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Tailoring the Medium and Bioreactor for Biocatalysis

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Introduction

Water as essential reaction medium for biocatalysts has been advocated for many years as one of the major advantages for biocatalysis. However, this so-called advantage has proven to be one of the severest limitations for broadening the scope of applications of biocatalysts. Especially when the involved reactants are poorly water soluble, applying aqueous media is a major draw-back. Therefore, much effort has been devoted in the last decade to the development of biocatalysis in non-conventional media, in particular organic solvents and to a lesser extent also supercritical fluids [1][2].

The introduction of an organic solvent in the reaction system has numerous advantages. The organic solvent may improve the solubility of the reactants, thereby improving the volumetric productivity of the reaction system. When water is one of the reaction products, the equilibrium of the reaction may be shifted by reduction of the water activity of the reaction mixture. This can be achieved by replacing the water in the reaction system by a water-miscible organic solvent, or by introducing polymers, sugars, or salts. In a two-phase system consisting of an organic and an aqueous phase, the equilibrium of a reaction can also be shifted in favour of the product when this product is preferentially extracted into the organic phase. High product concentrations may also be achieved by reduction of possible substrate and/or product inhibition and prevention of unwanted side-reactions such as hydrolysis of the substrate and/or product. When the product is extracted into the organic-solvent phase while the biocatalyst remains in the aqueous phase, product and biocatalyst recovery will be facilitated.

Obviously there are not only advantages of using organic solvents. Disadvantages also exist, *e.g.* the biocatalyst may be denaturated or inhibited by the solvent. In addition, introduction of an organic-solvent phase usually leads to an increasing complexity of the reaction system.

Epoxidation of Short-Chain Alkenes

It is clear that situations exist where the advantages of using organic solvents outweigh the disadvantages. The epoxidation of propene and 1-butene (*Fig. 1*) is a biotransformation where we expected this to be the case for the following reasons. Both substrates, oxygen and alkene, are poorly soluble in water, while the product, alkene oxide, is toxic to the biocatalyst. Introduction of an organic-solvent phase as substrate reservoir and product extractant allows high overall substrate and product concentrations and facilitates downstream processing of the product. This biotransformation was, therefore, used as a model system to study some fundamental aspects of two-liquid-phase biocatalytic conversions [3]. Because a cofactor, NADH, is needed, whole cells of *Mycobacterium* and *Xanthobacter* were used as biocatalysts. In a free-cell suspension, introduction of a water-immiscible organic-solvent phase gave rise to a series of undesired phenomena, such as clotting of the biomass and aggregation of the cells at the liquid-liquid interface, usually with loss of activity. This so-called phase toxicity [4] could be prevented by immobilization of the biocatalysts in hydrophillic gels. Toxicity as result of solvent molecules dissolved in the aqueous phase (so-called molecular toxicity [4]), remained in spite of the gel entrapment of the cells.

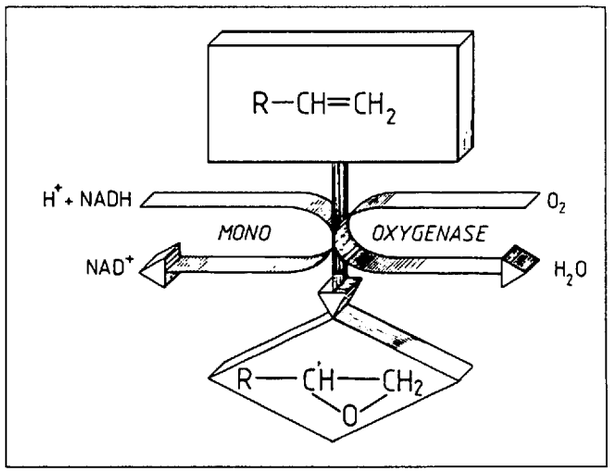

Fig. 1. *Microbial epoxidation of short-chain alkenes*

Bioconversion of Tetralin

Another example in which the application of a two-liquid-phase system was expected to be attractive, is the bioconversion of tetralin. Tetralin is a very apolar substrate for among others *Arthrobacter* and *Acinetobacter* [5]. It is toxic for these bacteria, at concentrations below its maximum solubility in water (< 150 mM) [5]. This system was used as a model to set-up a general strategy for selecting a suitable solvent for this kind of bioconversions [6].

The points to consider for the selection of a suitable solvent for bioconversion of apolar, toxic compounds in a two-liquid-phase system, are:

1) Toxicity of the solvent for the biocatalyst.
2) Biodegradability of the solvent by the biocatalyst.
3) Toxicity of the substrate/products for the biocatalyst.
4) Effect of the solvent on the toxicity of the substrate/product.
5) Immobilization of the biocatalyst.
6) Metabolic activity of the biocatalyst in the bioreactor.
7) Bioconversion by the biocatalyst in the bioreactor.

Some of these points are discussed below in more detail.

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Solvent Toxicity and Biodegradability

One of the parameters that was used to predict the toxicity of a solvent is the polarity of the solvent as expressed by the *Hildebrandt* solubility parameter combined with its molecular size. It was found by *Brink et al.* [3] that high activity retention of immobilized epoxidizing cells was favoured by a low polarity in combination with a high molecular weight. A better correlation was found for the polarity of the solvent expressed by its $\log P_{octanol}$ value [7]. $\text{Log}P_{octanol}$ is defined as the logarithm of the partition coefficient of the solvent in a standard two-phase system of 1-octanol and water. Several publications report a similar relation between activity retention of the biocatalyst and $\log P_{octanol}$ of the organic solvent used [6–10]. *Fig. 2* shows this relation for the metabolic activity of tetralin degrading cells of *Arthrobacter* and *Acinetobacter*. Generally speaking the solvents having a $\log P_{octanol}$ value of *ca.* 5 and higher are bio-compatible with cellular biocatalysts. This plot also shows that in the presence of a solvent in many cases a relative metabolic activity > 100% was found, especially for *Arthrobacter* cells. This bacterium proved to be able to use many of the nontoxic solvents as sole ernergy and carbon source [6]. This illustrates the importance of point 2 in the selection strategy.

Understanding Solvent Toxicity

Many attempts have been made to explain the empirical correlation between $\log P_{octanol}$ and the activity retention of cellular biocatalysts, but so far the mechanisms of solvent-caused toxicity are poorly understood. *Bar* [4] suggested that solvent toxicity in a two-liquid-phase system is caused by the presence of a second phase (phase toxicity) on the one hand, and by the solvent molecules that dissolve in the aqueous phase (molecular toxicity) on the other hand. Phase toxicity may result from extraction of nutrients and cell-wall components, or from limited availability of nutrients as a result of adherence of the cells to the interface, or from entrapment in an emulsion. Molecular toxicity may result from enzyme inhibition, protein denaturation and membrane modification.

Critical Membrane Concentration

The molecular toxicity of solvents can be studied separately in the aqueous phase using organic solvents at levels below saturation. *Osborne et al.* [10] demonstrated recently that there is a correlation between the 11α-hydroxylase activity of *Rhizopus nigricans* and the concentration of solvent in the cell membrane. The concentration of solvent in the cell membrane that caused complete loss of bioactivity was called the critical membrane concentration. This critical solvent concentration in the membrane was hypothesized to be independent of the type of organic solvent. If the concentration in the aqueous phase is lower than the concentration needed to provoke the critical solvent concentration in the membrane, the solvent is not toxic. The same phenomenon was described by *Seeman* [11] who related the loss of nervous function to the concentration of anaesthetics in the cell membrane.

The $\log P_{octanol}$ value of the solvent can serve here to predict molecular toxicity of a solvent. The partition behaviour of the organic solvent in the cell-membrane/aqueous-buffer/organic-solvent system can be related *via* a *Collander* type of relationship [12]:

$$P_{membrane} = X \bullet P^{Y}_{octanol} \qquad (1)$$

in which $P_{membrane}$ is the partition coefficient of the organic solvent in the membrane/aqueous-buffer mixture while $P_{octanol}$ refers to the octanol/aqueous system; X and Y are constants.

The minimum concentration of solvent in the aqueous phase which causes toxic effects, $[solvent_{aq,cr}]$, is related to the critical solvent concentration in the membrane, $[solvent_{membrane,cr}]$ *via* $P_{membrane}$ and *Eqn. 1* can thus be rearranged into

$$\log[solvent_{aq,cr}] = \log \{ \frac{[solvent_{membrane,cr}]}{X} \} - Y \bullet \log P_{octanol} \qquad (2)$$

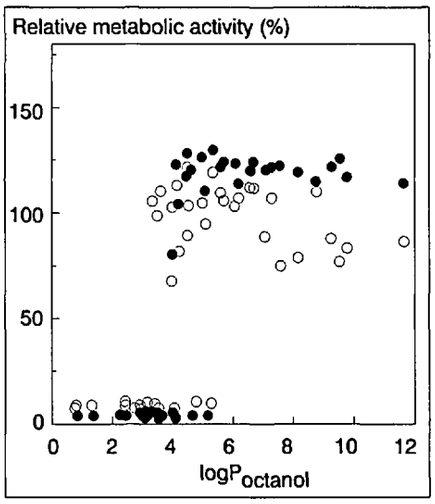

Fig. 2. *Relationship between the relative metabolic activity of* Arthrobacter (●) *and* Acinetobacter (○), *exposed to 10% (v/v) organic solvent, and* $logP_{octanol}$ (from [13])

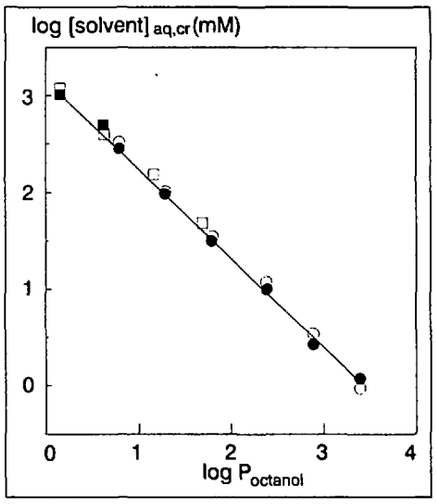

Fig. 3. *Relationship between the logarithm of the aqueous solvent concentration at which 50% of the initial oxygen-consumption rate of* Arthrobacter (closed symbols) *and* Acinetobacter (open symbols) *is inhibited, and the* $logP_{octanol}$ *of the solvents.* The circles refer to alkanols, the squares to alkyl acetates (from [13]).

A plot of the logarithm of the critical concentration of solvent in the aqueous phase against $\log P_{octanol}$ results according to the hypothesis in a straight line. From the intercept with the *y*-axe the critical solvent concentration in the membrane divided by X is obtained.

Osborne et al. [10] indeed found this linear relation for the hydroxylase activity of *Rhizopus nigricans*. They used a constant value for X (0.19) such as was also used by *Seeman* [11] for mammalian cells. A similar linear relation we found [13] for the metabolic activity of *Arthrobacter* and *Acinetobacter*, which were exposed to subsaturating concentrations of n-alkanols and n-alkylacetates as shown in *Fig. 3*. This figure also illustrates that no difference was found between the tolerance of the gram-positive *Arthrobacter* and the gram-negative *Acinetobacter* towards molecular toxicity of the alcohols and acetates, while in a two-liquid-phase system the solvent tolerance of the latter was better. This indicates that the generally better solvent tolerance of gram-negative bacteria probably is caused by a better tolerance towards phase-toxicity effects.

Although the concept of the existence of one critical membrane concentration for similar cell types is very appealing, we doubt if the critical membrane concentration can be considered constant for all solvents. X represents the partition of a solvent between a hypothetical membrane/octanol system and since the membrane/

solvent interactions and the octanol/solvent interactions will be different for each solvent, it is highly unlikely that the value of X is constant. Nevertheless, a linear plot between the $\log[solvent_{aq,\ cr}]$ and $\log P_{octanol}$ was found (*Fig. 3*), which indicates that in any case the value of the $\log([solvent_{membrane,\ cr}]/X)$ is constant. If X is considered to vary with the type of solvent used, it means that the critical solvent concentration in the membrane has to vary in the same way.

Although the question, whether or not the critical solvent concentration in the membrane is constant, remains unanswered, the plot can still be used to predict the molecular toxicity of any given solvent, since the critical concentration of the solvent in the aqueous phase can be estimated. If this critical concentration is lower than the aqueous solubility of the solvent, the solvent will show molecular toxicity.

Effect of the Solvent on the Toxicity of the Substrate

If a toxic substrate is used, its concentration in the aqueous phase should be optimized to obtain maximum bioconversion rates. The addition of a water-immiscible, bio-compatible organic solvent may help to minimize the damaging effects of the substrate and in the mean time to achieve a high overall substrate concentration. *Fig. 4* shows the beneficial effect of replacing part of the aqueous medium by the inert organic solvent fluoro carbon, FC-40, on the metabolic activity of *Arthrobacter* cells supplied with different overall concentrations of tetralin. In just aqueous buffer the toxic concentration of tetralin appeared to be 0.11 mM. By adding 20% (*v/v*) FC-40, the toxic concentration is increased to 0.17 mM. Based on a measured partition coefficient of tetralin over FC-40 and aqueous buffer of 15, one would expect in this case that the overall concentration of tetralin could be increased to concentrations up to 0.42 mM. Additional phase-toxicity effects, is the only explanation we have at the moment for this discrepancy.

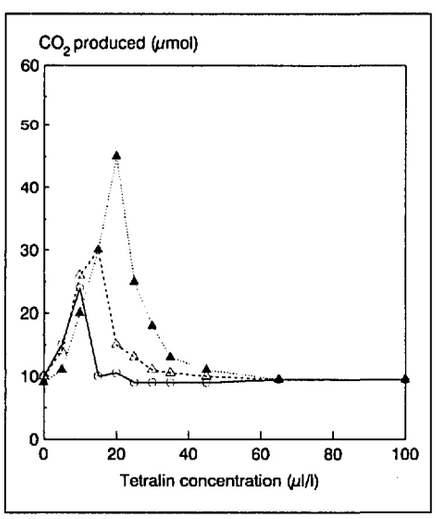

Fig. 4. *Effects of the addition of fluoro compound, FC-40 on the CO_2 production from various tetralin concentrations by* Arthrobacter. CO_2 was monitored after 7 days of incubation. Mineral salts medium in the absence of solvent (○), and in the presence of 10% FC-40 (△) and 20% FC-40 (▲).

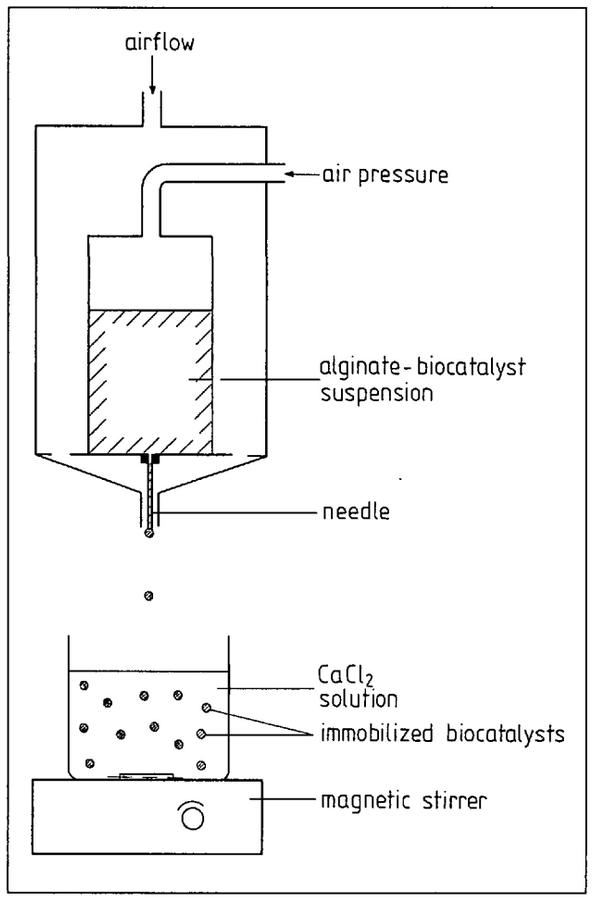

Fig. 5. *Laboratory-scale set-up for immobilization of cells in gels such as alginate*

Immobilization

Solvents that do not exhibit molecular toxicity, may exhibit phase toxicity when a separate phase of organic solvent is present. This effect was already mentioned for the epoxidizing cells and for the tetralin degrading cells. It was also extensively described by *Hocknull* and *Lilly* [9], who compared the metabolic activity of free and immobilized *Arthrobacter simplex* in organic-solvent/aqueous two-liquid-phase systems. Immobilization of the cells by entrapment in a gel such as alginate and *K*-carrageenan will largely prevent such interfacial inactivation phenomena. The immobilization procedure itself must be mild enough to retain high biocatalytic

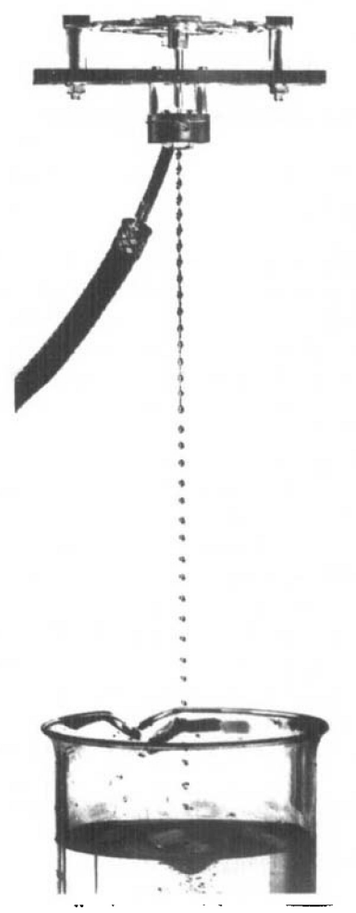

Fig. 6. *Rapid immobilization of cells by means of a resonance nozzle* [4]

activity and provide the cells with a matrix stable in the presence of solvents. As long as the water activity of the system is close to one, such as in a two-liquid-phase system of solvent and dilute aqueous buffer, both alginate and *K*-carageenan gel beads are stable. However, in systems with a low water activity, the gel beads dry out and shrink and biocatalytic activity is lost [16]. Another requirement we demand from the immobilization procedure is that it can be easily scaled up. Production of immobilized cells by entrapment in a gel is conveniently done on lab scale in the system schematized in *Fig. 5*. For production of large quantities of immobilized cells, this dripping method is tedious. The capacity can be improved by several orders of magnitude by use of a resonance nozzle (*Fig. 6*). In this case, the immobilization material, containing the biocatalyst, is pressed through a nozzle and forms a stream. A vibrator, connected to the membrane of the nozzle, causes the jet to breakup into droplets [14].

The Bioreactor

A schematic drawing of the system in which the measurements on the immobilized epoxidizing cells were executed, is shown in *Fig. 7*. In this system substrate and product concentrations were measured automatically at regular intervals and if necessary controlled at a constant level. Both batch and continuous experiments in bubble-column and packed-bed reactors were run. *Fig. 8* shows an example of the output from a batch run. After an initial increase in epoxide concentration, this increase levels off when substrates become depleted. Eventually even a decrease in product concentration is observed as a result of the capacity of the cells to slowly metabolize the epoxide. The latter effect can be retarded by using immobilized cells as can be clearly seen in *Fig. 8*.

Another type of bioreactor, specially designed for biocatalysis in organic solvents, is the liquid-impelled loop reactor (*Fig. 9*). This reactor combines the good mixing and mass transfer properties of air-lift loop reactors with the advantages of the introduction of an organic solvent as a second liquid phase. This new type of bioreactor has been described with respect to hydrodynamics, mixing and mass transfer [15] and is currently tested for the bioconversion of tetralin using FC-40 as organic solvent. This type of reactor is especially advantageous in case shear sensitive cells are used, such as plant cells [8]. In addition, transfer of toxic substrates from the organic-solvent phase into the aqueous phase, is better controlled in this

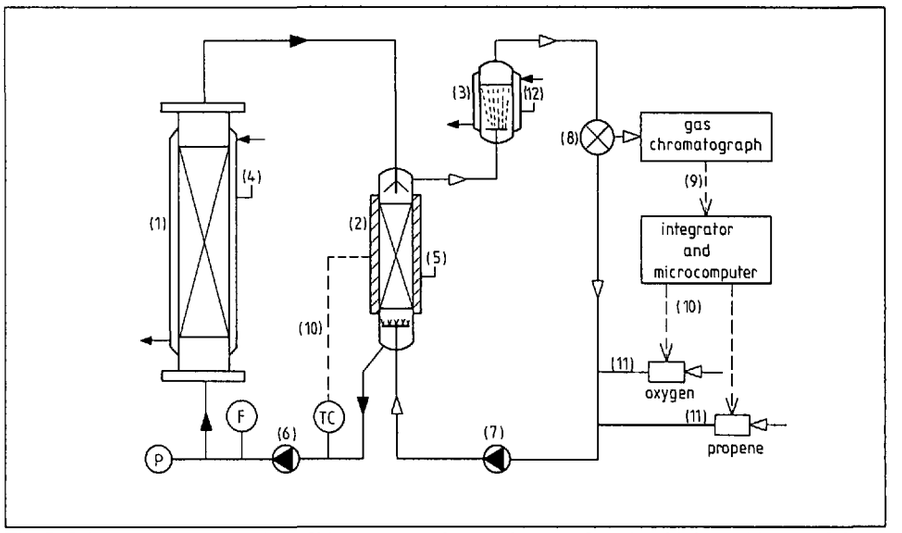

Fig. 7. *Schematic representation of bioreactor and control system for microbial epoxidation of short-chain alkenes* (from [16])

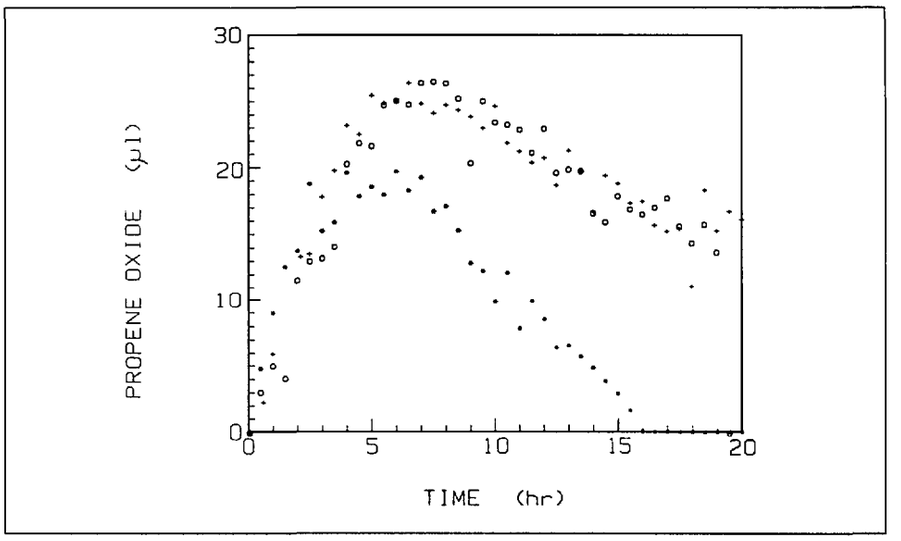

Fig. 8. *Amount of propene oxide produced by a* Mycobacterium *strain:* (*) *free cells;* (O) *cells immobilized in alginate;* (+) *cells immobilized in K-carrageenan* (from [16])

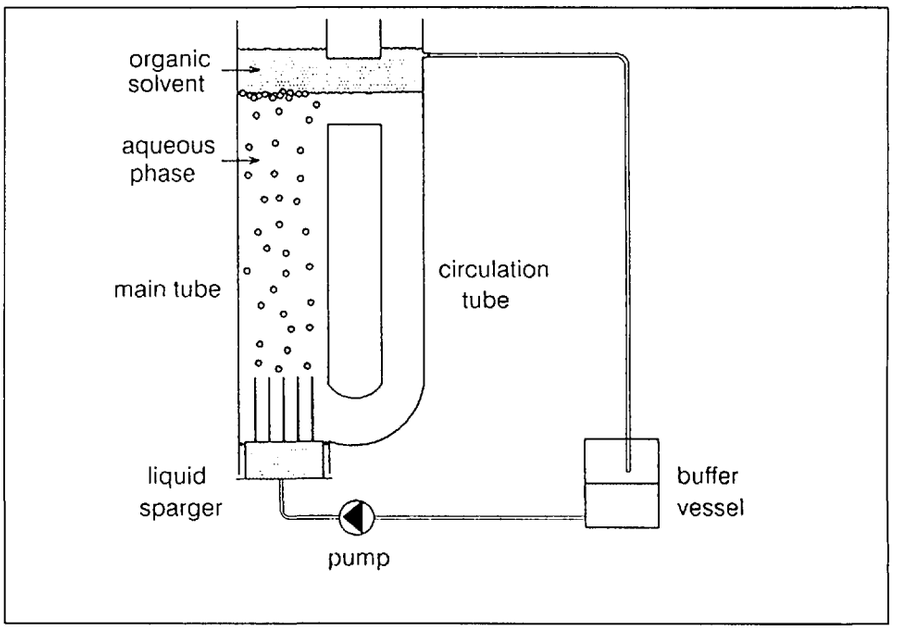

Fig. 9. *The liquid-impelled loop reactor* (from [15])

type of reactor compared with mass transfer in more commonly used reactor types, such as in the stirred-tank reactor, the packed-bed-reactor or the bubble column.

Modelling

Introduction of a second liquid phase makes the entire system more complex, in particular from the point of view of mass transfer. *Fig. 10* shows the various diffusion barriers the substrates have to take before reaching the cells. Mass transfer limitation is particularly severe inside the immobilization matrix. To account for this diffusion limitation we introduced the effective diffusion coefficient η from classical heterogeneous catalysis. Other components in our model were biocatalyst inactivation described by a first-order inactivation constant (k_d), a product inhibition term (K_p) and the further oxidation of the product by the cells (r_p).

The basic equations thus become

$$-\frac{dC_s}{dt} = \frac{\eta \cdot C_b \cdot r_s \cdot \exp(-k_d \cdot t)}{1 + \frac{C_p}{K_p}} \quad (3)$$

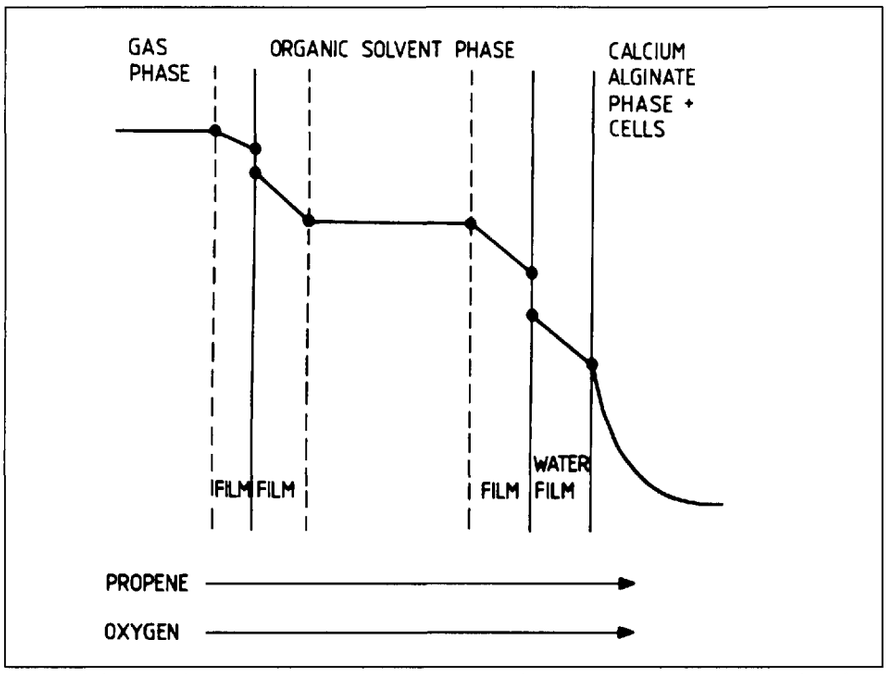

Fig. 10. *Schematic drawing of the transport of gaseous substrates to immobilized biocatalyst* (from [16])

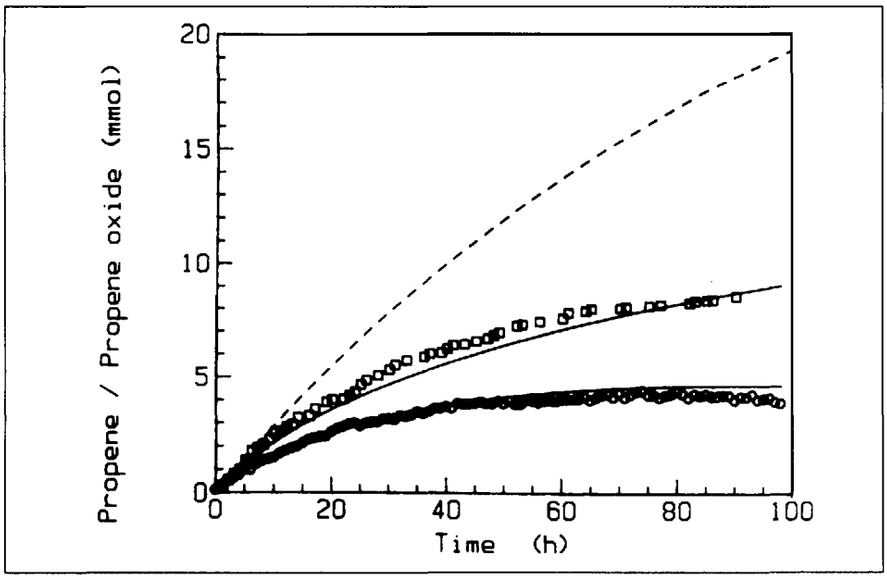

Fig. 11. *Predicted* (solid lines) *and actual* (points) *consumption of propene* (□) *and production of propene oxide* (○) *in the bioreactor system of* Fig. 7. (——) Calculated values when only the effect of deactivation is considered (from [16]).

and

$$\frac{dC_p}{dt} = \frac{dC_s}{dt} - r_p \quad (4)$$

with C_s, C_p, and C_b representing substrate, product and biocatalyst concentration, resp., t the time, and r_s the specific reaction rate described by *Michaelis-Menten* kinetics. With this model we were able to predict (not fit) the behaviour of the immobilized epoxidizing cells in continuous experiments very well. K_p, k_d and the *Michaelis-Menten* constants in r_s and r_p were determined in separate experiments, while η was calculated using the *Thiele* modulus concept. *Figs. 11* and *12* are examples of continuous runs and it is clearly shown that the above model predicts the experimental data well.

Scale Up

Using the model, a numerical exercise of the scale up of the system was executed. Based on our experience from lab-scale reactors, the following assumptions were made for the model calculations of the scale up of the continuous packed-bed reactor.

- $V_{reactor} = 10\ m^3$
- $\varepsilon = 0.35$
- $d_{bead} = 10^{-3}\ m$
- $X_{cells} = 70\ kg \cdot m^{-3}$
- $V_{max} = 5\mu mol\ O_2 \cdot min^{-1} \cdot g^{-1}$

In addition to these assumptions we also formulated 3 requirements under which we wanted the system to operate:

1) The oxygen conversion in the reactor should be less than 20% to prevent reduction in the conversion rate due to oxygen depletion.

2) The pressure drop over the packed bed should not exceed 1 bar to prevent compression of the gel beads (to accomplish a regular flow, the gel beads should be diluted with glass beads resulting in the relatively low void fraction ε of 0.35).

3) To accomplish an even liquid-flow distribution, the packed bed should be relatively high (height/diameter > 1).

Fig. 13 visualizes the importance of these three design criteria of the packed-bed immobilized-cell reactor in a graph of the supercritical velocity, U, *vs.* the bed height, H. Using water as continuous medium (*Fig. 13a*), it can be observed that no

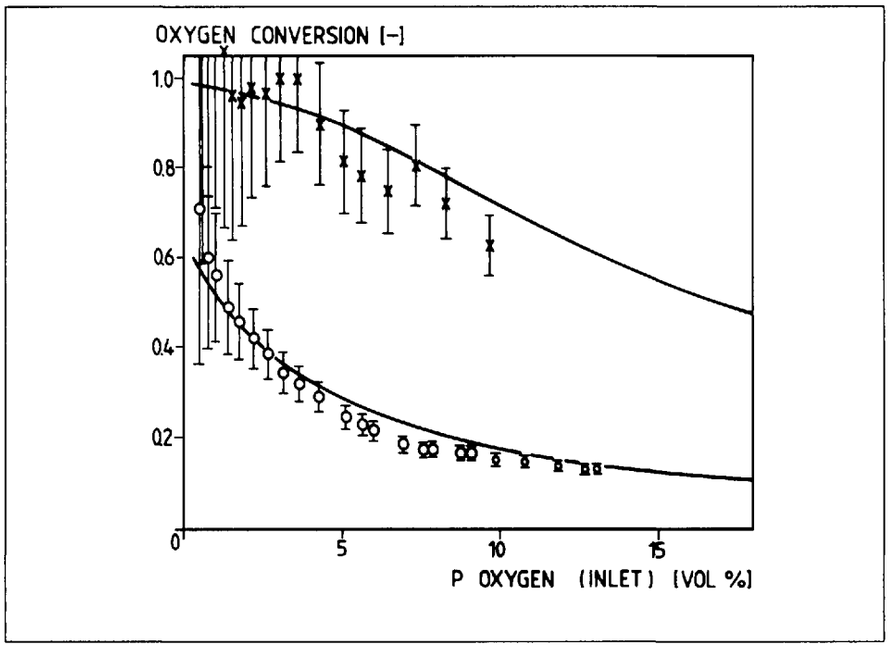

Fig. 12. *Predicted* (solid lines) *and actual* (point) *oxygen consumption in the bioreactor of* Fig. 7 *with water* (○) *or hexadecane* (+) *as continuous phase*. Because of the low oxygen solubility in water, rapid exhaustion occurs (from [16].

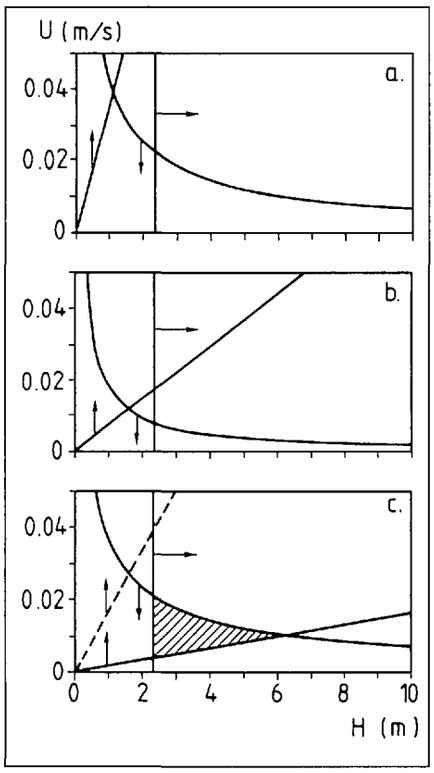

Fig. 13. *Three design criteria of a packed-bed immobilized-cell reactor* (oxygen depletion (↑) pressure drop (↓), height to diameter ratio (→)) *visualized in a graph of the superficial velocity, U,* vs. *the bed height, H; reaction medium: water* (a), *n-hexadecane* (b), *perfluoro hydrocarbon* (c). The hatched area represents combinations of U and H, which satisfy the three design criteria. The dashed line represents the oxygen-depletion criterium in case of a higher oxygen-consumption rate (from [16]).

combination of U and H can be found, which fulfills all the above-mentioned criteria. The same applies with hexadecane as continuous phase (*Fig. 13b*). Only if the fluoro compound with its high capacity for oxygen is used, an area of combinations of U and H can be distinguished, which meets all three requirements. However, when the specific oxygen-consumption rate (V_m) of the immobilized cells is increased by a factor 10 – which from a microbial point of view is not unfeasible – even operation with the fluoro compound as transport medium is not possible anymore.

These model calculations illustrate that organic solvents can, in some cases, be beneficial also on a larger scale, using a continuous packed-bed reactor type.

Conclusions

The main message we want to convey is that the introduction of an organic-solvent phase in addition to an aqueous phase can be beneficial for biocatalysis. It is shown that all three components, *i.e.* biocatalyst, medium and bioreactor, can be tailored with respect to optimal production. Finally, it is clear that biocatalysis in organic media is not confined to the use of enzymes but applies to microbial cells as well.

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