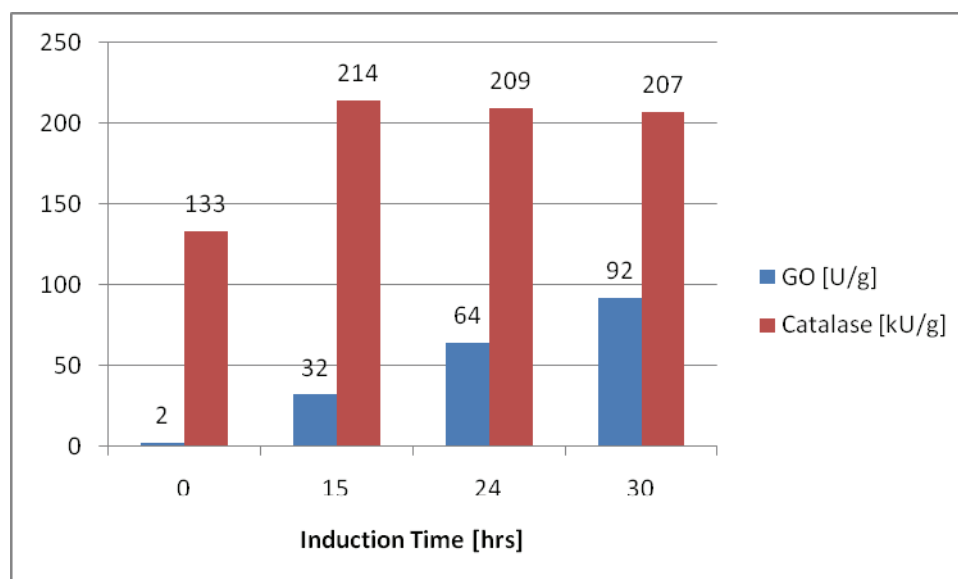


Figure 60. GO and catalase activities of spray-dried samples from fermentation 27016-1h plotted as a function of induction time. Fermentation conditions were *P. pastoris* MSP 8.6 new seed bank, 30°C, pH 5.0, 40% dissolved oxygen, 14 h glycerol fed-batch, and 10 – 20 g/L methanol induction for 30 h.

To quantify GO and catalase expression, all post-induction samples were spray-dried and analyzed for GO and catalase activity. Enzyme activities of spray-dried samples vs. induction time were plotted for fermentation 27016-1h (Figure 60). SDS-PAGE results yielded bands of increasing intensity vs. induction time for GO and bands of similar intensity vs. induction time for catalase. Consistent with SDS-PAGE results, GO activity increased throughout methanol induction up to 92 U/g measured by DCIP assay at the final harvest. Catalase activity peaked at 214,000 U/g at 15 h post-induction and slowly decreased to 207,000 U/g by fermentation harvest. Final biomass recovered was 2.87 kg from 21 L working volume or an equivalent of 137 g/L wet cell weight. Results for batch 27016-1h using the new seed bank achieved target outputs defined for the process, including 80 – 100 U/g for GO, 180,000 – 220,000 U/g for catalase, and 120 – 140 g/L wet biomass weight. The combined GO activity and wet cell weight resulted in an overall GO yield of 12,600 U/L as expected.

Table 16. 15 Reaction cycles using spray-dried biocatalyst from new seed bank to verify reaction conditions and determine upper limit for “enzyme to product” ratio

Reactor	pH	Lactate [M]	Biocatalyst [g/L]	Cycles	Medium	Temperature [°C]
1	7.0	1.0	15 + 2	15	water	25
2	7.0	1.0	15 + 2	15	water	25
3	7.0	1.0	12 + 2	15	water	25
4	7.0	1.0	12 + 2	15	water	25

Fermentation outputs were confirmed for the new seed bank, and batch 27016-1h was spray-dried to yield approximately 1.25 kg dry cells (almost 45% of the initial wet biomass weight) with 92 U/g GO activity and 207,000 U/g catalase activity in preparation for the enzyme reaction. The enzyme reaction was carried-out at optimal conditions from

Chapter 4. Due to strong substrate and product inhibition, 1.0 M initial L-lactate in water was chosen for batch conversion with cell recycling. The reaction was controlled at pH 7.0 and the near-ambient temperature of 25°C for reduced cooling costs. To determine the upper limit on “enzyme to product” ratio, starting biocatalyst concentrations of 12 g/L or 15 g/L were recycled 15 times with addition of 2 g/L of fresh cells as needed to maintain activity. Experimental conditions are given in Table 16.

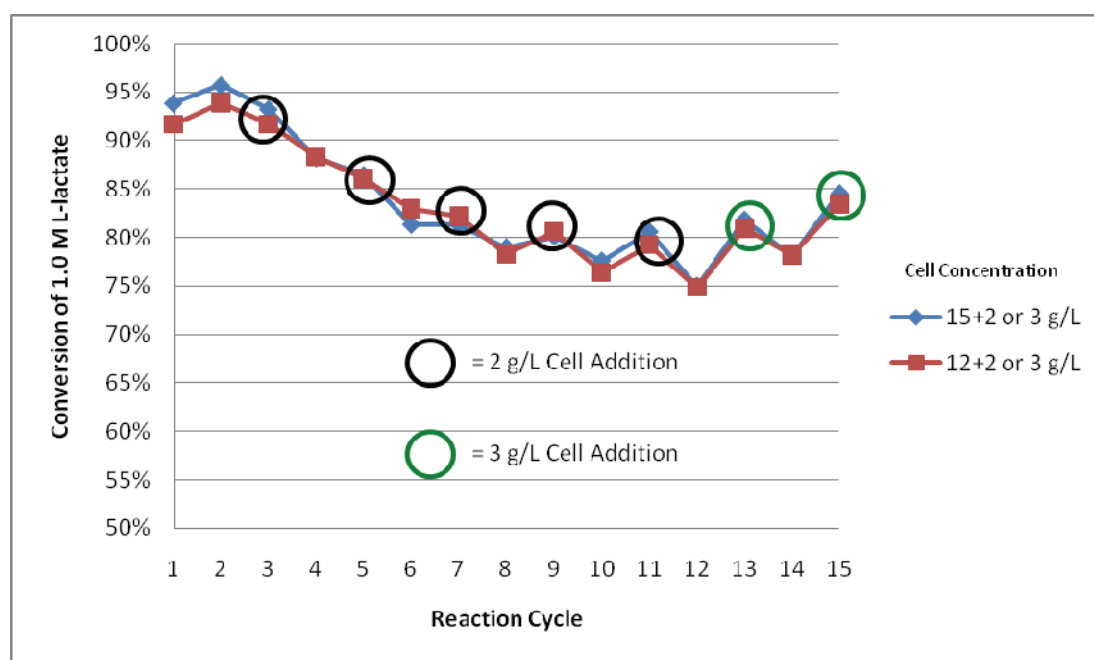


Figure 61. Percent conversion of 1.0 M lactate at the end of each 12 – 16 h reaction cycle. Reaction conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 15 reaction cycles. Initial cell concentrations were supplemented with either 2 g/L fresh cells or 3 g/L fresh cells at the start of the indicated reaction cycle.

After the 12 – 16 h reaction cycles, cells were removed from the product solution by centrifugation at 7,000 x g and 4°C for 10 min. A large batch of substrate solution was prepared for the entire experiment to keep consistency among cycles and stored at

4°C until needed. The recovered cells were then re-suspended in fresh substrate solution, and the process was repeated for 15 cycles. Throughout the recycling experiment, small amounts of fresh cells were added to maintain enzyme productivity. For all reactors, 2 g/L of fresh cells were added at the start of cycles 3, 5, 7, 9, and 11, while 3 g/L of fresh cells were added at the beginning of cycles 13 and 15. Initial and final samples were taken for Analox measurement of lactate and pyruvate concentrations. All reactors gave similar results over the 15 cycles, so one representation of each initial cell concentration is shown in Figure 61.

The 15 g/L initial cell concentration yielded about 2% higher lactate conversions vs. the 12 g/L condition for the first three cycles at 94%, 96%, and 93%, respectively. The percent conversion for cycle 2 increased as the reaction time was extended from 12 h at the first cycle to 13 h for cycles 2 – 7. At the beginning of cycle 3, 2 g/L of fresh cells were added to all reactors to help maintain conversions > 90% for cycle 3. However, conversions fell from 93% at cycle 3 to around 82% at cycle 7 even with 2 g/L fresh cell additions at the start of cycles 5 and 7. To offset this loss, reaction time was increased to 14 h for cycles 8 – 9, 15 h for cycles 10 – 11, and 16 h for the remaining cycles 12 – 15. In addition to increasing reaction time, 2 g/L of fresh cells were added at the beginning of cycles 9 and 11, which resulted in 75 – 80% conversions. The combination of 16 h reaction time and 3 g/L fresh cell additions helped reverse the trend and raise conversions to 78 – 85% for the last three cycles.

Pyruvate product formation was confirmed by HPLC analysis and results are shown in Figure 62. HPLC chromatograms are shown for the 98% pyruvic acid standard obtained from Sigma as well as the pyruvic acid product from completed cycles 1, 4, 8, 12, and 15 for reactor 1. The first reactor had an initial cell concentration of 15 g/L with 2 g/L fresh cell additions at the beginning of cycles 3, 5, 7, and 9, while 3 g/L of fresh cells were added at the start of cycles 11 and 15.

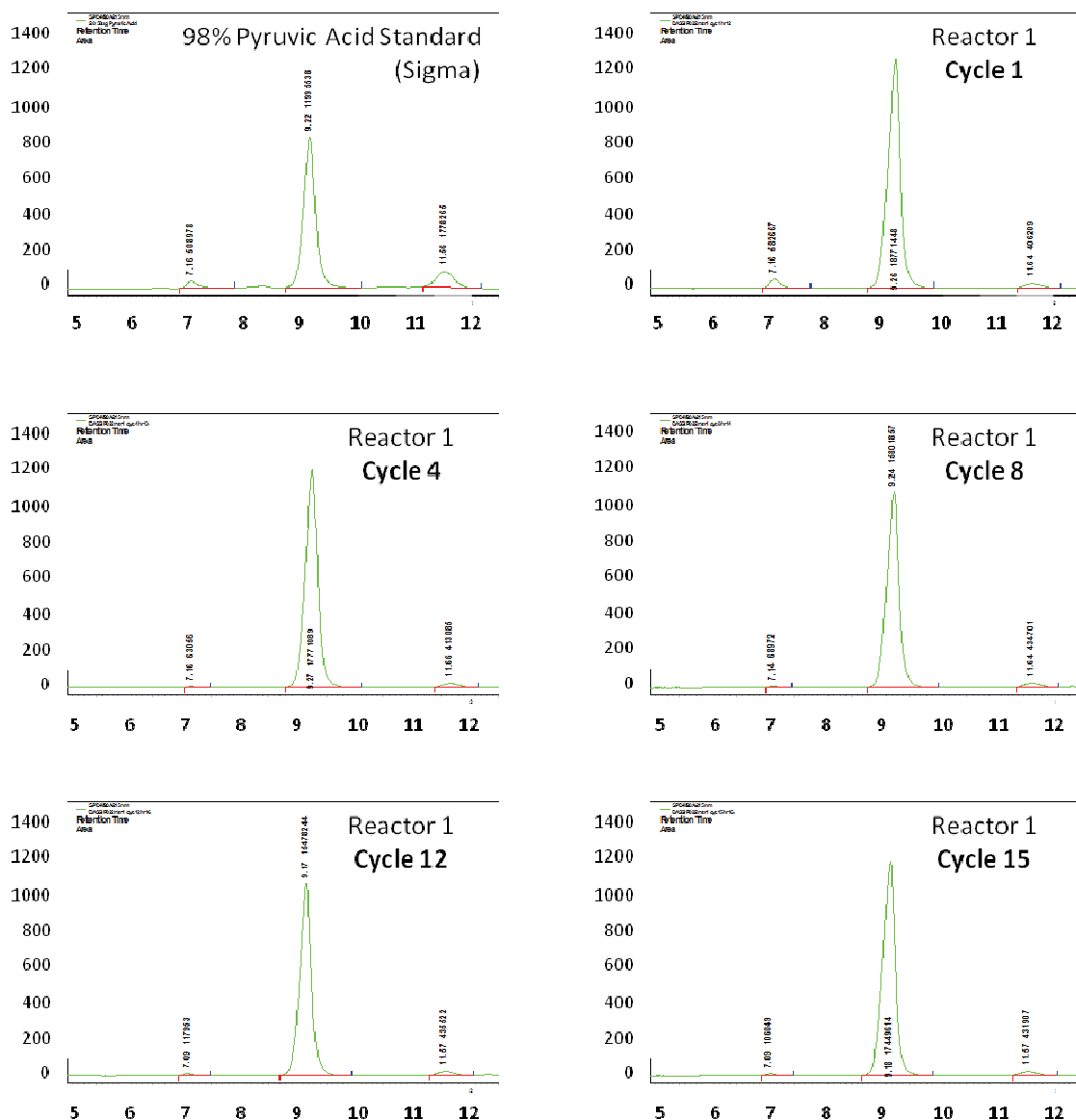


Figure 62. HPLC chromatograms of pyruvic acid product from reactor 1 at completed cycles 1, 4, 8, 12, and 15 compared to 98% pyruvic acid standard from Sigma. Reactor conditions were 1.0 M L-lactic acid in water, pH 7.0, 25°C, 1 L/min air flow, 1250 rpm, and 15 reaction cycles. Initial 15 g/L cell concentration was supplemented with 2 g/L of fresh cells at cycles 3, 5, 7 and 9, and fresh cell additions of 3 g/L at cycles 11 and 15. HPLC conditions were BioRad Aminex column at 30°C with 0.008 N sulfuric acid solvent at 0.6 mL/min and refractive index detection at 215 nm.

For HPLC analysis at the given conditions, approximate retention times were 9.2 min for pyruvate and 11.6 min for lactate. Therefore, it was observed that lactic acid was the main contaminant in the 98% Sigma pyruvic acid. However, this lactic acid peak is less intense in the pyruvic acid product from cycles 1, 4, 8, 12, and 15 for reactor 1 vs. the 98% Sigma pyruvic acid standard, suggesting high purity. Furthermore, these chromatograms are consistent throughout all 15 reaction cycles showing strong pyruvic acid peaks with minimal byproducts. Although the conversions fell below 90%, no lactate or other impurities were found when pyruvate was analyzed by HPLC.

Conclusions for Specific Aim 3

Early work at the University of Iowa's Center for Biocatalysis and Bioprocessing used benzalkonium chloride (BAC) to permeabilize *P. pastoris* cells for biocatalysis (Gough, et al., 2005). These BAC treated cells were recycled three times for conversion of 0.5 M lactate, yielding an "enzyme to product" ratio of approximately 1:2 (Gough, et al., 2005). Major process changes were implemented by replacing BAC treatment with spray-drying and optimizing the enzyme reaction, including biocatalyst recycling, to improve overall "enzyme to product" ratio. The improved process was carried-out at the optimal conditions from Chapters 3 and 4, including spray-dried *P. pastoris* MSP 8.6 from the old seed bank, 1.0 M L-lactate without substrate feeding, simplified L-lactate/water medium, pH 7.0, and 25°C. Spray-dried cells at 15 g/L were recycled 10 times with either 2 g/L fresh cell additions at the start of cycles 4, 6, and 8, or 5 g/L fresh cell additions at the beginning of cycle 4 only. The 2 g/L fresh cell additions helped boost catalytic power throughout the 10 reaction cycles to yield an improved "enzyme to product" ratio of 1:38.

The enzyme reaction was repeated with spray-dried cells from batch 27016-1h using the new seed bank. Again, the process was completed with optimized conditions from Chapters 3 and 4. To determine the upper limit on "enzyme to product" ratio,

starting biocatalyst concentrations of 12 g/L or 15 g/L were recycled 15 times. Fresh cells at 2 g/L were supplemented at the start of cycles 3, 5, 7, and 9, while 3 g/L of fresh cells were added at the beginning of cycles 11 and 13. All reactors gave similar results over the 15 cycles, so the 12 g/L initial cell concentration resulted in a maximum “enzyme to product” ratio of 1:41 at completion of the 15th cycle, therefore exceeding the target ratio of 1:38 established with the old seed bank and 10 enzyme cycles.

CHAPTER 6: SUMMARY OF COMPLETED RESEARCH AND SUGGESTED FUTURE WORK

Second-Generation Process Summary

Initial tests demonstrated comparable or better enzyme activities in spray-dried cells compared to detergent permeabilized cells. Both GO and catalase activities were stable for more than 20 days when stored at room temperature, and over 90% activity remained after 30 days. In addition, tests showed that there was no significant loss of enzyme activity due to leaching. The robustness of spray-drying was further confirmed by repeating the process under optimized conditions (150°C, 15 mL/min feed rate, and 600 mg cells/mL liquid feed). The spray-dried powder yielded enzyme activities of 80 – 100 U/g for GO and 160,000 – 220,000 U/g for catalase. Thus, it was concluded that spray-drying *P. pastoris* for use as biocatalyst was an effective process model.

Initial enzyme reaction set-points were determined from published results where Gough et al. achieved an “enzyme to product” ratio slightly above 1:2 (Gough, et al., 2005). Based on these parameters, experiments were performed in DASGIP reactors to maximize “enzyme to product” ratio and pyruvate yield. The DASGIP system contained four parallel reactors with control of temperature, pH, and dissolved oxygen. Other key variables included substrate loading, conducting the reaction in buffer or water, minimizing enzyme concentration, and maximizing the number of enzyme recycles. Optimal performance was achieved in water at pH 7.0 with an operating temperature of 25°C and 1.0 M substrate loading.

Major process changes were implemented by replacing BAC treatment with spray-drying and optimizing the enzyme reaction, including biocatalyst recycling, to improve overall “enzyme to product” ratio. The improved process was carried-out under optimal conditions using a new seed bank. Enzyme loading was at 12 g/L for the first two cycles, and subsequently, 2 – 3 g/L of fresh cells were added every alternate cycle to

reach 15 cycles. Under these conditions, a maximum “enzyme to product” ratio of 1:41 was achieved, therefore exceeding the ratio established by Gough et al.

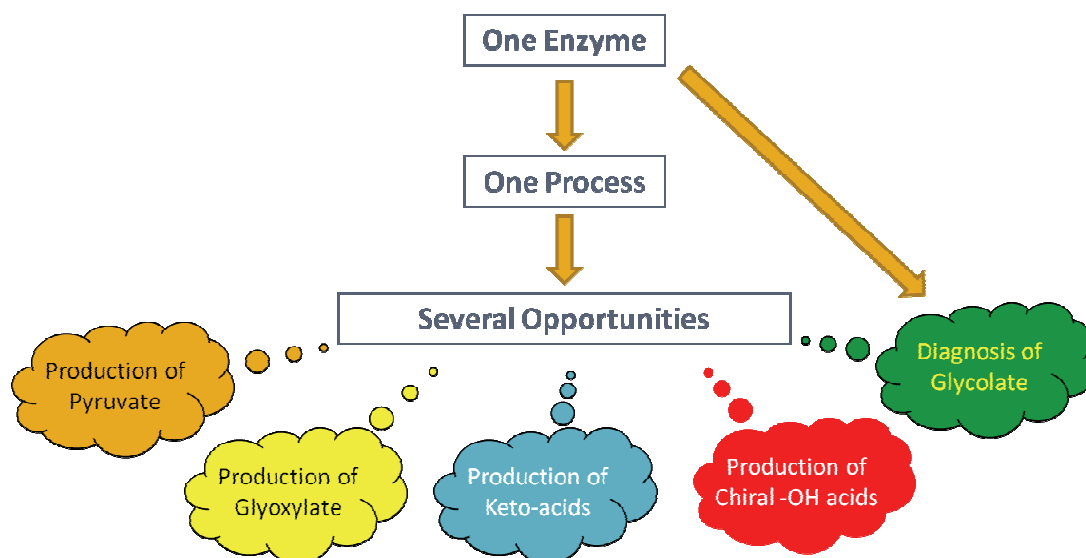


Figure 63. Spray-drying as a platform technology to yield *P. pastoris* biocatalyst for production of several keto-acids, resolution of chiral hydroxy-acids, and enzyme activity tests such as the diagnosis of glycolate.

Lastly, spray-dried *P. pastoris* expressing GO was tested for activity on other hydroxy-acids for resolution of these hydroxy-acids or production of their corresponding keto-acids (Appendix A). Glycolate oxidase activity was observed with several other substrates, including β -chlorolactic acid, 2-hydroxybutyric acid, and glycolic acid to name a few. High selectivity to S enantiomers of 3-phenyllactic acid, lactic acid, and 2-hydroxy-butryic acid was detected. Spray-drying did not alter GO enantiospecificity. Therefore, the spray-dried GO biocatalyst has new opportunities to produce R-specific chiral hydroxyacids from racemic mixtures (Figure 63).

Suggested Future Work

The conversion of lactate to pyruvate via spray-dried *P. pastoris* expressing GO and catalase has been optimized at the laboratory scale. Several key findings can be translated to the production scale including, application of the spray-drying process to prepare active GO and catalase, biocatalyst storage for use “as needed,” no detectable enzyme leaching from cells, and ability to reuse the biocatalyst for improved “enzyme to product” ratios. However, unit-operations must be resized for large scale production.

Fermentations have been completed up to 100 L with final harvest at 30 – 35 h and biomass yields of 7.2 kg. However, scaling to larger fermentations may result in reduced growth rates or cell yields due to limited oxygen transfer rates. Thus, further optimization could be needed to maintain high cell yields from larger fermentations. Spray-drying conditions were optimized for the Buchi B-190 spray-dryer with a maximum feed rate of 15 mL/min. From laboratory results, higher feed rates should be tested, and the unit can be scaled-up based on residence time and evaporation capacity. Enzyme reaction rate will also be dictated by oxygen transfer capacity, and reaction temperature should be controlled at 25°C to increase enzyme life. For cell recycling, centrifugation was easily done using a model with fixed-angle rotor at laboratory scale. Other cell separation techniques, such as tangential flow filtration, would be a better choice for efficient recycling of cells at larger volumes. Additionally, increased time for cell recovery at large scale may influence calculations for “enzyme to product” ratio and pyruvate yield, due to enzyme instabilities. This should be addressed at the next level.

Further process improvement can be accomplished through directed evolution of the GO gene to withstand 2.0 – 4.0 M substrate loading. Increased GO activity at high lactate concentrations could yield concentrated product solutions and minimize separation costs. X-ray crystallography of evolved GO would be valuable for tracking changes in active site structure and selecting additional substrates for chiral hydroxy-acid resolution or keto-acid production. Using spray-dried cells in biphasic solvents could be

another research opportunity as many aromatic substrates will likely have low water solubility. In addition, application of the spray-drying technology can be demonstrated with other enzymes in both yeast and bacterial backgrounds.

APPENDIX A: ENANTIOSELECTIVITY OF GLYCOLATE OXIDASE FROM SPRAY-DRIED *PICHTIA PASTORIS*

Importance of Chiral 2-Hydroxy Acids

The conversion of L-lactic acid to pyruvic acid by spray-dried *P. pastoris* expressing GO has been demonstrated. This selective oxidation of one enantiomer can also achieve resolution of optically active 2-hydroxy acids from racemic mixtures. Optically active 2-hydroxy acids are important for the synthesis of bioactive molecules, such as virus protease and angiotensin converting (ACE) inhibitors, beta blockers, A2 antagonists, and calcium channel blockers (Larissegger-Schnell, et al., 2006a). Optically pure 2-hydroxy acids are used for making chiral synthons like glycols (Prelog, et al., 1954), halo esters (Lee & Downie, 1967), and epoxides (Mori, et al., 1979). Derivatives of optically active 2-hydroxy acids, such as 3-cyclohexyl-2-hydroxy acids, are used in sialyl Lewis analogs, which are currently being tested for treatment of inflammatory disorders (Storz, et al., 2003).

Enantioselectivity of GO from Spray-Dried *P. pastoris*

Adam et al. tried the synthesis of (R)-2-hydroxy acids by enantio-selective oxidation of racemic 2-hydroxy acids using GO extracted from spinach (Adam, et al., 1997; Adam, et al., 1998). The group used soluble GO, an expensive FMN cofactor to maintain GO activity, and exogenous catalase to break-down the hydrogen peroxide byproduct. However, a more robust and economical route for the synthesis of these optically active 2-hydroxy acids is desirable. Thus, *P. pastoris* whole-cells expressing GO were tested for activity on several industrially relevant 2-hydroxy acids. *P. pastoris* was generated by fermentation process, and cells were prepared for biocatalysis either by detergent permeabilization or spray-drying as described in Materials and Methods. GO activity for the permeabilized or spray-dried cells was measured by DCIP assay and expressed in terms of lactic acid activity (Table 17).

Table A1. GO activity for racemic 2-hydroxy carboxylic acids where the optically active enantiomer is industrially important.

Racemic 2-hydroxy acid	GO Activity Relative to Lactic Acid	
	Permeabilized Cells	Spray-dried Cells
Lactic acid	100	100
3-Indolelactic acid	18	26
3-Phenyllactic acid	25	37
<i>p</i> -Hydroxyphenyllactic acid	26	38
β -Chlorolactic acid	100	121
Trifluorolactic acid	11	2
2-Hydroxybutyric acid	91	129
2-Hydroxy-3-methylbutyric acid	1	2
Glycolic acid	90	94
Mandelic acid	3	3
2-Hydroxydecanoic acid	40	49

Note: *P. pastoris* biocatalyst was either prepared by detergent permeabilization or spray-drying and GO activity was measured by DCIP assay as described in Materials and Methods. Results are expressed in terms of lactic acid where lactic acid is 100%.

Compared to the lactic acid control, equimolar concentrations of β -chlorolactic acid, 2-hydroxybutyric acid, and glycolic acid resulted in similar GO activities. Moderate GO activities were observed with 3-indolelactic acid, 3-phenyllactic acid, *p*-hydroxyphenyllactic acid, and 2-hydroxydecanoic acid. As a follow-up to substrate specificity testing, GO enantioselectivity was tested in the cases where (a) appreciable GO activity was observed in the racemic mixture, and (b) the substrate was commercially available as optically pure isomers. For enantioselectivity testing, only spray-dried cells were used. The spray-dried biocatalyst was prepared as described in Materials and Methods with optimal conditions determined from Chapter 3. GO activity was measured and the UV-VIS spectrometer trace of Δ absorbance/time is shown in Figure 64.

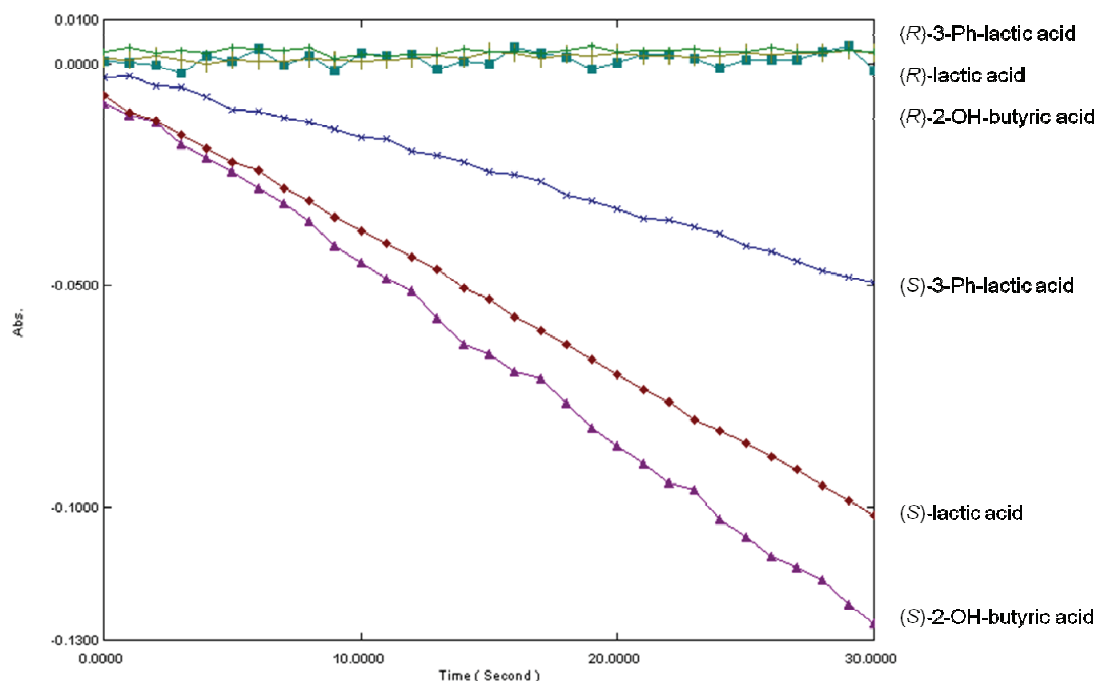


Figure A1. UV-VIS spectrometer trace of Δ absorbance/time for optically pure 2-hydroxy acids. *P. pastoris* biocatalyst was prepared by spray-drying and GO activity was measured by DCIP enzyme assay.

The enantioselectivity of GO has been confirmed in spray-dried cells with no observable Δ absorbance/time for the R enantiomers of 3-phenyllactic acid, lactic acid, and 2-hydroxy butyric acid. Spray-dried cells retained absolute specificity for S hydroxy acids in spite of high temperatures and shear forces associated with spray-drying. Therefore, some racemic 2-hydroxy acids may be resolved to chiral 2-hydroxy acids using biocatalyst prepared by the established process. In contrast to Adam et al., the spray-dried *P. pastoris* does not require GO extraction from plants, additions of expensive FMN cofactor or exogenous catalase. These modifications yield a simplified and more economical synthesis of important chiral 2-hydroxy acids.

APPENDIX B: PUBLICATIONS AND PRESENTATIONS

“New Process for Glycolate Oxidase Based Production of Pyruvic Acid and Chiral Hydroxyacids.” V. Subramanian, J. Glenn, and S. Das. PCT/US07/24074; filed November 16, 2007. The PCT application was published in May, 2009, and assigned a publication number of WO 2009/064277.

“Enantioselective Oxidation of 2-hydroxy Carboxylic Acids by Glycolate Oxidase and Catalase Coexpressed in Methylotrophic *Pichia pastoris*.” S. Das, J. Glenn, and V. Subramanian. Biotechnology Progress. Accepted: September, 2009.

“Resolution of Racemic 2-Hydroxy Carboxylic Acids by Glycolate Oxidase.” S. Das, J. Glenn, and V. Subramanian. *237th ACS Meeting*. Salt Lake City, UT (March, 2009).

“Reaction Optimization for Biocatalytic Production of Pyruvate from Lactate in DASGIP Fed-batch Pro System.” J. Glenn, S. Das, and V. Subramanian. *100th AIChE National Meeting*. Philadelphia, PA (November, 2008).

“Glycolate Oxidase Technology for Production of Pyruvic Acid.” J. Glenn, S. Das, and V. Subramanian. *17th Biocatalysis and Bioprocessing Conference*. The University of Iowa, Iowa City, IA (October, 2008).

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