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Supported Aqueous-Phase Enzymatic Catalysis in Organic Media

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ABSTRACT

The use of partially hydrated porous silica particles has been studied as a support for cofactor dependent enzymatic catalysis in organic solvents. At an optimal pore hydration corresponding to 70% pore volume, horse liver alcohol dehydrogenase catalyzes the oxidation and reduction of alcohols and aldehydes, respectively, with rates sixfold higher than with nonporous glass beads as the enzymatic support and with cofactor recycling numbers in excess of 10^5. Thus, supported aqueous-phase enzymatic catalysis makes highly effective use of the enzyme and cofactor by coimmobilization and by providing a high interfacial area for reactions in organic media.

Index Entries: Supported liquid-phase catalysis; alcohol dehydrogenase, organic media

INTRODUCTION

Although the use of enzymes in organic media has seen some remarkable successes (*1,2*), the problem of reaction optimization remains largely unaddressed. For example, it is well known that the amount of water required for catalysis is enzyme-, solvent-, and reaction-dependent (*3,4*). In many syntheses, the water in the reaction mixture must be minimized to

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shift the thermodynamic equilibria in favor of synthesis as opposed to hydrolysis (e.g., for lipase/protease-catalyzed esterification, transesterification, peptide synthesis, and so forth), and reaction optimization is really a compromise between the minimal amount of water that supports catalytic activity and the maximal amount of water that does not adversely affect reaction yield. Many enzymes, however, do not face such stringent thermodynamic constraints, and the amount of water tolerated by the system can be optimized with respect to enzymatic catalysis. An excellent example of this is the use of alcohol dehydrogenase (LADH) in nonaqueous media. The thermodynamic equilibria of LADH-catalyzed oxidation or reduction of alcohols or aldehydes and ketones, respectively, is not significantly dependent on the water concentration. The advantage of an organic reaction medium in LADH catalysis is the increased solubility of nonpolar substrates. The major limitation of LADH in organic solvents, however, is the poor interaction between the enzyme and the organic-insoluble nicotinamide (NAD(H)) cofactor. In order to provide an environment conducive to enzyme-cofactor interaction, enzyme immobilization has been employed (*5,6*). For example, LADH and NAD(H) have been coimmobilized onto nonporous glass beads in polar, water-immiscible solvents such as ethyl acetate and isopropyl ether (*7*). The solvents contained a saturating amount of water that enabled the surface of the glass beads to maintain a microaqueous environment that further enabled the LADH and NAD(H) to interact directly. The rates of catalysis were slow, however, probably owing to both the poor level of hydration of the enzyme on such supports and the low interfacial area for mass transfer between the aqueous and bulk organic phases. Entrapping LADH and NAD(H) within an aqueous phase of porous supports (*8*) assures the enzyme of existing in its proper hydration state for optimal catalysis. However, if the pores of the support are completely water-filled, severe diffusional limitations can result from the relatively small surface area of contact between the internal aqueous phase of the pores and the bulk organic phase, as in the case of nonporous supports. In this work, we describe a novel methodology (denoted as supported aqueous-phase enzymatic catalysis, or SAPEC) to enable biocatalysis to function optimally in predominantly organic media by using porous supports equilibrated at optimal hydration levels with large interfacial areas.

MATERIALS AND METHODS

Materials

Horse liver alcohol dehydrogenase, NAD(H), nonporous glass beads (75–150 μm), and porous glass beads (2000 Å) were purchased from Sigma Chemical Co. (St. Louis, MO). Porous silica gel (150 Å) and all substrates were obtained from Aldrich Chemical Co. (Milwaukee, WI). Tritiated water was obtained from Amersham (Arlington Heights, IL) and had a

specific activity 5 mci/mL. Ultima Gold scintillation cocktail was purchased from Packard (Downers Grove, IL). All other chemicals and solvents used in this work were of the highest quality commercially available.

Immobilization of LADH

Different amounts of LADH and NAD(H) were dissolved in Tris-HCl buffer (50 m*M*, pH 7.1) and the solution was mixed with dehydrated silica gel (150 Å pore diameter) at a ratio of 3 mL solution/g silica. The suspension was left at 25°C for 4 h, after which the gel was washed with buffer. No enzyme or cofactor was detected (via the Bradford protein assay and LADH assays) in the wash, suggesting that the silica gel adsorbed all of the enzyme and cofactor, and that the enzyme was probably physically adsorbed on the silica gel. Final drying to achieve different internal pore hydration levels was achieved under vacuum (25 mm Hg) at 25°C for different time periods depending on the desired hydration levels. For 70% internal pore hydration, the drying time was ca. 3.5 h. Other supports were also utilized in a similar manner.

Measurement of Internal Hydration Levels

The degree of internal hydration was determined gravimetrically. Following the final drying (*see above*), the weight of the silica gel was measured and compared to the weight of the initially dehydrated silica gel. The difference was assumed to be the weight of water within the pores of the silica support. The percentage of pore volume taken up by water can be calculated from the final weight of water in the gel and the pore volume of the gel (1.15 cm^3/g). The fraction of internal water lost during a typical reaction was determined both gravimetrically and via desorption of T_2O. The former was performed by removing the silica gel from the reaction mixture, drying at 25°C under vacuum (25 mm Hg) for 3 h, and weighing the water remaining in the support. Only solvents with substantially higher vapor pressures than water could be used for the gravimetric method, such that only the organic solvent and not internal water would evaporate.

Tritiated water desorption was carried out as follows: T_2O from Amersham was diluted tenfold in aqueous buffer to give a specific activity of 1.11×10^6 dpm/μL. The incorporation of T_2O into the porous silica was performed by suspending 0.25 g silica in 0.375 mL aqueous buffer plus 0.125 mL diluted T_2O, for 4 h. The silica was washed with cold aqueous buffer, as previously described for the nonradioactive water incorporation. The silica was vacuum dried for 3.5 h until an internal pore hydration level of 70% was reached (as confirmed by gravimetric analysis). Total T_2O desorbed from the silica into a given reaction mixture was quantitated by adding 50 μL of the reaction mixture to 10 mL of the liquid scintillation cocktail. The T_2O desorbed was measured with a Packard 1600CA Tri-carb Liquid Scintillation Analyzer.

Reaction Conditions

A typical small-scale reduction reaction was performed as follows: 25 m*M* of an aldehyde or ketone was dissolved in 10 mL of an organic solvent containing 0.4*M* ethanol and 2.5 g silica (containing 10 mg LADH [final concentration in reaction of 1 mg/mL] and 1 μg NADH [final concentration in reaction of 0.1 μg/mL]) was added to initiate the reaction. The suspension was shaken at 200 rpm with an orbital shaker at 25°C. Aliquots were removed periodically and analyzed by gas chromatography using an Econo cap carbowax capillary column from Alltech (Deerfield, IL) (30 m × 0.25 mm, 0.25 μm film thickness; N_2 carrier gas at 30 mL/min, isothermal column temperature of 100°C and injection and detection port temperatures of 250°C). The retention times of the 2-methylvaleraldehyde and 2-methyl-1-pentanol under these conditions were 2.46 and 4.87 min, respectively.

Enzyme kinetics were determined using fixed concentrations of one substrate and varying the concentration of the other substrate and vice-versa. For determination of the kinetic constants for NADH, 25 m*M* 2-methylvaleraldehyde was used with NADH concentrations ranging from 0.07–12.25 μ*M*. For aldehydic or alcoholic substrates, NAD(H) was fixed at 0.14 μ*M* with aldehyde/alcohol concentrations ranging from 5–100 m*M*. In all cases, ethanol (0.4*M*) or isobutyraldehyde (0.1*M*, for alcohol oxidation) was used and the LADH concentration was 1 mg/mL. Michaelis-Menten parameters were determined by a nonlinear fit of the data.

Preparative-Scale Synthesis of 2-Phenyl-1-Propanol

2-Phenylpropionaldehyde (0.15*M* was dissolved in 25 mL isooctane containing 1*M* ethanol) and 6.25 g silica gel (containing 25 mg LADH and 2.5 μg NADH) was added. The reaction was terminated after 48 h by filtering off the silica gel, and the isooctane and residual ethanol (and acetaldehyde produced from ethanol oxidation) were removed by rotary evaporation. The resulting oil was dissolved in methylene chloride and purified by silica gel chromatography (methylene chloride as eluant). The optical rotation of the product was measured using a DIP-Jasco optical polarimeter (sodium line at 546 nm).

RESULTS AND DISCUSSION

Design of Supported Aqueous-Phase Enzymatic Catalysis

We describe here a novel mode of conducting cofactor-requiring enzymatic catalysis in organic media. SAPEC involves a layer of water containing enzyme and cofactor that is coated on the internal surface of a

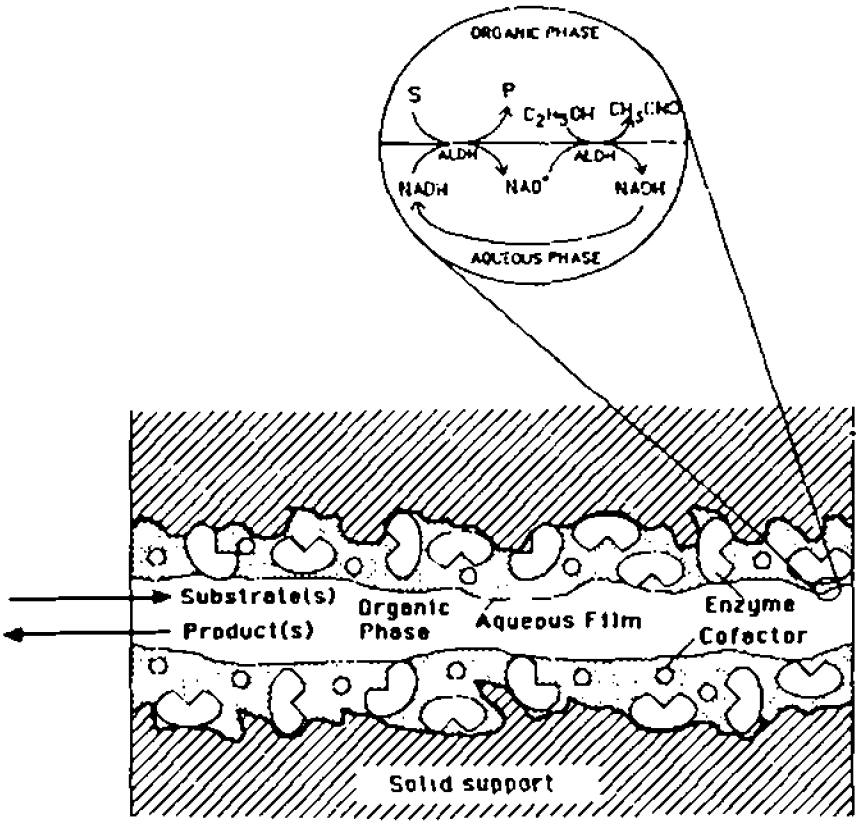

Fig. 1. A schematic of supported aqueous-phase enzyme/cofactor catalyst. S and P indicate aldehyde/ketone substrate and alcohol product, respectively.

porous, high surface area hydrophilic support. The enzyme may or may not be immobilized to the support. A general schematic of the SAPEC technique is shown in Fig. 1. The relative size and shape of enzyme, cofactor, and pore shown in the figure are not necessarily meant to be typical or correspond to those in this study. The SAPEC particles are contacted with a water-immiscible organic solvent (presaturated with water) containing the substrate(s). The substrate molecules diffuse from the bulk organic phase into the pores and react at the water-organic interface. The depth of the reaction zone in the aqueous film would depend on the partition coefficient of the substrate, any partitioning of the enzyme onto the interface, and the orientation of the enzyme at the interface. For relatively polar substrates, the reaction takes place wholly within the aqueous film. The products partition into the organic phase and diffuse out of the pores into the bulk organic liquid. Thus, the SAPEC technique not only immobilizes the biocatalyst and the cofactor to facilitate their interaction, but provides a large interfacial area between the aqueous and organic phases and, consequently, higher reaction rates. The features of high reaction

rates coupled with enhanced cofactor stability imparted by *in situ* cofactor regeneration should prove to be attractive commercially.

Alcohol Dehydrogenase Catalysis Using the SAPEC Technique

Alcohol dehydrogenase from horse liver was chosen for this study because of its wide substrate specificity and ability to catalyze oxidoreductions in both aqueous and organic media. Porous silica particles were used with a pore size of 150 Å, a pore volume of 1.15 cm^3/g, and a pore surface area of 300 m^2/g. In order to test the effectiveness of this porous support for LADH catalysis, 2-methylvaleraldehyde was used as the model substrate. This aldehyde is highly soluble in organic media, although not highly soluble in water, and is known to be an excellent substrate for the horse liver enzyme (*7*). A high interfacial surface area between the internal aqueous phase and the bulk organic phase was achieved by only partially hydrating the silica particles. Vacuum drying the silica beads containing various amounts of LADH (0.4–4.0 mg LADH/g bead) and 0.4 μg NADH (per g bead) for 3 h resulted in an internal pore hydration level of 70% (and hence, a hydrated volume of 0.805 mL/g silica). The partially hydrated silica (2.5 g) containing the enzyme and cofactor was added to 10 mL buffer-saturated ethyl acetate containing 25 m*M* 2-methyl-valeraldehyde. Ethanol (0.4*M*) was added to regenerate the NADH consumed during the reaction. The reaction was linearly dependent on the bulk reaction LADH concentration (data not shown) in the range of 0.1–1 mg/mL, suggesting that the oxidation of 2-methylvaleraldehyde was not limited by internal diffusion. The reduction of 2-methylvaleraldehyde to the corresponding alcohol proceeded quickly and with 1 mg/mL LADH—nearly 95% conversion was achieved after 3.2 h (Fig. 2). Michaelis-Menten kinetics were observed for the oxidation of NADH with a catalytic efficiency $(V_{max}/K_m)_{NADH}$ of 3640 h^{-1} (with 25 m*M* 2-methylvaleraldehyde) as well as for the reduction of 2-methylvaleraldehyde with a $(V_{max}/K_m)_{aldehyde}$ of 0.18 h^{-1} (with 0.14 μ*M* NADH bulk concentration).

The total water content of the reaction system was ca. 22% v/v (including the water content of the ethyl acetate); a relatively large concentration owing to the large amount of silica used in the reaction (2.5 g). This water content was lowered to 4% v/v by reducing the amount of silica tenfold while retaining the overall concentration of LADH and NADH. It should be noted that this modification did not affect the internal pore hydration. No loss in catalytic efficiency of LADH was observed by the reduction in the total water content, and therefore, the lower amount of silica gel was used in all subsequent kinetic experiments. The porous silica support was superior to nonporous glass beads (75–150 μm; *7*). The rate of reduction of 2-methylvaleraldehyde using 1 mg/mL LADH and 0.1 μg/mL NADH was ca. sixfold faster with the porous silica (Fig. 2). Moreover, increasing the pore size of the support by going to 2000 Å glass

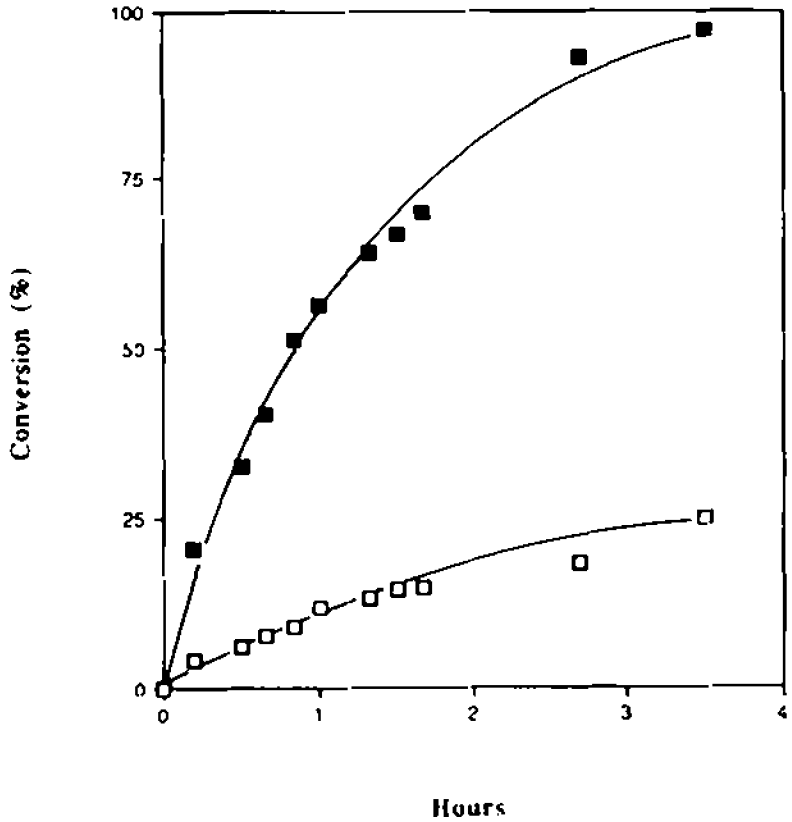

Fig. 2. Comparison of porous silica (■) to nonporous glass beads (□) for the LADH-catalyzed reduction of 2-methylvaleraldehyde. Conditions: 25 m*M* 2-methylvaleraldehyde, 1 mg/mL LADH, 0.1 μg/mL NADH, 0.25 g total silica gel, ethyl acetate as solvent (presaturated with aqeuous buffer), pore volume 70% filled with aqueous buffer.

beads (while retaining 70% internal pore hydration) resulted in a fivefold reduction in LADH activity. This may be speculated to be attributable to the decreased interfacial aqueous/organic surface area of the larger glass pores (9.5 m^2/g) as compared to the 150 Å silica gel or any deleterious effect of the porous glass beads on the enzyme, e.g., strong adsorption. Decreasing the silica gel pore size to 60 Å (with washing of the silica gel following incubation with enzyme as previously described), however, did not support catalysis, presumably because the enzyme (75 KDa) could not enter the small pores of this silica gel and was washed off during preparation.

Stability of Pore Hydration

To determine the stability of the internal pore hydration in organic solvents (e.g., whether desorption of water from the porous silica into the

organic solvents takes place), gravimetric and tritium desorption experiments were commenced. Figure 3A depicts the water desorption profiles for gravimetric analysis of 70% internal pore hydration. No significant loss in internal water content in buffer-saturated ethyl acetate was evident over a period of 48 h, suggesting that the internal pore hydration level was stable throughout the enzymatic reaction. An alternative to gravimetric analysis is to measure the desorption of T_2O from the silica pores into the reaction mixture. To that end, silica gel was prepared to 70% hydration with T_2O added to the water as described in Materials and Methods. Figure 3B depicts the fraction of total T_2O desorbed into buffer-saturated ethyl acetate and isooctane. As a control, the fraction of T_2O desorbed using aqueous buffer is also shown in Fig. 3B. Clearly, the organic reaction media were less capable of removing T_2O from the silica gel than was the aqeuous reaction medium, and with isooctane, less than 0.01% of the total T_2O was desorbed from the silica. It should be noted that the total fraction of T_2O desorbed was low, even in the aqueous buffer case (ca. 4.5% after 31 h). This may be the result of tritium exchange with the hydrogens of the silica gel, thereby incorporating radioactive label into the gel matrix. Hence, the propensity of T_2O to desorb from the silica gel should be measured by comparison to the T_2O desorbed from the silica gel in aqueous buffer. In either ethyl acetate or isooctane, only a small fraction of the desorbable water is removed over a period of 31 h.

Having established that the internal pore hydration level remains constant during the time-course of the enzymatic reaction, it was possible to evaluate the effect of internal pore hydration on enzymatic catalysis. The level of hydration inside the porous silica is expected to affect the rate of catalysis by (1) altering the interfacial surface area to pore volume ratio and consequently diffusional resistances, and (2) through kinetic and equilibrium considerations (e.g., by providing an optimal level of enzymic hydration). The effect of pore hydration is shown in Fig. 4. Reduction in pore hydration from 100 to 75% increased the catalytic efficiency of LADH in ethyl acetate by 15%. Below 75% pore hydration, the catalytic efficiency of LADH dropped, and with dry silica gel containing the enzyme, no activity was observed. LADH in butyl acetate was even more sensitive to the internal degree of hydration—an increase in V_{max}/K_m of nearly 3.5-fold upon reduction in internal hydration from 100 to 75%. The increase in activity observed in ethyl and butyl acetate upon a decrease in pore hydration level from 100 to 75% may be owing to the increase in the aqueous/organic interfacial area. As discussed in the Introduction, the interfacial surface area for completely water-filled pores is at the pore opening, only, whereas for lower degrees of hydration, the interfacial area increases such that with nearly dry silica, it is equivalent to that of the surface area of the pores. Below 75%, however, the enzyme may not have enough water to remain soluble and may be in direct contact with the

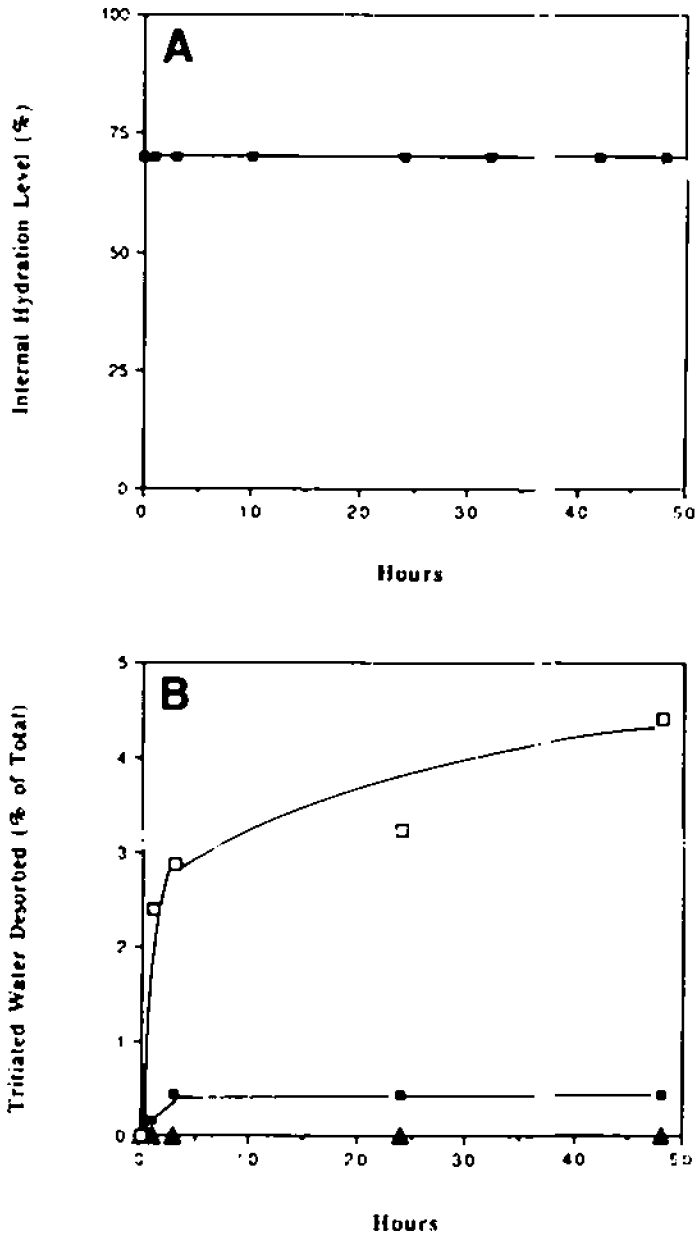

Fig. 3. Desorption of water from porous silica in various organic solvents via: (A) Gravimetric analysis in buffer-saturated ethyl acetate; and (B) Tritiated water technique with (□), aqueous buffer; (■), buffer-saturated ethyl acetate; and (▲), buffer-saturated isooctane.

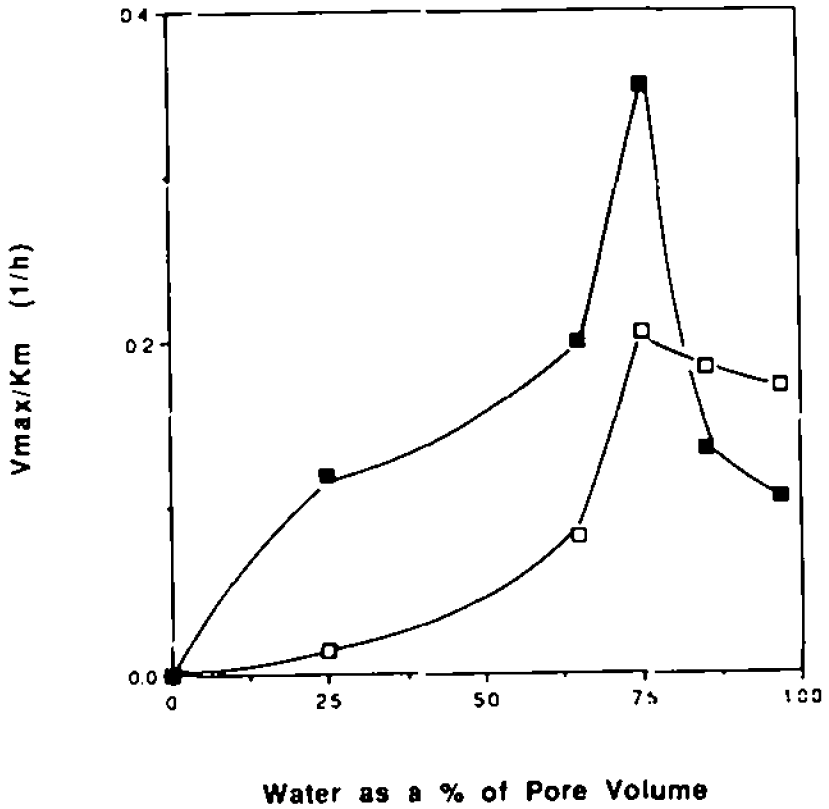

Fig. 4. Effect of internal hydration on LADH catalysis in ethyl acetate (□) and butyl acetate (■). Conditions: 5-100 mM 2-methylvaleraldehyde, 0.1 μg/mL NADH, 0.4M ethanol, and the reaction mixtures were shaken at 200 rpm at 25°C.

organic solvent, thereby leading to some deactivation. Alternatively, the loss in water reduces the polarity of the internal silica environment, thereby lowering the catalytic activity of the enzyme. A similar effect was observed by Deetz and Rozzell for yeast alcohol dehydrogenase in which catalytic activity could be increased 40-fold by using the polar thiolated glycophase controlled pore glass instead of relatively nonpolar untreated controlled pore glass (9). In any event, dehydrated silica cannot support catalysis. In contrast to internal hydration levels, the water content of the organic solvent (in the case of ethyl and butyl acetates) had little effect on LADH catalysis and only at water contents well below saturation did LADH lose catalytic activity, probably because of partitioning of water out of the silica pores and into the organic solvent.

LADH catalysis was investigated in a number of solvents ranging from the hydrophilic tetrahydrofuran to the hydrophobic isooctane. As shown in Table 1, solvents with the greatest degrees of hydrophobicity, and least solubilities in water (e.g., hexane, isooctane, and isopropyl

Table
Effect of Organic Solvents
on the Catalytic Efficiency of LADH in Hydrated Porous Silica

Solvent	Log P	V_{max}/K_m (h^{-1})	Optimal pore hydration, %
Isooctane	4.5	0.72	75
Isopropyl ether	1.9	0.67	75
Hexane	3.5	0.65	75
Butyl acetate	1.7	0.38	75
Ethyl acetate	0.68	0.21	75
Tetrahydrofuran	0.49	0	

Conditions: All water-immiscible solvents were presaturated with aqueous buffer prior to use. The reaction mixtures (10 mL) contained 0.1 μg/mL NADH, 1 mg/mL LADH, 0.4*M* ethanol, 5–100 m*M* 2-methylvaleraldehyde and the silica (0.25 g) was hydrated to 75% pore volume. The reaction mixtures were shaken at 200 rpm at 25°C. Log P values from Laane et al. (*17*) where P is the partition coefficient of a given solvent between water and 1-octanol.

ether) sustained the greatest catalytic efficiencies. In contrast, LADH was inactive in tetrahydrofuran. The solvent independence in optimal pore hydration indicates that the enzyme remains fully within the aqueous phase of the silica gel. Lower activity in water-immiscible polar organic solvents, as compared to nonpolar solvents, was not attributable to the stripping of water out of the pores, and hence, reduction in the degree of pore hydration, because no significant loss in internal hydration was observed in buffer-saturated organic solvents (*see above*). Instead, it may be speculated that polar solvents can dissolve easily in the internal aqueous phase and inhibit the enzyme. This phenomenon is a common cause of enzyme inhibition in aqueous-organic biphasic reaction mixtures (*10*). This phenomenon may also explain the increased activity of LADH in butyl acetate as compared to the more polar ethyl acetate (Fig. 4) as pore hydration is reduced. The lack of measureable LADH activity in THF may have been attributable to both solvent-induced enzyme inhibition and stripping of the internal water out of the silica gel.

Cofactor Recycling

A significant problem in using LADH for preparative biotransformations in aqueous solutions is the instability of the nicotinamide cofactor. For example, typical turnover numbers for NADH recycling in aqueous solutions range from 100–10,000 with the higher turnover numbers being observed upon tethering the NADH to a soluble polymeric support (*11*). Therefore, it was of interest to determine the NADH recycling turnover numbers using LADH within porous silica in organic media. To that end, 2.5 g silica, containing 10 mg LADH and 1.0 μg NADH, were added to 10

Table 2
Substrate Specificity for LADH
in Optimally Hydrated Silica

Substrate	V_{max}/K_m (h^{-1})
2-Methylvaleraldehyde[a]	0.72
2-Phenylpropionaldehyde[a]	0.35
2-Chlorocyclohexanone[a]	0.006
Trans-cinnamaldehyde[a]	0.003
Trans-cinnamyl alcohol[b]	0.013

Conditions: [a]Reduction reaction mixtures (10 mL) containing 0.1 μg/mL NADH, 1 mg/mL LADH and 0.4*M* ethanol, [b]Oxidation reaction mixture (10 mL) containing 0.1 μg/mL NAD^+, 1 mg/mL LADH, and 0.4*M* butyraldehyde. Substrate concentrations ranged from 5–100 m*M*. All reactions were shaken at 200 rpm at 25°C and the solvent was isooctane. The silica (0.25 g) was hydrated at 75% pore volume.

mL isooctane containing 0.1*M* 2-methylvaleraldehyde and 1.0*M* ethanol. The reaction reached 48% conversion after 34 h. Very little further reaction was observed over an additional 24 h. Based on this conversion of the 2-methylvaleraldehyde, the recycle turnover number for NADH was 3.41×10^5. This indicates that the internal aqueous microenvironment does not destroy the nicotinamide cofactor as in conventional aqueous-based catalysis. This is a significant advantage of the SAPEC technique.

Substate Specificity

A variety of substrates were examined in the porous silica/isooctane system (Table 2). The higher activity afforded by 2 methylvaleraldehyde as compared to the other substrates is consistent with LADH catalysis in aqueous solutions wherein alkyl, noncyclic molecules are more reactive than cyclic or aromatic substrates (*12*).

Preparative Synthesis

From a preparative standpoint, the reduction of 2-phenylpropionaldehyde was of particular interest because it had been performed stereoselectively with nonporous glass beads (*7*). Gram-scale synthesis of the 2-phenyl-1-propanol was carried out in isooctane as described in Materials and Methods. The reaction was terminated after 48 h after a conversion of 25.5 m*M* (17%) of the aldehyde to the alcohol. Purification of the alcohol via silica gel chromatography resulted in 0.70 g alcohol with a purity >98% (as measured by gas chromatography). The alcohol product was analyzed by optical polarimetry and found to have an α_D^{25} value of −14.5 (the literature value is −15.25 for the R isomer, *13*). Hence, 2-phenyl-1-propanol was produced with enantiomeric purity of 95%.

CONCLUSIONS

The SAPEC technique presented here is analogous in concept to the supported liquid-phase catalysis technique (*14, 15*) for homogeneous catalyzed gas phase reactions and the supported aqueous-phase catalysis technique for organic phase reactions with aqueous homogeneous catalysis (*16*). This article, however, is the first report of such a technique applied to enzymatic catalysis in organic media where the water content inside a porous catalyst is controlled to optimize between chemical, thermodynamic, and physical (surface area) factors. The regeneration efficiency of the nicotinamide cofactor is of particular interest in that it is greater than three orders of magnitude higher than in water with the free cofactor, and nearly two orders of magnitude higher than NAD(H) bound to a water-soluble polymer. Similar approaches may be useful for other cofactor requiring enzymes (e.g., other oxidoreductases as well as ligases and their ATP cofactor)

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REFERENCES

1. Dordick, J. S. (1989), *Enz. Microb. Technol.* **11**, 194-211.
2. Klibanov, A. M. (1990), *Acc. Chem. Res* **23**, 114-120.
3. Zaks, A. and Klibanov, A. M. (1988), *J. Biol. Chem.* **263**, 8017-8021.
4. Gorman, L. S. and Dordick, J. S. (1992), *Biotechnol. Bioeng.* **38**, 392-397.
5. Clark, D. S., Skerker, P. S., Creagh, L., Guinn, M., Prausnitz, J., and Blanch, H. W. (1989), *ACS Symp. Ser.* **392**.
6. Deetz, J.S. and Rozzell, J. D. (1988), *Trends Biotechnol.* **6**, 15.
7. Grunwald, J., Wirtz, B., Scollar, M. P., and Klibanov, A. M. (1986), *J. Am. Chem. Soc.* **108**, 6732-6742.
8. Wong, C.-H. (1987), *Biocatalysis in Organic Media*, Laane, C., Tramper, J., and Lilly, M. D., eds., pp. 197-208.
9. Deetz, J. S. and Rozzell, J. D. (1991), *Biocatalysts for Industry*, Dordick, J. S., ed., pp. 181-191.
10. Carrea, G. (1984), *Trends Biotechnol.* **2**, 102-106.
11. Buckmann, A. F., Morr, M., and Kula, M. R. (1987), *Biotechnol. Appl. Biochem.* **9**, 258-268.
12. Sekhar, V. C. and Plapp, B. V. (1990), *Biochemistry* **29**, 3289-4295.
13. Eliel, E. L. and Freeman, J. P. (1952), *J. Am. Chem. Soc.* **74**, 923-927.

14. Rony, P. R. (1969), *J. Catal.* **14,** 142.
15. Datta, R. and Rinker, R. G. (1985), *J. Catal.* **95,** 181–192.
16. Ashamut, J. P., Davis, M. E., Mirola, J. S., and Hanson, B. E. (1989), *Nature* **339,** 454–455.
17. Laane, C., Boeren, S., Hilhorst, R., and Veeger, C. (1987), *Biocatalysis in Organic Media*, Laane, C., Tramper, J., and Lilly, M. D., eds., pp. 65–84.