Engineering 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 of biocatalysis. However, this so-called advantage has proven to be one of the severest limitations for broadening the scope of applications of biocatalysts. 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 (Tramper et al., 1992).

The question obviously is: What advantages can the introduction of an organic solvent (phase) have over the use of just an aqueous medium? Table 1 shows these possible advantages, while Table 2 shows some disadvantages. Clerly there exist situations where the advantages outweigh the disadvantages.

TABLE 1
POSSIBLE ADVANTAGES OF ORGANIC SOLVENTS VERSUS AQUEOUS SOLUTIONS AS MEDIA IN BIOCATALYSIS

— High concentration of poorly water–soluble substrates/products
— Shift of reaction equilibria due to partitioning
— Shift of reaction equilibria due to reduced water-activity
— Reduction of substrate and product inhibition
— Prevention of hydrolysis of substrates/products
— Facilitated recovery of products and biocatalysts
— Less risk of microbial contamination
— Stabilization of the biocatalyst

The epoxidation of short-chain alkenes (Fig. 1) is a biotransformation where we expected this to be the case and which we studied for that reason extensively (Brink et al., 1988, and references cited therein). Both substrates,

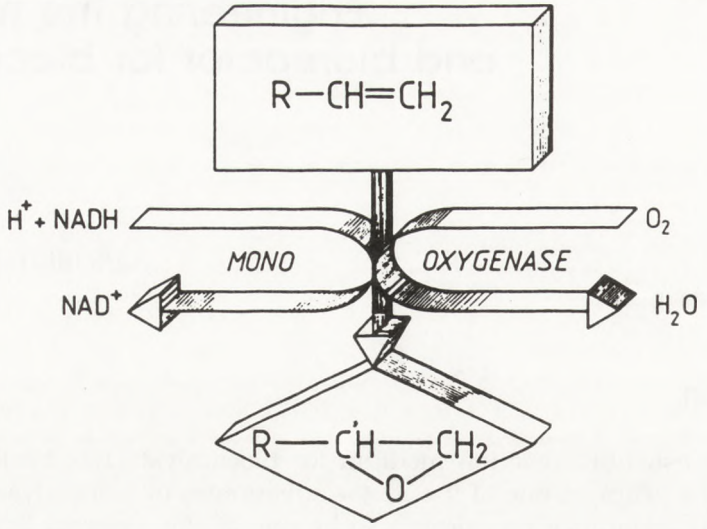


Fig. 1. Microbial epoxidation of short-chain alkenes.

oxygen and alkene, are poorly soluble in water, while the product, alkene oxyde, is toxic to the biocatalyst. Introduction of an organic-solvent phase as substrate reservoir and product extractant could therefore in principle allow high overall substrate and product concentrations and facilitate recovery of the product. Because of the need of a cofactor, NADH, we used whole cells, a *Mycobacterium* strain, as biocatalyst.

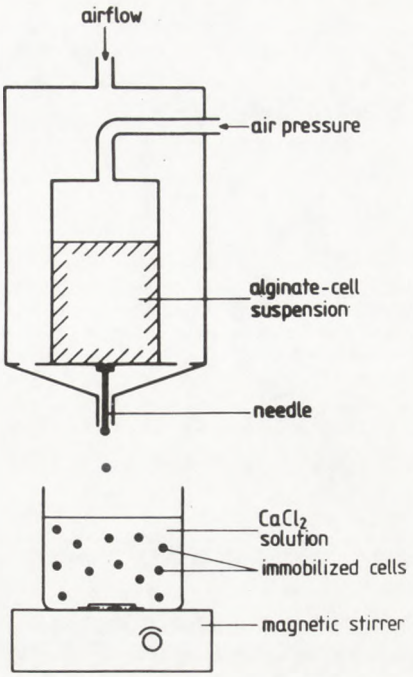
TABLE 2
POSSIBLE DISADVANTAGES OF ORGANIC SOLVENTS AS MEDIA IN BIOCATALYSIS

— Biocatalyst denaturation
— Biocatalyst inhibition
— Increasing complexity of the reaction system

Immobilization

Preliminary experiments showed that introduction of an organic-solvent phase, in addition to the aqueous phase, often resulted in inactivation of the cells at the interface. Immobilization of the cells by entrapment in a gel such as alginate and κ-carrageenan prevented such interfacial inactivation phenomena (Brink and Tramper, 1985). In addition to this requirement, the immobilization procedure must be mild enough to retain high biocatalytic activity and provide the cells with a support stable in the presence of solvents.

Fig. 2. Laboratory-scale set-up for immobilization of cells in gels such as alginate.



As long as the water activity of the system was close to one, thus certainly when there was a diluted aqueous phase present, both alginate and carrageenan gel beads were stable in the presence of organic solvents. However, when the water activity dropped, the gel beads dried out and shrunk, and biocatalytic activity was lost. Another requirement we demanded from the immobilization procedure was that it could be easily scaled up. Production of immobilized cells by entrapment in a gel was conveniently done on lab scale in the system schematized in Fig. 2. For production of large quantities of immobilized cells this dripping method was too tedious. By use of a resonance nozzle (Fig. 3.) the capacity could be improved by several orders of magnitude (Hulst et al., 1985; Hunik and Tramper, 1992).

Bioreactor

A schematic drawing of the system in which the measurements on the immobilized cells were executed is shown in Fig. 4. In this system, substrate and product concentrations were measured automatically at regular intervals and at desire controlled at a constant level (Brink et al., 1984). Both batch and continuous experiments in bubble-column and packed-bed reactors were run. Fig. 5 shows an example of the output from a batch run. After an initial increase in product concentration, this increase leveled off when substrates became depleted. Eventually even a decrease in product concentration was observed as a result of the capacity of the cells to slowly metabolize the epoxide. The figure clearly shows that this was much less so for the immobilized cell, which is advantageous if one is interested in epoxide production.

Type of solvent

Table 3 shows the requirements an organic solvent should meet for this particular system of epoxidation. At the time when we started these studies there were no guidelines available for selecting a suitable solvent. Therefore,

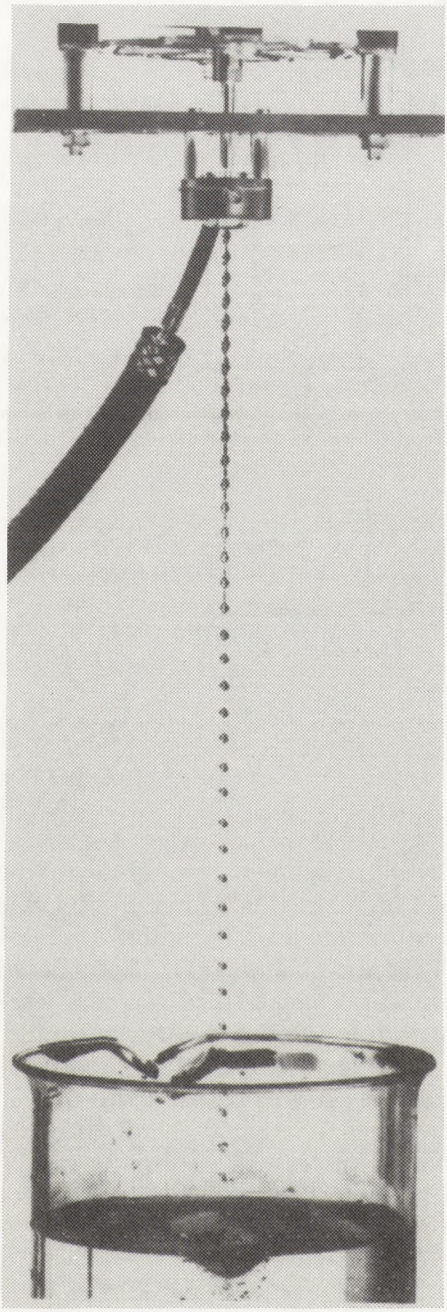


Fig. 3. Rapid immobilization of cells by means of a resonance nozzle.

we adopted a systematic approach and investigated the effect of about 40 solvents from various classes on the epoxidizing activity of the cells. Although we considered several physical-chemical parameters of the solvents, we were not able to find a clear correlations with one of them. The best rule of thumb we distilled from our experiments was that solvent with a Hildebrandt solubility parameter (measure for hydrophobicity) lower than 8 and a molecular mass greater than 200, had the least inactivating effect on the cells (Brink and Tramper, 1985). However, the log P concept (Fig. 6) introduced later by Laane et al., 1987) is a much better selection criterium.

TABLE 3
REQUIREMENTS SOLVENT SHOULD MEET AS MEDIUM FOR MICROBIAL EPOXIDATION

SOLVENT REQIREMENTS:
— WATER IMMISCIBLE
— HIGH CAPACITY FOR: * OXYGEN * PROPENE * PROPENE OXIDE
— NO CELL INACTIVATION
— NO INTERACTION WITH GEL MATRIX
— HIGH BOILING POINT
— LOW VISCOSITY
— NON-FLAMMABLE, NON-TOXIC
— CHEAP

Modelling

Introduction of a second liquid phase makes the system more complex, in particular from the point of view of mass transfer. Fig. 7 shows the various diffusion barriers the substrates have to

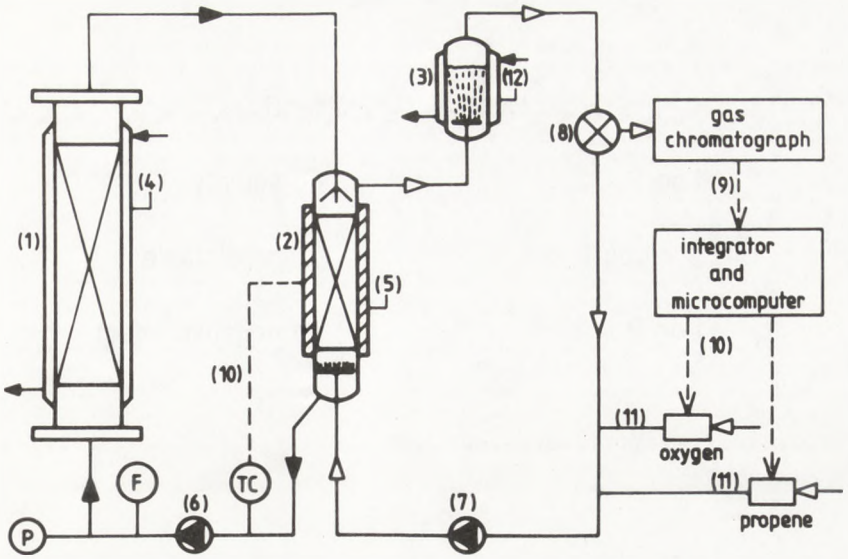


Fig. 4. Schematic representation of bioreactor and control system for microbial epoxidation of short-chain alkenes.

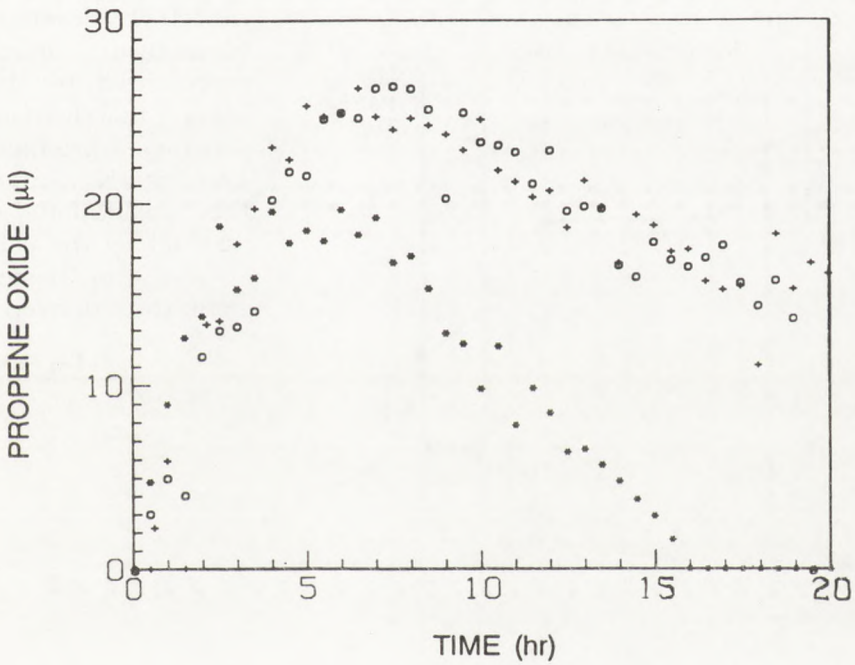


Fig. 5. Amount of propene oxide produced by a *Mycobacterium* strain: (*) free cells; (o) cells immobilized in alginate; (+) cells immobilized in κ-carrageenan.

LOG P CONCEPT:

$$P = [\text{solvent}]_{\text{octanol}}/[\text{solvent}]_{\text{water}}$$

Log P < 2	no activity
2 < Log P < 4	unpredictable
Log P > 4	no negative effect

Fig. 6. The log P concept of Laane et al., 1987.

take before reaching the cells. Mass-transfer limitation is particularly severe inside the support. To account for this diffusion limitation we introduced in our model the effective diffusion coefficient η from classical heterogeneous catalysis (Brink and Tramper, 1986 and 1987). Other components in the model we developed, are biocatalyst inactivation represented by the 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 two basic equations thus derived are

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

and

$$\frac{dC_p}{dt} = -\frac{dC_s}{dt} - r_p$$

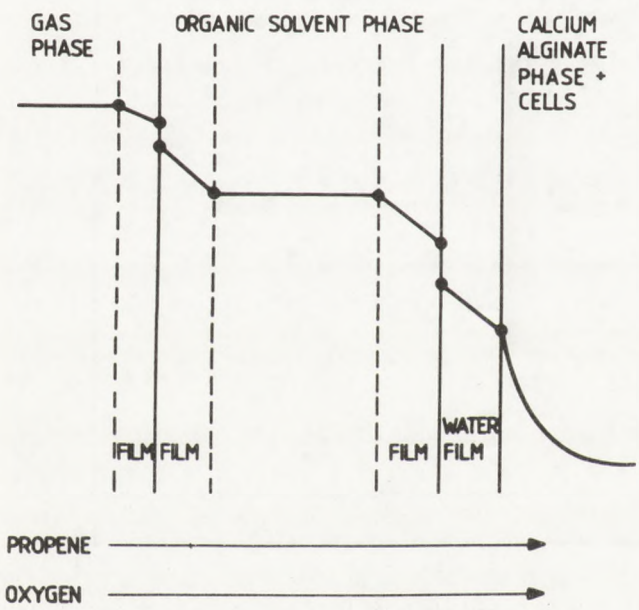


Fig. 7. Schematic drawing of the transport of gaseous substrates to immobilized biocatalyst.

with C_s, C_p and C_b representing substrate, product

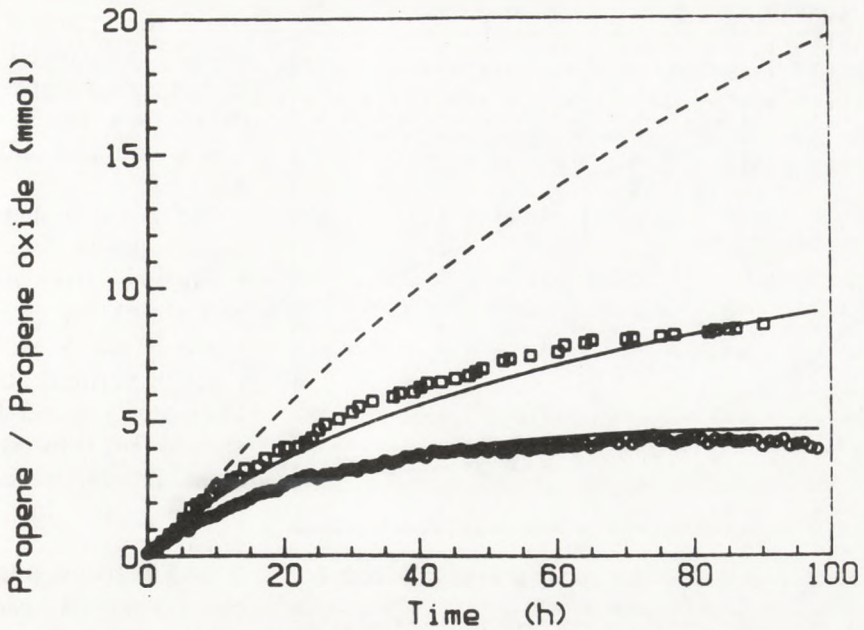


Fig. 8. Predicted (solid lines) and actual consumption of propene (❑) and production of propene oxide (o) in the bioreactor system of Fig. 7. (– – –) Calculated values when only the effect of deactivation is considered.

and biocatalyst concentration, respectively, 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) very well the behaviour of the immobilized cells in batch and continuous experiments. For that, K_p, k_d and the constants in r_s and r_p were determined in separate experiments, while η was calculated using the Thiele modulus concept. Fig. 8 and 9 are examples from continuous runs and it is clear that the model predicts the experimental data well.

Scale up

Using the above model, a numerical exercise of the scale up of the system was executed. Based on our experience with lab-scale reactors, we made the assumptions shown in Table 4. In addition to these assumptions we also formulated 3 requirements under which we wanted the system to operate (Brink, 1986):

1. The conversion of oxygen in the air-saturated solvent flowing through the packed-bed reactor should be less than 20% to prevent reduction in the rate of epoxidation due to oxygen depletion (area above slanted straight line in Fig. 10).

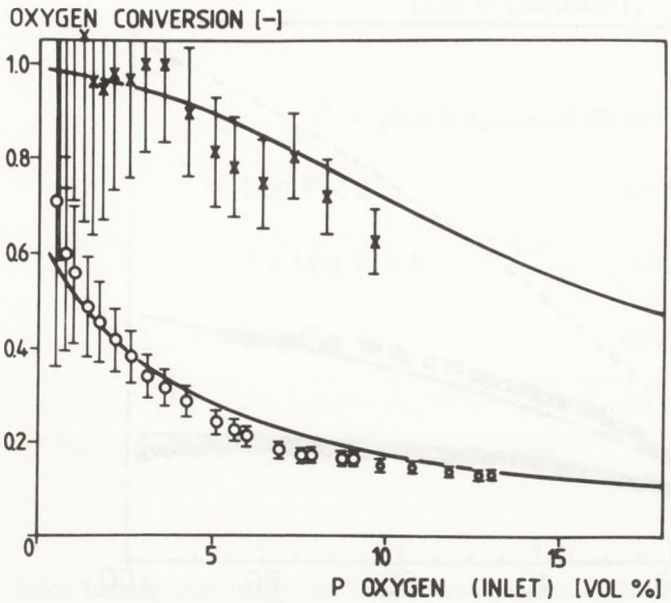


Fig. 9. Predicted (solid lines) and actual oxygen consumption in the bioreactor of Fig. 7 with water (o) or hexadecane (+) as continuous phase. Because of the low oxygen solubility in water exhaustion occurs.

2. The pressure drop over the packed bed should not exceed 1 bar to prevent compression of the gel beads (area below curved line in Fig. 10).

3. To accomplish a regular liquid-flow distribution, the packed bed should be relatively high (H/D > 1; area right of vertical straight line) and the gel beads should be diluted with glass beads, which explains the low bed porosity ε of 0,3.

Fig. 10 symbolizes the latter 3 requirements for 3 situations. This figure shows that only in the last case with the perfluorcarbon as continuous phase, because of the high oxygen capacity, all three requirements can be satisfied. However, when the specific activity of the cells in the bead is increased by a factor 10 — which from a microbial point of view is quite feasible — even in the fluorcarbon case the requirements are not fulfilled anymore. Clearly, however, this exercise illustrates that solvents can be beneficial also on a larger scale.

TABLE 4
ASSUMPTIONS MADE IN SCALE-UP EXERCISE

* $V_{reactor}$	=	10^3
* $\epsilon_{alginaat}$	=	0,3
* d_{bead}	=	10^{-3} m
* X_{cells}	=	70 kg m^{-3}
* V_m = 5μmol		$O_2 \cdot min^{-1} \cdot g^{-1}$

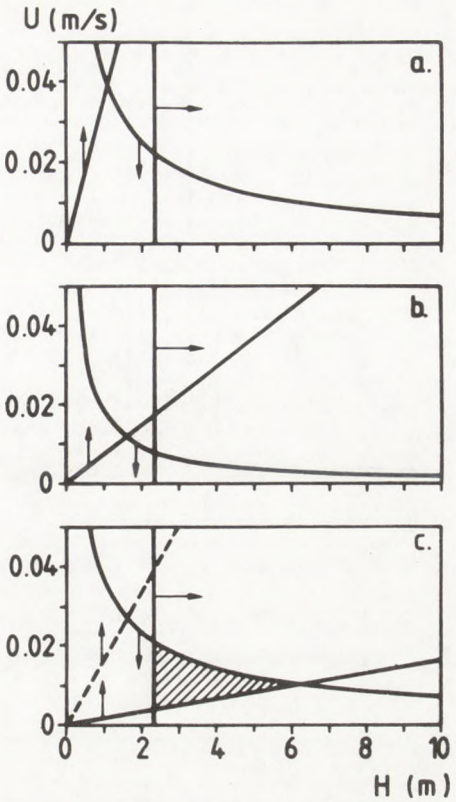


Fig. 10. Three design criteria of a packed-bed immobilized-cell reactor (oxygen depletion (↑), pressure drop (↓), height to diameter ratio (→)) visualized in a graph of the superficial velocity, U, versus the bed height, H; reaction medium: water (a), n-hexadecane (b), perfluoro hydrocarbon (c). The 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.

The liquid-impelled loop reactor

A reactor specially designed for biocatalysis in organic solvents is the liquid-impelled loop reactor (Fig. 11). This reactor integrates the advantages of air-lift loop reactors (Fig. 12) and of the introduction of organic solvents as a second liquid phase. Similar to the air-lift loop reactor (Verlaan, 1987) we have described this new-generation bioreactor with respect to hydrodynamics, mixing and mass-transfer (Van Sonsbeek, 1992). Table 5 shows the applications of the LLR which we study in our laboratory. Two of these are discussed below.

TABLE 5
APPLICATIONS UNDER STUDY IN THE LIQUID-IMPELLED LOOP REACTOR

* Production of secondary metabolites by plant cells
* Production of cis-benzene glycol from benzene by a mutant of *Pseudomonas sp.*
* Production of 1-tetralol from tetralin by *Acinetobacter*

Plant cells

Tagetes spp. (marigolds) produce thiophenes (Fig. 13) as secondary metabolites. Thiophenes are rather hydrophobic, intracellular compounds, poorly soluble in water, which are of interest as bioinsecticide. We have studied both free and immobilized *Tagetes* cells in LLR's taking air-lift loop reactors as reference (Buitelaar, 1991). The effects of solvents on growth and thiphene

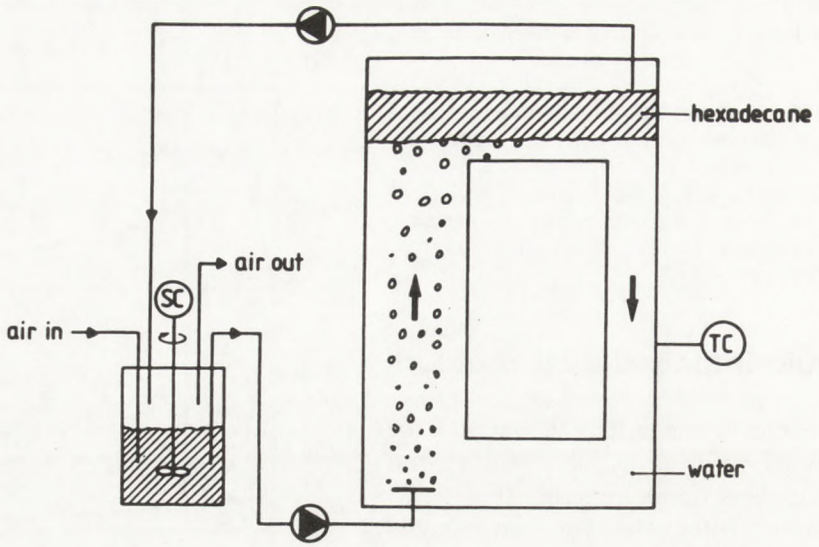
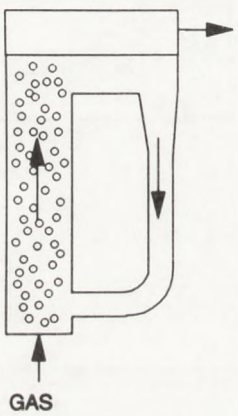


Fig. 11. Schematic representation of the liquid-impelled loop reactor.



Advantages of an Airlift-Loop Reactor

- efficient mixing
- efficient mass transfer of oxygen
- controlled liquid flow
- absence of mechanical agitators

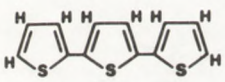
Fig. 12. The air-lift loop reactor and the advantages of its use.

production and excretion were investigated. Table 6 shows the effect of solvents on the growth. The log P rule applies, expect that log P has to be above 5 to retain full growth. The production of thiophenes did not differ for the one and two-phase systems; both produced 3 – 5 micromoles per gram of cells. However, a clear difference in excretion was observed. In the one-phase system less than 1% of the thiophenes was excreted, while in the two-phase system with hexadecane as organic phase about 50% of the thiophenes were excreted.

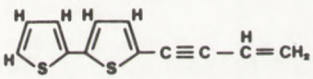
TABLE 6
SOLVENTS TESTED WITH THEIR LOG P VALUES AND GROWTH RESULTS OF PLANT-CELL (MARIGOLDS) CULTURES

Solvent	log P	Growth 1%	Growth 5%	Growth 10%
Alkanes				
Hexane	3,5	+	–	–
Heptane	4,0	–	nd	nd
Octane	4,5	–	nd	nd
Decane	5,6	+	+	+
Dodecane	6,6	+	+	+
Hexadecane	8,8	+	+	+
Phthalates				
Diethylphthalate	3,3	–	nd	nd
Dibutylphthalate	5,4	+	–	–
Dioctylphthalate	9,6	+	+	+
Didecylphthalate	9,8	+	+	+
Alcohol				
Nonanol	3,4	–	nd	nd
Ether				
Diisopentylether	3,9	–	nd	nd
Perfluorcarbon				
FC40	about 11	+	+	+

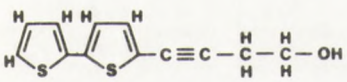
Legend: + is growth
– is no growth
nd is not determinated



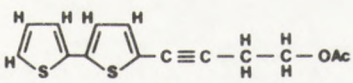
α-T
α-terthienyl



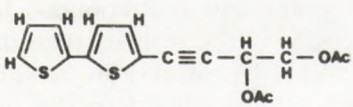
BBT
butenenylbithiophene



BBTOH
hydroxybutenylbithiophene



BBTOAc
acetoxybutenylbithiophene



$BBT(OAc)_2$
diacetoxybutenylbithiophene

Fig. 13. Tiophenes.

Tetralol production

Fig. 14 shows the conversion of tetralin into 1-tetralol by an *Acinetobacter sp.* Both substrate and product are poorly soluble in water and toxic to the biocatalyst. Conversion in a two-liquid-phase systems seems therefore attractive. Table 7 shows the points in the strategy we follow to determine if this is indeed case (Vermuë and Tramper, 1990). Fig 15 shows that with respect to point the log P concept is applicable. The cells show metabolic activity on hexadecane, nonane and undecanol, so clearly also 2 must be taken seriously.

TABLE 7
STRATEGY FOR SELECTING A SUITABLE SOLVENT: POINTS TO CONSIDER

1. Toxic effect of the solvent on the biocatalyst
2. Solvent as carbon or energy source for biocatalyst
3. Toxicity of substrate/product for biocatalyst
4. Effect of solvent on toxicity of substrate/product
5. Immobilization of biocatalyst
6. Metabolic activity of biocatalyst in a LILR
7. Product formation in a LILR

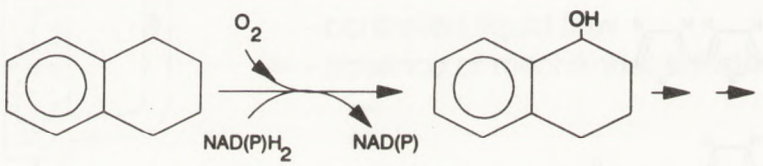


Fig. 14. Bioconversion of tetralin into 1-tetralol.

Conclusions

The main message of this presentation 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 just confined to the use of enzymes but applies to microbial and plant cells as well.

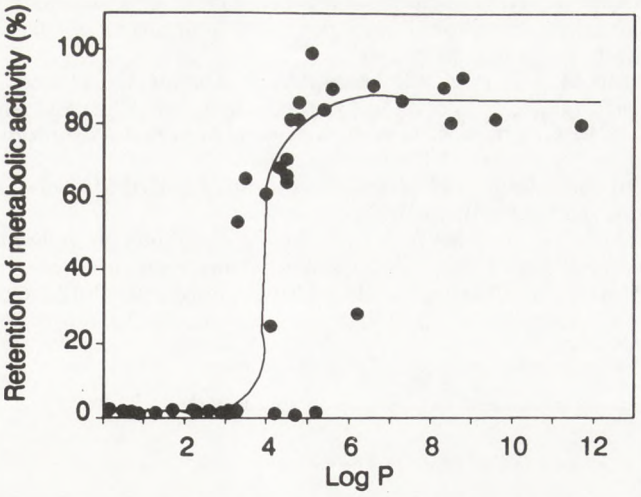


Fig. 15. Relationship between the relative metabolic activity of *Arthrobacter* (●) and *Acinetobacter* (○), exposed to 10% (v/v) organic solvent, and $\log P_{octanol}$.

References

Brink L. E. S., (1986), *Design of an organic-solvent-phase/immobilized-cell reactor for the microbial epoxidation of propene*, Ph.D. Thesis, Wageningen Agricultural University.

Brink L. E. S., Tramper J., (1985), *Optimization and modelling of the microbial epoxidation of propene in an organic-liquid-phase/immobilized-cell system* in Modelling and control of biotechnological processes (A. Johnson, ed.) Pergamon Press, Oxford, 111 – 117.

Brink L. E. S., Tramper J., (1985), *Optimization of organic solvent in multiphase biocatalysis*, Biotechnol. Bioeng., 27, 1258 – 1269.

Brink L. E. S., Tramper J., (1986), *Modelling the effects of mass transfer on kinetics of propene epoxidation of immobilized Mycobacterium cells: 1. Pseudo-one-substrate conditions and negligible product inhibition. 2. Product inhibition.*, Enzyme Microb. Technol., 8, 281-288 and 334 – 340.

Brink L. E. S., Tramper J., (1987), *Facilitated mass transfer in a packed-bed immobilized-cell reactor by using an organic solvent as substrate reservoir*, J. Chem. Tech. Biotechnol., 37, 21 – 44.

Brink L. E. S., Tramper J., Luyben K. Ch. A. M., Van't Riet K., (1988), *Biocatalysis in organic media*, Enzyme Microb. Technol., 10, 736 – 743.

Brink L. E. S., Tramper J., Van't Riet K., Luyben K. Ch. A. M., (1984), *Automation of an experimental system for the microbial epoxidation of propene and 1-butene*, Anal. Chim. Acta, 163, 207 – 217.

Buitelaar R. M., (1991), *Production and excretion of secondary metabolites by plant cell cultures*, Ph.D. Thesis, Wageningen Agricultural University.

Hulst A. C., Tramper J., Van't Riet K., Westerbeek J. M. M., (1985), *A ne technique for the production of immobilized biocatalyst in large quantities*, Biotechnol. Bioeng., 27, 870 – 876.

Hunik J. H., Tramper J., (1992), *Large-scale production of k-carrageenan droplets for gel bead production: theoretical and practical limitations of size and production rate*, Biotechnol. Progress, in press.
Tramper J., Vermuë M. H., Beeftink H. H., Von Stockar U., (1992), *Biocatalysis in nonconventional media*, Progress in Biotechnology, 8, Elsevier, Amsterdam, 763.
Van Sonsbeek H., (1992), *Physical aspects of liquid-impelled loop reactors*, Ph.D. Thesis, Wageningen Agricultural University.
Verlaan P., (1987), *Modelling and characterization of an airlift-loop bioreactor*, Ph.D. Thesis, Wageningen Agricultural University.
Vermuë M. H., Tramper J., (1990), *Extractive biocatalysis in a liquid-impelled loop reactor* in Proceedings of the 5th European Congress on Biotechnology (C. Cristiansen, l. Munck, J. Villadsen, eds.) Munskgaard Int. Publ., Copenhagen, 243 – 246.

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Modelowanie środowiska i bioreaktora dla procesu biokatalizy

Streszczenie

W artykule przedstawiono w syntetyczny sposób etapy badań koniecznych do opracowania procesu biotransformacji, na przykładzie epoksydacji alkenów. Opisano kryteria wyboru formy biokatalizatora (unieruchomione komórki drobnoustroju) i typu środowiska reakcji (rozpuszczalnik organiczny) oraz jego składu (rodzaj rozpuszczalnika). Opracowano matematyczny model procesu biotransformacji, w oparciu o który powiększono skalę i skonstruowano specjalny bioreaktor (10 m^3), zapewniający założoną wydajność procesu. Wykazano, że ten reaktor można z powodzeniem wykorzystać do innych biotransformacji (produkcja tiofenów przez komórki roślinne i 1-tetralolu przez bakterie).

Key words:
applied biocatalysis, bioreactor design, organic solvents, reaction medium tailoring.

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