Chimia 47 (1993) 93–96

ISSN 0009-4293

Microbiological Hydroxylations: Myths and Realities

Robert Azerad*

Abstract. Microorganisms such as filamentous fungi are known to carry out regio- and stereoselective hydroxylations of a wide range of natural or synthetic hydrophobic organic compounds, probably as part of a detoxification mechanism. The potential and limitations of such reactions, as a tool for performing preparative hydroxylation and functionalization, will be examined through selected examples: a quinidine derived alkaloid, a monoterpenic prochiral compound (1,8-cineole), and synthetic polyclic enones.

1. Introduction

Our group has been engaged for a long time in the use and application of enzymatic and biological systems in organic synthesis. More recently, we have been interested in investigating the potential of hydroxylation reactions, catalyzed by whole cells of microorganisms, for the large scale preparation of regio- and stereoselectively hydroxylated metabolites of natural or synthetic complex molecules of pharmacological interest and, further, for the preparation of functionalized asymmetric synthons of high optical purity.

These reactions, which, for pharmacological and toxicological reasons, have been extensively studied in the mammalian microsomal hepatic detoxification system of xenobiotics, can be frequently mimicked by simple microorganisms like filamentous fungi, which possess a similar enzymatic equipment and may provide an economical alternative for the large-scale production of metabolites, which otherwise would be difficult to synthesize chemically: this is the concept of 'microbial models of mammalian metabolism', first proposed by *Smith* and *Rosazza* [1], and now recognized to be a valid proposal through a number of comparative studies [2]. Beyond this concept, biohydroxylation reactions may represent a powerful method for the introduction of functional groups into already elaborated molecules, with the additional benefit of the usual regio- and stereolectivity of enzymic reactions [3]. Starting from easily accessible natural materials (alkaloids [4], steroids [5], terpenes [6]...) it is possible to obtain new complex molecules which can be tested for new biological activities or used as organic asymmetric synthons or synthetic intermediates. Typically, such monooxygenase-catalyzed reactions can hydroxylate aromatic rings, 'activated' (allylic or benzylic) methylenic carbon atoms, and most interestingly, 'unactivated' methyl or methylenic groups. The field of application of these reactions has been now extended to useful transformations of several purely synthetic organic materials of chemical interest.

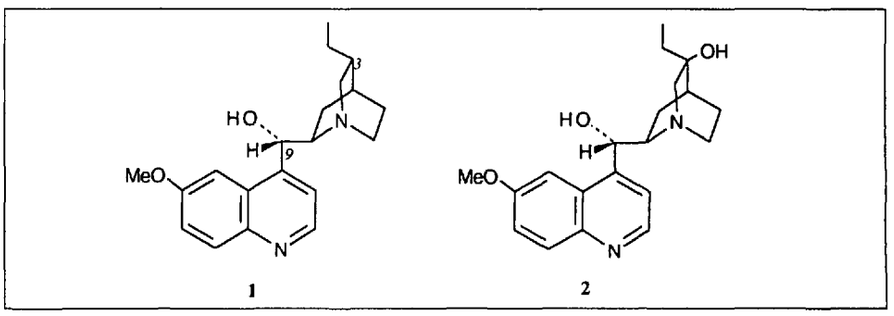
However, in each case, an empirical specific approach was employed and much remains to be done in order to make the regio- and stereochemical course of these reactions general and predictable. As in other fields of applied enzymology, this fact strenghthens the need for the elaboration of models, taking into account the generally observed flexibility and lack of specificity of detoxification-dedicated P450-monoxygenases [7]. Moreover, the general use of whole bacterial cells instead of pure enzymes introduces intrinsic limitations: number of enzymes involved, inductive or constitutive enzymes, toxicity of substrate and product(s), permeability and sequestration problems... Several of these problems and limitations will be evoked, together with the potential of the method, through a brief description of the results obtained in three examples of hydroxylation reactions concerning different substrate categories.

2. Hydroxylation of Quinidine Derivatives

Dihydroquinidine (**1**) is currently used as an antiarhythmic compound, and, as other quinine/quinidine-family alkaloids, is actively metabolized in man [8] to give a number of metabolites among which a 3(*S*)-hydroxy derivative **2** has been identified [9] and proposed as a potential substitution drug [10]. However, a chemical stereospecific hydroxylation of dihydroquinidine is not an obvious reaction and we have tried to design an alternative large-scale microbial process to produce such a hydroxy derivative in convenient economical conditions.

As the only known corresponding microbial conversions of quinidine, for example, were very uneffective (1–3% in 14 d) [11], we were induced to carry out an extensive screening of *ca.* 150 collection strains, which resulted in the selection of a unique species of mould, *Mucor plumbeus*, several strains of which had the unique property of metabolizing compounds of this alkaloid family to the desired derivatives, initially in moderate yields, but with the required stereochemistry. Optimization of growth conditions, of bioconversion parameters, and mainly esterification of the 9-OH group allowed to reach high conversion values (80–90%, see *Fig.*), at initial concentrations of *ca.* 1g/l, and for incubation periods of 3–5 d [12].

Several lessons may be derived from this new example of the concept of microbial models of mammalian metabolism: *i)* while in most cases, the same bioconversion properties are shared by a number of different microbial families, in this case, only a unique species was active, making necessary an extensive screening; *ii)* even



*Correspondence: Dr. R. Azerad
Laboratoire de Chimie et de Biochimie
Pharmacologiques et Toxicologiques
URA CNRS N° 400
Université René Descartes
45, rue des Saints-Pères
F-75270 Paris Cedex 06, France

inside the same taxonomical species, bioconversion activities are very different from one strain (corresponding to a geographical isolate) to another (*Fig.*); *iii)* manipulation of growth, incubation conditions, and substrate structural features (making it more hydrophobic, for example) may provide significant changes in the nature and yield of metabolites; *iv)* even after optimization, only low maximal concentrations of toxic substrates may be used (typically 0.5–1 g/l) and the hydroxylation job is generally a slow process, with low productivity.

3. Hydroxylation of a Prochiral Monoterpene

Many natural terpenoid compounds are common inexpensive substances which are already used as starting materials for organic synthesis. One of the possible valorizations of such compounds is their conversion to increasingly functionalized (hydroxylated) derivatives which may be used themselves as bioactive substances or as intermediates in the preparation of bioactive substances. A bicyclic monoterpene such as 1,8-cineole (**3**, 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane), the main component of several *Eucalyptus* essential oils [13], presents interesting structural features: hydroxylation at C(5) or (6) will afford position isomers of a monohydroxylated derivative; in each case, depending on the orientation of the reaction relative to the oxygen-bridge, *endo* or *exo* isomers may be formed. At last, the introduction of a hydroxyl group may occur at one of the enantiotopic edges of this prochiral molecule, giving rise to a single enantiomeric compound (*formulae* **3–7**). So, we can imagine that regio- and stereospecific biohydroxylations of **3** by different microorganisms may afford a full range of stereoisomeric monohydroxylated derivatives constituting new and interesting asymmetric synthons or chiral auxiliaries.

A few reports about 1,8-cineole metabolism are available (*Table*), and most of the microorganisms previously selected for these studies were bacteria, isolated from soil, using 1,8-cineole as sole carbon source and generally able to effect, in addition, the opening of the carbocyclic ring and of the ether linkage; this generally results in the complete degradation of the whole molecule, keeping the yield of eventually formed hydroxylated derivatives low or negligible [14][15]. At the contrary, some bacteria (*B.cereus*) [16] and mould strains (mainly *Aspergillus* strains) [17] [18] stop at the hydroxylation stage and allow isolation of several of the possible isomeric derivatives in respectable yields. However, optically pure compounds are not frequently obtained, indicating a defective recognition of the enantiotopic edges of the carbon cycle of the substrate. Moreover, only low concentrations of the terpenic compound (typically 0.1–0.5 g/l) are tolerated by the microorganisms, but the selection of a terpene-resistant *Aspergillus sp.* strain [18] allowed higher substrate concentrations (1–1.5 g/l). Several of the possible isomeric derivatives have already been obtained, and a more extensive screening would probably give access to new (stereo)isomers.

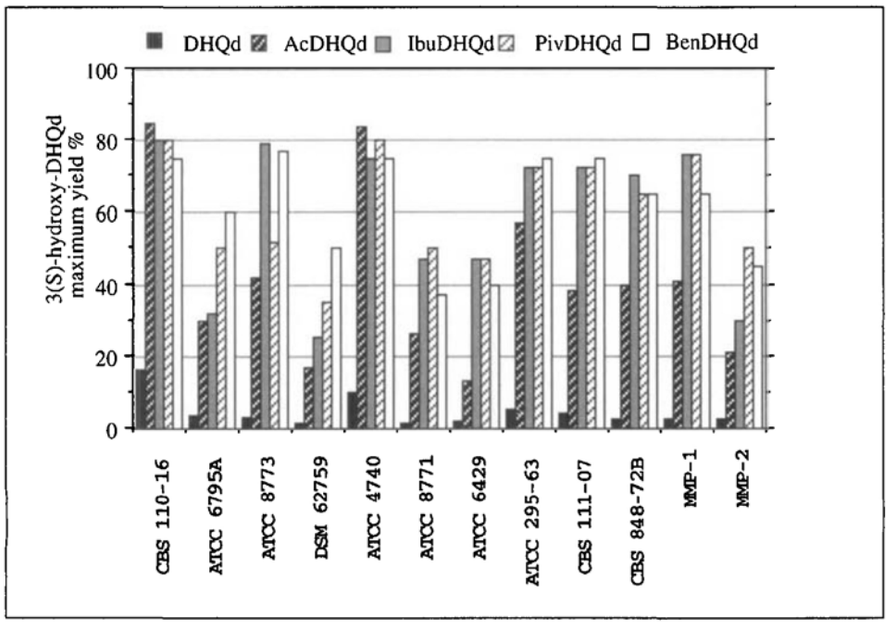
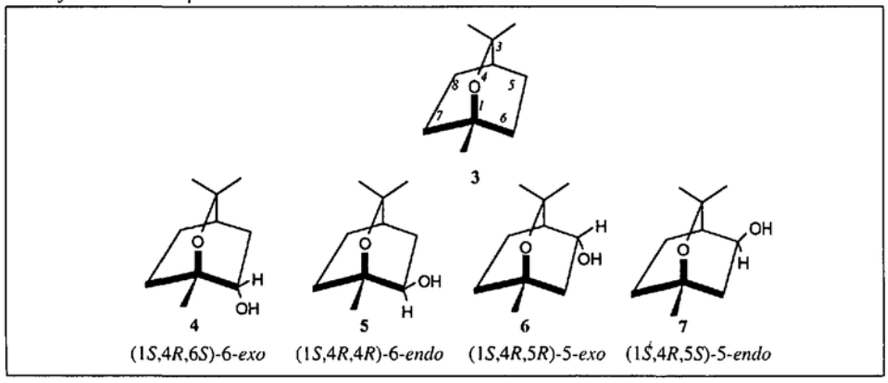


Figure. *Maximal conversion of dihydroquinidine* **1** (DHQd) *and its 9-O-acyl derivatives* (AcDHQd = acetyl, IBu = isobutyryl, Piv = pivaloyl, Ben = benzoyl) *into the corresponding (3S)-hydroxy derivatives by selected strains of* Mucor plumbeus

1,8-Cineole and Isomeric Derivatives Eventually Obtained by Different Hydroxylation Reactions at One of Its Enantiotopic Faces



4. Hydroxylation of Bicyclic Enones

Another example of the use of these reactions for the synthesis of asymmetric synthons has been developed. Starting from almost enantiomerically pure octalones **9–11** [19][20], phenanthrenones **12** [21], or hydrindenones **13** [19] with an angular methyl group, obtained by asymmetric synthesis, the unescapable problem of the functionalization of the B-ring was resolved through fungal hydroxylation reactions [22], illustrated in the *Scheme* and *formulae* **10–13** with products obtained by using again *M. plumbeus*.

As expected, allylic (benzylic) hydroxylation is a major pathway [23], but in some examples other unexpected hydroxylated products are formed, which may represent new useful functionalized synthons for total steroid or terpene syntheses. However, depending on the stereochemistry or small structural changes of the substrate, the regioselectivity of the introduction of the hydroxyl group may be deeply modified, preventing at this moment any kind of modelization studies and strengthening again the idea of the flexibility of the active site of cytochromes P450 involved in such reactions.

5. Conclusion

From the few examples which have been evoked, somewhat inconsistent pictures of fungal hydroxylations may be drawn: sometimes a high regio- and stereoselectivity is observed, sometimes mixtures of regio- (and/or stereo)isomers are produced. Nevertheless, the preparative potential of such methods, through the exceeding variety of existing microorganisms (and independently of yet unexplored possible genetic modifications) is obvious. It is clear that it will be still necessary to test a limited number of active microorganisms on a larger set of apparented substrate families, in order to gain more information about these reactions and, with the help of molecular modelization, have access to predictive methods. This approach, which has been initiated in our laboratory, may introduce in the future a 'new synthetic chemistry' (mimicking usual biosynthetic pathways) where only simple unfunctionalized compounds need to be primarily synthesized, while the introduction of critical functional groups will then be effected through selective microbial hydroxylation reactions. Selected target molecules, such as forskolin (**14**) or taxol (**15**), or some intermediate corresponding synthons, are particularly suitable for testing this concept.

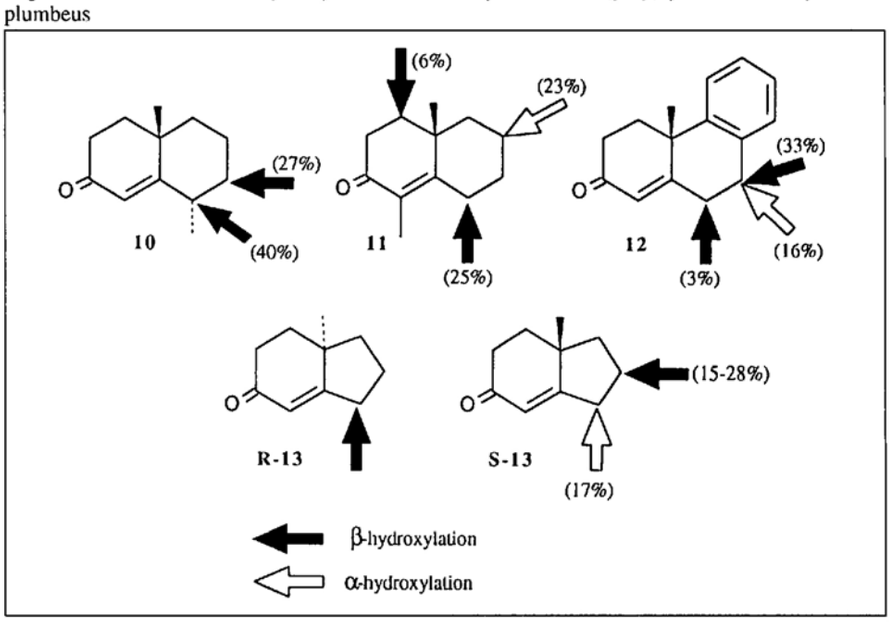
Financial support of this work by the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de l'Espace and, partly, by *Procter & Gamble Pharmaceuticals* (Laboratoire Nativelle, France) is gratefully aknowledged.

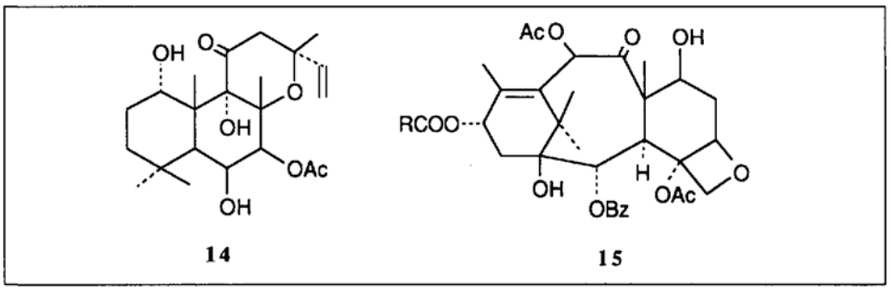
[1] R.V. Smith, J.P. Rosazza, *J. Nat. Prod.* **1983**, *46*, 79.
[2] D.A. Griffiths, D.J. Best, S.G. Jezequel, *Appl. Microbiol. Biotechnol.* **1991**, *35*, 373.
[3] H.L. Holland, in 'Organic Synthesis with Oxidative Enzymes', VCH Publisher, New York, 1992, pp. 463.
[4] H.L. Holland, in 'The Alkaloids', Eds. R.H.F. Manske and R.G.A. Rodrigo, Academic Press, New York, 1981, Vol. XVIII, p. 323.
[5] H. Iizuka, A. Naito, in 'Microbial Conversion of Steroids and Alkaloids', University of Tokyo Press, Springer-Verlag, Berlin, 1981, pp. 396.
[6] V. Krasnobajew, in 'Biotechnology', Ed. K. Kieslich, Verlag Chemie, Weinheim, 1984, Vol. 6a, Biotransformations, p. 31; V. Lamare, R. Furstoss, *Tetrahedron* **1990**, *46*, 4109.
[7] F.S. Sariaslani, *Adv. Appl. Microbiol.* **1991**, *36*, 133.
[8] B.B. Brodie, J.E. Baer, L.C. Craig, *J. Biol. Chem.* **1951**, *188*, 567; K.H. Palmer, B. Martin, B. Baggett, M.E. Wall, *Biochem. Pharmacol.* **1969**, *18*, 1845; B. Beerman, K. Leander, B. Lindström, *Acta Chem.*

Table. *Isomeric Monohydroxylated Products* (see *Formulae* **3–7**) *Obtained by Microbial Hydroxylation of 1,8-Cineole*

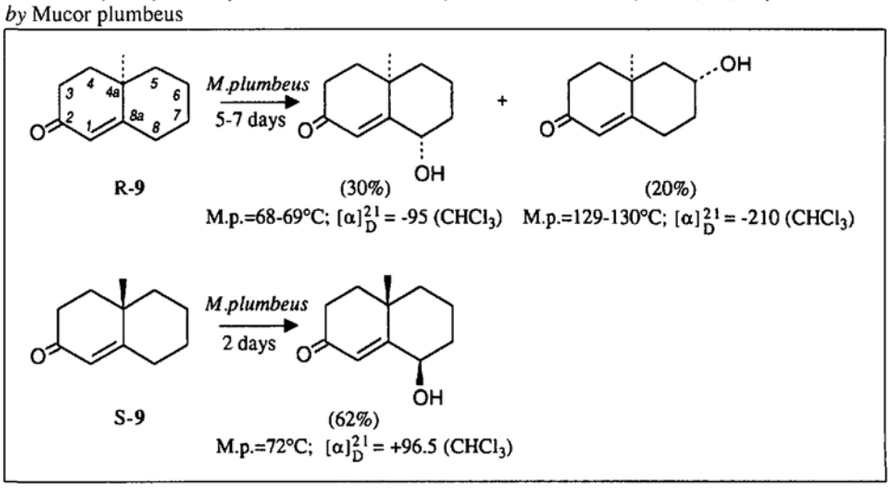
	Isolated products	Total yield	Ref.
Pseudomonas flava UQM-1742	**4** and **5**	< 10%	*Carman et al.* [14]
Rhodococcus sp. (C1)	*ent*-**5**	–	*Williams et al.* [15]
Bacillus cereus UI-1477	*ent*-**4**	74%	*Liu* and *Rosazza* [16]
Aspergillus niger	(±)-**4**, (±)-**6**, and (±)-**7**	< 50%	*Nishimura et al.* [17]
Aspergillus sp.	(±)-**4** and *ent*-**6**	60%	*Ismaili-Alaoui et al.* [18]

Regio- and Stereoselective Hydroxylation Patterns of Selected Bi(poly)cyclic Enones by Mucor plumbeus





Scheme. *Hydroxylation of Enantiomeric 4a-Methyl-4,4a,5,6,7,8-hexahydro-2(3H)-naphthalenones by* Mucor plumbeus



Scand., Ser. B **1976**, *30*, 465; B. Flouvat, G. Resplandy, A. Roux, P. Friocourt, C. Viel, M. Plat, *Therapie* **1988**, *43*, 255; G. Resplandy, A. Roux, I. Dupas, C. Viel, M. Plat, B. Flouvat, *J. Liq. Chromatogr.* **1988**, *11*, 1495.
[9] F.I. Caroll, D. Smith, M.E. Wall, C.G. Moreland, *J. Med. Chem.* **1974**, *17*, 985; F.I. Caroll, A. Philip, M.C. Coleman, *Tetrahedron Lett.* **1976**, 1757; F.I. Carroll, P. Abraham, K. Gaetano, S.W. Mascarella, R.A. Wohl, J. Lind, K. Petzoldt, *J. Chem. Soc., Perkin Trans. 1* **1991**, 3017.
[10] S. Fenard, J.-J. Koenig, P. Jaillon, F.X. Jarreau, *J. Pharmacol. (Paris)* **1982**, *13*, 129.
[11] F.M. Eckenrode, *J. Nat. Prod.* **1984**, *47*, 882.
[12] P. Wirsta, K. Regnard, M.C. Huet, B. Bartet, F. Deschamps, T. Ogerau, R. Azerad, unpublished results.
[13] H. Nishimura, D.M. Paton, M. Calvin, *Agric. Biol. Chem.* **1980**, *44*, 2495.
[14] I.C. MacRae, V. Alberts, R.M. Carman, I.M. Shaw, *Aust. J. Chem.* **1979**, *32*, 917; R.M. Carman, I.C. MacRae, M.V. Perkins, *ibid.* **1986**, *39*, 1739.
[15] D.R. Williams, P.W. Trudgill, D.G. Taylor, *J. Gen. Microbiol.* **1989**, *135*, 1957.
[16] W.G. Liu, J.P.N. Rosazza, *Tetrahedron Lett.* **1990**, *31*, 2833.
[17] H. Nishimura, Y. Noma, J. Mizutani, *Agric. Biol. Chem.* **1982**, *46*, 2601.
[18] M. Ismaili-Alaoui, B. Benjilali, D. Buisson, R. Azerad, unpublished results.
[19] M. Pfau, G. Revial, A. Guingant, J. d'Angelo, *J. Am. Chem. Soc.* **1985**, *107*, 273.
[20] G. Revial, *Tetrahedron Lett.* **1989**, *30*, 4121.
[21] T. Volpe, G. Revial, M. Pfau, J. d'Angelo, *Tetrahedron Lett.* **1987**, *28*, 2367; J. d'Angelo, G. Revial, T. Volpe, M. Pfau, *ibid.* **1988**, *29*, 4427.
[22] A. Hammoumi, G. Revial, J. D'Angelo, J.-P. Girault, R. Azerad, *Tetrahedron Lett.* **1991**, *32*, 651; A. Hammoumi, J.-P. Girault, R. Azerad, G. Revial, J. D'Angelo, *TetRahedron Asymmetry* **1993**, in press.
[23] H.L. Holland, B.J. Auret, *Can. J. Chem.* **1975**, *53*, 2041; J. Ouazzani, S. Arséniyadis, R. Alvarez-Manzaneda, A. Rumbero, G. Ourisson , *Tetrahedron Lett.* **1991**, *32*, 1983; J. Ouazzani, S. Arséniyadis, R. Alvarez-Manzaneda, E. Cabrera, G. Ourisson, *ibid.* **1991**, *32*, 647; S. Arséniyadis, J. Ouazzani, R. Rodriguez, A. Rumbero, G. Ourisson, *ibid.* **1991**, *32*, 3573.

Chimia 47 (1993) 96–99

ISSN 0009–4293

Enzyme Reaction Engineering

Christian Wandrey*

1. Introduction

Engineering aspects may become decisive in enzyme technology if enzymatically catalyzed enzyme reactions reach a preparative or productive scale. With the increasing number of enzymes or microorganisms available for biotransformations it is not sufficient to prepare useful biocatalysts. Additionally, methods of reaction engineering have to be employed in order to design a process competitive [1–11].

2. Reaction Conditions

First of all, thermodynamics of a given reaction system have to be analyzed. Next, suitable reaction conditions must be specified with respect to pH, temperature, substrate concentration and enzyme concentration. These reaction conditions have to be suitable for the biocatalyst involved. Important parameters are mechanical fragility, activity, and stability of the catalyst. Reaction conditions may also influence the achievable selectivity and enantioselectivity. For instance a high catalyst concentration (and correspondingly a short residence time) can discriminate non-desired parallel or consecutive reactions. After reaction conditions have been set, kinetic parameters can be identified. Using this information the final reactor design may follow to predict a suitable residence time, achievable conversion, space-time-yield and product specific enzyme consumption (*Fig. 1*).

3. Kinetics

Kinetic measurements have to be carried out under initial rate conditions in order to achieve information independent of product concentrations. Furthermore, one has to follow the reaction progress along the entire range of conversion. This is of special importance if several enzymes are analyzed since reactants for one enzyme may be inhibitors for another enzyme. Parameter estimation should be

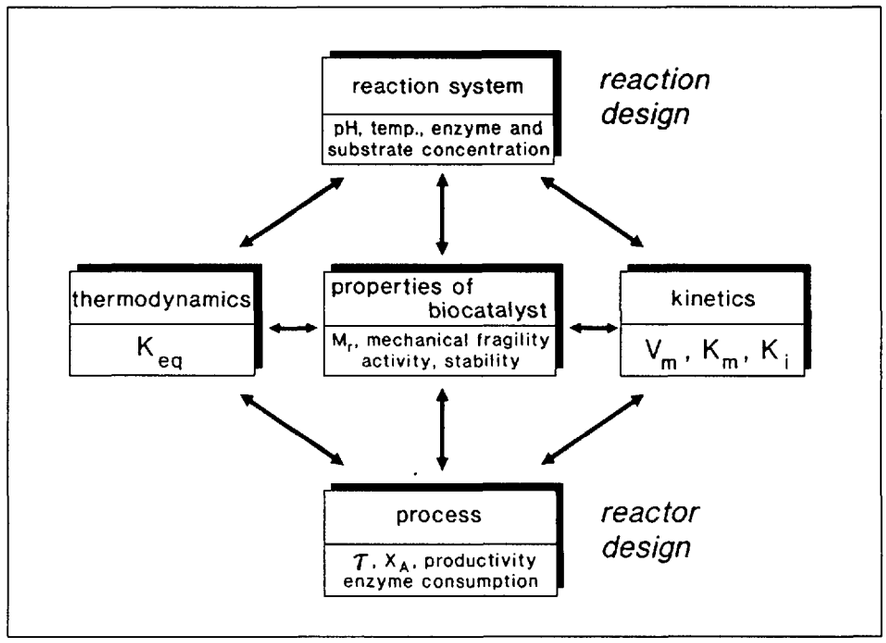


Fig. 1. *Influence of reaction conditions on reaction design and reactor design*

**Correspondence*: Prof. Dr. C. Wandrey
Institute of Biotechnology of the Research Centre Jülich
P.O. Box 19 13
D–5170 Jülich, Germany

Scand., Ser. B **1976**, *30*, 465; B. Flouvat, G. Resplandy, A. Roux, P. Friocourt, C. Viel, M. Plat, *Therapie* **1988**, *43*, 255; G. Resplandy, A. Roux, I. Dupas, C. Viel, M. Plat, B. Flouvat, *J. Liq. Chromatogr.* **1988**, *11*, 1495.
[9] F.I. Caroll, D. Smith, M.E. Wall, C.G. Moreland, *J. Med. Chem.* **1974**, *17*, 985; F.I. Caroll, A. Philip, M.C. Coleman, *Tetrahedron Lett.* **1976**, 1757; F.I. Carroll, P. Abraham, K. Gaetano, S.W. Mascarella, R.A. Wohl, J. Lind, K. Petzoldt, *J. Chem. Soc., Perkin Trans. 1* **1991**, 3017.
[10] S. Fenard, J.-J. Koenig, P. Jaillon, F.X. Jarreau, *J. Pharmacol. (Paris)* **1982**, *13*, 129.
[11] F.M. Eckenrode, *J. Nat. Prod.* **1984**, *47*, 882.
[12] P. Wirsta, K. Regnard, M.C. Huet, B. Bartet, F. Deschamps, T. Ogerau, R. Azerad, unpublished results.
[13] H. Nishimura, D.M. Paton, M. Calvin, *Agric. Biol. Chem.* **1980**, *44*, 2495.
[14] I.C. MacRae, V. Alberts, R.M. Carman, I.M. Shaw, *Aust. J. Chem.* **1979**, *32*, 917; R.M. Carman, I.C. MacRae, M.V. Perkins, *ibid.* **1986**, *39*, 1739.
[15] D.R. Williams, P.W. Trudgill, D.G. Taylor, *J. Gen. Microbiol.* **1989**, *135*, 1957.
[16] W.G. Liu, J.P.N. Rosazza, *Tetrahedron Lett.* **1990**, *31*, 2833.
[17] H. Nishimura, Y. Noma, J. Mizutani, *Agric. Biol. Chem.* **1982**, *46*, 2601.
[18] M. Ismaili-Alaoui, B. Benjilali, D. Buisson, R. Azerad, unpublished results.
[19] M. Pfau, G. Revial, A. Guingant, J. d'Angelo, *J. Am. Chem. Soc.* **1985**, *107*, 273.
[20] G. Revial, *Tetrahedron Lett.* **1989**, *30*, 4121.
[21] T. Volpe, G. Revial, M. Pfau, J. d'Angelo, *Tetrahedron Lett.* **1987**, *28*, 2367; J. d'Angelo, G. Revial, T. Volpe, M. Pfau, *ibid.* **1988**, *29*, 4427.
[22] A. Hammoumi, G. Revial, J. D'Angelo, J.-P. Girault, R. Azerad, *Tetrahedron Lett.* **1991**, *32*, 651; A. Hammoumi, J.-P. Girault, R. Azerad, G. Revial, J. D'Angelo, *TetRahedron Asymmetry* **1993**, in press.
[23] H.L. Holland, B.J. Auret, *Can. J. Chem.* **1975**, *53*, 2041; J. Ouazzani, S. Arséniyadis, R. Alvarez-Manzaneda, A. Rumbero, G. Ourisson , *Tetrahedron Lett.* **1991**, *32*, 1983; J. Ouazzani, S. Arséniyadis, R. Alvarez-Manzaneda, E. Cabrera, G. Ourisson, *ibid.* **1991**, *32*, 647; S. Arséniyadis, J. Ouazzani, R. Rodriguez, A. Rumbero, G. Ourisson, *ibid.* **1991**, *32*, 3573.

Chimia 47 (1993) 96–99

ISSN 0009–4293

Enzyme Reaction Engineering

Christian Wandrey*

1. Introduction

Engineering aspects may become decisive in enzyme technology if enzymatically catalyzed enzyme reactions reach a preparative or productive scale. With the increasing number of enzymes or microorganisms available for biotransformations it is not sufficient to prepare useful biocatalysts. Additionally, methods of reaction engineering have to be employed in order to design a process competitive [1–11].

2. Reaction Conditions

First of all, thermodynamics of a given reaction system have to be analyzed. Next, suitable reaction conditions must be specified with respect to pH, temperature, substrate concentration and enzyme concentration. These reaction conditions have to be suitable for the biocatalyst involved. Important parameters are mechanical fragility, activity, and stability of the catalyst. Reaction conditions may also influence the achievable selectivity and enantioselectivity. For instance a high catalyst concentration (and correspondingly a short residence time) can discriminate non-desired parallel or consecutive reactions. After reaction conditions have been set, kinetic parameters can be identified. Using this information the final reactor design may follow to predict a suitable residence time, achievable conversion, space-time-yield and product specific enzyme consumption (*Fig. 1*).

3. Kinetics

Kinetic measurements have to be carried out under initial rate conditions in order to achieve information independent of product concentrations. Furthermore, one has to follow the reaction progress along the entire range of conversion. This is of special importance if several enzymes are analyzed since reactants for one enzyme may be inhibitors for another enzyme. Parameter estimation should be

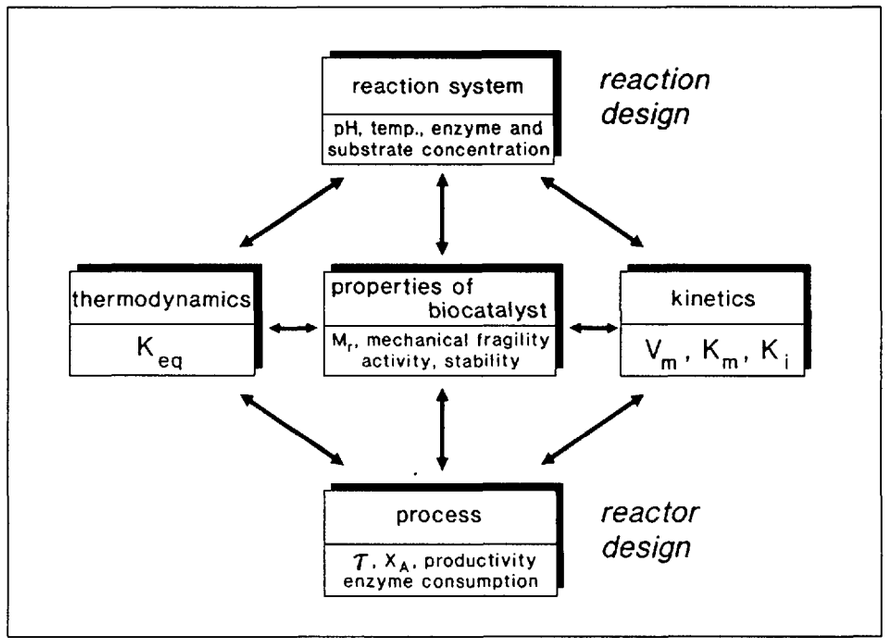


Fig. 1. *Influence of reaction conditions on reaction design and reactor design*

**Correspondence*: Prof. Dr. C. Wandrey
Institute of Biotechnology of the Research Centre Jülich
P.O. Box 19 13
D–5170 Jülich, Germany