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Whole cell biocatalysis in nonconventional media

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SUMMARY

In this paper biocatalytic reactions carried out by whole cells in nonconventional media are reviewed. Similar relationships are observed between solvent hydrophobicity and catalytic activity in reactions carried out by isolated enzymes and whole cells. In addition to the effect of organic solvent on biocatalyst stability, microbial cells are susceptible to damaging effects caused by the organic phase. In general, more hydrophobic solvents manifest lower toxicity towards the cells. Whole cell biocatalysts require more water than isolated enzymes and two-phase systems have been most widely used to study whole cell biocatalysis. Immobilization makes cell biocatalysts more resistant to organic solvents and helps achieve homogeneous biocatalyst dispersion. Cell entrapment methods have been widely used with organic solvent systems and mixtures of natural and/or synthetic polymers allow adjustment of the hydrophobicity–hydrophilicity balance of the support matrix. Some examples of stereoselective catalysis using microbial cells in organic solvent media are presented.

INTRODUCTION

The advantages of using enzymes in chemical synthesis relate to their versatility with respect to the range of reactions which can be catalysed, high observed reaction rates, enzyme regio- and stereo-specificity and the ability to carry out reactions under mild reaction conditions. Exploitation of regiospecificity is important in synthesis or modification of complex chemical structures with a number of reactive groups which may not be distinguished in conventional chemical reactions. Stereospecificity of enzymes is especially important in pharmaceutical manufacture. Often in chemical synthesis, different enantiomeric forms of molecules are produced. Because of the precise interaction between pharmaceuticals and biological receptors, usually only one enantiomeric form is biologically active and stereospecific enzymes may be exploited to select or produce the correct form.

Biocatalysis may be carried out using whole cells or isolated enzymes in free or immobilized form. Use of isolated purified enzymes is advantageous in that undesirable byproduct formation mediated by contaminating enzymes is avoided. However, extraction and purification of the enzyme is costly and, in biocatalytic reactions, enzymes are frequently less stable in the purified form than in a crude preparation or when present in whole cells [56]. Thus, in many industrial biotransformation processes, for greater cost effectiveness the biocatalyst used is in the form of whole cells. Whole cell biotransformations can manifest problems related to undesirable byproduct formation due to the presence of other enzymes. In these cases, environmental reaction conditions have to be manipulated to minimize byproduct formation or product degradation, or alternatively the activity of the undesirable enzyme may be reduced or eliminated by repression of enzyme synthesis, mutation of the cells or denaturation of the undesired enzymes. Cellular biotransformation systems are especially advantageous where enzyme cofactors participate in the biotransformation reaction and need to be regenerated. Metabolic conditions can often be designed to promote cofactor regeneration, thus avoiding the problems associated with cofactor recycling and regeneration which are encountered in isolated enzyme type biotransformations.

Until recently the majority of biocatalytic processes were carried out in aqueous media. However, observations that enzymes can catalyse reactions in organic media has led to intense research and development activity related to applications of enzymes in organic synthesis. A wide range of reactions may be carried out in such systems including oxidation-reductions, hydroxylations, esterifications and hydrolysis [23,25–27]. Because the vast majority of organic chemicals are insoluble in water, implementation of biocatalysis in non-conventional media has dramatically increased the number of potential organic precursors which may be utilized in enzymatic reactions. Some of the key advantages of biocatalytic reactions carried out in nonconventional media are: enhanced solubility of poorly water soluble substrates; suppression of undesirable side reactions; reduction of substrate and product inhibition; enhanced stability of the biocatalyst; ease of product and catalyst recovery; shift of thermodynamic equilibria in favor of synthesis and ability to manipulate enantioselectivity in organic solvents.

Most investigations on the applications and properties of

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enzymes in nonconventional media have involved use of isolated enzymes. In this paper we attempt to review reactions carried out in nonconventional media using whole cells as biocatalyst because of the practical and economic advantages described above related to use of whole cells rather than isolated enzymes. Examples of whole cell biocatalytic reactions carried out in organic solvent media are summarized in Table 1.

Some of the important issues regarding implementation of biocatalytic reactions in nonconventional media relate to choice of solvent, solvent toxicity, moisture content and biocatalyst immobilization and manipulation of the stereospecificity by the organic solvents and support matrices.

TABLE 1

Biocatalytic reactions carried out by cells in organic solvent media

Catalyst	Product	Substrate	References
Bacillus subtils	l-Methyl ester	d,l-Methyl acetate	[97]
B. subtilis	l-Menthol	d,l-Methyl acetate	[15]
Rhodotorula minuta	l-Menthol	d,l-Methyl succinate	[73]
Nocardia corallina	Epoxides	Alkanes	[38]
Pseudomonas oleovorans	1,2-Epoxyoctane	1-Octane	[24]
Pseudomonas putida	7,8-epoxy-1-octane	1,7-Octadiene	[43]
P. oleovorans	7,8-epoxy-1-octane	1,7-Octadiene	[83]
Nocardia rhodochrous	Cholest-4-ene-3-one	Cholesterol, Sterol	[18]
N. rhodochrous	4-Androstene-3,17-dione	Dehydroepiandrosterone	[72]
N. rhodochrous	Progesterone	Pregnenalone	[72]
N. rhodochrous	β-Sitostenone	β-Sitosterol	[71]
N. rhodochrous	Stigmastenone	Stigmasterol	[71]
Nocardia erythropolis	Cholestenone	Cholesterol	[3]
N. rhodochrous	4-Androst-3,17-dione	Testosterone	[92]
N. rhodochrous	Androst-1,4-diene-3,17-dione	Androstenedione	[99]
N. rhodochrous	Δ^1-Dehydrotestosterone	Testosterone	[92]
Arthrobacter simplex	Δ^1-Dehydroprednisolone	Hydrocortisone	[47]
Aspergillus ochraceus	11α-Hydroxyprogesterone	Progesterone	[20]
Rhizopus nigricans	11α-Hydroxyprogesterone	Progesterone	[74]
Flavobacterium dehydrogenans	4-Androstene-3,17-dione	Androstenoloneacetate	[10]
Mycobacterium sp.	Androstene-3-oxime	Cholesterol derivatives	[1]
Mycobacterium sp.	4-Androstene-3,17-dione	Dehydroepiandrosterone	[71]
Rhizopus arrhizus	Fatty acids	Olive oil	[8]
Mycobacterium sp.	Propene oxide	Propene	[14]
Saccharomyces cerevisiae	Benzyl alcohol	Benzaldehyde	[64]
S. cerevisiae	l-Phenylacetyl carbinol	Benzaldehyde & Pyruvate	[68]
Acetobacter aceti	Acetic acid	Ethanol	[6]
Penicillium roquefortii	2-Heptanone	Octanoic acid	[60]
Curvularia lunata	Hydrocortisone	Cortexolone	[87]
P. putida	Naphthalene 1,2-dihydrodiol	Naphthalene	[44]
Enterobacter aerogenes	Adenine arabinoside	Uracil & adenine	[100]
Corynebacterium equi	Tetradecanone	Tetradecanols	[90]
Cryptococcus neoformans	Dioic acid	n-Pentadecane	[22]
Pseudomonas aeruginosa	Dioic acid	n-Pentadecane	[22]
P. oleovorans	PHAs	Aliphatic hydrocarbones	[79]
E. coli	l-tryptophan	Indole and L-serine	[80]
Corynebacterium sp. S401	Fatty acids esters	Fatty acids & alcohols	[84]
P. putida	Toluene *cis*-glycol	Toluene	[42]
Aspergillus niger	β-Ionone derivatives	β-Ionone	[85]
S. cerevisiae	Ethanol	Glucose	[5]
A. niger	Citric acid	Glucose	[6]
Botryococcus braunii (alga)	Isoprenoid hydrocarbones	Glucose	[33]
Morinda citrifolia (plant)	Anthraquinones	Glucose	[7]
Tagetes patula (plant)	Thiophenes	Glucose	[19]
Lithospermum erythrorizon (plant)	Shikonin	Glucose	[55]

CHOICE OF ORGANIC SOLVENT

A wide range of water-miscible and immiscible organic solvents have been used with biocatalysts both as isolated enzymes or as whole cells. There are many factors to be considered when a suitable organic solvent has to be selected. Some of the factors influencing this choice are: reactant/product capacity; partition coefficient; melting/boiling points; surface tension; viscosity; toxicity; flammability; waste disposal; solvent availability and price [16,61]. In general, high activity is observed with hydrophobic solvents and low activity occurs with hydrophilic solvents [59].

Different solvent parameters have been introduced in order to correlate the biocatalytic activity and solvent hydrophobicity. Some of the most important ones are: dipole moment (μ); Hildebrand solubility parameter (δ); dielectric constant (ϵ); solvatochromism of dye (E_t) and logarithm of partition coefficient (log P) [12,58,59,82]. The most widely used parameter, Log P, is defined as the partition coefficient in a standard octanol : water mixture [58,59]. A general pattern is emerging, namely biocatalysis in organic solvents is low in polar solvents having a Log P < 2, moderate in solvents having Log P between 2 and 4, and high in apolar solvents having a Log P > 4 [58]. When the corresponding Log P values of the solvents used were plotted versus the catalytic activity, sigmoidal-shaped or S-shaped curves were obtained. The correlation between Log P and catalytic activity was valid for both isolated enzymes and cells. Here are some examples where the phenomenon applies to cells:

(1) The Δ^1-dehydrogenation of hydrocortisone to prednisolone by *Arthrobacter simplex* in organic–aqueous two-liquid phase [47].
(2) The production of L-tryptophan in two-liquid phase system from indole and L-serine by free- and immobilized cells of *Escherchia coli* [80].
(3) Steroid conversion in two-liquid-phase systems by *Flavobacterium dehydrogenans* [10].
(4) Propane epoxidation of immobilized *Mycobacterium* cells in biphasic solvent–water systems [82].
(5) Reduction of benzaldehyde by *Saccharomyces cerevisiae* in aqueous–organic solvent biphasic system [67].
(6) 11α-Hydroxylation by *Rhizopus nigricans* [74].
(7) Production of naphthalene-*cis*-glycol by *Pseudomonas putida* or naphthalene hydroxylation in two-liquid phase systems [45].

SOLVENT TOXICITY

The influence of organic solvents on biocatalyst activity retention has attracted wide attention. This emphasis is especially important with microbial cells because of the additional susceptibility of the cells to the damaging effect caused by the organic phase [61]. Hocknull and Lilly [47] have reported that conditions which favor high initial activities (e.g. good mass transfer) result in poor stability of the cells. They suggested that the decrease in stability of the cells in solvents with high Log P might be related to the increased agitation rate in the bioreactors. For example, Δ^1-dehydrogenation activity of free *A. simplex* cells in two-liquid phase environments show the typical S-shape curve in shake flasks but, in stirred tank bioreactors, the curve was altered with no activity observed in solvents having Log P < 2. High activity was observed only in one solvent, di-n-octylphthalate (Log P = 9.6).

Although solvents with high Log P and high molecular weight tend to favour the retention of biocatalytic activity, no simple relation apparently exists between these parameters when different classes of solvents are considered [12]. Therefore the problem of cell inactivation can only be addressed by selection of appropriate solvents and a better understanding of the damaging effect on cells must be sought to enhance cell stability [61]. The potential effects of organic solvents on microorganisms include: loss of cell viability; changes in cell morphology (cytoplasmic shrinkage, loss of membrane organization, ultrastructural changes); and physiological changes (inhibition of nutrient or oxygen uptake, loss of membrane permeability barrier, alteration of intermediary metabolism, inhibition of DNA synthesis) [61].

Bar [4] suggested that distinction had to be made between molecular and phase toxicity when microbial systems are used in two-liquid phase media. Molecular toxicity is caused by the dissolved organic solvent molecules in the aqueous medium, while phase toxicity occurs at the interface between the two solvent systems.

Toxicity to cells of organic solvents present in excess have more negative effects on biocatalytic activity than when present at saturation level [78].

Osborne et al. [74] correlated the biocatalytic activity of the membrane bound enzyme, 11α-dehydrogenase of *R. nigricans*, with solvent concentration in cell membrane. The concentration of the solvent in the cell membrane that caused complete loss of biocatalytic activity (~200 mM for most solvents tested) was termed critical membrane concentration. Alterations in membrane fluidity may be primarily responsible for overall loss of catalytic activity, especially at high membrane solvent concentrations [74].

Cell stability in solvents depends mainly on the cell type [46]. In a two-liquid phase bioreactor, hydroxylation of naphthalene by gram-negative *P. putida* can be performed in a wider range of organic solvents with lower Log P values than can steroid Δ^1-dehydrogenation by gram-positive *A. simplex*. The presence of a liquid interface appeared to cause inactivation of the Δ^1-dehydrogenation system of *A. simplex*, but did not cause significant inactivation of the hydroxylation system of *P. putida*. This latter effect may have been due to the protective effect of the outer membrane in the gram-negative strain. The finding that *P. putida* is tolerant to a wider range of organic solvents having lower Log P than *A. simplex* correlates well with the results of Inoue and Horikoshi [48], who described the solvent-tolerance limits of bacteria in terms of the solvent Log P.

Among all the bacteria tested, the *Pseudomonas* group showed highest solvent tolerance while the tolerance of *Chromobacterium* and some species within the genus *Bacillus, Corynebacterium, Brevibacterium* and *Rhodococcus* was relatively lower. It was concluded that the polarity of the solvents affected the cell-surface characteristics and hence the growth of the organisms [48].

Solvent toxicity tests, with a mixed culture of facultatively anaerobic, acid-producing bacteria, indicated that the nontoxic solvents included the paraffins (C_6–C_{12}), phthalates, organophosphorus compounds, Freon 113, Aliquat 336, di-isoamyl ether and trioctylamine, and toxic solvents included alcohols (C_5–C_{12}), ketones (C_5–C_8), benzene derivatives, isoamyl acetate and di-isopropyl ether. Generally, the chemicals were nontoxic unless present at levels in excess of that required to saturate the aqueous phase. Brazier and Lilly [11] reported that the oxidation of toluene to the corresponding *cis*-glycol by a mutant strain of *P. putida* was inhibited when the aqueous phase was saturated with toluene and a separate toluene phase existed.

Solvents having a relatively high Log P were not harmful to aerobic bacteria but great differences existed among the various different bacteria tested. Two *Pseudomonas* species, for example, were less susceptible to damage than others while a *Bacillus* species was much more susceptible. It was suggested that direct contact between the bacteria and the solvent did not necessarily lead to cell damage, because immobilized cells demonstrated similar behaviour to free cell suspensions. Ceen et al. [21] reported on the importance of assessment of the intrinsic cell stability with respect to the solvents when considering possible ways of protection. Study of solvent tolerance of the progesterone 11α-dehydrogenase system of *Aspergillus ochraceus* emphasized that the solvents must not interact strongly with cellular lipids. In general, solvents of greater solubility in water showed a trend of more rapid disruption on the cells [12,47]. The toxicity of n-alkanes, n-alk-1-enes, n-alkyl-1-ols and n-alkyl-1-bromides towards *Candida tropicalis, Candida* sp. 107 and *Saccharomyces carlsbergensis* was related to their solubilities in an aqueous medium [39]. n-Alkanes, and 1-alkenes of chain lengths shorter than 13, inhibited respiration of *Candida* sp. 107 and *S. carlsbergensis*. Implementation of strategies to reduce the solubility of the toxic compound reduced inhibition. A similar effect was observed in the epoxidation of 1,7-octadiene to 7,8-epoxy-1-octane and 1,2-7,8-diepoxyoctane by *Pseudomonas olevorans* in a two-phase system with cyclohexane as the second phase [83]. Addition of cylohexane decreased the concentration of monoepoxide in the aqueous phase and hence reduced the inhibition. In the production of epoxides by *Nocardia corallina* B-276, n-hexadecane reduced susbtrate inhibition by styrene and product inhibition by styrene oxide and 1,2-epoxyalkanes [38].

The hydrophobicity of reactant and product should also be taken into consideration [21]. In the conversion of toluene to toluene *cis*-glycol by *P. putida*, the substrate is a liquid with low aqueous solubility, while the product is very water soluble and thus is found largely in the aqueous phase (bottom) where the cells are partitioned. In aqueous two-liquid phase systems the cells are partitioned based on their surface properties such as interfacial energy and partition coefficient of the cells [2]. Cells may also be partitioned in the top phase in the production of androst-4-ene-3,17-dione (AD), androst-1,4-diene-3,17-dione (ADD) by *Mycobacterium* sp. [2]; and with *S. cerevisiae* in the production of benzyl alcohol and phenylacetyl carbinol (PAC) in two-liquid phase system containing chloroform (Nikolova, unpublished data).

While toxicity of any given solvent depends on the cell type and perhaps physiological state, it is difficult to predict the best solvent for a reaction of interest. Thus a screening technique such as dielectric spectroscopy may be a convenient rapid on-line assessment of biocompatibility of the solvents for the bioconversion of interest [89].

A systematic approach has been suggested to simplify the search for appropriate solvents [16,17]. A database system of 1500 solvents has been created and tested for ethanol recovery and the different solvents selected in that process were tested for toxicity towards the micro alga *Botryococcus braunii* in the efficiency of hydrocarbon recovery [33]. Different classes of solvents, including alkanes, alcohols, esters, ethers, and ketones were tested and, with the exception of the alkanes, high molecular weight and high boiling points solvents proved to be biocompatible with the *B. braunii*. Partitioning of the solvents among the three phase systems (solvent, aqueous media and intracellular) appeared to play a key role in toxicity considering that the least polar solvents were compatible to *B. braunii*. The degree of toxicity was evaluated based on relative impact on the cells, ranging from complete biocompatibility to inhibition to complete toxicity [57].

In electron scanning microscopy examination of *S. cerevisiae* cells, used to produce phenylacetyl carbinol in a range of different organic two-liquid phase systems, the effect of organic solvents on the cell structure was investigated [67]. In solvents such as ethyl acetate, butyl acetate and chloroform, having relatively low Log P, the cells appeared to be damaged, while in solvents with higher Log P such as toluene, hexane and decane, no apparent cells distortions were observed [67,68].

Ceen [20] speculated that initial contact with ethyl acetate caused permeabilization of the cell membrane with subsequent damage occurring to the membrane-bound enzyme of interest. In microbial oxidation of tetradecanols and related compounds by intact cells of *Corynebacterium equi*, from the solvents tested (isooctane, toluene, hexane, carbon tetrachloride and chloroform) only chloroform was inhibitory [90]. Reaction rate and extent of product formation were largely dependent on the type of organic solvents used and hydrophobic solvents such as isooctane and toluene were not inhibitory.

When the effect of homologous series of hydrophobic organic solvents on *F. dehydrogenans* in two-liquid phases was investigated, cell viability was high even in alkane-

TABLE 2

Some examples of immobilized cells used in nonconventional media

Catalyst	Application	Support material	References
E. aerogenes	Synthesis of adenine arabinoside	ENT-4000, PU-6	[100]
R. nigricans	9α-Hydroxylation of 4-androsten-3,17-dione	Polyacrilamide, alginate, agar	[64]
C. lunata	11β-Hydroxylation of cortexolone	ENT-4000, ENTP-4000	[65]
A. simplex	Δ^1-Dehydrogenation of hydrocortisone	Alginate	[96]
A. simplex	Δ^1-Dehydrogenation of hydrocortisone	ENT-4000, ENTP-2000	[86]
A. simplex	Δ^1-Dehydrogenation of hydrocortisone	Isopropylacrylamide/acrylamide	[75]
A. simplex	Δ^1-Dehydrogenation of hydrocortisone	Polyacrylamide, alginate, PEG/dextran, photocrosslinkable resin	[51]
N. erythropolis	Dehydrogenation of 3β-OH-steroids	DEAE-cellulose	[3]
N. rhodochrous	Dehydrogenation of 17β-OH-steroids	ENT, ENTP, PU-3, PU-6	[92,99]
N. rhodochrous	Dehydrogenation of 3β-OH-steroids	ENTP-1000, 2000, PU-3, 6, ENT-4000, PMB-2000	[29,71,72]
R. minuta	Ester hydrolysis of dl-metyl succinate	PU-3, PU-6, ENT-4000	[73]
N. corallina	Epoxidation of 1-octane	Siicone and/or alginate	[53]
S. cerevisiae	Reduction of benzaldehyde	Silicone and/or alginate	[66]
Mycobaterium	Epoxidation of propane and 1-butane	k-Carraginan	[12]
A. simplex	Δ^1-Dehydrogenation of steroids	Polyacrylamide, colagene	[30]
Bacterium cyclooxydans	Δ^1-Dehydrogenation of steroids	Polyacrilamide, colagene	[30]
A. ochraceus	11α-Hydroxylation of progesterone	Alginate	[20]
E. coli	Synthesis of L-tryptophan	K-Carraginan	[80]
Mycobaterium fortuitum	Transformation of β-sitosterol to AD, ADD	Alginate, polyanhydride resin, chitosan and eudragit 100, silicone, polyurethane	[88]
P. roquefortii	Synthesis of 2-heptanone	Alginate	[60]
A. niger	Conversion of β-ionones to it derivatives	PU-3	[85]
S. cerevisiae	Reduction of benzaldehyde	Celite	[67]
S. cerevisiae	Synthesis of L-phenylacetyl carbinol	Celite	[68]

substituted solvents having Log P values between 2 and 4 if the hydrophobicity of the substituent was low enough [10]. It was concluded that:

(1) Cell growth stage at the moment of solvent addition must be chosen properly since changes in membrane structure and function occurring during growth may effect cell viability.
(2) High viabilities can be achieved over a wide range of solvent hydrophobicity when the solvent used is of the alkyl-X type where X is a hydrophilic substituent.
(3) Cell viability together with substrate and product solubility determined the catalytic activity.

EFFECT OF THE MOISTURE CONTENT ON BIOCATALYTIC REACTION

The moisture content of organic solvents is important in biocatalysis whether the solvent is water-soluble or water-immiscible, or whether the biocatalyst is in the form of free or immobilized cells or isolated enzymes [98]. Complete depletion of water from the reaction system results in no biocatalytic activity [25–28,40]. The amount of water necessary varies considerably with the organic solvent used, most probably due to the different abilities of the solvents to compete with the water for the hydration sites of the catalyst. Yamane [98] suggested that differentiation of the states of water, such as freely dissolved water in the organic solvent in addition to the water bound to the catalyst, should be made because only the catalyst-bound water would appear to affect the catalytic activity. A dynamic equilibrium exists between the amount of water bound to the protein and that of the free water so that optimum moisture content for biocatalysis must be determined. Depending upon the moisture content present in the organic solvent and the miscibility of the organic solvent with water, the nonconventional biocatalytic systems are classified as miscible, two-phase or microaqueous. Reactions using water-miscible organic solvents are classified as miscible or monophasic, those using water-immiscible solvents with water levels less than organic phase saturation are defined as microaqueous organic solvent systems, while in two-phase systems the aqueous phases are present in excess of mutual saturation [61,82,90].

The choice of the particular system is dependent upon the nature of the biocatalytic reaction and the requirements or nature of the biocatalyst. For example, biotransformations involving poorly water-soluble as well as water-insoluble substrates will require a two-phase system. Also the choice

of the biocatalyst (isolated enzyme or whole cells) will determine to a large extent the type of system to be used. Since whole cell biocatalysts require more water than the isolated enzymes, two-phase systems may be the more appropriate [90]. When *N. corallina* cells were suspended in insufficient volume of buffer to reconstitute the cells, their biotransformation activity was largely reduced [18].

Two-phase systems have been most widely studied with whole cell biocatalysts. Apart from satisfying the greater moisture requirement of cells, partitioning of the biocatalyst in the aqueous phase can reduce the toxic effects of the substrate, product and/or organic solvent. The difficulty of partitioning isolated enzymes totally in one phase, and product and/or the substrate in the other phase, can sometimes be overcome by using whole cells as biocatalyst rather than isolated enzymes, making the choice of the biocatalyst of key importance [2].

The whole cells are usually used as: wet or dry cells suspended in water-immiscible [9,52,61,67–70,74] or water-miscible organic solvents [32]; wet or dry immobilized cells (see Tables 2–5). Some examples of whole cell systems using bioreactor configurations are provided in Table 6.

IMMOBILIZATION

Immobilization is one of the most promising approaches to rendering biocatalysts resistant to organic solvents [34–37, 91,92]. Immobilization also helps to achieve homogeneous dispersion of biocatalyst in organic solvents and facilitates continuous operation and biocatalyst recovery from the reaction media. However, in order to be able to utilize immobilized biocatalysts for conversion of hydrophobic compounds in organic solvent systems, the affinity of hydrophobic substrates for the support matrix and diffusion of the reactant through the matrix should not be limiting to the system. Hence, the use of suitable matrices with appropriate physicochemical properties (network size, ionic nature, hydrophobicity–hydrophilicity balance) might be preferable depending upon the hydrophobicity of substrate(s), product(s) and organic solvent(s) used [35]. Immobilization also seems to retain the water around the catalyst which is essential for biocatalytic function in organic solvent media. Hydrophilic supports may compete with the biocatalyst for the available water in the reaction system and therefore hydrophobic supports appear to be more suitable for organic solvents systems.

Various reactions have been successfully carried out by using immobilized microbial cells either in water organic cosolvent systems or in organic solvent system (Table 2). The most common method of immobilization of cells is entrapment. A wide range of support materials for immobilization (natural and synthetic polymers or a mixture of both) have been used as a means of adjusting the

TABLE 3

Effect of support matrix hydrophobicity on catalytic activity in biotransformation of benzaldehyde by *S. cerevisiae* [66]

Support matrix	Relative activity (%)	
	Hexane (2% moisture)	Aqueous phase
PU-3	6.80	9.02
PU-6	4.26	21.50
ENT-4000	16.00	119.92
ENTP-2000	47.73	193.46
Alginate	24.24	103.50
50% Silicone : 50% alginate	53.16	159.83
25% Silicone : 75% alginate	43.38	147.90
75% Silicone : 25% alginate	—	90.25
Silicone	—	96.58

TABLE 4

Some examples of support materials used in different organic solvent systems

Catalyst	Support materials	Solvent	References
A. simplex	Polyurethane k-Carraginan Celite Agar	Chloroform n-Decan-1-ol	[77]
S. cerevisiae	Silicone & Alginate Celite PU-3 PU-6 ENT-4000 ENTP-2000	Hexane Chloroform Ethyl acetate Butyl acetate Toluene Decane	[66]
N. rhodocrous	ENTP-2000 ENT-4000 PU-3 PU-6 PU-9	Chloroform/heptane Benzene/heptane	[72]

TABLE 5

Effect of the solvents on catalytic activity in cholesterol transformation by *N. rhodocrous* [72]

Solvent (1 : 1)	Activity (μmol h^{-1} g $cells^{-1}$)	
	Free cells	Entrapped in ENT-4000
Carbon tetrachloride-heptane	68	0
Benzene-heptane	57	0
Toluene-heptane	68	0
Chloroform-heptane	42	29
Methylene chloride-heptane	19	15
Ethyl acetate-heptane	27	0
Acetone-heptane	trace	0
Ethanol-heptane	0	0
Methanol-heptane	0	0

TABLE 6

Bioreactors used for bioconversions carried out by cells

Reactor type	Catalyst	Solvent	Operation time	References
Stirred-tank	*R. minuta*	N-Heptane	55–63 days (half-life)	[73]
Packed-bed	*Mycobaterium*	n-Hexadecane	1–3 days (half-life)	[13]
Fed batch	*P. roquefortii*	n-Hexane N-Hexadecane hydrosol PW, IP	12.5 days	[60]
Stirred-tank	*Nocardia* sp.	Carbon tetrachloride	2.9 days	[18]
Packed bed	*R. arrhizus*	Di-isopropyl ether	7 days	[8]
Stirred-tank	*R. arrhizus*	Di-isopropyl ether	7 days	[8]
Stirred-tank	*N. corallina*	n-Hexadecane	10 days	[53]
Stirred-tank	*B. subtilis*	Methyl acetate	n a	[15]
Stirred-tank	*P. oleovorans*	n-Octane	2 days	[95]
Fed-batch	*P. oleovorans*	n-Octane	8–12.5 days	[79]
Loop	*R. arrhizus*	n-Heptane	12.5 days	[56]

hydrophobicity–hydrophilicity balance. This balance derives from the intrinsic properties of the prepolymers and is affected by the water content of the gels as well as the polarity of the solvents used. Polymers, having hydrophobic properties, which have been used include photocrosslinkable resin prepolymers such as poly(propylene glycol) or ENTP or mixtures of poly(ethylene glycol) or ENT with ENTP; urethane prepolymers such as PU-3; silicone polymer or poly(dimethylsiloxane).

Recently, models of immobilized cell biocatalysts utilizing hydrophobic and/or hydrophilic matrices have been developed for organic solvent systems [53,75]. The researchers expressed the notion that the cell orientation and location in the matrix is dependent upon the nature of the matrix and its microenvironment. The observation that highest steroid conversion activity of *A. simplex* occurred in a hydrophobic thermally reversible matrix was attributed to the microenvironmental differences around the biocatalyst rather than enhancement of mass transfer of the substrate [75]. Electron scanning micrographs showed that, in the case of a mixed matrix (50% silicone : 50% alginate), the beads consisted of finely dispersed spherical silicone particles surrounded by a continuous alginate phase [53]. The cells appeared to be located on the surface of the silicon particles while, in the excess of alginate, the cells seem to be scattered more into the alginate matrix. With excess silicone, fine particles of alginate were dispersed in the silicone phase and the cells appeared to be partially embedded under the silicon phase adjacent to the interface. Kawakami et al. [53] reported that immobilized cells of *N. corallina* in a silicone polymer matrix or in a mixed matrix of silicon polymer and calcium alginate can carry out the epoxidation of 1-octane to 1,2-epoxyoctane in the presence of n-hexadecane. The effect of matrix composition on product formation is presented in Fig. 1 and Table 3.

Product formation in a mixed matrix (50 : 50) was approximately two and seven times, respectively, that observed when separate silicone and alginate matrices were used [53]. Such an optimized mixed matrix could be used in a continuous stirred-tank bioreactor. The immobilized cells retained desirable operational stability and exhibited 65% of the maximum productivity even after 250 h. The results suggested that it is essential to optimize the hydrophobicity/hydrophilicity balance of the support matrix. Different solvents have been used with a wide range of support materials. Some examples are presented in Tables 4 and 5.

The amount of water present in the support material will have an important role in the biocatalytic reaction. Large amounts of water retained by the support matrix could enhance the stability of *A. simplex* dehydrogenation activity in the organic solvent, but, at the same time, may limit the access of the hydrophobic substrate to the catalyst [77]. The effect of the volume fraction of an internal aqueous phase dispersed in a silicone matrix in the production of epoxides by nongrowing cells of *N. corallina* has been studied. The results showed that the product yield increased if the volume of internal water was increased and the best performance was observed when the water content was 30% [52–54].

STEREOSPECIFICITY

Catalytic properties of enzymes can be manipulated by use of organic solvents. Enzyme enantioselectivity in nonaqueous media largely depends on the nature of the organic solvent [81]. Since then, this phenomenon was observed for various asymmetric enzymatic processes in organic solvents and has attracted wide attention [31,41,93]. Also, computer-assisted modelling of enzyme enantioselectivity in organic solvents has been developed using asymmetric transesterification of achiral esters with chiral alcohols by subtilisn as a model system.

Biocatalytic reactions, carried out by microorganisms or isolated enzymes, have been used extensively in the preparation of chiral compounds, the building blocks in organic synthesis, and are of great interest to the pharmaceutical and fine chemical industries [31,50,76]. While more

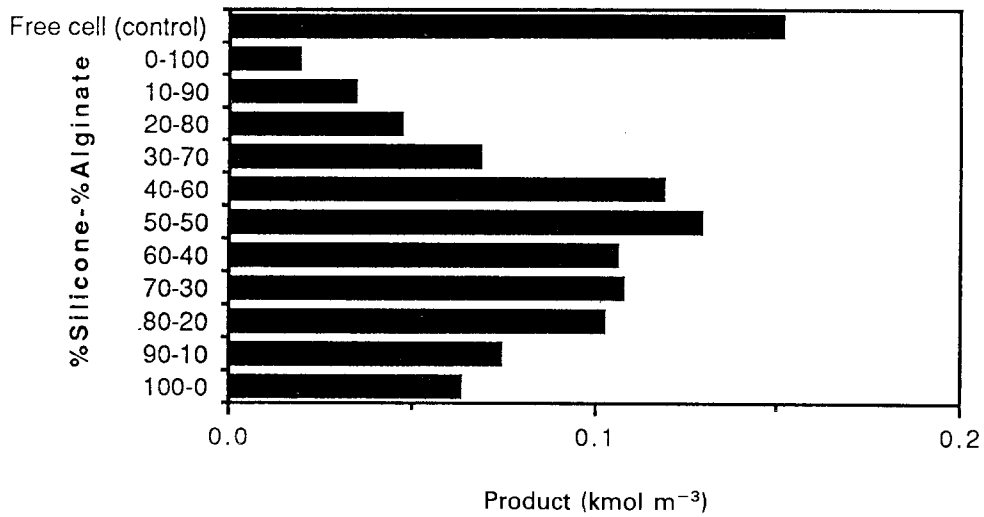

Fig. 1. Effect of the hydrophobicity of mixed matrices on catalytic activity of *N. corallina* epoxidation. Data taken from Kawakami et al. [53].

data is available for isolated enzymes, some examples of stereoselective catalysis by microbial cells in organic solvents are presented in Table 7. It will be of great interest to establish whether stereoselectivity of whole cell systems can be manipulated through modification of medium and support.

CONCLUSIONS

Although much work has been published on biocatalysis utilizing whole cells in nonconventional media, there are very few examples of practical applications. Nevertheless, some experimental studies have already been implemented in different types of bioreactors [13,14,62,94] and a brief summary is presented in Table 6. The results from these studies appear promising although few systems have so far been commercialized. Examples of whole cell catalysts used in industrial applications in nonconventional media include steroid dehydrogenation by *A. simplex* in toluene medium [30] and epoxidation of 1-octane in hexane [63]. It is expected that a significant increase in the number of industrial applications using whole cell biotransformations in nonconventional media will occur in the years ahead.

TABLE 7

Examples of stereoselective catalysis carried out by microbial cells

Catalyst	Application	References
B. subtilis	Hydrolysis of d,l-methyl acetate	[15]
B. subtilis	Esterification of d,l-methyl acetate	[97]
R. minuta	Hydrolysis of d,l-methyl succinate	[73]
S. cerevisiae	l-Phenylacetyl carbinol synthesis	[68]
Mycobaterium WMI	Synthesis of β-blockers	[49]
P. oleovorans	Hydroxylation of S-1,2-epoxyoctane	[24]

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