Supercritical Biocatalysis

Anita J. Mesiano, Eric J. Beckman,* and Alan J. Russell

Department of Chemical and Petroleum Engineering and the Center for Biotechnology and Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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Contents

I. Introduction

The use of supercritical fluids as nonaqueous solvents for enzyme-catalyzed reactions, first investigated in 1985 by Randolph et al., Hammond et al., and Nakamura et al., has been a fertile area of research for the past decade.[1−3] The ability to manipulate the physical properties of the solvent by simply changing the pressure or temperature is unique to supercritical systems.[4−8] A decade of research has also clearly demonstrated that the activity of enzymes in nonaqueous media is dependent on solvent properties.[9,10] What naturally follows is that supercritical fluids are attractive media in which to perform and, more importantly, control biocatalytic reactions.

Supercritical fluids are materials above their critical temperature, T_c, and critical pressure, P_c (Figure 1). The properties of supercritical fluids lie between the properties of liquids and gases. For example, supercritical fluid densities are comparable to those of liquids, while the diffusivities and viscosities are comparable to those of gases. The gaslike diffusivities and low viscosities enhance mass transfer rates of reactants to the active sites on enzymes dispersed in supercritical fluids (enzymes are insoluble in all supercritical fluids).[4] Reactions which are limited by the rates of diffusion, rather than intrinsic kinetics, will proceed faster in supercritical fluids than in liquids. For example, the diffusion coefficients of benzene and naphthalene increase by an order of magnitude in supercritical carbon dioxide or ethane as when compared to the liquid state.[11] Higher substrate concentrations can also increase observed reaction rates and improve the utility of the system.

As mentioned above, a key feature of biocatalysis in supercritical fluids is the tunability of the solvent. The density of a supercritical fluid is sensitive to both temperature and pressure, especially near the critical point. Small changes in pressure lead to significant changes in density, which in turn alters all density-dependent solvent properties, such as dielectric constant, solubility parameter, and partition coefficient.[12,13] Since the changes in properties are predictable and have been studied for many solvents and densities, one can rationally control all aspects of the reaction environment.

However, while solvent tunability has been suggested to be the main advantage of using supercritical fluids in place of traditional organic solvents, this has not been well demonstrated as of yet. This is most likely due to the fact that the types of processes which have employed supercritical fluids as solvents thus far have not exhibited certain characteristics which would render supercritical fluid use favorable. Some examples of supercritical fluids which have been used in biocatalysis to date and their critical constants are given in Table 1.[14]

The most popular supercritical fluid, carbon dioxide, has the added benefit of being a natural, unregulated solvent, with low toxicity and high availability.[15] Although supercritical carbon dioxide has been touted as a modern remedy for many commercial problems, the use of carbon dioxide as a solvent is complicated by the low solubility of many reactants under even supercritical conditions.[16] Therefore, many industrial applications are hindered by this obstacle, as well as the fact that high-pressure equipment can be quite costly. Despite these difficulties, the attraction of combining natural catalysts with natural solvents has been the driving force behind a growing body of literature concerning the stability, activity, and specificity of enzymes in supercritical carbon dioxide.[3,11,17]

Enzymes possess unique substrate specificity, while requiring only mild reaction conditions in order to facilitate their activity. A consequence of the combination of nonaggressive reaction conditions and selectivity is that it reduces the chances of undesir-

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Anita Mesiano is a graduate student at the University of Pittsburgh in the department of Chemical and Petroleum Engineering. She obtained her B.S. in chemical engineering from the University of Pittsburgh. Her interests include biocatalysis, supercritical fluid technology, and polymer science.

Eric Beckman received his B.S. degree in chemical engineering from MIT in 1980. Following a short industrial career (first at Monsanto's Plastics & Resins Division, then Union Carbide's Silicones and Urethane Intermediates Group), he attended the University of Massachusetts—Amherst, where he received a Ph.D. in Polymer Science & Engineering under the direction of Roger Porter. Following a postdoctoral appointment at Battelle's Pacific Northwest Laboratories, he joined the University of Pittsburgh in 1989, where he is currently William Whiteford Professor of Chemical Engineering. Dr. Beckman's group has published over 125 papers (and 15 patents) on supercritical fluid processing of polymers, use of CO_2 as both solvent and raw material in polymer science, biocatalytic production of polyesters, microcellular foam production using CO_2, and affinity extraction of polar compounds into CO_2. He currently supervises a research group of approximately 10 graduate students and postdocs. He is a member of both American Chemical Society and AIChE.

Dr. Alan J. Russell is Professor and Chairman of the Department of Chemical and Petroleum Engineering at the University of Pittsburgh. He is also Professor of Molecular Genetics and Biochemistry and holds associate directorships in the Center for Biotechnology and Bioengineering and the Pittsburgh Tissue Engineering Initiative. Dr. Russell received his baccalaureate degree in Biochemistry and Applied Molecular Biology from the University of Manchester Institute of Science and Technology (United Kingdom) in 1984 and his doctorate in Biological Chemistry from Imperial College, the University London, in 1987. Dr. Russell's research has focused on the symbiotic interface between enzymes and materials, specifically biotechnological chemical weapon defense; the study of proteins in extreme environments; biocatalytic polymer synthesis; and the development of rational approaches the biomaterial syntheses. His seminal work has been performed in the area of the decontamination of chemical weapons using enzymes.

able byproduct synthesis. Interestingly, the activity[18] and selectivity[19] of enzymes can be modulated by changes in the pressure or temperature of a supercritical fluid, increasing the range of products which a single enzyme can form.

In this review, we will focus our attention on how biocatalytic reactions in supercritical fluids are affected by factors such as water concentration, density, and the solvent employed in the reaction. Rational control of enzyme activity, specificity, and stability can be achieved by predictable changes in the reaction environment. It should be clear that once we understand the kinetic, thermodynamic, and transport phenomena which exert their effects on the reaction system we can apply the lessons learned to enzyme-catalyzed reactions of commercial relevance.

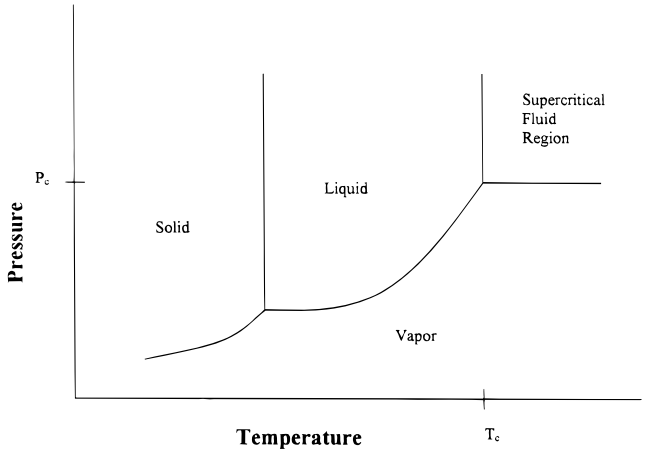

Figure 1. Typical phase diagram demonstrating where the supercritical fluid region is located.

Table 1. Critical Constants of Supercritical Fluids That Have Been Used in Biocatalysis

fluid	critical temperature (K)	critical pressure (MPa)
carbon dioxide	304	7.38
ethane	305	4.88
ethylene	282	5.04
fluoroform	299	4.84
sulfur hexafluoride	319	3.76

By developing an understanding of such enzyme–structure–function–environment relationships, we will be in a position to alter radically the future of solvent engineering of enzyme activity. Indeed, supercritical fluids may be uniquely able to offer a window into the mystery of nonaqueous enzymology.

Table 2. Solubility of Water in Supercritical Carbon Dioxide (Reproduced from ref 26. Copyright 1995 American Chemical Society)

temperature (°C)	pressure (bar)	water solubility (wt %)	water solubility (mol %)
105.0	344.8	0.19	0.90
50.0	344.8	0.31	0.75
75.0	344.8	0.55	1.33

II. Parameters Affecting Enzymatic Catalysis in Supercritical Fluids

A. Effect of Water Content on Enzyme Function in Supercritical Fluids

For many years, it was believed that enzymes could function only in aqueous environments. Although water is vitally important in maintaining enzyme activity and stability, research has demonstrated that enzymes can be vigorous catalysts in a wide variety of essentially nonaqueous systems.[10,20,21] The question is: how much water is enough? Many biochemists are surprised that a monolayer of water on the surface of an enzyme molecule is often sufficient to support enzyme activity and prevent denaturation of the enzyme.[22]

Water plays a vital role in the noncovalent interactions that allow the enzyme to retain its native conformation. In the complete absence of water, enzymes cannot maintain an active conformation, thus hindering their ability to function as catalysts.[23] The amount of water needed is specific to each solvent−substrate−enzyme system that is employed.[24]

Because of the small amount of water that must be present to support enzymatic activity, careful attention must be paid to the solubility of water in supercritical fluids to ensure that products and reactants do not separate out into individual phases of solvent and water.[25] Also, because water acts as a solubility modifier in many supercritical fluid reactions, it has the ability to change the achievable concentrations of both reactants and products. Results of studies on the solubility of water in carbon dioxide have shown that water does exhibit low solubility, as one would expect when trying to dissolve a polar substrate in a nonpolar solvent (Table 2).[26]

In general, it has been shown that enzymes exhibit increased specific activity in supercritical fluids when water is added to the system. However, an excess of water can hinder the synthesis of esters or transesterification due to the occurrence of hydrolysis. Therefore, it is vital to find an optimum water content for the reaction system. Randolph et al.[27] examined the effect of water content on the activity of cholesterol oxidase in supercritical carbon dioxide and found that the enzyme was 10-fold less active in dry carbon dioxide than in a system in which water was also present. The reduced activity in the absence of water was also found to be reversible since the enzyme regained full activity once 1% (v/v) water was added.

Factors such as the type of reaction occurring, the enzyme support, and the fluid that is being employed determine the optimal water content required for the system. The type of reaction occurring is an important factor due to the fact that in a reaction such as esterification, water is produced. Conversely, in hydrolysis, water is consumed. For example, Miller et al.[28] studied the interesterification of myristic acid with trilaurin at 9.5 MPa and 308 K in supercritical carbon dioxide and water-saturated supercritical carbon dioxide with an immobilized lipase and found that a higher enzyme activity was achieved in the low water content supercritical carbon dioxide. This was attributed to the decrease in solubility of trilaurin as the water content increased. Conversely, Dumont et al.[29] demonstrated that for the esterification of myristic acid by ethanol at 12.5 MPa and 323 K, the maximum reaction rate was achieved when a significant amount of water was present. These apparently incompatible results reflect the complexity of experimental nonaqueous enzymology.

The type of enzyme support has also been shown to affect the optimum water content required for biocatalytic reactions in supercritical fluids. An enzyme−immobilization matrix will affect the partitioning of water between the enzyme, support, and solvent and thereby disturb any water-dependent properties. Pore size, surface area, and support hydrophobicity will all affect the water adsorption isotherms, and thus the local water concentration in the vicinity of the enzyme. For example, Marty et al.[17,30] performed an extensive study on the lipase-catalyzed esterification of oleic acid by ethanol. It was found that the lipase from *Mucor miehei*, commercially known as Lipozyme, required approximately 10% (w/w) water content in order to achieve maximum activity. In this case, the enzyme was immobilized by macroporous anionic resin beads.

Another important factor that determines the optimal water content is the supercritical fluid that is being employed as the reaction medium. Hydrophilic solvents tend to partition water away from the enzyme to the solvent. In general, enzymes have been shown to exhibit higher activity in hydrophobic solvents than in hydrophilic solvents since more water stays associated with the enzymes. This was verified by Kamat et al.,[31] who showed that for the lipase-catalyzed alcoholysis of methyl methacrylate (MMA), hydrophobic solvents such as supercritical ethane or ethylene are superior to supercritical carbon dioxide. However, the same authors showed that for the transesterification reaction between 2-ethylhexanol and MMA in supercritical fluoroform, enzyme activity did not significantly change when water was added to the system as shown in Figure 2.[13]

Some reactions are also more sensitive to water than others. For example, if the goal of an experiment is to perform transesterification with an esterase, relatively high water concentrations will enhance the activity of the enzyme but also cause substantial biocatalytic ester hydrolysis. Thus, there is a balance between the positive and negative impact of varying water content. Marty et al.[30] investigated the effect of water content of the enzymatic support on the activity of Lipozyme, a commercial immobilized lipase

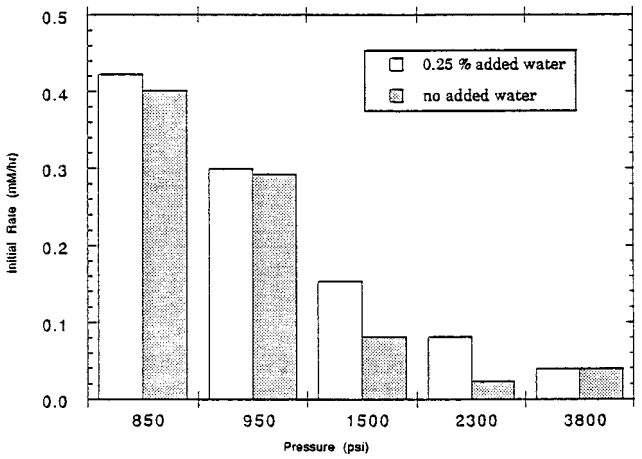

Figure 2. Effect of water content on lipase activity in fluoroform. (Reproduced with permission from ref 13. Copyright 1993 National Academy of Sciences, U.S.A.)

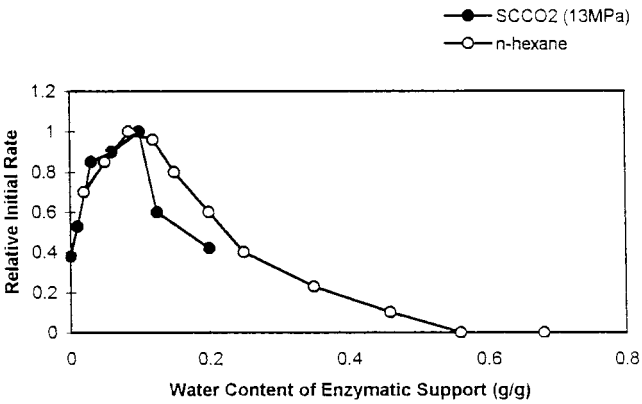

Figure 3. Effect of water content of enzymatic support on enzyme activity in supercritical carbon dioxide (13 MPa, 40 °C) and *n*-hexane (40 °C) (oleic acid, 8 mM; ethanol, 150 mM). (Reproduced with permission from ref 30. Copyright 1992 John Wiley and Sons, Inc.)

from *Mucor miehei*, in supercritical carbon dioxide and found that the optimum water content for enzyme activity was found to be approximately 10% (w/w). However, enzyme activity was reduced when the water concentration exceeded 200 mM and continued to decrease as more water was added (Figure 3).

The authors hypothesized that the negative effects caused by increasing the water content were related to the hydrophilic hindrance of the hydrophobic substrate as it tries to make its way to the enzyme.

The manner in which water is added to a reaction medium can also affect the activity of the enzyme. For example, Steytler et al.[32] performed the synthesis of butyl laurate from butanol and lauric acid with Candida Lipase B at 40 °C and 30 000 kPa bar in near-critical carbon dioxide. The authors showed that the method in which water was added to the reactor clearly affected the activity of the enzyme. The enzyme displayed a higher activity when water was added to the catalyst bed after the reactor was loaded as opposed to adding water directly to the enzyme. In this case, the decreased activity was attributed to the occurrence of hydrolysis.

While it is difficult to model water partitioning in systems that contain enzymes, several attempts have been made to address this issue. The partitioning of water between an enzyme particle and the solvent in which it is suspended is best addressed by considering the thermodynamic activity of water. Halling and colleagues[33,34] have meticulously investigated the effects of water activity in nonaqueous enzymology, including supercritical fluids, and have designed straightforward ways to control the water activity in nonaqueos biocatalytic systems. In nonaqueous media, water activity (a_w) is defined as the product of the activity coefficient of water in the solvent and the mole fraction of water in the solvent. By maintaining constant water activity in the system, the adverse effects that would occur due to the competition with the enzyme for available water would most likely be eliminated. Other advantages of maintaining a constant, known value of a_w include being able to predict enzyme activity when changes are made in solvent, reactants, support, and enzyme concentration and being able to determine the water mass action on hydrolytic equilibria.

To maintain constant water activity in the solvent, water may be added directly to the system or it can also be added through salt hydrates. This is accomplished through the ability of the salt to establish an equilibrium between hydrated forms. Halling[33] has also addressed the issue of adding salt hydrates directly to the reaction mixture and concluded that salt hydrates provide the added advantage of acting as ideal buffers that maintain constant water activity. There have been some issues raised as to whether the addition of the salt hydrates has negative effects on the enzyme. However, it has been concluded that this is not a serious problem.

B. Effect of Pressure

As stated earlier, supercritical fluids are compressible. A small change in pressure is accompanied by a dramatic change in density, thus altering the physical properties of the supercritical fluid. Since the properties of the fluid may modulate enzyme properties suspended therein, the effect of pressure on enzyme-catalyzed reactions in supercritical fluids is an important area of investigation.

Before considering how pressure-derived changes in solvent physical properties can effect enzyme properties, we must fully describe whether pressure itself can have an intrinsic effect on reaction rate. The Eyring Transition-State Theory[35] is used to explain the direct effect of pressure on the rates of reactions in supercritical fluids:

$$k = r(k_B T/h)K^* \quad (1)$$

where k is the rate constant, k_B is the Boltzmann constant (in J/K), h is Planck's constant (in J/s), T is the temperature (in K), r is a pressure and temperature independent coefficient, and K^* is an equilibrium constant that is related to the difference in free energies between the transition-state and the reactants.

As previously described,[36] a relationship can be derived between the activation volume and the reaction rate constant, k:

$$\delta \ln k/\delta P = -\Delta V^*/RT \quad (2)$$

where ΔV^* is defined as

$$\Delta V^* = V_c - \nu_A V_A - \nu_B V_B \quad (3)$$

where V_c is the partial molar volume of the activated complex (in m^3/mol), ν is the stoichiometric coefficient of each of the reactants, and V is the partial molar volume of each of the reactants (in m^3/mol). Equation 2 assumes that the rate constant is expressed in pressure-independent units. If the rate constant is expressed in terms of concentration units, isothermal compressibility must be added to the equation:

$$\delta \ln k/\delta P = -\Delta V^*/RT + \sum \beta \nu_i \quad (4)$$

where β is the solvent compressibility coefficient. For supercritical fluids near their critical points, their compressibility is very high and thus the second term of eq 4 is significant. However, for unimolecular reactions, where $\sum \nu_i = 0$, the second term of eq 4 becomes zero.

The theory described above has been extended to enzymatic reactions in supercritical fluids. However, this approach should be exercised with caution due to the fact that the mass transfer effects associated with enzymatic reactions can complicate the interpretation of the reaction rate data.

For example, a typical enzymatic reaction involving one substrate and one product can be written in the following manner:[36]

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} EP \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} E + P \quad (5)$$

where E, S, and P are the enzyme, substrate, and product, respectively. Each k represents the reaction rate constant for each given step in the process. ES is the enzyme−substrate complex and EP is the enzyme−product complex. If k_{-2} and k_{-3} are small enough to be neglected and assuming that the substrate concentrations are high, the remaining kinetic constants can be combined into a single variable, k_t, and the rate can then be defined as the product of the total enzyme concentration and k_t. Once this relationship has been established, the appropriate pressure derivatives can be taken and an expression for the overall activation volume change can be formulated:

$$\Delta V^*_{tot} = (k_3 \Delta V^*_2 + k_2 \Delta V^*_3)/(k_2 + k_3) \quad (6)$$

where ΔV^*_2 and ΔV^*_3 are the activation volumes associated with steps 2 and 3 in eq 5, respectively. If the rate-determining step in the reaction process is known, the rate equation may be further simplified. Also, if the rate constants are expressed in units independent of pressure, the reaction rate may be expressed as a function of pressure and the kinetic constant of the rate-determining step. For example, if the step in which EP forms E + P is the rate-determining step, the reaction rate may be written as follows:

$$\text{rate}(p) = k_3(0)\,[E]_0 \exp(-p\Delta V^*_3/RT) \quad (7)$$

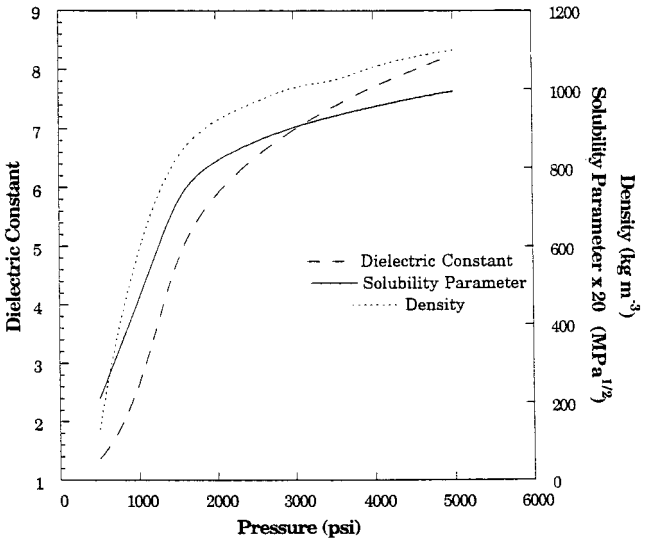

Figure 4. The effect of pressure on the physical properties of fluoroform. (Reproduced from ref 19. Copyright 1993 American Chemical Society.)

Table 3. Effect of Pressure on Enantioselectivity of Subtilisin *Carlsberg* and *Aspergillus* Protease in Supercritical Fluoroform at 50 °C (Reproduced with permission from ref 42. Copyright 1996 University of Pittsburgh)

pressure (MPa)	$(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$	
	subtilisin *Carlsberg*	*Aspergillus* protease
6.50	109	5.73
10.30	100	5.81
12.40	186	5.68
16.50	214	6.67
20.70	229	8.00
28.90	217	9.17

where $k_3(0)$ is the rate constant at atmospheric conditions.

Pressure not only affects the kinetics of reactions in supercritical fluids, but also the physical properties of the solvent. A small change in pressure made corresponds to a change in all density dependent properties such as the partition coefficient, dielectric constant, and Hildebrand solubility parameter, which is a first approximation for the solvating power of a given material. Extensive research in conventional solvents has shown that solvent physical properties such as dielectric constant, dipole moment, log P (where P is the partition coefficient), and hydrophobicity have various effects on enzyme activity, specificity and enantioselectivity.[37−41]

Kamat et al.[19] studied the effects of pressure on the physical properties of supercritical fluoroform. This effect can be substantial, as shown in Figure 4, where an increase of 13.8 MPa results in a 4-fold increase in the dielectric constant for fluoroform.

The pressure-induced increase in dielectric is sufficient to cause a dramatic change in enantioselectivity of subtilisin and *Aspergillus* protease for the transesterification of *N*-acetyl-(L or D)-phenylalanine ethyl ester (25 mM) with methanol (1 M) at 50 °C. As shown in Table 3,[42] both enzymes become more stereoselective as the pressure is increased. In other words, as fluoroform becomes more hydrophilic, the enantioselectivties increase.

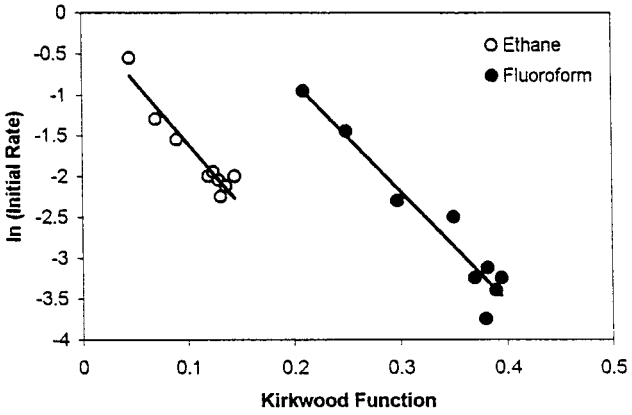

Figure 5. The relationships between solvent dielectric constant and activity of lipase in supercritical fluoroform and ethane. Note: The Kirkwood Function is $(\epsilon - 1)/(2\epsilon + 1)$, where ϵ is the dielectric constant. (Reproduced with permission from ref 13. Copyright 1993 National Academy of Sciences, U.S.A.)

Kamat et al.[13] were also able to demonstrate that the effect of the supercritical fluid on the activity of lipase is strongly dependent on the dielectric constant of the solvent. The effect of the solvent on the reaction rate constants can be approximated by the Kirkwood expression[43] for homogeneous reactions:

$$\ln \text{ rate} \propto (\epsilon - 1)/(2\epsilon + 1) \qquad (18)$$

where ϵ is the solvent dielectric constant. As shown in Figure 5, there appears to be a relationship between the solvent dielectric constant and the activity of the lipase in supercritical fluoroform and supercritical ethane when the natural log of the initial rate is plotted against the Kirkwood function, $(\epsilon - 1)/(2\epsilon + 1)$. This same approach was later used by Michels et al.[44]

Other studies were conducted by Chaudhary et al.[45] that further demonstrated that both the activity and specificity of subtilisin changed as the pressure of supercritical fluoroform changed. This was attributed not to a direct pressure effect or a change of water solubility, but to changes in the physical properties of the solvent. This conclusion was made due to the fact that both the activity and specificity of the enzyme paralleled the change in the physical properties, such as dielectric constant and log *P*, of supercritical fluoroform.

Pressure has also been shown to affect the stabilities of some enzymes. This phenomena was first observed by Penniston,[46] who noticed that for aqueous systems, when pressure was kept below 100 MPa, there was no significant change in enzyme activity. However, as the pressure was increased, some enzymes' activities increased, whereas others decreased. Other studies have observed similar phenomena. Yang et al.[47] demonstrated that when lipase, glucoamylase, and α-amylase were treated with supercritical carbon dioxide, changes in pressure had no significant effects on the stability of any of the enzymes.

Randolph[27] studied conformational changes of cholesterol oxidase in supercritical carbon dioxide and various mixtures of supercritical carbon dioxide and cosolvents using high-pressure electron paramagnetic resonance spectroscopy. Small changes in the EPR spectra indicated that conformational changes in the enzyme associated with pressure changes were minimal.

Conversely, Kasche et al.[48] demonstrated that the enzymes trypsin, chymotrypsin, and penicillin amidase underwent conformational changes when exposed to supercritical carbon dioxide. This attributed to the depressurization rate of the carbon dioxide, as well as other factors such as water content. Slow depressurization caused only partial inactivation in chymotrypsin and trypsin. However, as the number of pressurization−depressurization steps increased, the degree to which the enzymes were inactivated increased.

C. Effect of Solvent

1. Mass Transfer

As described above, the physical properties of supercritical fluids can have a dramatic effect on enzyme activity and stability. In nonaqueous media, enzymes are heterogeneous with respect to the solvent. As a result, such enzyme-catalyzed reactions can be influenced by external mass transfer (diffusion of the substrate from the bulk solvent to the surface of the enzyme particle) and internal mass transfer (diffusion of the substrate through the enzyme particle to an active site). Because the rate of mass transfer depends on factors such as solvent physical properties and enzyme powder morphology, the rate of mass transfer will change from solvent to solvent. However, because the physical properties of supercritical fluids can be altered by merely changing the pressure or temperature,[3−7] the rate of mass transfer can be manipulated in supercritical fluids. This is especially advantageous for reactions that are diffusionally limited since supercritical fluids exhibit high diffusivities.[11]

The effect of diffusion on heterogeneous reactions has been previously studied.[49,50] More specifically, Kamat et al.[51] studied the role of diffusion in nonaqueous enzymology. The authors found that 2 orders of magnitude less agitation was required for systems that employed supercritical fluids as the reaction medium as opposed to organic solvents. This was attributed to the high diffusivities of supercritical fluids. Therefore, supercritical fluids can enhance the rates of mass transfer in systems that are limited by external mass transfer.

In continuous flow systems, external mass transfer is dependent on the flow rate of the system. In batch systems, external mass transfer is dependent upon system agitation. However, internal mass transfer is dependent on the morphology of the enzyme powder, as well as the fluid employed. This is due to the fact that the size of the enzyme particle varies with solvent since different solvents promote clustering of enzyme particles to different degrees.[52] In supercritical fluids, one expects that enzyme powders would undergo morphological changes which depend on the solvent, temperature, and pressure.

Studies have been conducted to examine the effects of both internal and external mass transfer in su-

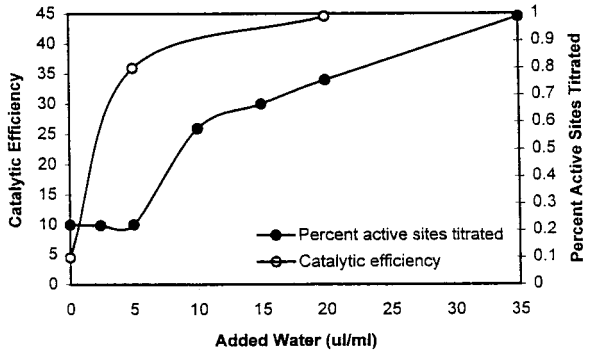

Figure 6. The influence of water content on the fraction of catalytically competent active sites (●). Catalytic efficiency (○) is originally from ref 56. (Reproduced with permission from ref 55. Copyright 1996 John Wiley and Sons, Inc.)

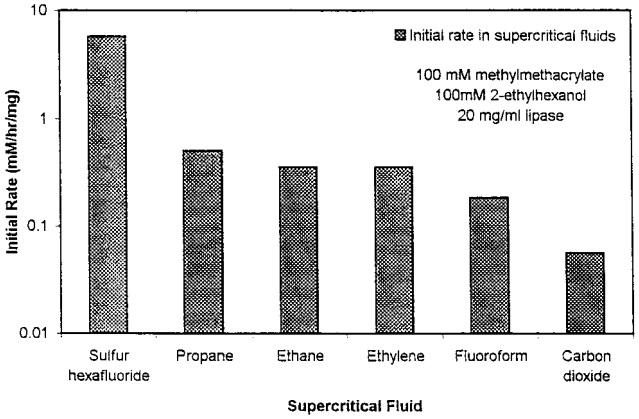

Figure 7. Comparison of lipase activity in supercritical fluids for the lipase-catalyzed reaction between 2-ethylhexanol (100 mM) and methymethacrylate (100 mM). All fluids were at 45 °C, except for sulfur hexafluoride, which was at 50 °C. (Reproduced with permission from ref 31. Copyright 1992 John Wiley and Sons, Inc.)

percritical fluids on observed enzyme activity. For example, Erickson et al.[6] calculated the Damkohler number and Thiele modulus, which give ratios of the characteristic reaction rate to the characteristic external diffusion rate and internal diffusion rate, respectively. The results indicated that mass transfer effects for the particular reaction studied, a transesterification reaction of laurin with palmitic acid, were negligible. Dumont et al.[53] also calculated the Thiele modulus to for myristic acid esterification in supercritical carbon dioxide and *n*-hexane at 12.5 MPa and 313 K. The authors found that the Thiele modulus for *n*-hexane was much greater than that for supercritical carbon dioxide. No external diffusion limitations were detected since the fact that altering the speed of the stirrer did not have an impact on the rate.

2. Active-Site Content

In nonaqueous media, solvent variation can alter the availability of an enzyme's active sites. Because enzymes are insoluble in organic media, it is probable that a fraction of the enzyme molecules do not actually participate in the reaction. The concentration of active sites that can take part in a reaction plays an important role in calculating both k_{cat} (the catalytic turnover number) and k_{cat}/K_m (the catalytic efficiency).[23] Therefore, a knowledge of the active site concentration is important for nonaqueous biocatalysis.[54] Wangikar et al.[55] used an active-site titration method in order to examine the parameters that active-site concentration is dependent upon. The authors found that active-site concentration is dependent upon the nature of the enzyme and how the enzyme is prepared, as well as the hydrophobicity and water content of the solvent. It should be noted that this study did not employ supercritical fluids as solvents. Active-site titrations have not yet been reported under supercritical conditions. The dependence of active site concentration on water content can be seen in Figure 6.[55,56]

3. Intrinsic Enzyme Activity

Originally, it was thought that as long as the essential water molecules required for catalytic activity were bound to the enzyme, the enzyme would maintain full activity.[23] However, the nature of the solvent is vital for maintaining the layer of essential water.[22] The more hydrophobic the solvent is, the less likely it is for the water to partition into it, thus preventing stripping of the water from the enzyme. For example, Kamat et al.[31] studied the lipase-catalyzed alcoholysis of methyl methacrylate and tested the activity of the enzyme in supercritical sulfur hexafluoride, ethane, ethylene, fluoroform, carbon dioxide, and near-critical propane. As seen in Figure 7, sulfur hexafluoride, the most hydrophobic of the solvents used, displayed the highest amount of enzyme activity, whereas the enzyme was the least active in carbon dioxide.

Although each of the reactions were performed at the same pressure, the density varied from one fluid to the other. Sulfur hexafluoride, an inorganic supercritical fluid, was the most hydrophobic of the fluids used and has an unusually high density, 0.75 g/cm^3.

Although supercritical carbon dioxide is a popular solvent due to its low toxicity and cost, enzyme activity and stability have been shown to be adversely affected when exposed to supercritical carbon dioxide. Zagrobelny et al.[57] studied the conformation of trypsin in situ as a function of the density of carbon dioxide using steady-state fluorescence spectroscopy. The results indicated that significant changes in protein conformation occurred during compression. However, this unfolded form was only slightly less stable than the native form of trypsin. This was in contrast to the previous work of Randolph,[27] in which the enzyme cholesterol oxidase was spin-labeled and studied using electron paramagnetic resonance spectroscopy. These experiments concluded that supercritical carbon dioxide had no effect on the conformation of the enzyme. What can be concluded, in general, is that the effect supercritical carbon dioxide has on a given enzyme's conformation is dependent upon the specific type of enzyme employed in the medium.

The effect of carbon dioxide was found to have a significant effect on the lipase-catalyzed alcoholysis reaction between 2-ethylhexanol and methyl meth-

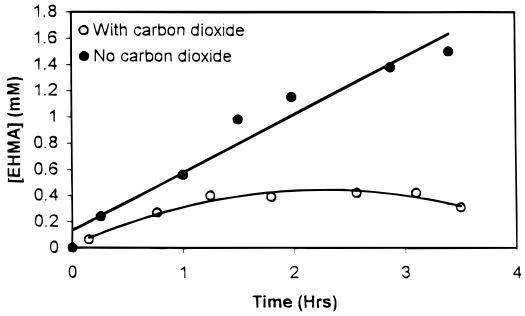

Figure 8. The effect of carbon dioxide on lipase activity in hexane. The reaction mixture contained 2-ethylhexanol (100 mM) and methyl methacrylate (100 mM), [EHMA], and was incubated at 30 °C in a shaker at 300 rpm. (Reproduced with permission from ref 31. Copyright 1992 John Wiley and Sons, Inc.)

acrylate. Kamat et al.[31] performed the reaction in hexane, but the activity of the lipase decreased significantly when carbon dioxide was bubbled through the reaction mixture, as shown in Figure 8.

This decrease in enzyme activity could be attributed to two observations. The carbon dioxide can form covalent complexes, carbamates,[58,59] with the free amine groups on the surface of the enzyme, thus inhibiting the enzyme. These complexes are stable at low temperatures. However, as temperature increases, the lipase stability also increases. Therefore, the effects of the carbamate structures can be reversed as the temperature increases, thus restoring enzyme activity.

Also, Kamat et al.[60] provided direct evidence of the formation of these structures. By using laser desorption mass spectroscopy (LD-MS), the effect of carbon dioxide on subtilisin was studied. Subtilisin was chosen due to the abundance of lysine groups on its surface and since it is a well-characterized enzyme. Because LD-MS can measure protein molecular weight with a high degree of accuracy, samples of subtilisin were analyzed before and after exposure to carbon dioxide. The unexposed sample fell within the expected range. However, after exposure to carbon dioxide, the molecular weight of the protein increased by 176 atomic mass units, which is equivalent to four carbon dioxide molecules.

Another possible reason for the decrease in enzyme activity can be attributed to the effect of pH since enzyme activity is sensitive to pH. Although it is not possible to directly measure pH in nonaqueous media,[9] a local pH in the aqueous layer around the enzyme still exists. The local pH of the hydration layer may be altered by the carbon dioxide dissolved in the layer.[5,31]

However, this effect can be minimized by the addition of buffering salts. The change in pH can then be calculated using the Henderson−Hasselback equation.[61] These salts can be concentrated by lyophilizing the enzyme and the overall effect of dissolved carbon dioxide is negligible, assuming that both the fluid and the salts are able to exchange protons. The latter assumption was proven by the work of Yang et al.[62] Further studies by Chulalasananukul et al.[5] were able to support the hypothesis that pH effects were not the main reason that enzyme activity decreases in the presence of carbon dioxide.

Borges de Carvalho et al.[63] also studied the effect of carbon dioxide on the transesterification of vinyl butyrate by benzyl alcohol and found that carbon dioxide had negative effects on the catalytic activity of subtilisin, which agreed with the results of Kamat.[60] The study compared carbon dioxide with propane, and it was found that propane was a better solvent for the reaction. This was attributed to the fact that enzyme hydration, which greatly affects the enzyme's activity, is higher in propane than in carbon dioxide since water is not very soluble in carbon dioxide.

Solvent effects on enzymatic reactions can also be described in terms of transition-state analysis.[64] The change in the rate constant, k, of a reaction associated with pressure changes can be described by eq 2. If the activation volume is positive, then the reaction will be hindered by pressure. However, if the activation volume is negative, then the rate of the reaction will improve at higher pressures. Therefore, supercritical fluids that exhibit very high negative activation volumes for certain reactions will improve the rates of these reactions.

Solvent effects can also be enhanced by small additions of cosolvents. The presence of small percentages of compounds such as ethanol, acetone, and methanol can increase the solubilities of different compounds in supercritical fluids. For example, Lemert and Johnston[65] found that the addition of 2% tri-*n*-butyl phosphate to supercritical carbon dioxide increases the solubility of hydroquinone by more than 2 orders of magnitude with respect to pure supercritical carbon dioxide. This phenomena is due to the formation of a charge-transfer complex between the cosolvent and the solute. Cosolvent addition can be used to even further fine-tune the physical properties of supercritical fluids.

III. Enzymatic Reactions in Supercritical Carbon Dioxide

The focal point of the majority of research being conducted in the area of enzymatic catalysis in supercritical fluids is that of employing supercritical carbon dioxide as the reaction medium. Supercritical carbon dioxide possesses some apparent advantages over other supercritical fluids, such as low cost and toxicity. However, research has indicated that other supercritical fluids, such as fluoroform and ethane are better suited to act as the reaction medium for biocatalytic reactions.

Some of the earliest work with supercritical carbon dioxide was carried out by Randolph et al.[1] The enzyme alkaline phosphatase was found to be active in a batch reaction system that employed supercritical carbon dioxide as the solvent. The enzyme catalyzed the reaction of disodium *p*-nitrophenyl phosphate in which *p*-nitrophenol was produced, but was limited by the solubility of disodium *p*-nitrophenyl phosphate in supercritical carbon dioxide. However, the enzyme was shown to be active after exposure to supercritical carbon dioxide for 24 h.

The effect of pressure on the activity of subtilisin *Carlsberg* was studied by Barreiros et al.[66] in supercritical carbon dioxide, ethane, and compressed pro-

pane. Although an increase in pressure was found to decrease the catalytic activity of the enzyme, the most profound effect was in supercritical carbon dioxide, thus further supporting the hypothesis that carbon dioxide has an adverse effect on the activity of subtilisin.

Polyphenol oxidase, which oxidizes *p*-cresol and *p*-chlorophenol to the corresponding *o*-benzoquinones, was studied by Hammond et al.[2] and was found to be active in both supercritical carbon dioxide and fluoroform. The reaction was performed in a reactor under both batch and flow conditions. As flow rate increased, the conversion decreased due to the shorter residence times in the reactor. However, the enzyme was inactive by the end of the oxidation process.

The enzymes employed in most of the work involving supercritical fluids, and more specifically, carbon dioxide have been lipases. Extensive work has been carried out by various research groups on a wide variety of reactions. Nakamura et al.[3,25,67] studied the acidolysis of triolein with stearic acid in supercritical carbon dioxide in both a batch and continuous reactor. Four lipases were used, three of which were immobilized. The enzymes were found to be stable in supercritical carbon dioxide and the combination of a high substrate concentration, low water content, and short residence time resulted in better productivity from the reaction.

Dumont and Barth[53] performed and esterification reaction with myristic acid using an immobilized lipase from *Mucor miehei* in both *n*-hexane and supercritical carbon dioxide. Although the reaction in supercritical carbon dioxide exhibited a higher maximum velocity, the myristic acid was found to be more soluble in hexane, thus calling into question whether the carbon dioxide was a better solvent for this particular reaction.

The issue of substrate solubility in supercritical carbon dioxide was also studied by Yoon et al.[68] The transesterification reaction between triolein and either behenic acid or its ethyl ester was carried out with an immobilized lipase. The ethyl ester of behenic acid was found to be approximately 1000 times more soluble in supercritical carbon dioxide than that of behenic acid. Therefore, the rate of the reaction in which the ethyl ester was used was higher than the one that used behenic acid.

Performing enzymatic reactions is supercritical carbon dioxide can also be used to produce optical isomers via chiral synthesis or resolution of a racemic mixture. Ikushima et al.[69] studied the lipase (*Candida cylindracea*) catalyzed transesterification of (±)-citronellol with oleic acid. As the pressure of supercritical carbon dioxide was increased, the rate of the reaction increased. This effect was especially noticeable at the critical point. Also, the optical purity of the product was found to be sensitive to pressure. Around the critical point, the *S* ester was stereoselectively formed. However, at higher pressures, the optical purity was much less. Endo et al.[70] also produced chiral esters from secondary alcohols and short-chain fatty acids using two immobilized lipases in supercritical carbon dioxide. The reactions catalyzed by lipase OF produced both the *R* and *S* forms of the ester, while the Lipozyme-catalyzed reactions yielded only the *R* form of the ester.

Barreiros et al.[71] studied the activity of Novozym 435 (immobilized *Candida antarctica* lipase B) in supercritical carbon dioxide, supercritical ethane, and compressed propane. The enzyme was used to catalyze the transesterification reaction of butyl acetate by *n*-hexanol. It was found that at 35 °C and 10 000 kPa, the activity of Novozym was similar in supercritical ethane and compressed propane but approximately 1 order of magnitude lower in carbon dioxide. However, the reaction rate did increase with temperature in the supercritical carbon dioxide, whereas it did not in the compressed propane and increased only slightly in supercritical ethane.

Liaw et al.[72] investigated the continuous synthesis of phenylethyl acetate by the lipase-catalyzed esterification of phenylethanol with acetic acid in supercritical carbon dioxide. By varying parameters such as water content, reaction temperature and pressure, substrate concentration, and gas flow rate, optimum operating conditions were found for the reaction. When water content was kept below 3% (w/w), the conversion rate was approximately 70%. However, the enzyme was irreversibly inactivated when water content was above 8%.

As previously mentioned, Kamat et al.[31] studied the lipase (*Candida rugosa*)-catalyzed transesterification of methyl methacrylate with 2-ethylhexanol in a variety of supercritical fluids. For this particular reaction system, supercritical carbon dioxide was found to be a very poor solvent when compared to other supercritical fluids. This was not surprising due to the fact that when the alcoholysis of methyl methacrylate was carried out in hexane, the presence of carbon dioxide inhibited the activity of the lipase. It was proposed that the carbon dioxide formed reversible carbamate complexes with the free amine groups on the surface of the enzyme. Direct evidence for carbamate formation was later shown through laser desorption mass spectroscopy (LD-MS).[60]

IV. Enzymatic Reactions in Other Supercritical Fluids

Although supercritical carbon dioxide is the most frequently used supercritical fluid for a reaction medium, there are a variety of other supercritical fluids that can be used as solvents for biocatalytic reactions, such as fluoroform, ethane, sulfur hexafluoride, and near-critical propane.

Some of the first work performed in supercritical fluids other than carbon dioxide was performed by Hammond et al.,[2] who used supercritical fluoroform as the reaction medium for the oxidation of *p*-cresol and *p*-chlorophenol by the enzyme polyphenol oxidase. The reaction was performed in batch mode and under flow conditions. Oxygen was required to be present for the reaction to occur. However, when fluoroform was used under flow conditions (34 471.5 kPa psi at 1 L (STP)/min), approximately 70% of the substrate was oxidized by the polyphenol oxidase.

As stated earlier, Borges de Carvalho et al.[63] studied the effects of high-pressure propane, carbon dioxide, and a mixture of the two gases on the

catalytic activity of subtilisin. The activity was the highest in propane, followed by the mixture of carbon dioxide and propane. The activity was at its lowest in the carbon dioxide. The solvation ability of each of the various solvents tested was analyzed and further supported the notion that supercritical fluids, other than carbon dioxide would be a more suitable choice for biocatalytic reactions.

Also previously mentioned, Barreiros et al.[66,71] studied the catalytic activity of subtilisin *Carlsberg* and Novozym 535 in compressed propane and supercritical ethane. As pressure was increased, the catalytic activity of subtilisin decreased. However, when compared to supercritical carbon dioxide, the effect of pressure was not as drastic in the compressed propane and supercritical ethane. The catalytic activity of Novozym 435 was about 10 times higher in supercritical ethane and compressed propane when compared to supercritical carbon dioxide. Also, the activity of the enzyme in supercritical ethane increased, although not drastically, as the temperature increased.

Randolph et al.[73] were able to demonstrate that small changes in the pressure of supercritical ethane produced large, positive activation volumes, which are a measure of how pressure dependent the rate-limiting step of a reaction is. The effects of varying the solvent's physical properties and varying the concentration of the reactants, cosolvents, and products were studied in the Heisenberg spin-exchange reaction between nitroxide free radicals. Both effects were evident since the reaction rates varied as the parameters were varied. Activation volumes as large as 7 L s/mol were reported for supercritical ethane.

Kamat et al.[13] were also able to show how solvent properties, such as Hildebrand solubility parameter and dielectric constant, can be changed by changing the pressure of the supercritical fluid. Once again, supercritical fluoroform, ethane, sulfur hexafluoride, and near-critical propane were tested. The dielectric constant of supercritical fluoroform changes from 1 to 8 by increasing the pressure from 5860 to 27 577 kPa. The ability to tune the physical properties of solvents by merely changing the pressure provides for the activity of the enzyme, in this case lipase from *Candida rugosa*, to be manipulated as well.

Chaudhary et al.[74] performed a lipase-catalyzed transesterification between bis(2,2,2-trichloroethyl)-adipate and 1,4-butanediol in supercritical fluoroform. By varying the pressure of the fluoroform, it was possible to separate the low dispersity polymer fractions from the synthesized polymer. It was also demonstrated that polymer molecular weight and dispersity could by controlled and predicted by varying the pressure of the supercritical fluoroform. As the pressure increased, so did the average molecular weight of the soluble polymer and the precipitated polymer, as seen in Table 4.[76]

Table 4. Effect of Pressure on Molecular Weight and Dispersity during Lipase-Catalyzed Polymerization in Supercritical Fluoroform (Reproduced from ref 76. Copyright 1995 American Chemical Society)

pressure (psi)	maximum molecular weight of soluble polymer	average molecular weight (dispersity)	
		synthesized polymer	precipitated polymer
900	739	701 (1.07)	764 (1.02)
1600	1076	778 (1.11)	1272 (1.03)
2400	1982	1035 (1.18)	2130 (1.03)
3000	2189	1338 (1.23)	2590 (1.05)

V. Conclusions

For over a decade, research has been conducted in the field of enzymatic reactions in supercritical fluids.[1−3] It has been stated that the main advantage of using supercritical fluids in place of organic solvents is that the physical properties of supercritical fluids have the ability to be manipulated by merely changing the temperature or pressure of the reaction system.[3−7]

Much of the work that has been performed in the field of biocatalysis in supercritical fluids has employed supercritical carbon dioxide as the solvent. Supercritical carbon dioxide is attractive due to its low toxicity and cost, as well as its environmental friendliness.[15] However, it has been shown that in many cases, carbon dioxide is perhaps the worst supercritical fluid to use as a solvent. This is most likely due to the fact that the processes which have employed carbon dioxide as a solvent do not possess certain characteristics which would render carbon dioxide use favorable. However, there are several instances in which carbon dioxide would be advantageous to use as a solvent in a biocatalytic reaction.

For example, a process that would use an enzyme to convert a hydrophobic substrate into a hydrophilic product could be performed in carbon dioxide and then stripped away into water, thereby allowing the reactants to partition into the carbon dioxide phase and the products to partition into the aqueous phase. Additionally, water-saturated carbon dioxide could be recycled back to the original reactor, thus providing a favorable environment for the enzyme to maintain its activity. Other instances where carbon dioxide could be advantageous are when gaseous reactants are used in a process due to the fact that the solubility of gases in most liquids is poor and in the food and pharmaceuticals industry since carbon dioxide is nontoxic and unregulated.

While there are examples that clearly demonstrate that the use of carbon dioxide can be advantageous, one must still realize that there are certain economic issues that may prevent the implementation of carbon dioxide in some processes due to high capital and operating costs. However, there are certain constraints that if used, will help to minimize the large costs associated with the use of carbon dioxide. These include minimizing the operating pressure via the use of materials that demonstrate high solubility in carbon dioxide, thus reducing the size of needed equipment; eliminating large pressure drops which would in turn eliminate the large cost of recompressing the gas; employing continuous processing and minimizing the flow rate of carbon dioxide, which both reduce equipment size; recycling those materials with high carbon dioxide solubility since they are almost always expensive.

Although extensive research has been conducted in the field of supercritical biocatalysis since 1985,

the advantages of replacing conventional organic solvents with supercritical fluids have not fully been demonstrated yet. However, if one follows the guidelines stated above, the attractive combination of natural catalysts with natural solvents will hopefully one day live up to its potential.

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