BENIGN TUNABLE SOLVENTS FOR IMPROVED PROCESSING OF PHARMACEUTICALLY RELEVANT PRODUCTS AND CATALYSTS

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BENIGN TUNABLE SOLVENTS FOR IMPROVED PROCESSING OF PHARMACEUTICALLY RELEVANT PRODUCTS AND CATALYSTS

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To my parents who taught me to treat school like my job and to always be proud of the job I do.

And also to Frank, for being my best friend.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACN	Acetonitrile
ASES	Aerosol Solvent Extraction System
2BP	2-benzoylpyridie
BSG	Brewer's Spent Grain
CAL B	*Candida antarctica* lipase B
CST	Critical solution temperature
DELOS	Depressurization of an Expanded Liquid Organic Solution
DC	Denaturation capacity
DHAP	Dihydroxyacetone phosphate
DLS	Dynamic light scattering
E	Electromotive force
FA	Ferulic Acid
FAXX	0-[5-0(trans-feruloyl)- α -L-arabinofuranosyl]-(1→3)-0-β-xylopyranosyl-(1→4)-D-xylopyranose
FID	Flame ionization detector
FruA	1,6-bisphosphotate aldolase
GC	Gas chromatograph
GDH	Glucose dehydrogenase
GAS	Gas-antisolvent
GXL	Gas expanded liquid
Hepes	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography

k_{GL}	Slope of the linear E vs. pH correlation
K_m	Michaelis constant
KRED	Ketoreductase
K_w	Self dissociation constant of water
LCST	Lower critical solution temperature
M	Miscible
MS	Mass Spectrometer
N.A.	Data not available
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase tetrasodium salt
NCW	Near Critical Water
OATS	Organic aqueous tunable solvent
P	Partition coefficient between octanol and water
1PE	1-phenylethanol
2PE	2-phenylethanol
PEA	Phenyethyl acetate
PEG	Polyethylene glycol
PEG-DME	Poly(ethylene glycol) dimethyl ether
PPM	Phenyl-pyridin-2-yl-methanol
PVE	Palmitic acid vinyl ester
S	Substrate concentration
SHOP	Shell higher olefins process
$scCO_2$	Super critical carbon dioxide

STP	Standard temperature and pressure
T	Temperature
THF	Tetrahydrofuran
UCST	Upper critical solution temperature
UV-Vis	Ultra violet - visible
v	Rate of reaction
V_{max}	Maximum rate of reaction
VOC	Volatile organic compound
VLLE	Vapor-liquid-liquid-equilibrium
ε	Extinction coefficient
λ	wavelength

SUMMARY

Sustainable technologies are vital to reducing the environmental impact of chemical enterprises. Solvents are often seen as just a medium in which a reaction takes place; however they can also play a dominant role in the overall toxicity of a typical pharmaceutical/fine chemicals batch chemical operation. Further, careful solvent selection for a reaction may also lead to more facile separation and purification of products, thus reducing the overall cost of a chemical process.

This thesis presents an environmentally benign processing technique for sustainable biocatalytic reactions coupled with facile built-in separation. An organic aqueous tunable solvent (OATS) system allows access to a hydrophobic substrate which is transformed with a homogeneous enzymatic catalyst in a single liquid phase. Subsequent CO_2 addition produces a biphasic mixture where the hydrophobic product partitions preferentially into the organic rich phase for separation while the hydrophilic enzyme catalyst partitions into the aqueous rich phase, where it is recyclable. Processing parameters in OATS systems are discussed and an overall product recovery of 80% is observed after six reaction cycles. Additionally, greater than 99% enantiomeric excess (ee) is shown for catalyzed hydrolysis of *rac*-1-phenylethyl acetate with *Candida antarctica* lipase B (CAL B) both before and after CO_2-induced separation.

CHAPTER I

INTRODUCTION

In a typical pharmaceutical/fine chemicals (non-polymer) batch operation, solvent use consistently accounts for 80 - 90% of mass utilization and often plays the dominant role in the overall toxicity profile of any given process [1]. Previous life cycle inventories and assessments of pharmaceutical synthesis have shown that solvent use consumes about 60% of the overall energy used to produce an active pharmaceutical ingredient (API) and accounts for 50% of the total post-treatment greenhouse gas emissions [2]. Therefore, there is a broadly recognized need for reduced solvent use, the development of environmentally benign processing techniques, and investigation of alternative solvents [3, 4].

Organic aqueous tunable solvent (OATS) systems are engineered to couple a reaction and separation in order to reduce the overall environmental impact of a process. OATS systems allow reactions between hydrophobic and hydrophilic components to be carried out in a single phase. Post reaction, a biphasic system is generated by the addition of CO_2 to split the reaction mixture into a gas-expanded organic phase containing hydrophobic components and an aqueous phase containing the hydrophilic catalyst [5, 6]. The CO_2-induced phase separation allows for a one-pot reaction and separation scheme that offers facile separation of hydrophobic products with simultaneous recycle of hydrophilic catalysts. OATS systems can reduce the ecological footprint and cost associated with asymmetric transformations of hydrophobic substrates by:

1. Using highly selective homogeneous enzymatic catalysts to reduce the production of byproducts requiring downstream purification;

2. Recycling both catalysts and solvents;

3. Replacing a portion of the volatile organic solvent used in reactions with environmentally and chemically benign water and CO_2, which are both virtually non-toxic, non-flammable, and relatively inert;

4. Applying modest CO_2 pressure as a phase switch for product recovery, in contrast to the massive amounts of organics consumed in conventional liquid–liquid extractions.

The enantioselective properties of many enzymes are unmatched by other catalysts; thus they allow for the production of many pharmaceutically relevant compounds and other fine chemicals. Enzymes are attractive for organic synthesis, as they display high substrate specificity and in some cases, catalyze reactions that are not possible in a single step via traditional synthesis. Examples include enantioselective asymmetrization of diesters with proteases or esterases [7] and regioselective reductions of steroids [8]. Non-biological catalysts often contain heavy metals or may require energy-intensive high-temperature processes to achieve catalysis. On the other hand, biocatalysts are environmentally benign and usually function at relatively mild aqueous conditions with moderate temperature, pressure, and pH. Thus, biocatalysis can allow for the development and implementation of “greener” processes that can potentially replace less environmentally friendly steps in chemical synthesis.

Homogeneous catalysts offer superior activity and selectivity, possess high atom efficiency, and their catalytic properties are well defined allowing fine tuning of

capabilities [9]. Additionally, homogeneous catalysts avoid limitations such as active-site heterogeneity or mass transport limitations of reactants and products to and from the active site. Unfortunately, separation of homogeneous catalysts from the product mixture is difficult, thus preventing their use in many large-scale industrial processes [9]. Table 1-1 presents the features of homogeneous verses heterogeneous catalysts.

Table 1-1: Homogeneous vs. Heterogeneous Catalysis [9].

	Homogeneous	Heterogeneous
Activity	+++	-
Selectivity	+++	-
Selectivity	+++	-
Catalyst recycling	-	+++
Quantity of catalyst	++	+++
Total turnover number	+	+++

OATS combines the benefits of homogeneous and heterogeneous catalysis into one chemical process which is advantageous from both an environmental and commercial point of view. Catalyst recycling in OATS systems allows an efficient use of the generally expensive homogeneous biocatalyst making such a process commercially feasible. Additionally, phase separation caused by environmentally benign CO_2 reduces the amount of organic solvent needed as compared to traditional liquid-liquid extractions. This is important because moderate reductions in organic solvent use can result in improvement to the associated environmental life cycle impacts. For example, reduction of tetrahydrofuran from 1 kg to 0.75 kg avoids approximately 4 kg green house gas emissions if one accounts for avoided disposal or recovery emissions [1]. Cost and

environmental consequence can also be mitigated through recycling of the solvents used in OATS systems.

The water used in OATS systems is an attractive solvent because it is non toxic, low cost, and naturally abundant. Like many thermodynamic properties, the solubility of organic compounds in ambient water has been studied extensively and has resulted in a acquisition of many data in multiple volumes [10, 11]. Water does not "swell" with CO_2 addition while most organic liquids do "swell" with CO_2 addition. Thus, miscible mixtures of organic solvents and water become immiscible under CO_2 pressure. This forms the basis for the extension of aqueous organic phase behavior in OATS systems, which are compared to other aqueous biphasic systems in Chapter II.

CO_2 is a safe and inexpensive medium, with adjustable physical properties that can be continuously tuned from gas-like to liquid-like by temperature and pressure changes. Binary phase behavior of CO_2 and many organic liquids was measured [12-14] extending from recent interest in the solubility of carbon dioxide in organic liquids to create gas-expanded liquids (GXLs) as described in Chapter II. CO_2 is easily removed by depressurization, and is an environmentally benign medium that has undergone intensive investigation as an organic solvent alternative during the past two decades [15].

When a miscible organic-aqueous tunable solvent (OATS) system is exposed to even modest pressures of CO_2, it will split into two phases -- a water phase with minimal CO_2 concentration and an organic phase rich in CO_2. Hydrophobic organic products preferentially partition to the organic phase of OATS systems, phase split by CO_2, for facile product recovery. Many factors contribute to this hydrophobic effect including, polarity and water self-association [16]. Conversely, the enzyme biocatalyst will

distribute into the aqueous phase of pressurized OATS systems. Additionally, water is well suited for the dissolution of ionic species such as salt buffers necessary to maintain adequate pH for the enzymatic biocatalyst because of it is a high-density, polar, high dielectric medium.

In Chapter III, specific processing challenges with regards to buffer addition for enzyme stability are addressed. The use of CO_2 as a separating agent for simultaneous biocatalyst recycle and product isolation requires two processing considerations to be addressed. First, the solution equilibria of the water-solvent-CO_2 system must be well characterized so that as the product-laden organic phase is removed from the reactor, an accurate make-up stream can be added to maintain the appropriate OATS mixture. This will allow for repeatable separation of successive reaction cycles. Second, the pH of the aqueous phase must be carefully maintained to preserve optimal biocatalyst activity.

Chapter IV further discusses the advantages to using hydrophobic substrates in conjunction with enzymatic catalysts. A comparison of OATS systems to alternative techniques, and demonstrations of two successful examples of reaction coupled with separation in OATS systems using the biocatalyst *Candida antarctica* Lipase B (CAL B) are presented. In Chapter V, the challenges of extending OATS systems to a ketoreduction with *in situ* co-factor regeneration are examined.

Recommendations for continuing work on OATS systems are in Chapter VI. Some suggestions are made for the improvement of both enzymatic catalyst and solvents used in OATS systems. Additionally, criteria for a specialized moderate pressure vessel are given. Finally, Appendices A thru C extend the notion of tunable solvent processing of pharmaceutically relevant compounds to other solvent classes.

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CHAPTER II

BACKGROUND

Introduction

A brief overview of multiphase approaches to homogeneous catalyst recovery and recycle is given to introduce the idea of holistic processing through novel solvent systems presented in this thesis. Background on gas-expanded liquids (GXLs) is also included to frame discussion of their utility towards the facile, built-in separation inherent to organic aqueous tunable solvent (OATS) systems examined in Chapter III and explore the processing challenges faced when using GXLs in conjunction with a biocatalyst in Chapter IV.

Multiphase (biphasic, liquid-liquid) catalysis

The industrial importance of homogeneous catalysis has magnified over the last decade because homogeneous catalysts offer increased selectivity, activity, and often more mild reaction conditions [1, 2]. The problems associated with catalyst recovery after reaction presents a serious drawback to applications of homogeneous catalysis for large scale operations and is the main reason that heterogeneous catalysis are used for more than 80% of industrial processes [3]. To address the separation concerns posed by homogeneous catalysis, a number of methodologies have been studied including thermal and chemical recovery such as the distillation of acetic acid in the Monsanto process [4]; catalyst heterogenization through covalent binding, adsorption, ion pair formation or

entrapment [5, 6]; membrane technology [7, 8]; and various multiphase approaches [1, 9-11].

Multiphase, also known as biphasic or liquid-liquid, approaches allow catalyst restriction into one liquid phase while the products separate into a second immiscible phase, like oil and water. The product phase is easily decanted away from the catalyst phase which meets the requirements of the "green solvent" concept because the solvent and catalyst are recycled and do not enter the environment. Four cases may be considered for this approach:

1. Products separate during catalysis;
2. Catalysis is followed by extraction;
3. Solvent for extraction is already added during catalysis;
4. Monophasic catalysis with induced biphasic conditions for separation.

The Shell higher olefins process (SHOP) [12], developed in 1965, is the classic example of approach 1, and is in fact the first example of multiphase catalysis. In this process, a nickel catalyst dissolved in 1,4-butanediol is used to produce α-olefins. The α-olefins are immiscible with 1,4-butanediol and therefore form a second liquid phase which is easily "spooned" off, or decanted. Today, this biphasic concept is demonstrated industrially for various systems such as the hydroformylation of propene and butane with soluble sulfonated R_3P ligands in water [13] and fluor based solvents for the dimerization of 1-butene [14], but is limited in scope to those products that are able to form a second phase in which the given catalyst will not be miscible.

In the second case, conventional homogeneous catalysis is followed by the extraction of products into a second phase. This approach can be used to recover products, or the costly ligands and metals commonly used in enantioselective reactions. As long as the appropriate extraction solvent may be found, in principle, this approach can be applied to any homogeneous catalysis. In practice; however, it can be difficult to find a good solvent that selectively extracts the product.

For the third case, the extractant is already added during catalysis, thus, there are two immiscible phases in the reactor. The product formed during reaction is continuously removed into this second phase, which makes this concept very useful when product inhibition occurs in a chemical reaction. Normally, homogeneous catalysis requires high conversion and high selectivity to make the process economic [5]; however, product removal at lower conversion via solvent extraction may have economic benefits. This is true for avoiding consecutive reactions as illustrated by the telomerization of butadiene with ammonia [15].

Demands on the catalyst for the first three cases are present because of the often small but always present mutual solubility between phases. The addition of a second organic phase can be detrimental to activity because the organic solvent may chemically react with the catalyst or, as discussed further in Chapter III, biocatalysts may be denatured. For the fourth case, the catalyst operates in single phase composed of mixed solvents which can be tailored to cater to the needs of a particular catalyst. Upon perturbation, a second phase forms allowing for facile separation. As such, care must also be taken to ensure the given perturbation will not harm the catalyst. These systems can be run completely homogeneously, avoiding the problems caused by mass transfer

limitations, while the separation post-reaction is preformed under biphasic conditions. This second phase formation can be induced by temperature or composition changes.

Composition changes via the addition of solute, the formation of a different product, the consumption of starting material, or the addition of a small volume percent of another solvent, salt or water is sufficient to slightly perturb the original miscible mixture and induce phase separation forming a biphasic system [9]. Alternatively, thermally induced systems, also known as thermomorphic solvent mixtures, promote miscibility with the application of heat and/or immisciblilize upon cooling.

Representative schemes to exploit these perturbation techniques are depicted in Figure 2-1 where S represents substrates, P represents products, and c represents the catalyst. Specific examples of reactions used are presented, and the homogeneous catalyst used for the reaction is shown. These schemes illustrate a holistic approach to chemical processing because the reaction solvent is specifically designed in order to plan for facile down stream purification. Additional examples may be found in the literature [1, 9, 11, 16]. These systems, as well as approaches 2 and 3, can also be used in conjunction with phase transfer catalysts and soluble polymer supported catalysts [9, 17, 18].

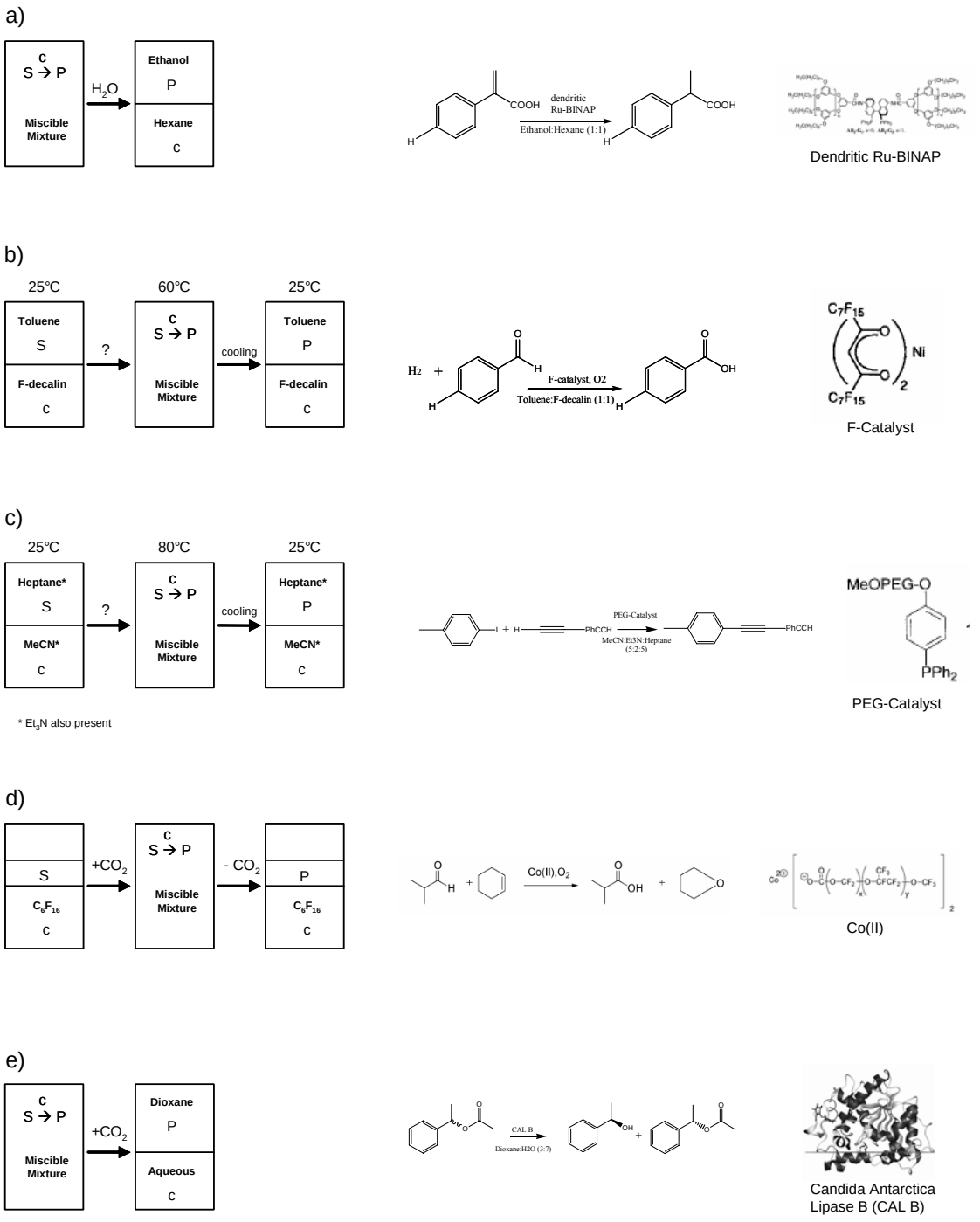

Figure 2-1: Depiction of a) water as perturbing agent [19]; b) thermomorphic system with fluorus catalyst and solvent [20]; c) thermomorphic system with 3 component mixed solvent [21]; d) CO_2 induced fluorus solvent system [22]; and e) CO_2 induced organic aqueous tunable solvent (OATS) system.

A representative phase diagram of a strictly regular solution typical of thermomorphic systems is shown in Figure 2-2. A reaction could be run at a temperature in the one phase region and then increased to immisciblilize the mixture for separation. The parabola shown represents the compositions of coexisting phases. A tie-line may be constructed as is shown between point A and B to give the equilibrium composition of the two phases at a particular temperature. It is clear from this diagram that thermomorphic schemes do not necessarily consist of two pure liquid phases, and often require a large change in temperature to obtain a biphasic system for separation. We are interested in biphasic separation of homogeneous enzyme catalysts. Enzymes inactivate at high temperatures in aqueous media due to both partial unfolding and covalent alterations in the primary structure [23]; therefore we chose to look instead at composition changes.

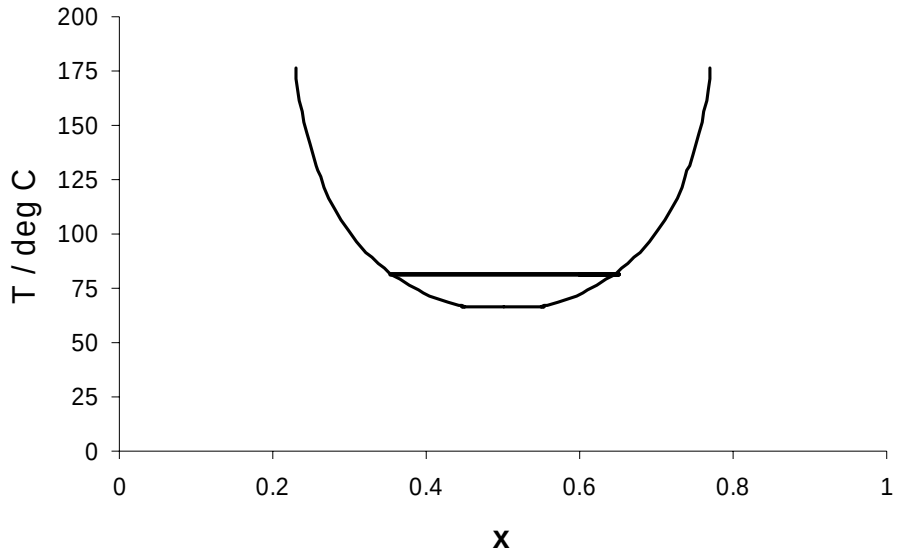

Figure 2-2: Solubility diagram of temperature (T) versus mole fraction (x) of a strictly regular solution exhibiting a lower critical solution temperature.

With the exception of CO_2 as a solute, composition changes can be hard to reverse making recycle and reuse of the homogeneous catalyst difficult. If, for instance, the component used to induce phase separation is still in solution, then a second reaction could be hindered by the component, or a miscible mixture may not be obtainable in the presence of the component. CO_2 is advantageous as a solute to induce phase separation because it can easily be vented off post separation of the product, allowing one to more easily return to the original reaction composition for subsequent reactions and separations. The additional benefits of gas-expanded liquids forming the organic phase of such CO_2 induced schemes are discussed below.

Gas Expanded Liquids (GXLs)

A gas-expanded liquid or GXL is formed by the pressurized dissolution of a gas into an organic solvent. CO_2 is an ideal gas to use in GXL formation because it is relatively inert, benign, and its low critical temperature makes it highly useful for the processing of many heat sensitive compounds such as pharmaceuticals. At moderate pressures (3 to 8 MPa), CO_2 has considerable solubility in a number of organic solvents such as acetates, alcohols, ethers, and ketones. Dependent on the amount of CO_2 dissolved in the organic solvent, the system will exhibit both gas-like and liquid-like properties. Figure 2-3 shows qualitatively the relative transport and solvent strengths of several different solvent systems. If increased solvation power is desired, the dissolved CO_2 should be minimized to make the GXL more liquid-like. Conversely, if more transport power is needed the amount of dissolved CO_2 is increased making the GXL more gas-like. Physiochemical properties such as density, dielectric constant, and

polarity can be varied by adjusting the gas pressure [24-26]. Therefore, GXL systems are pressure-tunable solvent systems because one can change the solvent characteristics at will to tailor conditions for a specific reaction or separation. Variation in temperature of GXLs offers even further versatility.

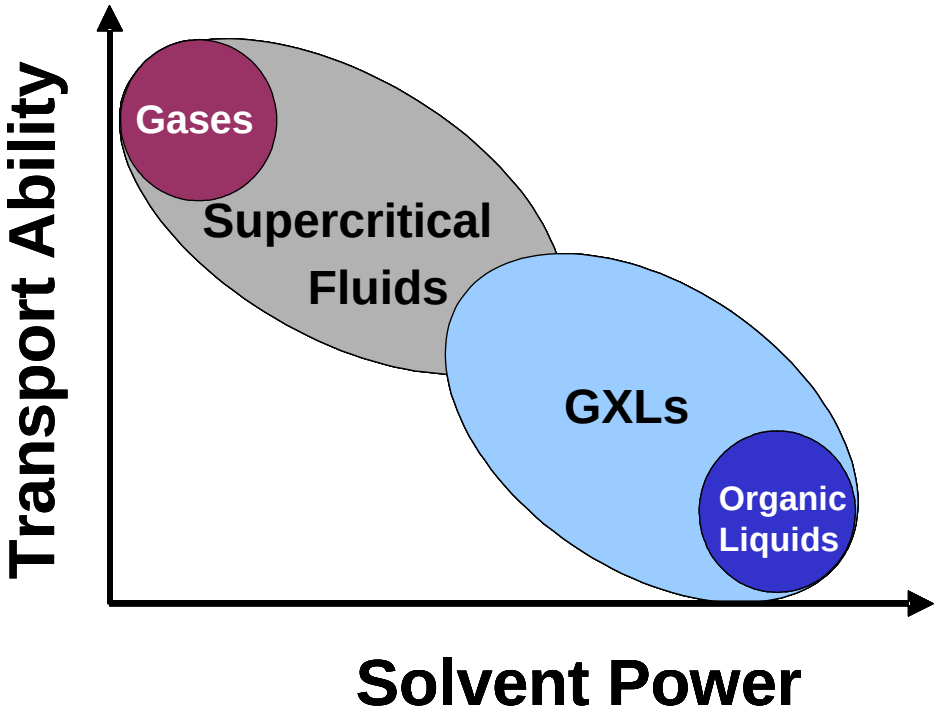

Figure 2-3: Qualitative look at the tunablity of GXLs with relative transport ability and solvent power as compared to other classes of solvents.

Benefits of GXLs in terms of both improved behavior of fundamental chemical processes and potential environmental benefits include [27-29]:

- improved turnover frequencies;
- enhanced gas solubility in the liquid phase of gases such as H_2, O_2, and CO which are important for oxidation, hydroformylation, hydrogenation, and carbonylation reactions;

- comparable or better product selectivity than neat organic solvents or $scCO_2$;
- substantial (up to 80% by volume) replacement of volatile organic solvents with dense-phase CO_2;
- enhanced reaction rates and low process pressures yield process economics that are more favorable than those for $scCO_2$;
- reduced risk of reaction runaway and explosions via operation within CO_2-based media in the near-critical region (where specific heat increases with isothermal pressure increases);
- milder processing pressure (tens of bars) as compared to $scCO_2$ (hundreds of bars);
- facilitated catalyst separation schemes.

As such, GXLs have been investigated as novel solvents in different homogeneously catalyzed reactions [27-32] as well as heterogeneously catalyzed oxidation reactions [33, 34]. Improved turnover frequency in GX-methanol as compared to neat methanol has been shown [30], and it was suggested the presence of large amounts of CO_2 correlated with increased H_2 solubility in ionic liquids for improved reaction rates during enantioselective hydrogenation of imines [32]. However, the enhancement of (O_2 and CO) gas solubility observed in CO_2-expanded systems (acetonitrile, acetone, and methanol) is modest when compared to the gas solubility at comparable pressures (or gas fugacity), and this enhancement occurs only at the cost of higher total pressure [35].

Gas-antisolvent (GAS) crystallization is a common technique utilizing GXLs as an antisolvent for separations [36] and the production of micronized therapeutic particles [37]. Traditional crystallization uses cooling to supersaturate the system for induced nucleation and crystal growth. This results in reliance on heat transfer from a surface which can lead to nucleation at the cooler surface only and inconsistent particle formation, not to mention problems of fouling. GAS crystallization is advantageous over traditional crystallization techniques because supersaturation occurs homogeneously throughout the solution yielding the potential to nucleate crystals throughout a solution resulting in small uniform particles [37]. Similar techniques for uniform particle formation, such as Depressurization of an Expanded Liquid Organic Solution (DELOS) and Aerosol Solvent Extraction Systems (ASES), also use GXLs and, like GAS crystallization, benefit from the elimination of a separate stage for recovery of the antisolvent [37].

Other uses of GXLs for separation include size-selective nanoparticle precipitation [38] and gas assisted mechanical recovery of vegetable oils from seeds [39]. In this work, GXLs are used to facilitate homogeneous catalyst recovery while coupling reaction with separation as shown in Figure 2-1e. Monophasic catalysis with induced biphasic conditions for separation is possible because the hydrophobicity of CO_2 serves to lower the solubility of a hydrophilic solvent in water. Thus, CO_2 solute addition phase separates a miscible mixture of water with many common organic solvents including acetone, acetonitrile, (1,4)-dioxane, ethyl acetate, and tetrahydrofuran.

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CHAPTER III

BUFFER EFFECTS ON ORGANIC AQUEOUS TUNABLE SOLVENT (OATS) SYSTEMS

Introduction

Organic aqueous tunable solvent (OATS) systems are a simple method for homogeneous catalyst recycle derived from the water/organic biphasic techniques popularized by the Ruhrchemie/Rhône-Poulenc process [1]. In OATS systems, a polar organic cosolvent added to water forms a homogeneous reaction solution that may be coupled with subsequent CO_2-induced phase splitting for facile heterogeneous separation as illustrated in Figure 3-1. Here we are interested in using homogeneous biocatalysts, which often require a buffer for stringent control of pH to maintain the proper conformation for catalytic activity. The utility of biocatalysts are further discussed in Chapters IV and V.

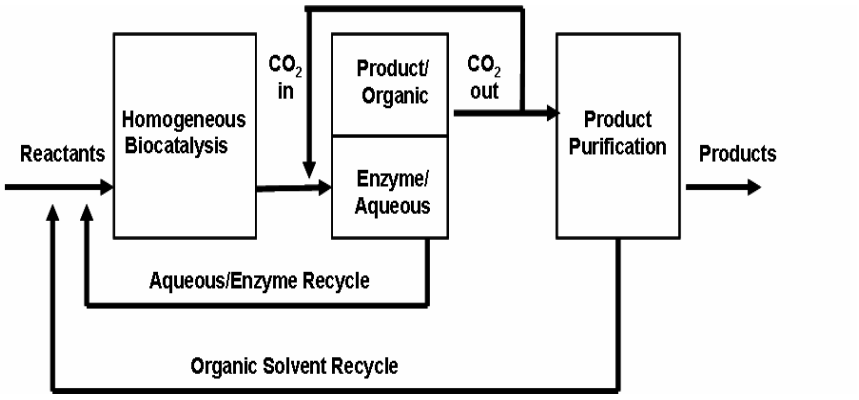

Figure 3-1: OATS process schematic for homogeneous biocatalysis with tunable solvent separation and recycle.

Measurements of pH in OATS systems are complicated by two factors: the use of organic cosolvents and high pressure. To survive the CO_2–pressurized separation process, the enzyme must withstand the pressure used for separation as well as the associated pH fluctuations. The pressure required to unfold enzymes (>2000 bar) [2] is one to two orders of magnitude higher than that required to separate OATS mixtures (10-50 bar), so it is unlikely that hydrostatic pressure alone will damage the enzyme. However, CO_2 addition to aqueous mixtures leads to a dramatic drop in solution pH, due to carbonic acid formation [3, 4] and such low pH values can have detrimental effects on enzyme activity and stability.

The pH of Mixed Solvents

Barbosa et al. [5] describe a method for assignment of reference buffer solutions for standardization of potentiometric sensors in mixed solvents THF-water and acetonitrile-water. In a mixed solvent (i.e. water plus the desired organic cosolvent), the measurement of pH is estimated by:

$$pH_x = pH_s + (E_s - E_x) / k_{GL}$$

where pH_x is the desired pH of the mixed solvent, and is related to the pH of a standard reference solution, pH_s as well as the electromotive force of the cell (E_x or E_s) given by:

Reference Electrode| Salt Bridge| Sample solution at pHx or Buffer Solution at pHs| Glass Electrode

where k_{GL} is the practical slope of the linear E vs. pH correlation. Reference pH_s values of the standard solutions in mixed solvents were found and subjected to target factor

analysis to obtain equations that allow calculation of the pH_s values of the buffer solutions permitting pH measurements in the usual way [6].

An alternate technique, described by Tucker et al. [7] uses acid-base indicator transition colors as compared to the pH of standard mixtures measured with a glass combination saturated calomel electrode. The behavior of nine indicator dyes were studied and the transition range of a given indicator dye was found to vary with the amount of organic cosolvent present, as illustrated in Figure 3-2 for bromocresol green in (1,4)-dioxane aqueous mixtures.

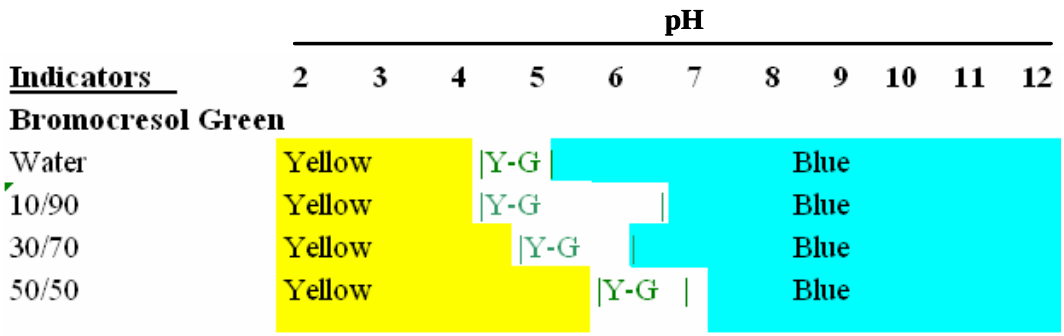

Figure 3-2: Reproduction of data [7] shows change in indicator appearance with organic solvent (1,4)-dioxane (v/v) content for indicator dye bromocresol green. Y-G is yellow-green, and ranges represent the average of eight independent determinations.

The method of Barbosa et al. would require specialized electrode assemblies [8] for high pressure in situ measurements. Therefore, in this study, pH was monitored using indicator dyes, taking care to run calibrations at equivalent organic cosolvent concentrations to account for the variance in transition range. In this way, the transition range of an indicator dye (measured via UV-Vis spectroscopy) can be compared against standard solutions of known pH in order to determine the pH of an unknown solution under pressure.

Ternary Phase Data of OATS

Vapor-liquid-liquid-equilibria (VLLE) phase data of OATS systems previously studied include polar organic solvents tetrahydrofruran (THF), acetonitrile, and (1,4)-dioxane with water and CO_2 [9]; however, it was experimentally observed that phase equilibria of these CO_2 pressurized OATS mixtures did not exactly match the previously reported data when there was an addition of salt buffer. Characterization of VLLE of the water-solvent-CO_2 system is vital for repeatable separation of successive reaction cycles.

The phase equilibria of OATS systems are best illustrated on a ternary phase diagrams. Figure 3-3 illustrates a typical diagram for a type 1 ternary made up of two partially immiscible solvents and a solute (CO_2) which is completely miscible in either solvent. The area under the curved line is the two-phase region; outside of this region there exists a single miscible phase. In the absence of solute, the mutual solubilities are given by points marked A and B. As solute is added to the first tie line, the mutual solubilities become C and D, and for the second E and F. At some point enough solute is added to give complete miscibility at a point G called the consolute point. Tin an optimal OATS system, we would use a tie line that gives maximum separation, well below any consolute point.

We are interested in understanding the role of buffers in the immiscibility of water and polar organic solvents under CO_2 pressure. With this information, as the product-laden organic phase is removed from the reactor, an accurate make-up stream may be calculated and added to maintain the appropriate OATS mixture for subsequent facile reaction and separation.

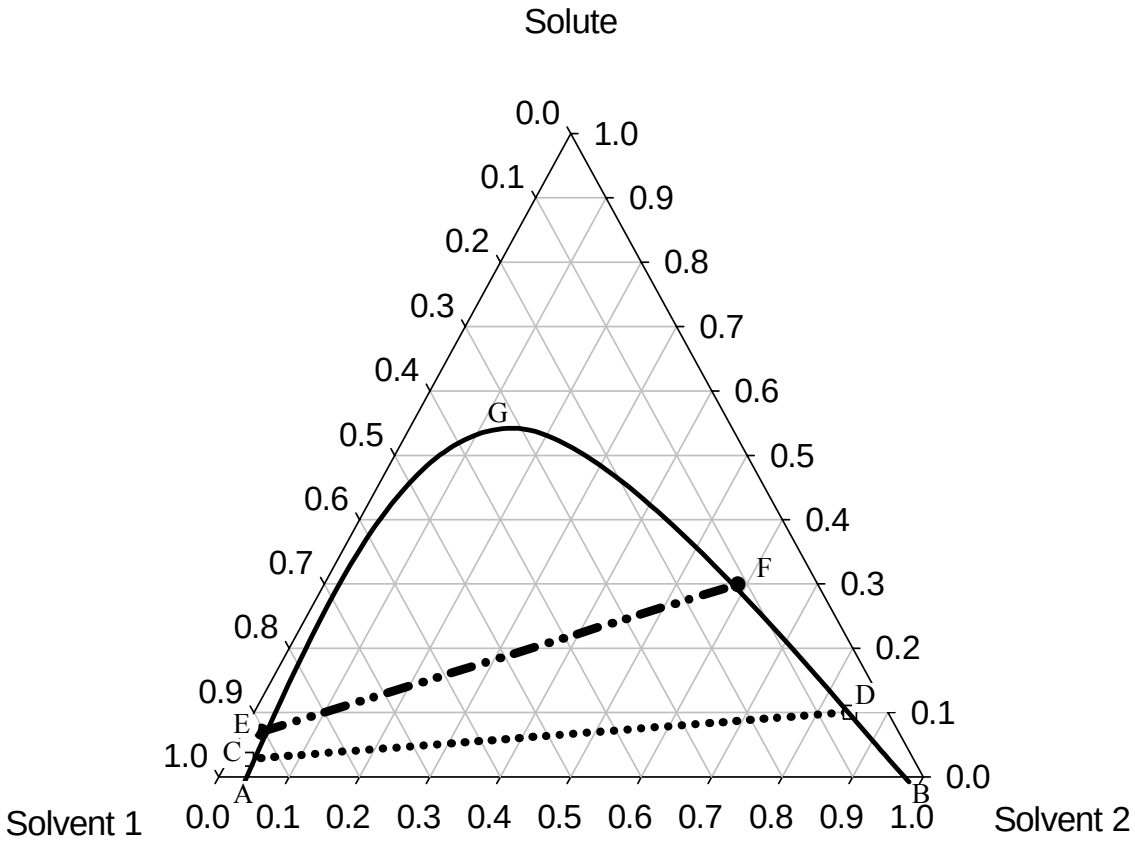

Figure 3-3: Representative ternary diagram showing envelope of two phase region and tie lines indicating composition of immiscible liquids.

Experimental Methods

Materials

Candida antarctica lipase B, CAL B, (SOL-101; ≥8 kU/mL) was a kind gift from Biocatalytics (Pasadena, CA). HPLC grade solvents (99.9%) (1,4)-dioxane, acetonitrile, tetrahydrofuran, and water were purchased from Sigma-Aldrich and were used without further purification. ACS grade sodium phosphate monobasic monohydrate and (2-hydroxyethyl)-1-piperazineethanesulfonic acid from EMD Chemicals were also used as received and are abbreviated as phosphate buffer and Hepes buffer respectively.

Pressurized pH Measurements

The pH of the pressurized aqueous phase was correlated with the extinction of a soluble dye similar to the method of Holmes et al. [10]. Solutions of indicator dyes bromocresol green ($pK_a = 4.7$) and bromothymol blue ($pK_a = 7.1$) from Sigma were made in varying amounts of organic cosolvent and pH was measured via a glass combination saturated calomel electrode (± 0.5 pH units). Absorbance peaks of the acid and base forms of these solutions were also measured with a Hewlett–Packard 8453 spectrophotometer to generate calibration curves experimentally. The spectroscopic measurements of pressurized OATS mixtures were preformed using a high-pressure 10 mL barrel cell with quartz windows set to path length of 2.3 cm. The cell was jacketed with ethylene glycol to control temperature and equipped with a pressure gauge (uncertainty ± 0.14 bar), thermocouple (uncertainty ± 0.1 K), and magnetic stir bar. The ratio of the measured absorbance peaks in the pressurized aqueous phase was compared to the experimentally constructed calibration curves to determine the pH. The amount of

organic solvent in the aqueous phase varies with pressure and this concentration variance can change the color transition range of acid-base indicators [7]. Separate calibration curves of pH vs. absorbance were made for each organic solvent concentration studied. Tertiary phase data of water-(1,4)-dioxane-CO_2 mixtures [9] was used to estimate the amount of organic solvent present in the water rich phase at a given pressure, thus allowing the pH under various pressures to be spectroscopically evaluated.

Enzyme activity Assays

To test the effect of exposure to reduced pH, liquid CAL B stock was diluted 1:100 into 50 mM sodium formate (pH 3) or 50 mM sodium acetate (pH 4 or 5) solutions and incubated at room temperature for the desired time. 20 uL aliquots were removed periodically and added to 1 mL of aqueous assay solution containing 0.1 mM p-nitrophenyl butyrate (Sigma 99%) dissolved in 50 mM Hepes buffer (pH 7). Remaining activity was monitored by measuring absorbance increase at 400 nm on a DU-800 spectrophotometer (Beckman)and specific activity (umol/min/mg enzyme) was calculated using an extinction coefficient of $\varepsilon = 8.656$ (mM^{-1} cm^{-1}).

For the evaluation of CAL B dependence on pH, OATS reactions mixtures containing the desired amount (1,4)-dioxane (99%, Sigma), phenethyl acetate (99%, Alfa Aesar), and 150 mM sodium phosphate were prepared and 1/100 diluted CAL B in pH 7.1 phosphate buffer was added in a ratio of 0.5 mL enzyme per 9.5 mL OATS mixture. 0.25 mL samples were removed periodically and immediately mixed 1:1 with a mixture of 1:1 glacial acetic acid:(1,4)-dioxane to quench the reaction. Reaction progress was

followed by measuring the phenethyl acetate and 2-phenyl ethanol content of samples using an Agilent GC-FID with a DB17 column (Agilent model 6890).

Ternary Phase Data Measurements

For the unbuffered CO_2 – organic solvent – water systems reported here, a synthetic method of high-pressure vapor-liquid-liquid-equilibria (VLLE) was used, as previously reported [9]. VLLE for the buffered systems was determined by a direct sampling technique. A schematic of the equilibrium cell apparatus is shown in Figure 3-4. A T316 windowed Parr reactor with a Magnadrive impeller for mixing was used. Pressure in the cell was monitored with a pressure transducer with digital readout (Druck, DPI 260, PDCR 910, GE Infrastructure Sensing, Billerica, MA) calibrated against a hydraulic piston pressure gauge (Ruska, GE Infrastructure Sensing, Billerica, MA) to an uncertainty of +/- 0.1 bar. Samples were taken via a sample loop affixed to a 6-way valve, allowing capture of fixed sample volume regardless of operating pressure. These samples are depressurized into a dilution solvent and analyzed via GC-FID to determine organic solvent content and Karl Fischer titration to determine water content. The content of the 6-way valve is diverted to an inverted burette placed in a water bath to measure CO_2, instead of being depressurized into the dilution solvent. The volume of carbon dioxide at STP is determined by the displacement of water in the burette. The sample should not be bubbled through the water as there is an appreciable solubility of carbon dioxide in the water. Without any mixing, the rate of dissolution of carbon dioxide into the water is negligible so long as the volume is determined rapidly. The line is flushed with rinse solvent to ensure all the carbon dioxide is in the burette. The

sampling is repeated three times for liquid content and three times for CO_2 content per phase for a given pressure and the results averaged to mitigate error in the sampling procedure. This direct sampling technique was validated by first applying it to an unbuffered system and comparing those results with previous data taken via the synthetic method [9]. The buffer concentration was kept below the solubility limit of the gas-free solvent mixture being tested, and we did not observe any salt precipitation upon depressurization.

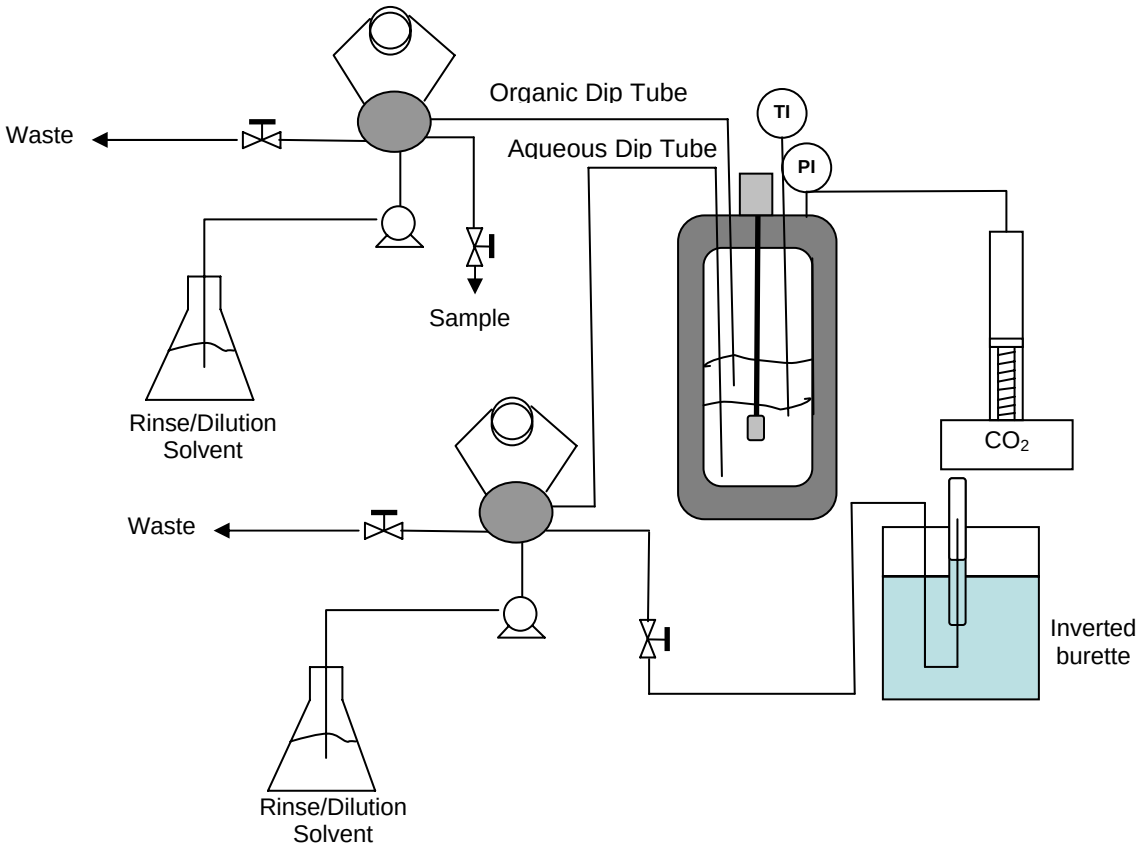

Figure 3-4: Schematic of the apparatus used for direct sampling of organic and aqueous phase composition for liquid-liquid phase equilibria data.

Results and Discussion

CO_2 Effects on pH and CAL B Activity:

Buffer addition can prevent the pH of aqueous phases of CO_2 – water systems from dropping below pH 5 [11]. Here we examine the pH of water CO_2 - (1,4)-dioxane - water systems. Figure 3-5 shows pH values of the aqueous phase of CO_2 separated OATS mixtures as a function of CO_2 pressure. In unbuffered mixtures, the addition of only 5 bar of CO_2 dramatically lowers the pH. When 150 mM phosphate buffer is included in the aqueous component, the pH decreases and levels off at approximately pH 5 under 50 bar CO_2. Since the typical time required to pressurize, mix, and separate OATS mixtures with CO_2 is approximately 30 minutes, the aqueous phase containing enzyme is exposed to pH 5 for a similar amount of time.

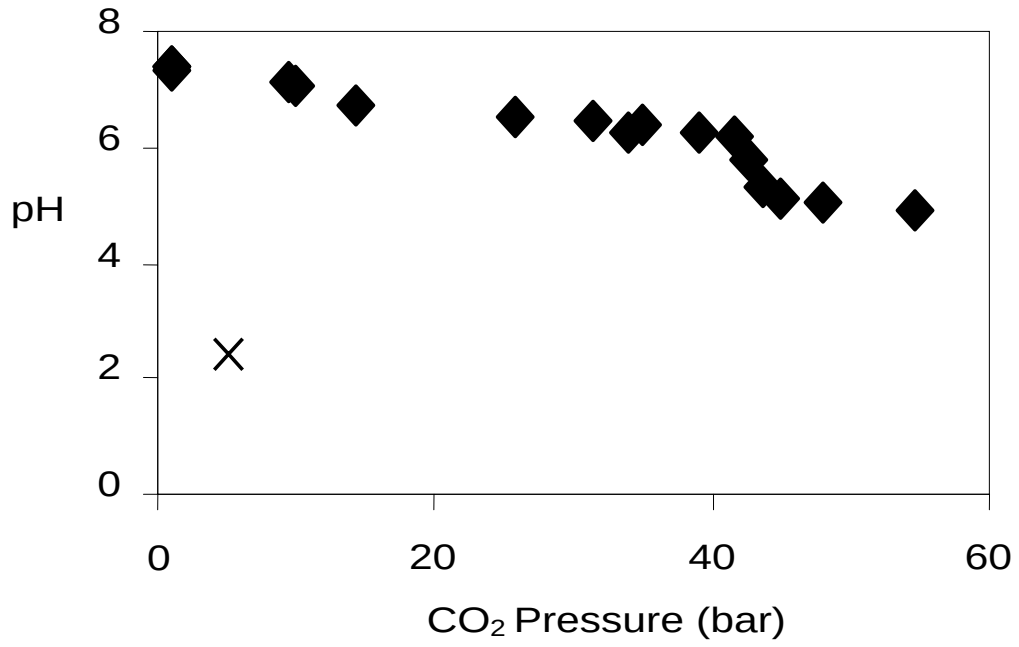

Figure 3-5: The pH of unbuffered (×) and buffered (150 mM phosphate) (◆) 30/70 (1,4)-dioxane/water v/v solutions.

The addition of CO_2 and accumulation of acetate ion (a reaction product) can lower the pH of the reaction media for the model hydrolysis reaction of phenethyl acetate described in Chapter IV. The reaction conversions in the appropriate pH solution were measured after two hours (typical OATS reaction time) and are shown in Figure 3-6. As pH was reduced from 8 to 5, the observed conversions decreased from 55% to 40% showing a clear dependence on pH.

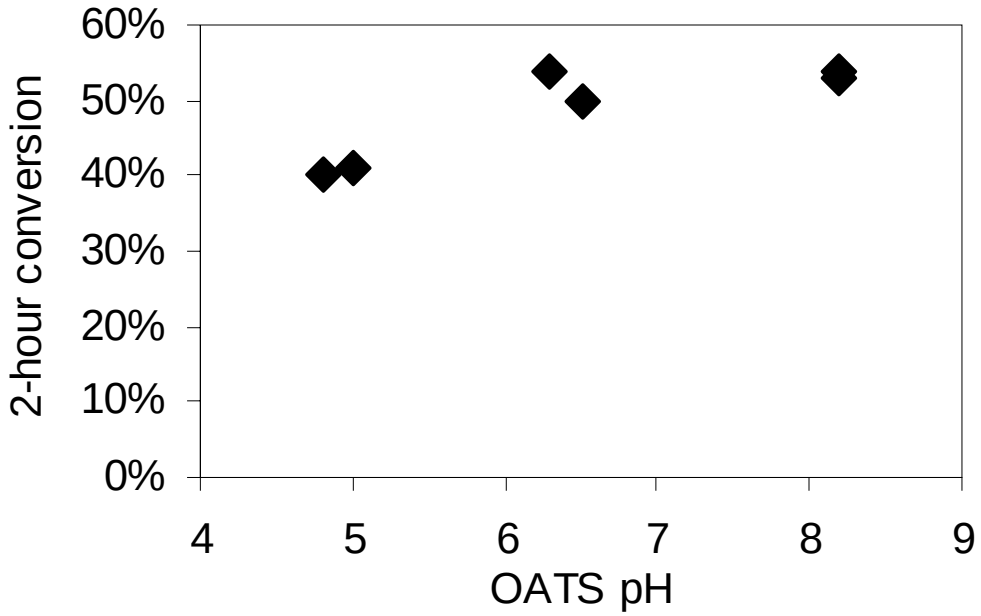

Figure 3-6: CAL B pH dependence in OATS, 40/60 (1,4)-dioxane/water v/v solutions.

However, in OATS, the enzyme needs only to survive the reduced pH condition, because the pH may be again increased before subsequent reactions by removing CO_2 from solution. Therefore, CAL B was also studied to assess the enzyme tolerance to low-pH exposure experienced during the separation portion of the OATS process. CAL B samples were incubated in buffered low-pH solutions and assayed for activity at pH 7 (see Figure 3-7). Even after 2 hours of exposure to solution of pH 4, CAL B samples

showed no loss of activity. Clearly, the buffer is effective at preventing the pH of the pressurized aqueous phase from decreasing to low levels and CAL B is not detrimentally affected by short exposures to pH 5 that are encountered during phase separation.

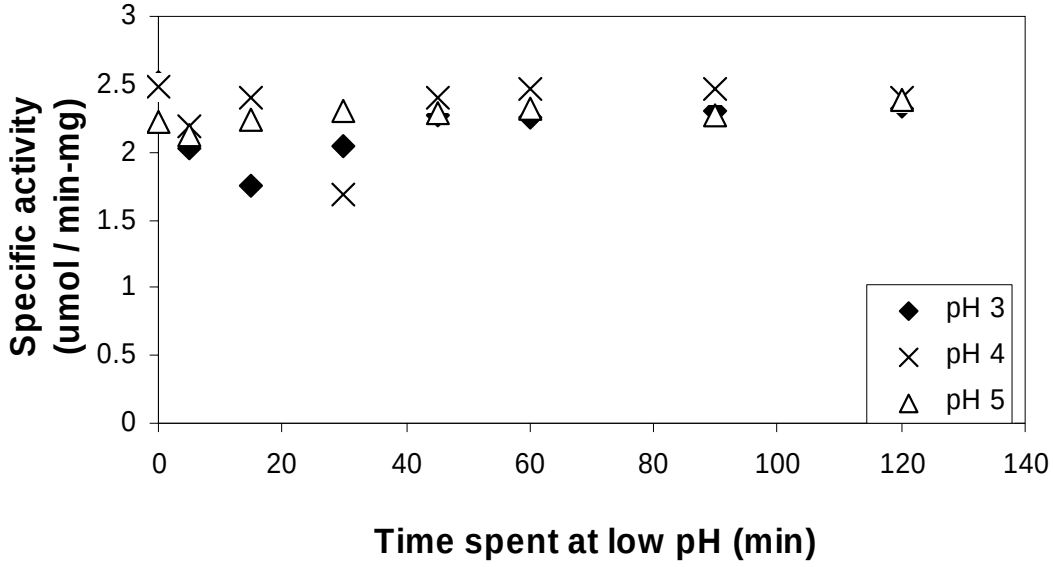

Figure 3-7: CAL B was incubated in solutions of pH 3, 4, or 5 and then assayed for activity in pH 7 solutions after various exposure times.

Buffer Effects on Ternary Phase Behavior:

Two commonly used buffers for biocatalytic systems are monobasic sodium phosphate (phosphate buffer) and (2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes buffer). Both can buffer adequately against carbonic acid formed in water-CO_2 mixtures [10, 12], and the phosphate buffer has been shown to adequately buffer recycled OATS mixtures over six cycles [12].

Ternary phase diagrams at 25 ºC are shown for CO_2 - (1,4)-dioxane - water systems in Figures 3-8 thru 3-10. While both buffers alter the two-phase region critical to

separation in the OATS system, it is clear that the Hepes has a much greater effect. One may better appreciate this effect by comparing the amount of CO_2 pressure required to reach 0.1 mole fraction of water in the organic rich phase (i.e. the pressure required to remove 90% of the water from the organic phase). For the unbuffered system only 3.1 MPa is required, however, in the Hepes case the pressure must be raised to 4.4 MPa to achieve a water content of 0.1 mole fraction. The intermediate pressure of 3.7 MPa for the phosphate buffered case illustrates that this effect also occurs for inorganic salts. We surmise that the effect is increased because the organic salt (Hepes buffer) is more soluble in the organic solvent than the inorganic salt (phosphate buffer).

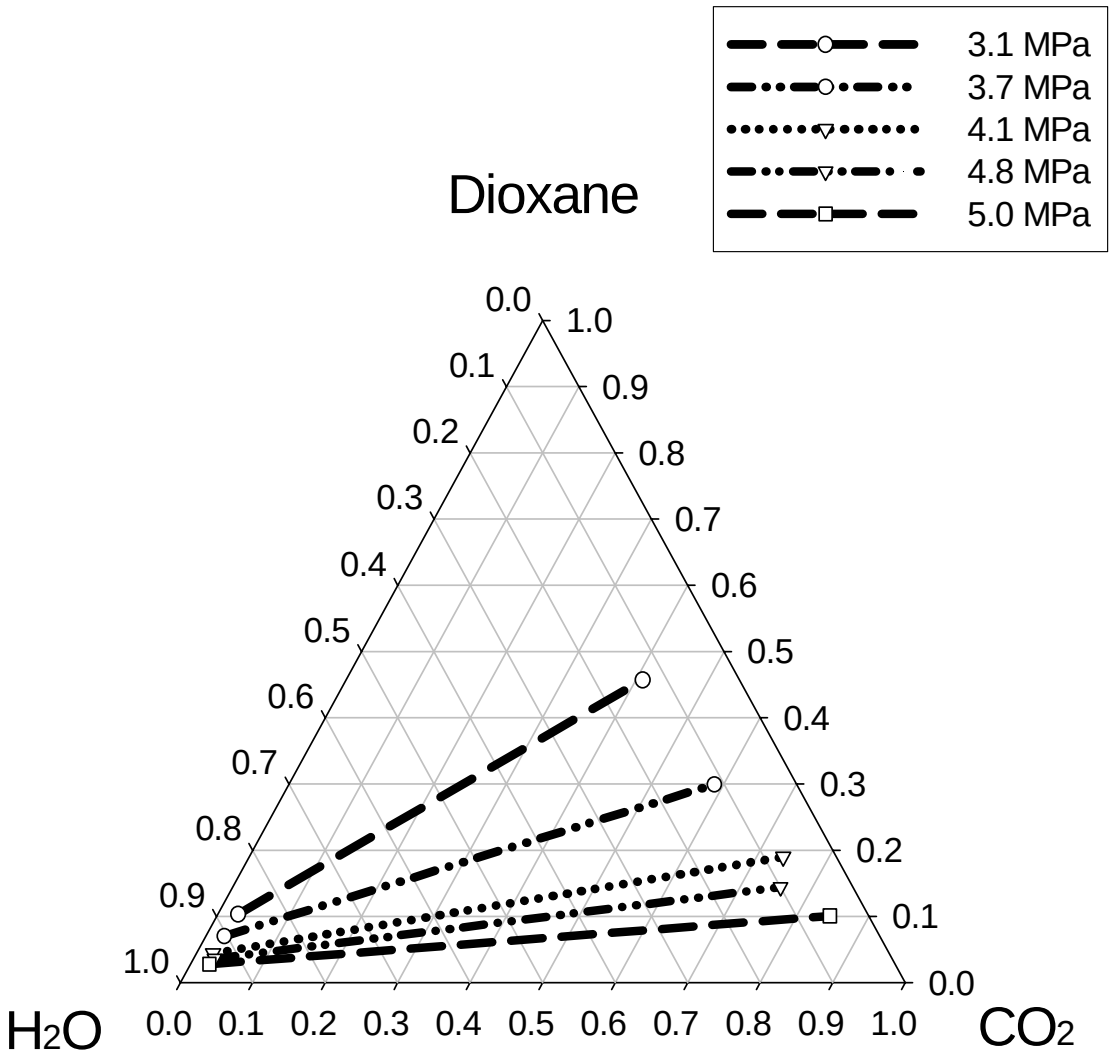

Figure 3-8: Ternary phase behavior of CO_2 – (1,4)-dioxane – water system with 150mM phosphate buffer.

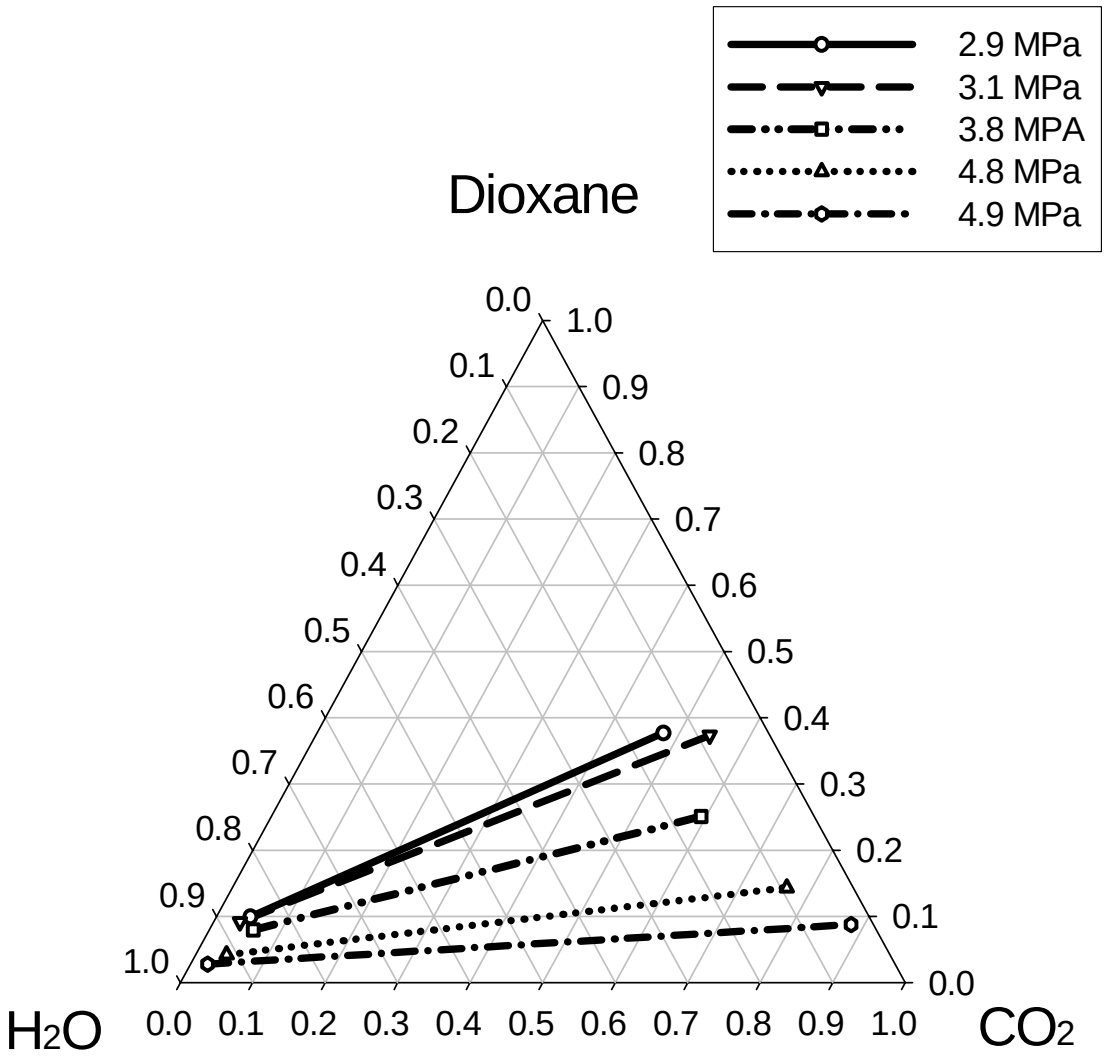

Figure 3-9: Ternary phase behavior of CO_2 – (1,4)-dioxane – water system with 150 mM phosphate buffer.

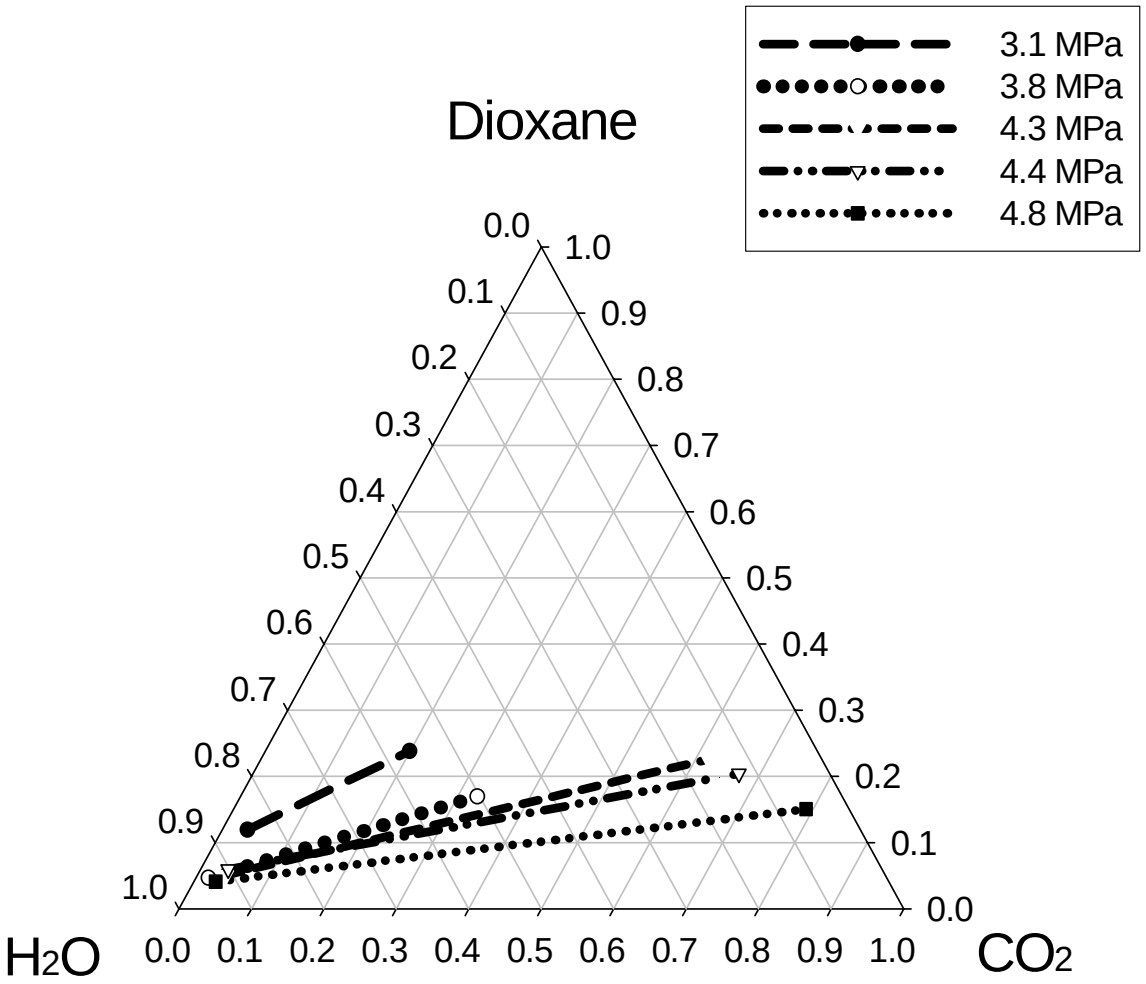

Figure 3-10: Ternary phase behavior of CO_2 – (1,4)-dioxane – water system with 150 mM phosphate buffer.

Hepes buffer was considered undesirable and was not studied further due to its high cost and the large reduction in two phase region for separation processing illustrated in the above CO_2 - (1,4)-dioxane - water systems. A comparison of both phosphate buffered and unbuffered CO_2 - THF - water and CO_2 - acetonitrile - water systems is shown in Figure 3-11 thru 3-14. For all cases, the two liquid phases can be made purer (relative to the amount of water and organic solvent) by the addition of more CO_2 with a resulting increase in pressure. We postulate that the addition of a buffer causes a "salting out" effect of CO_2 similar to the observations of Perez-Salado Kamps et al. in a miscible mixtures of CO_2 – methanol – water containing salts [13]. Salting-out does not refer to precipitation of salt, but rather a reduction in the amount of CO_2 in an organic species in the presence of salt at a given pressure. Thus, for the buffered cases, the solubility of CO_2 is reduced, causing a lower purity of the phases.

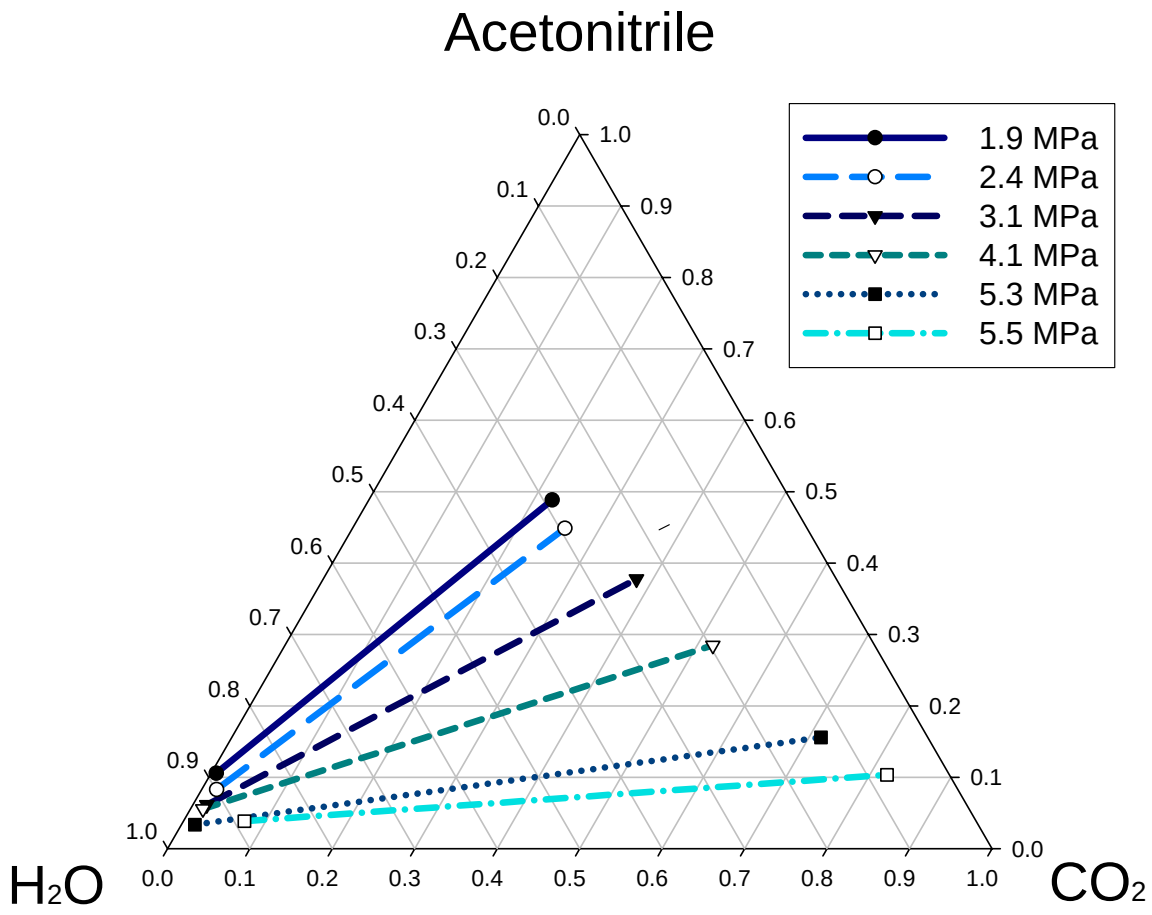

Figure 3-11: Ternary phase behavior of CO_2 – acetonitrile – water system with no buffer.

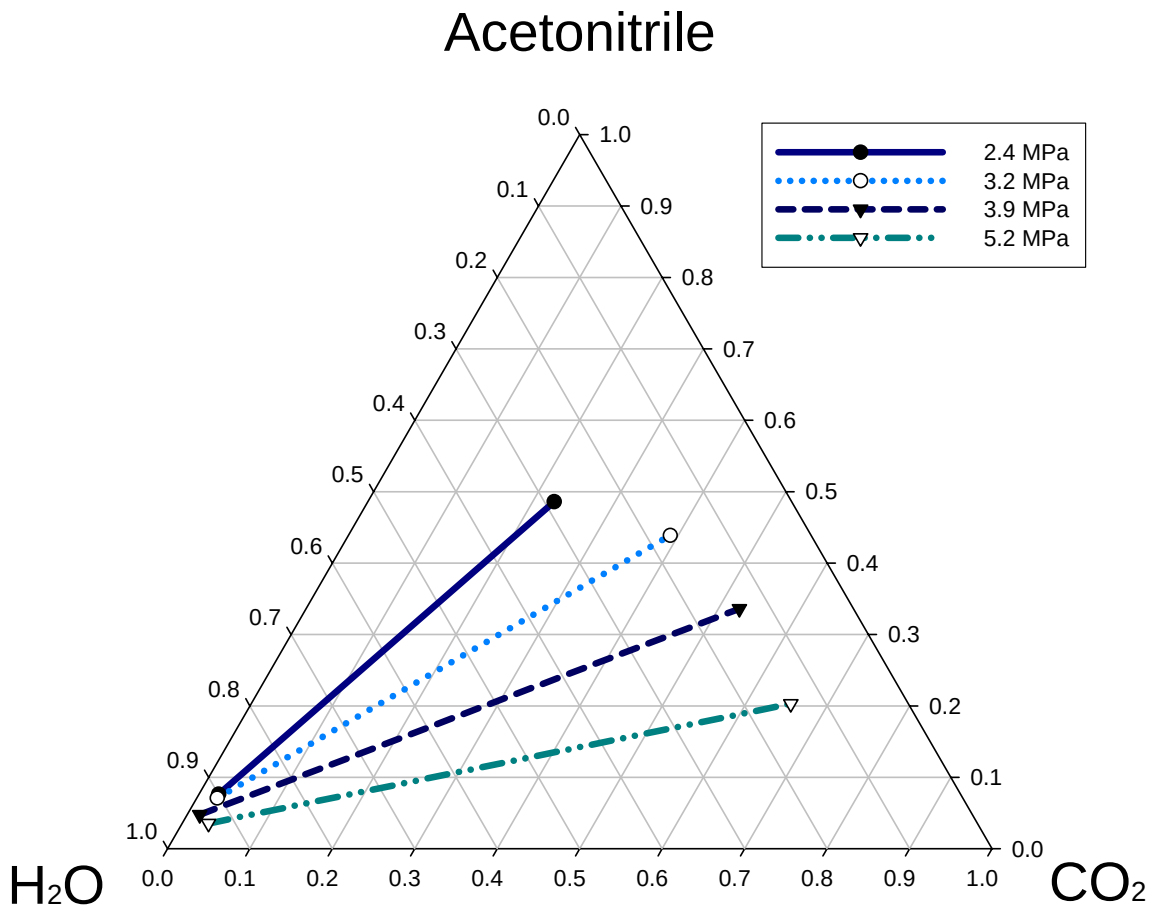

Figure 3-12: Ternary phase behavior of CO_2 – acetonitrile – water system with 150mM phosphate buffer.

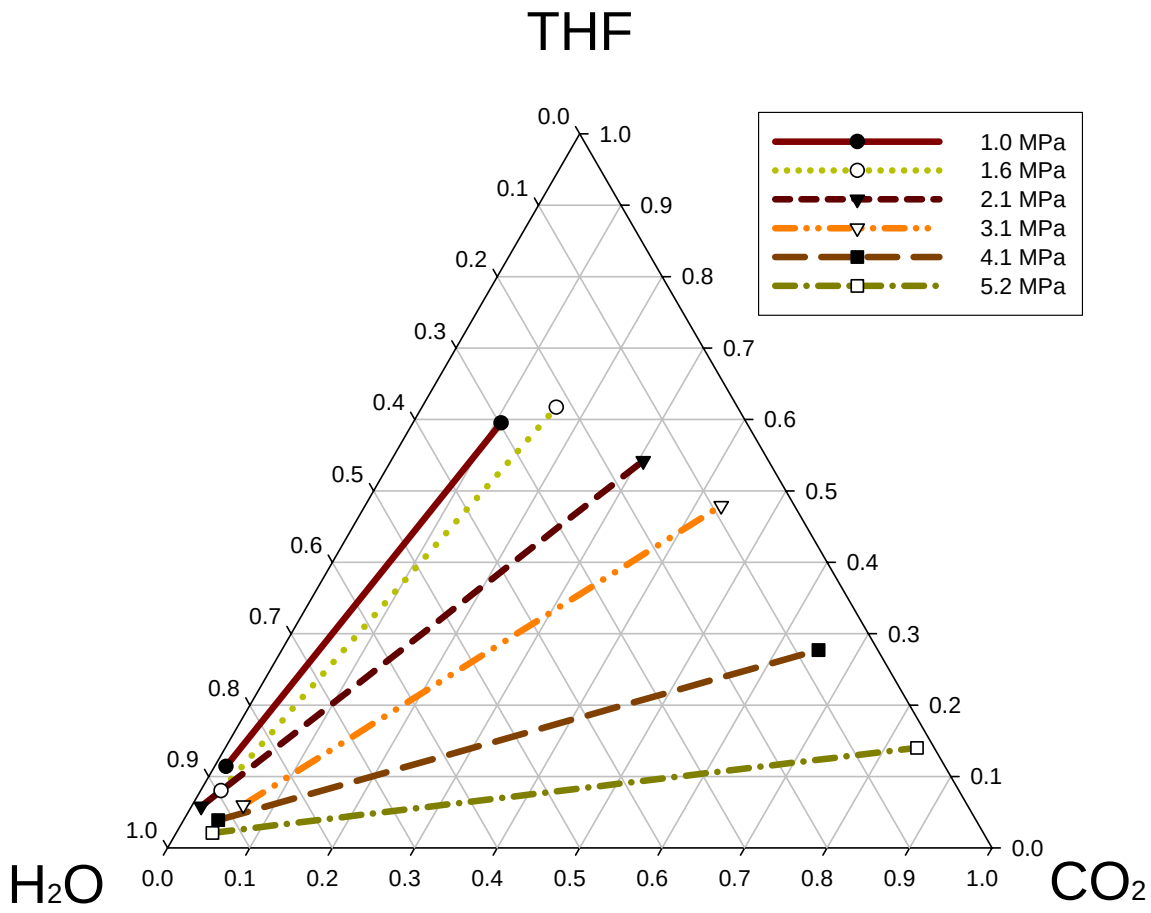

Figure 3-13: Ternary phase behavior of CO_2 – THF – water system with no buffer.

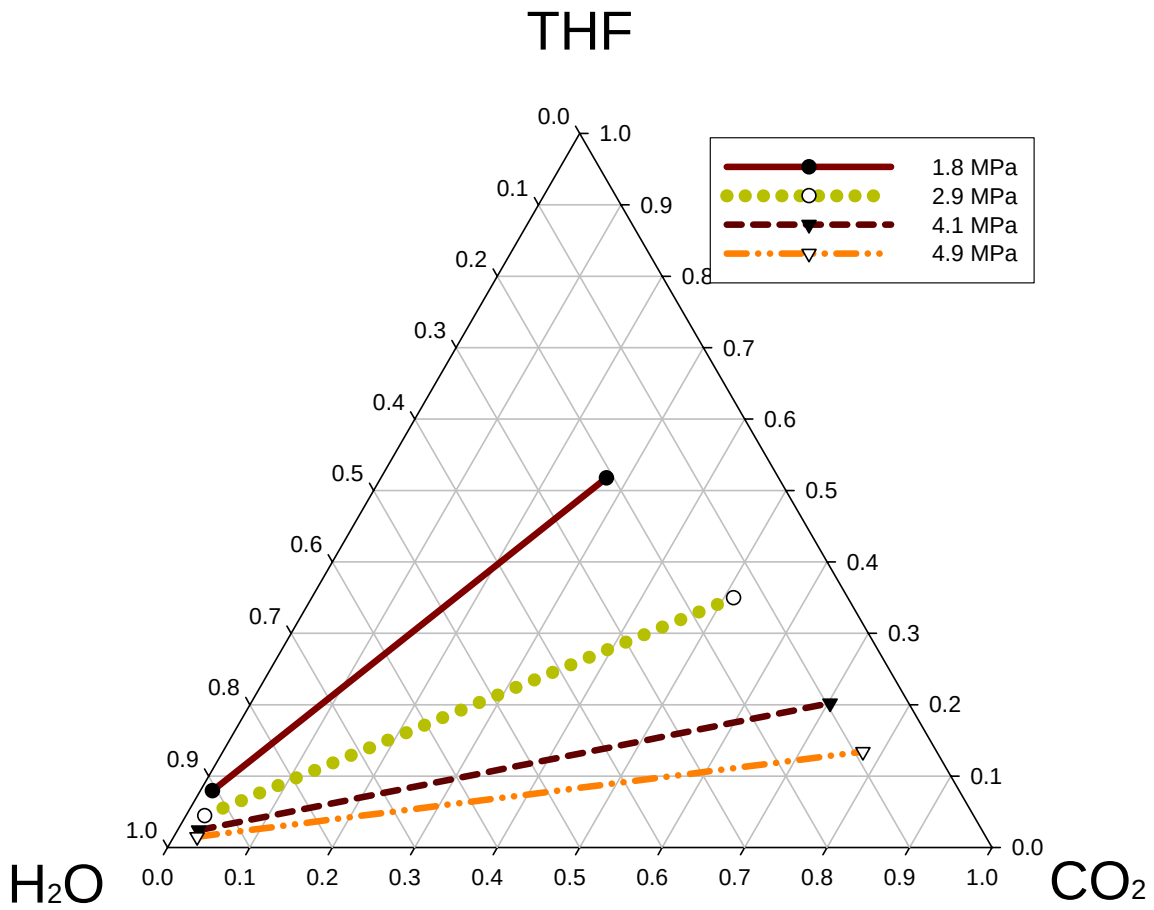

Figure 3-14: Ternary phase behavior of CO_2 – THF – water system with150mM phosphate buffer.

Conclusions and Recommendations

We demonstrate the range of applicability of our novel technique coupling enzymatic reaction with product purification and catalyst recycle. We show that in our OATS systems, a reaction can be run homogeneously in an organic/aqueous miscible mixture, followed by a CO_2-induced phase separation giving a product-containing organic phase and a catalyst-containing aqueous phase at modest pressures. The phase behavior critical to this separation is dramatically affected by the buffer salt Hepes and is mildly altered with the addition of phosphate buffer. Compared to the unbuffered OATS mixtures, both buffers studied require additional CO_2 pressure to achieve "clean" organic and aqueous phases during separation.

Detailed ternary phase data can be tedious to obtain experimentally. Therefore, the next logical extension to this work would be the incorporation of modeling to reduce the amount of experimental data required to characterize VLLE in OATS system with buffers. Thermodynamic properties of multicomponent liquid mixed– solvent mixtures containing salts and gases may be described by Gibbs free energy expression. Unbuffered systems were well predicted with "the Peng-Robinson Equation of State with Huron– Vidal type mixing rules from correlations of the binary systems, with the modified Huron– Vidal 1 (MHV1) and Huron– Vidal– Orbey– Sandler (HVOS) model with UNIQUAC excess energy model performing the best [9]." Incorporation of the solubility of salts with gases in aqueous/organic solutions complicates this prediction.

In buffered OATS systems, the concentrations of the solvent components are high in comparison to the solute components (i.e. the buffer salt). In such cases, the reference states for the chemical potentials of the solvent and solute species are often normalized according to the asymmetric convention described by means of the molality scale [13]. Recent expansion of Pitzer's molality-scale-based model for Gibbs excess energy [14, 15] extends to mixed-solvent electrolyte- and gas-containing systems [16]. In such, the Gibbs energy of transfer of a solute from one pure solvent to the solvent mixture was shown to play a very important role in modeling the solubility of gases and salts in aqueous/organic solutions. However, the UNIQUAC model does not explicitly take into account the temperature and pressure dependent Gibbs energy of transfer, and therefore may not be best suited for dealing with buffer solutions in OATS mixtures. Further work to examine reliable numerical values or the Gibbs energy of transfer of the desired buffer salts is needed.

To ensure the activity of the biocatalyst gives the highest possible reaction rates the pH should be carefully controlled in OATS, as illustrated by the above example of enzyme CAL B. Clearly, use of 150 mM phosphate buffer is sufficient to prevent the pH of the pressurized aqueous phase from decreasing to low levels, and CAL B is not detrimentally affected by short exposures to pH 5 that are encountered during phase separation. However, better pH management could be achieved through automated monitoring and control. This would allow for the addition of acid or base upon fluctuations in pH due to either acidic/basic product formation, or CO_2 accumulation upon subsequent reaction cycles. Specialized equipment such as the Mettler Toledo LabMax® or MultiMax™ are available "off the shelf" for ambient pH control. These

systems could prove useful for the reaction portion of the OATS process; however, after reaction the solution would need to be transferred to a pressure vessel for separation. Alternatively, one could build an in situ pH monitoring and control system into an existing pressure vessel. Better post reaction stirring or bubbling gaseous N_2 to displace the remaining CO_2 would also help to reduce pH drop between cycles.

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CHAPTER IV

HOMOGENEOUS BIOCATALYSIS AND RECYCLE OF MODEL REACTIONS WITH *CANDIDA ANTARCTICA* LIPASE B IN OATS SYSTEMS

Introduction

Enzymatic catalysts are at the forefront of emerging sustainable synthesis and processing techniques. The enormous potential of enzymes for the transformation of synthetic chemicals with high chemo-, regio- and enantioselectivities is well established [1, 2], making enzymes useful for the production of single enantiomers of drug precursors or intermediates of increasing importance in the pharmaceutical industry [1-3]. Additionally, chiral intermediates are also now in high demand for the production of bulk agricultural products [3-5].

A chiral molecule and its mirror image form a pair of enantiomers and a mixture of enantiomers in equal proportions is called a racemic mixture. (Most chemical reactions that exhibit chirality are nonspecific and result in a racemic mixture.) With the exception of their optical rotation, enantiomers share all the same physical properties so they can not be easily separated by boiling point, melting point, etc. In addition, enantiomers are chemically identical except when reacted with other chiral molecules. Two enantiomers may exhibit completely different properties. This can be as trivial as the simple fragrance difference of the limonene enantiomers; one has a citrus fragrance the other turpentine [6], or as important as one enantiomer being toxic while the other holds medicinal value as is the case for the sedative thalidomide [7]. For this reason, enantioselective synthesis is an important facet of pharmaceutical discovery.

Many synthetic routes to chiral molecules are tedious due multiple steps which often have many undesired side products, and chiral molecules are extremely difficult to separate. The use of enzymes for chemical transformations increases specificity and enantioselectivity. Some enzymes exhibit greater than 99% enantiomeric excess (ee) and therefore do not require further resolution of enantiomers. Additionally, enzymes usually function at relatively mild aqueous conditions with moderate temperature, pressure, and pH allowing for process routes that can potentially replace less environmentally friendly steps in chemical synthesis. Notably, for the past four years recipients of the Presidential Green Chemistry Challenge Award [8] have used enzyme as a part of green processing and synthesis schemes.

Often the need to function in relatively mild reaction solutions can also limit the utility of enzyme catalysts. Consider, for example, the final step to produce a prototype anti-asthma drug 4-ethyl-(2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl))-phenoxy acetic acid from its methyl ester (Figure 4-1). Enzymatic catalysis can attain the selective hydrolysis of the ester bond while ensuring that the active ring of the molecule (phtalimide ring) is preserved [9]; however, this substrate is water insoluble making it unavailable for reaction in aqueous solution. Initial work on enzymatic reactions in organic solvents to increase hydrophobic substrate miscibility was originally met with some skepticism as to its practical usefulness[10]; however, research in this area over the past two decades has shown marked success and led to an array of new biocatalytic processing techniques.

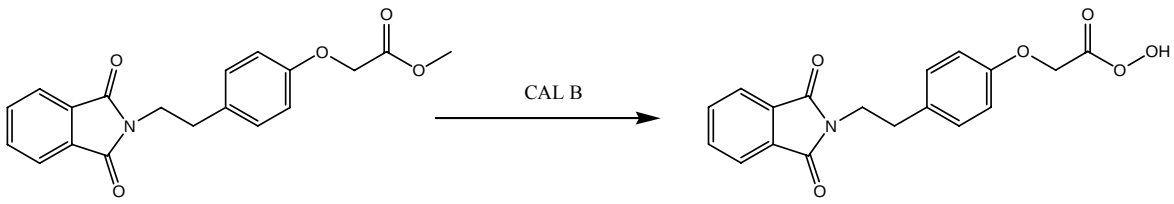


Figure 4-1: CAL B may be used for selective hydrolysis of the ester bond in the final step of the production of an anti-asthma drug 4-ethyl-(2-(1,3-dioxo-1,3-dihydro-2-isoindoylyl))-phenoxy acetic acid.

Enzymatic Transformations of Hydrophobic Substrates in Mixed Solvents

Numerous schemes which employ either organic solvents or ionic liquids with water have been developed to allow enzymatic catalysis to be used with hydrophobic substrates [4, 11, 12]. Included are water-in-oil microemulsion systems and aqueous biphasic catalysis, both of which utilize multiple liquid phases for reaction. Solid enzyme preparations, for example lyophilized enzymes or enzymes adsorbed on an inert support suspended in an organic solvent or ionic liquid, use a single liquid phase (although, in the presence of a solid catalyst phase) and are therefore most closely related to our own OATS system, which couples monophasic liquid biocatalysis with a biphasic separation.

These three solvent types (pure organic liquids, ionic liquids, and OATS) are compared to illuminate better which of these tools is best suited for a particular application. What is common to all three of these solvent systems is that they allow enzymatic transformation of hydrophobic substrates not accessible via traditional aqueous media. As mentioned above, the ability to transform such substrates is an extremely desirable asset for the pharmaceutical and agrochemical industries. Organic solvents affect enzymatic performance via three routes [13]:

1. They can strip off the essential water that is associated with the enzyme;
2. They can interact with the enzyme by changing the protein dynamics, conformation, and/or active site;
3. They can interact with the substrates and products by direct reaction.

Medium Engineering has also been investigated as a means to influence or even reverse an enzyme's selectivity [14]. However, there is no general paradigm that adequately describes an approach to medium engineering, most likely because multiple factors are involved, among which water content is a critical component [15-17]. One could assume that both ionic liquids and OATS systems might have similar effects on the enzyme; however, the extent would depend greatly on the anion/cation or amount/type of organic cosolvent, respectively.

A clear disadvantage of pure organic solvents is the requirement of the enzyme to be immobilized [4] to achieve high reaction yields. This need for immobilization adds time and effort to already laborious isolation techniques. Furthermore, immobilized enzymes are susceptible to activity loss due to the immobilization process and leaching of the enzyme from the solid support. At present, enzymatic reactions in ionic liquids also require immobilized enzymes, although some research is being conducted to tailor ionic liquids to prevent dissolved enzymes from denaturing [18].

Ionic liquids are suggested as possible alternative green solvents due to their nonvolatile nature, thermal stability, and ability to be tuned via anion/cation selection. This unique ability to be tuned for certain physical and chemical properties, such as polarity, hydrophobicity, viscosity, and solvent miscibility, renders ionic liquids

particularly useful for dissolving highly polar substrates such as carbohydrates and amino acids [19]. Conversely, the important issues of toxicity and ecological impact of most ionic liquids are not well known [19, 20], and neither are means to effectively recycle the used ionic liquid. For biocatalysis, purification of the ionic liquid is often necessary for good yields [21], and the practical challenge of developing efficient methods for product isolation from ionic liquids remains. Very volatile products may be evaporated off, products of intermediate volatility have been removed with supercritical CO_2 extraction [20, 22], and ways to isolate nonvolatile products have begun to be addressed through liquid-liquid extraction [12, 22, 23].

A major advantage of the OATS system is the inherent sequence of separation and recycle, discussed in Chapter III. Such systems support the dissolution of salt buffers to maintain an adequate pH for completely homogeneous enzymatic catalysis. As in ionic liquids, OATS can be tuned for increased substrate solubility, in this case, through careful selection of the type and amount of organic cosolvent. OATS bridges the best of both worlds of catalysis: it offers the increased selectivity, yields, and ee values seen in homogeneous catalysis [24], while affording the facile separation of heterogeneous catalysis. Furthermore, OATS systems are beneficial because they have a reduced amount of organic solvent in reaction mixtures, and the CO_2-induced separation eliminates the need for large volume solvent extractions for product recovery [25].

Here we show this coupled reaction and separation scheme for both an achiral and chiral model reaction with robust enzyme catalyst *Candida antarctica* lipase B (CAL B) in OATS. This versatile enzyme has been used to catalyze esterifications of hydrophobic

acids and alcohols as well as ester hydrolyses in a range of solvents [26-28] (including supercritical CO_2 [29-32]), rendering it an attractive choice for use in an OATS process.

Experimental Methods

Materials and General Experimental

Candida antarctica lipase B, CAL B, (E.C. 3.1.1.3; SOL-101; ≥8 kU/mL) was a kind gift from Biocatalytics (Pasadena, CA). HPLC grade solvents (99.5%) 1,4-dioxane, acetone, acetonitrile, tetrahydrofuran, and water were purchased from Sigma-Aldrich and were used without further purification. Reagents, used as received, include: *rac*-1-phenylethyl acetate, PEA, (Acros 99%), *rac-1*-phenylethanol, 1PE, (Acros 98%), *(R)*-1-phenylethanol (Alfa Aesar 99%), *(S)*-1-phenylethanol (Sigma 98%) phenethyl acetate (Alfa Aesar 99%), 2-phenethanol, 2PE, (Acros 99%), and CO_2 (Airgas SFC grade). Reaction progress was followed by measuring the product and substrate content of samples as compared to calibration standards. Kinetic tests were done with 150 mM phosphate buffer (pH 7.1) mixed with the appropriate amount of solvent and a stock solution of substrate dissolved in organic solvent to yield a final reaction mixture of 6 mL at the desired volume percentage of organic solvent. For example, to make a 15 mM 1-phenylethyl acetate solution in 6 mL of a 30% (1,4)-dioxane OATS system, we mixed 0.45 mL of stock (200mM) 1-phenylethyl acetate in (1,4)-dioxane, 1.35 mL of pure (1,4)-dioxane, and 4.2 mL of buffered water. 1.75 mL of this reaction mixture was aliquoited to three test tubes for reaction and the remainder 0.75 mL was taken as an initial sample. 0.1 mL of 1/100x enzyme was added to each test tube to initiate the reaction and 0.25 mL was removed periodically for analysis. Each condition was repeated three to five times.

Reaction progress was monitored via GC-FID and enantiomeric excess via LC. Yields were found using an Agilent gas chromatograph (GC-FID model 6890) with a DB17 column (Agilent, Palo Alto, CA). Enantioselectivity was monitored by analytical high performance liquid chromatography (LC) at 245nm using an Agilent 1100 series LC and a Chiralcel OD-RH (Diacel, Inc., Fort Lee, NJ) with a 0.46 cm I.D. x 15 cm L column set at 35 ºC and a flow rate of 0.5 mL/min (40% acetonitrile / 60% water) for 25 minutes. Both R and S enantiomers were resolved for the purchased racemic mixture; however, the S enantiomer was not present in detectable amounts for any of the reaction samples. Therefore, ee is reported as 100% minus the limit of detection, 1%. All samples were immediately mixed 1:1 with a mixture of 1:1 glacial acetic acid:organic solvent of interest to quench the reaction before either GC or HPLC analysis.

Solubility Tests

The solubility limits for the substrates were tested in various OATS systems, starting at 10% organic solvent and increasing in increments of 5% until a cloud point was reached. A 5 mL solution of the desired OATS system was prepared. For example, a 30% (1,4)-dioxane OATS system is made by combining 1.5 mL of (1,4)-dioxane with 3.5 mL of buffered water. To this 5 mL solution, 1 mL of 1-phenylethyl acetate was added. The mixture was placed in an oil bath at 25 ºC and magnetically stirred for 1 hour. The mixture was then allowed to let stand, without agitation, for 10 minutes while it separated into two phases. The top phase, undissolved 1-phenylethyl acetate, and the bottom phase the OATS system saturated with 1-phenylethyl acetate. The bottom phase

was sampled and analyzed via GC-FID for concentration as compared to a calibration made from standard solutions of 1-phenylethyl acetate.

Separation Experiments

Reactions were carried out in a 50 mL Jurgeson pressure vessel (Figure 4-2), stirred with a magnetic stir bar. The cell was mounted on a perpendicular rotating shaft so that the cell could be agitated under pressure for more vigorous mixing of the CO_2. Pressure in the cell was measured by a pressure transducer with digital readout (Druck, DPI 260, PDCR 910, GE Infrastructure Sensing, Billerica, MA) which was calibrated against a hydraulic piston pressure gauge (Ruska, GE Infrastructure Sensing, Billerica, MA) to an uncertainty of +/- 0.1 bar. While pressurized, the cell was kept behind a 1/4" polycarbonate enclosure as a safety precaution.

All samples taken were quenched with a 1:1 mixture of glacial acetic acid:(1,4)-(1,4)-dioxane before further analysis. Reactions were carried out at ambient room temperature (22-25 ºC). 15.92 mL of 150 mM phosphate buffer (pH 7.1) was mixed with 5.117 mL (1,4)-dioxane and 1.706 mL of (1,4)-dioxane containing 200 mM 1-phenylethyl acetate. 2 mL of this mixture was withdrawn as an initial (t = 0) sample, and 1.3 mL of 1/100x enzyme was added to initiate the reaction. The reaction mixture was loaded into the pressure cell and stirred with a magnetic stir bar for 1 hour, after which 0.5 mL was removed and assayed for conversion via GC-FID and enantiomeric excess via LC. The cell was sealed and pressurized with CO_2 to 50 bar. CO_2 was first added through a dip tube at the side of the cell so that it could bubble directly through the liquid phase, thus decreasing the time to reach equilibrium.

While pressurized, the cell was rocked to mix the contents well and aid CO_2 dissolution into the reaction mixture. After several inversions, the cell was turned upright and stirred with the magnetic stir bar to dislodge any organic droplets that may adhere to the cell wall in the aqueous region of the cell. Stirring was then stopped and the phases were allowed to settle. When the cell pressure equilibrated at 50 bar, the CO_2 line was moved to the top port and allowed to continually flow from the supplier's gas cylinder regulated at 50 bar. Once a clear phase boundary was formed, the organic layer was removed by opening the valve on the dip tube and by bubbling the organic layer into a pre-weighed flask containing a known amount of (1,4)-dioxane until the top of the organic layer was just above the dip tube height. The flask was re-weighed to determine the amount organic layer collected and the flask contents were measured via GC-FID and LC to determine the amount of product and reactant in the collected organic layer. After removal of the organic layer, the CO_2 supply line was removed from the cell and the CO_2 was vented to the atmosphere to depressurize. The vessel top was removed and a 0.5 mL sample was taken and measured via GC-FID and LC to determine the amount of product and reactant in the remaining in the aqueous layer.

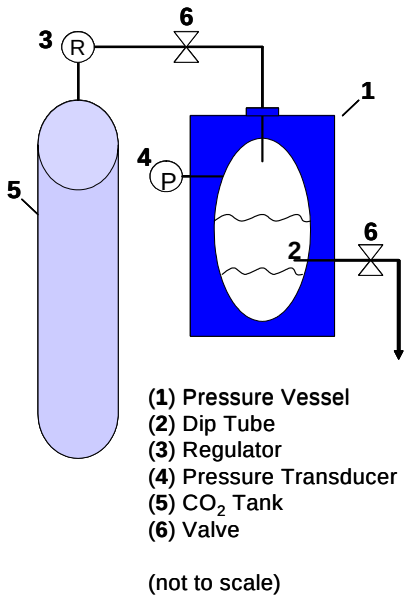


Figure 4-2: Schematic of pressure vessel used for separation and recycle experiments.

Recycle Experiments

All reactions were carried out at ambient room temperature (25 ºC) in the same Jerguson pressure apparatus described above for the separation experiments. 12 mL of 150 mM phosphate buffer (pH 7.1) was mixed with 8 mL of dioxane containing 140 mM PEA. 0.75 mL of this mixture was withdrawn as an initial (t = 0) sample, and 1.132 mL of 1/100x CALB was added to initiate the reaction. The reaction mixture was loaded into the pressure cell and stirred by a magnetic stir bar for 2 hours, after which 0.382 mL was removed and assayed for conversion via GC. The cell was sealed and pressurized with CO_2 to 50 bar. While pressurized, the cell was rotated to mix the contents well and aid CO_2 dissolution into the reaction mixture. After several inversions, the cell was turned upright and stirred with the magnetic stir bar to dislodge any organic droplets that may adhere to the cell wall in the aqueous region of the cell. Stirring was then stopped and the phases were allowed to settle. Once a clear phase boundary was formed, the organic

layer was removed by opening the valve on the dip tube and bubbled into a pre-weighed flask containing 5 mL of dioxane until the top of the organic layer was just above the dip tube height. The flask was re-weighed to determine the amount organic layer collected and the flask contents were measured via GC to determine the amount of product and reactant in the collected organic layer. Karl Fischer titration was used to determine the amount of water present in the organic phase. After removal of the organic layer, the CO_2 supply line was removed from the cell and the pressure cell was vented to the atmosphere to depressurize. After removing the cell cap, magnetic stirring was used to help remove dissolved CO_2 from the reaction mixture, and 0.25 mL of reaction mixture was withdrawn and assayed via GC to determine reactant and product concentrations.

To begin the successive reaction cycles, the volumes of water and dioxane removed from the cell in the organic layer and due to reaction sampling were replaced with 150 mM phosphate buffer and dioxane containing 300 mM PEA to bring the cell volume to 20.5 mL and 40% (v/v) dioxane. After this addition, 0.25 mL was removed as an initial sample for each new cycle. Following two hours of reaction with stirring, 0.25 mL was removed and sampled for conversion via GC. The reaction mixture was then separated with CO_2 and the organic layer removed and sampled as described above. Following cell depressurization, 0.25 mL was removed and assayed by GC. The average cycle time, from addition of enzyme to the addition of more substrate and buffer, was three hours. Cycles 1-4 were conducted within one day. Following removal of the organic layer of cycle 4, the cell was depressurized, 0.25 mL sampled and the reaction mixture was left in the cell overnight. The following morning, 0.25 mL was removed and

assayed for conversion and then buffer and 300 mM PEA in dioxane were added to bring the reaction mixture to 20.5 mL and 40% dioxane to begin cycle 5.

Results and Discussion

We first tested the feasibility of OATS mixtures for biocatalytic reactions and separations with soluble *Candida antarctica* lipase B (CAL B). The hydrolysis of 2-phenethyl acetate (PEA) to 2-phenylethanol (2PE) and acetate (Figure 4-3) was selected as a model reaction because both PEA and 2PE both have extremely low water solubility and can be easily detected via gas chromatography to assay reaction progress.

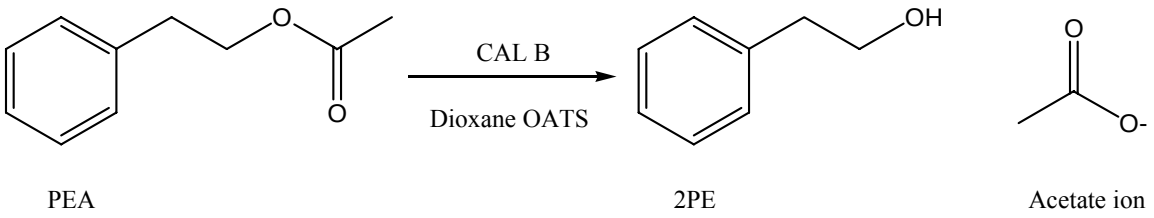


Figure 4-3: Hydrolysis of 2-phenyl acetate (PEA) to 2-phenylethanol (2PE).

Enzymatic reaction rates in aqueous buffer-(1,4)-dioxane mixtures were evaluated to choose a suitable solvent composition for future recycle experiments as shown in Figure 4-4. By taking advantage of higher substrate solubility afforded by the addition of water-miscible organic solvents, higher specific reaction rates can be obtained than in purely aqueous solvent. While rates at 8 mM PEA in water-(1,4)-dioxane mixtures decreased as (1,4)-dioxane content increased, the specific rate in water-(1,4)-dioxane

mixtures nearly saturated with PEA substrate improved over the range of (1,4)-dioxane content tested due to the higher substrate solubility (up to 56 mM PEA in 40% (1,4)-dioxane).

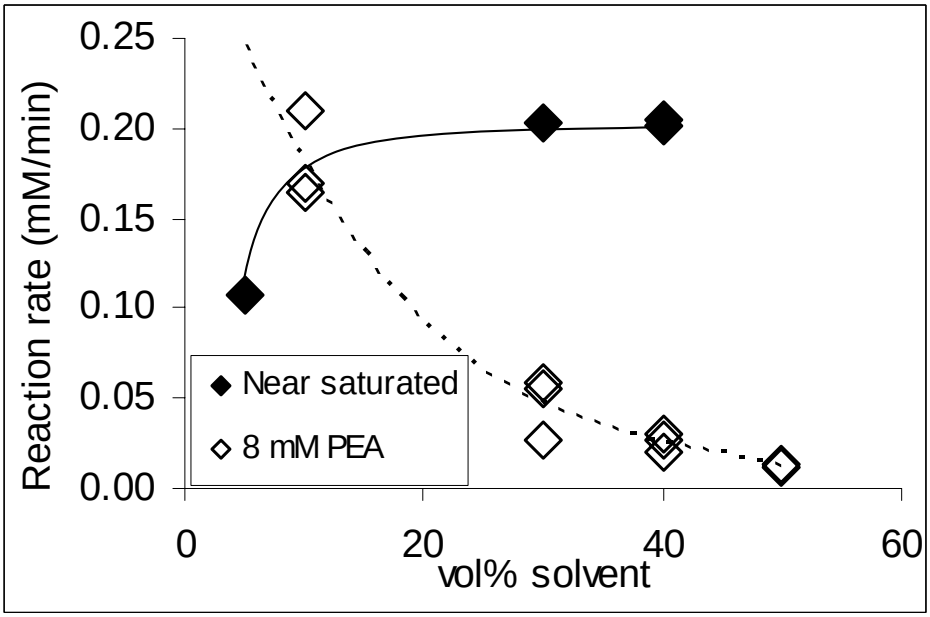


Figure 4-4: Reaction rates in (1,4)-dioxane OATS mixtures. In 10% (1,4)-dioxane, the 8 mM PEA is near saturation.

A 40% (1,4)-dioxane mixture was chosen for use in further experiments because this mixture provides increased substrate solubility and achieves the highest observable reaction rate. The 40% (1,4)-dioxane mixture is also favorable for the phase separation. Upon CO_2 addition it provides a larger organic phase volume to aid extraction of reaction products than lower (1,4)-dioxane levels, thus improving separation. At greater than 40% (1,4)-dioxane concentration, buffer precipitated out of solution and further study is needed to see if this lower buffer concentration can adequately maintain sufficiently high pH levels. The importance of adequately buffering the reaction mixture is discussed in Chapter III.

Having demonstrated improved reaction rates water-(1,4)-dioxane mixtures nearly saturated with substrate and the absence of adverse effects on CALB resulting from pH changes during separation, the feasibility of implementing a process using (1,4)-dioxane OATS mixtures and CO_2 for separation and recycle of the homogeneous biocatalyst was tested in 40:60 (1,4)-dioxane:buffer (v:v) mixtures containing PEA. After two hours of reaction, CO_2 was added to separate the mixture. The organic layer was decanted under pressure, and after CO_2 was removed from the cell, the next cycle began by adding buffer solution and (1,4)-dioxane containing 300 mM PEA to the remaining reaction mixture to begin a new reaction cycle. The cycle time from the beginning of one reaction to the next averaged three hours. Two trials of six consecutive reactions and separations were conducted. In each trial, the reaction mixture remaining in the cell was left overnight after the fourth separation, and the fifth reaction was initiated the following morning by adding more buffer and 300 mM PEA in (1,4)-dioxane.

The observed conversion for each two-hour reaction of both trials is shown in Figure 4-5. Over the concentration ranges seen during reactions, the CALB-catalyzed reaction in 40% (1,4)-dioxane mixtures is first order with respect to PEA concentration, with an apparent rate constant of 0.0067 min^{-1}, indicating that the enzyme kinetics are not substrate-saturated at these concentrations. With the given rate constant (Figure 4-6), a conversion of 55% is predicted for two hours of reaction, and the observed conversions for the first reaction of both trials are very close to this value (58% and 54%). An overall process conversion (total moles of 2PE formed / total moles of PEA added) of 61% was obtained for both runs.

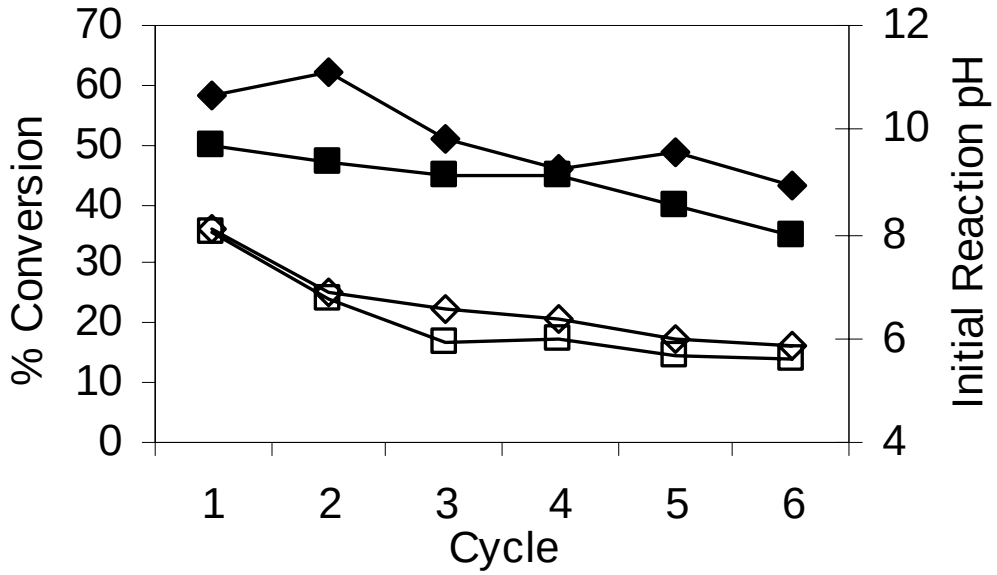


Figure 4-5: Conversions (filled symbols) and starting pH (open symbols) of recycled OATS reactions for Trial A (◆) and Trial B (■).

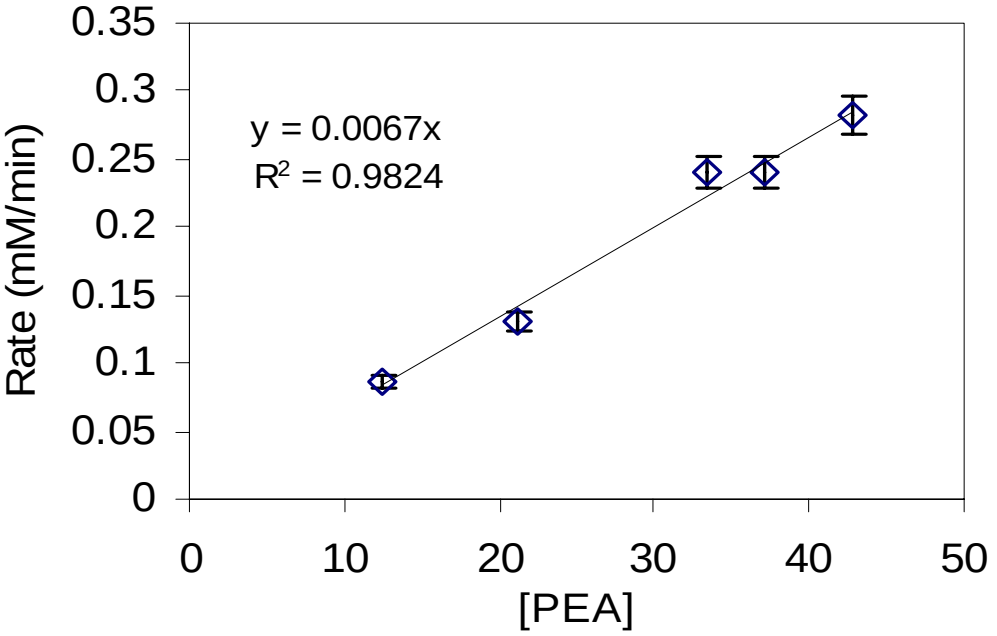


Figure 4-6: The linear plot of reaction rate vs. substrate concentration, [PEA], demonstrates a first order reaction ($r = -k\ C_A$) with rate constant $k = 0.0067\ min^{-1}$. Integrating the concentration over t = 2 hours (120 min) gives conversion, $x = 1-C_A/C_{Ao} = (1- e^{-kt}) = 0.55 = 55\%$.

The conversion in each trial decreases by 10 to 15 percentage points by the sixth reaction, suggesting deactivation of CAL B. However, since some enzyme is removed as reactor contents are sampled, the total enzyme concentration in the reactor will be diluted as new solution is added to begin new cycles. This dilution accounts for 11 of the 15 percentage points of conversion lost by the sixth reaction. Furthermore, the pH of the reaction mixture decreased from 8 to 5.5 over six cycles (Figure 4-5). This was most likely due to residual CO_2 that remains saturated (or even supersaturated) in the OATS mixture following the initial separation and the accumulation of acetate in the aqueous phase during successive runs. As discussed earlier in Chapter II, CAL B reaction rates decrease by up to 20% from pH 8 to 5; thus, the pH drift likely explains the remaining observed conversion loss, and better pH control should reduce this effect.

The concentration of PEA and 2-PE in the separated organic and aqueous layers was measured as well as the concentration of water in the organic layer. The organic layer contained approximately 4 wt% water for all cycles, in agreement with previous results [33]. The distribution of PEA and 2PE between the separated organic and aqueous phases in each cycle was measured in both trials and is shown in Figure 4-7. Distribution coefficients in trial A ranged between 20-55 for PEA and between 11-15 for the more hydrophilic 2PE. Trial B showed lower distribution coefficients for both PEA (8-16) and 2PE (6-9). A higher starting concentration of PEA was used in trial A (72 mM) as compared to trial B (56 mM), so it appears that the distributions may be concentration-dependent. Regardless, the observed distribution coefficients are favorable and show that the separated organic phase can be used to extract product from the reaction mixture: we recover 80% of the 2PE product in the organic layer for both trials.

By addressing concerns of reactivity, enzyme stability in separated media, and favorable partitioning, a biocatalytic OATS scheme for processing hydrophobic substrates with product separation and biocatalyst recycle has been developed. Given a cycle time of three hours, 56 mM starting concentration for PEA in 40% (1,4)-dioxane OATS, and an average of 50% conversion per cycle, we calculate a volumetric productivity, expressed by the space-time-yield, of 27.4 g/(L·d). Even with an equivalent residence time of 2 hours in the reactor and assuming complete conversion, the space-time yield in PEA-saturated (4 mM) aqueous mixture cannot exceed 5.9 g/(L·d), almost five times less than that observed in the OATS system. In addition, we find that the enzyme can be recycled with very little activity loss between cycles. The activity loss observed is entirely accounted for by the dilution of the enzyme and pH decrease of the reaction medium.

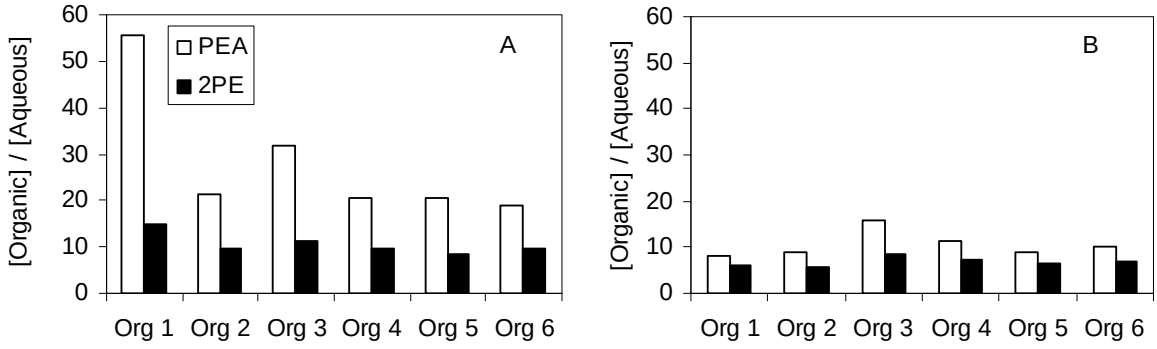
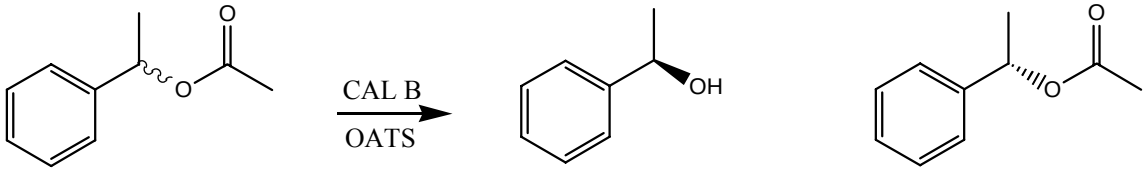


Figure 4-7: OATS distribution coefficients in the organic phase (Org) of the specified cycle number for (A) Trial 1 and (B) Trial 2.

Next, the chiral resolution shown in Figure 4-8 was investigated to elucidate the effect of CO_2-induced separation on the overall ee value of the reaction as well as to examine alternate OATS solvents for effectiveness of biocatalyst reaction and separation. Acetone, acetonitrile, (1,4)-dioxane, and tetrahydrofuran (THF) were each investigated as

OATS solvents. THF showed buffer precipitation even at concentrations as low as 5% by volume and was therefore eliminated from the study. Cloud points (indicating either the substrate or buffer precipitation) were found for organic-aqueous mixtures of acetone, acetonitrile, and (1,4)-dioxane to be at 35%, 50%, and 60% by volume, respectively. Table 4-1 compares the substrate saturation concentration, rate constant, and ee value of the product for each of these solvents at a fixed volume percent organic solvent.



rac-1-phenylethyl acetate *(R)-1*-phenylethanol *(S)*-1-phenylethyl acetate

Figure 4-8: Chiral resolution using CAL B.

Table 4-1: Comparison of OATS solvents and their effectiveness for the chiral resolution of 1-phenylethyl acetate with *Candida antarctica* lipase B (CAL B)

Solvent	% Volume in OATS	Substrate Saturation (mM)	Pseudo 1st Order Rate Constant $(sec)^{-1}$	Product Enantiomeric Excess (ee)
Acetone	30	18.1 ± 0.9	0.003 ± 0.001	> 99%
Acetonitrile	30	9.1 ± 0.5	0.009 ± 0.001	> 99%
(1,4)-Dioxane	30	17.7 ± 0.8	0.014 ± 0.001	> 99%

Excellent ee values are observed for all of the solvents studied with this reaction. It is apparent from Table 4-1 that a (1,4)-dioxane OATS system is best suited for this reaction because of its high saturation concentration as compared to acetonitrile and increased rate constant as compared to acetone. Figures 4-9 and 4-10 show detailed kinetic and solubility data in various (1,4)-dioxane mixtures. The reaction rate drops with increasing (1,4)-dioxane concentration (even when the reaction is run at the solubility limit of each solution), therefore a 30% (1,4)-dioxane mixture was chosen for separation experiments.

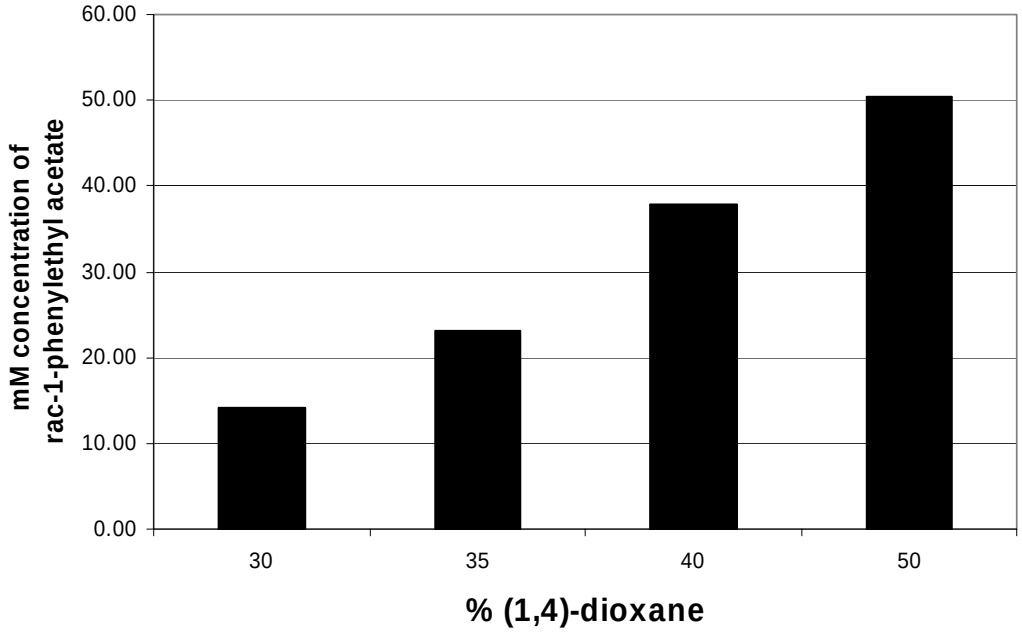


Figure: 4-9: Solubility limit of the substrate *rac*-1-phenylethyl acetate in (1,4)-dioxane.

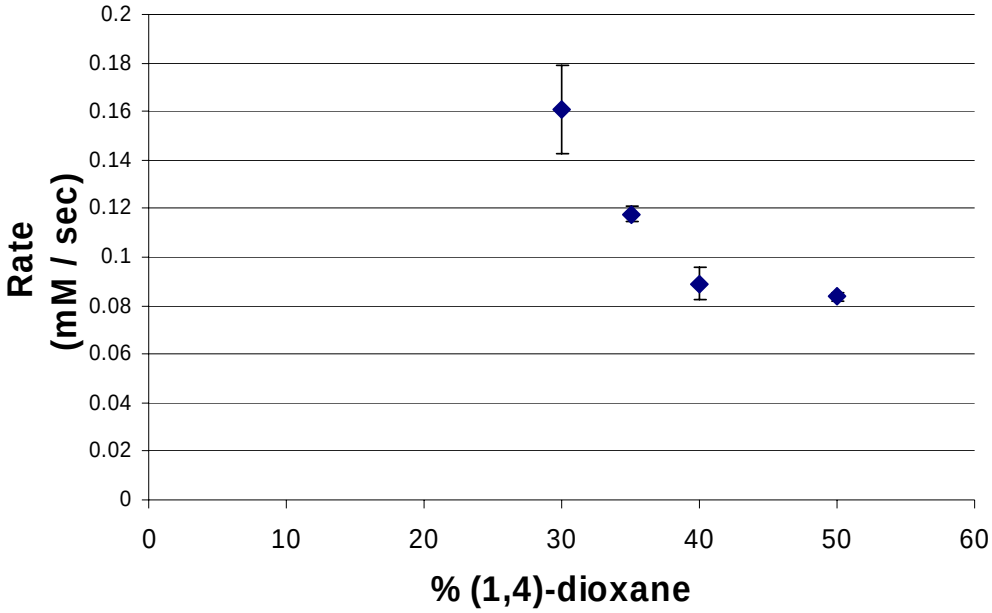


Figure: 4-10: Substrate conversion with initial substrate concentration of the reaction specified as the saturation limit of the (1,4)-dioxane mixture at the given volume percent (1,4)-dioxane.

The 30% (1,4)-dioxane OATS system was tested with subsequent separation as described in the experimental section and compared to a control reaction that did not undergo CO_2-induced separation. The overall ee value of the *(R)*-1-phenylethanol without separation (> 99%) is the same as the ee value of the organic and aqueous phases indicating the CO_2-induced separation has no significant effect on the overall ee value of the product.

Conclusions and Recommendations

OATS systems facilitate catalysis of reactions that are unfavorable in water due to solubility constraints, however, they are not appropriate to reduce water-induced side reactions because the miscible mixture still requires a high water content (generally, around 30-70% water by volume). In OATS systems, a reaction can be run homogeneously in an organic/aqueous miscible mixture, followed by a CO_2-induced phase separation giving a product-containing organic phase and a catalyst-containing aqueous phase at modest pressures. We suggest that this is a promising avenue given the success of mixed aqueous-organic solvents for reaction [34, 35] and our ability to also tune our separation with buffer, temperature, and pressure variation.

An OATS process will be most effective when three requirements are met:

1. The use of an OATS mixture must provide acceptable enzyme reactivity in the reaction medium.
2. The biocatalyst must survive the CO_2 –pressurized separation process.
3. The biocatalyst should be retained in the aqueous phase and reaction products should partition favorably into the organic phase.

Thus, the reactivity, pH stability, recycleability of the biocatalyst, and partitioning behavior of the substrate and product in a water-(1,4)-dioxane OATS mixture were tested. By satisfying these requirements, we have developed a system that allows 80% recovery of reaction products in the organic phase, and displays less than 10% apparent biocatalyst activity loss after six recycles. We also show the application of CO_2-switched

OATS systems for enantioselective reactions of hydrophobic substrates. The overall ee value of the product is the same before and after OATS separation, at > 99%.

Biocatalytic OATS reaction-separation schemes fulfil an identified need [36] to develop new options to meet current challenges in biochemical synthesis. By integrating reaction and separation, simpler and simultaneously more efficient processes with reduced ecological footprint can be designed. The work here shows that biocatalysis in OATS is feasible and can be an effective option for designing biocatalytic processes, especially when hydrophobic substrates are involved. The preservation of ee value opens the door to combining OATS with other biocatalysts and their array of unique chemistries to efficiently synthesize pharmaceutically relevant chiral products. A larger reaction volume, decreased sampling, and improved pH control should minimize enzyme activity loss further. Even better product recoveries could be obtained by using a reaction with more hydrophobic products.

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CHAPTER V

ENZYME-CATALYZED DIARYL KETONE REDUCTION IN OATS SYSTEMS

Introduction

Chiral diarylmethanols are useful as bioactive compounds and precursors for pharmaceutically important antihistaminics, anaesthetics, diuretics, antidepressives, antiarrhythmics, and anticholinergics [1-4]. High selectivity for many diarylmethanols has been shown via addition of an aryl nuceleophile to aromatic aldehydes; however the most common aryl donors are expensive and not readily available for large scale use [3, 5]. Alternatively, inexpensive prochiral ketones may be reduced to their corresponding alcohols to introduce chirality in a molecule and form the desired diarylmethanol [3].

Truppo et. al. [5] make a strong case for the use of isolated enzyme catalysts as opposed to chemical or whole cell catalysts for ketone reduction. They state that chemical catalysts for prochiral ketone reduction have a very limited substrate range and the known examples of highly selective diarylketone reductions rely on both electronic and steric effects to determine the degree of enantioselectivity limiting them to substrates that are either highly electronically dissymmetric or have an ortho-substituted aryl group. Additionally, they concede that whole cell catalysts may offer excellent stereoselectivity for substrates difficult to reduce with chemical catalysts, but whole cells require more difficult separations as well as low substrate loading, which result in unsatisfactory volumetric productivity.

Although isolated enzymes can be more selective than whole cells or chemically catalyzed reductions, their recovery and purification can be prohibitively expensive [6].

Another limitation specific to the formation of diarylmethanols is the low solubility of hydrophobic phenyl containing ketone substrates in aqueous media. Here we investigate the feasibility of using OATS systems to increase substrate solubility, recycle the hydrophilic homogeneous enzymes (and cofactor), and simultaneously remove the desired hydrophobic diarylmethanol product.

Experimental Methods

Materials

The ketoreductase (KRED 101) and glucose dehydrogenase (GDH 103) were manufactured by Biocatalytics (Pasadena, CA) and kindly provided to us by David Rozzell from Merck. The following compounds were used as received: acetonitrile (Sigma-Aldrich, 99.9%), 2-benzoylpyridie, 2BP, (Alfa Aesar, 99%), (1,4)-dioxane (Sigma-Aldrich, 99.9%), glucose (EM Science, ACS Grade), methyl ethyl ketone (Aldrich, 99%), nicotinamide adenine dinucleotide phosphate, NADP, (Amresco, Reagent grade), nicotinamide adenine dinucleotide phosphate-oxidase tetrasodium salt, NADPH, (Calbiochem 94.9% by HPLC), phenyl-pyridin-2-yl-methanol, PPM, (Sigma Aldrich), sodium phosphate monobasic monohydrate, phosphate buffer, (EMD Chemicals, ACS grade), and tetrahydrofuran, THF, (Sigma-Aldrich, 99.9%).

Instrumentation

A Hewlett Packard Gas Chromatograph with Mass Selective Detector model 5973 (GC-MS) with a HP-5MS column (Agilent, Palo Alto, CA) and an analytical high performance liquid chromatography (HPLC) were used to determine 2BP and PPM

concentration as compared to standard calibration curves. For solubility tests, an Agilent 1100 series HPLC with a Chiralcel OD-RH (Diacel, Inc., Fort Lee, NJ) , 0.46 cm I.D. x 15 cm L column set at 35 ºC and a flow rate of 0.7 mL/min (30% acetonitrile / 70% water) with 30 minutes runs was used at λ = 254nm. All other HPLC measurements were obtained on a Beckman Coulter System Gold HPLC with a Beckman Coulter ODS Ultrasphere C18 column (Beckman Coulter, Inc., Fullerton, CA) , 0.46 mm I.D. x 25 cm L column set at a flow rate of 1 mL/min (50% acetonitrile / 50% water at a pH of 3 with 5 mM potassium phosphate buffer) with 10 minutes runs was used. At λ = 340 nm. A Beckman Coulter DU 800 Spectrophotometer was used to observe GDH reactions by monitoring NADPH (extinction coefficient of approximately 6300 cm L mol^{-1} [7]) formation at 340nm .

Solubility Tests

In 2 mL total volume, solutions of the appropriate volume percent (1,4)-dioxane (5% - 40%) were made with DI water. Solid 2BP was added until the solution became supersaturated and either a solid precipitate or a second liquid phase was observed. The samples were mixed at 23 ºC in a thermostatic water bath for 45 minutes then centrifuged for approximately 1.5 minutes. The supernatant was removed and directly assayed for 2BP concentration via HPLC.

Screening Assay

In a method adapted from Truppo, et al. [5], ketones were run at 1g scale in 150mM phosphate buffer using the following conditions and concentrations: 30 ºC, pH of

7.15, 2 g/L KRED 101, 2 g/L GDH 103, 20 g/L glucose, 1 g/L NADP cofactor, 25 g/L ketone, 10% v/v tetrahydrofuran (THF), (1-4)-dioxane, acetonitrile, or no solvent. The enzymes, cofactor and glucose were first dissolved in the buffer. Next the substrate was dissolved in organic solvent and the substrate solution was then added to the reaction mixtures or added as a solid for no solvent case. The reactions were run for 4 hours at 30 ºC with periodic pH titration using NaOH. Upon completion, the reaction solution was extracted with 2X volumes of methyl ethyl ketone and analyzed via GC.

Kinetic Assays

In order to evaluate the Michaelis-Menton parameters of the multiple-enzyme and multiple-substrate system of interest, we examined each half reaction independently. K_m and $V_{\max}$ for each substrate in the half reaction was determined by varying one substrate concentration while taking care have a constant excess of the second substrate. The rate of reaction was determined by NADPH turnover at 340nm. All reactions were initiated with the addition of enzyme to reaction solvent containing 10% (1,4)-dioxane and 90% 150mM phosphate buffer titrated to a pH of 7.15 by volume. When 2BP was varied, 2.5 mM NADPH and 122 μg/mL KRED 101 were used. When NADP was varied, 1 mL total volume with 100 mM glucose and 1 μg/mL were used. When glucose was varied, 1 mL total volume with 1mM NADP and 1 μg/mL GDH 103 were used.

For the GDH half reaction the NADPH peak at 340 nm could be monitored *in-situ* via UV-Vis with 1mL total reaction volume. Each substrate concentration examined was run in triplicate. For the KRED half reaction the UV spectra of the substrate 2BP overlapped that of NADPH, therefore the HPLC was used to monitor the reaction rate.

From 2.75 mL total reaction volume, 300 µl was sampled every two minutes for 10 minutes, quenched with 1:1 (1,4)-dioxane, and placed in boiling water for 1 minute before HPLC analysis. All reactions were initiated with the addition and the reaction solvent contained 10% (1,4)-dioxane and 90% 150mM phosphate buffer titrated to a pH of 7.15 by volume.

Organic Solvent Tolerance Tests

All reactions were initiated with the addition of enzyme to reaction solvent containing the desired percentage of (1,4)-dioxane with the remainder of the total volume being 150 mM phosphate buffer titrated to a pH of 7.15. The KRED 101 solvent tolerance tests contained 20mM 2BP, 2.5 mM NADPH, and 122 µg/ml KRED 101 in 2.82 mL total reaction volume. 300 µl samples were taken every two minutes for 10 minutes, quenched 1:1 with (1,4)-dioxane, and placed in boiling water for 1 minute before HPLC analysis. The GDH 103 solvent tolerance tests contained 100mM glucose, 1mM NADP, 1 µg/ml GDH 103 and were done in 1mL quartz cuvettes with 1 cm path length assayed via UV-Vis at 340 nm.

Exposure and Recovery Tests

The half reactions were studied to determine the effect of incubation in high volume percent organic solvent on enzyme activity with the previously described assay conditions. The enzyme was placed into solutions containing 10-50% (1,4)-dioxane by volume and then assayed for remaining activity immediately. The reversibility of solvent

induced deactivation was tested by diluting solvent exposed enzyme 1:20 for KRED 101 and 1:40 for GDH 103 in 150mM phosphate buffer for one hour before being assayed.

Results and Discussion

The motivation to use an OATS system for the reduction of diaryl ketones stemmed from the need to use at least 5-10% organic solvent to solublize these hydrophobic substrates for biocatalytic reaction with homogeneous ketoreductases. It was thought that since the ketoreductases of interest appeared to survive 10% organic solvent, they may also be amenable to the higher concentrations of organic solvent necessary for OATS systems. The reaction shown in Figure 5-1 was previously screened against an in-house library of commercially available ketoreductases (KREDs) at Merck and the highest ee obtained for the R-alcohol was with KRED 101 used in conjunction with the cofactor regenerating enzyme GDH 103 [5]. This reaction is used as a tests case for OATS in this work.

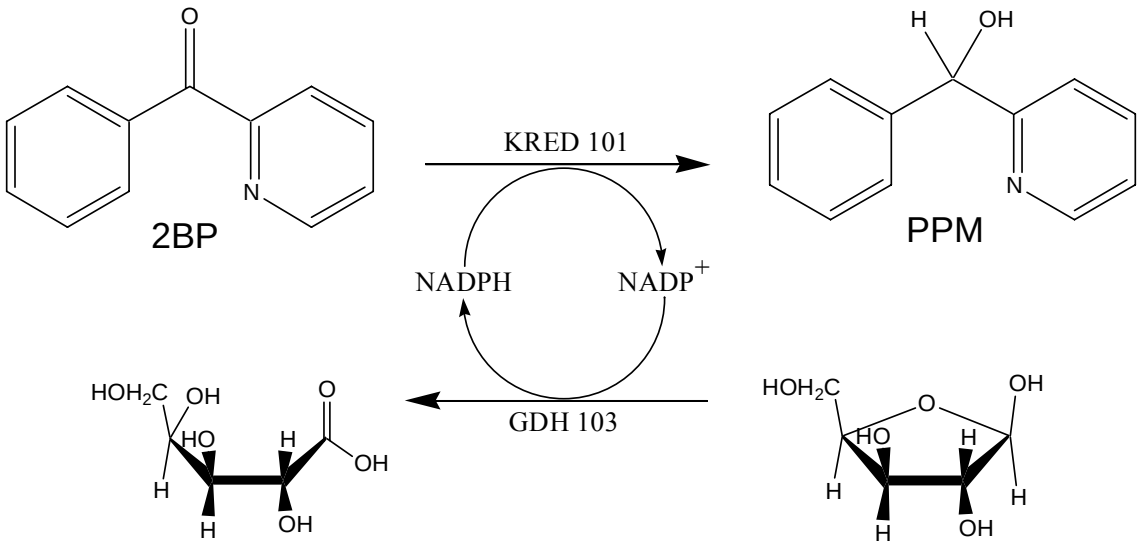

Figure 5-1: Reduction of 2-benzoylpyridie (2BP) to phenyl-pyridin-2-yl-methanol (PPM) using KRED 101 coupled with cofactor regenerating enzyme GDH 103.

We first repeated the reaction conditions specified in the screening assay by Merck [5] to ensure that we were able to obtain comparable yields. In addition to using THF as organic solvent for substrate solublization, we also screened reaction mixtures containing (1,4)-dioxane, acetonitrile, and no organic solvent. The literature reaction conditions formed a slurry and not a homogeneous mixture; therefore, we are able to report only yields and not rates of these reactions in Table 5-2. From this table, we found (1,4)-dioxane to have a slightly elevated yield. Due to this elevated yield and our previous experience with this solvent in OATS systems, (1,4)-dioxane was chosen for further study over the other solvents examined.

Table 5-1: Percent yield of product, PPM, after 4 hours reaction time.

Reaction Solvent	% Yield (4hr)
10% Acetonitrile	95
10% (1,4)-Dioxane	96
10% THF	92
No Added Solvent	44

In order to determine the maximum 2BP concentration resulting in homogeneous solutions and to gauge the effectiveness of better solubilizing the substrate by increasing the content of (1,4)-dioxane, we tested the solubility of 2BP in various solutions. The results are shown in Figure 5-2, and although the solubility increases it is still well below that of the operating concentration used in the Merck screening (25 g/L).

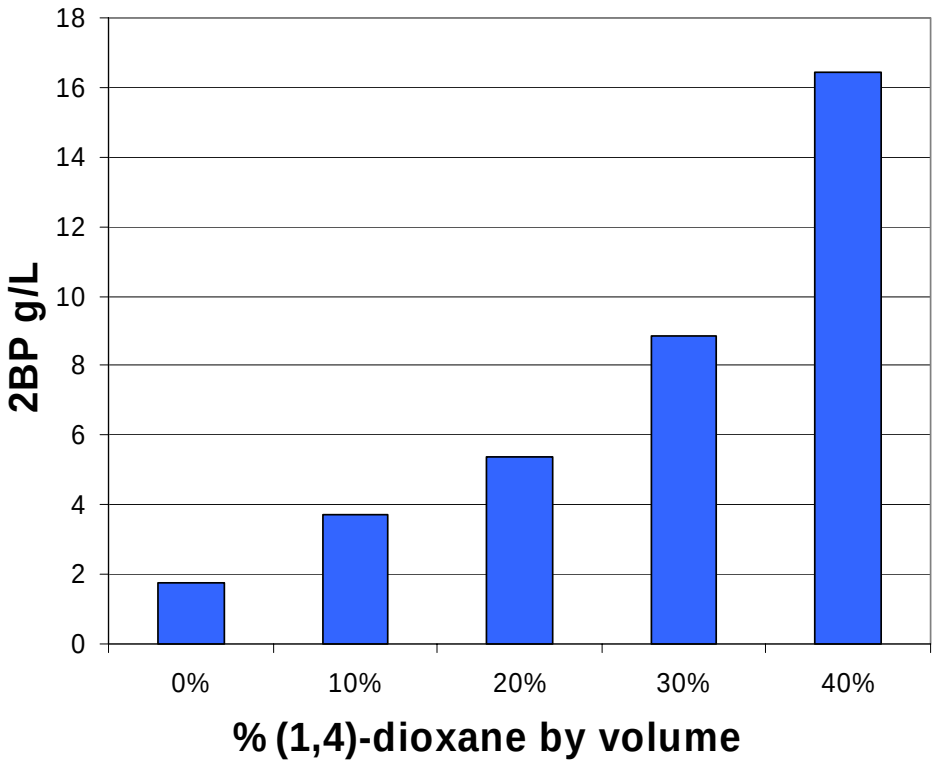

Figure 5-2: Solubility of 2BP in various solutions of (1-4)-dioxane.

Next, the minimum substrate concentration for which the rate of the reaction is maximized was found for 2BP, glucose, and NADP in order to facilitate reproducibility of later kinetic analysis in varying solvents. The reactions were evaluated in 10% (1,4)-dioxane solutions to allow adequate 2BP dissolution for analysis. Experimentally observed reaction rates as a function of substrate concentration are shown in Figures 5-3 thru 5-5. The Michaelis constant, K_m for each substrate was determined using Sigma Plot's nonlinear regression function (ligand binding) given by the Michaelis-Menten equation:

$$\mathrm{v} = \frac{V_{\max} + S}{K_m + S}$$

Where V_{max} is the maximum rate of reaction for a given total enzyme concentration, v is the observed rate of disappearance of the substrate, and S is the substrate concentration of interest. Curve fits are shown as lines.

In order to ensure rate is independent of substrate concentration, we must operate in the range where v = V_{max}. Generally using a substrate concentration of approximately 10 times the value of Km will be sufficient for ensuring the maximum rate of reaction is occurring. It is also important to ensure that stoichiometric amounts of substrate are used over the reaction times chosen. Given the K_m values presented in Figures 5-3 thru 5-4, and given that K_m ranges from 0.004 to 0.017 mM for the 3 KREDS (where a K_m for NADPH is reported on the enzyme database BRENDA [8]), further reactions were run using 20mM 2BP, 100mM glucose, 1mM NADP and 2.5 mM NADPH.

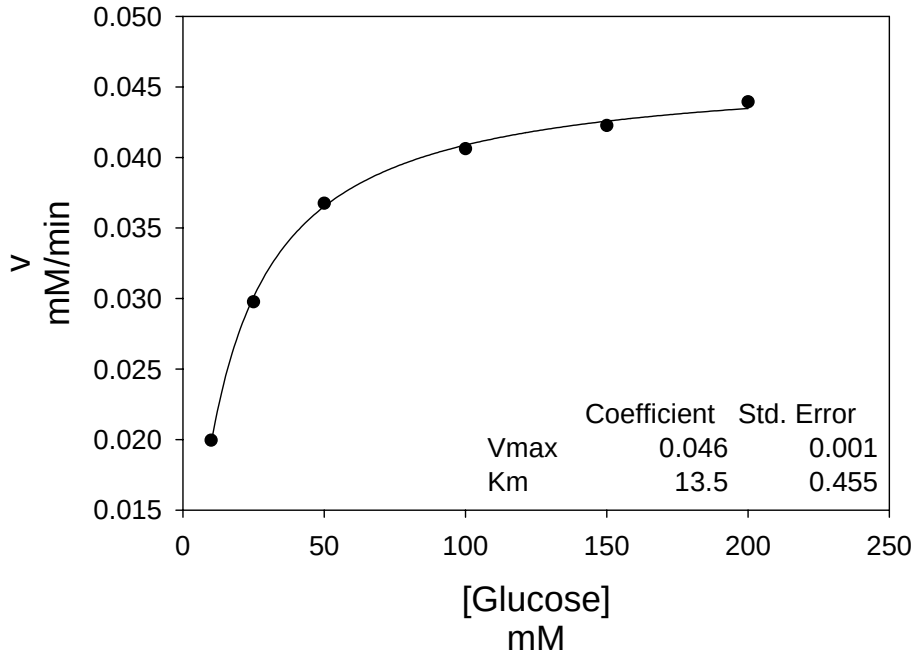

Figure 5-3: Mechalis-Menten plot for GDH 103 with varying glucose concentrations.

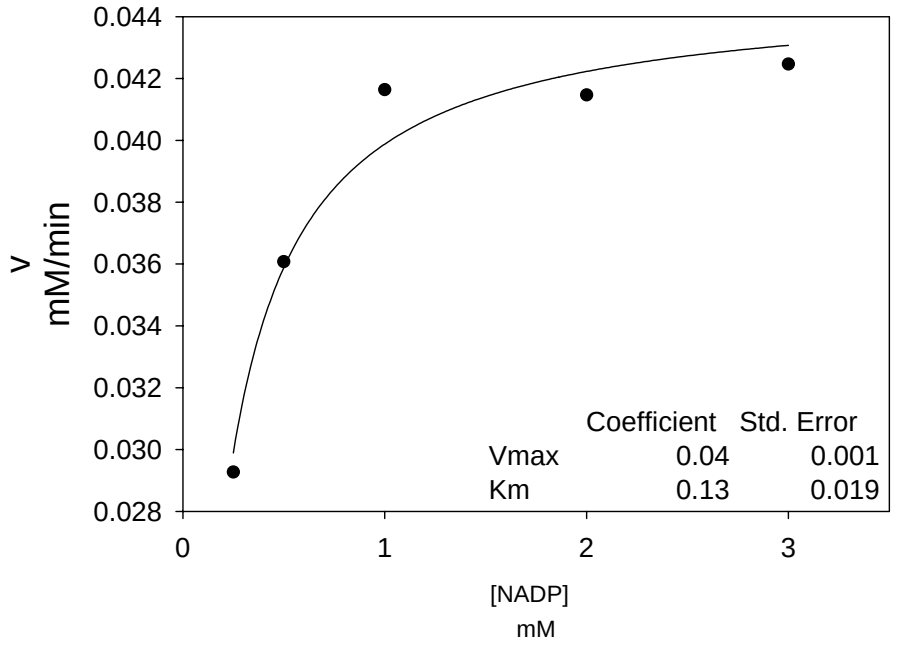

Figure 5-4: Mechalis-Menten plot for GDH 103 with varying NADP concentrations.

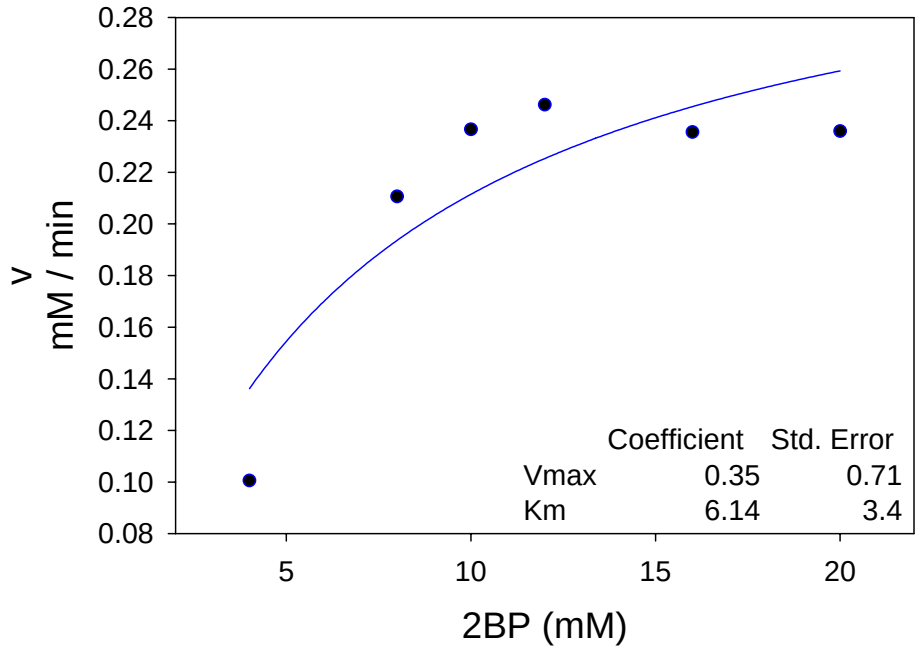

Figure 5-5: Mechalis-Menten plot for KRED 101 with varying 2BP concentrations.

Figures 5-6 and 5-7 show both enzymes exhibited reduced rates of reaction in solutions of increased (1,4)-dioxane concentration. Prolonged exposure to (1,4)- dioxane also proved detrimental to enzyme activity. Corresponding decreased rates were observed after incubation in solutions of (1,4)-dioxane with subsequent assay in 10% (1,4)-dioxane solution, and the enzyme activity was not recovered after one hour incubation in buffered water (Figures 5-9 and 5-10). In both cases, GDH 103 appeared a more solvent tolerant because its overall drop in reaction rate was lower than that of the KRED 101. The specific activity reported in for 50% (1,4)-dioxane solutions in the GDH exposure test may be artificially high due to buffer precipitation at this concentration causing a slight amount of turbidity in the solution.

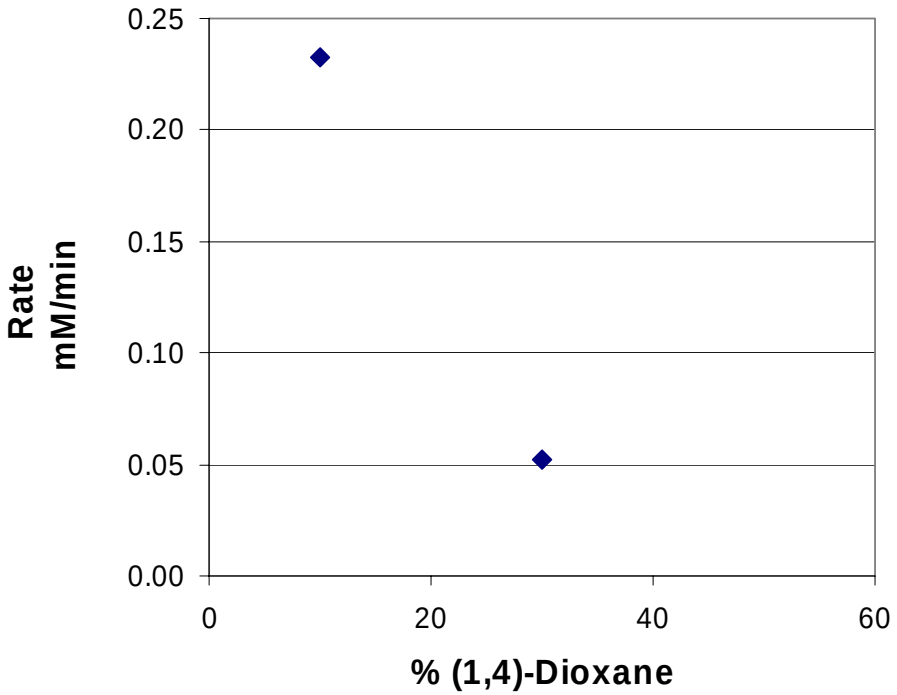

Figure 5-6: Rate of KRED 101 as a function of increasing (1,4)-dioxane concentration.

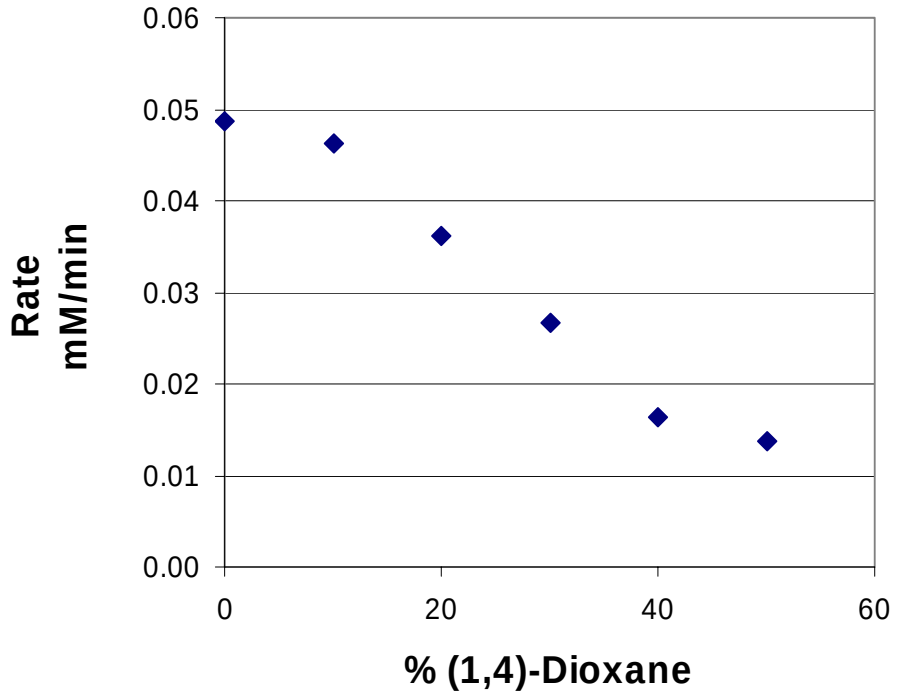

Figure 5-7: Rate of GDH 103 as a function of increasing (1,4)-dioxane concentration.

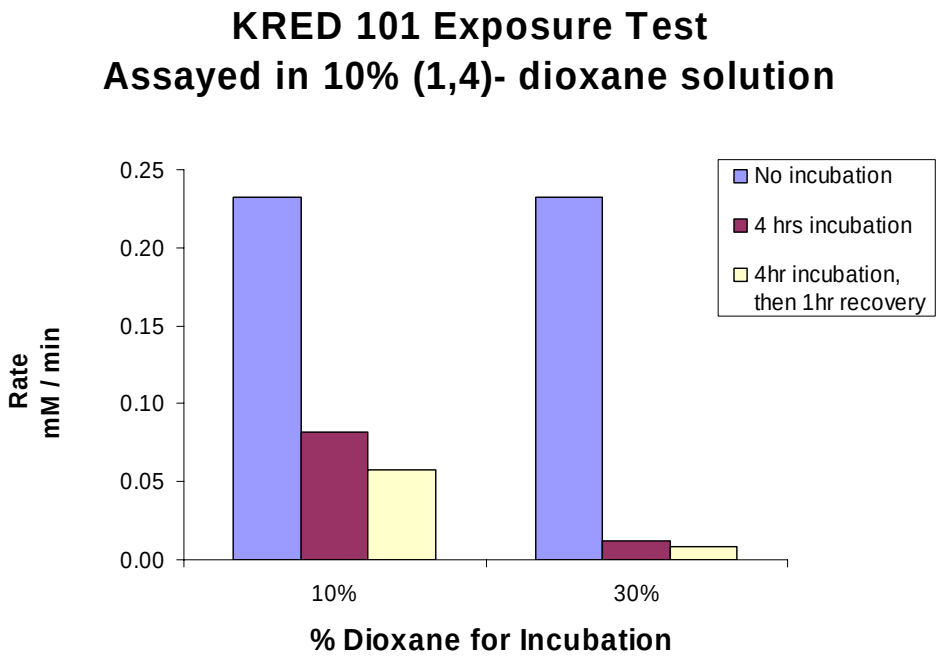

Figure 5-8: Exposure and recovery test for KRED 101.

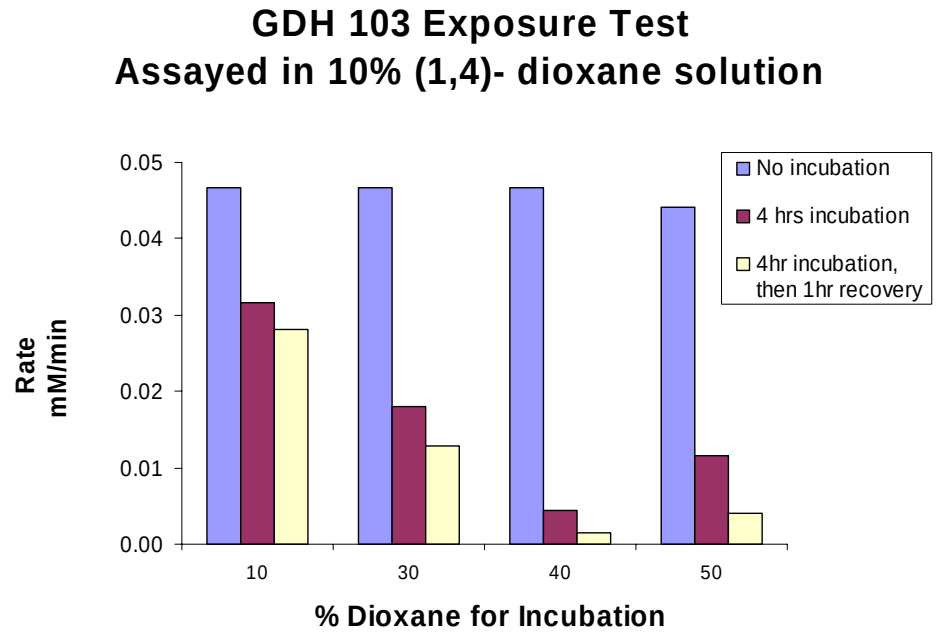

Figure 5-9: Exposure and recovery test for GDH 103.

Conclusions and Recommendations

We do not recommend that this reaction be pursued further in OATS systems. Even if reactions were run at low organic solvent concentration and more solvent were added for the separation only, the enzyme would at the very least need to survive prolonged exposure (~4 hours) to organic solvents or be able to be recovered after exposure to buffered water in order to be recycled. The inability of either KRED 101 or GDH 103 to tolerate high levels of (1,4)-dioxane makes this reaction a poor candidate for OATS.

One factor that is worth further investigating is degradation of the cofactor, NADPH in the presence of mixed aqueous-organic solvents. Cofactor degradation could be an additional factor for the reduced rates of reaction shown at high organic solvent content. An activity assay or standard destruction experiment using capillary electrophoresis of NADPH and other similar co-factors would lead to more facile selection of organic solvent for future reactions requiring co-factor regeneration in both OATS systems and organic solvent content containing reactions in general. The co-factor was fresh, and not incubated, during the exposure and recovery tests; therefore, it was not likely a contributing factor to the observed decrease shown in these experiments.

To continue to pursue OATS systems for pharmaceutically relevant synthesis coupled with facile product separation, we suggest investigating:

1. Enhancement of the stability of biocatalysts in organic solvents.
2. Use of biocatalysts with the proven ability to withstand exposure to high organic solvent concentration.
3. Expansion of the repertoire of OATS solvents.

4. Better prediction of the appropriate level and type of organic solvent for a given enzyme.

Recommendations for future work to meet one or more of these criteria are detailed in Chapter VI.

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CHAPTER VI

RECOMMENDATIONS

Introduction

The investigations in this thesis indicate that Organic Aqueous Tunable Solvent (OATS) systems support chiral enzymatic reactions and offer a holistic approach to processing with the potential for running commercial reactions in a more sustainable fashion. The largest barrier to general industrial application of biocatalytic OATS systems is reduced stability and activity of enzyme catalysts with pH variance and/or exposure to high organic solvent concentration; therefore, further work is recommended to mitigate these effects. Additionally, the use of CO_2 pressure, even at the moderate levels required in OATS systems is a challenging adoption for a laboratory not familiar with the design or function of pressure vessels. Design of an "off the shelf" unit to use with OATS and other GXL systems is suggested to ease permeation of process schemes requiring moderate pressure into to the industrial research culture. This would also pave the way for adaptation of OATS systems to couple multiple reactions in a single reactor vessel.

Biocatalyst Stability and Activity

Strategies for addressing pH maintenance in OATS as well as the associated phase behavior changes with the addition of a salt buffer were addressed in Chapter III. In Chapter IV we revealed OATS systems to be viable as a homogeneous enzyme recycle system that allows increased access to hydrophobic substrates when using robust CAL B

as biocatalyst. Nevertheless, as shown in Chapter V, there is still a need to address the fragility of many enzyme classes in the presence of organic solvents.

The most common method for addressing this problem is immobilization of the enzyme on a support; however, this approach does not make the best sense for OATS systems. If we were to immobilize the enzyme, we would suppress on the benefits of homogenous catalysis discussed in Chapter I, and the concept of coupling the reaction and separation via a tunable solvent system would not be as pertinent because immobilized catalysts may be centrifuged or passed over filters for separation and recycle. In place of immobilization for increased enzyme stability and activity, we recommend applying one or more of the following criteria, described in detail in the following sections, to allow OATS systems to become more generally useful:

1. Enhancement of the stability of biocatalysts in organic solvents.
2. Use of biocatalysts with proven activity at high organic solvent concentration or the ability to be recovered after incubation.
3. Expansion of the repertoire of OATS solvents.
4. Better prediction of the appropriate level and type of organic solvent.

Enhancement of the stability of biocatalysts in organic solvents

There are a number of described techniques for preparing organic-soluble enzyme systems to provide a microenvironment conducive to catalysis which also protects the enzyme from solvent-induced inactivation. These techniques include: chemical modification of proteins [1] such as PEGylation [2] or glycosidation [3]; lyophilization in the presence of additives; and addition of protectants through precipitation from lipid-

water dispersions [4], non-covalent surfactant coating [5, 6], surfactant ion paring via liquid-liquid extraction [7], or direct solubilization (DS) in organic solution containing surfactant and trace amounts of water [8]. Of the chemical additions, DS appears to offer the most versatility (for the tested enzymes DS yielded consistent conversions across a spectrum of solvent polarities) and it requires far less preparation time and effort than lyophilization or chemical modification. It is not recommended that additives be used in OATS because they can present added separation complexity, especially for the case where the additives are organic solvent soluble. However, study of systems with additives can be useful in understanding why a particular enzyme is so inactive in organic solvents.

Alternatively, protein engineering is a powerful approach to enhancing protein stability which provides permanently altered "improved" biocatalysts through rational, combinatorial, and/or data-driven designs of these proteins [9, 10]. (Stable variants of proteins may also be obtained through mining the genomes of extremophilic organisms, or cloning and characterization of enzymes from meta-genomic libraries.) Data suggest that there may be a tendency that proteins which are stable to one type of denaturing condition are also stable towards other types [11]; therefore, we recommend that organic solvent tolerance be tested on an enzyme which has already been intentionally selected for temperature or pH stability. Protein engineering methods are very time intensive, often requiring multiple screening and selection rounds, so continued close collaboration with the Bommarius group in this arena is recommended. A possible enzyme target to screen is a GDH variant selected for temperature stability. This enzyme is highly interesting for regeneration of expensive cofactors (i.e. NADPH) which are coupled with

a number of industrially relevant enzymatic systems such as the ketoreductase discussed in Chapter V. Its use in OATS systems would allow facile cofactor recycle, thus further reducing the processing costs.

Use of biocatalysts with the proven activity in high organic solvent concentration or ability to be recovered after incubation

Although the KRED evaluated in Chapter V was able to tolerate low levels of organic solvent (10% by volume) this did not translate into tolerance to exposure of high concentrations organic solvents used in OATS. Some alternative biocatalysts which have shown stability towards high levels of polar organic solvents are given in Table 6-1, along with the substrate(s) and product(s) used for assay.

The reactions shown in Figure 6-1 are suggested for future work using fructose 1,6-bisphosphotate aldolase (FruA) in OATS systems. Chiral asymmetric aldol reactions are very interesting due to the importance of carbon-carbon bond formation in synthetic organic chemistry [12]. Enantioselectivity is particularly outstanding for aldol reactions catalyzed by enzymes, which also work on non-chiral starting materials, as compared to simple base catalysis, which is enantioselective only with stoichiometric amounts of chiral starting materials. The donor dihydroxyacetone phosphate (DHAP) is insoluble in many organic solvents while the acceptor is generally insoluble in water, making this an ideal case for use with the aqueous cosolvent mixtures used in OATS. This reaction has previously been shown to work with FruA from rabbit muscle [13]; however, substrate specificity would need to be tested in the organic solvent tolerant variant mentioned in Table 6-1 which is derived from *Eschericha coli* and *Edwardsiella ictluri*. If this variant

is unattainable, the stable monomeric FruA from *Staphylococcus carnosus* may be examined.

Table 6-1: Proposed enzymes for use in OATS with known organic solvent tolerance.

Enzyme	Solvent Tolerance	Substrate(s)	Product(s)
Alanine Racemase [14]	Maintains more than 90% of its activity in 50% acetonitrile.	L-2-aminobutyric acid	D-2-aminobutyric acid
		L-Alanine	D-Alanine
		L-Serine	D-Serine
Carboxypeptidase Y [15]	Stable at pH 7.0 for at lease 15 minutes in 30% (1,4)dioxane.	Poly-alpha-L-glutamic acid, H_2O	Glutamioc acid
Variant of fructose bisphosphate aldolase [12]	Maintains more than 60% of its activity after 24 hour incubation in 20% acetonitrile, and regained 100% of activity upon recovery in aqueous media.	Fructose 1,6-bisphosphate, hydrazine dichloride hydrate	D-glyceraldehyde -3-phosphate, dihydroxyacetone phosphate
6-phosphogluconate dehydrogenase [16]	Shows 15-20% enhanced activity in solutions of acetone and (1,4)-dioxane	6-phospho-D-gluconate, NADP	D-ribulose 5-phosphate, CO_2, NADPH
Lipase from *Streptomyces rimosus* [17]	Stable in mixtures containing 50% (v/v) ethanol, (1,4)-dioxane, acetonitrile or acetone. (18 hours incubation with residual activity assayed in aqueous solution.)	p-nitrophenyl caprylate	4-nitrophenol

The option exists to run the OATS system as described in Chapter IV, or in a modified way wherein the reaction may be run at low concentrations of organic cosolvent with additional solvent added only for CO_2-induced separation. After separation the solution is again at low organic solvent concentration (2-4%) which may allow the enzyme a relaxation period to regain activity. This method may be favorable because the FruA variant showed full recovery of activity when placed in aqueous media [12].

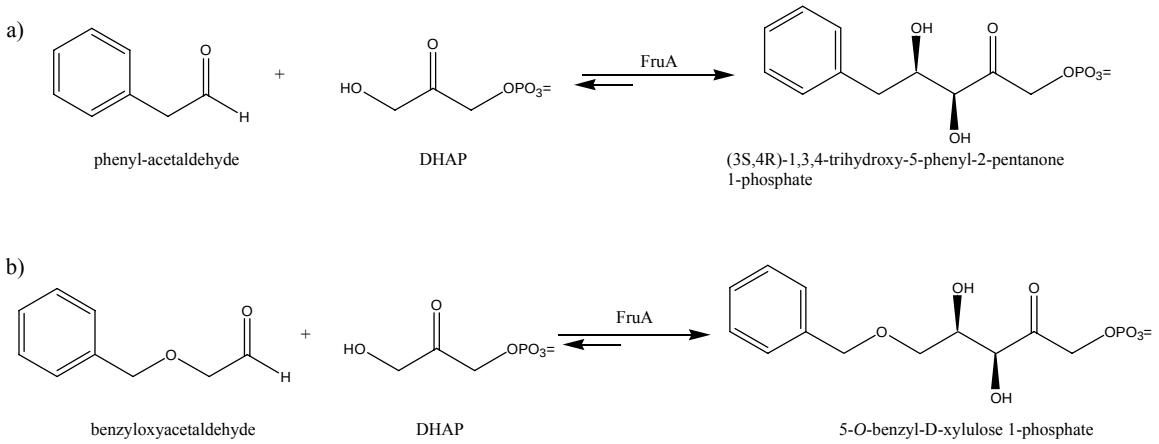

Figure 6-1: Reversible aldol reaction between donor DHAP and acceptor aldehydes a) phenyl-acetaldehye and b)benzyloxyacetaldehyde.

Expansion of the repertoire of OATS solvents

In this thesis, OATS systems containing acetonitrile, (1,4)-dioxane, and tetrahydrofuran were investigated as recycle systems for homogeneous catalysts. Additionally, acetone and ethyl acetate, when mixed with water and the solute CO_2, have been shown to exhibit the type 1 ternary phase behavior characteristic to OATS systems. Properties of these solvents are listed in Table 6-2. The pressures required are a function of the binary liquid mixture composition. This phase behavior has been reviewed in

detail for water – alcohol – CO_2 mixtures [18, 19]. Further utility of OATS systems may be obtained by investigating new solvents for similar phase behavior.

We are interested in the cases for which the second derivative of the Gibbs free energy of mixing in a binary solvent mixture containing water is negative, and two phases exhist. The term lower critical solution temperature (LCST) indicates that the complete mixing occurs below the listed temperature but not immediately above it. When CO_2 pressure is added and GX-tetrahydrofuran forms, this LCST is effectively lowered allowing a two phase region to exists at room temperature. An upper critical solution temperature (UCST) indicates that complete mixing occurs above the listed temperature, but not immediately below it. For the cases of acetonitrile and dioxane water mixtures, the addition of CO_2 effectively raises the UCST also allowing a two phase region to exist at room temperature. Thus, the existence of a critical solution temperature (CST) and the ability to form a GXL in the given organic solvent are useful indicators that phase separation with type 1 ternary behavior may occur.

Table 6-2: Solvent properties of current organic cosolvents used in OATS.

Solvent [references]	acetonitrile [20-23]	acetone [21-24]	ethyl acetate [21-23, 25]	(1,4)-dioxane [20-23]	tetrahydrofuran [21-24]
Boiling Point (ºC)	81	56.1	77.2	74.1	66
Melting Point (ºC)	-43.8	-94.7	-83.6	-30.4	-108.4
Flask Point (ºC)	12.8	-18	-4	12	-14.5
Viscosity (cP, 25 ºC)	0.39	0.295	0.894	1.05	0.53
Log P	-0.34	-0.24	0.71[a]	-0.27	0.46

In order to mitigate the detrimental effects on the enzyme a number of solvent parameters have been examined. P is the partition coefficient of a solvent in a biphasic water/octanol system, is the most common parameter used. The higher the log P the greater solvent denaturation power for solvents of the same functionality, e.g. alcohols and polyols. Log P is not, however, a universal criterion for solvent selection because the correlation breaks down upon addition of different solvent classes. Khmelnitsky et al. [22] assessed qualitatively the relative ability of different organic solvents to exert denaturing effect on proteins with a denaturation capacity (DC) scale. The DC scale is a parameterized model which takes into account Log P, $E_T(30)$ which is a measure of solvent polarity proposed by Reichardt [26], and n, the ratio of molar surface area of water to that of the corresponding organic solvent. The higher the DC value, the more of the solvent is a denaturant.

Table 6-3 lists some common organic solvents in order of decreasing DC with their various solvent properties. From this table, we recommend 2-methyl-2-propanol as a good candidate for OATS systems due to is low DC value and the existence of a CST similar to that of current OATS solvents.

Table 6-3: Characteristics of organic solvents. CST is given for mixtures of the organic solvent with water.

Solvent [references]	Log P [22]	$E_T(30)$ [22]	n [22]	DC [22]	CST [27, 28]
Formamide	-1.65	237	0.444	0.0	M.
Ethylene glycol	-1.43	236	0.264	18.7	$<20^{U}$
Glycerol	-2.50	238	0.194	20.2	$<-23^{U}$
Methanol	-0.74	232	0.414	30.5	M
N-Methylformamide	-1.13	226	0.314	32.4	N.A.
1,2-Propanediol	-1.35	226	0.213	38.8	$<20^{U}$
1,3-Butanediol	-1.02	221	0.178	49.1	$<20^{U}$
Ethanol	-0.32	213	0.301	54.4	M
Dimethyl sulfoxide	-1.35	188	0.199	60.3	N.A.
N,N-Dimethylformamide	-1.01	183	0.244	63.3	M
Acetonitrile	-0.34	192	0.344	64.3	-0.9^{U}
1-Propanol	0.34	212	0.231	69.2	$-23^{U,e}$
Sulfolane	-0.77	184	0.199	69.3	N.A.
2-Propanol	0.14	203	0.237	70.2	$<-23^{U}$
1-Butanol	0.89	210	0.194	77.2	127^{U}
Acetone	-0.24	177	0.254	78.2	$<-11^{U}$
2-Methyl-1-propanol	0.83	205	0.194	79.2	129^{U}
2-Butanol	0.61	197	0.194	80.4	-2.6^{L} 112^{U}
Hexamethylphosphoramide	0.28	171	0.099	90.1	N.A.
1,4-Dioxane	-0.27	151	0.240	92.1	$<-15^{U}$
2-Methyl-2-propanol	0.37	184	0.190	92.2	$<0^{U}$
Tetrahydrofuran	0.46	156	0.241	100	71^{L} 138^{U}

N.A.) data not available M) miscible, no CST observed e) extrapolated
L) lower critical solution temperature U) upper critical solution temperature

Organic carbonates are also recommended for investigation as alternative solvents for OATS systems. A glance at the properties of some organic carbonates in Table 6-4 as compared to the current OATS solvents shown in Table 6-2, illustrates why they are of interest. These compounds are often colorless liquids and most are biodegradable with low toxicities [29], a pleasant odor [30], and often have eco-friendly production methods [31, 32]. Propylene carbonate shows a favorable solubility of 0.558 mole fraction CO_2 at 54.8 atm and 26.7 ºC [33]; however, phase behavior should be assessed for ternary mixtures of this and other organic carbonates with water and CO_2.

Table 6-4: Solvent properties of some organic carbonates.

Solvent [references]	diethyl carbonate [34, 35]	dimethyl carbonate [32]	ethylene carbonate [29]	propylene carbonate [29]
Boiling Point (ºC)	126	90.3	248	242
Melting Point (ºC)	-43	4.6	36.4	-49
Flask Point (ºC)	31.1[a]	21.7	160	135
Viscosity (cP, 25 ºC)	0.749	0.625	2.56[b]	2.5
Log P[a]	1.21	0.147	-0.897	-0.405

a) Calculated [36] b) Supercooled liquid

Organic carbonates have high solvency and are commonly used as cleaning solvents or carrier solvents for cosmetics and medications [29, 30]. However, their use as reaction solvents can be hindered by their reactivity. The carbonate group is commonly subject to nucleophillic attack. Non-nucleophillic substrates may be pursued in neutral solutions, or this drawback can be an advantage if one envisions the organic carbonate as

both solvent and reactant. For instance, paraoxonase (PON1; EC 3.1.8.1; formerly EC 3.1.1.2), hydrolyzes cyclic carbonate esters [37] such as propylene carbonate. Such hydrolysis is useful in the activation of the carbonate ester prodrug prulifloxacin [38]. Before using an organic carbonate with an enzymatic catalyst in OATS, it is suggested that the DC value be calculated and compared against the list of solvents given by Khmelnitsky et al. [22] for an approximation of the denaturation power of that organic carbonate.

Better prediction of the appropriate level and type of organic solvent

The model for the relative denaturation capacity (DC) of organic solvents presented by Khmelnitsky et al. [22] was derived with the assumption that the organic solvent inactivation was fully reversible (Figure 6-2). It this work, we show KRED 101 and GDH 103 activity is not recoverable upon exposure to aqueous solution. This discrepancy may have occurred because of the low concentration of enzyme solutions studied by Khmelnitsky et al. [22] which was approximately 0.006 - 0.025 μg/mL whereas we investigated enzymes at 1 – 122 μg/mL in our experiments. If this is the case, then a new schematic for organic solvent denaturation should be postulated. For example, one might speculate that the binding of the organic cosolvent by partially dehydrated protein may not be reversible. New models could be incorporated to look at interactions between an organic solvent and specific functional groups in an enzyme. Perhaps dynamic modeling could be used to look at preferential solvation on a molecular level to determine if irreversible binding is caused by the organic solvent, creating a more stable peptide conformation than water.

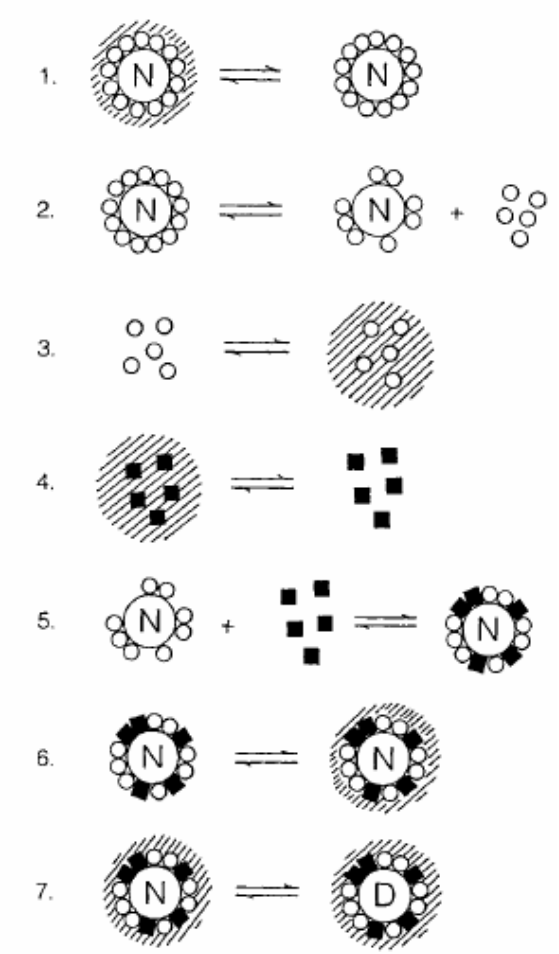

Figure 6-2: Molecular steps involved in the process of reversible protein denaturation by organic solvents as described by [22]. ○ water ■ organic cosolvent molecule, ▒ surrounding solvent.

It is also important to evaluate the difference between enzyme stability and enzyme activity. The DC scale may offer insight on an organic solvent's level of denaturation, however a stable enzyme does not guarantee good activity. Salt/buffer effects, pH, temperature, and substrate selection are all critical components to ensuring good enzymatic activity.

“Off the Shelf” Reaction Vessel

Four separate high pressure reaction vessels were used in this work due to various constraints of each model. For example, the pressure vessel used to take UV measurements for pH determination could not be used for recycle experiments because of the difficulty in viewing a clear phase boundary through the small windows. Design of a single reaction vessel that could be marketed to industrial research labs would be useful in proliferating processing technologies that require moderate to high pressure such as OATS and other schemes involving the use of GXLs. Such a reaction vessel would also facilitate future work in our own laboratory. Suggestions for the high pressure vessel include:

1. Tall rectangle or cylinder shape to give room for solvent expansion.
2. *In situ* pH titration
    a. Selection of probe to accommodate use with organic solvents
    b. Preferably with the capabilities to function under pressure
3. A large viewing area on the side and a window at either the top or bottom.
    a. Adaptors for fiber optic leads to allow *in situ* time resolved UV-Vis, and/or florescence measurements.
4. Capabilities to sample at adjustable depths.
5. Jacketing for facile temperature control.
6. An impeller for easy control of stirring rates during reaction with vigorous mixing allowing faster CO_2 dissolution for separation.

Multiple Reaction Steps in a Single Vessel

One proposal to take advantage of the versatile reaction vessel described above is the combination of an OATS system with our group's fluorous-silica project for multi-step reactions in a single vessel. Figure 6-3 illustrates how such a combination could allow an enzyme catalyzed reaction to be succeeded by a metal catalyzed reaction in a single reaction vessel. The product from the biocatalytic reaction is only able to react after the addition of CO_2 causes the fluorous tails on the silica to unfold and release the metal catalyst into solution. The pressurized system will need to be well mixed as that the fluorous silica will be present in both phases.

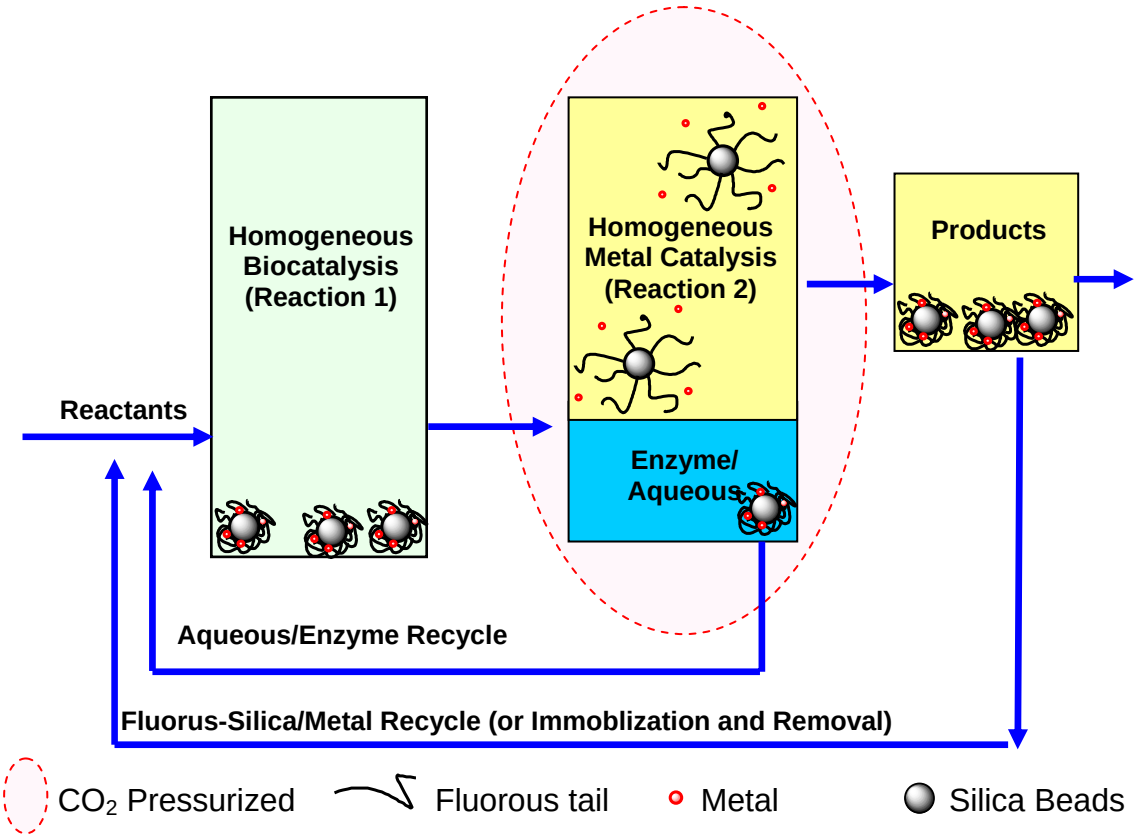

Figure 6-3: Schematic of biocatalytic OATS coupled with CO_2-induced phase separation which simultaneously allows release of metal from fluorous-silica.

Figure 6-4 provides two examples of staged reactions that may be used in this process scheme. Reaction 1a and 1b both make use of an enzymatic hydrolysis to illustrate the ability to selectively work on one side of a molecule and form a chiral center. The recycle of the enzyme catalyst is the same as in a regular OATS process. Reaction 2a is meant as general example wherein a metal catalyst is easily recycled upon CO_2 depressurization and collection of the fluorous silica. This reaction was selected because the mild reaction conditions will not denature the enzyme; therefore, reuse of both catalysts is possible.

Reaction 2b is interesting from a green synthesis standpoint because there are no byproducts, and the tin (Sn) can be easily removed upon depressurization and collection of the fluorous silica, thus eliminating metal contamination from the product. Further, the classical method for production of tetrazoles generates HN_3, an explosion hazard especially on a large scale. Tetrazoles are an important class of heterocycles often featured in medical chemistry studies [39, 40]. This reaction uses a commercially available fluorinated wilkinson catalyst, F-Wilkinson, Rh(PAr3)3 where Ar = C6H4-(CH2)2-(CF2)6F. The two caveats to this reaction are the that, the reaction is run at 100 ºC and the fluorous tin tetrazole is treated with 1M HCl then extracted with organic solvent (from a fluorous solvent) to free it of fluorous impurities [41]. It is not likely that the enzyme would withstand this treatment. However, the reaction could be attempted at reduced temperature, and due to the presence of CO_2 and water, the organic portion would have dissolved carbonic acid which could potentially work as the acid treatment. Further, the aqueous phase could be removed under pressure before the heating and/or acid treatment takes place.

Future work in this OATS/fluorous-silica system may make use of staged reactions with multiple metal catalysts (one catalyst with a water soluble ligand, and the other with the ligand tethered to the fluorous silica) to increase the variety of reactions which are able to be run in this process. For example, Suzuki coupling reactions using palladium with fluorous ligands would work well as a second reaction. This coupling of OATS systems with fluorous-silica for CO_2^-mediated metal release could reduce solvent needs would be an interesting extension to the work presented in this thesis. It could decrease time spent on work-up of intermediates, provide opportunities for staged reactions of complex molecules, facilitate catalyst recycle, and/or remove metal contaminants used for reaction.

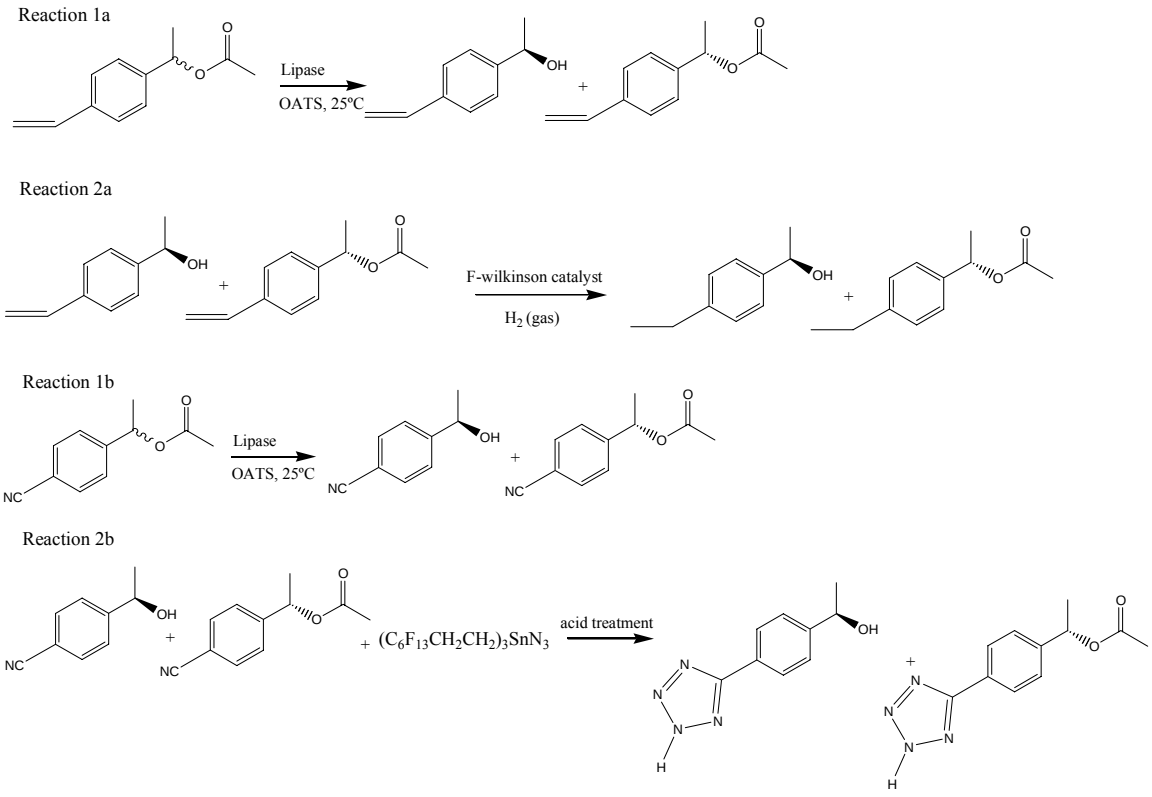

Figure 6-4: Staged reaction for use with the OATS/fluorous silica system a) for simultaneous recycle of multiple homogeneous catalysts b) for immobilization of a metal contaminant.

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APPENDIX A

NEAR-CRITICAL WATER PROCESSING OF FERULIC ACID AND BREWER'S SPENT GRAIN

Introduction

Brewer's Spent Grain (BSG) is the residual biomass from the elaboration of beer and spirits such as whiskey, and is commonly composed of a mixture of wheat, barley and/or hops. Currently, BSG is primarily used for fertilizer or as feed stock rich in protein and fiber [1, 2]. Its relatively low cost and wide availability (57 million pounds per year produced for a typical large brewery [3]) makes BSG an attractive biomass source for extraction of phenolic compounds such as ferulic acid. Near-Critical Water (NCW) extractions of Brewer's Spent Grain (BSG) are investigated for use as a sustainable carbon source for high value added chemicals and biodegradable polymer building blocks.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is the major low molecular weight phenolic found in various cereal grains including wheat [4] and barley [5] in both free and bound forms[6]. Bound ferulic acid is attached to the arabinose backbone of arabinozylans through ester linkages concentrated on the outer shells of plant cell walls, and to lignin mainly by ether bonds (Figure A-1) [7, 8]. Ferulic acid is a high value added chemical used as a pharmaceutical agent due to its antioxidant and anti-inflammatory properties, a food preservative, and a food precursor for the production of vanillin (4-hydroxy-3-methoxybenzaldehyde). Natural vanillin is valued at $30/kg while synthetic vanillin is $13-$20/kg [9]. The term "natural" refers to guidelines given by the

USDA specifying that "products contain no artificial ingredients, coloring ingredients, or chemical preservatives; and the product and its ingredients are not more than minimally processed [10]." There is an increasing demand for "all natural" products in the global market [11] and the cost difference in natural versus synthetic vanillin is illustrative of profit motivation for chemical producers to cater to this demand. NCW extraction is considered a natural processing technique offering some benefits over traditional process procedures

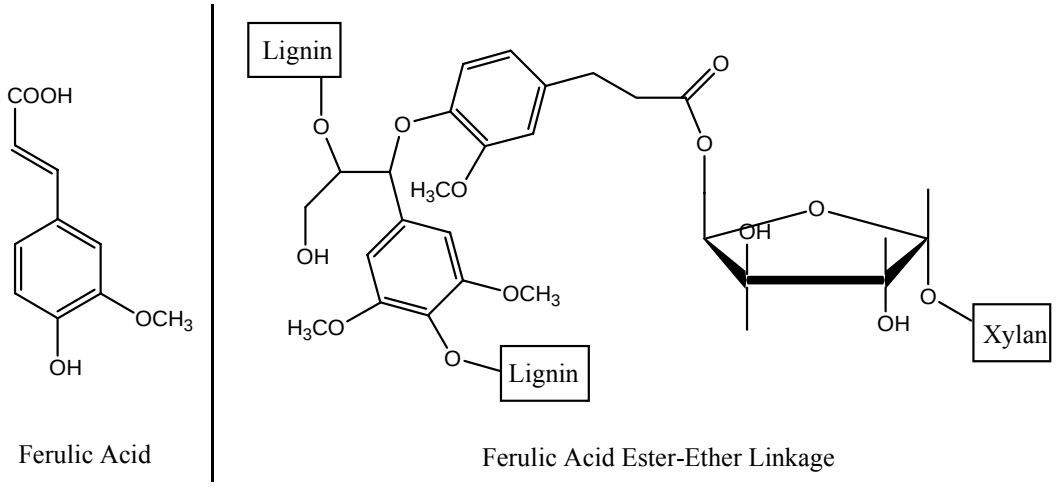

Figure A-1: Ferulic acid and bound ferulic acid with cell wall constituents.

Free ferulic acid may easily be removed from plant stuff via non-natural processing techniques such as solid liquid extraction with methanol or acetone, while bound ferulic acid must first be hydrolyzed via either acid or base catalysis before liquid extraction [6]. The most commonly explored "natural" processing technique for the release of added-value chemicals such as ferulic acid is biological treatment of BSG, via microbial or fungal esterases, and is reviewed elsewhere [12, 13]. The ability to remove all portions of ferulic acid is dependent on the microorganism or enzyme being used.

Many microbial and enzymatic processes face product inhibition; therefore, the product must be continually removed to allow further reaction. This removal is not trivial because conditions must be mild enough to keep from denaturing the enzyme and the process must be economical to afford industrial use. Isolation and purification of product from microbial broth often requires many of costly separation steps to remove the cell components and purify the product. This can consist of centrifugation to remove cell particulates followed by further enzymatic processing to convert to the desired product, such as is the case for ferulic acid processing to obtain vanillin [14]. Additionally, because most biological entities are active at near physiological conditions (moderate pH, temperature, and pressure), constraints are placed on the robustness of the process limiting the ability to optimize the system. As such, microbial and fungal systems also suffer from a number of drawbacks to scale-up and commercialization.

An alternative to natural processing without the associated issues of biological systems is NCW extraction. NCW extraction has the potential to remove both free and bound ferulic acid because at temperatures of 200-300 ºC, NCW has a self dissociation constant, K_w, three orders of magnitude larger than that of ambient water [15]. This promotes acid/base catalyzed reactions, such as the necessary hydrolysis of ester linkages, due to the increased source of hydronium and hydroxide ions [16]. Contrary to the case of acid or base addition, the reacting system in NCW requires no neutralization, thus avoiding the need for disposal of salt waste. Other benefits to NCW extraction is the elimination of volatile organic solvents, and the tunable nature of the system by manipulation of temperature and pressure as is illustrated by the change in the dielectric constant of water at various temperatures (Figure A-2).

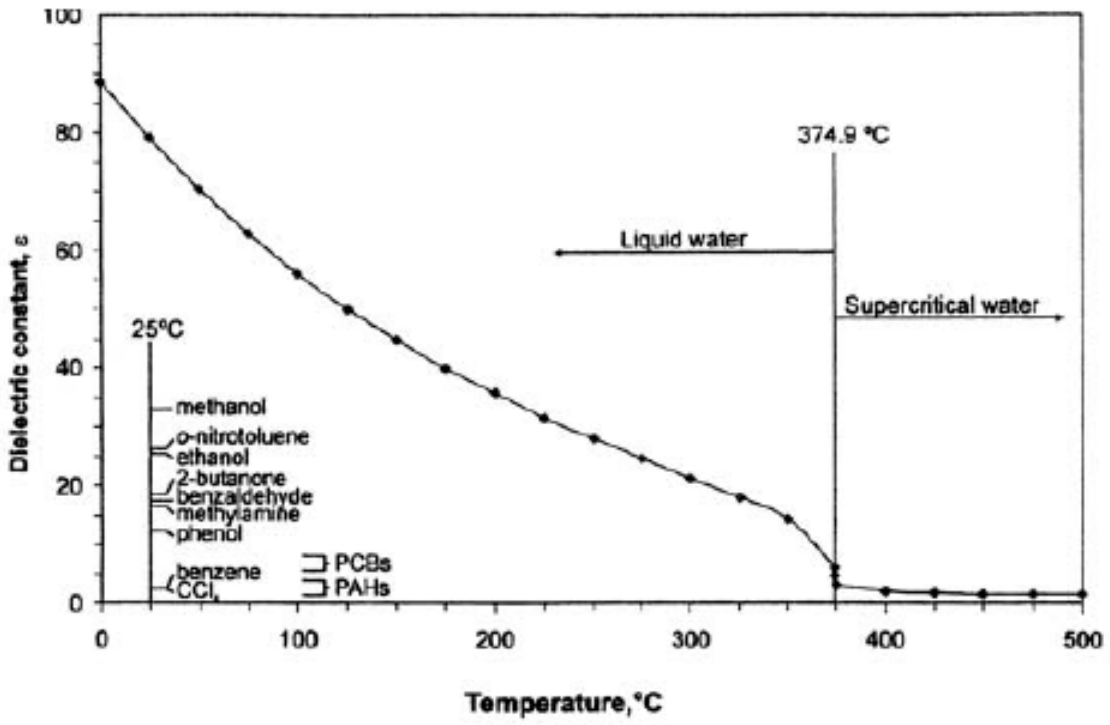

Figure A-2: Dielectric Constant of water with changing temperature [17].

One drawback of NCW processing is the possible degradation of phenolic compounds at high temperature [18, 19], such as the decarboxilation of ferulic acid to 4-vinyl guiacol (4-hydroxy-3-methoxystyrene), or further transformation to vanillin and/or vanillic acid (4-hydroxy-3-methoxy benzoic acid) shown in Figure A-3. Degradation presents a challenge to optimizing conditions for ferulic acid production, but also presents an opportunity for tuning the processing conditions to obtain the desired product because the degradation products can also be of value. For this reason, we study both solutions of pure ferulic acid in NCW as well as BSG extractions with NCW.

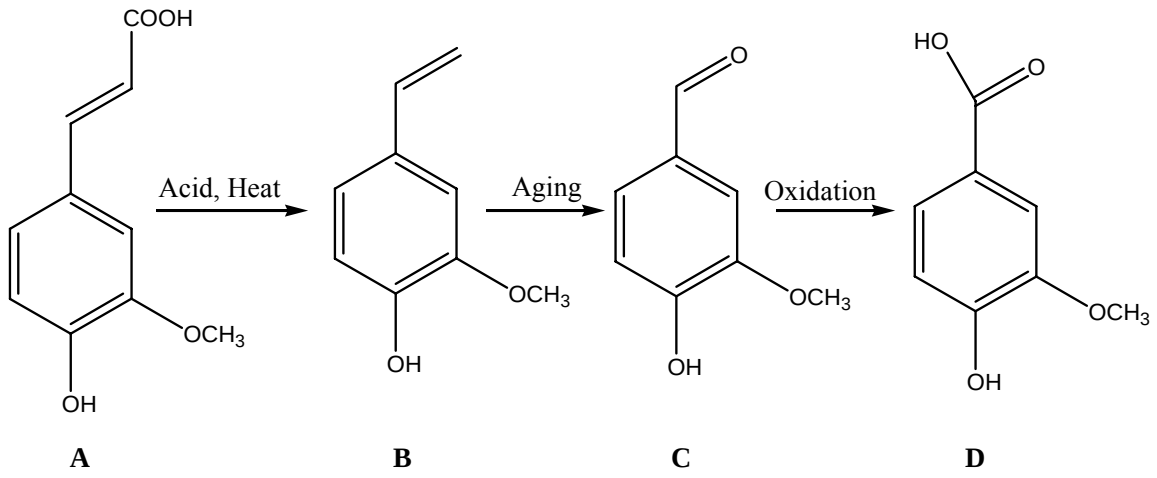

Figure A-3: Proposed pathway for the conversion of A, ferulic acid (4-hydroxy-3-methoxycinnamic acid); B, 4-vinylguaiacol (4-hydroxy-3-methoxystyrene); C, vanillin (4-hydroxy-3-methoxybenzaldehyde); D, vanillic acid (4-hydroxy-3-methoxy benzoic acid). (Adapted from Koseki 1996 [7]).

Experimental Methods

Materials

The Brewers Spent Grain (BSG) was graciously donated by Five Seasons Brewing Company in Sandy Springs, GA and Sweetwater Brewing Company in Atlanta, GA. Water and Methanol were purchased from Sigma-Aldrich (99.9% Chromasolv HPLC Grade). Trifluroacetic acid (98%), 4-vinylguaiacol (98%), vanillin (99%), vanillic acid (97%), were obtained from Aldrich, while ferulic acid (99%) was obtained from Acros Oganics. All were used as received.

Solid ferulic acid, a stock solution of ferulic acid dissolved in methanol, and a stock solution of ferulic acid dissolved in water at pH 11.9 were used for ferulic acid degradation experiments. The first batch of BSG (Five Seasons #1) was used as given and its analysis is described elsewere.[14] The BSG from the second batch (Five Seasons #2) was not further processed before use. The 3rd batch of BSG (Sweetwater #3) was

milled to a fine particle size (via a wiley mill, with a number 3 standard sieve) in order to use a more homogeneous mixture of grain particles for batch reactions. This was tried because samples of BSG are heterogeneous mixtures of large, unknown grain composition and small quantities are necessary for the reaction vessels (0.15-0.3g), making it hard to get exact same mixture of particle grains for each reactor without first milling the samples.

Degassing Procedures

To prevent oxidation of the phenolic compounds, water is degassed for all ferulic acid and BSG extraction experiments by either bubbling nitrogen through the water for one hour or via the freeze-purge procedure for degassing solvents on a schlenk line as described below:

Schlenk Line procedure for degassing solvents:

1) Fill condenser tubes on schlenk line with liquid N_2 and submerge condensers.
2) Place solvent into air free glassware for degassing.
3) Pour liquid N_2 into a dewar and place the flask of solvent into the liquid N_2.
4) Close all lines on the schlenk line then turn on the vacuum pump and N_2.
5) Freeze all the liquid in the flask
6) Attach fill/vacuum line to the flask
7) Open vacuum line valve then open flask valve to vacuum all the air out of the flask. Leave this open for ~10-15 seconds.
8) Close the vacuum line valve; open the N_2 line valve thus refilling the flask with N_2. Leave open for ~5 seconds.

9) Cycle between vacuum and N_2 (steps 7-8) three times, refilling with N_2 in the last step.
10) Close the flask valve and close the N_2 line valve.
11) Thaw completely.
12) Repeat steps 5-11 three times.
13) Open the purge valve before turning off the pump to keep the pump oils from entering the schlenk line. Turn off the pump and N_2.

NCW Procedures

A sample of ferulic acid or BSG is weighed and deposited into 3mL titanium reactor tubes as pictured in Figure A-4 and as used previously by our group [14, 20-22]. The titanium reactor was filled with water up to a total volume of 2 ml, then sealed with a NPT plug with threads wrapped in teflon tape. The volume of these reactors should never be filled more than 2/3 full due to the expansion of water with increased temperature. The titanium tube is placed in an aluminum heating block at the desired temperature. Temperature is controlled with 4 cartridge heaters (Omega Technologies Co.) and a Model CN9000A temperature controller (Omega Technologies Co.). Calibrations for the thermocouples within the heating block are made by testing against an external thermocouple placed within one of the titanium tubes. The titanium reactor tubes take approximately 5 minutes to reach the desired temperature as previously calculated by our group [21] and shown in Figure A-5. As such, this apparatus is limited in its ability to run extractions at times less than 5 minutes. After the desired residence

time, the tube is removed from the heating block and quenched in a room temperature water bath.

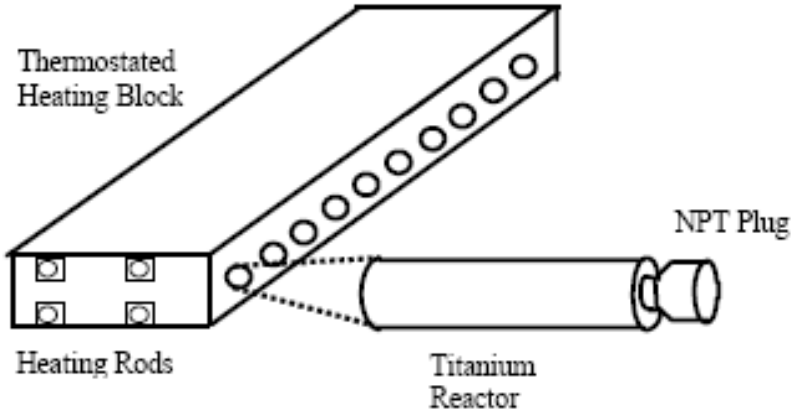

Figure A-4: Near-critical water reactor and heating block apparatus [14].

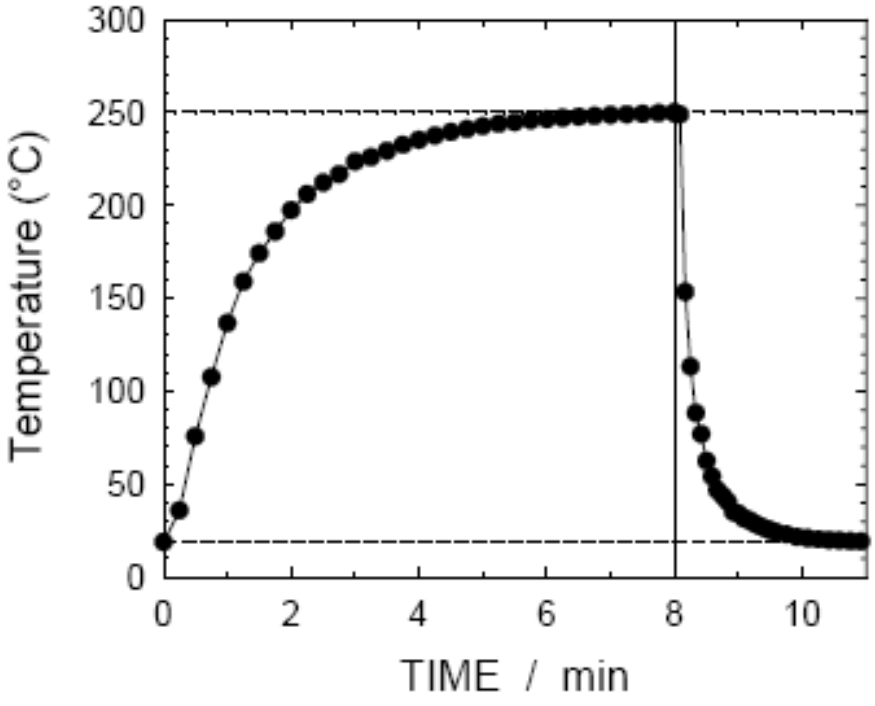

Figure A-5: Internal temperature of NCW reactor heat up and quenching [17].

The resulting extractant is removed and the titanium tube is rinsed 2x with methanol, letting the methanol sit in the tube for 5 minutes to dissolve any residual organic material. Methanol was used for analysis specifically for its solubility of both ferulic acid and 4-vinyl guiacol. The extactant and methanol rinses were combined and centrifuged to remove the biomass or any other solid particulates (i.e. tephlon tape used to seal tubes). Before analysis the supernatant was filtered through 0.45 μm non-sterile hydrophobic fluorophore Millex-FH filters from Millipore. Samples of filtered supernatant were analyzed using a Hewlett Packard Gas Chromatograph with Mass Selective Detector model 5973 (GC-MS) with a HP-5MS column (Agilent, Palo Alto, CA) and by analytical high performance liquid chromatography (HPLC). We monitored both 310nm and 354nm using an Agilent 1100 series HPLC affixed with a Synergy 4μ Hydro-RP column (Phenomenex, Torrance, CA) 80Å, 250 x 4.6mm set at 30 ºC and a flow rate of 1.0 mL/min (32% Methanol / 68% water with 1M TFA) for 90 minutes. HPLC samples were tested as compared to standard solutions.

Results and Discussion

NCW Reactions of Ferulic Acid

The degradation of pure ferulic acid and formation of 4-vinyl guiacol are shown at 250 ˚C under various batch extraction conditions in NCW are shown in Figures A-6 and A-7. The focus of this study was to understand better how NCW affects ferulic acid once it is freed from the plant cell wall. Solid ferulic acid and solutions of ferulic acid in methanol and basic water were used to ensure that all of the ferulic acid was in solution while reacting. Ferulic acid has a very low solubility in water at neutral pH (6.63 g/ml [23]); therefore, base or methanol was added to increase the solubility. Not surprisingly, as shown below, addition of base dramatically increased the degradation of the ferulic acid because acid or base can catalyze the decarboxylation [6, 7].

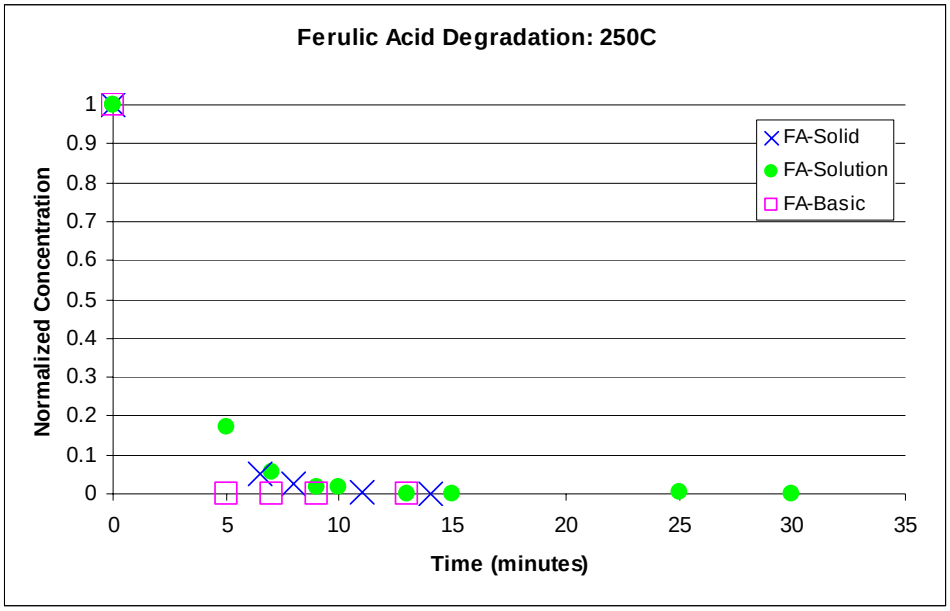

Figure A-6: Ferulic acid degradation in NCW batch reactors at given time for solid ferulic acid, ferulic acid in methanol solutions, and ferulic acid in pH 11.9 water solutions.

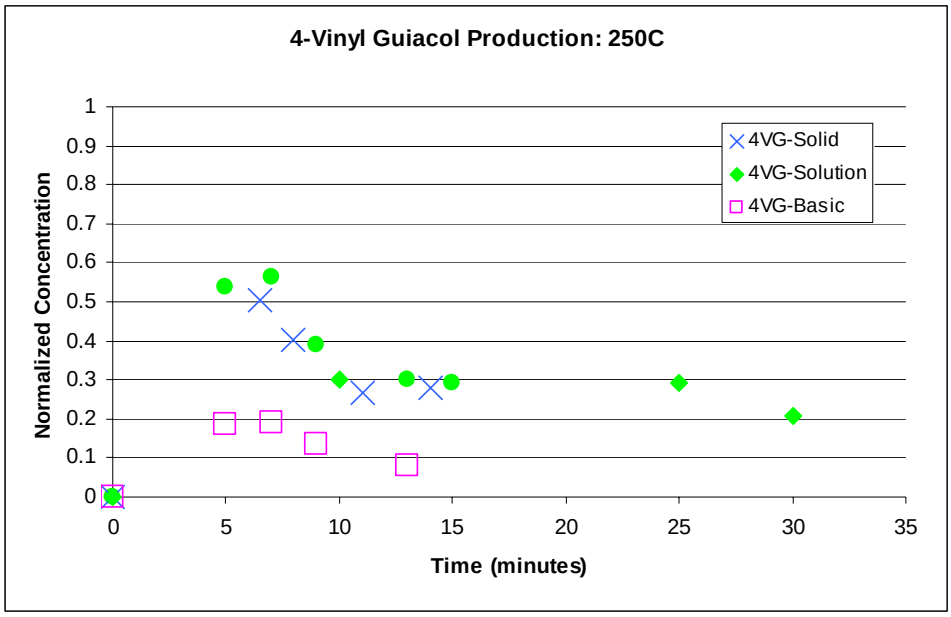

Figure A-7: 4-vinyl guiacol formation in NCW batch reactors at given time for solid ferulic acid, ferulic acid in methanol solutions, and ferulic acid in pH 11.9 water solutions.

In addition to the major components shown above, a clearly identifiable vanillin peak indicating further degradation at this temperature was seen as well as some unidentifiable higher molecular weight peaks, believed to be dimers or trimers of the above components (see Table A-1 for further details). It is clear from these data that the majority of the ferulic acid has transformed within the first 5 minutes of NCW reaction. Heat-up and quench time for the NCW reactors is constrained, as detailed in the experimental methods section; therefore, experiments at times less than 5 minutes cannot be studied with the given apparatus. Below 200 ˚C water does not attain the necessary self-dissociation to achieve acid/base catalysis required to remove the "bound" ferulic acid from the BSG; although the rate may be slow enough to determine the kinetics at a lower temperature in the given apparatus, this information would not be helpful to the current study.

Table A-1: Effect of NCW on ferulic acid for *Solid* ferulic acid used as received, *Solution* which is ferulic acid dissolved in methanol, and *Basic* describing ferulic dissolved in water at a pH of 11.9. *Observation of additional peaks as these retention times on the LC likely indicates that some ferulic acid was either degrading or that one of more of the above phenolics had dimerized.

Form	Residence Time (min)	Normalized Concentrations			Retention time of other peaks observed*
		Ferulic Acid	4-Vinyl Guiacol	Vanillin	
Solid	0	1	0	0	none
Solid	6.5	0.05	0.5	0.03	6, 9, 19
Solid	8	0.03	0.4	0.05	7, 19
Solid	11	0.01	0.27	0.05	7, 19
Solid	14	0	0.28	0.05	7, 19
Solution	0	1	0	0	none
Solution	5	0.17	.54	0.02	7, 9
Solution	7	0.06	.56	.02	7, 10, 11, 19
Solution	9	0.02	.39	.03	7, 10, 11, 19
Solution	10	.02	.3	.03	19
Solution	13	0	.3	.03	7, 10, 11, 19
Solution	15	0	.3	.03	4, 7, 10, 19
Solution	25	0	.29	.05	19
Basic	0	1	0	0	none
Basic	5	0.001	0.19	0.01	19
Basic	7	0	0.19	0.01	19
Basic	9	0	0.14	0.01	19
Basic	13	0	0.08	0.02	19

NCW Extractions of BSG

A previous proof of concept study by our laboratory showed that NCW could successfully extract ferulic acid, vanillic acid, and 4-vinyl guiacol from BSG (Five Seasons #1) [14]. During the current study, vanillic acid was extracted from the BSG, and although there were a number of LC-MS peaks in the phenolics region, no other products were successfully identified. Table A-2 shows representative data for comparison of these studies. The most striking difference is the absence of both ferulic

acid and 4-vinyl guiacol for the 2nd and 3rd batches of BSG. The two major reasons for the discrepancy in these results are batch-to-batch variation in BSG and differences in analytical techniques used to evaluate extractants.

Table A-2: Representative results from NCW extraction of various BSG batches indicating the presence (+) or absence (-) of ferulic acid (FA), 4-vinyl guiacol(4VG), and Vanillic Acid (VA) at the given conditions.

1st Batch Five Seasons #1					2nd Batch Five Seasons #2					3rd Batch Sweetwater #3				
min	ºC	FA +/-	4VG +/-	VA +/-	min	ºC	FA +/-	4VG +/-	VA +/-	min	ºC	FA +/-	4VG +/-	VA +/-
70	25	-	-	-	30	25	-	-	-	30	25	-	-	-
10	175	+	+	+	10	150	-	-	-	15	250	-	-	+
10	275	+	+	+	10	275	-	-	-	30	250	-	-	+
70	175	+	+	+	30	150	-	-	+					
70	275	+	+	+	30	275	-	-	+					

The batch of BSG used in the initial proof of concept experiments could not be retested because it was tainted by mold growth from improper storage. BSG variability from batch to batch is well known [19], and for the given BSG batches, we were not aware of grain type or composition, nor the operating temperatures used for fermentation. Either or both of these factors could have contributed to the increased ferulic acid and/or 4-vinyl guiacol present in the NCW extractant from this batch. For these reasons, it is reasonable to assume that the first batch obtained could have been composed of grains with higher ferulic acid content and fermented at lower temperatures than the second or third batch.

Additionally, the LC column used to analyze the first batch of BSG extractant was clogged and a new more efficient column was purchased to replace it. Extractant from

the first batch of BSG was analyzed with a simple Zorbax C18 column[14] while a Phenomenex Synergi™ Hydro-RP, a C18 column with polar end capping, was used to analyze extractants from 2nd and 3rd batches of BSG. Figures A-8 and A-9 show spiking experiments with FA added to samples of BSG extractant then analyzed with the two different columns. It is clear that the peak separation is much more efficient with the Phenomax column. For this reason, it is possible that the original analyses of ferulic acid and 4VG may have been artificially high due to peak overlap of similar phenolics on the Zorbax column. This peak overlapping is further suggested by the inaccuracy conveyed in the results for vanillic acid as discussed in Newton's thesis [14].

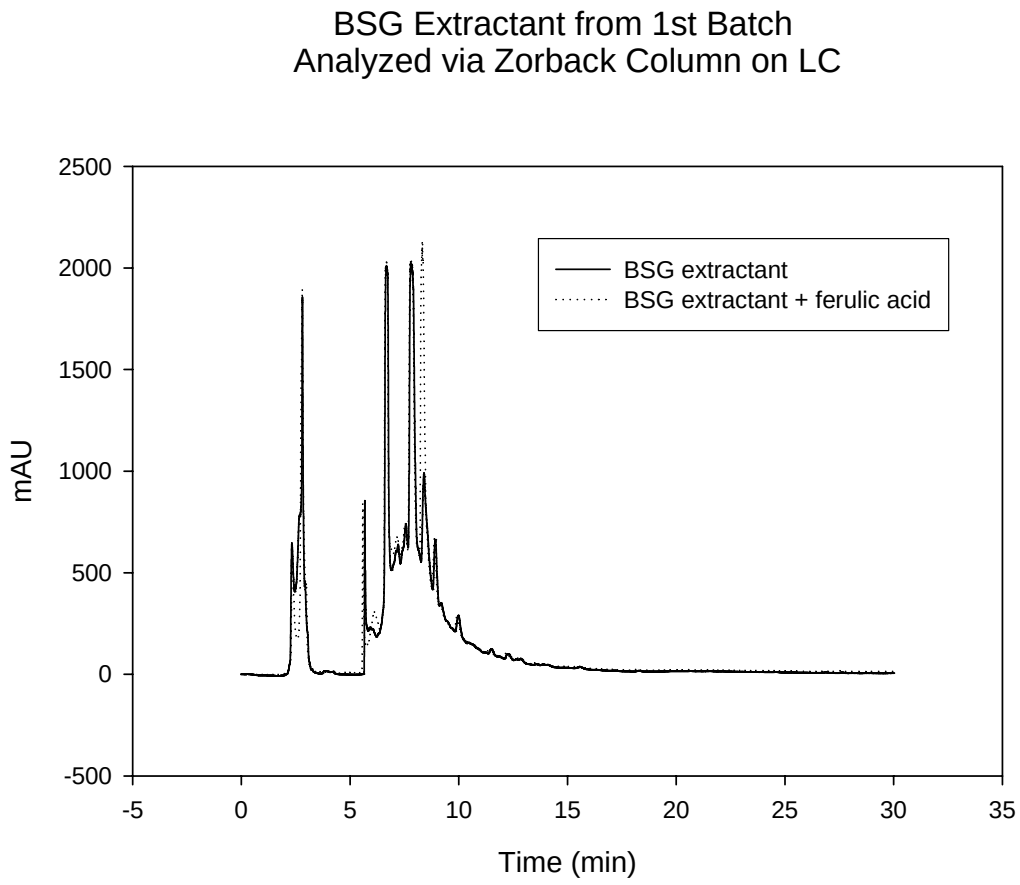

Figure A-8: BSG extractant from 1st batch with and without ferulic acid spiking.

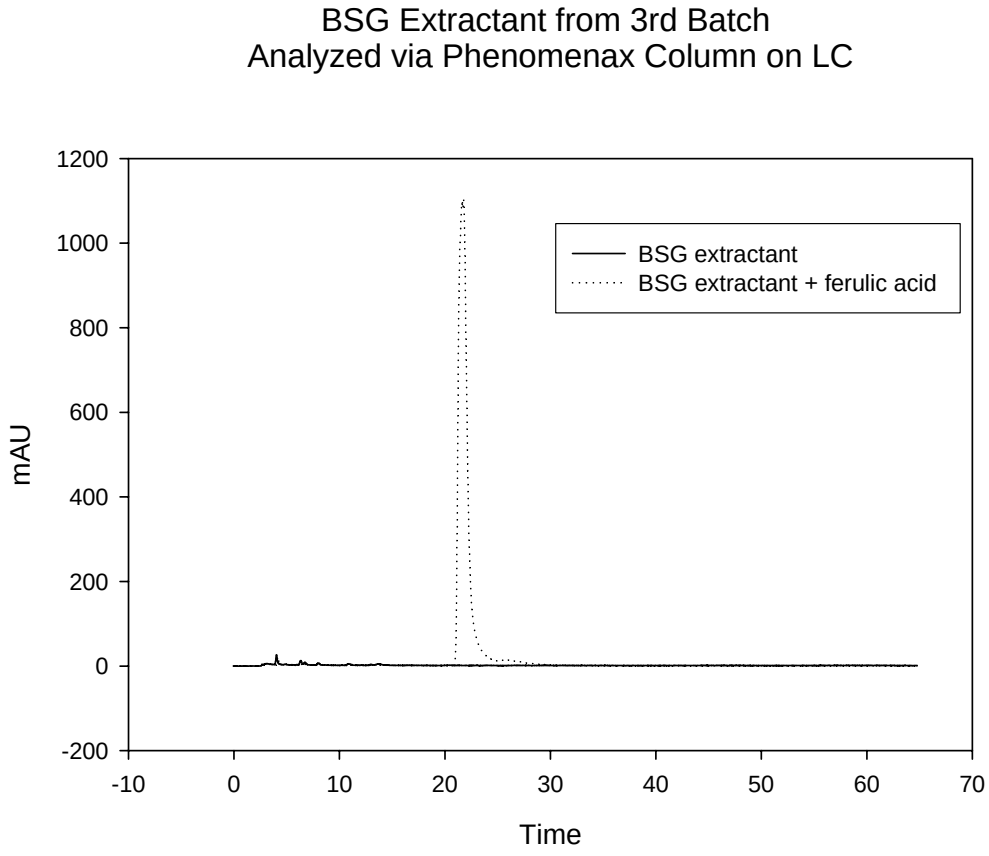

Figure A-9: BSG extractant from 3rd batch with and without ferulic acid spiking.

Nonetheless, conformation that the NCW can extract 4-vinyl guiacol was shown for the first batch of BSG via GC-MS analysis [14]. 4-vinyl guiacol is a derivative of ferulic acid and is not itself present in the plant; therefore, it is presumed that ferulic acid was first extracted and the thermal decarboxalation process described above transformed it into 4-vinyl guiacol. Thus, NCW is an effective way to extract hydroxycinnamic acids from BSG; however, much work is needed to elucidate how to best do so, as will be described in the recommendations section.

Conclusions / Recommendations

BSG is viable source of high value added phenolic compounds; however, batch to batch variations in BSG grain composition make this an inadequate material for studying the general effectiveness of NCW as an extraction solvent ferulic acid and its degradation products.. Additionally, the current batch reactor set-up lacks the heating capability to study NCW effects on ferulic acid and the other phenolic compounds of interest shown in Figure A-3. The limitations exhibited could be addressed by the addition of stirring to a batch reaction to increase heat transfer; however, this is not feasible in the current large aluminum block heating apparatus. Alternatives include, a continuous flow apparatus, microwave heating, or use of a somewhat limited (in terms of operating temperature, 200ºC and pressure, 100 bar) commercially available extraction units such as those available through Dionex® [24].

A more homogeneous and well defined starting material is recommended for examining the efficiency of NCW as an extraction solvent for phenolic compounds from biomass. Future research in our laboratory will look at the effect of NCW and CO_2 enhanced NCW extraction on cellulose, hemicellulose, and lignin, three main components of plant cell walls. CO_2 addition is a potentially beneficial benign and reversible way to add acidity. In order to specifically investigate the ester linkage of ferulic acid, one could use FAXX (0-[5-0(trans-feruloyl)- α -L-arabinofuranosyl]-(1→3)-0-β-xylopyranosyl-(1→4)-D-xylopyranose) shown in Figure A-10. With further understanding of the chemical degradation of these more defined systems, better initial operating parameters (temperature, pressure, co-solvent, etc) may be examined on actual biomass samples.

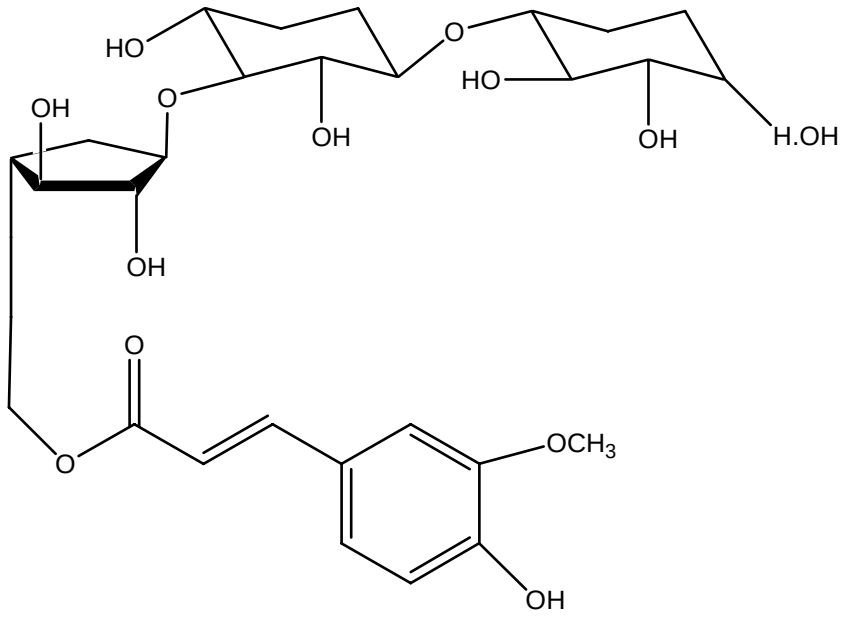

Figure A-10: Structure of FAXX [25].

Additionally, a move towards agriculture / forestry products, as opposed BSG as biomass source, could be beneficial for integration of this process into current biorefinery development. Figure A-11 represents the biorefinery concept [26], including the addition of a high value added chemical extraction unit using NCW extraction for co-production of biofuels and high value added chemicals. Lignin, a main component of the waste still solids stream below, cannot be converted to ethanol. Thus, lignin is a potential source of biomass for high value added chemical extraction in the biorefinery process scheme.

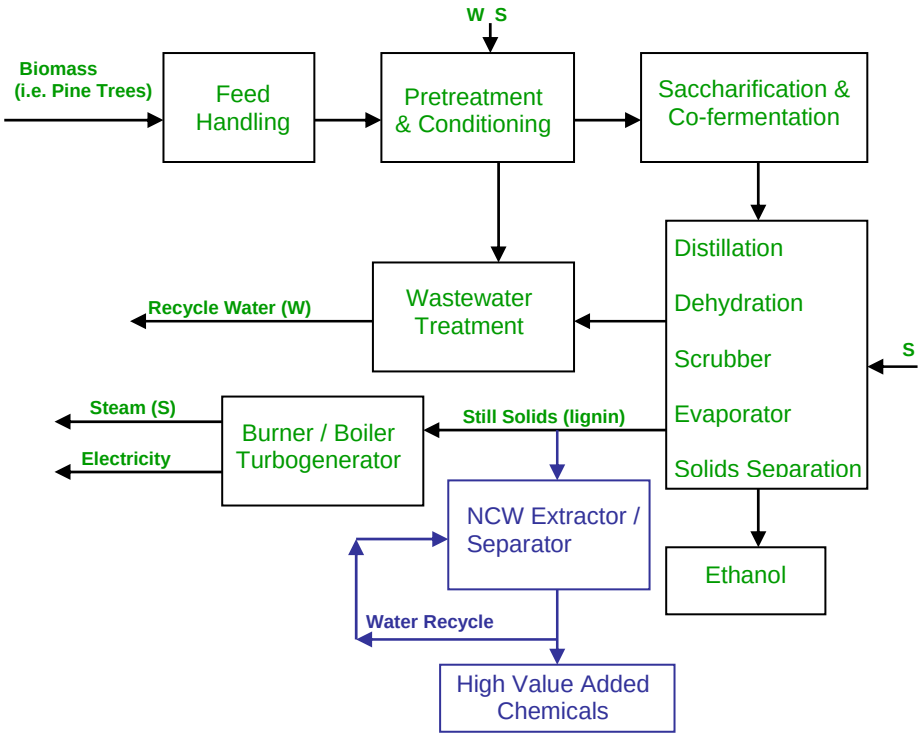

Figure A-11: Schematic of a typical Biorefining process with addition of unit for production of high value added chemicals in blue. (Adapted from NREL 2007)

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26. National Renewable Energy Labratory www.nrel.gov/biomass/biorefinery.html (February 23rd, 2007)

APPENDIX B

CONTROLLED POLYMER FLOCCULATION IN GAS-EXPANDED LIQUIDS

Introduction

Studies have been reported for dispersions of metal nanoparticles in pressurized solvents [1], and there has been significant work on polymers partitioning in supercritical CO_2 [2, 3]. Using gas-expanded liquids (GXLs) to form reversible polymer dispersion is a novel way of combining the technology and modeling from both of these areas. One can imagine being able to capture a desired species from solution by adding functionalized tags to a polymer and using a CO_2 "switch" to cause both the polymer and desired species to flocculate. The desired species (be it product, catalyst, or contaminant) would be captured by the polymer due to its interaction with the functionalized groups placed onto the polymer. This desired species could then be filtered away from the solvent for removal, or re-dispersion in fresh medium. Possible applications include catalyst recovery/recycle, remediation of metals, and chiral separations.

Like OATS systems, polymer dispersions can be tuned with CO_2 pressure. In this process, polymers may be dispersed in an organic solvent at atmospheric pressure and made to flocculate with the addition of CO_2. As such, reversible dispersions of polymers in GXLs offer another opportunity to look at a reaction and separation simultaneously. A functional group may be added to the polymer in order to create an affinity to a particular substance. For example, sulfonated polystyrene's negative charge may attract a cationic catalyst such as 1-butananinium tributyl-bromide. By manipulating charge-charge interactions, it may be possible to precipitate selectively a positively

charged particle, such as a catalyst, out of solution when the polymer flocculates with CO_2 pressure. Other affinity tags may be added to polymers to take advantage of ionic or covalent interactions to address metal remediation or chiral product separation.

Experimental Methods

Materials

ACS grade (99%) Cyclohexane and Hexane from Sigma were used as received. Polystyrene standard (Molecular weight = 30,000) with a polydispersity of 1.06 was obtained from Alfa Aesar for initial co-flocculation proof of concept. Polysulfonated polystyrene was synthesized by Kristen Kitagawa using the method outlined by Carvalho and Curvelo [4] to obtain two polysulfonated polystyrene samples one at 25% sulfonation and the other at 50% sulfonatioin. Both had a molecular weight of 8000 and polydispersity of 1.1.

Ambient Dispersion Tests

All measurements were performed on a Hewlett-Packard 8453 diode array UV-Vis spectrophotometer (1 nm resolution and ±0.2 nm wavelength accuracy). The UV signature of the above polystyrene and sulfonated polystyrenes were tested in solutions of hexane and cyclohexane in a 1 mL cuvette with a path length of 1 cm. Polymer, 0.01 g< was mixed with 40 mL solvent for one hour. Mixing was stopped and the UV signature of the solution was compared against that of the pure solvent to determine if a styrene peak was apparent at 260nm, indicating either a dissolution or stable dispersion of the polymer in solution.

Pressurized Co-Flocculation Experiments:

A stainless steel jacketed vessel with two sapphire windows (6.4 mm thick) was constructed for use in high pressure UV-Vis spectroscopy. The windows were sealed with Teflon gaskets capable of withstanding pressures over 250 bar. The cell has a path length of 2.3 cm and an internal volume of 13 mL. Temperature was controlled with a refrigerated constant temperature flow bath (VWR 1150) with a mixture of ethylene glycol and water as the heat transfer fluid and monitored with a thermocouple and readout (Omega). The temperature variation was maintained within ±0.1°C of the set point. Pressure was monitored by a pressure transducer and readout (Druck, DPI 260, PDCR 910, GE Infrastructure Sensing, Billerica, MA) calibrated against a hydraulic piston pressure gauge (Ruska, GE Infrastructure Sensing, Billerica, MA) to an uncertainty of +/- 0.1 bar. A Teflon-coated, magnetic stir bar was used to agitate the contents of the cell to facilitate equilibrium. All measurements were performed on a Hewlett-Packard 8453 diode array UV-Vis spectrophotometer (1 nm resolution and ±0.2 nm wavelength accuracy).

4 mL of 0.046 mM polystyrene in cyclohexane was added to the pressure vessel and mixed for 20 minutes. The mixing was stopped and traces of 260nm and 800nm were followed for 10 minutes with scans at 10 second intervals. CO_2 was added volumetrically to achieve the desired pressure via a 260D Syringe Pump (Teledyne Isco, Lincoln, NE), and the cell was mixed for 2 hours to allow time to come to equilibrium. The mixing was stopped and traces of 260nm and 800nm were followed for 10 minutes with scans at 10 second intervals. This process of CO_2 addition (increasing pressure), mixing, and UV scan was repeated until the desired maximum pressure was reached. At

such time the cell was partially depressurized by allowing CO_2 to vent, mixed for 2 hours and traces of 260nm and 800nm were followed for 10 minutes with scans at 10 second intervals. This process of CO_2 venting (decreasing pressure), mixing and UV scans was repeated until the cell reached ambient pressure.

Results and Discussion

Both the Flory-Huggins χ-interaction parameter [5, 6] and the van der Waals (VDW) interaction potential [1] were modeled for polystyrene in hexane and cyclohexane GXLs. The VDW interaction energy predicts the attractive forces between two polystyrene particles in solution. The VDW attractions became more negative as pressure increased, showing the potential for dispersion in ambient solvent and flocculation in a GXL. The χ-interaction parameter was also evaluated as shown in Figure B-1. A χ value greater than 0.5 suggests an unstable dispersion and probable flocculation. From the χ-interaction parameter, cyclohexane GXL is predicted to be a good solvent at low CO_2 pressure and a bad solvent at high CO_2 pressure for polystyrene. Hexane GXL is predicted to be a poor solvent over the entire pressure range examined and therefore a bad choice for this process.

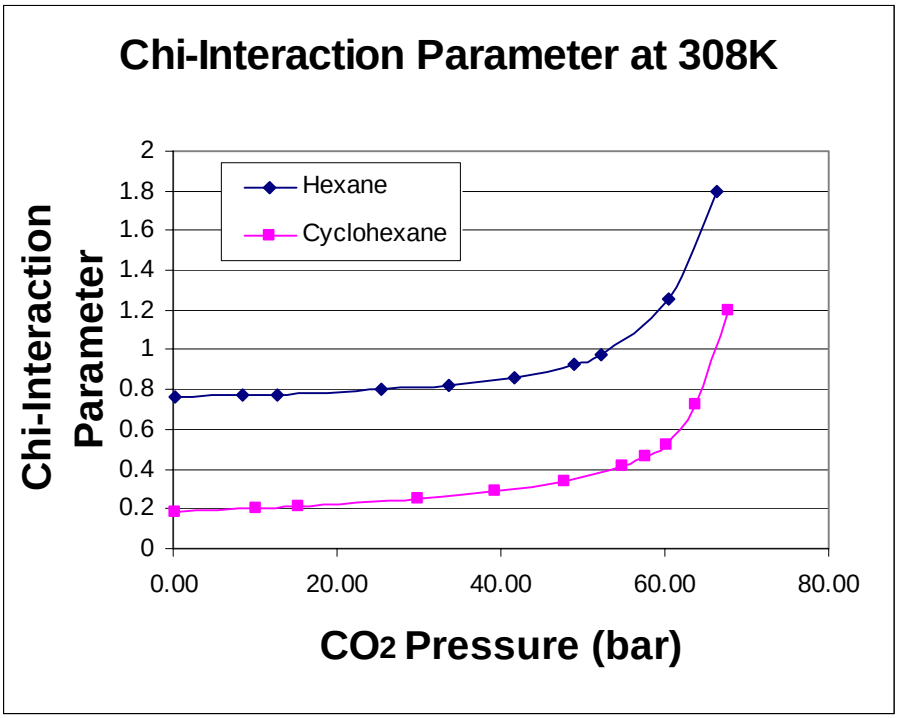

Figure B-1: Predicted χ interaction parameters over a range of pressures at 308 K.

Polystyrene could not be dispersed in hexane at room temperature, as predicted by the χ interaction parameter. Laboratory experiments were initially thought to confirm the prediction for polystyrene dispersion in ambient cyclohexane and flocculation in cyclohexane with approximately 60 bar CO_2 pressure (Figure B-2); however when the volume expansion was accounted for, it was shown that the absorbance drop was artificial (Figure B-3). Additionally, preliminary work of this selective precipitation technique for proof of concept with a cationic dye and sulfonated polystyrene showed that the sulfonated polystyrene was not able to be dispersed in cyclohexane at the molecular weights examined.

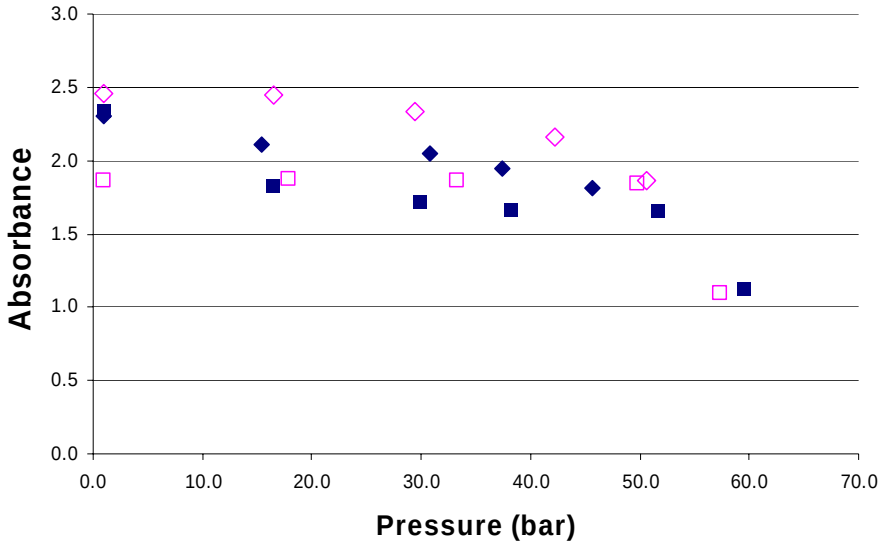

Figure B-2: Polystyrene in cyclohexane at 252 seconds and 308K. Blue indicates increasing pressure while pink represents decreasing. Absorbance **has not** been corrected for volume expansion due to CO_2.

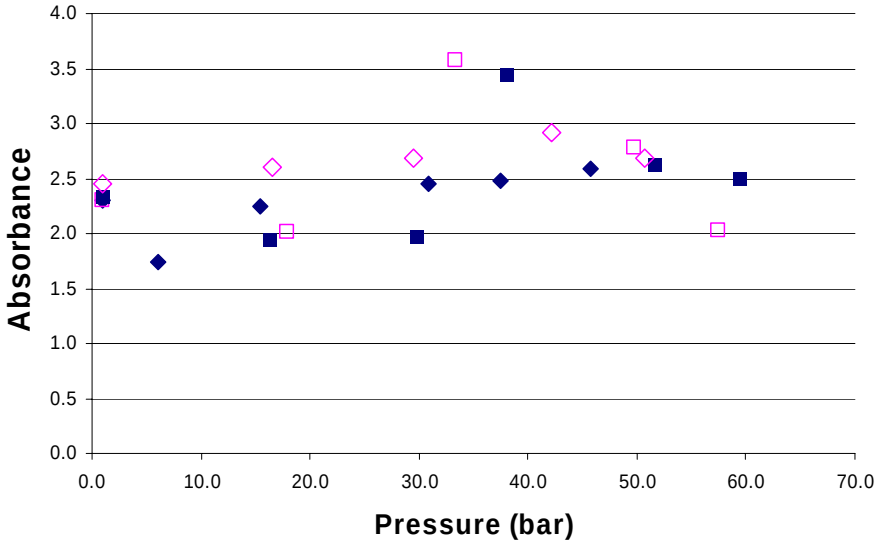

Figure B-3: Polystyrene in cyclohexane at 252 seconds and 308K. Blue indicates increasing pressure while pink represents decreasing. Absorbance **has** been corrected for volume expansion due to CO_2 using binary phase data [7].

Conclusions and Recommendations

The van der Waals attractions did not give an accurate picture of the behavior of the polymers in solutions of increasing CO_2 pressure. This model was previously used to predict dispersion of nano sized metal particles, and assumptions for negligible repulsion forces may not apply to the macro sized polymer species examined here. A more complex model for interactions between polymer species should be examined and/or dynamic light scattering (DLS) experiments could better elucidate the polymers behavior in the solvent.

Should a proper model be developed, charge interaction is still the simplest affinity tag to begin with because it does not require a complicated polymer synthesis, but rather a simple addition of sulfur groups to a commercially available polystyrene standard. The polydispersivity of the functionalized polystyrene will not change from the original, thus saving time on material characterization. A cationic dye is a good starting place because its concentration will be in catalytic amounts simulating catalyst recovery, and the concentration can be observed by a UV-Vis spectrometer.

DLS can be used to better determine particle interactions under pressure. It can give information on polydispersity and elucidate the mechanism of precipitation; specifically, whether particles simply fall out or if they agglomerate to form larger clusters then precipitate. In addition, it would offer an opportunity for joint research as that the facilities to measure light scattering under pressure are not available at Georgia Tech. Mark McHugh at Virginia Commonwealth University is one possible collaborator.

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APPENDIX C

LOW MOLECULAR WEIGHT POLYETHYLENE GLYCOL AS AN ALTERNATIVE SOLVENT FOR ENZYME CATALYZED REACTIONS

Introduction

The demand for safe, environmentally-friendly solvents continues to grow, and economical rationales exist to justify the use of these alternative solvents such as the estimated $115 billion dollars spent annually on pollution control and treatment [1]. Low molecular weight polyethylene glycol (PEG) is desirable as a green reaction media because it is nonvolatile [2], has a toxicity low enough to be approved as a food additive for humans [3], and is biodegradable [4, 5].

Liquid PEG was first used as a solvent for homogeneous catalysis in 1995 by Naughton and Drago [6]. Due to its nonvolatility, low toxicity, and biodegradability, PEG has been investigated as a replacement solvent for volatile organic compounds (VOC's) for pharmaceutical crystallization/precipitations [7] and chemical synthesis [2, 8, 9]. VOC's are often flammable, can have adverse health affects upon inhalation, and are known smog contributors. PEG eliminates these problems; however, it can also introduce a whole new set of issues including difficulty in separating products post reaction, mass transfer limitations due to higher viscosity, and undesired solvent substrate interactions. For the case of enzymatic catalysts, the effect on enzyme stability is also a concern in PEG.

PEG has been used to enhance enzyme activity in organic solvents through PEGylation (covalent modification of the enzyme with PEG to increase stability [10-12]),

as an additive to reactions within reverse micelles to decrease surfactant interaction with the enzyme catalyst [13], and as a solvent for immobilized enzyme preparations [14, 15]. However the effect of PEG on soluble enzyme solutions in low water content PEG has not as yet been investigated. In this work, low molecular weight PEG is studied as a possible alternative solvent for fine chemical synthesis with the soluble enzyme *Candida antarctica* lipase B (CAL B) as biocatalyst. The benefits of using a soluble biocatalyst such as CAL B are discussed in Chapter IV.

Separation from the liquid polymer PEG is not a trivial issue and use of VOC's with a traditional liquid-liquid extraction of products post reaction defeats the purpose of using a nonvolatile solvent for reaction. Here we also examine the feasibility of using an OATS-like mixture to partition preferentially products into a CO_2-expanded liquid such that the total amount of VOC required is reduced compared to liquid-liquid extraction. The enzyme is placed in an organic/PEG miscible solution and substrate is added. After the desired reaction time, CO_2 pressure is added to induce a phase separation. This separation occurs because the organic co-solvent swells under CO_2 pressure to create a GXL. The phase envelope is therefore moved from the one-phase to two-phase region when CO_2 is added. The PEG phase is only slightly miscible with CO_2, thus its swelling is negligible [16]. Hydrophobic products should partition preferentially to the organic top phase. The enzymatic catalyst will partition favorably into the bottom phase, rich in PEG, because enzymes prefer polar hydrophilic environments

Experimental Methods

Materials

The following compounds were used as received: acetonitrile (Sigma-Aldrich, HPLC grade 99.5%); benzoic acid (Sigma); benzoic acid ethyl ester (TCI, TCI grade); 1-butanol (Aldrich, 99.8%); butyl acetate (Aldrich); n-butyl cinnamate (Lancaster, 98%); ethanol (Sigma-Aldrich, ACS grade); ethyl palmitate (Sigma); ferulic acid (Acros, 99%); methyl sulfoxide-d6,99 +99% deuterium enrichment (Acros, 99%); octanol (Sigma-Aldrich, 99+%); palmitic acid vinyl ester (TCI, TCI grade); *rac*-1-phenylethyl acetate (Acros, 99%), *rac*-1-phenylethanol (Acros, 98%),; polyethylene glycol 300, PEG 300, (Aldrich, typical Mn = 300); polyethylene glycol 400, PEG 400, (Aldrich, typical Mn = 400); poly(ethylene glycol) dimethyl ether, PEG-DME, (Aldrich, typical Mn = 250); sodium phosphate monobasic monohydrate, phosphate buffer, (EMD chemicals, ACS grade); vanillin (Aldrich, 99%) and vinyl acetate (Acros). *Candida antarctica* lipase B, CAL B, was used in a lyophilized and solubilized form, ICR-110 and SOL-101 respectively (Biocatalytics) as well as an immobilized form on acrylic resin, Novozyme 435 (Aldrich).

Partitioning Experiment

A stainless steel jacketed vessel with two sapphire windows (6.4 mm thick) was constructed for use in high pressure UV-Vis spectroscopy. The windows were sealed with Teflon gaskets capable of withstanding pressures over 250 bar. The cell had a path length of 2.3 cm and an internal volume of 13 mL. Temperature was controlled with a refrigerated constant temperature flow bath (VWR 1150) with a mixture of ethylene

glycol and water as the heat transfer fluid and monitored with a thermocouple and readout (Omega). The temperature variation was maintained within ±0.1°C of the set point. Pressure was monitored by a pressure transducer and readout (Druck, DPI 260, PDCR 910, GE Infrastructure Sensing, Billerica, MA) calibrated against a hydraulic piston pressure gauge (Ruska, GE Infrastructure Sensing, Billerica, MA) to an uncertainty of +/- 0.1 bar. A Teflon-coated, magnetic stir bar was used to agitate the contents of the cell to facilitate equilibrium.

All measurements were performed on a Hewlett-Packard 8453 diode array UV-Vis spectrophotometer (1 nm resolution and ±0.2 nm wavelength accuracy). The UV response was calibrated at ambient conditions using solutions containing known concentrations of the solute of interest. Concentrations of solute in the pressurized cell are not corrected for volume expansion because phase data measurements for acetonitrile-CO_2-PEG 400 are still being acquired by other members of the group.

Reactions

The hydrolysis of 1-phenylethyl acetate was preformed in both PEG 300 and PEG 400. The lyophilized formulation of CAL B, ICR-110, dissolved in 150 mM phosphate buffer titrated to a pH of 7.1 was added such that the total volume of enzyme solution added would make up 1% of the total reaction volume. As that the PEG contained 0.5% +/- 0.07% water by volume (as determined by Karl Fisher titrator, Mettler Toledo DL31) the final reaction mixture was approximately 1.5% water.

GC analysis could not be preformed for the hydrolysis reaction because the fractions of PEG were not able to be moved far enough from the product and substrate to

identify them independently. Reaction progress for the hydrolysis of 1-phenylethyl acetate was analyzed qualitatively via analytical high performance liquid chromatography (HPLC) at 245nm using an Agilent 1100 series HPLC and a Chiralcel OD-RH (Diacel, Inc., Fort Lee, NJ) with a 0.46 cm I.D. x 15 cm L column set at 35 ºC and a flow rate of 0.5 mL/min (40% acetonitrile / 60% water) for one hour. For HPLC analysis, even when reaction samples were diluted up to 90% and blanks were run between samples (i.e. one hour equilibration time between samples), the PEG residues on the column eluted at unpredictable residence times that interfered with reproducible quantitative calculation of product or substrate concentration.

FT-IR spectra were measured on a Vector 22 instrument using a Golden Gate diamond ATR accessory with a DTGS detector at a resolution of 2 cm^{-1}. All NMR were carried out on a Varian Mercury Vx 400 MHz using CD_3SOCD_3 as both the solvent and the reference. Reaction progress for esterification of vinyl acetate and palmitic acid vinyl ester (PVE) with ethanol was follow via a Hewlett Packard Gas Chromatograph with Mass Selective Detector model 5973 (GC-MS) with a HP-5MS column (Agilent, Palo Alto, CA). Reactions preformed in PEG 400 and toluene were quenched by submersion of GC vials into liquid nitrogen for 5 minutes. The toluene samples were analyzed directly while the PEG samples were extracted 3 times with hexane before GC analysis to minimize the amount of PEG sent to the GC.

Fractionation of PVE was observed; thus the peak indicating PVE was found at molecular weight 239 instead of its true value of 282. PEG fractionation was observed in the GC wherein the GC area of peaks corresponding to the PEG fractions altered but the retention times were approximately the same from run to run as shown in Figure C-1.

After careful method development, the fractions did not overlap the product or substrate peaks thus allowing analysis via GC.

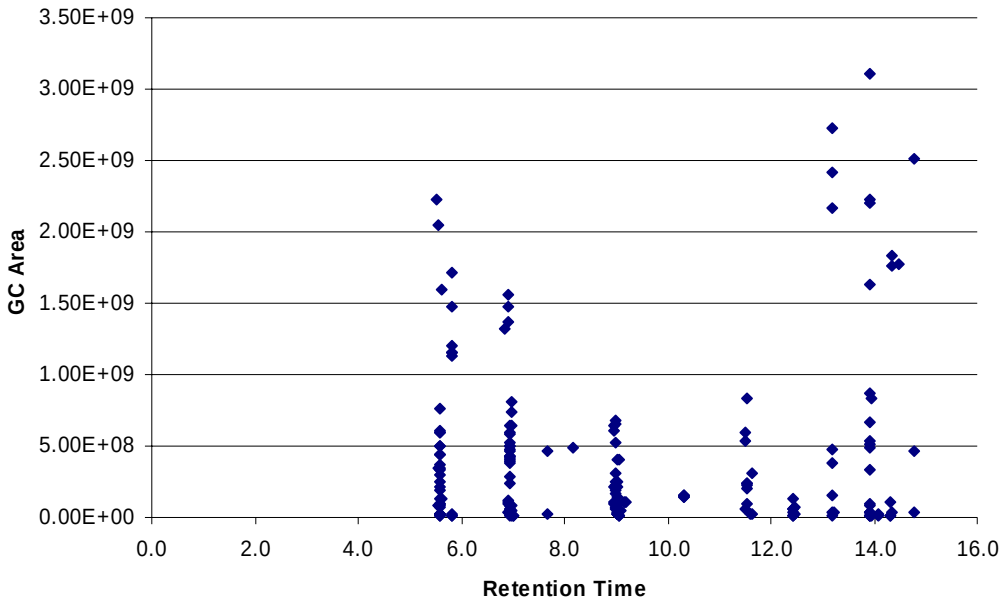
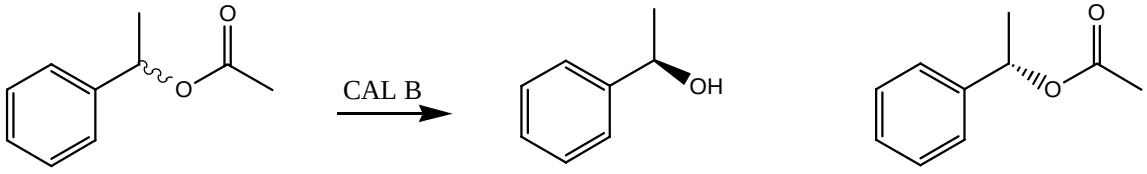


Figure C-1: GC areas resultant from PEG fractionation at the given retention time for the group of 72 samples used in this work.

Results and Discussion

The hydrolysis reaction shown in Figure C-2 was first investigated because the results in PEG and PEG-organic systems could easily be compared with data taken in water and water-organic systems which were being run in parallel experiments for OATS systems. Distribution of the product 1-phenylethanol was examined to determine if the product would preferentially partition into the organic rich phase of CO_2 pressurized PEG-acetonitrile (PEG-ACN) mixtures for facile separation. These data are compared to distribution in an analogous OATS separation scheme in Figure C-3. The PEG-ACN scheme achieves comparable separation of the 1-phenylethanol into the organic rich

phase at slightly elevated pressure as compared to the OATS system. Volume expansion corrections in the PEG-ACN mixture were not calculated because pertinent phase data were not yet available. We know corrections for this expansion will result in more favorable partitioning numbers because the observed concentration in the organic rich phase is more diluted due to expansion than the observed concentration in the PEG rich phase. This principle is illustrated for the OATS data which is presented with volume expansion correction in Figure C-4.



rac-1-phenylethyl acetate *(R)-1*-phenylethanol *(S)*-1-phenylethyl acetate

Figure C-2: Hydrolysis reaction studies using CAL B for a chiral resolution of phenylethyl acetate.

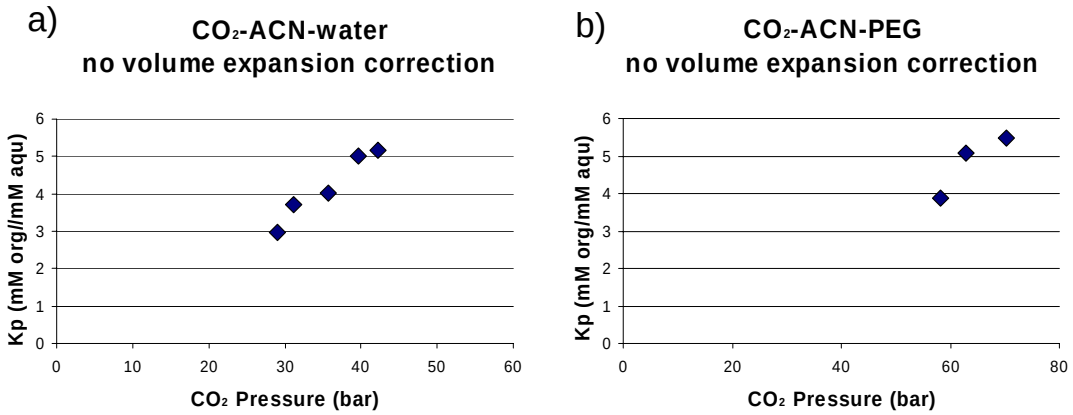


Figure C-3: Distribution of *(rac)*-1-phenylethanol in 50:50 (v:v) mixtures of a) ACN:water and b) ACN: PEG under CO_2 pressure.

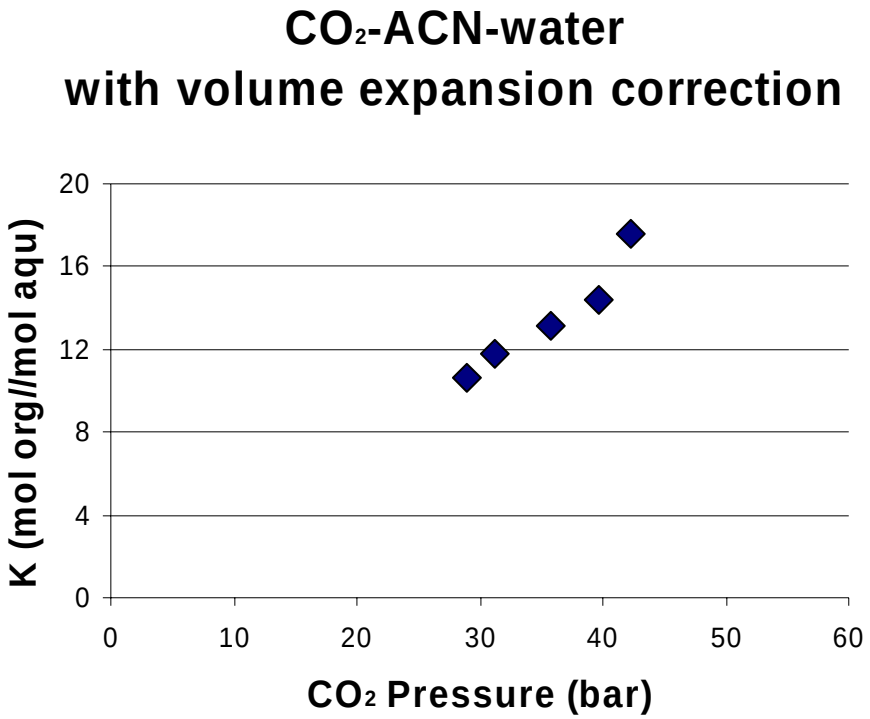


Figure C-4: Data or the ACN:water distribution of *(rac)*-1-phenylethanol replotted to include the volume expansion correction.

Quantitative analysis of neat and/or diluted samples proved problematic due to residual PEG fractions in the HPLC. For conversion of 1-phenylethyl acetate, the product was seen after two days reaction in PEG solution. Although this was faster than an unanalyzed reaction in PEG (product appeared after 4 days), it was much slower than the timescale of the reaction in water, which is shown in Chapter IV to occur in just minutes. Increasing the water content could help increase the reaction rate of the hydrolysis reaction; however, that would place the reaction into a more complex mixed solvent system which could increase the difficulty of separation and/or catalyst recycle. For these reasons, better analytical techniques were not pursued to characterize further the kinetics of this reaction. Instead, the water sensitive esterifictaion reactions shown in Figure C-5 were next investigated.

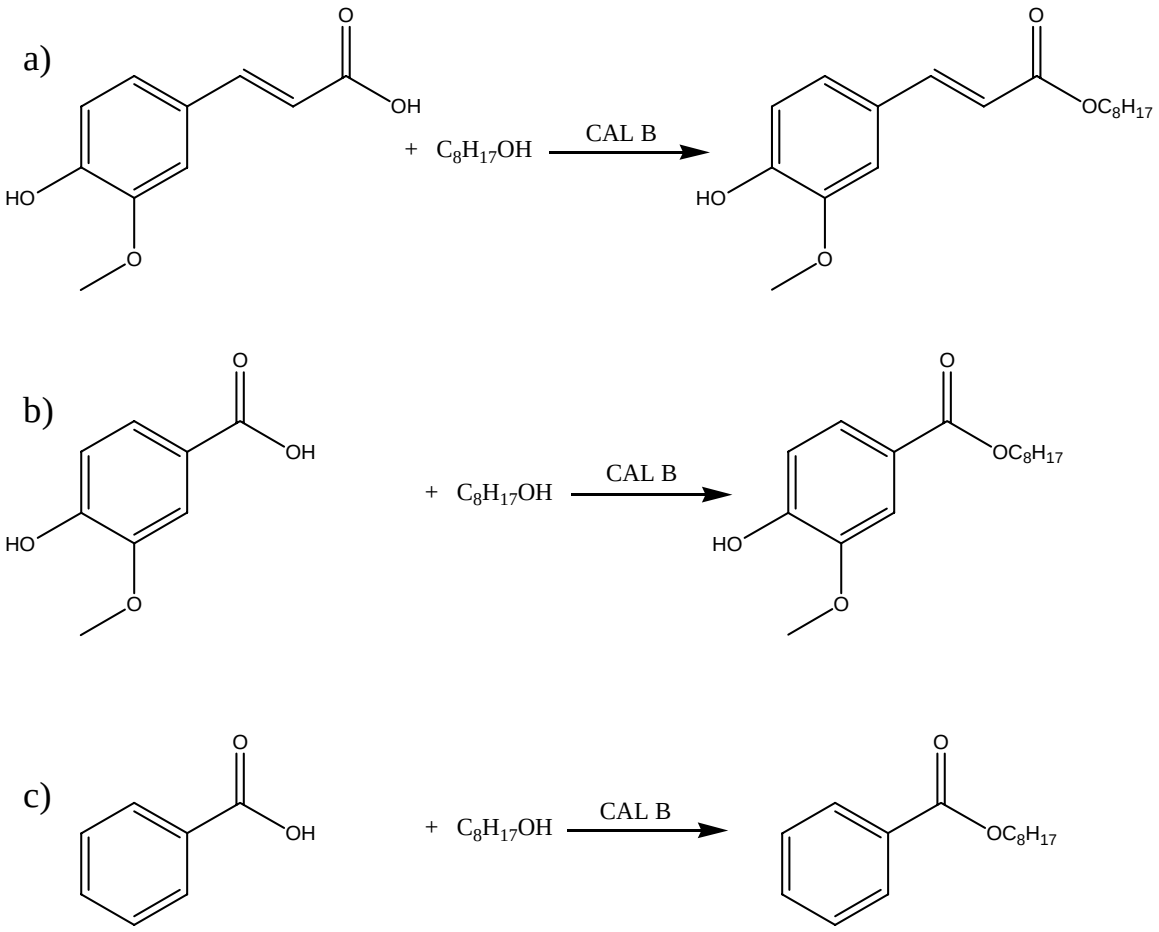


Figure C-5: Attempted esterification of a) ferulic acid, b) vanillic acid, and c) benzoic acid with octanol in PEG 400 with *Candida antartica* lipase B as catalyst.

These esterification reactions were selected because the substrates shown in a and c are both products of the NCW extractions illustrated in Appendix A, while the substrate in c is a less complex analogous molecule. Facile processing of water sensitive reactions with these substrates is interesting from the perspective of a processing scheme for coproducts in biorefineries to increase profits. The reactions were attempted with lyophilized (ICR-110) and immobilized (Novozyme 435) CAL B in PEG 400. The reactions in PEG did not show product via NMR analysis for either enzyme formulation.

Additionally, IR analysis showed a peak shift in the 1550-1750 cm^{-1} range of the pure components ferulic acid and vanillic acid after a few hours dissolution in PEG (Figure C-6). This is most likely due to hydrogen bonding between the PEG and the hydroxyl groups of these molecules. No shift is seen in this region of IR for benzoic acid because there is no hydroxyl group directly off of the phenolic ring. Reaction of benzoic acid and butanol was next examined. For comparison sake, the reaction was attempted in both PEG 400 and toluene. The reactions in PEG did not show product via NMR analysis, however the reaction was apparent in toluene for both enzyme formulations.

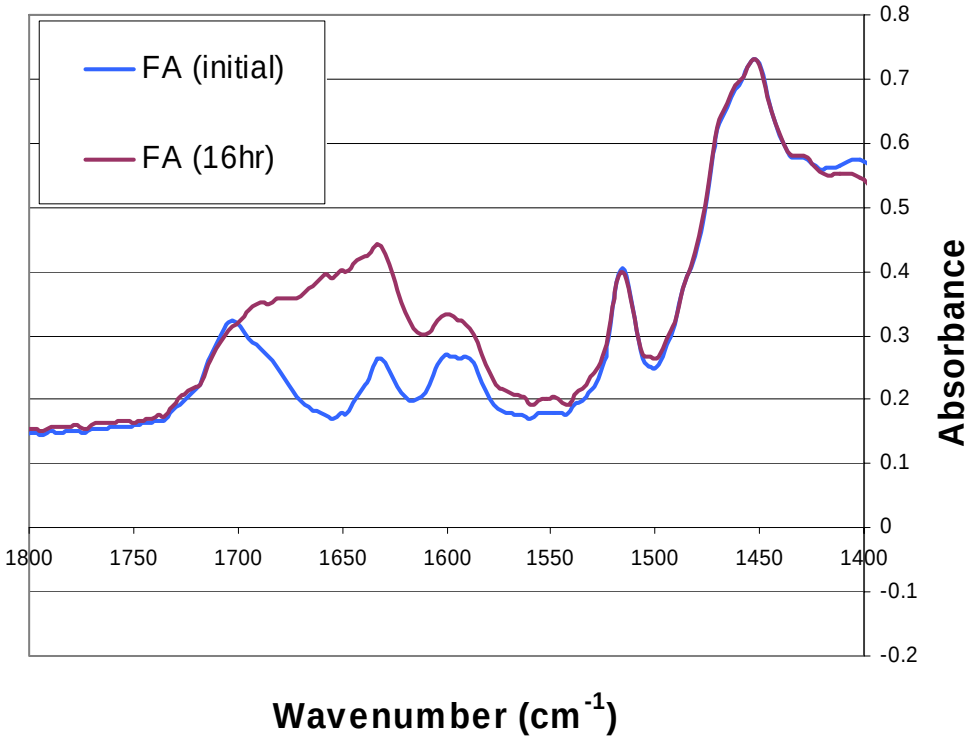


Figure C-6: The ATR-IR spectra of ferulic acid (FA) in solution of PEG 400 tested immediately upon dissolution and after sitting for 16 hours shows a shift due to interaction with the phenolic hydroxyl group.

The esterifiction of vinyl acetate with butanol was attempted next because CAL B is known to be very active towards vinyl groups. The conversions, shown in Table C-1, clearly indicate reaction is possible in PEG, but the background reaction with no enzyme in toluene occurs at a comparable rate.

Table C-1: Conversion to butyl acetate in PEG 400 and toluene for lyophilized (ICR-101), immobilized (Novozyme 435) and control reaction (no enzyme) after 24 hours.

Solvent	**Enzyme**	**Conversion**
PEG 400	ICR-101	5.9 %
PEG 400	Novozyme 435	11.2 %
PEG 400	No Enzyme	0.0 %
Toluene	ICR-101	92.5 %
Toluene	Novozyme 435	91.4 %
Toluene	No Enzyme	12.56 %

A more interesting reaction that did not show a background reaction in toluene after 24 hours is the tranesterification of palmitic acid vinyl ester with ethanol shown in Figure C-7. Ethanol was used instead of 1-butanol because the product of the ethanol tranesterification, ethyl palmitate is commercially available for comparison to reaction samples, and PEG-DME was used as solvent because palmitic acid vinyl ester was not soluble in PEG 400. Figure C-8 show appearance of product, ethyl palmitate. This was first thought to be a good model reaction for further study of separation techniques for product recovery; however, the palmitic acid vinyl ester disappeared in reaction solutions that did not contain ethanol, the second substrate (Figure C-9). This is likely due to the dimethyl ether end caps on PEG-DME reacting with palmitic acid vinyl ester.

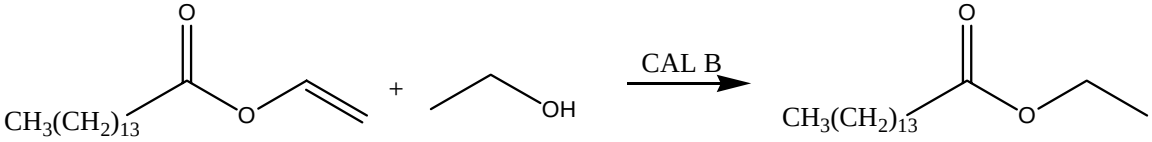


Figure C-7: Transesterification reaction of palmitic acid vinyl ester with ethanol using biocatalyst *Candida antartica* lipase B to obtain ethyl palmitate.

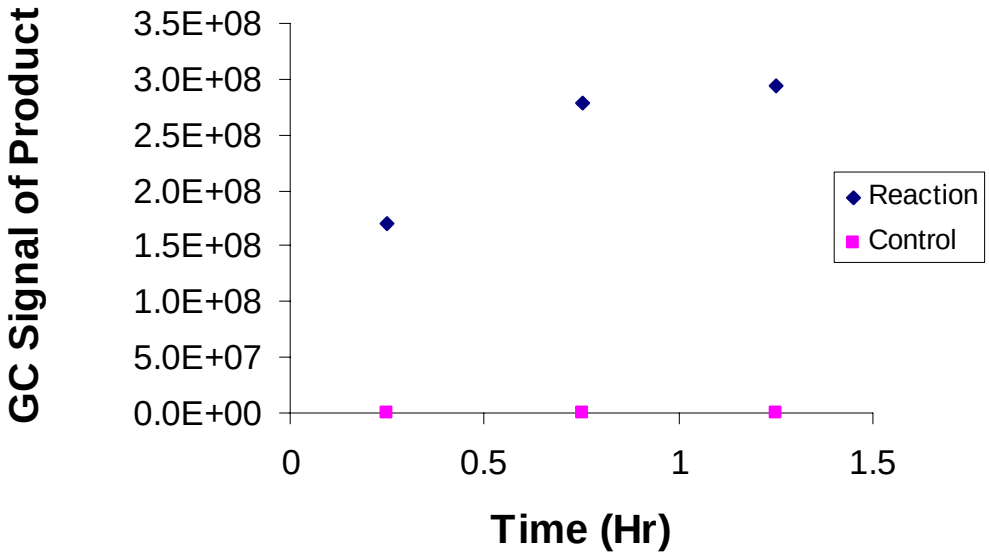


Figure C-8: Formation of product, ethyl palmitate, in the transesterification of palmitic acid vinyl ester with ethanol using lyophilized CAL B as catalyst.

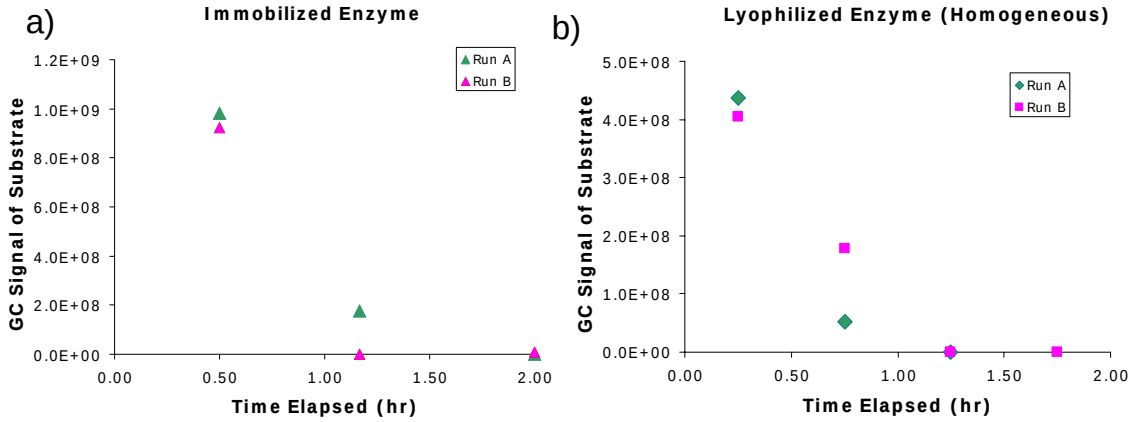


Figure C-9: Substrate, palmitic acid vinyl ester, disappearance in solutions of PEG-DME with a) immobilized and b) lyophilized CAL B.

Conclusions and Recommendations

Initial partitioning studies with PEG-ACN were comparable to OATS-like systems; however, as compared to a hydrolysis reaction done in water and esterification reactions done in toluene, CAL B catalyzed reactions in PEG showed reduced rate of reaction. The reduced enzymatic activity in pure PEG will be further reduced upon addition of an organic cosolvent, which is necessary for an OATS-like separation scheme. Additionally, it is clear from this work and others [17, 18] that PEG is not an inert substance. Interactions with the solute are likely attributed to hydrogen bonding with the PEG backbone, reaction with the hydroxyl end groups in PEG 400, or the dimethyl ether end caps in PEG-DME. This reduced activity, coupled with the reactivity of PEG and inability to easily assay reactions in PEG, lead to a decision to discontinue this work.

Although solutions of PEG and PEG-DME did not prove to be good solvents for the reactions studied here, this does not preclude the utility of PEG and other liquid polymers as solvents. The advantages of low toxicity, non-volatility, and biodegradability are still very important in pursuing green solvent systems. The challenges observed here may be overcome by enhancing the ability of the biocatalyst to react in the PEG solution. One approach to enhance activity might be examination of PEGylated enzyme preparations which are known to show enhanced solubilization and activity in organic solvents as compared to their non PEGylated counterparts [11, 19]. Alternatively, mixed solvents of PEG or non-biocatalytic reactions may be a better route to take as that they have already shown success in the literature [2, 8, 9, 14].

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Publications

J. M. Broering, E. M. Hill, J. P. Hallett, C. L. Liotta, C. A. Eckert, and A. S. Bommarius "Biocatalytic Reaction and Recycle Using CO_2-Induced Organic-Aqueous Tunable Solvents (OATS)." *Angew. Chem. Int. Ed.* 2006, 118 (26) 4786-4789.

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Presentations

2006 AIChE Annual Meeting; *Using Tunable Solvents to Couple Biphasic Extraction with Homogeneous Reactions*, San Francisco, CA (November 2006, oral)

231st American Chemical Society Annual Meeting; *Biocatalysis and CO_2-Induced Separation and Recycle Using Organic-Aqueous Tunable Solvents (OATS),* Atlanta, GA (March 2006, poster)

18th Annual Georgia Institute of Technology Chemical & Biomolecular Engineering Graduate Student Symposium *Recycle of Homogeneous Bicatalysts Using Organic-Aqueous Tunable Solvents (OATS),* Atlanta GA (March 2006, oral and poster)

2005 International Symposium on Supercritical Fluids; *Enzymatic Reactions and Catalyst Recycle with Benign Tunable Solvents,* Orlando, FL (May 2005, poster)