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IMMOBILIZED ENZYMES IN ORGANIC MEDIA: DETERMINANTS OF WATER DEPENDENCE

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INTERIM REPORT

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EXECUTIVE SUMMARY

The overall goals of our DOE-funded project are to investigate the critical factors that limit commercial scale applications of enzymes in organic solvents, and to scale-up a process for the production of a precursor to optically active polymers. Initially, we are attempting to quantify the effect of intra-particle dynamics on overall process performance. This involves examination of coupled enzyme kinetics, solubility, wetting and mass transfer effects toward support selection, reactor design/scheme and scale-up. For this study we selected a pure enzyme preparation: a lipase from *C. Viscosum*. The enzyme has been immobilized on celite and the catalyzed interesterification kinetics in petroleum ether and its dependence on water concentration have been determined. The results are very similar to those obtained in the past with crude *Pseudomonas Cepacia*. So far we have formulated a model for tripalmitin depletion, based on taking mass balances. A parameter has been included which accounts for the effect of water content inside the celite particle. The initial rate of depletion can be described by Monod-like kinetics. Our experimental data, at 0.003 mg H₂O per cm², fit a model and parameters presented in the literature. Because of the small size of the support, the relatively low amount of water, and the low reaction rates, our calculations predict that the lipase system at 0.003-0.01 mgH₂O/cm² is reaction rate limited. The mass transfer through the organic phase within the support particles is not limiting. In order to test the model, we studied the affect of particle morphology on the interesterification kinetics. Fully dense particles with varying diameter but the same total surface area have been tested and compared to the porous celite. The water content dependence with the fully dense particles suggest that mass transfer limitations due to inter-particle aggregation are the main cause of slowed down kinetics beyond a water level of 0.01 mgH₂O/cm². With porous supports such as celite, the onset of rate decrease occurs at a higher water content (0.03 mgH₂O/cm² for celite 560) as the inner volume of the pore accommodates more water, thus preventing it from accumulating on the outer particle surface and causing aggregation. The implications of these findings for reactor design are discussed.

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MILESTONE STATUS

The overall goals of our DOE-funded project are to investigate the critical factors that limit commercial scale applications of enzymes in organic solvents, and to scale-up a process for the production of a precursor to optically active polymers. The first 12 months of the project were to be dedicated to the complete understanding of low water systems and the mass transfer modelling with initial experiments on "dry" systems:

Task 1 Low Water System (completion in 18 months)

Demonstrate the applicability of the Biphasic theory on a new low-water system: pure enzyme, immobilized vs. suspended enzyme, covalent vs non-covalent immobilization.

Task 2 Mass-Transfer (completion in 24 months)

Investigate the mass transfer contributions to the observed kinetics experimentally and theoretically.

Task 3 "Dry" Systems (completion in 30 months)

Study the water dependence in "dry" systems with water content lower than the solubility in solvent.

After working on the project for eight months we are ahead of schedule in both Task 1 and 2, and on schedule on Task 3.

INTRODUCTION

In the past we have studied the effect of water content on the enzymatic interesterification of tripalmitin with stearic acid in a non-polar solvent, petroleum ether. A crude enzyme preparation, Amano P lipase, was immobilized non-covalently on a porous inorganic support. These studies yielded a correlation between the water dependent peak activity and the support particle's pore volume. The system was, in fact, a biphasic system with a large organic phase and a minute aqueous phase, in which we attributed the decline in enzyme activity at the higher water levels to mass transfer limitations. As this correlation was demonstrated for one crude enzyme, we felt that in order to support our case, we need to demonstrate that the same conclusions apply to other enzymes, to pure enzyme and to various enzyme configurations (Task 1). Also included in the initial target of this phase of the project has been to model mass transfer in the system and quantify the effect of inter- and intra-particle dynamics on overall process performance (Task 2). This involves examination of coupled enzyme kinetics, solubility, wetting and mass transfer effects toward support selection, reactor design/scheme and scale-up. A preliminary model of tripalmitin depletion has been formulated and shown to fit our experimental data, at very low water levels. In parallel, we have tested the interesterification rate dependence on water content with a pure lipase immobilized on non-porous particles. The results shed more light on the major factors controlling the overall reaction rate, and thus provide guidance towards reactor designs with maximal efficiency.

MATERIALS AND EXPERIMENTAL METHODS

Enzyme immobilization

Immobilization of the lipase onto the support was accomplished by deposition from a buffered solution. Typically, 5 mg lipase from *C. Viscosum* was dissolved in 1-2.5 ml of 0.005M potassium phosphate buffer. The volume of the enzyme solution depended on the support's pore volume, less was needed for non-porous supports. The enzyme solution was added to 500mg of 35-80 mesh support particles. A polystyrene petrie dish was utilized for preparation of the enzyme/support slurry, since in a glass container some of the enzyme adsorbs to the glass rather than the support. The mixture was air-dried in the refrigerator and then lyophilized, to give a dry support with 1% w/w enzyme loading (a different loading was used with sand as the support). This immobilization procedure was accurate within about 20%. Variation in the loading could be significant from one batch to another especially for supports which did not adsorb the crude enzyme as well as the better adsorbers.

Interesterification rate measurements

The rate of tripalmitin interesterification with stearic acid was monitored by Gas Chromatography, using a RSL-300 fused silica capillary column from Alltech, and a Perkin Elmer 8500 Gas Chromatograph equipped with a flame ionization detector, and PE Nelson 2600 Chromatography Software. All kinetic measurements were conducted with samples containing 4ml of petroleum ether, 10mg of supported lipase, 0.03M tripalmitin, 0.08M stearic acid, and 20 μ l of tetradecane or pentadecane, an internal standard for the GC analysis. All of the components were placed in a teflon/silicon capped vial at room temperature. At that temperature the tripalmitin and the stearic acid were insoluble in the organic solvent. The reaction was initiated by warming the mixture up to 45°C which caused the substrates to go into solution, and then adding the water. The water was injected into the sample and placed on the vial wall above the support particles. The mixture was then swirled gently to allow the water to migrate homogeneously into the support. The support particles never clumped in the process. The vials were placed on a shaker at 45 degrees Celsius between sampling times. Liquid samples were removed from the reaction mixture by capillary action at appropriate time intervals using approximately 2.4 cm sections of non-heparinized microhematocrit tubes. Each sample filled tube was immediately placed in a GC autosampler vial. The sample was then derivatized by first adding 780 μ l dry chloroform and 10 μ l N-methyl-N-(trimethylsilyl)-trifluoroacetamide to the autosampler vial. Next, the vial was sealed, vortexed to mix its contents, and allowed to incubate at room temperature for 20 minutes. The derivatizing agent reacted with the mono and di-glycerides to form their silyl ethers, and with palmitic and stearic acid to form their silyl esters. Each component was quantified using the appropriate standard. All triglyceride and diglyceride standards were obtained from Sigma. In one or two cases, where a standard could not be obtained or it was too contaminated to use, a response factor of a similar component was applied. The rate of stearate incorporation was calculated as the rate of change of the ratio of stearate moles incorporated into the di- and tri-glycerides, divided by the total moles of stearate and palmitate in the di- and tri-glycerides. Each chromatogram provided a value of S/Glycerides for a specific time.

Spectroscopic kinetic assays

Kinetic measurements of the lipase's esterase activity with p-nitrophenol-caproate (PNP-caproate) were performed spectrophotometrically in aqueous buffer, using Hewlett Packard's 8451A Diode-Array spectrophotometer. Release of p-nitrophenol was monitored at 400nm.

LOW WATER SYSTEMS: WATER DEPENDENCE STUDIES

Task 1

Pure Lipase Kinetics

We have studied the effect of water content on the enzymatic interesterification of tripalmitin with stearic acid in a non-polar solvent, petroleum ether. For this study we selected a pure enzyme preparation: a lipase from *C. Viscosum*. The enzyme was non-covalently immobilized on celite 560 by mixing the support with a buffered solution of concentrated enzyme, and drying it in air, followed by lyophilization. The loading level was 1% by weight. The interesterification kinetics of this enzyme in petroleum ether and its dependence on water concentration has been determined. The results are very similar to those obtained in the past with crude *Pseudomonas Cepacia*, confirming the generality of the phenomenon. The plots of the initial rate of stearate incorporation into the glycerides vs. water content for the two enzymes immobilized (deposited) on celite 560, are given in Figures 1 and 2. The maximal rate in both cases is obtained at a water level that reflects half filled pores. The differences in absolute rates are due to different loadings.

Controls

The interesterification reaction was attempted with all ingredients present except for the enzyme. No reaction was detected.

Some glycerol could be generated in the process as a by-product of hydrolysis. Theoretically, a significant concentration of glycerol in the water might increase the solubility of the glycerides and fatty acids in the aqueous phase, and thus affect the reaction rate. We have determined that no significant change of solubility is occurring in the presence of up to 60% glycerol, a concentration which is orders of magnitude higher than experimentally suggested.

Enzyme Adsorption to the Support

For the mathematical modeling of the mass transfer it is important to know where the enzyme is; whether it is adsorbed on the support side of the aqueous phase, dispersed (dissolved) in the bulk water or concentrated at the aqueous / organic solvent interface. We have so far concluded that under conditions which represent the kinetic experiments, namely, completely hydrated supported enzyme, no significant adsorption of lipase to the support surface occurs. This was determined by incubating various amounts of celite with a concentrated enzyme solution, removing the supernatant, and testing it for protein content and esterase activity. Both tests showed no change from the original enzyme concentration, even as the celite amount was high enough to make a very thick slurry, Figures 3 and 4.

Morphology Effects on Interesterification Rates

Initially, we had planned to try variations in the support particle size distribution and pore size distribution as a means for estimating the importance of microscopic geometry. Hence, mercury intrusion and SEM analysis have been performed on the celite 560 originally used as the microaqueous environment support. The results suggested that the pore size and shape distributions are very inhomogeneous. In order to simplify the analysis and at the same time separate intra-particle from inter-particle effects, non-porous particles were tested. We employed two supports: proprietary monodisperse silica microspheres with 1μ diameter, and sand with average diameter of 250μ . The weight of support used for kinetic experiments was chosen so that it will provide the same surface area as provided by 10 mg of celite 560. Surface area was estimated from the average particle diameter rather than measured, and therefore, might contain significant error. The CV lipase was immobilized on these supports with loadings that resulted in the same absolute enzyme concentration as in the celite experiments. Different techniques had to be developed for each of these supports. The 1μ microspheres do not settle and stay in solution in a very fine suspension. Thus they had to be dispersed and hydrated while sonicating. The heavy sand particles needed a special mixing arrangement to make sure they disperse properly. In both cases, despite the mixing, aggregation could be easily observed starting at a rather low water level: approximately $0.012 \text{ mgH}_2\text{O}/\text{cm}^2$, and resulting in a slurry above $0.03 \text{ mgH}_2\text{O}/\text{cm}^2$. This is not surprising since all the water added is on the outer surface. Initial rates of stearate incorporation into the glycerides was measured as a function of added water. The results are presented in Figures 5 and 6. The water content is expressed in weight per unit surface: mg/cm^2 . Figure 7 presents the celite data replotted on the same scale for comparison. It is clear that there is a direct correlation between the aggregation and the decrease in rate. The results clearly demonstrate, that with the fully dense particles, mass transfer limitations due to inter-particle aggregation are the main cause of slowed down kinetics beyond a water level of $0.01 \text{ mgH}_2\text{O}/\text{cm}^2$. With porous supports such as celite, the onset of rate decrease occurs at a higher water content ($0.03 \text{ mgH}_2\text{O}/\text{cm}^2$ for celite 560) as the inner volume of the pore accommodates more water, thus preventing its accumulation on the outer particle surface and delaying aggregation. Intra-particle mass transfer through the aqueous phase is also an important contributor to the delayed decrease in interesterification rate in the porous particle case.

MODELING KINETICS AND MASS TRANSFER EFFECTS

Task 2

Mass Balance

Mathematical models can be used to predict the reaction yields and selectivity, and optimize reaction conditions. We will take an evolutionary approach to model building: We will start with simple models then add more detail if required for a more complete description. As a first step, we considered the mass balance of tripalmitin. The depletion of tripalmitin is an approximate indicator of the incorporation of stearic acid by lipase, especially in the initial stages of the reaction.

To demonstrate the mass balance approach to modeling, consider a balance on tripalmitin (TP). TP must travel from the bulk phase to the enzyme-containing celite. Assume that the organic bulk phase is well-mixed throughout the reaction period. Therefore, for a batch system, the accumulation of TP is equal to the depletion:

$$\frac{dC_p}{dt} = -v_{obs} \quad (1)$$

where C_p = concentration of tripalmitin (M)
 t = time (min)
 v_{obs} = observed reaction rate (M/min).

Each of the celite beads are surrounded by a static film of the organic phase. Since the beads themselves act as a sink for TP (through reaction), the concentration difference of TP in the bulk phase and at the support surface is the driving force for the mass transfer of TP through this static film:

$$v_{obs} = k_i a (C_p - c_p^s) \quad (2)$$

where k_i = mass transfer coefficient (cm²/min)
 a = external surface area of support (cm²)
 C_p^s = concentration of tripalmitin at surface (M).

Diffusion is the dominant mechanism of mass transfer through the organic phase inside the celite beads. The concentration profile of TP is a function of radial distance from the center, as well as time. Assume a uniform distribution of enzymes inside the support. The accumulation of TP inside the support then equals the diffusion of TP in the support minus the depletion of TP:

$$\frac{\partial C_{ps}}{\partial t} = \frac{1}{r^2} \frac{\partial}{\partial r} \left(D_e r^2 \frac{\partial C_{ps}}{\partial r} \right) - v' a_{sw} \quad (3)$$

where

- C_{ps} = concentration of tripalmitin inside support (M)
- r = radial distance from center of support (cm)
- D_e = diffusion coefficient (cm²/min)
- v' = reaction rate of TP inside support per active surface area (M/min/cm²)
- a_{sw} = active surface area (cm²)

The "active" surface area, a_{sw} , accounts for the effect of water content inside the celite support. When no water is present, the enzyme is inactive and a_{sw} is zero. The enzymes become more active as water wets the pores, eventually reaching a maximum (corresponding to a maximum a_{sw}). "Activation" by water may be due to the solubilizing of the enzyme. The water-insoluble substrates cannot diffuse into the water-filled pores. So as water fills the pores in higher than optimal concentrations, less enzyme becomes accessible to the substrate, thereby lowering the observable enzyme activity. Also, since water-filled pores reduce the area of the organic/water interface, fewer enzymes will be available at the interface.

The active surface area can be estimated experimentally. Over time, a_{sw} depends on pore size distribution, pore shapes, wettability and surface energy of Celite; and enzyme activity, diffusion, and solubility. To obtain a theoretical expression for a_{sw} , a better understanding is needed of how the enzyme is distributed in the two liquid phases, at the liquid interface, and on the solid surface.

The effectiveness factor (η) is defined as ratio of the observed reaction rate to the reaction rate without mass transfer limitations. Use of η allows us to relate the intrinsic reaction rate with the observed reaction rate:

$$v' = \frac{v_{obs}}{\eta a_{sw}} \quad (4)$$

To complete the mass balance of tripalmitin (equations 1-4), we need to develop an expression for the intrinsic rate (v') in terms of concentrations and rate constants.

Reaction Rate Model

Miller et al. (1991) studied the kinetics of an unsupported lipase in cyclohexane plus 0.05% water to interesterify trilaurin with lauric acid. They found that esterification is three times faster than hydrolysis. After applying the appropriate corrections, the model and

parameters of Miller et al. can be used to estimate the reaction rate for our tripalmitin/stearic acid system. The following equations represent the hydrolysis step:

where E = enzyme, TP = tripalmitin, E-P = acyl-enzyme, P = palmitic acid, 1,2PP = dipalmitin. Before equilibrium is reached, all reactions proceed from left to right. Miller derived the rate for these reactions as

$$v = \frac{v_{\max}[TP]}{K_m + [TP]} \quad (5)$$

where

$$v = -\frac{d[TP]}{dt} \quad (6)$$

or the rate of tripalmitin disappearance. The square brackets represent concentrations (in M). Also:

$$v_{\max} = \frac{[E_0]k_2k_3}{K_2 + k_3} \quad (7)$$

where $[E_0]$ is the initial enzyme concentration. And

$$K_m = \frac{K_1k_3}{k_2 + k_3} \quad (8)$$

Adjusting for the different temperature, and for the different enzyme quantity and activity, the constants of Miller et al. become $K_m = 7.8 \times 10^{-3}$ M, and $v_{\max} = 2.46 \times 10^{-5}$ M/min. Figure 8 compares the experimental tripalmitin profile with the model prediction. Our tripalmitin profile fits the model for 10% hydration. Higher hydrations have faster rates. The Miller model describes unsupported enzymes containing only 0.06% water. Water may wet celite surfaces not containing enzymes. Therefore, we expect enzyme

solvation by water to be more efficient in an unsupported system such as Miler et al.'s. We will modify the model to account for these water effects.

Mass Transfer versus Reaction Rate Limited

Bailey and Ollis (1977) derived an "observable" Thiele modulus:

$$\Phi_{obs} = \frac{v_{obs}}{D_s S_0} \left(\frac{V_p}{A_p} \right)^2 \quad (9)$$

where v_{obs} = observed reaction rate (M/min)
 D_s = substrate diffusivity in the support (cm²/min)
 S_0 = initial tripalmitin concentration (M)
 V_p = volume of single support pellet (cm³)
 A_p = external surface area of support particle (cm²).

Φ_{obs} is a measure of reaction rate versus diffusion rate. High values of Φ_{obs} indicate diffusion limitations. The substrate diffusivity in hexane inside the support (D_s) depends on the porosity, which is controlled by the water content. For a liquid, the pore diffusivity in the support (D_s) and the free diffusion coefficient (D) are related by (Vermeulen, et al.):

$$D_s = \frac{D X_{eff}}{\tau} \quad (10)$$

where X_{eff} = the effective internal porosity (pore volume filled with organic phase/support volume)
 τ = tortuosity.

The tortuosity can be approximated as $1/X_{eff}$, giving

$$D_s = D X_{eff}^2 \quad (11)$$

The effective porosity depends on the hydration as follows:

$$X_{eff} = X - h\rho_s \quad (12)$$

where X = porosity (pore volume/support volume)
 h = hydration (g water/g support)
 ρ_s = piece density of support (g support/support volume)

The piece density of celite 560 is 0.87 g/cc and the porosity is 62%. At 30%

hydration ($h=0.3$), the effective porosity is 0.36. Please note that when the celite pores are completely filled with water, $h=0.71$ g/g, and the effective porosity becomes zero. This analysis is valid only when most of the reaction occurs inside the support, and not at the surface.

Assuming that trilaurin diffusivity in cyclohexane is on the order of tripalmitin in hexane, we can adjust the Miller et al. value for temperature to obtain $D = 4.76 \times 10^{-6}$ cm²/sec. At 30% hydration, $v_{\text{obs}} = 6.59 \times 10^{-5}$ M/min when $S_0 = 0.028$ M. For 30-80 mesh Celite, the average particle size is 0.0192 cm. These numbers give us a value of $\Phi_{\text{obs}} = 0.00262$. Such a low Thiele modulus means that the effectiveness factor is very close to one. Therefore, the observed reaction rate is nearly equal to the intrinsic reaction rate.

Because of the small size of the support, the relatively low amount of water, and the low reaction rates, our calculations predict that the lipase system at 10-30% hydration is reaction rate limited. The mass transfer through the organic phase is not limiting.

Conclusions

A Monod-type model for tripalmitin describes the initial reaction rate well. For longer times, the accumulation of other compounds must be considered. This can be accomplished by taking a mass balance on all of the significant products. The effect of water on enzyme activity and solubility must also be determined.

DISCUSSION

The above mathematical model deals with intra particle diffusion through the organic phase. It describes the kinetics for tripalmitin depletion, based on taking mass balances. A parameter has been included which accounts for the effect of water content inside the celite particle. The initial rate of depletion can be described by Monod-like kinetics. Our experimental data, at 0.003 mg H₂O per cm², fit a model and parameters presented in the literature. Because of the small size of the support, the relatively low amount of water, and the low reaction rates, our calculations predict that at low water levels, 0.003-0.01 mgH₂O/cm², interesterification will be hydrolysis reaction rate limited and not mass transfer limited. The mass transfer through the organic phase within the support particles is not rate limiting. Thus one expects to see no difference in the rate behavior between the porous and the non-porous supports at a water level below 0.01 mgH₂O/cm². Within experimental error the data at this water level is probably in agreement with the model's prediction. At higher water contents it is clear that there is a large difference between the porous and the non-porous supports. The experimental observation of aggregation in this region for the fully dense particles clearly accounts for this difference, and identifies the inter-particle aqueous pools as the cause for mass transfer limitations. With porous supports such as celite, the onset of rate decrease occurs at a higher water content (0.03 mgH₂O/cm² for celite 560) as the inner volume of the pore accommodates more water, thus preventing its accumulation on the outer particle surface and delaying aggregation. The various porous supports that we have tested in the past showed a correlation of the onset of rate decrease (or rate maximum) with the pore volume of the support (Figure 0). The higher the pore volume, the more water can be accommodated within the pores as compared to the outer surface, and the larger is the delay in rate decrease. In addition, as the water fills up the pores, intra-particle mass transfer effects are also expected.

A similar water dependence has been reported for suspended enzyme (Affleck, 1992). It is very probable that the same aggregation phenomenon due to added water is responsible for the rate decrease under those conditions as well. We will be testing free suspended enzyme in our system shortly.

The aggregation causes a major decrease in the interesterification rate. For practical applications, this could be overcome by appropriate reactor design. We will be examining various reactor designs that should eliminate this problem, i.e. various configurations in which the hydrophilic support particles are isolated from one another, either physically, or by effective fluidization.

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FIGURE 1

Intersesterification Rate vs. Water Content

CV Lipase on Celite 560

6332-117-2

FIGURE 2
INTERESTERIFICATION RATE VS. WATER CONC.
10mg Celite supported P.F. Lipase, 10/89

FIGURE 3

Enzyme Concentration vs. Amount Celite560

Determined by Biorad Assay

6058-136

FIGURE 4
PNPC Assay Determination of Celite Absorption of CV Lipase

FIGURE 5

Interesterification Rate vs. Water Concentration

1% CV lipase loaded 1 μ silica spheres

FIGURE 6
Interesterification Rate vs. Water Concentration

0.004% CV Lipase loaded Sand

6058-159

FIGURE 7
Interesterification Rate vs. Water Concentration

1% CV Lipase loaded Celite 560

6332-117-2

FIGURE 8

Lipase on Celite in Hexane

Tripalmitin Profile

Hydration
(g water per
g support,
%):

■ 10

▲ 20

● 30

— 0.06 (Miller et al.)