[8] A.A. Klyosov, 'Enzyme Engineering at the Industrial Level – Present Status and Future Prospects', United Nations Industrial Development Organisation, Wien, 1989.
[9] K. Schügerl, 'Bioreaktionstechnik', Verlag Sauerländer, Aarau, 1985, Vol. 1, 1991, Vol. 2.
[10] S. Servi, Ed., 'Microbial Reagents in Organic Synthesis', Kluwer Academic Publishers, Dordrecht, 1992 (Proceeding of the NATO Advanced Reserarch Workshop on Microbial Reagents in Organic Synsthesis, Sestri Levante, Italy, March 23–27, 1992).
[11] S.C. Stinson, 'Chiral Drugs', *Chem. News*, **1992**, *28*, 46.
[12] M. Biselli, 'Enzymkatalysierte Racematspaltung von Aminosäuren mit integrierter Rückführung – dargestellt am Beispiel der Umsetzung von D,L-Alanin zu L-Alanin', Ph.D. Thesis, University of Bonn (published as a Report of the Research Centre Jülich, 2581, ISSN 0366-0885), 1991.
[13] S. Fukui, Ed., 'Special Issue on Biotechnology in Japan: Bioreactor system for the chemical industry', *J. Biotechnol.* **1990**, *14*.
[14] U. Kragl, D. Vasic-Racki, C. Wandrey, 'Kontinuierliche Reaktionsführung mit löslichen Enzymen', *Chem.-Ing.-Tech.* **1992**, *64*, 499 'Continous Processes with Soluble Enzymes', *Indian. J. Chem., Sect. B* **1993**, *32*, 103.
[15] P. Miethe, I. Jansen, U. Niedermeyer, U. Kragl, R. Haftendorn, M.-R. Kula, C. Wandrey, K.-H. Mohr, H.-W. Meyer, 'Continuous synthesis of (+)-(*R*)-benzaldehydecyanohydrin using (*R*)-oxynitrilase in lyotropic liquid crystals', *Biocatalysis* **1992**, *7*, 61.
[16] D. Rozzell, F. Wagner, Eds., 'Biocatalytic Production of Amino Acids and Derivatives', Hanser Publishers, Munich, 1992.
[17] C. Wandrey, A. Fischer, B. Joksch, A. Schwarz, 'Enzyme Reaction Engineering for Peptide Synthesis' Eds. D.S. Clark, and D. Estell, *Ann. N. Y. Acad. Sci.* **1992**, *672*, 528 (Enzyme Engineering 11).
[18] U. Kragl, D. Gygax, O. Ghisalba, C. Wandrey, 'Enzymatic Two-Step Synthesis of *N*-Acetylneuraminic Acid in the Enzyme Membrane Reactor', *Angew. Chem. Int. Ed.* **1991**, *30*, 827.

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How to Select a Useful Biocatalyst

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Early euphoria in biotransformations has declined in recent years to have a more realistic assessment of the uses in chemical syntheses. Reservations concerning this sector can be summarized as follows:

a) Finding a biological system suitable for the intended enzymatical reaction is mainly *empirical.*

b) When using living material biological deviations of unknown origin severly hamper the *reproducibility.*

c) The multivarious parameters and factors present, when investigating variables for growth and propagation of cells, formation and activity of required enzymes, and for the technical performance of the transformation reaction, demand *time consuming experimental series.*

d) Whereas initially *low yields*, due to *incomplete transformation*, can be partially increased by the results of these studies, the improvement of low yields, due to *undesired side reactions*, or *further degradations* by selective inhibition, requires some knowledge of the properties of the different enzymes or broad mutation programs.

e) The *purification of the metabolite*, with the separation of components of the nutrient media or of products formed in parallel by biosynthesis, is sometimes laborious.

f) In order to achieve economic production, the *yield* of a biotransformation, in relation to *volume and time*, requires high educt concentration, short conversion time and high level of the formed product using cheap nutrient media and low cost methods of recovery and purification.

g) Some of these problems can be decreased or eliminated by using *cell-free enzymes*, preferably in immobilized form for repeated use. This technique, however, is *limited* to stable enzymes without a cofactor requirement or with cofactors which can be regenerated in economic systems.

h) In general, the application of cell-free enzymes could eliminate a further problem, namely the *health risk*, using microorganisms which are virtually harmless or fungi forming spores.

i) A significant improvement in the process is achieveable by using gene transfer of a single usable enzyme from any cell, perhaps containing several undesired enzymes, into one host cell having the best properties for cultivation and increased enzyme production. However, this excellent method, needs special laboratory conditions required by the regulations *governing the use of rDNA-strains.*

k) Finally, the controlled *synthesis of an enzyme*, with all desired specifities and high activity is thought likely in the future taking the protein design path. This strategy requires either knowledge or cast-iron predictions of the active side and the tertiary structure. However, this condition is fulfilled in only a few cases and only in two examples in the field of the numerous P450-enzymes catalyzing multifold reactions of various important educts. *Plenty of time and patience* is apparently necessary for enlarging on the first successes with relatively simply regulated enzymes.

Some of these problems would be eliminated by the selection and use of a good biocatalyst.

There are various routes to this objective, all of unknown efficiency.

1. Search for Novel Biocatalytic Systems

The cultivation of mammalian and plant cells in submerged culture is made more difficult: by the exclusion of various cell types; by the requirement for expensive media; by the amount of time required for propagation; and by the sensitivity of higher cells against shear forces. Therefore, the search for novel biocatalytic systems is focussed on testing new isolated microorganisms.

Such screening programs (*Cheetham* 1987) are generally oriented towards finding the concrete enzymatic conversion of one substrate (educt) into one single de-

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fined target product (metabolite). The chance of success is similar to roulette, coupled with the test of a very large number of samples. Analytical methods on agar slants, based on biological activities, special growth conditions or selected staining procedures, which offer high test capacities in the earlist stage of the screening, have unfortunately only developed in very rare cases.

Propagation of the isolates and testing their capabilities for the desired biotransformations in shaking flàsks or small vials on the one hand and the analysis of the culture broth directly (or their extracts) with organic solvents with either TLC-, HPLC-, GC-, spectroscopically or other methods on the other hand are the well-known burdens of such programs.

The potential would be better, if only microorganisms are searched for which can use the added substrate (educt) as sole carbon- and energy source. These strains are obviously able to attack the compound in any way, however, they consume it *via* total degradation. This undesired sequence of reactions can sometimes be stopped at various stages of the degradation by selective inhibiton of responsible enzymes. Use of different reaction conditions, addition of selective inhibitors or mutagenization and rescreening of the mutants are procedures which can be implemented to accumulate the desired intermediate.

Active isolates forming undesired side products can be treated in the same manner for inhibition of the secondary reactions.

Various culture enrichment techniques are available for the isolation of the growth of novel microorganisms associated with the added substrate (educt). In this context samples of soil, effluent or waste were tested which originated from the environment of the substrate (educt). Hydrocarbon or sterol side-chain oxidizing microorganisms were taken *e.g.* from soil near gasoline stations, phenol oxidizing fungi were obtained from rotting wood, terpene transforming bacteria were isolated from relevant plants and from waste water from canning factories.

Although automation of such screening programs would be highly welcome, at present the collection and evaluation of analytical data with computer programs represents the main source of progress and increased economy. Unfortunately, the data consist primarily of results with respect to a target product, whereas side reactions or unexpected new biotransformations are often overlooked. Therefore, the search for novel useful microorganisms remains a laborious task and demands a great deal of effort and personnel in relation to the output.

2. Screening with Already Known Microorganisms from Type Culture Collections

Random screening programs are subject to additional problems:

a) Novel microorganisms can in principal be pathogenic to plants or mammals. Even minor effects couse problems, not least with the authorities.

b) The stability of the strain regarding the enzymatic activity is uncertain during extensive subcultivation.

c) The novel microorganism can turn out to be identical with an already known strain from an international type culture collection. (This would be an exception as only 1% of all assumed microorganisms of the world have been isolated to date.)

d) The procedure needs broad experience in microbiological techniques and labs and service rooms with relevant equipment, inconvenient for organic chemists.

On the other hand there are various national culture collections, which provide thousands of certified strains of microorganisms usable in principle for biotransformations. Additional collections of various research groups world wide are listed in a comprehensive directory (*Takishima et al.* 1989).

Broad screening programs for finding a useful biocatalyst for a desired reaction can be shortened by the availability of pure cultures of bacteria and fungi. The selection of microorganisms with the assumed relevant enzyme activity demands, however, some knowledge of past results.

Altough some books (*Davies et al.* 1989, *Roberts et al.* 1992, *Faber* 1992, *Poppe* and *Novak* 1992, *Holland* 1992, *Kieslich* (Ed.) 1987), proceedings (*Porter* and *Clark* (Eds.) 1984, *Tramper et al.* (Eds.) 1985, *Schneider* (Ed.) 1986, *Laane et al.* (Eds.) 1987, *Moody* and *Baker* (Eds.) 1987, *Whitaker* and *Sonet* (Eds.) 1989, *Copping et al.* (Eds.) 1990, *Abramovicz* (Ed.) 1990) and numerous review articles have been published in recent times and although databases on results of the last five years exist in the form of useful abstracts (*Crout* 1992) or other registration systems (*Königsberger* 1992), comprehensive treatments of biotransformations in catalogue form are limited to special classes of structures like steroids (*Charney* and *Herzog* 1967) or steroids and alkaloids (*Iizuka* and *Naito* 1981) and are, unfortunately, out of date (*Wallen* and *Stodola* 1959, *Kieslich* 1975, *Rosazza* (Ed.) 1982, *Laskin* and *Lechevalier* (Eds.) 1984).

In order to continue the comprehensive treatment of this field up to the state of the art, *i.e.* continuing a survey from 1957 up to 1972 (excluding steroids), we collected publications and patents through to 1992. From these 7500 references, 35000 single reactions were extracted and compiled in a computer databank.

The program permits searches:

a) for microorganisms used (including plant cells, microsomal fractions of mammalian cells) and cell-free enzymes

b) for their sources and availabilities from relevant culture collections or enzyme producing companies

c) for the substrates used (educts)

d) for the type of the enzymatic transformation, organized in classes and groups with subdivisions in more than one thousand reactions and finally

e) for some characteristic conditions of the fermentation, to example cell-free enzyme, immobilized cells, organic solvents.

This catalogue indicates roughly which taxonomic group of micro-organisms possess preference for a desired type reaction. In this manner screening programs can be limited in the first run to the test of collec-. tives of potentially useful strains.

In addition special strains can be selected for first experiments, which are usually described for the relevant target reaction.

Whereas the substrate specifity of many microorganisms and enzymes for several type reactions is relatively low, the regio- and stereospecifity of reaction with a given substrate can be quite high. Therefore, substrates with common structural features, but slightly different, were often biotransformed by one single microorganism with similar selectivities. Even substrates of greater structured difference can be converted in the same way, if the sterical shape and the charge of the surrounding of the point of attack is similar.

On the other hand, however, small differences of the structure near the centre of attack are able to inhibit the enzymatic reaction completely.

These imponderables can add up so that even after very careful selection of principally useful mircoorganisms, the impression of an accident can remain. Early, fairly reliable predections of the regio- and/or enantiomeric specifities of the reactions are possible with the *proviso* of some enzymes and substrate classes. The results of repeated or even systematic investigations of selected microorganisms have already resulted in some rules, like the *Bertrand-Hudson* rule for selective oxidation of one hydroxy group in aliphatic polyols (*Hann et al.* 1938) and the *Chargraff* rule for the same reaction at cyclitols (*Magasanik et al.* 1952), the *Prelog* rule for the reduction of various

ketones (*Prelog* 1964, *Ringold et al.* 1964) and to a postulation for the regioselective hydroxylation of various substrates (*Fonken et al.* 1967) and in particular for steroids (*Jones* 1973). Some of these rules and postulations have since been improved or extended to other enzymatic or microbial systems, for example hydroxylations with *Beauveria sulfurescens* (*Fourneron et al.* 1989), keto reductions (*Sih* and *Chen* 1981), esterhydrolysis (*Chen* and *Sih* 1989). However, the developed models for these reactions are exclusively effective for the special biocatalytic systems investigated. The rules for biotransformations with whole cells are always dubious, as different yet similar enzymes or isoenzymes can be responsible for overlapping and distinctive results. Unfortunately, to date the mechanism substrate specifity and selectivities are known for only few biocatalysts, investigated in the form of pure enzymes i.a. cytochrom P-450$_{cam}$, horse radish peroxidase, cyclohexanone oxigenase (see *Holland* 1992, *Sariaslani* 1989) and several lipases, esterases, amidases, proteases, and oxidoreductases (see *Whiteside* and *Wong* 1985, *Gerhartz* 1990). In addition, first spectacular results have been described on the chemical modification of an enzyme for improving the enantioselectivity (*Gu* and *Sih* 1992).

Dozens of lipases, esterases, and proteases can be purchased from enzyme manufacturing companies for the preparation of various optically active alcohols, carboxylic acids, amines, and amino acids. Very efficient biocatalysts are also known for the reduction of various ketones, whereby simple baker's yeast is a practical, though not always enantiospecific, potent system. Nearly 75% of all single biotransformations described in the last 12 years were hydrolytic reactions and keto reductions.

In addition to this broad knowledge we expect that numerous, preferably useful microorganisms and where possible their cell-free enzymes will be studied in more detail. In this case, investigations into the inducability of the enzymes and the influence of varied nutrient media would be essential, especially since broader screenings can test these factors only marginally.

These results will obviously enhance the databanks but cannot conceal the 'jigsaw puzzle' character of our field of biotransformations. But the acquired knowledge of preferred groups of microorganisms predestined for special type reactions and the accumulated experience on the borders of the selectivities of their enzymes, obtained by experts and colleagues throughout the world and compiled in a single databank, should facilitate the empiral finding and selection of useful biocatalysts.

[1] D. Abramowicz, Ed., 'Biocatalysis', Van Nostrand Reinold, New York, 1990.
[2] W. Charney, H.L. Herzog, 'Microbial Transformations of Steroids', Academic. Press, New York, 1967.
[3] P.S.J. Cheetham, 'Screening for novel biocatalysts', *Enzyme Microb. Technol.* **1987**, *9*, 194.
[4] C.S. Chen, C.J. Sih, 'Enantioselektive Biokatalyse in organischen Solventien am Beispiel lipase-katalysierter Reaktionen', *Angew. Chem.* **1989**, *101*, 711.
[5] L.G. Gopping, R. Martin, J.A. Pickett, C. Bucke, A.W. Bunch, Eds., 'Opportunities in Biotransformations', Elsevier, London, 1990.
[6] H.G. Crout, Warwick Biotransformation Club Database (Service for members only), Organic Chemical Inst., Univ. Warwick, U.K., 1992.
[7] H.G. Davies, R.H. Green, D.R. Kelly, S.M. Roberts, 'Biotransformations in preparative organic chemistry: The use of isolated enzymes and whole cell systems in synthesis', Academic Press, London, 1989.
[8] K. Faber, 'Biotransformations in Organic Chemistry', Springer Verlag, Berlin, 1992.
[9] G.S. Fonken, M.E. Herr, H.C. Murray, L.M. Reincke, 'Microbial Hydroxylation of Monocyclic Alcohols', *J. Am. Chem. Soc.* **1967**, *89*, 672.
[10] J.D. Fourneron, A. Archelas, R. Furstoss, 'Microbial Transformations 10. Evidence for a Carbon-Radical Intermediate in the Biohydroxylations Achieved by the Fungus *Beauveria sulfurescens*', *J. Org. Chem.* **1989**, *54*, 2478.
[11] W. Gerhartz, 'Enzymes in Industry, Production and Application', VCH Publishers, Weinheim, 1990.
[12] Q.-M. Gu, C.J. Sih, 'Improving the Enantioselectivity of the *Candida cylindracea* Lipase *via* Chemical Modification', *Biocatalysis* **1992**, *6*, 115.
[13] R.M. Hann, B. Tilden, C.S. Hudson, 'The Oxidation of Sugar Alcohols by *Acetobacter suboxidans*', *J. Am. Chem. Soc.* **1938**, *60*, 1201.
[14] H.L. Holland, *Chem. Rev.* **1988**, *88*, 473.
[15] H.L. Holland, 'Organic Synthesis with Oxidative Enzymes', VCH Publishers, Weinheim, 1992.
[16] H. Iizuka, A. Naito, 'Microbial Conversion of Steroids and Alkaloids', Univ. Tokyo Press, Springer Verlag, Heidelberg, 1981.
[17] E.R.H. Jones, 'The microbial hydroxylation of steroids and related compounds', *Pure Appl. Chem.* **1973**, *33*, 39.
[18] E. Keinan, E.K. Hafeli, K.K. Seth, R. Lamed, 'Thermostable Enzymes in Organic Synthesis: 2. Asymmetric Reduction of Ketones with Alcohol Dehydrogenase from *Thermoanaerobium brockii*', *J. Am. Chem. Soc.* **1986**, *108*, 162.
[19] K. Kieslich, 'Microbial Transformations of Nonsteroid Cyclic Compounds', Georg Thieme, Stuttgart; John Wiley, New York, 1975.
[20] K. Kieslich, 'Biotransformations', Eds. H.-J. Rehm and G. Reed, Biotechnology, Vol. 6a, Verlag Chemie, Weinheim, 1984.
[21] K. Königsberger, Biotransformation Database (non official service), Organic Chemical Inst., Univ. Graz, 1992.
[22] C. Laane, J. Tramper, M.D. Lilly, Eds., 'Biocatalysis in Organic Media', Elsevier, Amsterdam, 1987.
[23] A.I. Laskin, H.A. Lechevalier, Eds., 'Microbial Transformation', in CRC Handbook of Microbiology, CRC Press, Boca Raton, Florida, 1984.
[24] B. Magasanik, R.E. Franzl, E. Chargraff, 'The Stereochemical Specificity of the Oxidation of Cyclitos by *Acetobacter suboxydans*', *J. Am. Chem. Soc.* **1952**, *74*, 2681.
[25] G.W. Moody, P.B. Baker, Eds., 'Bioreactors and Biotransformations' (mainly technical aspects and procedures), Elsevier, London, 1987.
[26] Th. Oberhauser, K. Faber, H. Griengl, 'A Substrate model for the enzymatic resolution of esters of bicyclic alcohols by *Candida cylindracea* lipase', *Tetrahedron* **1989**, *45*, 1679.
[27] L. Poppe, L. Novak, 'Selective Biocatalysis; A Synthetic Approach', VCH Publishers, Weinheim, 992.
[28] R. Porter, S. Clard, Eds., 'Enzymes in Organic Synthesis', Ciba Foundation Symposium 111, Pitman, London, 1984
[29] V. Prelog, 'Specification of the stereospecifity of some oxido-reductases by dimond lattice sections', *Pure Appl. Chem.* **1964**, *9*, 119.
[30] H.J. Ringold, J.T. Bellas, A. Clark, 'Adamantanone as a probe for the dimensions and characteristics of the substrate binding pocket of certain alcohol dehydrogenases', *Biochem. Biophys. Res. Commun.* **1967**, *27*, 361.
[31] S.M. Roberts, K. Wiggins, G. Casy, 'Whole Cell and Isolated Enzymes in Organic Synthesis', JohnWiley, New York, 1992.
[32] J.P. Rosazza, Ed., 'Microbial Transformations of Bioactive Compounds', Vol. I and II (in parts lists on biotransformations), CRC Press, Boca Raton, Florida, 1982.
[33] F.S. Sariaslani, 'Microbial Enzymes for Oxydation of Organic Molecules', *Crit. Rev. Biotechnol.* **1989**, *9*, 171.
[34] M.P. Schneider, Ed., 'Enzymes as Catalysts in Organic Synthesis', Nato ASI Series C, Vol. 178, Reidel Publ. Co., Dordrecht, 1986.
[35] C.J. Sih, C.S. Chen, 'Mikrobielle asymmetrische Katalyse – enantioselektive Reduktion von Ketonen', *Angew. Chem.* **1984**, *96*, 556.
[36] Y. Takishima, J. Shimura, Y. Ugawa, H. Sugawara, 'Guide to World Data Center on Microorganism with List of Culture Collections in the World', 1st edn., WFCC World Data Center on Microorganisms, 1989.
[37] J. Tramper, H.C. van der Plas, P. Linko, Eds., 'Biocatalysis in Organic Synthesis', Elsevier, Amsterdam, 1985.
[38] L.L. Wallen, F.H. Stodola, R.W. Jackson, 'Type Reactions in Fermentation Chemistry', Agricultural Res. Serv./US Dept. Agriculture, ARS-17-13, 1959.
[39] G.M. Whiteside, C.H. Wong, 'Enzyme in der organischen Synthese', *Angew. Chem.* **1985**, *97*, 617.
[40] J.R. Whitaker, P.E. Sonet, Eds., 'Biocatalysis in Agricultural Biotechnology', ACS Symposium Series, Vo. 389, Washington, 1989.