

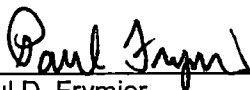
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I am submitting herewith a thesis written by Paula A. Cameron entitled "Gas Phase Enzyme Catalysis for Esterification and Transesterification Reactions Using Immobilized Lipase." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

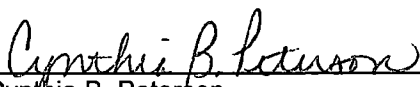


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**Gas Phase Enzyme Catalysis  
for Esterification and Transesterification Reactions  
Using Immobilized Lipase**

A Thesis  
Presented for the  
Master of Science  
Degree  
The University of Tennessee, Knoxville

Paula Ann Cameron  
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## Abstract

Since the early 1980s, work in the area of nonaqueous bioprocessing has revolutionized the field of biocatalysis. Once thought to function only in aqueous environments, enzymes have been proven to be catalytically active in organic solvents as well as in completely gaseous systems. Gas phase enzymatic systems are advantageous for several reasons, including reduced mass transfer limitations and increased thermal stability. In such systems, some water remains bound to the enzyme and can also be present in the form of relative humidity. This water plays a dual role: it is necessary for catalytic activity, but at the same time its presence facilitates thermal inactivation of the enzyme.

In order to provide a more fundamental understanding of the dual role of water in gas phase enzyme catalysis, the effects of relative humidity and temperature on several lipase catalyzed, gas phase transesterification reactions were investigated. Several different reactor configurations were used including single pass, continuous recycle, and batch reactor modes. Temperature and relative humidity were controlled both inside reactors and throughout the course of the reaction in an attempt to account for and optimize their effects.

Results indicated that, at low relative humidity, reaction rates increase with temperature up to a point. However, when relative humidity was increased, a similar increase in temperature resulted in the loss of nearly all enzyme activity. These results are consistent with the idea that dryer enzymes are more thermally stable. Furthermore, at constant ambient temperatures, production increased dramatically with an increase in relative humidity, confirming the idea that an increase in water increases catalytic activity.

A mass balance performed on reactors at higher relative humidity revealed that hydrolysis (rather than transesterification) of the ester was occurring. These hydrolysis rates should be determined separately from transesterification rates at all combinations of temperature and relative humidity. This would allow for the determination of transesterification rates

independent of hydrolysis and enable more accurate comparisons. An optimal combination of temperature and relative humidity for this reaction system could then be determined.

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## Chapter I

### Background and Motivation

Biocatalysts, which include enzymes and whole microbial cells, are used industrially for a variety of processes, including baking, brewing, dairy processing, and pharmaceutical and specialty chemical manufacturing (Klacik, 1988). Enzymes are highly specific and very effective catalysts and can enable much higher reaction rates than most synthetically catalyzed reactions. In many instances, a single-step enzymatic catalysis can replace a multiple-step chemical synthesis (for example, production of optically pure isomers; Lalonde, 1997). In addition, the products of enzymatic catalysis can often be labeled as "natural," thereby commanding a much higher price than their synthetically produced counterparts (McCoy, 1998). These combined factors have created considerable interest in the area of enzymatic catalysis.

In nature, enzymes are known to function in completely aqueous environments. An enzyme's specificity is a result of its extremely complex, three-dimensional structure, formed by hydrogen, salt, and covalent disulfide bonds, as well as hydrophobic and hydrophilic interactions (Bailey and Ollis, 1986). Water is absolutely necessary for an enzyme's catalytic function, as it is a participant (directly or indirectly) in all such bonding, and its removal distorts the enzyme structure, rendering it inactive. It is therefore not surprising that, for the greater part of the last century, studies in enzymatic catalysis have used water as the sole reaction medium (Zaks and Klivanov, 1985).

### Nonaqueous Biocatalysis

Since the early 1980s work in the area of nonaqueous biocatalysis has revolutionized the way we think about enzymatic catalysis. Nonaqueous biocatalysis, typically defined as reactions in which biocatalyst, substrate, and product are all present in an organic phase (Davison *et al.*, 1997), can include countless variations. Davison *et al.* (1997) discuss many of these, including

work in vapor and supercritical fluid phases. In many of these systems, a tightly bound shell of water may be retained around the enzyme molecule and is sufficient to maintain the catalytically active conformation of the enzyme (Klibanov, 1986).

The bulk of work in the area of nonaqueous biocatalysis has involved systems in which the biocatalyst, substrate, and product are present in an organic phase. Such systems offer many advantages over traditional aqueous-based systems (Zaks and Klibanov, 1985):

- Organic substrates are more soluble in organic media.
- Enzymes are insoluble in organic media and therefore more easily recovered and reused. Immobilization may not be necessary.
- Product recovery from aqueous solutions is typically more difficult and expensive than from organic solvents.
- New reactions, not possible in water due to thermodynamic equilibria or kinetic restrictions, may be carried out.
- Undesirable side reactions, such as hydrolysis of acid anhydrides and halogenates, are eliminated in the absence of water.
- Enzymes have been shown to exhibit increased thermal stability and altered substrate specificity in organic solvents

As mentioned above, nonaqueous biocatalysis also includes vapor phase systems, in which substrates are delivered and products recovered completely in the gaseous phase. For those situations in which substrates are gases or can be vaporized, gas phase biocatalysis offers many of the same advantages as organic phase biocatalysis (mentioned above) as well as the following (Barzana et al., 1989):

- Diffusivities in the gas phase are orders of magnitude higher than in water, and increase exponentially with temperature (as opposed to linearly in water). Consequently, mass transfer limitations are virtually eliminated.
- Most gaseous substrates have a poor solubility in water (which decreases with increasing temperature) thereby limiting their concentrations. By contrast, the

concentration of substrates in the gaseous phase can be directly increased by increasing system pressure.

- Gas phase products and unreacted substrates can be easily separated and recovered by fractional condensation.

Additionally, immobilization and recovery of the biocatalyst are considerably simplified since the biocatalyst is in a solid state (Lamare and Legoy, 1993).

The potential for applications of gas phase enzyme catalysis is great. Barzana et al. (1987) suggested the use of dehydrated enzymes, along with appropriate chromogenic reagents, for the detection of toxic gases in air (e.g., carbon monoxide) or breath (e.g., alcohol). They also suggested industrial applications, which could include purification of gas streams and chemical modifications. Other potential applications include bioremediation of gaseous streams, analysis of gaseous mixtures, and gas phase biosynthesis (Russell and Yang, 1996).

First, however, a more fundamental understanding of gas phase enzyme catalysis is necessary. Specifically, the role of water and its effects on enzymatic activity and thermal stability merit further investigation.

## **The role of water**

### *Thermal Stability*

As mentioned above, in nonaqueous enzymatic systems, a tightly bound shell of water is retained around the enzyme molecule, which is thought to be sufficient to maintain the catalytically active conformation of the enzyme (Klibanov, 1986). On the other hand, water also plays a part in the reactions resulting in enzyme inactivation, particularly thermal inactivation.

Irreversible thermal inactivation of enzymes occurs, after reversibly unfolding, as a result of one or more of the following reactions (Klibanov, 1986):

- Formation of incorrect, scrambled structures.
- Destruction of disulfide bonds via  $\beta$ -elimination.
- Deamidation of asparagine and glutamine residues.

- Hydrolysis of peptide bonds at aspartic acid residues.

Water is a required for each of these processes. It follows then, that in the absence of an aqueous phase, the reactions that govern thermal inactivation will be limited, and the enzyme should become more thermally stable.

Indeed, Zaks and Klibanov (1984) confirmed the phenomenon of increased thermal stability of enzymes in organic media. In their study, various lipases were placed in mixtures of solvents with different water concentrations at 100°C and their activities were measured over time (*Figure 1*). In 100 percent water at 100°C, the enzyme lost its activity almost immediately. In a tributyrin-heptanol mixture containing 0.8 percent water, thermal stability was significantly increased. When the solvent water content was reduced to 0.015 percent, thermal stability increased dramatically, with a half-life of more than 12 hours.

Increased thermal stability is advantageous for several reasons. The most obvious is the ability to operate at higher temperatures, thereby increasing reaction rates. Higher temperatures also help to eliminate the risk of microbial contamination, which results in the release of proteases that inactivate enzymes (Klibanov, 1986).

#### *Water activity, control and measurement*

In order to take full advantage of nonaqueous enzymatic catalysis, the dual role of water (enough to maintain activity, but as little as possible in order to exploit thermal stability) requires further elucidation. The concentration of water required by an enzyme for optimal catalytic activity varies with different systems, as well as with different enzymes. This makes comparisons between systems based on water concentration difficult. Water activity,  $a_w$ , is a thermodynamic parameter that has been utilized to more precisely correlate enzymatic water requirements with optimal catalytic activity. For example, in a study of organic phase biocatalysis by Valivety *et al.* (1992), *Mucor miehei* lipase showed a maximal enzymatic activity at  $a_w$  about 0.55 in various solvents (*Figure 2A*). Plots of catalytic activity vs. water concentration, on the other hand,

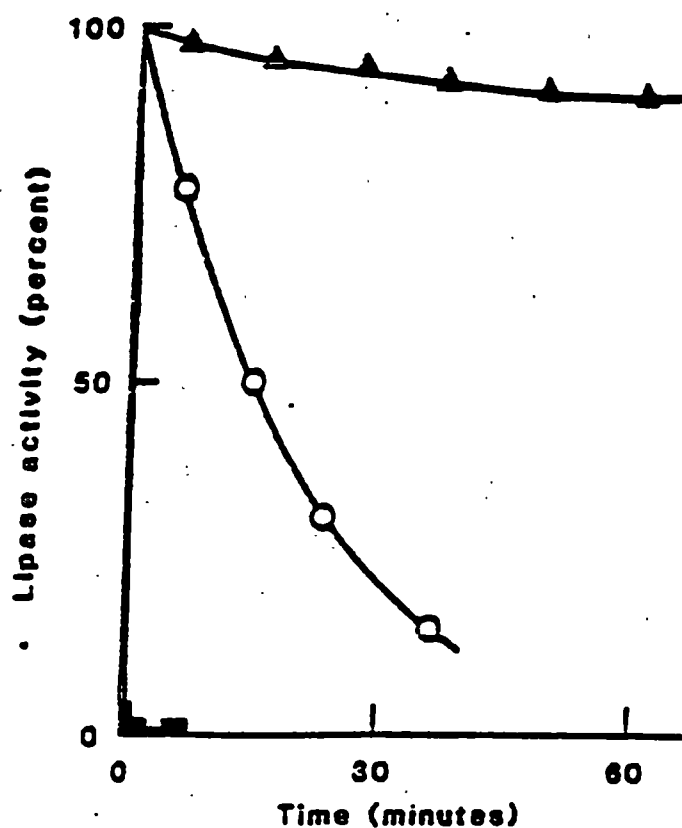
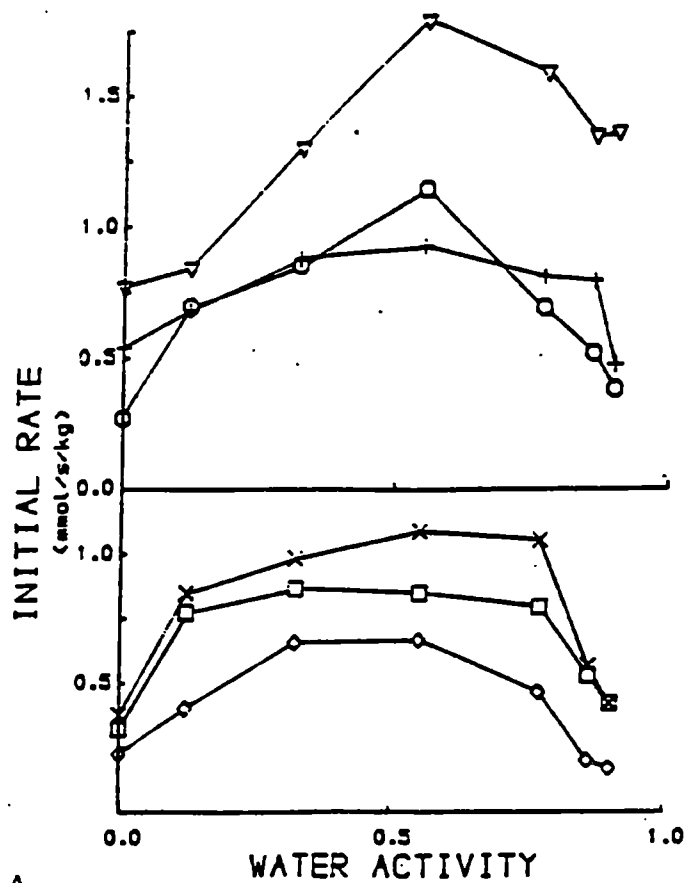
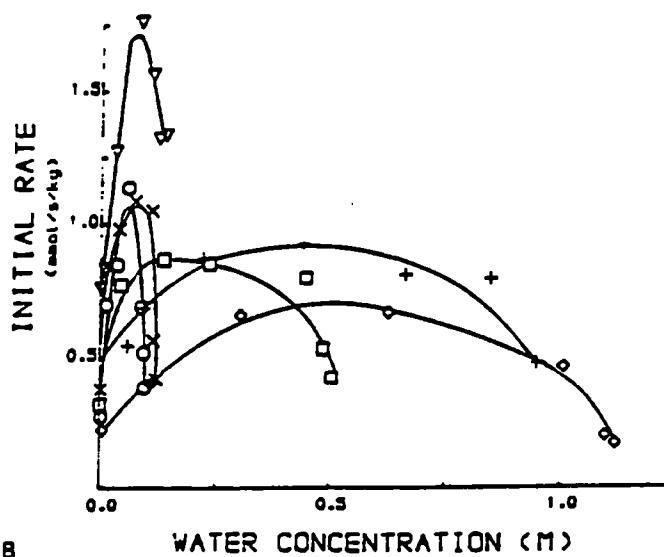


**Figure 1.** Time course of thermal inactivation at 100°C. Dried powder of porcine pancreatic lipase placed in water or 0.1 M phosphate buffer (pH 8.0) (■) and in 2M solution of heptanol in tributyrin containing 0.8 percent water (○) or 0.015 percent water (△). From Zaks and Klibanov, 1984.



A



B

**Figure 2.** Effect of water on activity of lipase catalyst in various solvents. (A) Presented in terms of water activity. (B) Presented in terms of water concentration in the organic phase. From Valivety *et al.*, 1992.

exhibited widely varying optima due to the differing solubility of water in the different solvents (Figure 2B).

Since  $a_w$  often reflects an unchanging optimum even as other aspects of a system are changed, it is important that this parameter be measured and/or controlled (Halling, 1994). Water activity may be measured in whatever phase is experimentally convenient; in a system at equilibrium, the activity of any component will be equal in each phase, although concentrations in each phase may be very different (Halling, 1994). Often the most convenient phase for measurement of  $a_w$  is the equilibrated gas phase, in which relative humidity ( $H_R$ ) is the most efficient measure of  $a_w$ . Halling (1994) reports that a number of sensors have been used successfully, although care must be taken with specific selection as some sensors can be adversely affected by certain organic vapors.

Halling (1994) has compiled several proven methods for control of  $a_w$ :

- Addition of preequilibrated silica gel to the reaction mixture.
- Preequilibration of all phases with saturated salt solutions of known  $a_w$ .
- Use of  $a_w$  sensors along with controlled drying or water addition.
- Addition of solid salt hydrate(s) to the reaction mixture.
- Equilibration of reactor headspace with saturated salt solutions of known  $a_w$ .

Wehtje *et al.* (1997) used semipermeable silicone tubing and saturated salt solutions along with various reactor configurations to successfully control  $a_w$ .

## Gas phase enzyme catalysis

There are several reports of attempts to optimize and account for the effects of temperature and  $a_w$  in gas phase systems. Barzana *et al.* (1989) explored the effects of  $a_w$  on the catalytic performance of alcohol oxidase acting on ethanol vapors. They found that enzyme activity increased by several orders of magnitude when  $a_w$  was increased from 0.11 to 0.97. At the same time, however, they found that thermostability decreased drastically. Parvaresh *et al.* (1992) used two different enzymes (porcine pancreatic lipase or PPL, and *Fusarium solani*

cutinase or PGS) to catalyze transesterification reactions between methyl propionate or ethyl propionate, and various alcohols. For the PGS, they found an increase in activity and a decrease in stability as  $a_w$  increased. However, they found the stability of PPL was not affected by  $a_w$ , hypothesizing that this was due to impurities present in the protein, creating a microenvironment able to retain water.

It is important to note that, in each of the above examples, no attempt was made to control  $a_w$  inside the reactor. Only the initial hydration level of the enzymes was controlled (see below). Since no water was added to the reactor, the initial water content of the enzyme decreased abruptly at the start of the reaction, resulting in a rapid loss of activity initially (Lamare and Legoy, 1993). This decrease in water content was observed in transesterification reactions as well (Parvaresh *et al.*, 1992) even though water is not directly involved in transesterification reactions. To fully understand the behavior of such systems, it would be necessary to work at a controlled hydration level inside the reactor, throughout the course of the reaction.

In each of the above cases, the initial water content of the enzymes was controlled by equilibration of dry enzyme powder over saturated salt solutions. However, Partridge *et al.* (1996) found that the catalytic activity of certain dried enzyme preparations could not be significantly improved upon equilibration over saturated salts. Instead, high catalytic activity could only be recovered upon returning the enzyme preparations to an aqueous buffer. Such a hysteresis effect may account for the observations of PPL activity found by Parvaresh *et al.* (1992), above.

Russell and Yang (1996) controlled water throughout the reaction (although not initial enzyme hydration) of allyl alcohol and butyraldehyde vapors catalyzed by alcohol dehydrogenase from baker's yeast (YADH). In their experiments, humidified gaseous substrates were passed over a dried biocatalytic plug (initial  $a_w = 0$ ) and changes in concentration of substrate, product, and gas humidity were measured. Activity was found to increase with increasing temperature and  $a_w$  over a temperature range of 22-50 °C and a relative humidity (of the inlet gaseous stream) range of 50-100 % (Table 1). Barzana *et al.* (1987) showed comparable results over similar ranges of temperature and enzyme hydration for a system of alcohol oxidase used to

**Table 1.** The effect of humidity on YADH performance. Modified from Russell and Yang; 1996.

| Temperature, °C              |  |  |    |    |     |    |    |     |    |    |     |
|------------------------------|--|--|----|----|-----|----|----|-----|----|----|-----|
| 22                           |  |  | 30 |    |     | 40 |    |     |    |    |     |
| Relative Humidity, %         |  |  | 50 | 85 | 100 | 50 | 85 | 100 | 50 | 85 | 100 |
| Conversion (steady state), % |  |  | 22 | 29 | 33  | 29 | 33 | 50  | 44 | 51 | 65  |
|                              |  |  |    |    |     |    |    |     | 56 | 69 | 76  |

catalyze the gas phase reaction of ethanol with molecular oxygen. However, in this case, hydration of the protein was controlled only initially (via equilibration over saturated salts).

The following work represents a detailed investigation of the effects of relative humidity (or  $a_w$ ) and temperature on several lipase-catalyzed, gas phase transesterification reactions. In an attempt to account for and optimize these effects, temperature and  $a_w$  are controlled both inside reactors and throughout the course of the reaction. Both batch and continuous systems are studied. The goal of this investigation is to provide a more fundamental understanding of gas phase enzyme catalysis, and to further clarify and optimize the dual role of water (enough to maintain activity, but as little as possible in order to exploit thermal stability).

## Chapter II

### Materials and Methods

#### Enzyme immobilization

Crude porcine pancreatic lipase (Sigma) was immobilized onto glass wool by first dissolving/suspending the lipase in a 50 mM  $K_2HPO_4/KH_2PO_4$  phosphate buffer at pH 7. Glass wool was then saturated with this solution (3 g lipase : 15 mL buffer : 2 g glass wool). In order to predetermine the water activity of the enzyme, the wool was allowed to dry for approximately two days at room temperature under forced air in a constant relative humidity ( $H_R$ ) environment.

$H_R$ , and thus enzyme water activity ( $a_w$ ), was controlled using molecular sieves (Sigma, Type 5A) or saturated salt (JT Baker) solutions as follows:

|                     |                |
|---------------------|----------------|
| Molecular Sieve, 5A | $H_R = 00.0\%$ |
| Lithium Chloride    | $H_R = 11.3\%$ |
| Magnesium Chloride  | $H_R = 33.0\%$ |
| Sodium Chloride     | $H_R = 75.3\%$ |

Solutions of saturated salts continuously absorb or release water vapor, thereby keeping a constant humidity within a closed space (Svensson, *et al.*, 1994). A closed space was created using a transparent plastic bag, into which the wet glass wool and a pan containing the saturated salt solution were placed. A fan was used inside the bag to quickly drive off any free liquid (water droplets) in order to prevent bacterial contamination of the enzyme. Humidity inside this closed space was measured using a Testo 610 Humidity Sensor, calibrated using the Testo Control and Calibration Set for Humidity Sensors. The bag was sealed with a rubber band around the fan and sensor cords. A state of constant humidity to within  $\pm 0.1\%$  was reached after approximately one hour.

The packing dried to  $H_R = 0\%$  and  $H_R = 11.3\%$  appeared to be completely dry and was brittle. At  $H_R = 33.0\%$ , the packing was only slightly less rigid. At  $H_R = 75.3\%$ , the packing was

completely malleable and sticky to the touch. In order to determine the water content of the immobilized enzyme at each  $H_R$ , samples of approximately 100 mg packing were analyzed by Karl-Fischer titration (Mettler DL 18 Karl-Fischer Titrator) in which water is converted stoichiometrically in the presence of sulfur dioxide, methanol, and base by addition of iodine.

Results follow ( $\pm 3$  mg/g):

$H_R = 00.0\%$       22.3 mg  $H_2O$  / g packing

$H_R = 11.3\%$       26.9 mg  $H_2O$  / g packing

$H_R = 33.0\%$       31.5 mg  $H_2O$  / g packing

$H_R = 75.3\%$       134.0 mg  $H_2O$  / g packing

After drying, the wool was randomly packed into reactors such that known quantities of both wool and lipase were present.

## Reactor Types

### *Single Pass Column*

Columns 50 cm long having a diameter of 5 cm were operated in single pass mode for a period of approximately one month. Air at either room humidity or dry (pumped by suction through Type 5A molecular sieve) was used to sparge substrates (JT Baker, *Table 2*) in separate containers. The two vapor phase substrate streams were then combined and delivered to the top of the column. Total flow was 40 mL/min, measured at the column outlet using a bubble flow meter and stopwatch. The column was operated at ambient temperature (20-25 °C) and either room humidity ( $H_R = 55-65\%$ ) or dry ( $H_R = 0\%$ ). Inlet and outlet vapor phase samples were taken at regular intervals and substrate concentrations measured via gas chromatography (GC) as described below.

### *Continuous Recycle Columns*

Jacketed columns 15 cm long with a diameter of 1 cm were operated in continuous recycle mode (*Figure 3*). Ambient air was pumped (suction) through Type 5A molecular sieve to

**Table 2. System components.**

| Reactor type       | Reaction                        |                                |
|--------------------|---------------------------------|--------------------------------|
|                    | Substrates                      | Products                       |
| Single Pass        | Isoamyl Alcohol + Vinyl Acetate | Acetaldehyde + Isoamyl Acetate |
| Continuous Recycle | Isoamyl Alcohol + Ethyl Acetate | Ethanol + Isoamyl Acetate      |
| Batch              | Isoamyl Alcohol + Vinyl Acetate | Acetaldehyde + Isoamyl Acetate |

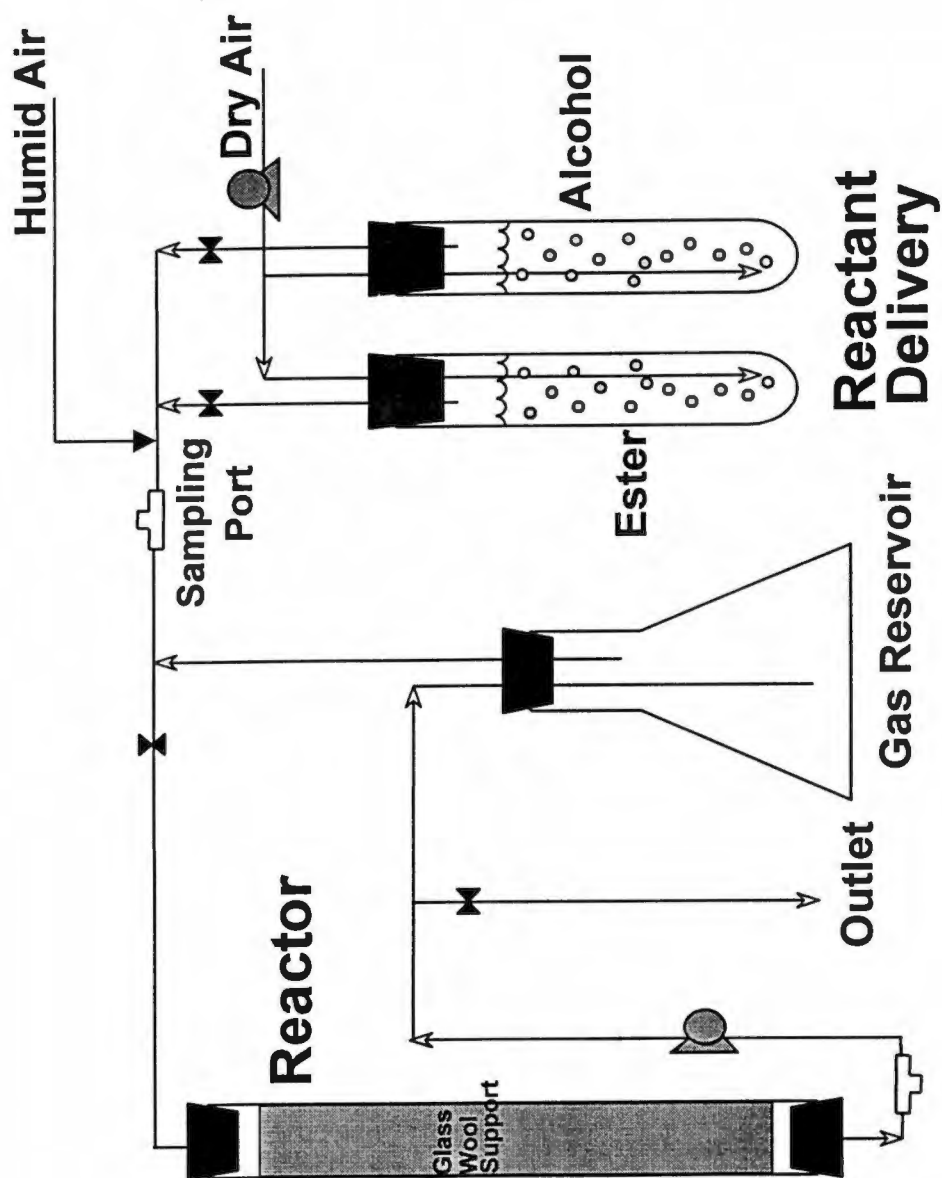


Figure 3. Schematic of continuous recycle experiments.

remove water vapor and used to sparge organic substrates. The resulting organic stream was then mixed with a humidified air stream in order to obtain an influent organic stream of known  $H_R$ , which corresponded to packing humidity, as mentioned above. Columns were packed with either immobilized lipase (1.5 g lipase on 1.0 g glass wool) or, as a control, clean and dry glass wool. Each was charged with the humidified organic stream for a period of approximately one hour, then closed off and operated in continuous recycle mode. A 250-mL Erlenmeyer flask was used in series to increase the entire system volume (*Figure 3*). Column temperature was controlled via a recirculating water bath connected to the column jacket. Vapor phase samples were taken at half-hour intervals and analyzed via GC, as described below.

#### *Batch Reactors*

Media bottles with a standard volume of 122 mL and serum closures were used for batch experiments. Reactors were loaded with immobilized enzyme (1.5 g lipase on 1.0 g glass wool) dried to a known humidity (or clean, dry glass wool as a control) along with a separate, upright 2 mL vial containing the corresponding saturated salt solution, as mentioned above. Reactors were then allowed to equilibrate overnight. Liquid reactants (5  $\mu$ L of each) were delivered through the stopper using a 25  $\mu$ L gas-tight syringe (Hamilton). Reactor temperature was controlled by immersion in a constant temperature water bath.

At designated time points, reactors were sacrificially sampled in the following manner:

- a vapor phase, headspace sample was taken
- in order to extract all system components for subsequent mass balance calculations, solvent (see below) was added, and shaken for 2 minutes; this effectively stopped any reaction by both stripping water from the enzyme, and by increasing diffusion limitations by at least three orders of magnitude
- a second vapor phase, headspace sample was then taken in order to verify the extraction of system components by the solvent from the vapor phase
- a 2 mL sample of the solvent phase was removed for analysis.

All samples were analyzed via GC as described below.

## **Extractive Solvent Selection**

Several solvents (*Table 3*) were examined in order to select an appropriate extraction solvent for batch reactors and subsequent mass balance calculations. Tests were performed to gauge the reactivity of solvents with the system components, and to determine optimum separation of the solvents from system components using GC.

In order to determine potential solvent interactions with system components, 20  $\mu\text{L}$  each of reactants or products were added to 2 mL solvent in a 2 mL autosampler vial with screw on caps and Teflon septa. Samples were analyzed using GC (see below) over a period of 48 hours for evidence of both forward and reverse reactions (*Table 3*).

Solvents were also evaluated on the basis of their chromatographic retention times, in order to select a solvent whose peak would not obscure peaks of system components.

Based on reactivity tests and chromatographic separation, benzene was selected as the best extraction solvent for batch reactors and subsequent mass balance calculations.

## **Analytical Methods**

All samples were analyzed using a Hewlett-Packard 5890 gas chromatograph (GC), with Hewlett-Packard 3365 Series II ChemStation Software, Version A.03.34. The GC was equipped with a flame ionization detector (FID) and a 30 m x 0.53 mm capillary column (J&W DB-624). Vapor samples were taken using a 0.5 mL gas tight syringe supplied with a valve (Hamilton). Solvent samples were analyzed with an autosampler (Hewlett-Packard 7673). See *Table 4* for method information specific to each type of analysis (vapor or solvent).

Instrument calibrations were performed in both vapor and solvent phases individually for all system components (*Table 2*). For vapor phase calibrations, 5  $\mu\text{L}$  of the component of interest was injected into a sealed 122 mL media bottle through the stopper. After equilibrating for

Table 3. Extractive solvent-system interactions.

| Solvent              | Addition of: | Time (hr:min) | Reactants          |                       |                    | Products           |                       |                       |
|----------------------|--------------|---------------|--------------------|-----------------------|--------------------|--------------------|-----------------------|-----------------------|
|                      |              |               | Vinyl Acetate (uM) | Iso-amyl Alcohol (uM) | Acet aldehyde (uM) | Acet aldehyde (uM) | Iso-amyl Acetate (uM) | Iso-amyl Acetate (uM) |
| Methanol             | Reactants    | 0:00          | 9.4 E+04           | 4.5 E+05              | 1.6 E+03           | 2.5 E+01           |                       |                       |
|                      |              | 1:00          | 8.0 E+04           | 4.3 E+05              | 4.1 E+03           | 2.3 E+01           |                       |                       |
|                      |              | 3:00          | 8.2 E+04           | 4.4 E+05              | 1.1 E+04           | 5.5 E+01           |                       |                       |
|                      |              | 48:00         | 2.9 E+04           | 4.5 E+05              | 7.1 E+04           | 7.0 E+01           |                       |                       |
|                      | Products     | 0:00          | 1.9 E+02           | 1.5 E+04              | 1.5 E+05           | 5.6 E+04           |                       |                       |
|                      |              | 1:00          | 2.4 E+02           | 1.5 E+04              | 1.5 E+05           | 5.6 E+04           |                       |                       |
|                      |              | 3:00          | 4.1 E+02           | 1.1 E+04              | 1.5 E+05           | 5.6 E+04           |                       |                       |
|                      |              | 48:00         | 2.6 E+03           | 1.5 E+04              | 1.5 E+05           | 5.7 E+04           |                       |                       |
|                      |              |               |                    |                       |                    |                    |                       |                       |
|                      |              |               |                    |                       |                    |                    |                       |                       |
| Carbon Tetrachloride | Reactants    | 0:01          | 1.9 E+05           | 6.9 E+04              | 1.9 E+02           | 1.9 E+02           |                       |                       |
|                      |              | 1:40          | 2.3 E+05           | 1.0 E+05              | 1.6 E+02           | 2.6 E+02           |                       |                       |
|                      |              | 4:49          | 2.3 E+05           | 1.0 E+05              | 1.5 E+02           | 2.4 E+02           |                       |                       |
|                      |              | 26:12         | 2.3 E+05           | 1.0 E+05              | 1.2 E+02           | 3.9 E+02           |                       |                       |
|                      | Products     | 31:22         | 2.4 E+05           | 1.1 E+05              | 1.3 E+02           | 3.8 E+02           |                       |                       |
|                      |              | 48:24         | 2.3 E+05           | 1.0 E+05              | 1.5 E+02           | 4.1 E+02           |                       |                       |
|                      |              | 0:04          | 0.0 E+00           | 3.0 E+03              | 2.7 E+05           | 8.7 E+04           |                       |                       |
|                      |              | 1:32          | 0.0 E+00           | 1.3 E+03              | 2.7 E+05           | 9.2 E+04           |                       |                       |
|                      |              | 4:56          | 0.0 E+00           | 1.2 E+03              | 2.4 E+05           | 9.2 E+04           |                       |                       |
|                      |              | 17:59         | 0.0 E+00           | 7.9 E+02              | 1.6 E+05           | 8.8 E+04           |                       |                       |
| Cyclohexane          | Reactants    | 23:17         | 0.0 E+00           | 6.9 E+02              | 1.6 E+05           | 8.7 E+04           |                       |                       |
|                      |              | 40:19         | 0.0 E+00           | 5.1 E+02              | 1.6 E+05           | 8.8 E+04           |                       |                       |
|                      |              | 0:10          | 2.3 E+05           | 1.0 E+05              | 1.4 E+02           | 2.8 E+02           |                       |                       |
|                      |              | 1:41          | 2.2 E+05           | 1.0 E+05              | 1.4 E+02           | 2.8 E+02           |                       |                       |
|                      | Products     | 4:51          | 2.3 E+05           | 1.0 E+05              | 1.2 E+02           | 2.7 E+02           |                       |                       |
|                      |              | 26:16         | 2.2 E+05           | 1.0 E+05              | 9.9 E+01           | 2.8 E+02           |                       |                       |
|                      |              | 31:26         | 2.2 E+05           | 1.0 E+05              | 9.4 E+01           | 2.7 E+02           |                       |                       |
|                      |              | 48:29         | 2.1 E+05           | 9.9 E+04              | 1.1 E+02           | 3.1 E+02           |                       |                       |
|                      |              | 0:02          | 0.0 E+00           | 8.8 E+03              | 2.6 E+05           | 1.9 E+05           |                       |                       |
|                      |              | 1:32          | 0.0 E+00           | 3.9 E+03              | 2.7 E+05           | 8.2 E+04           |                       |                       |
| Benzene              | Reactants    | 4:54          | 0.0 E+00           | 2.8 E+03              | 2.5 E+05           | 8.0 E+04           |                       |                       |
|                      |              | 18:00         | 0.0 E+00           | 2.9 E+03              | 2.4 E+05           | 8.3 E+04           |                       |                       |
|                      |              | 23:05         | 0.0 E+00           | 3.9 E+03              | 2.4 E+05           | 8.3 E+04           |                       |                       |
|                      |              | 40:11         | 0.0 E+00           | 2.7 E+02              | 2.1 E+05           | 8.0 E+04           |                       |                       |
|                      | Products     | 0:04          | 0.0 E+00           | 4.2 E+03              | 2.8 E+05           | 8.9 E+04           |                       |                       |
|                      |              | 1:43          | 0.0 E+00           | 4.0 E+03              | 2.7 E+05           | 8.5 E+04           |                       |                       |
|                      |              | 5:03          | 0.0 E+00           | 2.7 E+03              | 2.6 E+05           | 8.4 E+04           |                       |                       |
|                      |              | 18:10         | 0.0 E+00           | 2.6 E+03              | 2.5 E+05           | 9.1 E+04           |                       |                       |
|                      |              | 23:17         | 0.0 E+00           | 1.7 E+03              | 2.4 E+05           | 9.2 E+04           |                       |                       |
|                      |              | 40:20         | 0.0 E+00           | 1.6 E+03              | 2.3 E+05           | 8.7 E+04           |                       |                       |
| Chloroform           | Reactants    | 0:06          | 2.3 E+05           | ns*                   | 1.3 E+02           | 2.5 E+02           |                       |                       |
|                      |              | 1:39          | 2.4 E+05           | ns*                   | 1.2 E+02           | 2.6 E+02           |                       |                       |
|                      |              | 4:50          | 2.3 E+05           | ns*                   | 9.8 E+01           | 2.4 E+02           |                       |                       |
|                      |              | 26:19         | 2.3 E+05           | ns*                   | 9.2 E+01           | 2.4 E+02           |                       |                       |
|                      | Products     | 31:31         | 2.3 E+05           | ns*                   | 9.6 E+01           | 2.5 E+02           |                       |                       |
|                      |              | 48:36         | 2.3 E+05           | ns*                   | 1.0 E+02           | 2.7 E+02           |                       |                       |
|                      |              | 0:04          | 0.0 E+00           | 4.2 E+03              | 2.8 E+05           | 8.9 E+04           |                       |                       |
|                      |              | 1:43          | 0.0 E+00           | 4.0 E+03              | 2.7 E+05           | 8.5 E+04           |                       |                       |
|                      |              | 5:03          | 0.0 E+00           | 2.7 E+03              | 2.6 E+05           | 8.4 E+04           |                       |                       |
|                      |              | 18:10         | 0.0 E+00           | 2.6 E+03              | 2.5 E+05           | 9.1 E+04           |                       |                       |

\*ns = not separable

**Table 4. GC analysis methods.**

|                            | Vapor     | Solvent   |
|----------------------------|-----------|-----------|
| InjectionType              | Splitless | Splitless |
| Injection Temperature (°C) | 200       | 200       |
| Detector Temperature (°C)  | 200       | 200       |
| Initial Temperature (°C)   | 75        | 35        |
| Initial Time (min)         | 5         | 3         |
| Ramp 1 rate (°C/min)       | 20        | 10        |
| Final Temperature (°C)     | 120       | 120       |
| Final Time (min)           | 3         | 1         |
| Ramp 2 rate (°C/min)       | —         | 20        |
| Final Temperature (°C)     | —         | 150       |
| Total run time (min)       | 10.25     | 14.00     |

approximately 30 minutes, vapor samples in increasing increments were taken using a 0.5 mL gas tight syringe equipped with a valve, and manually injected into the instrument. Solvent phase calibrations were achieved by preparing stock solutions consisting of 5  $\mu$ L of the system component diluted in a volumetric flask to 25 mL with solvent. Increasing volumes of the stock solutions were then further diluted with solvent in 2 mL autosampler vials, and samples were analyzed using the autosampler.

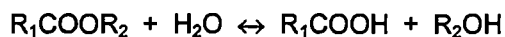
## Chapter III

### Results and Discussion

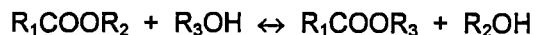
#### System Selection

As noted previously (*Table 2*), two different transesterification reactions were used to investigate the effects of temperature and water activity on enzymatic catalysis in gas phase systems. In both cases, crude porcine pancreatic lipase immobilized onto glass wool was the biocatalyst (see Materials and Methods).

These particular system components were chosen for several reasons. Lipase is a well-characterized enzyme, and is known to catalyze esterification and transesterification reactions in both aqueous and nonaqueous media (Zaks and Klibanov, 1984). Lipase is an esterase, part of a class of enzymes known as hydrolases, which catalyze hydrolysis reactions (Bailey and Ollis, 1986). Esterases carry out the cleavage of ester bonds (or synthesis, in the case of the reverse reaction) to yield an acid and an alcohol:



Or, in the case of a transesterification reaction, a different ester:



Glass wool was used as the immobilization matrix for the lipase, and was selected due to its high surface area, low cost and availability.

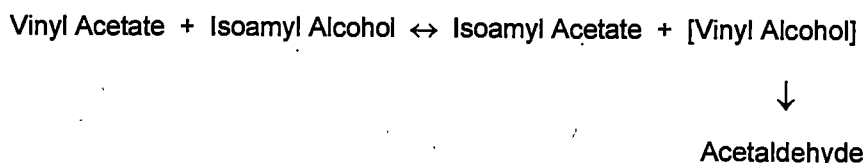
Isoamyl acetate, an ester commonly referred to as banana oil, can be produced using both esterification and transesterification reactions, and is used in many flavor and odor applications (Budavari, *et al.*, 1989). Production of this ester via a biocatalytic process could

allow end users to label the flavor enhancement as "natural," thereby increasing its commercial value (McCoy, 1998). Transesterification reactions were selected for analysis over esterifications based on previous work by Barton, *et al.* (1997), which indicated that 1) water produced by esterification reactions and 2) sorption of the acid substrate onto the enzyme, lowered the apparent pH of the enzyme, leading to irreversible denaturation.

Finally, enzymes are typically active only at ambient temperatures. It follows that an investigation of temperature effects on enzymatic systems should then include studies of systems at ambient temperatures. Therefore, in order to operate entirely in the gas phase, it was necessary to select substrates based on their capacity to be volatilized at relatively low temperatures.

### Single Pass Columns

Single pass columns were operated at room temperature (20-25 °C) and either room humidity ( $H_R = 55-65\%$ , Column 1) or dry ( $H_R = 0\%$ , Column 2). The following transesterification reaction was investigated for these columns (*Table 2*) in which vinyl acetate and isoamyl alcohol form isoamyl acetate and vinyl alcohol, which rapidly and irreversibly forms acetaldehyde :



In both columns, production of isoamyl acetate (*Figure 4*) and acetaldehyde (*Figure 5*) was observed (indicating that the enzyme remained active) for the duration of the experimental run, 28-36 days. This is in contrast to enzyme stability in the aqueous phase, which can be limited to a few hours (Russell and Yang, 1996). The variations in individual data points day-to-day are mainly due to daily adjustments in reactant delivery (refilling reactant reservoirs and replacing pump tubing as it became worn).

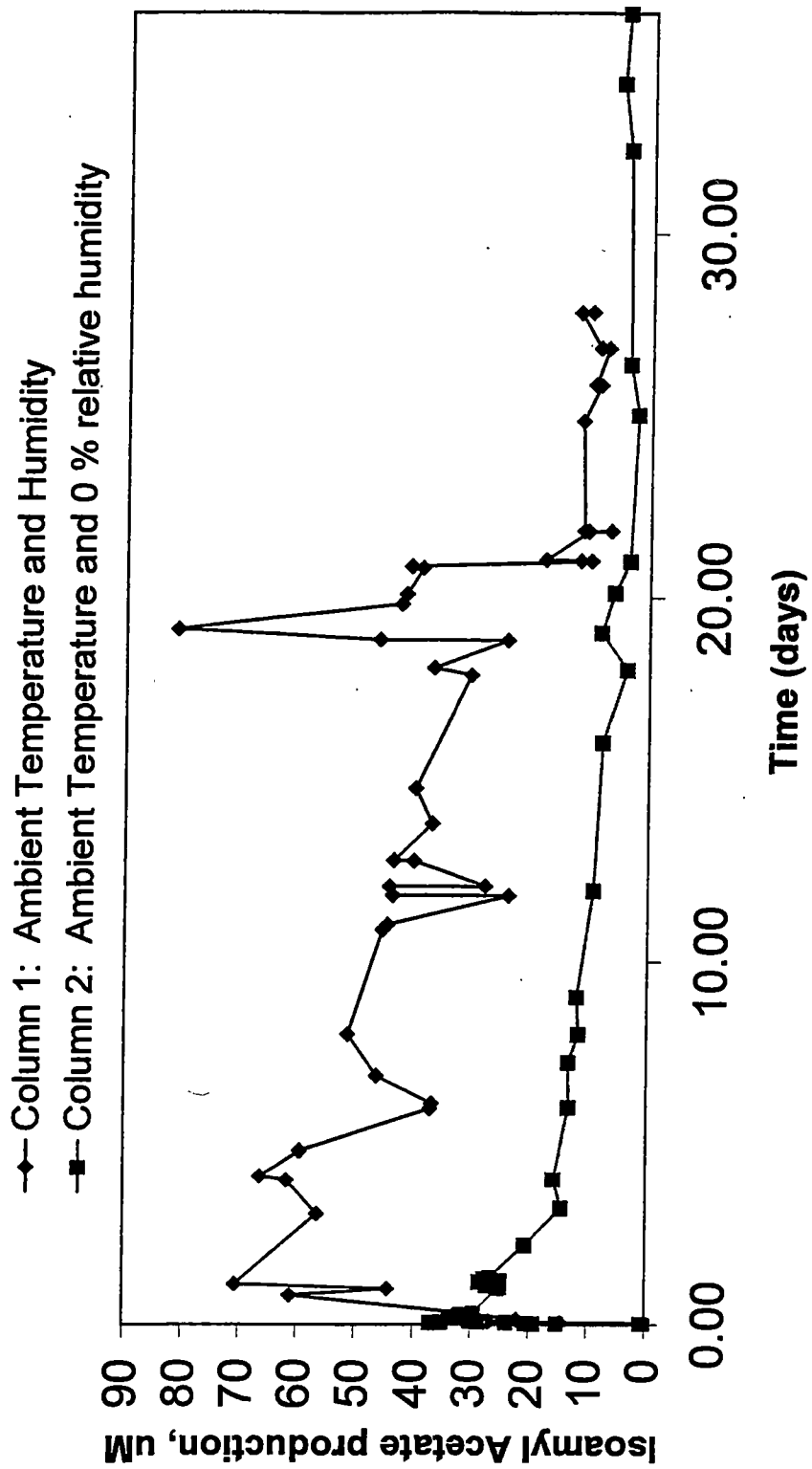


Figure 4. Production of Isoamyl Acetate in a single pass reactor.

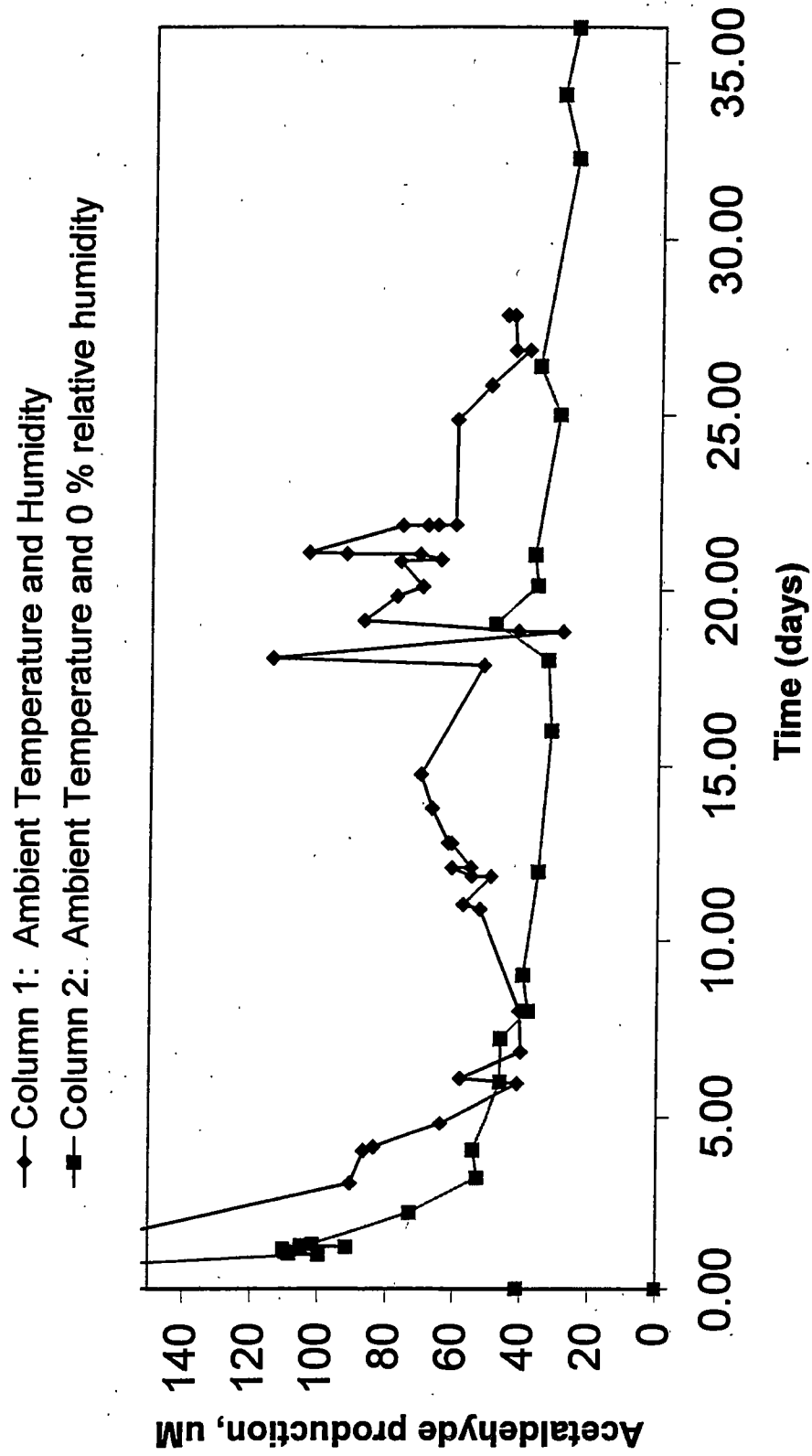


Figure 5. Production of Acetaldehyde in a single pass reactor.

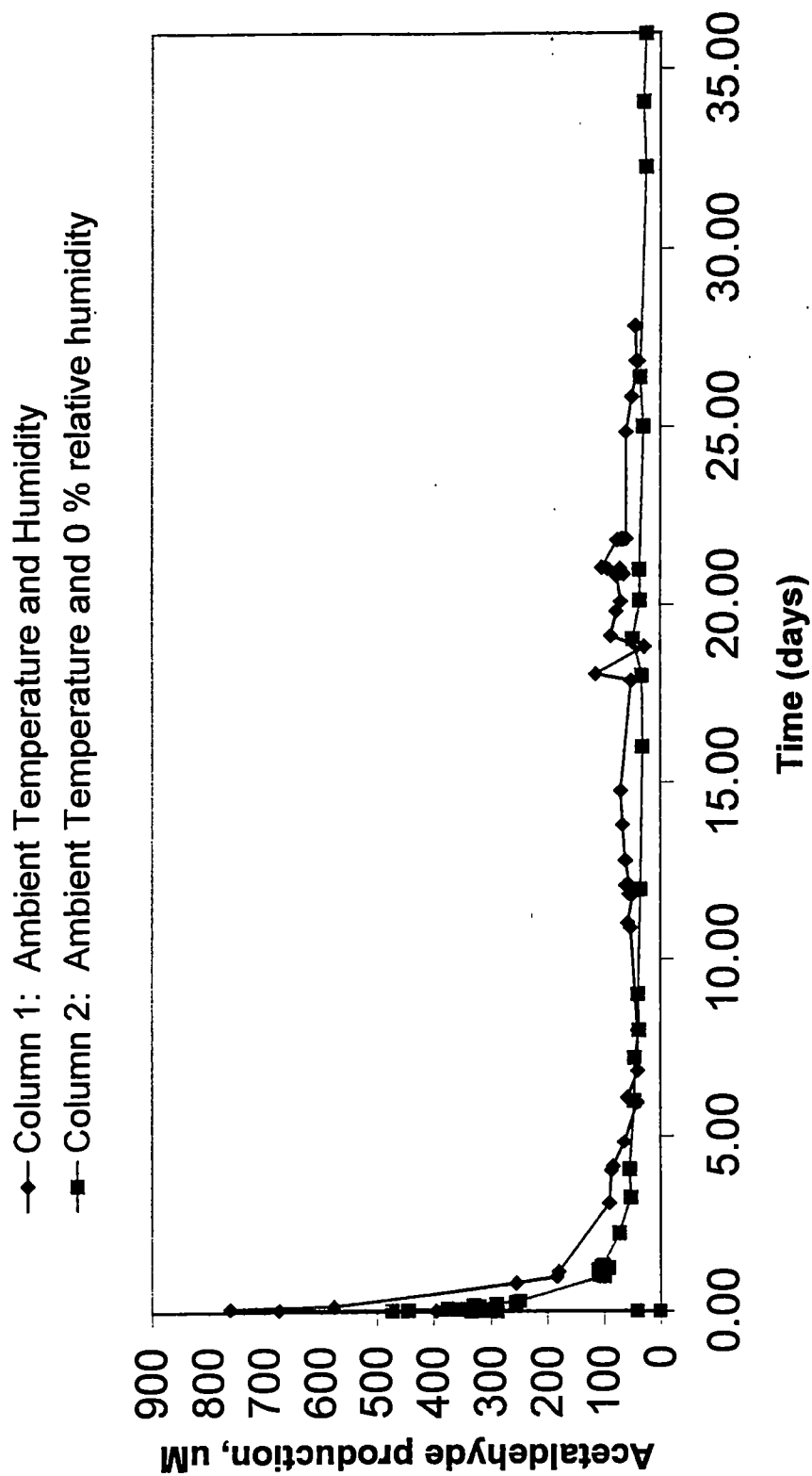


Figure 5 (continued). Production of Acetaldehyde in a single pass reactor.  
Initial "spike" of Acetaldehyde.

Although some water is present on packing even after drying to  $H_R = 0\%$  (see Materials and Methods) overall trends in *Figure 4* (which shows the production of isoamyl acetate for both columns) indicate that for Column 1 (ambient relative humidity) production is greater and drops off more slowly than in Column 2 ( $H_R = 0\%$ ). Production of acetaldehyde (*Figure 5*) is also greater when humidity is present in the gaseous stream. This is consistent with the idea that, in nonaqueous systems, there is an optimal amount of water necessary for enzymes to retain their maximum catalytic activity (Klibanov, 1986).

A color change was observed in the immobilized lipase packing down the length of both columns (*Figure 6*). In each case, the packing turned a pale pinkish-brown color, and was darker near the column outlet. This color change is probably due to the sorption of the system components to the column packing. Russell and Yang (1996) also observed a color change when using alcohol dehydrogenase to catalyze the gas phase reaction of allyl alcohol and butyraldehyde, and attributed this to reaction of the enzyme with the aldehyde products.

Another notable occurrence was the initial spike of acetaldehyde production, at over 10 times stoichiometric quantities (*Figure 5*). This occurred in both reactors, and while the phenomenon remains largely unexplained, a possible source could be production of acetaldehyde via hydrolysis of vinyl acetate (see below).

## Continuous Recycle Columns

In order to more thoroughly investigate the combined effects of temperature and water activity on gas phase transesterification, smaller columns (15 cm long with a diameter of 1 cm) were operated in continuous recycle mode at controlled temperatures and relative humidities. Reactors were charged with a reactant stream at controlled humidity, then closed off and the reactant stream was recirculated (*Figure 3*). In each case, a control column containing clean glass wool packing with no lipase was also run. No reaction was observed in these controls. The transesterification reaction for these columns was as follows (*Table 2*):

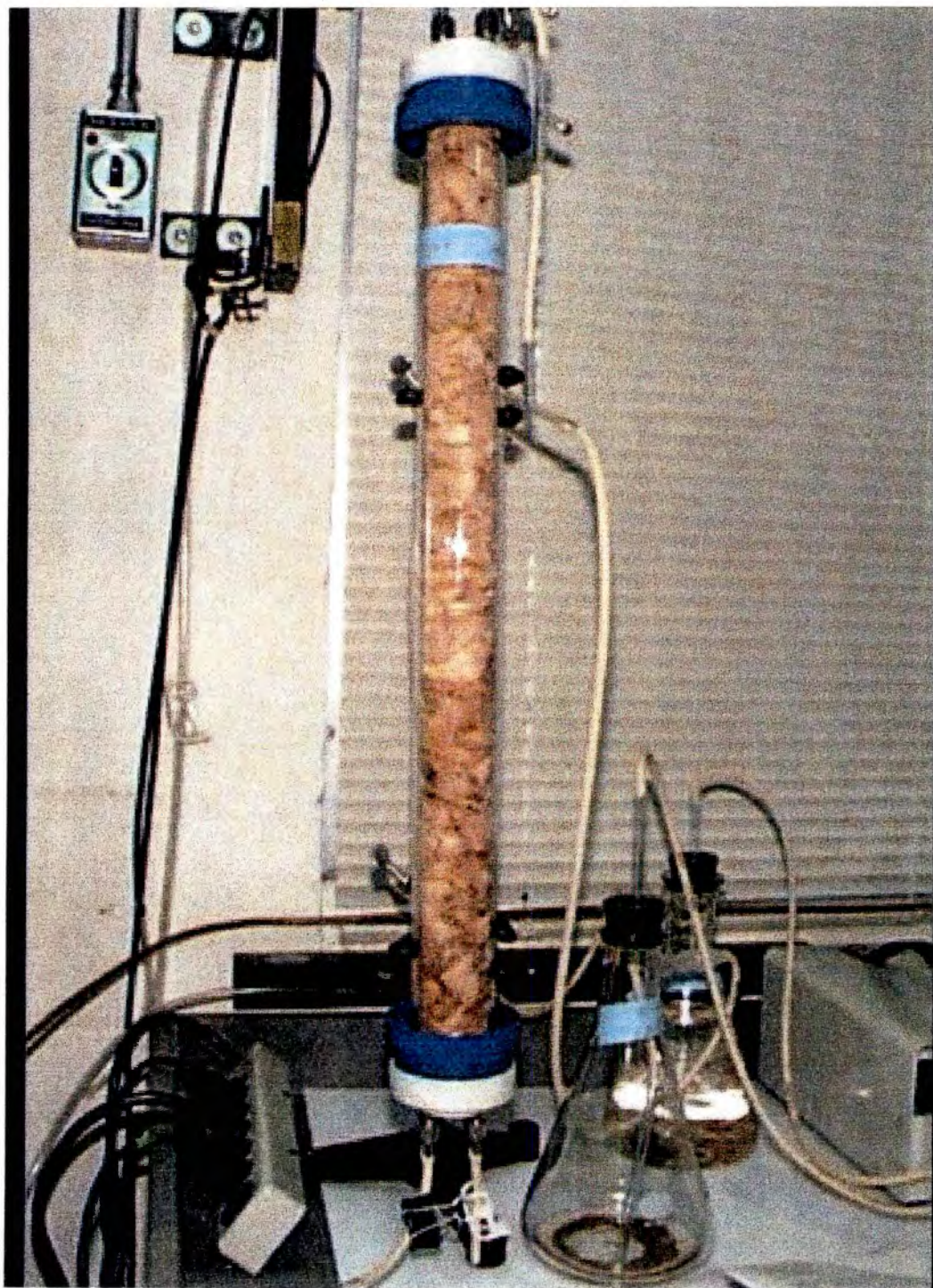


**Figure 6.** Color photograph of color change in a single pass column.



The columns were run at eight combinations of temperature and relative humidity (*Figure 7*), of which five (*Figure 8*) were selected for additional analysis. Initial substrate concentrations varied in each case, since inlet streams were controlled primarily for water content, or  $H_R$ , rather than organic vapor concentrations. For this reason, comparison on the basis of initial rates alone was not possible.

Assuming the reaction is second order, the rate of ethanol production is expressed as:

$$r_{\text{ethanol}} = k C_{\text{ethyl acetate}} C_{\text{isoamyl alcohol}} \quad (1)$$

However, since the concentration of ethyl acetate ( $C_{\text{ethyl acetate}}$ ) was more than 10 times the concentration of isoamyl alcohol ( $C_{\text{isoamyl alcohol}}$ ), for each case we can let:

$$k' = k C_{\text{ethyl acetate}} \quad (2)$$

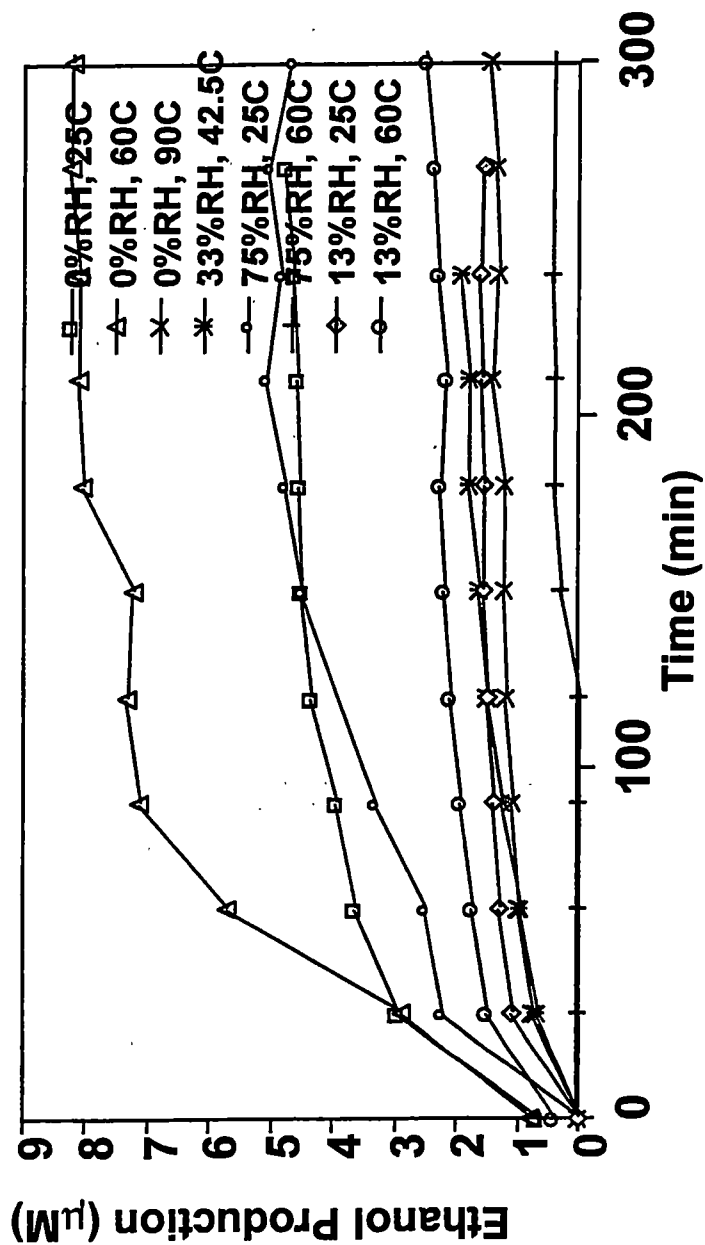
which leads to:

$$r_{\text{ethanol}} = k' C_{\text{isoamyl alcohol}} \quad (3)$$

This equation approximates the initial rate of reaction as pseudo first order. Based on this approximation, rate constants ( $k'$ ) were calculated for each set of experimental conditions in the following manner:

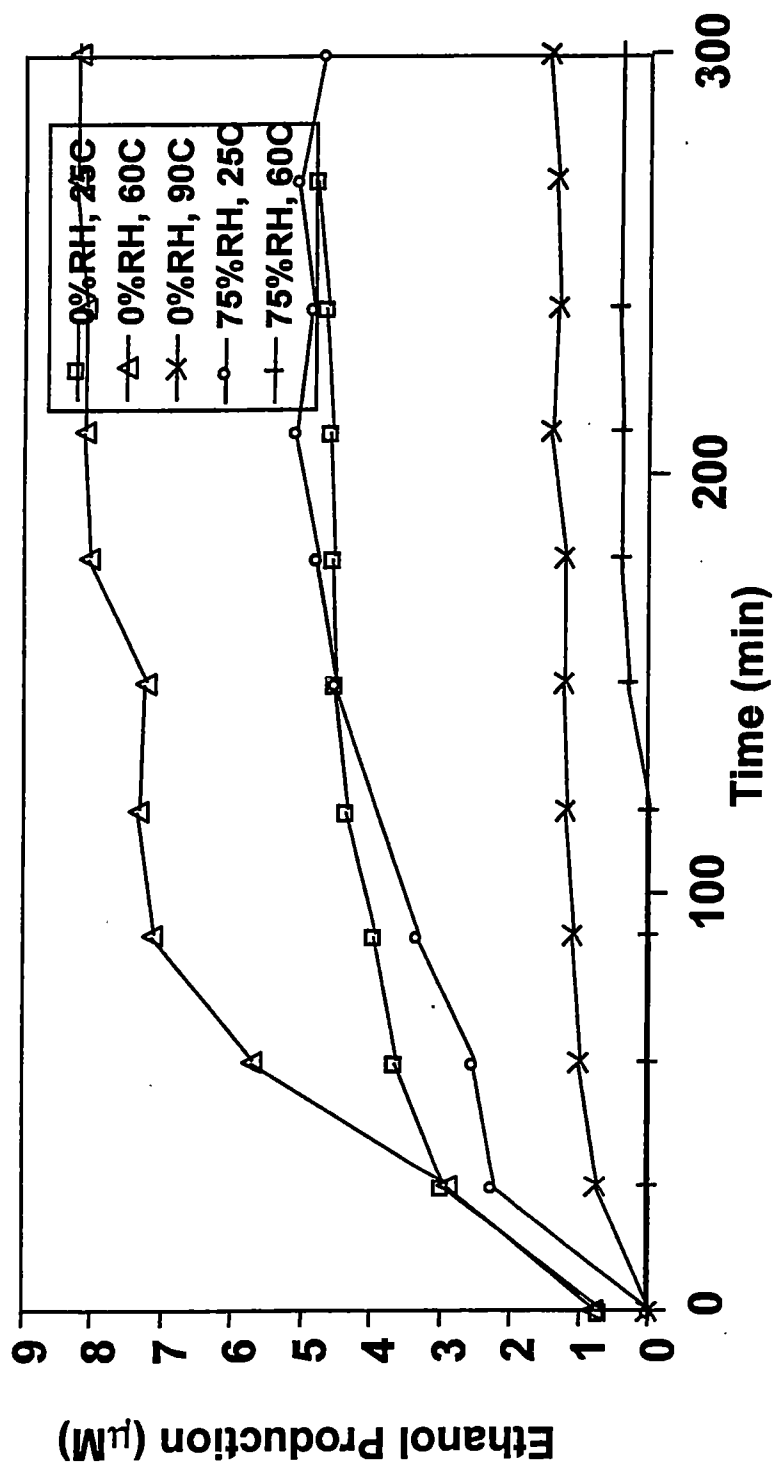
- Initial rate of ethanol production ( $r_{\text{ethanol}}$ ) was determined based on the first two data points, at 0 and 30 minutes
- Equation (3), above, was solved for  $k'$  using the average concentration of isoamyl alcohol ( $C_{\text{isoamyl alcohol}}$ ) over the first 30 minutes of reaction

Results are shown in *Table 5*.



Ethyl Acetate + Isoamyl Alcohol  $\rightarrow$  Ethanol + Isoamyl Acetate

Figure 7. Effects of relative humidity and temperature on ethanol production (all combinations).



**Ethyl Acetate + Isoamyl Alcohol → Ethanol + Isoamyl Acetate**

**Figure 8.** Effects of relative humidity and temperature on ethanol production (select combinations).

**Table 5. Pseudo first order rate constants.**

| Relative Humidity,<br>% | Temperature, °C | Rate Constant, k',<br>(1/h) |
|-------------------------|-----------------|-----------------------------|
| 0                       | 25              | 0.26                        |
| 0                       | 60              | 0.50                        |
| 0                       | 90              | 0.12                        |
| 75                      | 25              | 1.97                        |
| 75                      | 60              | 0.24                        |

At  $H_R = 0\%$ , values for  $k'$  increase when the temperature increases from  $25\text{ }^{\circ}\text{C}$  to  $60\text{ }^{\circ}\text{C}$ , dropping off only when the temperature is increased to  $90\text{ }^{\circ}\text{C}$  (although even at  $90\text{ }^{\circ}\text{C}$  the enzyme retains some activity). However, when water content is increased to  $H_R = 75\%$ ,  $k'$  falls dramatically with an increase in temperature from  $25\text{ }^{\circ}\text{C}$  to  $60\text{ }^{\circ}\text{C}$ , indicating that the "drier" enzyme is more stable at higher temperatures. These trends confirm the phenomenon of increased thermal stability of enzymes with decreasing water content. Conversely, at a constant temperature of  $25\text{ }^{\circ}\text{C}$ ,  $k'$  increases by more than one order of magnitude with an increase in  $H_R$ . Clearly, at this lower temperature, the increase in enzyme hydration leads to an increase in activity. As with the single pass results (above) this is consistent with the idea that, in nonaqueous systems, there is an optimal amount of water necessary for the enzyme to retain its maximum catalytic activity (Klibanov, 1986).

## Batch Reactors

### *Motivation*

While the data from continuous recycle reactors, above, were informative, there were concerns about sorption of system components to both the immobilized lipase and the immobilization matrix (glass wool) itself. Barton, *et al.* (1997) noted significant sorption of vapors to identical packing. In order to quantify this sorption and to perform a mass balance on the entire reaction system, batch reactors were studied. The batch reactors consisted of clear glass media bottles (122 mL) with gas-tight serum closures, loaded with immobilized lipase (or clean glass wool for controls) that had been equilibrated to a constant  $H_R$  over saturated salt solutions (see Materials and Methods section). A 2 mL vial containing the corresponding saturated salt solution was placed inside in order to keep a constant humidity within the reactor. Separate sacrificial reactors were used for each time point. The reaction was initiated by injecting substrates through the septa and into the vessel. The following transesterification reaction was investigated in these reactors (*Table 2*):



At designated time points, the headspace was sampled and then solvent was added in order to extract the entire reaction system, including reactor surfaces, packing and headspace. Mass balances were performed using values obtained from solvent extraction (total  $\mu\text{moles}$  of each component present in reactor). Sorption was quantified by subtracting headspace values from solvent extraction values:

$$\mu\text{moles sorbed} = \text{total } \mu\text{moles} - \mu\text{moles in headspace}$$

#### *Extractive Solvent Selection*

Reaction tests were performed on several different solvents in order to select one that would not react with the system components (*Table 3*). Reactants (vinyl acetate and isoamyl alcohol) or products (isoamyl acetate and acetaldehyde) were added to each solvent to check for evidence of either forward or reverse reaction.

Methanol was rejected as an extraction solvent since it rapidly reacted with vinyl acetate (reactant addition), forming acetaldehyde. Isoamyl acetate concentrations in carbon tetrachloride (reactant addition) appeared to be increasing over time. In cyclohexane, there was evidence of a decrease in isoamyl acetate (product addition). Chloroform was rejected because it was not separable from vinyl acetate via gas chromatography (see Materials and Methods).

Benzene was therefore selected as the extraction solvent. Concentrations of all components in benzene appear to be stable over time, with the exception of acetaldehyde, which decreases slightly after 18 hours. However, this was not significant since samples were typically extracted and analyzed within 18 hours.

## Results

Batch reactors at nine different combinations of temperature and  $H_R$  (*Table 6*) were utilized in an attempt to quantify sorption of system components, and to perform a mass balance. *Figure 9* shows typical results for low  $H_R$  experiments. For the 1:1 reaction (here, at  $H_R = 11\%$  and  $25^\circ\text{C}$ ), reactants (vinyl acetate and isoamyl alcohol) drop off at roughly the same rate as products (isoamyl acetate and acetaldehyde) are formed, indicating a closed mass balance. *Table 7* illustrates the sorption of each of the system components to the entire reactor, including the immobilized lipase packing. Both isoamyl alcohol and isoamyl acetate appear to sorb strongly, and controls indicate that most of the sorption is related to the immobilization matrix (glass wool) rather than the enzyme itself

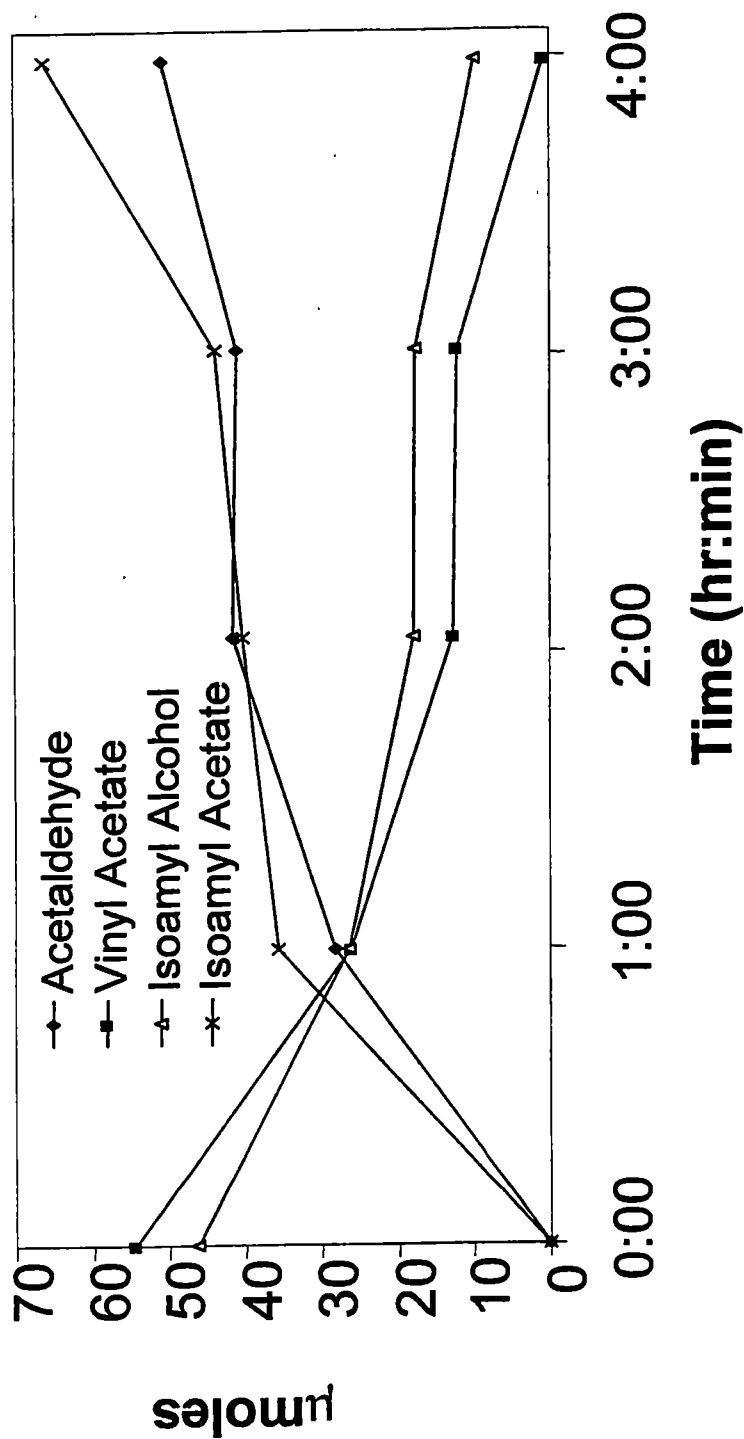
At higher values of  $H_R$ , an interesting trend was observed (*Figure 10*). In this case ( $H_R = 75\%$  and  $60^\circ\text{C}$ ) the vinyl acetate was completely gone after just 30 minutes, while the isoamyl alcohol had only partially reacted. Only minute quantities of isoamyl acetate were being formed. At the same time, a large amount of acetaldehyde was being produced. Results were similar at low temperatures. This lead to the conclusion that, when higher concentrations of water are present (i.e. at high values of  $H_R$ ), hydrolysis of vinyl acetate is favored over the transesterification:



In order to verify that hydrolysis was indeed occurring (and to what extent) batch reactors at  $H_R = 75\%$  (see Materials and Methods) and  $25^\circ\text{C}$  were injected with vinyl acetate only, and the reaction followed (*Figure 11*). In less than 20 minutes, 95% of the vinyl acetate had reacted, indicating that the hydrolysis reaction was extremely fast. The calculated rate for the disappearance of vinyl acetate ( $r_2$ ), using the first two data points (at zero and five minutes) was nearly  $1000 \mu\text{moles/hour}$ , almost an order of magnitude greater than rates for the overall transesterification reaction ( $r_T$ , see *Table 8*).

**Table 6.** Experimental conditions for batch reactors.

| Temperature,<br>°C | Relative Humidity, % |    |    |    |
|--------------------|----------------------|----|----|----|
|                    | 0                    | 11 | 33 | 75 |
| 25                 | x                    | x  | x  | x  |
| 60                 | x                    | x  | x  | x  |
| 90                 | x                    | —  | —  | —  |

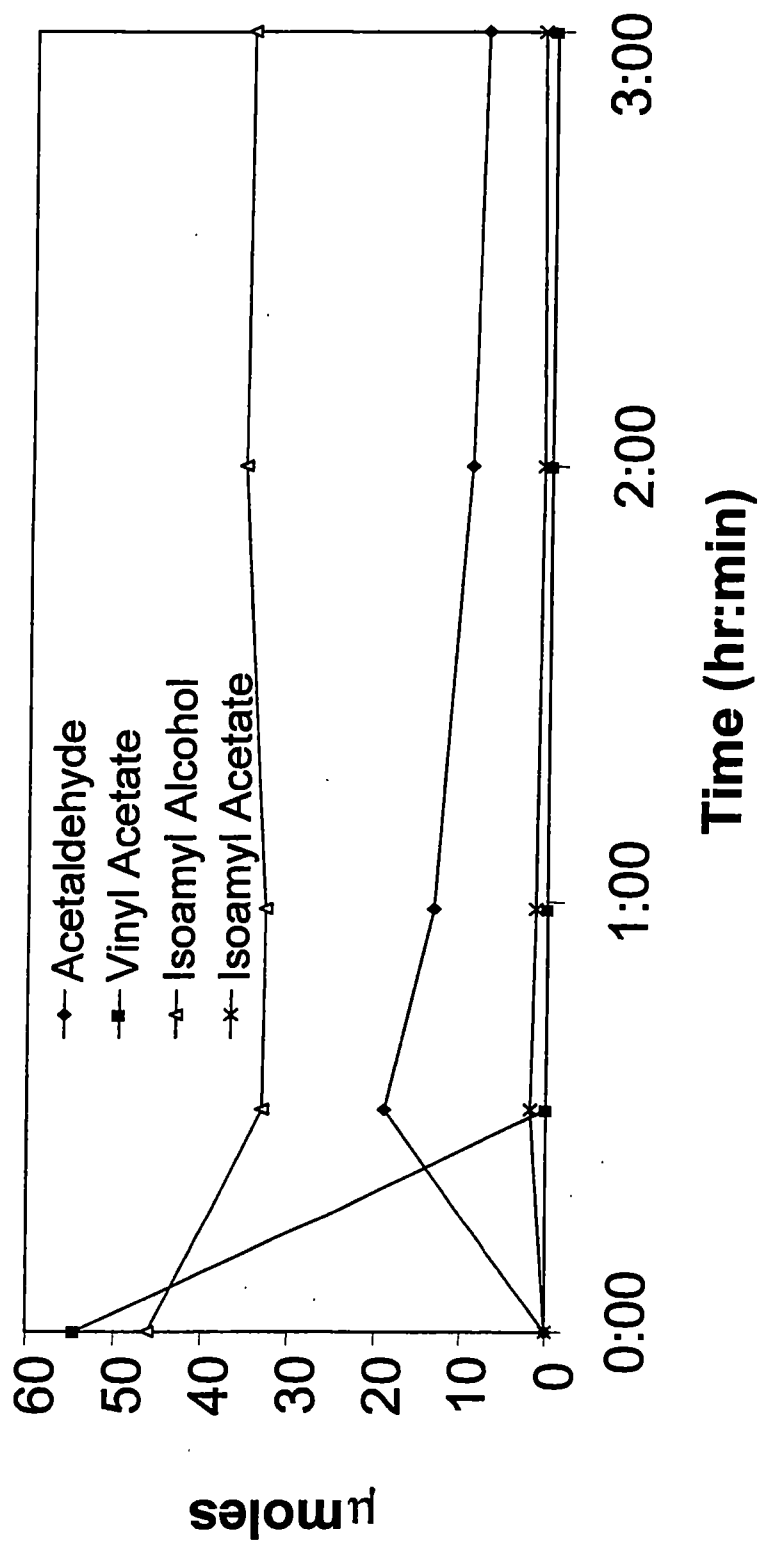


Vinyl Acetate + Isoamyl Alcohol → Isoamyl Acetate + Acetaldehyde

Figure 9. Batch reactors with benzene extraction, 11%  $H_R$  / 25 °C.

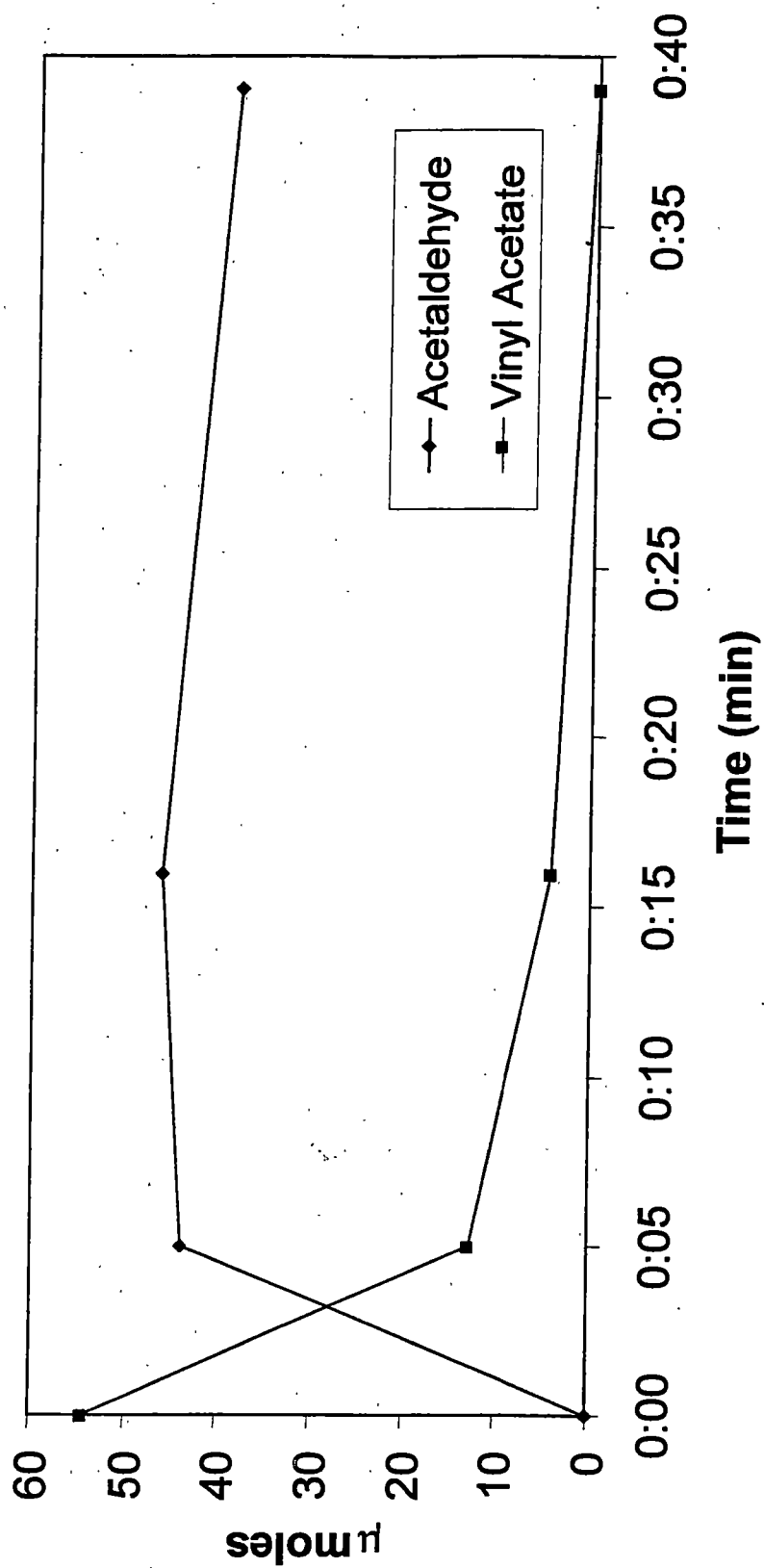
**Table 7. Sorption of system components to batch reactor and immobilized lipase packing. 11% H<sub>R</sub> / 25 °C.**

| Time, hr:min |                                   | Acetaldehyde | Vinyl Acetate | Isoamyl Acetate | Isoamyl Alcohol | Isoamyl Acetate |
|--------------|-----------------------------------|--------------|---------------|-----------------|-----------------|-----------------|
| 0:00         | Total in vapor phase, $\mu$ moles | 0.0          | 44.3          |                 | 5.8             | 0.0             |
|              | Total in reactor, $\mu$ moles     | 0.0          | 51.0          |                 | 44.7            | 0.0             |
|              | % sorbed                          | 0.0%         | 13.1%         |                 | 86.9%           | 0.0%            |
| 1:00         | Total in vapor phase, $\mu$ moles | 27.5         | 23.1          |                 | 8.5             | 17.4            |
|              | Total in reactor, $\mu$ moles     | 27.8         | 26.0          |                 | 26.1            | 35.3            |
|              | % sorbed                          | 0.9%         | 11.2%         |                 | 67.3%           | 50.7%           |
| 2:03         | Total in vapor phase, $\mu$ moles | 41.0         | 10.5          |                 | 7.0             | 25.0            |
|              | Total in reactor, $\mu$ moles     | 41.1         | 12.7          |                 | 17.2            | 38.8            |
|              | % sorbed                          | 0.1%         | 17.3%         |                 | 59.1%           | 35.5%           |
| 3:01         | Total in vapor phase, $\mu$ moles | 39.3         | 9.4           |                 | 5.9             | 23.6            |
|              | Total in reactor, $\mu$ moles     | 40.6         | 12.0          |                 | 16.8            | 41.3            |
|              | % sorbed                          | 3.2%         | 21.8%         |                 | 64.8%           | 42.8%           |
| 3:59         | Total in vapor phase, $\mu$ moles | 48.1         | 0.6           |                 | 3.8             | 23.5            |
|              | Total in reactor, $\mu$ moles     | 50.2         | 0.8           |                 | 9.5             | 65.2            |
|              | % sorbed                          | 4.3%         | 23.2%         |                 | 59.9%           | 63.9%           |



Vinyl Acetate + Isoamyl Alcohol  $\rightarrow$  Isoamyl Acetate + Acetaldehyde

Figure 10. Batch reactors with benzene extraction, 75%  $H_R$  / 60 °C.



Vinyl Acetate + Water  $\rightarrow$  Acetic Acid + Acetaldehyde

Figure 11. Hydrolysis of Vinyl Acetate, 75% H<sub>R</sub> / 25 °C.

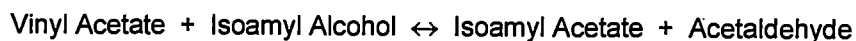
Table 8. Determination of transesterification rates.  $r_1 = r_T - r_2$ .

| Relative Humidity                                                                                    | Temperature (C) | $r_{T,VIAC}$                                             | $r_{2,VIAC}$                                                            | $r_{1,VIAC}$                             |                                          |
|------------------------------------------------------------------------------------------------------|-----------------|----------------------------------------------------------|-------------------------------------------------------------------------|------------------------------------------|------------------------------------------|
|                                                                                                      |                 | OVERALL REACTION                                         | HYDROLYSIS                                                              | TRANSESTERIFICATION                      |                                          |
|                                                                                                      |                 | Initial Overall Rate, $r_T$ ( $\mu\text{moles h}^{-1}$ ) | Specific Rate Constant, $k_2$ ( $\mu\text{moles}^{-1} \text{h}^{-1}$ )* | Rate, $r_2$ ( $\mu\text{moles h}^{-1}$ ) | Rate, $r_1$ ( $\mu\text{moles h}^{-1}$ ) |
| <u><math>r_2</math> DETERMINED OVER 5 MINUTES, WITH <math>k_2</math> TEMPERATURE DEPENDENCE:</u>     |                 |                                                          |                                                                         |                                          |                                          |
| 0%                                                                                                   | 25              | 116.38                                                   | 0.15                                                                    | 0.00                                     | 116.38                                   |
| 0%                                                                                                   | 60              | 215.70                                                   | 1.08                                                                    | 0.00                                     | 215.70                                   |
| 0%                                                                                                   | 90              | 46.20                                                    | 2.01                                                                    | 0.00                                     | 46.20                                    |
| 11%                                                                                                  | 25              | 48.89                                                    | 0.15                                                                    | 144.36                                   | -95.47                                   |
| 11%                                                                                                  | 60              | 96.39                                                    | 1.08                                                                    | 5687.87                                  | -5591.48                                 |
| 33%                                                                                                  | 25              | 105.02                                                   | 0.15                                                                    | 433.08                                   | -328.07                                  |
| 33%                                                                                                  | 60              | 198.18                                                   | 1.08                                                                    | 17063.61                                 | -16865.43                                |
| 75%                                                                                                  | 25              | 239.23                                                   | 0.15*                                                                   | 984.28                                   | -745.05                                  |
| 75%                                                                                                  | 60              | 245.73                                                   | 1.08                                                                    | 38780.92                                 | -38535.19                                |
| <u><math>r_2</math> DETERMINED OVER 39 MINUTES, WITH <math>k_2</math> TEMPERATURE DEPENDENCE:</u>    |                 |                                                          |                                                                         |                                          |                                          |
| 0%                                                                                                   | 25              | 116.38                                                   | 0.03                                                                    | 0.00                                     | 116.38                                   |
| 0%                                                                                                   | 60              | 215.70                                                   | 0.18                                                                    | 0.00                                     | 215.70                                   |
| 0%                                                                                                   | 90              | 46.20                                                    | 0.34                                                                    | 0.00                                     | 46.20                                    |
| 11%                                                                                                  | 25              | 48.89                                                    | 0.03                                                                    | 24.19                                    | 24.70                                    |
| 11%                                                                                                  | 60              | 96.39                                                    | 0.18                                                                    | 953.10                                   | -856.71                                  |
| 33%                                                                                                  | 25              | 105.02                                                   | 0.03                                                                    | 72.57                                    | 32.45                                    |
| 33%                                                                                                  | 60              | 198.18                                                   | 0.18                                                                    | 2859.29                                  | -2661.11                                 |
| 75%                                                                                                  | 25              | 239.23                                                   | 0.03*                                                                   | 164.93                                   | 74.30                                    |
| 75%                                                                                                  | 60              | 245.73                                                   | 0.18                                                                    | 6498.39                                  | -6252.66                                 |
| <u><math>r_2</math> DETERMINED OVER 39 MINUTES, WITH NO <math>k_2</math> TEMPERATURE DEPENDENCE:</u> |                 |                                                          |                                                                         |                                          |                                          |
| 0%                                                                                                   | 25              | 116.38                                                   | 0.03                                                                    | 0.00                                     | 116.38                                   |
| 0%                                                                                                   | 60              | 215.70                                                   | 0.03                                                                    | 0.00                                     | 215.70                                   |
| 0%                                                                                                   | 90              | 46.20                                                    | 0.03                                                                    | 0.00                                     | 46.20                                    |
| 11%                                                                                                  | 25              | 48.89                                                    | 0.03                                                                    | 24.19                                    | 24.70                                    |
| 11%                                                                                                  | 60              | 96.39                                                    | 0.03                                                                    | 136.16                                   | -39.77                                   |
| 33%                                                                                                  | 25              | 105.02                                                   | 0.03                                                                    | 72.57                                    | 32.45                                    |
| 33%                                                                                                  | 60              | 198.18                                                   | 0.03                                                                    | 408.47                                   | -210.29                                  |
| 75%                                                                                                  | 25              | 239.23                                                   | 0.03*                                                                   | 164.93                                   | 74.30                                    |
| 75%                                                                                                  | 60              | 245.73                                                   | 0.03                                                                    | 928.34                                   | -682.61                                  |

\* Experimentally determined at 75% RH and 25C; all other values adjusted for temperature increase.

### Rate Determinations

Two reactions contribute to the rate of disappearance of vinyl acetate in this reaction system—the reaction of interest, or transesterification ( $r_1$ ):



and the competing reverse esterification, or hydrolysis ( $r_2$ ):



For each combination of  $H_R$  and temperature (*Table 6*) the initial overall rate of disappearance of vinyl acetate ( $r_T$ ) was measured, where:

$$r_T = r_1 + r_2$$

The rate of transesterification ( $r_1$ ) at each combination of  $H_R$  and temperature can be determined by subtracting the rate of hydrolysis ( $r_2$ ) from the overall rate ( $r_T$ ):

$$r_1 = r_T - r_2$$

Then the corresponding rate constant ( $k_1$ ) can be calculated and comparisons between reaction conditions made.

Using  $r_2$ , and the average concentrations of water ( $C_{\text{Water}}$ ) and vinyl acetate ( $C_{\text{Vinyl Acetate}}$ ) over the first two data points (5 minutes, *Figure 11*), a specific rate constant ( $k_2$ ) was calculated for the hydrolysis of vinyl acetate:

$$r_2 = k_2 C_{\text{Water}} C_{\text{Vinyl Acetate}} \Rightarrow k_2 = r_2 / (C_{\text{Water}} C_{\text{Vinyl Acetate}})$$

The concentration of water corresponding to  $H_R = 75\%$  is 75% of the vapor pressure of water (at the temperature of interest) divided by the temperature and the gas constant (from the ideal gas law,  $PV = nRT \Rightarrow C = n/V = P/RT$ ). Since  $r_2$  was measured (as described above) at only one combination of temperature and  $H_R$  ( $H_R = 75\%$  and  $25^\circ\text{C}$ , *Figure 11*), attempts were made to correct for the effects of changes in water concentration and temperature as follows:

- Water concentrations were calculated at each  $H_R$  by taking an appropriate percentage of the saturated vapor pressure of water and dividing by the appropriate temperature and the gas constant, as described above.
- The specific rate constant ( $k_2$ ) was adjusted for each temperature based on a general "rule of thumb" in reaction kinetics, which states that the rate of reaction doubles for every  $10^\circ\text{C}$  increase in temperature (Fogler, 1992).
- At each combination of temperature and  $H_R$ , a new value for  $r_2$  was calculated using the temperature-adjusted  $k_2$  and the appropriate water and vinyl acetate concentrations:

$$r_2 = k_2 C_{\text{Water}} C_{\text{Vinyl Acetate}}$$

The rate of transesterification ( $r_1$ ) at each combination of  $H_R$  and temperature was then determined by subtracting the calculated hydrolysis rate ( $r_2$ ) from the measured initial overall rate ( $r_T$ ) as described above (*Table 8*).

As can be seen in *Table 8*, the above calculations resulted in extremely large numbers for the rate of hydrolysis,  $r_2$ , and consequently negative values of  $r_1$ . One explanation for this is the fact that the actual value for  $r_2$  (at  $H_R = 75\%$  and  $25^\circ\text{C}$ ) was measured over a much shorter time frame (5 minutes) than were the initial overall rates,  $r_T$  (15-60 minutes). Since all rates are minimum values, this means that the measured value for  $r_2$  is a far more accurate minimum than the measured values of  $r_T$ . However, even if the hydrolysis rate is calculated over a longer initial time (39 minutes), resulting in a less accurate minimum, values for  $r_1$  remain negative (*Table 8*).

Another source of error in the calculation of  $r_2$  may be the "rule of thumb" approximation for the temperature dependence of  $k_2$ . This was used as a best case estimate, but in reality holds only for very specific combinations of activation energy and temperature (Fogler, 1992). Therefore, this approximation of temperature dependence is most likely artificially inflating values of  $k_2$ . However, when this temperature dependence is removed, values for  $r_1$  remain negative, although less so (*Table 8*).

It is clear from the above calculations that, in order to accurately determine the rates of the transesterification reaction (independent of hydrolysis) at each  $H_R$  and temperature combination, the competing hydrolysis rates must also be experimentally determined at each combination of temperature and  $H_R$ . Furthermore, these rates should be determined over similar time spans so that their accuracies are comparable.

## Chapter IV

### Conclusions and Future Work

Several different reactor schemes were used to investigate the effects of temperature and water activity, or relative humidity, on lipase-catalyzed, gas phase transesterifications. In single pass reactors, considerable production was observed for a period of 28-36 days, indicating enzyme activity and stability well in excess of what is typical of aqueous systems. In addition, when some humidity was present in the gaseous stream, increased production was observed over reactors in which no humidity was present. This corroborates the fact that some water is necessary for enzymes to retain optimal catalytic activity.

Continuous recycle column reactors were used to more thoroughly investigate the combined effects of temperature and relative humidity in this gas phase system. Since one reactant was always in excess, a pseudo first order reaction was assumed, allowing calculation and comparison of specific reaction rates. At low relative humidity, reaction rates increased with temperature up to a point, although the enzyme retained some activity even at 90 °C. However, when relative humidity was increased, a similar increase in temperature resulted in the loss of nearly all enzyme activity. These results are consistent with the idea that dryer enzymes are more thermally stable. Concomitantly, at constant ambient temperatures, production increased dramatically with an increase in relative humidity. Again, this confirms the idea that there is an optimal amount of water necessary for an enzyme to retain its maximum catalytic activity. It is likely that these two competing factors can be optimized to achieve maximum production.

In order to perform a mass balance and to quantify sorption of system components to reactor packing, reactors were operated in batch mode and completely extracted with solvent. At low relative humidity, the mass balance was closed, and isoamyl alcohol and isoamyl acetate were found to sorb most strongly to the packing. Most of the sorption is related to the immobilization matrix (glass wool) rather than the enzyme itself, and alternatives should be

investigated. These could include plastic or Teflon wools, glass beads, or filtration membranes. Cross-linked enzyme crystals (CLECs) should also be investigated, as they are known to exhibit many of the same benefits as immobilized enzymes, such as increased activity and selectivity (Partridge *et al.*, 1998).

At high relative humidity (and therefore higher water concentrations), the reaction of the ester with water (hydrolysis) rather than the alcohol (transesterification) was favored. More data is needed in order to accurately determine the extent of these two competing reactions; hydrolysis rates should be determined separately from overall reaction rates at all combinations of temperature and relative humidity. This would allow for the determination of transesterification rates independent of hydrolysis and enable more accurate comparisons. An optimal combination of temperature and relative humidity for this reaction system could then be determined.

The ability to operate gas phase enzymatic systems at high temperatures is important if such systems are ever to have value industrially. High temperatures enable the volatilization of higher molecular weight (and typically more expensive) esters, alcohols and acids. Thermophilic enzymes would be ideal in such situations. There is also evidence that the cross-linking of enzymes (for instance with glutaraldehyde) improves thermal stability in nonaqueous systems (Bagi *et al.*, 1997).

Potential applications for gas phase catalysis extend beyond synthesis. Purification or bioremediation of gaseous streams is one such area. Another is the development of analytical devices and techniques that would allow for the direct determination of gases and vapors. Costly and time consuming sample pretreatments (such as absorption of gases onto solids, with subsequent extraction prior to analysis) could be avoided. A gas phase enzymatic reaction producing a corresponding color change (due to sorption of a product to an immobilization matrix, for instance) can be easily envisioned. Such devices could be used for the detection and destruction of toxic gases in closed environments.

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## Vita

Paula Ann Cameron was born in Tahlequah, Oklahoma, on March 17, 1970. She received her Bachelor of Science in Chemistry and Mathematics from Northeastern State University in December 1992. Upon graduation, she worked as an analytical chemist at the Oak Ridge National Laboratory in Oak Ridge, Tennessee, conducting research and development of bench and pilot scale activities related to the treatment of contaminants (such as VOC's, PCB's, heavy metals, and radionuclides) in water, soils and solids.

In addition to working full time, from January 1996 to August 1997 she attended the University of Tennessee, Knoxville, taking undergraduate courses in Chemical Engineering. In August 1997 she was accepted as a full time graduate student in Chemical Engineering at the University of Tennessee. As a member of the Biochemical Engineering Research Group, headed by Brian H. Davison, her research focused on gas phase enzyme catalysis.

Paula will graduate with a Master of Science in Chemical Engineering in May 1999 and has accepted a position with The Dow Chemical Company as member of their Research Assignments Program.