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Chemocatalysis and Biocatalysis (Biotransformation): Some Thoughts of a Chemist and of a Biotechnologist

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Abstract:

Biotransformation processes are far more diverse than therapeutic protein production processes. Thousands of different strains and enzymes are required to exploit the selective biotransformation potential for the conversion of a myriad of different substrates into the desired products, especially new optically active APIs. Timeline compressions in the development cycle of pharmaceuticals, in combination with a missing broad strain and enzyme choice, result in the fact that biotransformation typically represents the second generation process choice in the manufacturing of a small molecule pharmaceutical. As the enzyme is the first and foremost functional element in biotransformation for small molecules, novel biocatalysts, especially oxidoreductases and lyases, are needed. As targets in the pharma industry are so variable and complex, unusual nontechnical solutions should also be considered, for example, strain alliances between companies.

Introduction

Biotransformation processes have been used by mankind for several thousand years. For example the biotransformation of ethanol to acetic acid (vinegar) by *Acetobacter* was most likely developed concomitantly with ethanol production from fermentable sugars by our ancestors in Babylon (Mesopotamia), Egypt, Mexico, and the Sudan. Table 1 give some randomly chosen milestones in the history of applied biotransformations. As a matter of fact, the biotransformation of ethanol to vinegar was probably also the first true biotransformation process applied in an industrial manner. The glucose isomerase is an example of a biotransformation operated at scales typically seen in the petrochemical industry (~15 million tonnes per year). This reaction converts glucose of starchy materials into high fructose syrups, which have enhanced sweetening properties but lower calorific values.

Although biotransformation has become an established method in organic chemical synthesis,¹ biocatalysis could, from our perspective, have a much bigger impact in this area. The authors, a chemist and a biotechnologist working together in this area, would like to summarize their experience from a very practical point of view and to draw attention to the remaining bottlenecks hampering the wider use of biotransformation.

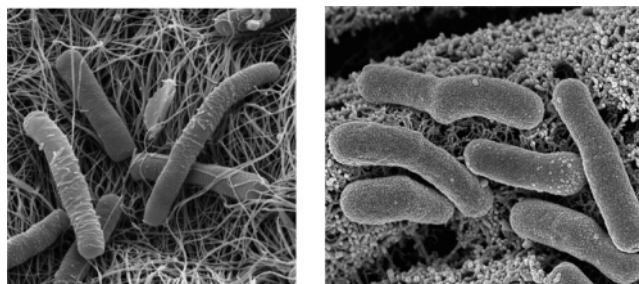


Figure 1. Examples of microbial strains used for biocatalysis. The photograph on the left shows *Escherichia coli*, one of the workhorses in biotechnology and the “standard” bacterium used for genetic engineering. The diameter of the *E. coli* cells varies between 0.5 and 1.5 μm . The bacterium on the right belongs to the genus *Rhodococcus*, a polymorphic organism able to grow into very long filaments.

Why Use Biotransformations?

There are two reasons, why one might want to use biotransformations:

1. The ability of microorganisms, e.g., bacteria, to produce large amounts of biomass and a great variety of different enzymes in a short time.

2. The chemo-, regio-, and enantioselectivity of enzymes.

Because of their small size (Figure 1) bacteria have by far the largest surface-to-volume ratio in the living world, which allows them to maximize their metabolic rates because of a high exchange of molecules and metabolites through their surface. With the right cultivation conditions, microorganisms grow exponentially according to the equation

$$X_t = X_0 e^{(\mu^* t)}$$

X_0 is the biomass concentration at time zero or the start of cultivation. X_t is the biomass concentration at the time of harvest. μ is a strain specific growth rate. Some of the fastest growing bacteria weighing maybe 10^{-12} g are theoretically able to duplicate and grow so fast that their biomass would reach the mass of the earth (9×10^{54} tons) in less than a week. This means, that if a bacterial strain produces an enzyme which can be used industrially, large amounts of enzymes can theoretically be produced economically.

Most microorganisms are also able to grow under varying conditions and on a great variety of substrates. This metabolic flexibility requires that these microorganisms are also able to produce hundreds of different enzymes for all sorts of reactions. However, these enzymes are not naturally overproduced but are regulated according to the physiological

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(1) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258.

Table 1. Some selected milestones of industrially relevant biotransformation and biocatalytic processes

year	process
5000 BC	Vinegar production
800 BC	Casein hydrolysis with chymosin for cheese production
1670	“Orlean” process for the industrial bio-oxidation of ethanol to acetic acid
1680	Antoni van Leeuwenhoek first to see microorganisms with his microscope.
1897	E. Buchner discovers yeast enzymes converting sugar into alcohol
1934	Regioselective biooxidation of sorbite to sorbose for the Reichstein Vitamin C synthesis
1940	Sucrose inversion using an invertase
1950	Bioconversion of steroids
1970	Hydrolysis of penicillin to 6-aminopenicillanic acid
1973	First successful genetic engineering experiments
1974	Glucose to fructose isomerisation with immobilized glucose isomerase
1985	Enzymatic process for the production of acrylamide
1990	Hydrolysis by protease (trypsin) of porcine insuline to human insuline
1995	3000 ton pa plant for the biotransformation of nicotinonitrile to nicotinamide

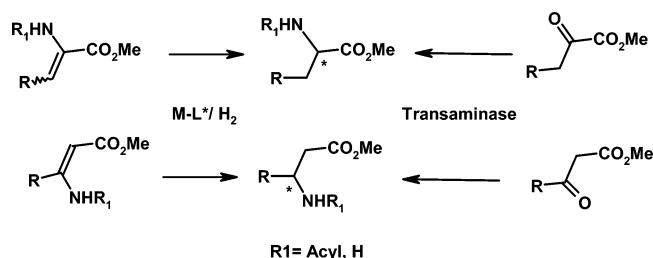


Figure 2.

needs of the cells. Also, enzymes are only produced and working under given environmental conditions at any point in time.

It is clear that the choice between a biotransformation and chemical technology will be driven by the commercial performance of the selected synthetic strategy on a specific target. Currently there are a number of well-established chemical technologies for introducing chirality into a molecule where biotransformations might not be competitive. On the other hand, biology might be useful in cases where there is no chemical solution or might allow extension of the arsenal of chemical transformations. The open question is which one of these two technologies will dominate for a particular chemical transformation.² For example:

Asymmetric hydrogenation of olefins, ketones, and enamines is well established with several examples realised on a commercial scale. Some specialized companies offer chiral catalysts as well as contract research support for the industrialisation of specific projects.³ A biotransformation alternative for ketone reduction might be considered if suitable conditions for hydrogenation of the substrate cannot be found. Enantioselective transaminase might be an attractive synthetic alternative for preparing α -amino acids and β -amino acids or chiral amines.⁴ One of the possible advantages of the biotransformation route is avoidance of

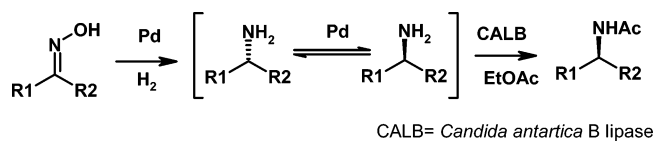


Figure 3.

the necessity for a protecting group, which was one of the main drawbacks of the current asymmetric hydrogenation of unsaturated β -amino acids, where an acyl protecting group on nitrogen is usually required. On the other hand, Merck process research chemists recently reported the first general method of high-yielding, highly enantioselective hydrogenation of unprotected β -enamino esters (Figure 2).⁵

Methods for preparing **asymmetric epoxides** and their derivatives, such as Jacobsen hydrolytic kinetic resolution, Sharpless allylic epoxidation and dihydroxylation, are remarkable scientific developments.⁶ To the best of our knowledge, the degree of success of the commercialisation of these technologies in the fine chemical industry has not equalled that reached by asymmetric hydrogenation. A biocatalytic platform might allow access to a single versatile, scalable, ecologically friendly technology.⁷

Chemical and bioresolution are both suboptimal, as half of the substrate is lost. However, if a dynamic resolution can be implemented via a chemical or biotech process, or a combination of both, these approaches become very attractive (Figure 3).⁸

In the above example the reduction/racemization process is catalyzed by palladium, and the lipase is the resolution

- (2) (a) Enantioselective catalysis in fine chemicals production. Blaser, H.-U. *Chem. Commun.* **2003**, 293. (b) *Asymmetric Catalysis on Industrial Scale: Challenges, Approaches and Solutions*; Blaser, H.-U., Schmidt, E., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2004; ISBN 3-527-30631-5.
- (3) Blaser, H.-U.; Brieden, W.; Pugin, B.; Spindler, F.; Studer, M.; Togni, A. *Solvias Siphos Ligands: From Discovery to Technical Applications. Topics in Catalysis* **2002**, 19, 3.
- (4) Stewart, J. D. *Curr. Opin. Chem. Biol.* **2001**, 5, 120.

- (5) Hsiao, Y.; Rivera, N. L.; Rosner, T.; Krska, S. W.; Njolito, E.; Wang, F.; Sun, Y.; Armstrong, J. D., III; Grabowski, E. J. J.; Tillyer, R. D.; Spindler, F.; Malans, C. *J. Am. Chem. Soc.* **2004**, 126, 9918.
- (6) (a) Tokunaga, M.; Larrow, J. F.; Kakiuchi, F.; Jacobsen, E. N. *Science* **1997**, 277, 936. (b) Aouni, L.; Hemberger, K. E.; Jasmin, S.; Kabir, H.; Larrow, J. F.; Le-Fur, I.; Morel, P.; Schlama, T. *Industrialisation Studies of the Jacobsen hydrolytic kinetic resolution of epichlorohydrin. In Asymmetric Catalysis on Industrial Scale: Challenges, Approaches and Solutions*; Blaser, H.-U., Schmidt, E., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2004; p 165. ISBN 3-527-30631-5. (c) Pfenninger, A. *Synthesis* **1986**, 89. (d) Kolb, H. C.; VanNieuwenhze, M. S.; Sharpless, K. B. *Chem. Rev.* **1994**, 94, 2483. (e) Lohray, B. B. *Tetrahedron Asymmetry* **1999**, 3, 1317.
- (7) Oxyrane aims high in biotechnology. Short, P. L. *C&EN* **2005**, October 24, 27.
- (8) (a) Choi, Y. K.; Kim, M. S.; Ahn, Y.; Kim, M.-S. *Org. Lett.* **2001**, 3, 4099. (b) Reetz, M. T.; Schimossek, K. *Chimia* **1996**, 50, 668.

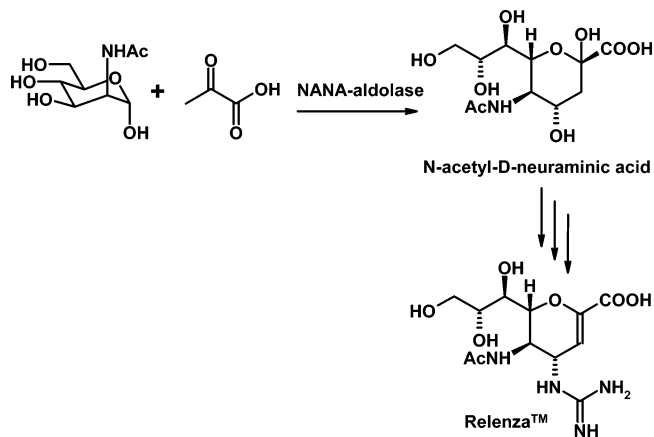


Figure 4.

catalyst. The mechanism of racemization consists of a combination of dehydrogenation of the undesired chiral amine and readdition of hydrogen to the imine; meanwhile acylation of the desired chiral amine is catalysed by the enzyme. Thus complete conversion of ketooximes to one single optically pure amine is possible.

Aldol reactions are not used in pharmaceutical synthesis as often as in academia. Usually such chemical transformations have been applied to the total synthesis of natural products, and therefore methods are described extensively in the literature.⁹ In industry, a natural product will usually be produced either by extraction or by fermentation. Nevertheless aldolases are interesting enzymes offering access to complex building blocks such as statin side chains or sugars.¹⁰ One example is the preparation of *N*-acetyl-D-neuraminic acid, one of the key intermediates in the synthesis of the influenza treatment Relenza (Zanamivir from GSK) where *N*-Acetylneuraminate pyruvate-lyase (NANA-aldolase) is involved (Figure 4).

Compared to chemical methods, **bio-oxidation** can be very effective for transformations such as the direct oxidation of a methyl group to a carboxylic acid function.¹¹ Chemically such a transformation usually requires several steps, and therefore a bioprocess might be more cost-effective. Besides C–C bonding enzymes, oxidoreductases belong to the enzymes where chemistry has most need for biotransformation options. Another example of a specific challenge with respect to oxidoreductases is how to stop the three-step enzymatic oxidation of a methyl group at the aldehyde oxidation state.

Organic chemistry looks back on a continuous and fruitful development, which started in the late 18th century, and

which continues today. This uninterrupted development provided the methods required for almost any synthesis, albeit ecologically and economically often still unfavourable.

In contrast, the enzymatic toolbox for organic synthesis is much younger and not yet routinely used by synthetic organic chemists. The ideal situation for biotransformations would be that thousands of different stable enzyme preparations were commercially available. Unfortunately the current situation is that biotransformation is routinely the second generation process and that the majority of processes are based on whole cell processes and not on commercially available enzymes.

In the past 5 years we have observed that the number of our customers' processes which involve biotransformation has decreased. This is surprising as the expected revenues¹² stemming from the application of chiral technologies to the production of enantiomerically pure molecules should have been a good trigger for using biological alternatives instead of the traditional technologies (chiral pool, chemical resolution) or the asymmetric tool box (chiral catalyst). On the other hand, our own route evaluation showed that there was potential for synthetic alternatives involving a biotransformation in these routes. Nitrile hydratase, aldolase, transaminase, hydrolases, oxidoreductases, etc. could be applied not only for the introduction of a chiral centre in a molecule but also for synthesis under mild conditions where chemoselectivity was a major concern in the process. An open question is also in which direction the pharma pipeline will develop in the future, and to what extent novel drugs based on recombinant therapeutic proteins and monoclonals continue to increase at the cost of small molecules.

“Why, after so much promise, and in the face of the prodigious flow of new products for medicinal and agricultural markets, has the harvest (of biotechnology) in the chemical area been so thin? The overall impact on the chemical process industry will be slight. Organic chemistry will continue as its mainstream.” Is this pessimistic statement by R. L. Hinman (1991)¹³ still valid? In addition, what could be the reasons for the underperformance of applied biotransformations, and what is most needed to debottleneck the situation?

What Are the Challenges in Biotransformations?

As a customer manufacturing organisation (CMO) dealing mostly with pharmaceutical companies, we are challenged with a broad spectrum of organic molecules. These organic molecules vary from very flat and small to very large structures in which the synthetic complexity can be increased by the presence of many chiral centres. The main challenge faced by a CMO is to deliver the necessary quantity and quality of a key intermediate or an API on time, and with a cost-competitive process throughout the life cycle of the product. Therefore the activities of a process development chemist should aim towards the development of a com-

(9) Carreira, E. M. Recent advances in asymmetric aldol addition reactions. In *Catalytic Asymmetric Synthesis*, 2nd ed.; Ojima, I., Ed.; Wiley-VCH: 2000; p 513; ISBN 0-471-29805-0.

(10) (a) *Enzymes in Synthetic Organic Chemistry*. Wong, C. H., Whitesides, G. M., Eds. Tetrahedron Organic Chemistry Series Vol. 12; Chapter 4, C–C bond formation, pp 195–242; ISBN 0 08 035941 8. (b) Bednarski, M. D.; Chenault, H. K.; Simon, E. S.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 1283. (c) Smith, P. W.; Sollis, S. L.; Howes, P. C.; Cherry, P. C.; Starkey, I. D.; Cobley, K. N.; Weston, H.; Scicinski, J.; Merritt, A.; Whittington, A.; Wyatt, P.; Taylor, N.; Green, D.; Bethell, R.; Madar, S.; Fenton, R. J.; Morley, P. J.; Pateman, T.; Beresford, A. *J. Med. Chem.* **1998**, *41*, 787.

(11) Shaw, N. M.; Robins, K. T.; Kiener, K. T. *Adv. Synth. Catal.* **2003**, *345*, 425

(12) In 2002 revenues generated were valued at \$7.0 billion. Information from Frost & Sullivan April 2003.

(13) Hinman, R. L. A Role for Biotech in Producing Chemical? *Bio/Technology* **1991**, *9*, 533.

R&D Challenge to build a profitable business



Figure 5.

mercial process where manufacturability, robustness, and best fit to the plant will be established (Figure 5).

CMOs are often “stuck” with the route selected by the customer and any change will be perceived by both parties as high risk for delivery time and quality (impurity profile change), not to mention the financial impact a process change implies. Often a bio option will only come in question if the chemical arsenal cannot achieve the synthesis of the target molecule or one of the key transformations. Why? Because the appropriate strain and enzymes are often missing. Biotransformation is often perceived as the last option when the process chemist sees no other alternative. Nevertheless, depending on the business situation of the new chemical entity, such change might be accepted but often as a 2nd generation process if significant cost improvement can be expected. For low volumes (early phase requirements 10 to 100 kg) the development of a biotransformation route is too time-consuming compared to organic chemistry, especially if speed is requested for delivering material for clinical studies.

An Example

An example demonstrating the issue is the request to make compound **2**. No technical package was provided, just the specifications with the quantities needed along with an aggressive delivery date. After a literature survey and technical evaluation followed by a first rough cost estimate, the chemist selected a synthetic route where an enzymatic reduction was chosen to introduce the chirality during the final stage¹⁴ (Figure 6).

After the substrate (**1**) had been efficiently prepared using a chemistry-based route, more than 40 strains from the Lonza strain collection were selected to test the enzymatic reduction. These organisms included yeasts and Gram+ microbial strains of different genera. The strain finally selected was used for subsequent work, which included the following:

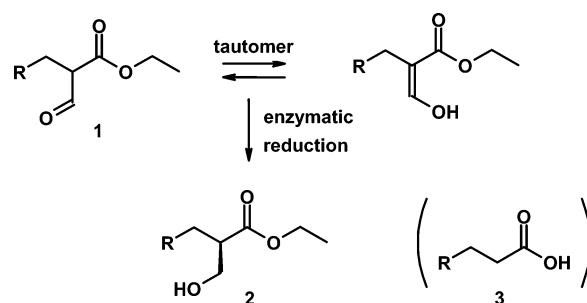


Figure 6.

1. Define fermentation conditions for biomass and enzyme production
2. Define biotransformation conditions
3. Define the conditions for isolation and purification
4. Pilot production of 100 kg of **2**

The biomass for the biotransformation was grown and induced on yeast universal medium (YM) supplemented with sucrose instead of glucose. The use of sucrose resulted in an increase in the yield of biomass from the fermentation, a good induction of the enoate reductase, the production of less undesired side product (**3**) in the biotransformation and a high enantiomeric excess of **2**. The harvested and washed biomass was used for the bioreduction, which was performed in a dilute phosphate buffer. The desired product (**2**) was extracted from the biosolution with ethyl acetate and washed with aqueous sodium carbonate to remove the acid impurity (**3**). Finally compound **2** was purified by distillation to afford a colourless liquid lab sample within the specifications,¹⁵ but with an unsatisfactory overall yield of 34%. It took a total of 5 months to carry out this feasibility study, which included the identification of the best candidate (strain), identification of a basic procedure for biomass and enzyme production (fermentation) and biotransformation, and production of a small sample on a 20 L scale.

At this stage and at the end of a feasibility study, a process is rarely ready for a 100 kg campaign in the pilot plant. Although process weaknesses were addressed and identified

(14) (a) Matzinger, P. K.; Leuenberger H. G. W. *Appl. Microbiol. Biotechnol.* **1985**, 22, 208. (b) Servi, S. *Synthesis* **1990**, 1. (c) Seebach, D.; Roggo, S.; Maetzke, T.; Braunschweiger, H.; Cercus, J. Krieger, M. *Helv. Chim. Acta* **1987**, 70, 1605. (d) Seebach, D.; Renaud, P.; Schweizer, W. B.; Züger, M. F.; Brienne, M. J. *Helv. Chim. Acta* **1984**, 67, 1843. (e) Ehrler, J.; Giovannini, F.; Lamatsch, B.; Seebach, D. *Chimia* **1986**, 40, 172.

(15) Optical purity: not less than 99.0 area% by HPLC and assay not less than 98.0%.

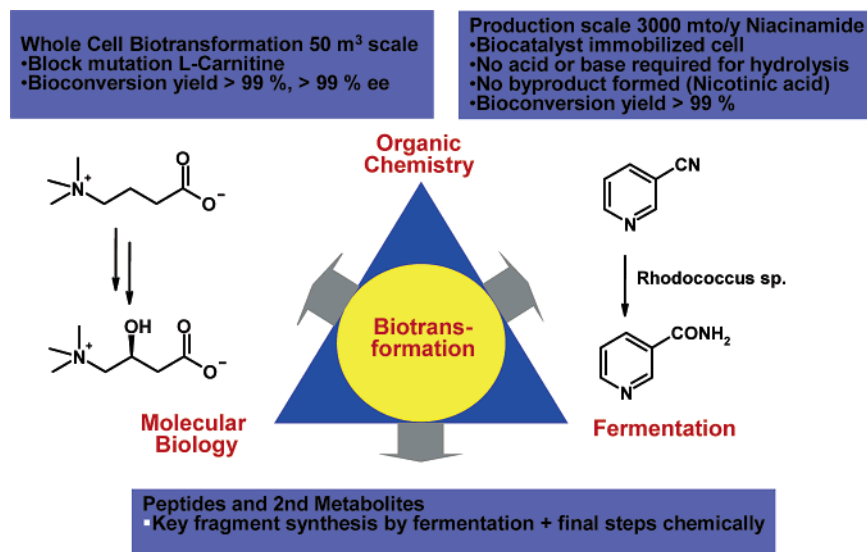


Figure 7.

during the feasibility study, an optimization of the whole process is still needed with a focus on the following milestones:

1. Increase the biomass titre (gram biomass per litre fermentation broth)
2. Increase the specific productivity of enzymes (g enzyme/ g biomass)
3. Maximize volumetric productivity and enantiomeric excess (if necessary) during biotransformation
4. Maximize yield during isolation and purification

This example shows also the importance of integrating chemistry and biology and of having multidisciplinary teams, since organic chemistry, molecular biology, fermentation, and QC must all deliver effective results in this technology. At Lonza, this synergy has contributed to several business success stories on important products such as L-carnitine, Niacinamide, and others (Figure 7).

Today's chemical toolbox allows most molecules to be made without the need to implement a biotransformation reaction, and the choice of applying classical methods is mainly driven by the requirement for speedy delivery of the materials required for early clinical trials. In the long term biotransformation technology can certainly be competitive in many cases; it is probably a matter of confidence, commitment, and culture to initiate the early use of biotransformation, even when the future of the new chemical entity is still uncertain.

What Are the “Burning Issues” in Biotransformation?

What is needed to include biocatalysis in the process development of an early stage project? From our experience, we put the burning issues in biotransformation in the following order.

1. Availability of strains and enzymes. This means stable strains or enzymes from the different enzyme classes ready for use in a screening for a new product. A broad strain library is a key success factor for the biotransformation industry, and innovation should focus on this aspect.

2. Biotransformation “environment” is of second priority. Although, for most problems more or less practicable solutions are available today, there remain exceptions: (i) **Product inhibition** still waits for a scalable technical multiproduct solution to remove the inhibiting product from a biotransformation broth (ISPR, **In Situ Product Removal**). (ii) **Solubility of reactants** requiring nonaqueous reaction media.

3. Down Stream Processing (DSP) is the least problematic aspect of biotransformation, as chemical processing technology for small molecules is available.

The lack of a broad strain and enzyme base is the factor most seriously impeding biotransformation. What makes the situation even more difficult is that a prospective or proactive enzyme search is very difficult as we do not know which biotransformation candidate goes into a feasibility study. Things are easier with established commercial products, where a switch from chemical synthesis to biotransformation is desired. It is also in this area where some remarkable biotransformation successes have been observed. As was mentioned earlier, timeline compressions in the development cycle of pharmaceuticals makes things even worse, as there is very little time for the screening of new enzymes for new products.

To overcome the strain shortage, creative technical as well as unusual nontechnical solutions are needed. The Swiss Industrial Biocatalysis Consortium is presently trying to establish an unconventional solution. Companies active in strain and enzyme screening have countless strains which they will never use in commercial processes. As everybody in the industry has the same problem, the consortium plans to share information on their strain and enzyme collections. In this way redundant strains might become valuable for both the strain receiver and the strain donor.

The consortium also investigated the actual enzyme toolbox for biotransformation.¹⁶ The following priorities formulated for the completion of the toolbox are summarized.

(16) Swiss Industrial Biocatalysis Consortium. Meyer, H. P.; Münch, T. *BioWorld EUROPE* **2005**, 1, 14.

Table 2. Analysis of the posters presented during the 7th Biotrans Symposium held in Delft (2005),¹⁷ one of the key events in Biotransformation; the numbers in parentheses are the same figures for the 6th Symposium held in Olomouc¹⁸

1. Oxidoreductases	24% (28%)	Redox reactions
2. Transferases	6% (3%)	Functional group transfer
3. Hydrolases	55% (58%)	Hydrolysis of functional groups
4. Lyases	12% (10%)	Nonhydrolytic addition/ removal of groups ^a
5. Isomerases	2% (1%)	Intramolecule rearrangements
6. Ligases	1% (0%)	Formation of C–O, C–S, C–N, or C–C bonds

^a C=C, C=O, C=N formation by elimination, e.g., decarboxylases, aldolases, etc.

In the area of R&D for new strain and novel enzymes, the following priorities were proposed by the Swiss Industrial Biocatalysis Consortium:

Oxidoreductases

Dehydrogenases

1. NADH-dependent dehydrogenases for the asymmetric reduction of ketones, ketoacids, and olefins.
2. Oxidations of alcohols with dehydrogenases have second priority.

Oxygenases

1. Monohydroxylations, especially hydroxylations of non-activated centers and of non-natural substrates, are important reactions. Improve the practicability and robustness of the in-vitro P-450 systems, or. develop an FMO based alternative system.
2. Peroxidases.
3. Other reactions mentioned were the transformation of ribonucleotides, stereospecific epoxidations, and the oxidation of ketones to esters and lactones (Baeyer–Villiger).

Lyases

1. Synthetically useful enzymes for C–C bond formation (preferably asymmetric) using aldolases and hydroxynitrile lyases.
2. C–N (aminolyases) and C–O (hydratases) bond formations. Find lyases with a broad substrate acceptance.

However, considering the actual research focus, one must conclude, that few research organisations are interested in the search for, and development of, novel enzymes rather than optimizing existing solutions. Table 2 shows that most publications still describe hydrolytic enzymes, and half of them lipases and esterases. On the other hand, only about every tenth paper deals with lyases, an enzyme group for which a requirement has been identified.

Indeed looking at a natural product like Vancomycin, nature shows how it is able to generate C–O bonds by oxidative ring closure to biaryl ether moieties (positions 2, 4, and 6) and the C–C bond of the biphenyl (position 5 and 7) in a efficient way for such a complex structure in comparison to the fantastic total synthesis achievements performed by chemists¹⁹ (Figure 8). Is the biosynthesis of a natural product not a good source of generating research

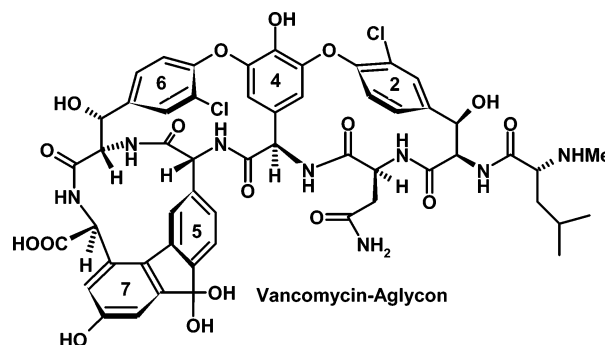


Figure 8.

ideas, as for example looking for strains or enzymes to enable C–C, C–N, or C–O bond formation? If such strains were identified this could bring complementary methodology to, for example, Suzuki–Miyaura coupling or Hartwig–Buchwald amination reactions.

Outlook and Conclusion

There is a growing interest in sustainable solutions for industrial manufacturing to replace nonrenewable fossil fuel based chemical feedstocks in the long term (Figure 9). Biosynthesis and biotransformation techniques will be needed to realise the switch to these new value chains. Biosynthesis will predominantly be used for the production of low value-added products, biotransformation predominantly for the higher value products. Biosynthesis will play the key role in providing the renewable feedstocks, using for example oil- and sugar-based biomaterials as starting materials. There exist several bulk building blocks which can be produced by biosynthesis from renewable biomaterials: examples are ethanol, butanol, succinic acid, maleic acid, glutamic acid, 3-hydroxybutyrolactone, and many others. Of course, biosynthesis remains also a source of high value-added components, such as microbial secondary metabolites, which are naturally validated (by evolution) lead structures for pharmaceuticals.

Highly selective biotransformations on the other hand will chemo-, regio-, or enantioselectively add value to biosynthetically manufactured building blocks.

The prospects for biotransformation are theoretically good, as a number of enabling technologies are available today, and genetic engineering will accelerate the impact of biotransformation on organic chemical synthesis.

Metagenomics. However, nothing can replace the need of finding and identifying new strains or novel enzyme activities. Unfortunately the standard microbiological cultivation and screening methods cannot deliver the wealth of novel strains and enzyme activities needed in an appropriate time frame. A common estimate among microbiologists is that

- (17) *Biotrans 2005 Symposium. The Key to Industrial Biotechnology. Delft July 3–8*; Straathof, A. J. J., van der Lans, R. G. J. M., Franssen, M. C. R., Sheldon, R. A., Eds.; Van Marken Delft Drukkers: Delft, The Netherlands, 2005; ISBN: 90 809691 17
- (18) 6th International Symposium on Biocatalysis and Biotransformation. Olomouc June 28th to July 3rd, 2003, *Chemické listky* **2003**, 97, 325.
- (19) (a) For a review: Nicolaou, K. C.; Boddy, C. N. C.; Bräse, S.; Winssinger, N. *Angew. Chem.* **1999**, *111*, 2230. (b) Stüssmuth, R. D.; Pelzer, S.; Nicholson, G.; Walk, T.; Wohleben, W.; Jung, G. *Angew. Chem.* **1999**, *111*, 2096.

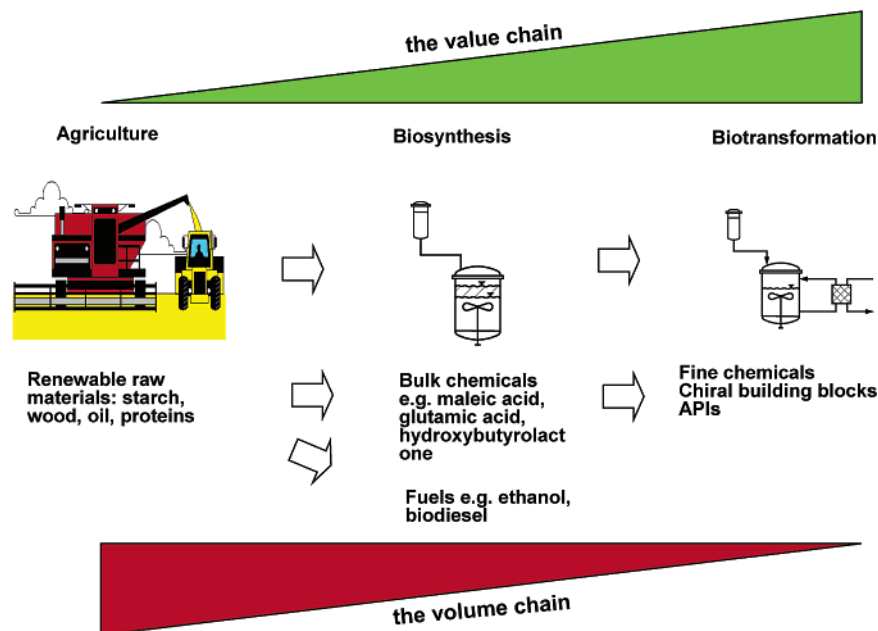


Figure 9. The realisation of sustainable (green) chemistry will be necessary for the future of coming generations. Biotransformation will play an important role in this new product chain, especially for the production of higher value-added fine chemicals.

the vast majority (99%) of prokaryotes has never been investigated in the laboratory and that their characteristics and potential are therefore unknown. Whether these organisms are “unculturable” or whether they just do not grow on the media and culture conditions used, this enormous untapped reservoir of potentially new enzyme activities could not be used. Metagenomic gene discovery is extracting genes directly from nucleic acids gathered in the environment, bypassing tedious microbiological cultivation and product search.²⁰ The potential of this direct “mining” for genes and novel enzymes can only be guessed at. Hitherto inaccessible biospheres will become accessible; an example is the exploitation of the microbial habitat of deep subsurface biospheres.²¹

Enzyme Evolution. While the metagenomic approach is searching for novel enzymes in the natural reservoir, directed evolution is a different approach to reach the same goal. With directed evolution, an existing and known enzyme is going through repeated cycles of variations and screening to finally select an enzyme protein with the desired functions. This is a quite well-established technology, and there are numerous companies worldwide who offer directed evolution, often with proprietary and specific patent-protected techniques. Directed evolution combines techniques such as different genetic engineering methods to create genetic diversity, high-throughput screening, and advanced analytical and computational methods. In contrast to the classical and linear enzyme improvement, directed evolution is a branched process which allows the fast creation of novel enzyme variants for industrial application. As the functional recombinant expression in a suitable microorganism is a prereq-

uisite, this shows also how important it is to have a choice of several microbial host and expression systems.

Cloning of Genes. Once an enzyme has been found and its amino acid sequence, or the nucleic acid sequence of an enzyme protein, analysed, one can make use of the surgical precision of genetic engineering or cloning of genes. The enzyme can be engineered, meaning that genetic variants can be constructed and selected based on new desired properties. The enzymes from the different classes vary greatly in their requirements. As a consequence, a broad range of production systems or hosts is needed. Today, however, the combination of different hosts (bacteria, yeast, and filamentous fungi), expression systems, and plasmids theoretically allows hundreds of different possibilities to express and produce an enzyme.²² This is a great advantage compared with the options available in the days when R. Hinman made his critical comment. This variety of possibilities of cloning of genes and enzyme production potentially allows solutions to be developed, for example, for productivity increase, relief of inhibition phenomenon, or the avoidance of the cultivation of harmful organisms.

Systems Biology. High-throughput experimental techniques and parallel fermentation techniques generate large amounts of data at various levels, which are routinely logged and stored. The development in bioinformatics will allow the study of complex biological systems by a combination of high-throughput experimental techniques with in silico model and experimental design. The integration of wet and in silico experimentation can be used for a new approach for strain improvement. The sequencing of the genomes and corresponding computer programs to search homologies are also a prerequisite, for example, for the above-mentioned

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metagenomics. Systems biology is the attempt to not only integrate and analyze a variety of biochemical information but also actually predict a phenotype based on models which are fed with data from disparate data sets.²³

Analytical Methods. Analytical methods are certainly an area where technology has advanced in great steps and progress has been made. Nevertheless, the single most important impediment to the use of high-throughput screening remains missing analytical methods which allow an on-line and rapid measurement of the enzymatic reaction. There are some methods for hydrolytic enzymes based on colour assays, but many of the other enzyme classes cannot be used in a high-throughput rapid screening, since a simple and rapid assay is not available. The bottleneck of the sophisticated solution is the analytical method.

Conclusion. Progress has also been made in other biotransformation areas which were not discussed here. For example innovative downstream processing such as microwave assisted extractions, supercritical CO₂ or ionic liquids as solvents, zeolites and solvent-free reactions, simulating moving bed technology, in situ product removal, or process integration in general – these process innovations will contribute to the success of biotransformation. But will this be enough? Is the pessimistic statement we cited in the chapter “Why use biocatalysis?” still true?

No. We believe that the future will show the opposite and that process chemistry will be fundamentally changed in the chase for more sustainable synthetic processes. Biotransformation is clearly a valuable technology, as it will reinforce and widen the options available to process research and development chemists in their quest for cost-competitive processes to manufacture key intermediates. However, the statement is unfortunately still valid for the pharma application. Biotransformation should be implemented right from the start during the route selection by the synthetic chemist and during early experimentation. Because of the myriad of structures of organic molecules and the time constraints, large and well characterised strain and enzyme collections must be readily available to the chemist for this purpose. We consider the microbial strain or the enzyme to be the critical functional element in biotransformation for new small molecule pharmaceuticals. The strain is not everything in biotransformation, but biotransformation does not start at all if there is not a good strain to begin with. For these reasons, we need three things: (1) The biotransformation toolbox must be filled with new strains and novel enzymes and enzyme activities. (2) As the challenge is too big to be realised within reasonable time lines, alliances (such as the Swiss Industrial Biocatalysis Consortium) are needed to relieve the shortage of strains. (3) We need an even better integration of chemists and biologists than we already have. Because of its complexity, biotransformation needs a true blending of both sciences. If we succeed we are convinced

that biotransformation in combination with biosynthesis will become one of the main tools in organic chemistry in the future.

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GLOSSARY

API	Active Pharmaceutical Ingredient.
Biocatalyst	Biological agents such as microorganisms or enzymes that activate or speed up a chemical reaction.
Bioconversion	Chemical conversion of a substance using biological methods (enzymes or whole cells, biocatalysis or biotransformation).
Biocatalysis	Chemical conversion of a substance (educt, defined starting material) into a desired product with the aid of a free or immobilized enzyme. The difference between biocatalysis and biotransformation is that biocatalysis uses an isolated enzyme.
Biotransformation	Chemical conversion of a substance (educt, defined starting material) into a desired product with the aid of a (usually) living whole cell, containing the necessary enzyme(s). Unless necessary for the context, we will use only the term biotransformation.
Biosynthesis	De novo production of an entire molecule by a living organism. Unlike biotransformation which acts on a starting substance or educt, biosynthesis is not dependent on educts or starting substances, but only on nutrients.
Building blocks	Compounds which can combine with other compounds to form a new molecule.
Clone	A population of genetically identical microorganisms derived from a single parent cell.
Cloning (of a gene)	<i>Terminus technicus</i> to describe the transfer of genes from one (micro)organism to another. Inserting a population of DNA molecules, known to contain the DNA of interest, into a population of vector DNA molecules in such a way that each vector molecule contains only a single DNA molecule from the original population.
DNA	Acronym for deoxyribonucleic acid, usually 2'-deoxy-5'-ribonucleic acid. DNA contains the genetic information of an organism, and the code in the genes contains the blueprints to form the different proteins.
Educt	Chemical starting compound for a chemical or biological conversion to a product.
Enzyme	Proteins which act as biological catalysts (biocatalysts), initiating all biochemical reactions. Enzymes are classified in six enzyme classes according to their mechanism (see also Table 2).

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Fermentation	Process for the cultivation of microorganisms in special vessels (fermenters) which allow the “monoseptique” propagation of a desired microorganism; only the desired microorganism is allowed to grow using sterile fermentation technology. Thus the result of a fermentation is microbial biomass, which contains the desired enzyme used in the following biotransformation. The biotransformation can take place during fermentation or after the fermentation in a separate vessel which often does not need to be operated in a sterile manner.	NCE	New Chemical Entity
Fine chemicals	Value-added intermediates and active substances used for example in pharmaceuticals.	Product inhibition	Finely tuned enzyme activities are often inhibited at higher concentrations of the resulting product of their catalytic activity. Unlike educt inhibition, which can be overcome using well regulated feeding techniques, product inhibition is more difficult to deal with in biotransformation.
Gene	Basic unit of the hereditary material DNA, an ordered sequence of nucleotide bases that encodes a protein. Genes are the blueprints for the enzymes used in biotransformation.	Secondary metabolite	A product of the secondary metabolism. Secondary metabolites are typically produced in tiny amounts, are very complex (sometimes with “dozens” of chiral centers), and are biologically very active. Secondary metabolites (e.g., vancomycin) are not essential for normal growth, development, or reproduction of a microorganism. On the other hand, the products of the primary metabolism (e.g., ethanol sugar fermentation) are essential for the survival of the microorganism.
Genome	The entire complement of genetic material in a (micro)organism.	Sequence	The order of amino acids in a protein or the order of nucleotides of a gene in DNA. Knowing the sequence allows the cloning, heterologous expression, and production of a protein in different hosts or microorganisms.
Green Chemistry	This term for sustainable (chemical) industrial manufacturing processes striving for, e.g., minimal waste production and energy consumption. ²⁴ Biosynthesis and biotransformation are assumed to play a key role in green chemistry in the future.	Strain	A genetically homogeneous population of (micro-)organisms of common origin. A strain can be biochemically or morphologically differentiated from other strains. Microbial strains are able to produce enzymes with distinctive chemo-, regio-, and enantioselectivity. Another characteristic of microbial strains is that they divide and grow very quickly and are well suited for rapid production of large amounts of enzymes. The growth of microorganisms (see example in photograph) can be exponential.
Host	Microorganism which is used for the expression of foreign DNA, consequently the production of a foreign protein (enzyme), which is encoded in a gene. The expression of a foreign gene for the production of a foreign protein is also called heterologous expression, because the gene does not belong naturally to the producing organism (host).	Strain collection	Microorganisms can be stored for several years in small ampules in frozen form (−80°C) or lyophilized. Individual strains can be revived easily for tests. Thus the larger a collection of different strains, the higher the success rate for a new biotransformation candidate.
Immobilized enzymes	The half-life and stability of enzymes can be prolonged by “associating” enzymes using cross-linking, covalently binding them to, e.g., polymer resins, by encapsulating them.	System biology	(<i>In silico biology</i>) Computational models of biological systems, where varying streams of biochemical information are integrated and modeled <i>in silico</i> . System biology is applied from drug discovery to metabolic pathway simulation.
Metagenomics	(Environmental Genomics) is the study of genomes recovered from environmental samples as opposed to from clonal cultures. This relatively new field of genetic research allows the genomic study of organisms that are not easily cultured in a laboratory.	White biotechnology	A term for industrial biotechnology, which includes a vast area of products, processes, and industries. Biocatalysis/biotransformation is one of many process technologies used in white biotechnology ²⁵ .
Monooxygenases	Cofactor dependent enzymes introducing one atom of atmospheric oxygen into a substrate, while reducing the second one to water. Dependent upon the cofactor on distinguishing for example between flavine nucleotide (FMO) and NAD dependent (P-450) or cytochrome monooxygenases.		
ISPR	In Situ Product Recovery. Removal of the product from the solution during the reaction. By removing the product, which inhibits cellular growth and/or an enzyme activity used for biotransformation, the productivity can be maximized.		

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