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IMMOBILIZED ENZYMES IN ORGANIC MEDIA:

DETERMINANTS OF WATER DEPENDENCE

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PROGRESS STATEMENT

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EXECUTIVE SUMMARY

The overall goals of this 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 a specialty polymer. The overall performance of an immobilized enzyme can be influenced by its intrinsic structure and by external factors such as water content, support, pH, etc.. We have investigated the interrelation between support morphology and water content, and its effect on overall enzyme performance.

Using a lipase catalyzed inter-esterification reaction as a model, we studied the controlling factors when water content in the organic solvent is such that a micro-aqueous phase is formed. In such an environment it was found that support particle aggregation is the major cause for decline in enzyme activity. We have shown that particle porosity, as well as the use of a particular non-woven fabric as an enzyme support, could alleviate this problem. These findings are being translated into a bioreactor design.

We have also studied two "dry" non-aqueous systems, where a water phase is not formed since the water content is below its solubility in the organic solvent. In one of the systems, Subtilisin catalyzed trans-esterification of vinyl acrylate with a chiral alcohol, we have demonstrated that the use of a proprietary fabric support provides a significant boost in enzyme activity. We suggest that this particular fabric with its hydrophilic fibers acts as a lyoprotectant in the process of drying the enzyme. The benefits of this material as an enzyme support and its use in a lab scale bioreactor are being studied.

Preliminary experiments have also been performed with a second "dry" reaction. This is the lipase catalyzed synthesis of AlliedSignal's new product, VECTomer 4010. The enzymatic synthesis of this product could potentially provide a cleaner route to a better-performing material. We have so far demonstrated that the product can indeed be made enzymatically in a non-aqueous environment, and that rates could be improved by adding 3% water and using PEG as a lyoprotectant. Although significant improvement has been achieved in the efficiency of this trans-esterification reaction, the rate of VECTomer production is still very low from the standpoint of a commercial process. In addition, under the best operating conditions, significant quantities of by-products are formed. We will be addressing these problems in the next few months.

MILESTONE STATUS

The overall goals of this 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 specialty polymers.

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.

Accomplishments: A major factor controlling the enzyme performance was determined to be the aggregation of support particles at high water content. Support requirements for minimization of aggregation were also determined and a new proprietary support with enhanced performance has been identified. Task completed.

Task 2 Mass-Transfer (completion in 24 months)

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

Accomplishments: Calculations predict that mass transfer through the organic phase within the particle is not rate limiting, and that at very low water levels, i.e. 10-30 mg/g of support, the initial inter-esterification step, hydrolysis, is rate limiting. These calculations enabled us to isolate the controlling operating factors. This task is completed.

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

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

Accomplishments: Two "dry" systems were investigated with different results. An enzymatic synthesis of AlliedSignal proprietary monomer was developed. Enhanced performance of proprietary support was demonstrated also under dry conditions. Task 3 is on schedule.

INTRODUCTION

The majority of United States manufacturing industries are facing an ever growing challenge by foreign competition to become more innovative and efficient. At the same time, increased environmental awareness is compelling these industries to convert to "green manufacturing" practices. These pressures are especially intense in our commodity and specialty chemical businesses. One of the more powerful tools for achieving those new goals is biotechnology. The promise of biotechnology has been successfully realized in the pharmaceutical industry and it is on its way to being demonstrated in the agricultural markets. In contrast, the impact of biotechnology on the greater chemical industry as a whole has been very limited. The poor utilization of biotechnology for the manufacture of specialties and commodities has not resulted from lack of potential on the part of the technology. On the contrary, while traditional chemical processing usually requires complex multi-step reactions resulting in a multitude of by-products, some of which end up as waste, biocatalysis could yield unique chemical products through more selective and therefore less wasteful processes.

The slow adoption of biomanufacturing technologies by the chemical industry is due to a number of technical barriers, one of which is the necessity to use dilute aqueous solutions in enzymatic reactions with hydrophobic substrates. The low substrate concentration imposes extra processing costs as a result of increased reactor size and water removal steps. The utilization of enzymes in organic solvents will remove this barrier by affording concentrated solutions of industrial substrates, or by eliminating the solvent altogether. A non aqueous environment could also minimize product inhibition when the products are water insoluble.

The application of enzymes in non-aqueous environments has been demonstrated for numerous enzymes and substrates. However, in most cases the overall rate and stability of the enzymes are not high enough to sustain a commercial process for production of a non-pharmaceutical product. The performance of the catalyst can be influenced by its intrinsic structure and by external factors such as water content, support, pH, etc.. This project is addressing some of these external factors with the goal of constructing a lab scale reactor for production of a specialty monomer. In particular we have studied the enzyme performance as a function of the interrelation between support morphology and water content. This study resulted in the identification of support aggregation as a major cause of reduced enzyme activity, and the elucidation of preferred support morphologies that could minimize this problem. We are also studying "dry" systems where aggregation is not a factor. In these systems we are producing specialty monomers. One of the monomers is VEctomer, an AlliedSignal new product which is produced in a lipase catalyzed trans-esterification reaction conducted in a mixture of the reactants containing no additional solvent. The other monomer, a chiral acrylate derivative, is produced in a dry solvent following the procedure developed by A. Klibanov. In both cases we have progressed significantly in determining the effects of water content, support, and enzyme configuration on overall performance.

MATERIALS AND EXPERIMENTAL METHODS

Subtilisin Carlsberg was purchased from Sigma, and *C. Viscosum* lipase from Genzyme. Enzyme immobilization onto the various supports 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 porosity, less was needed for non-porous supports. The solution was then added to 500mg of support. In the case of the fabric, the enzyme solution was pipetted onto a hanging piece of fabric. 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).

All enzymatic reactions were carried out at 45°C. The rate of tripalmitin inter-esterification 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 as an internal standard for the GC analysis. Liquid samples were removed from the reaction mixture and derivatized with N-methyl-N-(trimethylsilyl)-trifluoroacetamide. All triglyceride and diglyceride standards were obtained from Sigma. 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.

The kinetics of vinyl acrylate trans-esterification with (R,S)-sec-(2-naphthyl)ethyl alcohol was monitored by Gas chromatography, using a HP 5890 series 2 GC instrument equipped with a DB-1 30 meter long 0.25mm diameter column and a FID detector. Reaction conditions followed those reported by Margolin et al¹. Samples contained 0.5 M vinyl acrylate, 0.5 M racemic naphthyl ethanol, and 10mg/ml Subtilisin Carlsberg in 5 ml of *tert*-amyl alcohol.

VEctomer 4010 production was monitored by HPLC using a Shodex KF-802 GPC column, and a HP 1090 LC instrument equipped with a Diode Array detector. Reaction mixtures contained DMI dissolved in HBVE in a 1:3 molar ratio, and 40,000 units/ml of *C. Viscosum* lipase.

RESULTS AND DISCUSSION

A. LOW WATER SYSTEMS: SUPPORT MORPHOLOGY / WATER DEPENDENCE

(Tasks 1&2)

Model Reaction and Rate Measurements

The enzymatic reaction employed in this study was the inter-esterification of tripalmitin with stearic acid in a non-polar solvent, petroleum ether. The catalyst we selected was a rather pure enzyme preparation: a lipase from *C. Viscosum*. The enzyme was non-covalently immobilized on various supports 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 unless otherwise stated. The inter-esterification kinetics of this enzyme in petroleum ether and its dependence on water concentration was monitored and the initial rates of stearic acid incorporation into the glycerides was determined as a function of water content. Although in all cases the overall water content in the reaction mixture did not exceed 0.5%, it was enough to form a thin aqueous micro-phase on the enzyme support. A typical plot of the data, obtained with Celite 560 as a support, is shown in figure 1.

Porous Vs. Non-Porous Particles

In order to identify the relative contributions of inter-particle and intra-particle mass transfer to the kinetic behavior, we have studied a broad range of supports. In the past we looked at celite particles with varying pore volume and found that all these porous particles gave similar water dependence curves with a wide maximum (figure 2). These curves produced a linear dependence of the water content at maximum initial rate on the pore volume (figure 3). The Maximal rate in each case was observed when half of the pore volume was filled with water. Kinetic modeling calculations showed that in the absence of water or at very low water levels, the hydrolysis step, rather than the intra-particle mass transfer of substrate to enzyme, is rate limiting. However, as the water phase increases in thickness, the experimental results indicate a significant contribution from intra-particle mass transfer limitations due to water plugging the pores. Thus porosity seems to stretch the optimal performance range.

Although a good correlation with porosity has been observed, the results do not provide clear information about inter-particle effects, such as aggregation of the particles due to higher water phase thicknesses. To separate out possible effects other than porosity, we studied the performance of the lipase immobilized on non-porous particulate supports. We tested two fully dense particles with different size, sand and 1μ monodisperse silica microspheres

produced at AlliedSignal. The amount of support used in these experiments was selected so that it will provide the same surface area as the porous celite 560. All other parameters including the enzyme content have been kept the same. This was done to preclude surface area effects in the comparison between the porous and non-porous particles. The initial rates vs water content for these two supports are shown in figure 4. In both cases the observed inter-esterification rate reaches its maximum and drops abruptly. At the onset of the rate drop or immediately past it, particle aggregation was observed. It is clear from the results that the non-porous particles do not provide a useful range for the enzyme optimal performance. The experiment also demonstrates that the inter-particle aggregation is by far the major factor modulating the water effects on the initial reaction rate. The aggregation increases the dimensions of the water phase and slows down the diffusion of water insoluble substrates to the enzyme in the aqueous phase.

Non-Woven Fabric Supports

The previous results demonstrate the role of aggregation in slowing down the enzymatic reaction, and suggest one way of partially alleviating this problem, namely to use very porous supports. The surfaces within the particle cannot aggregate and thus provide a broader workable range of water levels. However, this solution does not prevent aggregation of the particles themselves. We have tested another support design that could provide a different solution. We selected non-woven fabrics as supports in which the fibers are physically held in place and cannot aggregate. In particular we have tested an AlliedSignal proprietary non-woven fabric which contains a mixture of hydrophilic and hydrophobic fibers. Pieces of the fabric were impregnated with an enzyme solution, air dried and then lyophilized. Kinetics of inter-esterification were monitored under the same conditions, and with the same absolute enzyme and water concentrations as in the particulate supports case. The results are shown in figure 4. Also shown are preliminary rate measurements obtained with Bounty paper towel as a support for comparison.

The high inter-esterification rates and wideness of the maximal activity range observed for the mixed polarity fabric is consistent with the lack of aggregation and thus supports this concept. The hydrophobic fibers provide channels for the organic solvent at higher water levels than is possible with porous particles. The preliminary paper towel results suggest that the positive effect is common to other non-woven fabric structures. In addition to the wide maximum observed with the mixed polar fabric, there is an apparent large overall rate increase by a factor of four as compared to celite 560. A slightly more modest increase was also observed for the Subtilisin system presented next. The source of this effect will be discussed later in the text.

These results are very encouraging and provide a foundation for a bioreactor design. In the next year we will look closer into these fabrics as enzyme supports in organic solvents and use the result to construct a bioreactor.

Free Suspended Enzyme

As an additional support for our conclusions we have run the reaction with free suspended lipase and monitored progress at the same water/enzyme ratios as in the immobilized enzyme experiments. Even at the lowest water concentrations we observed the aggregation of the enzyme. Accordingly no significant inter-esterification has been detected.

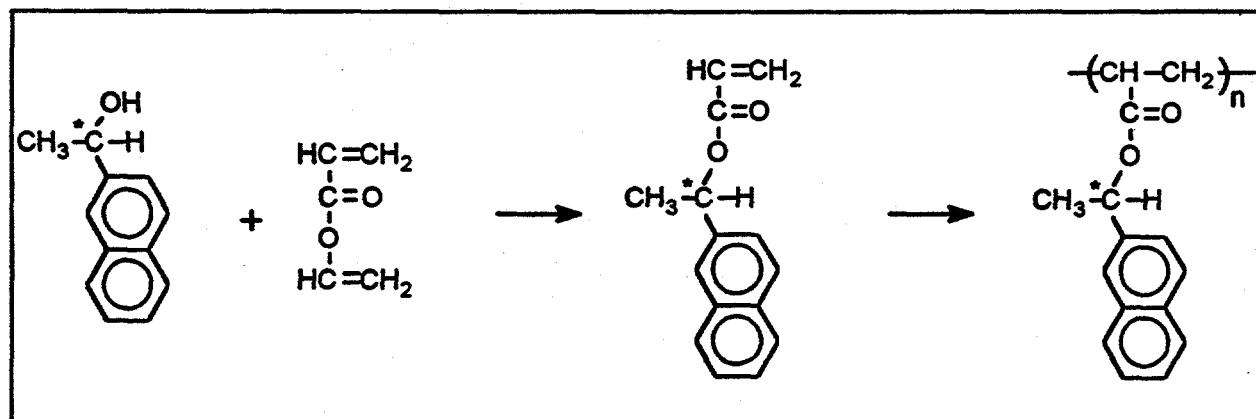
B. DRY SYSTEMS

(Task 3)

We refer to an enzyme/solvent system as "dry", if it has no added water, or its water content is below its solubility in the solvent. Under such conditions one does not expect the water to form a separate phase. We have begun studies on two different dry systems: synthesis of a chiral acrylate monomer, and synthesis of VEctomer 4010. The goal is to apply the knowledge we obtained on the relationship between support morphology and water dependence to the synthesis of monomers with commercial potential.

Chiral Acrylate Monomer

This reaction system has been reported by A. Klibanov¹. It involves the trans-esterification of vinyl acrylate with (R,S)-sec-(2-naphthyl)ethyl alcohol catalyzed by Subtilisin Carlsberg in *tert*-amyl-alcohol as a solvent. Only the S ester is produced. The chiral acrylate can then be polymerized to form chiral polymers. Other chiral alcohols might later be used to produce commercially viable polymer products.

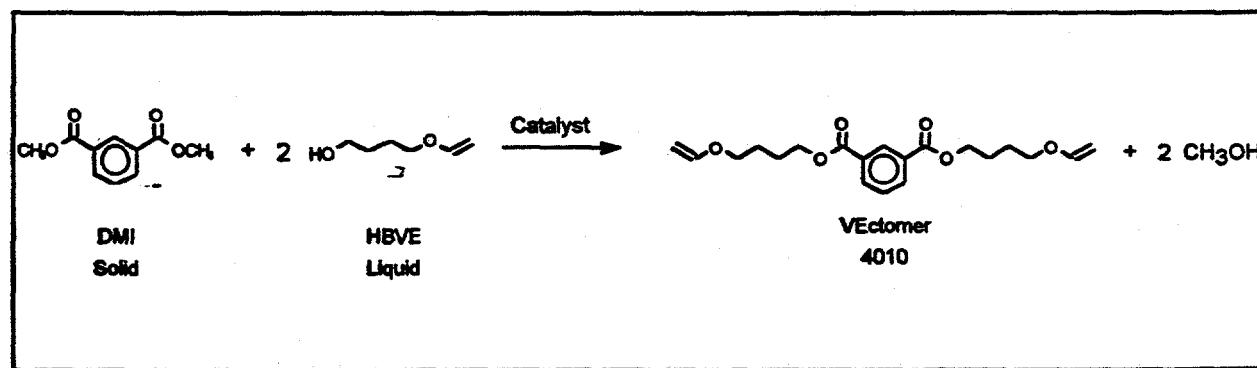


Preliminary experiments were conducted to optimize the reaction yield. The reactions were carried out under the same conditions as reported by Klibanov et al¹ except when mentioned otherwise. Water dependence of the trans-esterification initial rates were determined with free suspended enzyme, and results are given in figure 5. Water concentrations were kept way below the solubility of water in amyl-alcohol. The results show that there is no advantage in adding water. The linear decrease in conversion rate with increased water content probably reflects successful competition of the water with the alcohol for the acrylate resulting in hydrolysis.

We have demonstrated that an enzyme support has a major influence on enzyme performance in wet systems, where an aqueous micro-phase is present. As the trans-esterification reaction is conducted in a dry solvent, one does not expect the supports to exhibit the same performance trends. In this situation one expects the enzyme to perform the same as long as it is sufficiently dispersed. We examined this hypothesis with our porous, non-porous and fabric supports. Figure 6 shows the reaction progress for the immobilized as well as the free enzyme. In order to keep the absolute amount of enzyme the same as in the free suspended enzyme case, we used a very high loading of enzyme onto the supports. This might explain the reduced performance observed with the celite and silica microspheres as compared to the free enzyme. The extra enzyme solids probably formed a layer too thick for efficient mass transfer. This effect should be more apparent with the porous celite since the extra solids may clog some of the pores and cut access to a large amount of enzyme. Indeed the free enzyme provides better conversion rates than both celite and the silica microspheres. The porous celite exhibits the poorest performance. However, the enzyme immobilized on our proprietary fabric performs significantly better than the free enzyme (by a factor of two). The same large activity increase has been observed also with the wet inter-esterification system in addition to the mass transfer improvements. We suspect that this particular fabric with its hydrophilic fibers acts as a lyoprotectant² in the process of drying the enzyme. Another explanation might be that the hydrophilic fibers provide better, more uniform, partitioning of the water on the support, and making it available to all the enzyme. This extra benefit of the mixed polarity non-woven support will be further investigated.

VEctomer 4010

VEctomer 4010 is a new AlliedSignal monomer in a commercialization stage. The current synthesis of VECTomer 4010 involves a catalyzed trans-esterification reaction of dimethyl isophthalate (DMI) with excess hydroxybutylvinyl ether (HBVE).



Unreacted excess HBVE is removed by distillation, but catalyst residues remain behind in the

4010 product. These residues affect the polymerization step, resulting in a decrease in performance as well as inconsistent product quality. We have explored the use of an enzyme as the catalyst in the trans-esterification reaction. The excess HBVE reactant will serve as the solvent in which dimethylisophthalate, DMI, is dissolved. Reaction could be conducted in a continuous fashion with an immobilized enzyme configuration, or in a batch mode with free suspended enzyme. Since the catalyst will be completely insoluble in the organic mixture, it could be easily recovered and reused. It will thus eliminate the chemical catalyst residues, and the problems caused by their presence. We report here some preliminary results of utilizing *C. Viscosum* lipase as the catalyst for the above trans-esterification reaction.

Since the trans-esterification reaction does not require water as part of the catalytic mechanism, we have utilized the free suspended enzyme rather than immobilized enzyme. We hence screened 10 commercially available lipases, proteases and esterases for activity. Four enzymes: Genzyme's *C. Viscosum* lipase, Amano's lipase N, Novo's lipolase, and Subtilisin Carlsberg, showed some activity, with the lipase from *C. Viscosum* producing the best conversion rates. High enzyme concentrations, approximately 40,000 units per ml of reaction mixture, were necessary to obtain significant conversions. However, even at this high catalyst level, only marginal quantities of the final product, the di-ester, were observed.

The degree of conversion to VEctomer 4010 was found to depend on water content and on lyophilization conditions. Figure 7 depicts the reaction progress at various water concentrations. All water levels were below the solubility in the reaction mixture. The results demonstrate that water enhances the rate of conversion, with 3% being the optimal concentration. At higher water levels, the rate of DMI disappearance increases, but the rate of VEctomer formation decreases. The higher water concentrations also enhance the formation of by-products.

Additional improvements in the reaction rate were achieved by optimizing the lyophilization step. Two factors were examined: pH of the enzyme solution being lyophilized, and addition of polyethyleneglycol (PEG) as a lyoprotectant. The dependence of the reaction components' concentrations after 24 hours on pH is plotted in figure 8. The best conversions were obtained at pH 7.5. Dabulis et al² reported improvement in non-aqueous enzymatic activity when the enzyme was lyophilized from a solution containing lyoprotectants such as polyethyleneglycol. We have tested the effect of two PEG (MW 3400) concentrations on the enzyme performance, and the results are shown in figure 9. The difference between the effects of the two concentrations is negligible, however, when compared to the lyophilization in the absence of PEG, significant rate enhancement is observed (figure 10).

Although significant improvement has been achieved in the efficiency of this trans-esterification reaction, the rate of VEctomer production is still very low from the standpoint of a commercial process. In addition, under the best operating conditions, significant quantities of by-products are formed. We will be addressing these problems in the next few months.

FUTURE PLANS

The coming year will be devoted to designing and building a lab scale reactor for continuous enzymatic production of a specialty monomer. The non-woven proprietary fabric will serve as an enzyme support. As a part of this effort we will conduct the following work:

1. Study the effect of fabric composition on the immobilized enzyme performance. In collaboration with our Fibers Business unit we will produce fabrics with various compositions and test their performance.
2. Examine various reactor configurations for continuous operation.
3. Study and improve the long term operating parameters of the enzymatic reactor (weeks to months).
4. Determine commercial viability of enzymatic VEctomer 4010 production.
5. If a new monomer, other than VEctomer 4010, is produced in the bioreactor, the polymer will be synthesized and characterized.

References

1. Margolin, A. L., P. A. Fitzpatrick, P. L. Dubin, and A. M. Klibanov, "Chemoenzymatic Synthesis of Optically Active (Meth)acrylic Polymers", J. Am. Chem. Soc., **113**, pp. 4693-4694 (1991)
2. Dabulis, K. and A. M. Klibanov, "Dramatic Enhancement of Enzymatic Activity in Organic Solvents by Lyoprotectants", Biotechnol. Bioeng., **41**, pp. 566-571 (1993)

Figures

Figure 1: Dependence of tripalmitin inter-esterification rates on water content.

Figure 2: Effect of support on water dependence. Tripalmitin inter-esterification catalyzed by Amano Lipase P. Support particle size was 35-80 mesh.

Figure 3: Water content at maximal initial rate vs celite support particles' pore volume.

Figure 4: Effect of porous and non-porous supports on lipase catalyzed inter-esterification of tripalmitin.

Figure 5: Effect of water on Subtilisin catalyzed Vinyl-acrylate trans-esterification

Figure 6: Effect of support on enzyme activity in the Subtilisin catalyzed trans-esterification of vinyl acrylate.

Figure 7: Effect of water content on VEctomer production rates.

Figure 8: Effect of lyophilization pH on VEctomer production rates.

Figure 9: Effect of PEG concentration in the lyophilizing solution on lipase activity.

Figure 10. Comparison of water and PEG effects on the efficiency of VEctomer enzymatic synthesis.

Inter-esterification Rate vs. Water Content
1% CV Lipase on Celite 560

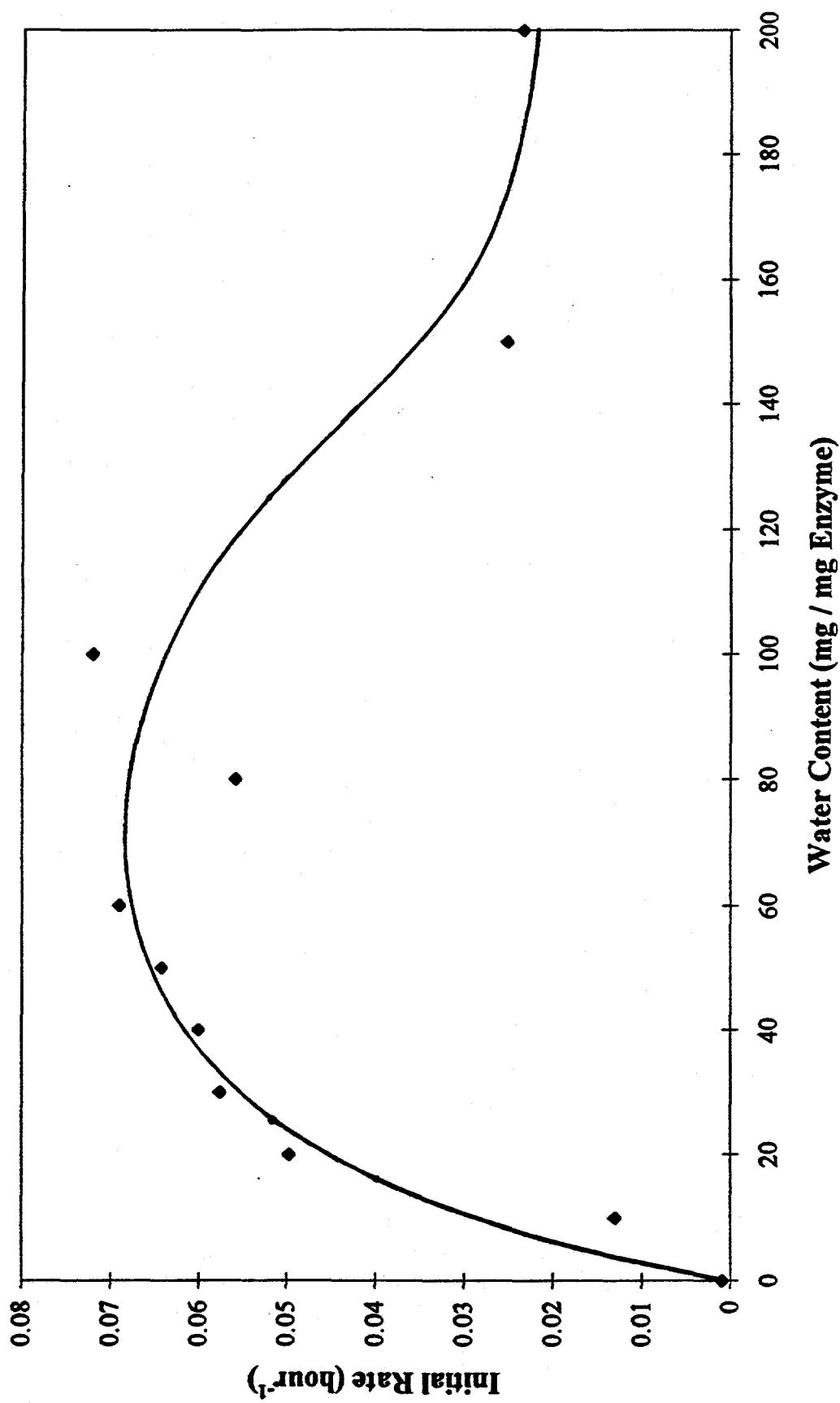


Figure 1

Effect of Support on Inter-esterification Water Dependence

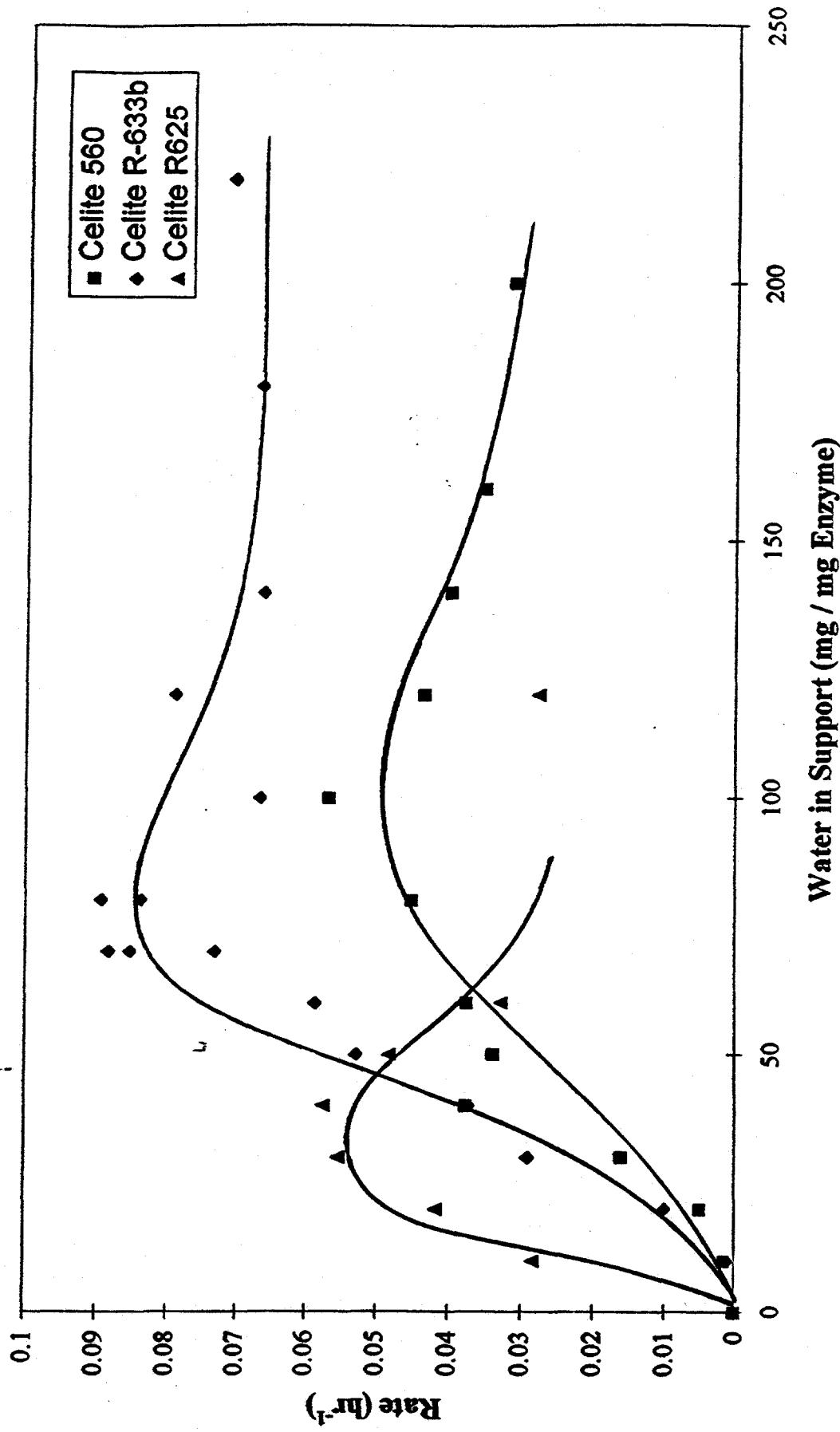


Figure 2

Water Content at Max. Rate vs. Celite Pore Volume

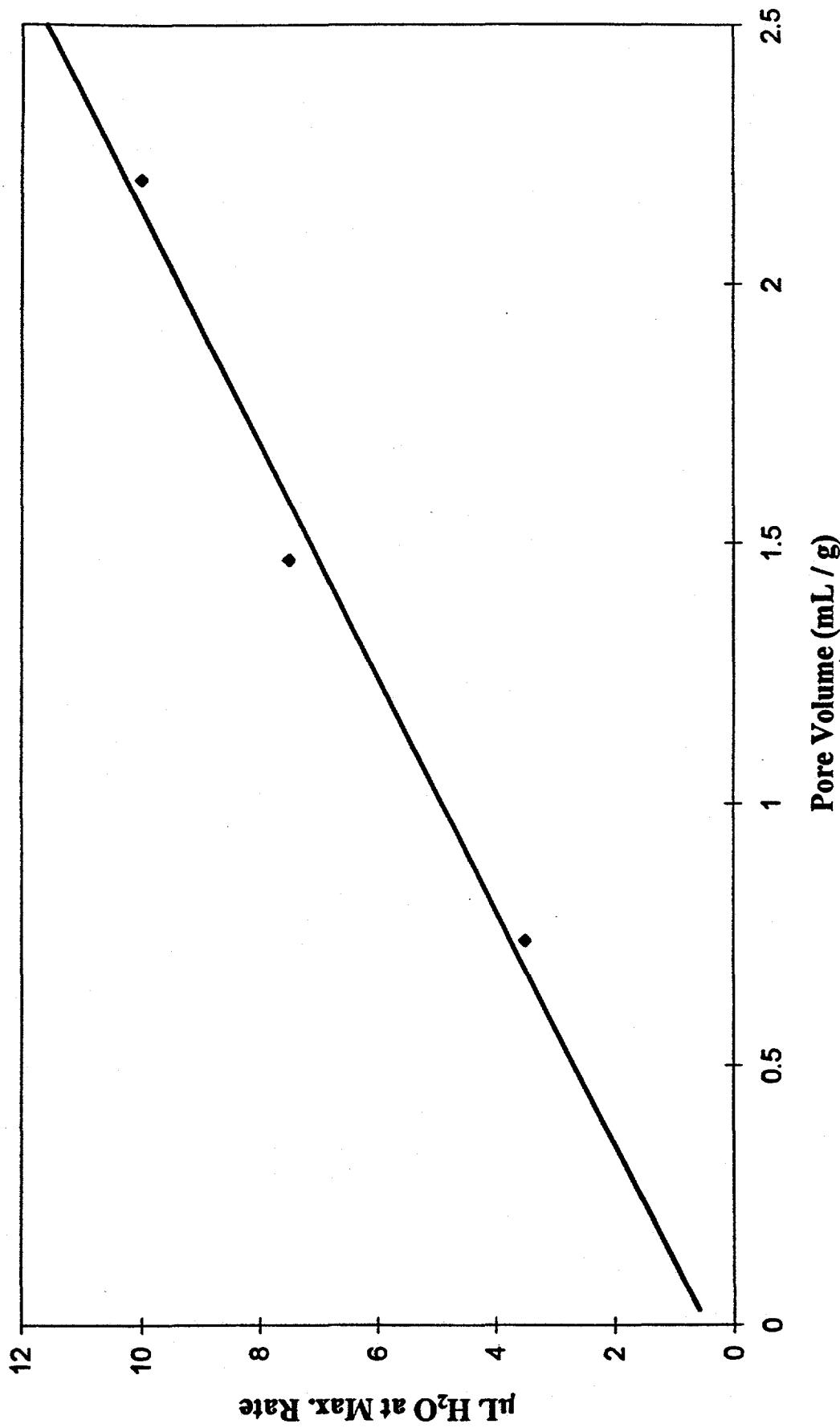


Figure 3

Effect of Support on Tripalmitin Inter-esterification

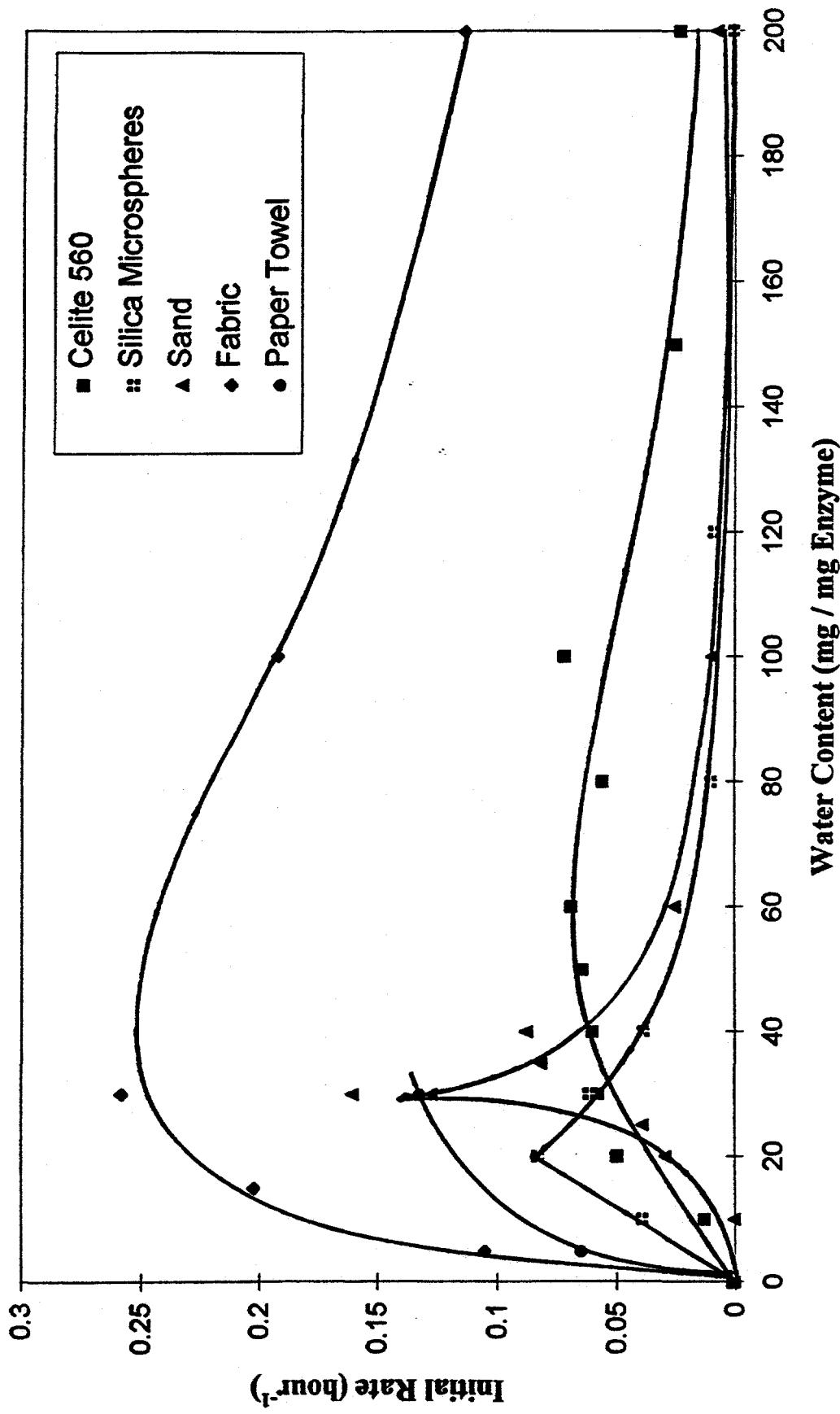


Figure 4

**Effect of Water on Vinyl-Acrylate
Trans-esterification
Free suspended Subtilisin**

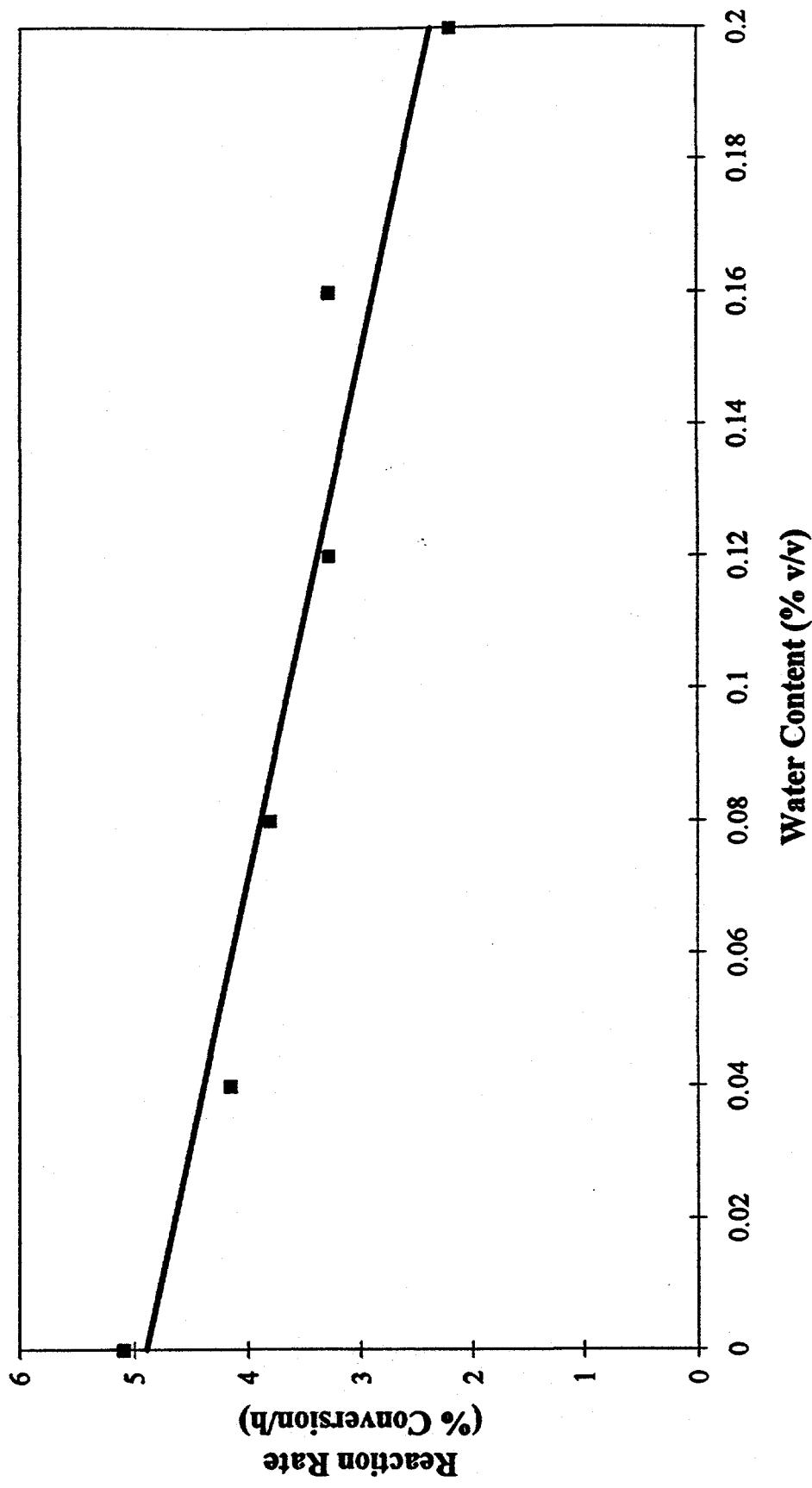


Figure 5

Effect of Support on Enzyme Activity
Subtilisin catalyzed Trans-esterification of Vinyl Acrylate

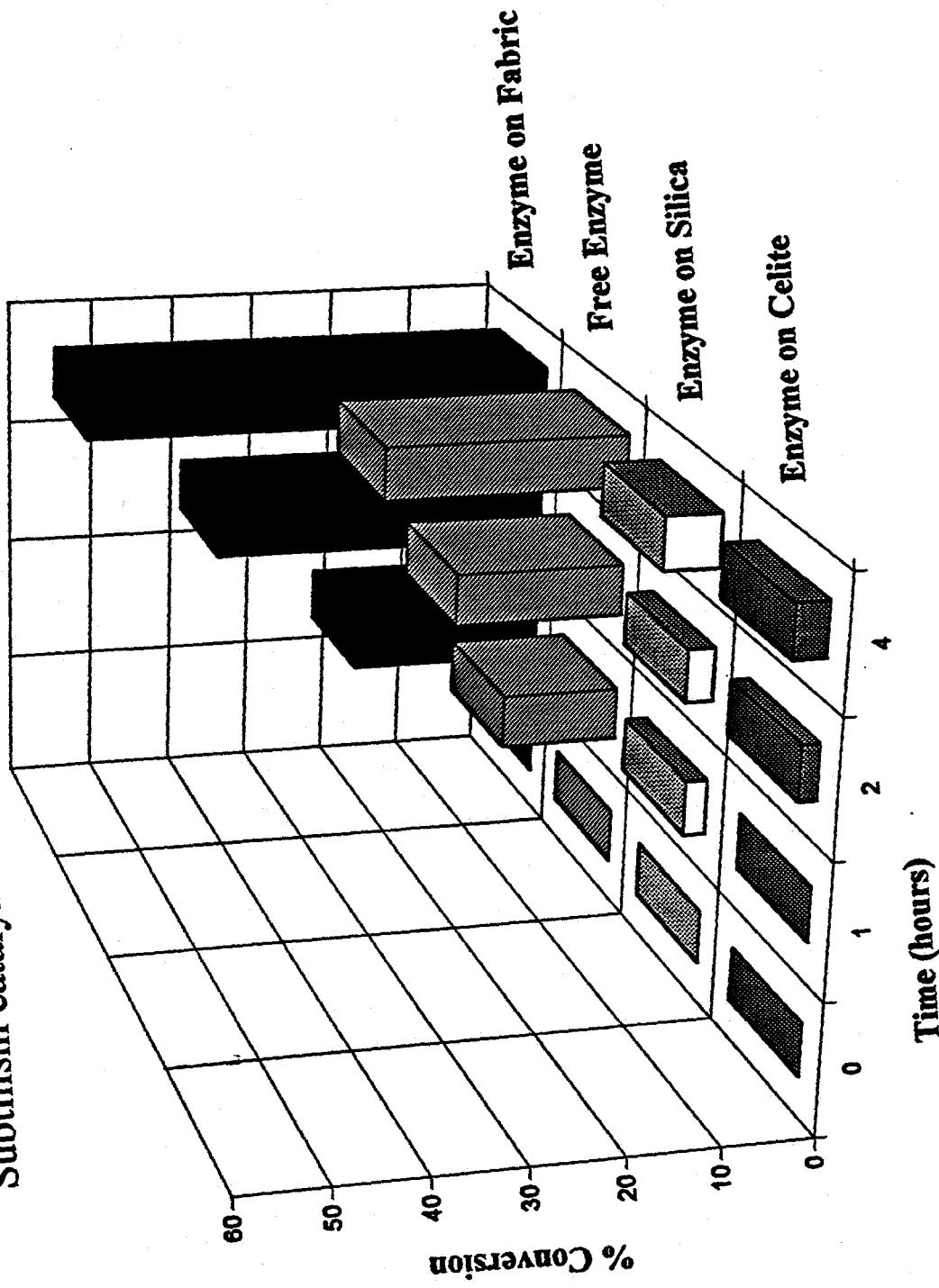


Figure 6

Effect of Water on VEcotorer Production

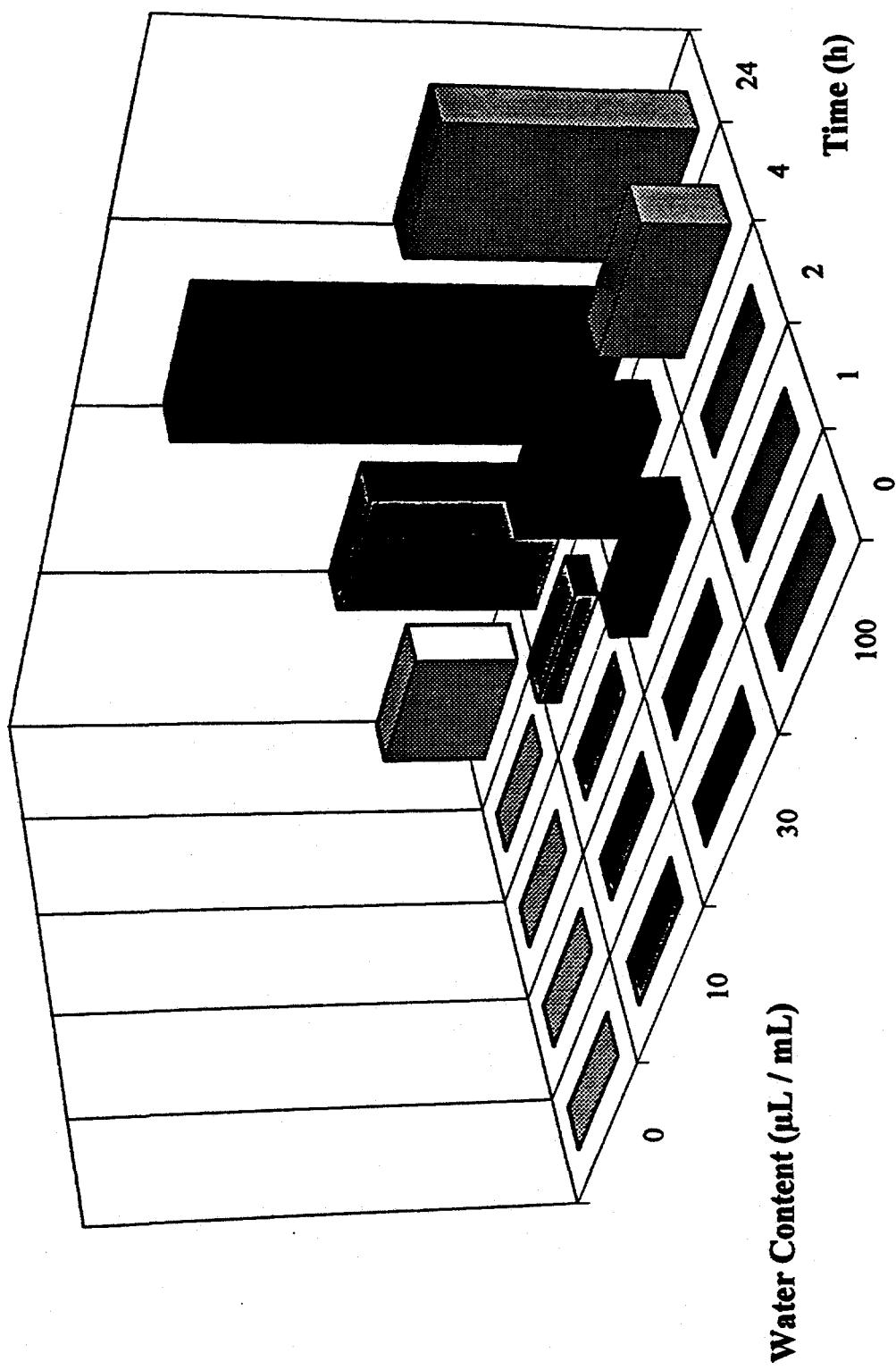


Figure 7

Effect of pH on Reaction Progress (24 hours)

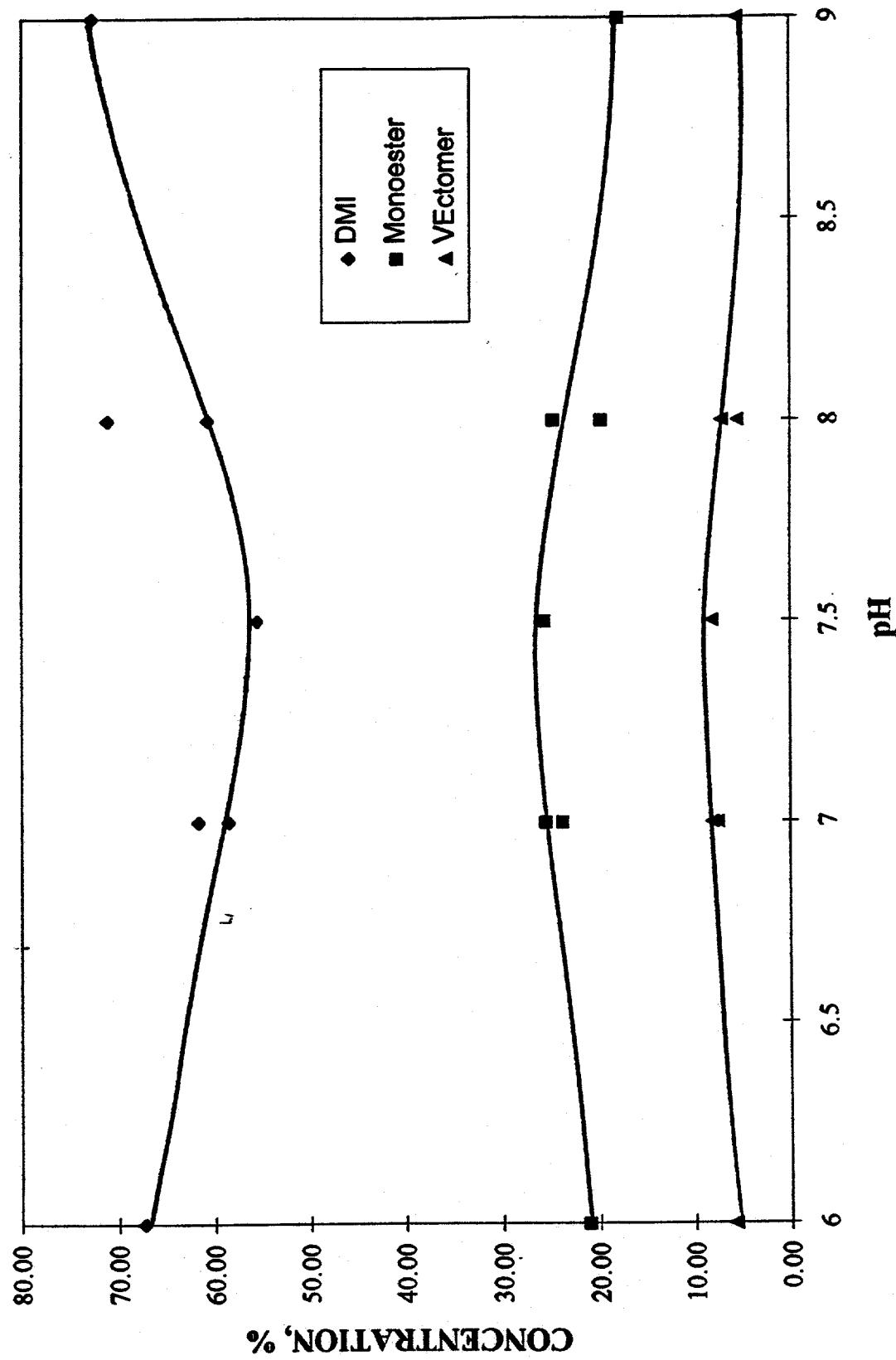


Figure 8

Effect of PEG on Vectomer Production

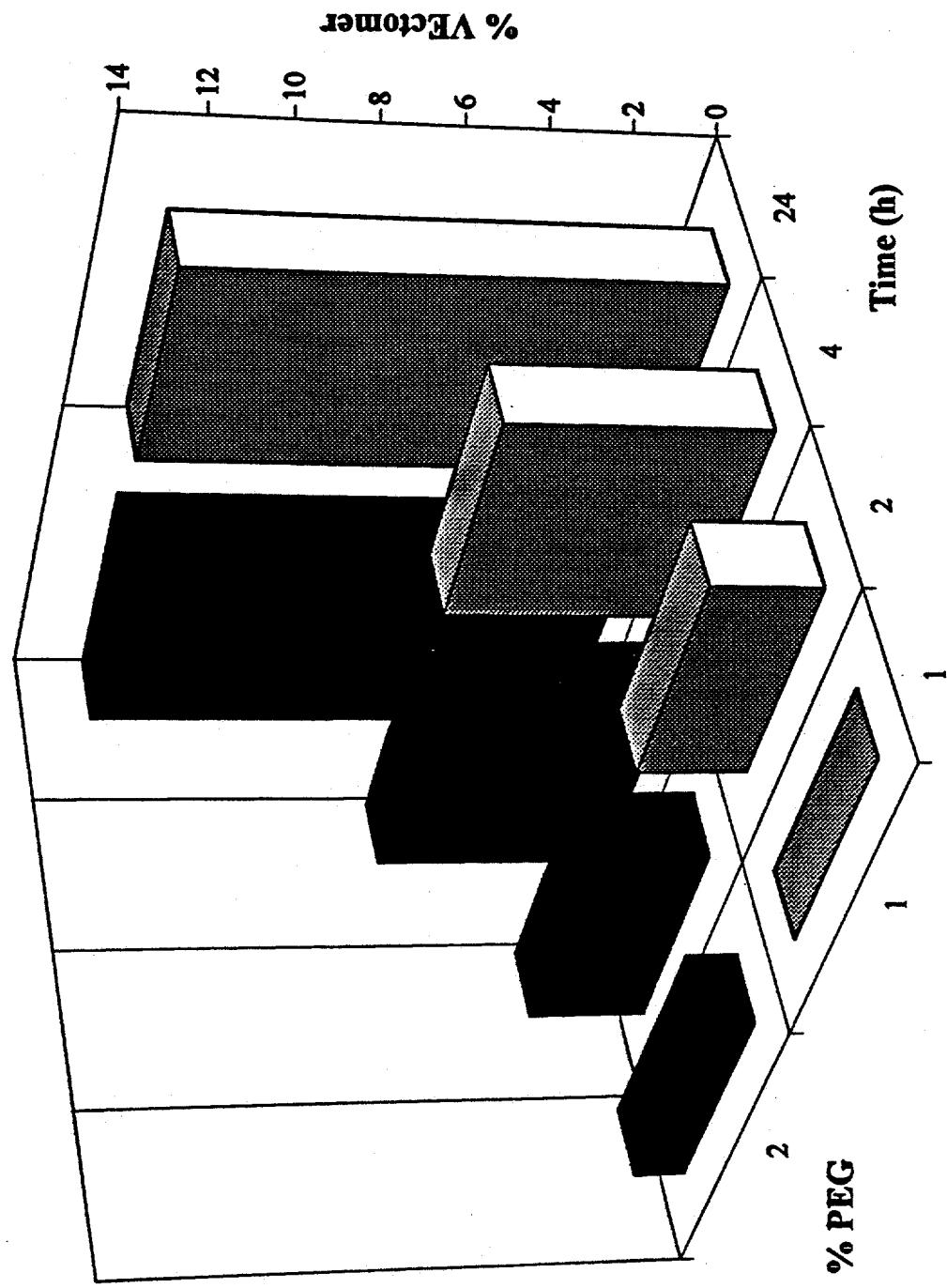


Figure 9

Lipase Catalyzed VEcotormer Production

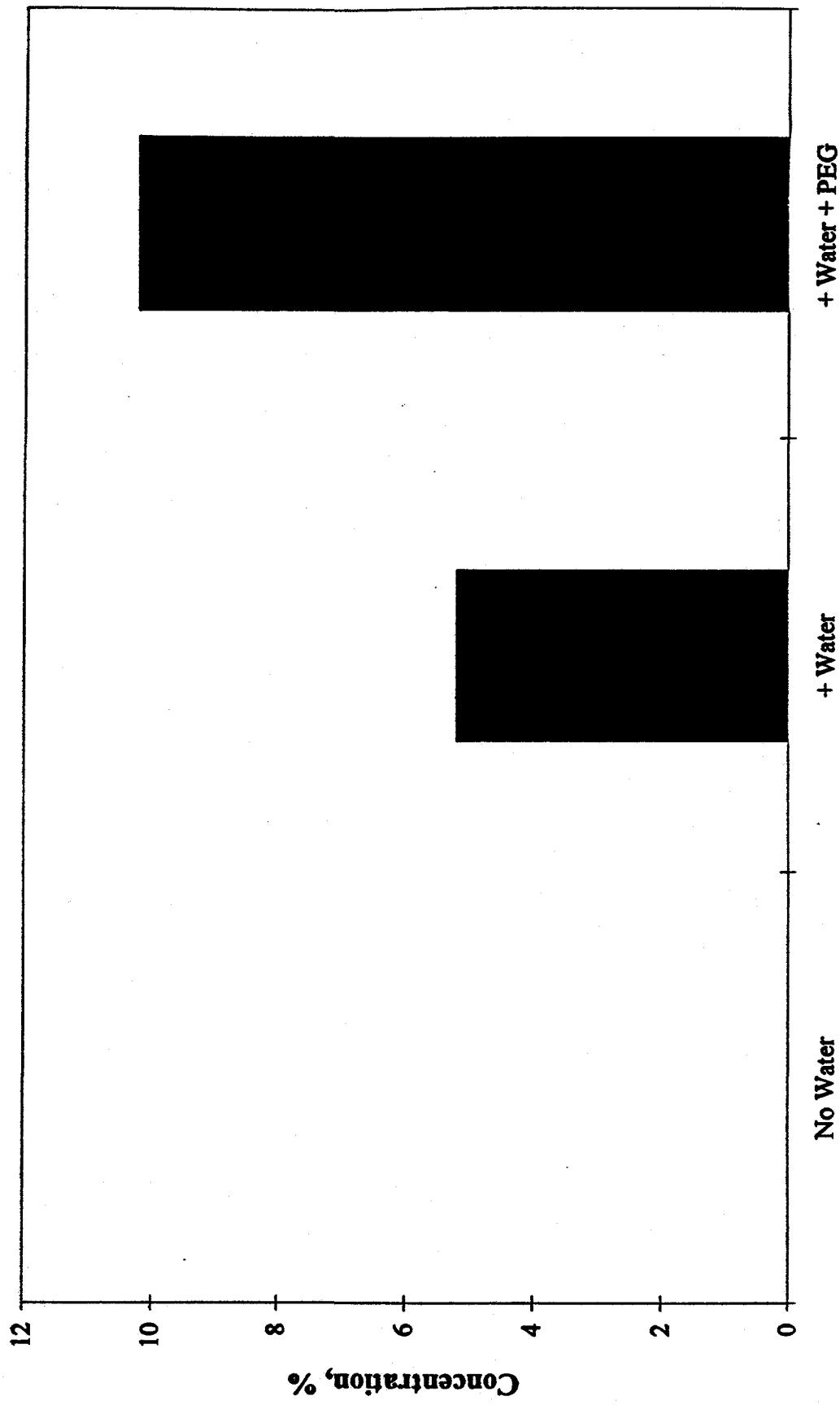


Figure 10