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Two-Liquid Phase Biocatalytic Reactors [and Discussion]

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Two-liquid phase biocatalytic reactors

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Two-liquid phase biocatalytic reactors in which a large proportion of the reaction volume is occupied by the organic phase have been investigated for steroid transformations and the modification of oils and fats. The organic phase may consist of the reactant alone or the reactant dissolved in a water-immiscible organic solvent. Under appropriate conditions, immobilized cells and enzymes have reasonable operational stabilities in such reactors, and some commercial processes are being developed.

Introduction

Many organic compounds of interest to the chemical and biological industries have low solubilities in water or aqueous solutions. There is now a growing interest in the use of biological catalysts to carry out selective and stereospecific conversions of some of these compounds. In some cases it is possible to increase their solubilities by addition of surface-active agents or water-miscible organic solvents. An alternative approach is to use a two-liquid phase reactor in which the organic phase consists of either the reactant alone or the reactant dissolved in a water-immiscible organic solvent (Lilly 1982). One particular advantage of this approach is the possibility of operating the reactor with a large proportion of the volume occupied by organic phase. This overcomes one of the major drawbacks of biological catalysts, the need to work in dilute aqueous solutions, with all the associated problems of product recovery. At the same time the biocatalyst can be readily separated from the product phase without the need for immobilization of the catalyst, although there may still be other good reasons for immobilization, such as increased operational stability and protection against the harmful effects of organic solvents on catalyst activity and stability.

Types of reaction system

During the early development of biochemical reactors, especially those involving immobilized enzymes or cells, it was useful to classify the various types of reaction system (Lilly & Dunnill 1971). The development of two-liquid phase reactors is now at about the same stage. Although only a few systems have been studied so far, it is worth distinguishing in a simple manner between the different types so that a rational approach can be made to a better understanding of their behaviour and the design of appropriate reactors (Lilly 1982).

There are four basic distinctions that can be made for classification purposes: (1) the liquid phase ratio (aqueous or organic continuous phase), (2) the presence or absence of organic solvent, (3) the site of reaction (at the liquid–liquid interface or in the aqueous phase), and (4) the form of the catalyst (free or immobilized). All four of these will influence the performance and characteristics of the reaction system.

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The organic phase

One of the advantages already mentioned is the possibility of using high ratios of organic to aqueous phases. Tables 1 and 2 give some examples for free and immobilized biocatalysts respectively. In some cases the organic phase is a very high proportion of the reaction volume and is the continuous phase with the remaining space occupied either by free microorganisms or immobilized biocatalyst. For reactions such as cholesterol oxidation by *Nocardia rhodochrous*, phase inversion occurs as the ratio of cell suspension to organic phase is increased to 0.6–0.8

Table 1. Two-liquid phase reactions with free biocatalysts

reaction	catalyst	organic phase: solvent	organic phase: volume (% of total)	reference
cholesterol oxidation	*N. rhodochrous*	tetrachloromethane	80–95	Buckland *et al.* (1975)
triglyceride hydrolysis	*Rh. arrhizus*	di-isopropyl ether	99	Bell *et al.* (1981)
octadiene epoxidation	*Ps. oleovorans*	cyclohexane	20	Schwartz & McCoy (1977)
1-octene epoxidation	*Ps. oleovorans*	none	10–80	de Smet *et al.* (1981)
menthyl ester hydrolysis	*B. subtilis*	none	5–70	I. Brookes & M. D. Lilly (unpublished results)

Table 2. Two-liquid phase reactions with immobilized biocatalysts

reaction	catalyst	organic phase: solvent	organic phase: volume (% of total)	reference
cholesterol oxidation	*N. rhodochrous*	benzene–heptane	67–70	Omata *et al.* (1979)
		trichloroethane	80	Duarte & Lilly (1980)
menthyl ester hydrolysis	*R. minuta*	heptane	67–70	Omata *et al.* (1981)
fat interesterification	lipase	hexane	96	Yokozeki *et al.* (1982)
hydroxysteroid dehydrogenation	HO-steroid dehydrogenase	ethyl acetate	33	Carrea *et al.* (1979)

Table 3. Water-immiscible organic solvents used in two-phase reactions

hexane	dichloromethane	diethyl ether
heptane	trichloromethane	di-isopropyl ether
hexadecane	tetrachloromethane	dibutyl ether
cyclohexane	dichloroethane	dipentyl ether
benzene	trichloroethane	
toluene		ethyl acetate
	petroleum ether	butyl acetate
	gas oil	

(Duarte 1982). Two reactions catalysed by suspended microorganisms, octene epoxidation and menthyl ester hydrolysis, have been done with only reactant present initially as the organic phase, but all the others include organic solvents. Table 3 lists water-immiscible organic solvents that have been tried with varying success. Some of the factors influencing the choice of solvent have been discussed elsewhere (Lilly 1982).

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Reaction kinetics and equilibria

The kinetics of reactors containing enzymes or microorganisms, either free or immobilized, acting on water-soluble reactants is now well documented (Wang *et al.* 1979). The presence of a second liquid phase makes the kinetics potentially more complex and may also affect the reaction equilibrium.

Packed-bed reactors have been operated for interesterification (Macrae 1981; R. Wisdom, P. Dunnill & M. D. Lilly, unpublished results) and fat hydrolysis (Bell *et al.* 1981) in which the catalyst packed in the bed acted as a stationary aqueous phase through which the organic phase was passed. Other types of reaction have normally been done in an agitated vessel, which is advantageous for such reactions as cholesterol oxidation because one of the reactants, oxygen, must be supplied from the gas phase. So far we have little knowledge of the mean size or distribution of droplets in these agitated reactors. Thus, for instance, in those reactions where the organic phase is the continuous one it is not clear whether each cell or catalyst particle is surrounded by organic phase or is dispersed in larger droplets containing many cells or particles. There has been much argument in the past about the exact mechanisms by which hydrocarbons are taken up by microorganisms (Reddy *et al.* 1982), and the same questions need to be answered for other two-phase systems. There is some evidence (Duarte 1982) that *Nocardia rhodochrous* releases surface-active agents when used for the oxidation of cholesterol dissolved in water-immiscible organic solvents, causing changes in the creaming properties (the ease of separation of the two liquid phases). The mechanism of transfer of the reactant from the organic phase to the catalyst has important implications for the design of suitable reactors, especially for immobilized catalysts.

Microorganisms or enzymes may be immobilized to give either a non-porous or porous solid catalyst. A non-porous structure will ensure that the biocatalyst is on the exterior surface and close to the liquid–liquid interface but will have a low activity per unit volume of immobilized catalyst unless very small particles or thin sheets are used. Unless a porous structure has large open pores, most of the biocatalyst will be in pores away from the liquid–liquid interface. The choice of a suitable immobilization support therefore depends on the site of action of the biocatalyst. For a lipase that acts at the interface, a non-porous or very open porous support will be necessary to achieve good reaction rates. It is interesting that celite has proved to be a good support material for immobilization of this enzyme (U.K. patent no. 1,577,933; Yokozeki *et al.* 1982). For reactions that take place in the aqueous phase, unless there is an adequate rate of transfer of reactant through the aqueous phase, catalysts immobilized in porous supports will be at a severe disadvantage. Many of the support materials that have been used successfully for water-soluble reactants such as Sepharose, porous glass, DEAE-cellulose, polyacrylamide and alginates have been tried. In addition a range of synthetic polymers have been examined by Fukui and coworkers (Yamane *et al.* 1979; Omata *et al.* 1979; Fukui *et al.* 1980). Existing data on the proportion of the catalyst's activity available after immobilization indicate that good reaction rates can be obtained (table 4), so that transfer of reactant from the interface to the catalyst does not appear to be a serious problem with present immobilized biocatalytic activities.

The observed activities are influenced by the way in which reactants partition between the support material used for immobilization and the external solvent. Fukui and his coworkers have made a detailed study of the effect of this partitioning on the reaction rates of immobilized

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cells acting in water-immiscible solvents. In particular they have examined polyurethanes containing a mixture of two prepolymers, one (PU-3) more hydrophobic than the other (PU-6) because its ethylene oxide content is lower (figure 1). In three cases the reaction rate increased with the PU-3 content of the polymer, as did the partitioning of the reactants in favour of the polymer. For *N. rhodochrous* there was a marked difference between the rates of oxidation of cholesterol and dehydroepiandrosterone, a reflection of the greater affinity of the latter for the support polymer. The activities for these two reactions given in figure 1 are expressed relative

TABLE 4. EFFECT OF IMMOBILIZATION ON THE OBSERVED ACTIVITIES IN WATER-IMMISCIBLE ORGANIC SOLVENTS

reaction	catalyst	support material	observed activity (% of free value)	reference
β-HO-steroid dehydrogenation	enzyme	Sepharose	60	Carrea *et al.* (1979)
20β-HO-steroid dehydrogenation	enzyme	Sepharose	44	Carrea *et al.* (1979)
cholesterol oxidation	*N. rhodochrous*	polyacrylamide	40	Duarte & Lilly (1980)
cholesterol oxidation	*N. erythropolis*	DEAE-cellulose	66	Atrat *et al.* (1980)
dehydroepiandrosterone oxidation	*N. rhodochrous*	polyurethane (PU-3)	105	Omata *et al.* (1979)
menthyl ester hydrolysis	*R. minuta*	polyurethane (PU-3)	42	Omata *et al.* (1981)

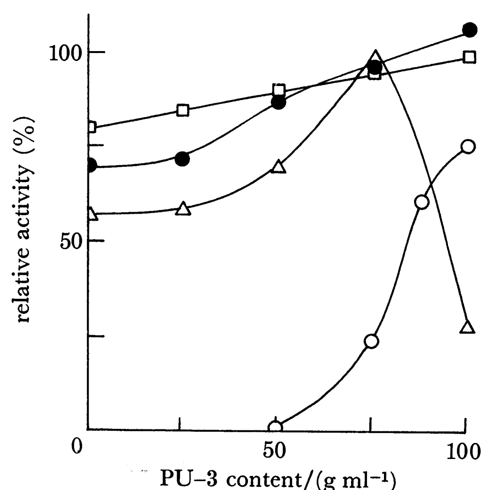

FIGURE 1. The effect of gel hydrophobicity on the activities of immobilized cells in water-immiscible organic solvents. The gels were made from mixtures of a hydrophilic prepolymer (PU-6) and a hydrophobic prepolymer (PU-3). Cholesterol oxidation (○) and dehydroepiandrosterone transformation (●) by *N. rhodochrous* (Omata *et al.* 1979); menthyl succinate hydrolysis (□) by *R. minuta* (Omata *et al.* 1981); testosterone oxidation (△) by *N. rhodochrous* in the presence of phenazine methosulphate (Fukui *et al.* 1980).

to the activity of the free cells. Thus, for dehydroepiandrosterone, the immobilized cells can attain an activity greater than the free cells owing to the partitioning of the reactant (table 4). The fourth case, testosterone oxidation, involved a water-soluble second reactant, phenazine methosulphate, and the reaction rate was optimal in a gel containing 75% of the PU-3.

In our own experiments on the oxidation of cholesterol to cholestenone with *N. rhodochrous* suspended in organic solvents (Duarte 1982), partitioning of the cholesterol and cholestenone between the cells and the solvent was observed. At the start of the reaction the rate of cholesterol

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disappearance and cholestenone production differed greatly when calculated on the basis of the concentration changes in the organic solvent.

In addition to influencing the reaction rates, organic solvents can also perturb the reaction equilibrium because of the different solubilities of reactants and products in the organic phase (Martinek & Semenov 1981). For instance, Semenov *et al.* (1981) have used chymotrypsin to catalyse the synthesis of *N*-acetyl-L-tryptophanyl-L-leucine amide in a biphasic mixture consisting of ethyl acetate:water (98:2 by volume). Schwartz & McCoy (1977) used the greater solubility of epoxide derivatives in cyclohexane compared with that in water to increase fivefold the conversion of octadiene to these inhibitory derivatives.

TABLE 5. OPERATIONAL STABILITIES FOR FREE AND IMMOBILIZED CELLS IN WATER-IMMISCIBLE SOLVENTS

catalyst	solvent	temperature °C	form	total time days	remaining activity (%)	reference
N. rhodochrous cholesterol oxidase	trichloroethane	30	free	6.2	80	Duarte & Lilly (1980)
			immobilized	12.5	50–68	
steroid Δ^1-dehydrogenase	benzene–heptane	20	free	0.13	2	Yamane *et al.* (1979)
			immobilized	0.25	50	
R. minuta menthyl esterase	heptane	30	free	16.7	25	Omata *et al.* (1981)
			immobilized	25	75	

Catalyst stability

There are now many reports of increased stability of enzymes and microorganisms resulting from immobilization (Lilly 1978; Klibanov 1979). Although for some microorganisms or enzymes in two-phase reactions (Lilly 1982) quite reasonable operational stabilities have been observed, immobilization often makes a considerable improvement (table 5). Whereas cholesterol oxidase is a relatively robust enzyme and immobilization only results in a small change in stability, there is a marked increase for the other two enzymes, steroid Δ^1-dehydrogenase and menthyl esterase. It is usually not clear whether the change is a result of immobilization *per se* or greater protection from the organic phase, especially if this contains organic solvent.

There is some evidence that immobilization protects enzymes from organic solvent from one of the conversions being examined in our laboratories. *N. rhodochrous* may be grown such that only the first enzyme of the cholesterol degradation pathway, cholesterol oxidase, is induced (Buckland *et al.* 1976). When these cells were used in the presence of water-immiscible solvents, cholesterol was converted quantitiatively to cholestenone (Buckland *et al.* 1975). If the culture was grown with cholesterol as the sole carbon source, harvested cells continued to oxidize cholesterol without accumulation of intermediates such as cholestenone, androst-4-en-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) (Lewis 1981). When a water-immiscible organic solvent or gas-oil containing additional cholesterol was added to the aqueous reaction mixture, degradation of cholestenone ceased and cholestenone accumulated at a rate similar to the rate of cholesterol disappearance (Lewis 1981). However, cells grown on cholesterol and immobilized in polyacrylamide were able to degrade cholesterol with only a slow rate of

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cholestenone accumulation (Duarte 1982) (figure 2). After 140 h only small amounts of AD and ADD were detected. These experiments demonstrate that immobilization of the cells protects the cholesterol degradation pathway from the deleterious effect of organic solvents. This is an encouraging result, especially when coupled with the good operational stabilities that have been reported for immobilized lipase (Macrae 1981; Yokozeki *et al.* 1982) and for immobilized cells catalysing menthyl ester hydrolysis (Omata *et al.* 1981).

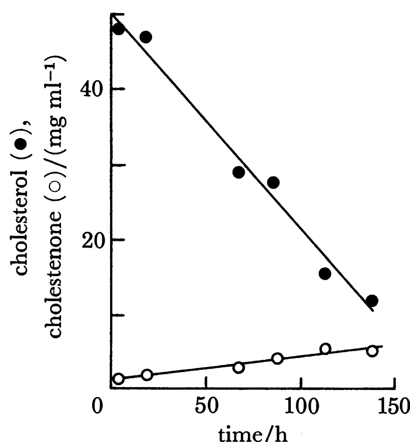

FIGURE 2. The degradation of cholesterol by *N. rhodochrous* immobilized in polyacrylamide. Reaction conditions: 50 ml of trichloroethane initially containing cholesterol at 50 mg ml^{-1}; 30 ml of polyacrylamide gel containing cells; 20 ml of aqueous medium containing yeast extract and glycerol. The concentrations of cholesterol (●) and cholestenone (○) in the organic phase are shown.

CONCLUDING REMARKS

It is evident from the results discussed briefly in this paper that work on two-liquid phase reactions has been concerned mainly with steroid transformations and the modification of oils and fats. Microorganisms are highly versatile catalysts able to attack a wide range of sparingly water-soluble aliphatic and aromatic compounds. However, these reactions have normally been studied in mixtures where the reactants are present in low concentration. It is possible that some at least of these could be done at high reactant concentrations in the same way as for steroids, oils and fats. In the future, therefore, a much wider range of conversions may be developed.

It is also possible to use other types of catalyst than the microbial cells and isolated enzymes described here in two-liquid phase reactions. For instance, we have isolated a subcellular fraction from *Pseudomonas putida* capable of oxidizing alkanes and alkenes with the organic phase occupying up to 20 % of the reaction volume (S. Harbron & M. D. Lilly, unpublished results).

In summary, two-liquid phase biocatalysis is far less advanced than many other areas of immobilized biocatalysis, particularly those involving water-soluble reactants. Although there are still many problems to be solved, we can expect to see new commercial processes using multi-liquid biocatalytic reactors during the next decade.

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Discussion

F. E. Young (*Department of Microbiology, University of Rochester Medical Center, U.S.A.*). Are the microorganisms able to grow in these two-liquid phase systems?

M. D. Lilly. In the microbial systems that we are investigating, microbial growth is not observed but it would be possible in some systems if appropriate nutrients were supplied.

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However, I should explain the approach that we have generally adopted in our group at U.C.L. towards microbial catalysis.

Traditionally both microbial growth and product formation have been carried out in the same fermentation vessel. Our objective has been to separate the two processes so that they can each be optimized and product formation can, if necessary, be done under conditions unsuitable for growth. I think it is valuable to pursue this concept of the extended use of micro-organisms to synthesize products. Unfortunately, one of the present constraints on this approach is our lack of understanding of microbial physiology. The popularity of molecular biology and genetic manipulation means that the importance of microbial physiology is being overlooked and there is a shortage of well trained microbial physiologists in many countries.

A. R. Thomson (*Biochemistry Group, A.E.R.E. Harwell, U.K.*). Professor Lilly's comment regarding the need to separate the growth phase from the production phase in bioreactors corresponds very closely with our own thinking in the development of our immobilized-cell programme. In this we have developed techniques for immobilizing plant cells (using *Vinca* as a model system), with a high degree of retention of viability (*ca.* 90%), and have maintained such cells under non-growth conditions so that alkaloids have been produced extracellularly for periods of up to 6 months. This suggests clearly that production of complex compounds by using immobilized eukaryotic cells in reactor systems is feasible. We also expect that this type of technology would simplify recovery and purification of products. It has not been found necessary in this case to permeabilize the cells, because sugars provide a carbon source for synthesis.

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