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Guidelines for Reporting of Biocatalytic Reactions

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Abstract

Applied biocatalysis is the general term for the transformation of natural and non-natural compounds by enzymes for preparative purposes. Because of this, the term biocatalysis is also used to refer to the application of enzymes in chemistry. There is a steadily rising number of publications reporting the use of biocatalysis. Unfortunately, the value of many of these publications is limited because essential information about the experiments is not presented. Recently, the scientific committee of the European Federation of Biotechnology Section on Applied Biocatalysis (ESAB), taking also inspiration from the STRENDA guidelines, prepared and published guidelines for the correct reporting of experiments in biocatalysis. The present manuscript would like to draw attention to some specific relevant experimental issues, which differentiate applied biocatalysis from fundamental enzymology and deserve particular methodological consideration.

Introduction

A wide variety of complex molecules are accepted by enzymes, including synthetic molecules with structures very different from the substrates found in nature. The surge in practical utilization of biocatalysts is driven by their versatility, regio-, chemo-, and

enantio-selectivity, along with the necessity for the chemical industry to move to environmentally compatible catalysts and processes [1, 2]. Biocatalysis already represents an important tool for the production of fine chemicals and especially pharmaceuticals.

As a discipline, applied biocatalysis touches upon different fields. Overall, the development of a complete biocatalytic process for practical industrial applications involves the contributions of disciplines as diverse as molecular biology, enzymology, microbiology, biotechnology, organic chemistry, materials chemistry and chemical engineering. Gaining a comprehensive and detailed knowledge of all aspects of biocatalyst behaviour is a very difficult proposition.

Recently, the scientific committee of the European Federation of Biotechnology Section on Applied Biocatalysis (ESAB) approved a document, which provides practical and schematic guidelines for reporting experimental data effectively while, most importantly, enabling other scientists to reproduce the experiments. The aim of standardized reporting is also to create value for many other scientific disciplines, especially where biocatalysis must be integrated in multi-step syntheses.

The ESAB guidelines take the STRENDA checklist as a starting point, trying to avoid an unnecessary duplication of standards and checklists in overlapping scientific areas. The resulting document [3] incorporates the STRENDA checklist (http://www.strenda.org/), and includes a list of some items specific to applied biocatalysis and explanations for those who are less familiar with the field.

Therefore, reports of experiments in applied biocatalysis should follow the STRENDA checklist, although not all items on the checklists will need to be specified for every study in biocatalysis, but they rather act to prompt for information that will be required where necessary for the type of experiments reported or that will be useful to supply if available.

Among the topics discussed in the ESAB guidelines, here we would like to put the emphasis on those issues that correspond to essential methodological differences between applied biocatalysis and fundamental enzymology. They can be schematically referred to four major conceptual differences: 1) In applied biocatalysis the emphasis is most often towards the preparative application of enzymes rather than on their full characterization; 2) Since the main priority is to make the enzyme/biocatalyst suitable for preparative applications, the identification of optimal experimental conditions as well as the possibility to make a clear comparison between different sets of conditions are generally of primary importance; 3) Biocatalysts most often are used under ‘‘non-physiological’’ – sometimes extreme – experimental conditions; 4) The audience of applied biocatalysis is multi-disciplinary, constituted not only by enzymologists or biochemists, so that the attention is focused on widely varying experimental issues. While fundamental scientists will be more interested in understanding the catalyst behaviour, synthetic chemists will be more focused on the reproducibility and

efficiency of the synthetic protocol, whereas process engineers will be concerned with the scalability of the process or mass transfer issues. The following paragraphs would like to provide a brief overview on the complexity of the experimental systems which are the object of study in applied biocatalysis, underlining the crucial concepts which must be taken into account in the reporting of experimental data. Most of these concepts have been developed thanks to fundamental researches carried out in the last two decades, and they enabled applied biocatalysis to abandon purely empirical investigative strategies, thus becoming a more rigorous discipline. Nevertheless, it emerges clearly that the complete understanding of enzyme behaviour in such complex systems still represents a formidable task.

Description of Experiments in Applied Biocatalysis: Correct Identification of the Biocatalyst

The development of fermentation processes and biochemical methods specifically aimed at the production of enzymes make it possible to manufacture enzymes as purified, well-characterized preparations even on a large scale. The use of recombinant gene technology has further improved manufacturing processes and enabled the commercialization of enzymes that could previously not be produced on large scale. Furthermore, modern biotechnologies, such as protein engineering and directed evolution, have further revolutionized the development of industrial enzymes. These advances have made it possible to provide tailor-made enzymes displaying new activities and adapted to new process conditions, enabling a further expansion of their industrial use [4, 5].

As mentioned above, most often in applied biocatalysis the attention is focused on the identification of the optimal experimental conditions that allow a biocatalyst to perform an efficient biotransformation for preparative purposes. Therefore, biocatalysts may often be less well characterised than is usual in pure academic enzymology. The only requirement, which is equally important for biocatalysis, is that the catalysts used must be specified unambiguously.

Commercial enzyme products need to be described by citing the manufacturer with name and address, complete product name, code from manufacturer, date of sample or batch number.

As the general industrial aim is to minimize the enzyme purification and operate the process with the crudest form of catalyst possible (usually a lysate), most studies are made with crude or partially purified enzymes. As a matter of fact, many commercial enzymatic preparations contain a quite low percentage of protein (sometimes $< 1\,\%$), whereas a considerable amount of stabilizing agents, mostly polyols and salts, are present. The detailed description of the impurities or additives is of primary importance for the reproducibility of the experiments. It must be underlined that the presence of impurities and additives in the original enzymatic preparation must also affect the immobilization protocols (see below).

Possible interference with enzymatic assays or protein determination procedures must be carefully evaluated. This is especially crucial when different enzymes or experimental conditions are compared in terms of efficiency. It is worth noting that many academic enzyme catalysis studies are carried out using pure enzymes and their results might not always be easily translatable at industrial scale.

Reproducibility of Experiments and Identification of the Biocatalyst: The Case of Immobilized Biocatalysts

Biocatalysts can be used as microbial or plant cells or as isolated enzymes. However, enzymes usable for a given reaction are often hampered by lack of long-term stability under process conditions, and also by difficulties in recovery and recycling. These problems can be overcome by immobilizing the enzymes on solid supports, so that the biocatalysts are used as insoluble particles.

Immoblization may provide the following advantages:

- repeated or continuous use,
- easy separation from the reaction mixture,
- enhanced stability,
- possible modulation of the catalytic properties,
- prevention of protein contamination in the product,
- easier prevention of microbial contaminations.

Since the first uses of biocatalysts in organic synthesis dating back almost a century, researchers have tried to identify methods for linking an enzyme to a carrier [6].

A single broadly applicable method for enzyme immobilization still needs to be discovered. The most frequently used immobilization techniques fall into four categories:

- non-covalent adsorption or deposition,
- covalent attachment (mostly used for isolated enzymes),
- entrapment in a polymeric gel (mostly used for whole cells), membrane or capsule,
- cross-linking of an enzyme.

All these approaches are a compromise between maintaining high catalytic activity while achieving the advantages of immobilization.

Support binding can simply exploit weak hydrophobic and van der Waals interactions, or stronger ones such as ionic. More appropriate for industrial applications is the covalent binding of the enzyme to the support since it has the advantage that the enzyme cannot be leached from the solid support.

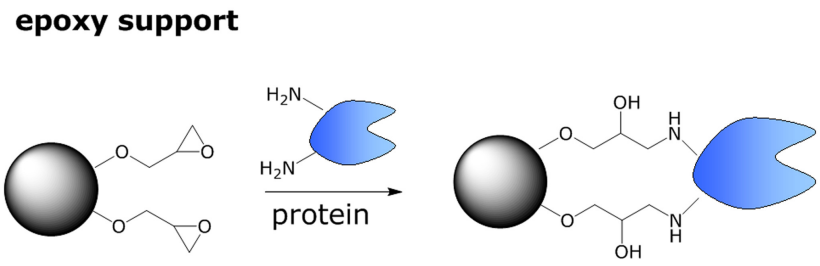

Figure 1. An example of covalent immobilization of enzymes on a functionalized solid support: amino groups of lysine side chains are used for the nucleophilic opening of the epoxy rings present on the surface of the support.

Enzymes can be also entrapped in polymer networks such as an organic polymer or a silica sol-gel, or a membrane device such as hollow fibres or a microcapsule. The physical restraints generally are too weak, however, to prevent enzyme leakage entirely. Hence, additional covalent attachment is often required.

Carrier-free immobilized enzymes are prepared by the cross-linking of enzyme aggregates or crystals, using a bifunctional reagent. This procedure leads to macroparticles, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs). Entrapment is more suited for the immobilization of whole cells. The increasing knowledge of enzyme structures and mechanism has enabled more controlled immobilizations. Information derived from protein sequences, 3D-structures, and reaction mechanism are combined with the properties of carriers (functional groups, hydrophobicity, magnetic properties) and physical/chemical methods in order to develop optimal immobilization strategies on a rational basis. As a matter of fact, a remarkable number of scientific publications describe protocols for developing efficient immobilized enzymes [7]. In these studies, a clear protocol of immobilization with full characterization of the biocatalyst should be reported, by specifying a) amount of support and enzyme used; b) amount of the enzyme that was actually bound to the support, (*e.g.* by measuring the free protein present in solution before and after immobilization); c) the activity of the immobilised preparation; d) the residual water content (to allow an estimation of the activity on a dry weight basis); e) data on the physical-chemical properties of the support (*e.g.* chemical nature, porosity, surface area, size of the particles, type of functional groups present on the surface and their density, when available); f) any further relevant information necessary to reproduce and compare different protocols, such as

the distribution of the enzyme within particles (when feasible) [8] or the diffusion of substrates and products between the pores of the particles and the bulk reaction medium, when mass transfer limitations are relevant.

The use of immobilized enzymes may imply that the enzymatic activity is measured under conditions of strong mass transfer limitation. This must be taken into account particularly in stability studies, since in that case the intrinsic activity of the catalyst can fall substantially with little change in measured activity – because this is still largely limited by the same mass transfer rate [9, 10].

Since the ultimate objective of enzyme immobilization is to obtain a re-cyclable biocatalyst, the evaluation of operational stability (i. e. under the conditions of the useful biocatalytic synthesis) is of major importance. This can be studied in a continuous reactor, or by recycling the catalyst in repeated batches [1]. In the latter case, the treatment (*e. g.* rinsing) of the biocatalyst after recovery can play a crucial role, so these procedures should be specified. Presentation of results must clearly distinguish total time spent under reaction conditions from elapsed time, particularly when the cycle includes an extended storage time between successive batch reactions.

Reproducibility of Experiments Carried out under Non-physiological Conditions: ''Non Conventional Media''

The economic feasibility of a biocatalytic process at industrial level depends on several factors. The usual requirement is to achieve product concentrations comparable to chemical processes, namely at least 50 – 100 g/l. In nature, enzymes usually work at millimolar levels of substrate so that such high concentrations are achievable only thanks to proper process development, as well as protein engineering allowing the enzyme to maintain sufficient activity. Most biocatalytic industrial processes still operate in aqueous environments that generally correspond to low product concentrations because of the poor solubility of most organic molecules in water.

For a long period it was thought that enzymes should be restricted to their natural environment: diluted aqueous reaction media at ambient pressure and temperatures. Indeed industrially enzymes were first employed only for hydrolytic processes. With an increase of the range of enzyme applications the aqueous medium became limiting. These observations led to the introduction of the so called ''non-conventional media'' in biocatalysis. By definition, a non-conventional medium is any system different from a dilute aqueous solution of an enzyme.

Among the ''non conventional systems'' those employing organic solvents are the most widely used and can be classified into three different categories:

- Enzyme suspended in a monophasic organic solution
- Monophasic aqueous/organic solution
- Biphasic aqueous/organic solution.

The first examples of biocatalysis in organic solvents actually date back to before 1900, and in the 1930s Ernest Alexander Sym published ground breaking work on the activity of pancreatic-lipase preparations in organic solvents, finding a correlation between the equilibrium position and the water concentration of the system [11, 12]. However, biocatalysis in organic solvents did not ''take off'' until the 1980s when the application of enzymes in monophasic organic solvents was finally studied in a systematic way [13].

These systems can be obtained by replacing the bulk water by a water immiscible organic solvent and this leads to a suspension of the solid enzyme in a monophasic organic solution. Although the biocatalyst seems to be dry from a macroscopic view, it must have necessary residual bound water to remain catalytically active. As a consequence, these systems are also referred to as ''low-water media''.

Indeed, a ''low-water medium'' can be constituted simply by the neat organic substrates, without the extra addition of solvents. As an example, immobilized lipases suspended in a mixture of triglycerides and alcohols catalyze the transesterification that produces biodiesel (alkylesters of fatty acids) and glycerol [14]. The same immobilized lipases are used also on industrial scale for the synthesis of polyesters *via* polycondensation: the biocatalyst is employed at temperatures above 60 °C and suspended in highly viscous mixture composed of the neat substrate monomers [15].

An essential understanding of the behaviour of enzymes in organic solvent came from the studies on the effect of water activity (a_w), a crucial parameter for determining the correct degree of hydration of enzymes in non-aqueous media [16]. The concept of water activity can be related to the ''available water'' present in the system, namely the water which is ''free'' to react, hydrate other molecules or partition in other phases. The ''availability'' of the water will be lower, for instance, in the presence of polar protic solvents, which are prone to establish hydrogen bonds with water molecules thus reducing their ''freedom''. When a system reaches the equilibrium, the water activity will be the same in all phases. Therefore, the reaction, the enzyme hydration and ultimately the enzyme activity will be affected by the a_w rather than by the overall water concentration in the system.

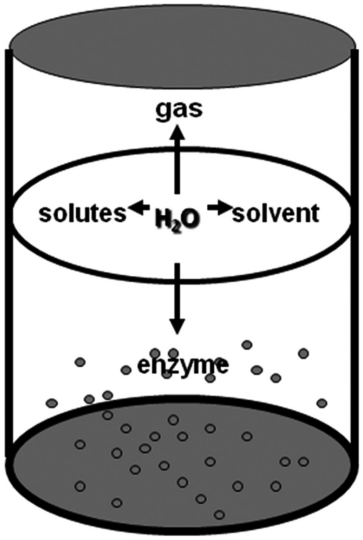

Figure 2. A schematic representation of the distribution of water among phases in a system employing enzymes suspended in mono-phasic organic medium. The hydration of the biocatalyst will depend on the amount of "available water" (i.e. water activity) rather then on the total amount of water present in the system. Since at equilibrium the water activity will be the same in all phases, it can be measured in the most accessible one, generally the gas phase *via* the measurement of vapour pressure of water.

To perform at its best each enzyme requires an optimal degree of hydration that is guaranteed by working at controlled water activity (a_w). Many lipases work efficiently at rather low values of water activities (< 0.2) whereas most other enzymes such as proteases require water activities values above $0.4 - 0.5$.

The water activity can be determined by measuring the vapour pressure in the gas phase of the system which has reached equilibrium. A hygrometer can be used for this purpose, although most of the instruments available on the market are easily damaged by organic solvents. Alternative options are: a) to dry or bring to a defined water content all the reaction components; b) to equilibrate defined parts of the reaction mixture to the desired value of water activity by making use, for instance, of couples of hydrated salts [17]. In general, enzymes work well in relatively non-polar organic solvents ($\log P > 2$), those that do not mix with water and therefore do not compete for the "free water" nor interfere with the hydrogen bonds responsible for the native active conformation of the enzyme.

Monophasic aqueous/organic solutions are usually employed for the transformation of lipophilic substrates which are poorly soluble in an aqueous system and which would therefore react at a low reaction rate. These systems consist of water and a water-miscible organic co-solvent such as dimethyl sulfoxide, dimethyl formamide, tetrahydrofuran, dioxane, acetone or a short chain alcohol. If the proportion of the solvent exceeds a certain threshold (which depends on the enzyme and the co-solvent used), the enzyme undergoes denaturation.

When dealing with these ''non-physiological'' reaction media, some further methodological precautions must be taken in the reporting of experimental conditions. For instance, when the medium is not a dilute aqueous solution, to get a true pH, the electrode needs to be calibrated with pH standards in the same medium (if suitable standards are known). More commonly, the electrode will have been calibrated as normal using dilute aqueous standards, in which case this should be stated clearly and the readings referred to as 'apparent pH'. Sometimes pH will be set or read before the medium is in the final form used for reaction (*e. g.* before heating or adding a co-solvent). Again this must be specified.

During the last decades much attention has been paid, by both academia and industry, to the development of further new solvents, possibly environmentally friendly. Two new classes of solvents are the most cited in the literature: ionic liquids (IL) and supercritical fluids (SF) [18]. Ionic liquids usually consist of an organic cation, often containing a nitrogen heterocycle, and an inorganic anion. First assays of biocatalysed reactions in these unusual media were remarkably successful showing that enzymes not only tolerate these solvents, but, indeed, that they are also stable and the activity is comparable or even better than in organic solvents [19]. However, so far most studies are of an exploratory nature. The relationships between structure of the IL and activity or stability of the enzyme is not yet clearly understood. This is, however, a prerequisite for this methodology to be developed to the full. Similarly to their behaviour in organic solvents, enzymes in ionic liquids require a certain degree of hydration. This should be guaranteed by controlling the water activity (a_w) of the system. This is particularly difficult in the case of the hygroscopic ionic liquids that, depending on the synthetic procedure, the drying process or the storage conditions, can contain very different amounts of water.

Supercritical fluids are materials above their critical temperature, T_c, and critical pressure, P_c. Properties of supercritical fluids lie between the properties of liquids and gases. The densities of supercritical fluids are comparable to those of liquids, while the viscosities are comparable to those of gases. Supercritical fluids are an environmentally friendly alternative to organic solvents as media for biocatalysis because they can be non flammable (e. g. CO_2) and at the end of enzymatic processes, traces of sc-fluids can be removed by depressurisation.

The combination of ILs with supercritical fluids can be a good strategy to circumvent the use of organic solvents to recover solutes. Thanks to the high solubility of supercritical fluids in the ILs, the mass transfer of solutes is increased, and it is possible to couple (bio)transformations in ILs with extraction by supercritical fluids.

The most used SF for biocatalysed processes is supercritical-CO_2, but also other supercritical fluids like supercritical-ethane have been used successfully. In biocatalytic processes, the gas-like viscosity enhances mass transfer rates of reactants to the active sites of enzymes that are dispersed in the supercritical fluid. In this way reactions that are limited by the rates of diffusion, rather than intrinsic kinetics, will proceed faster in supercritical fluids than in normal liquids. A key feature of biocatalysis in supercritical fluids is the tunability of the medium. Small changes in pressure lead to significant changes in density, thus altering all density-dependent solvent properties (dielectric constant, solubility parameter and partition coefficient).

Biocatalyzed Reactions in Multi-phase Systems: Correct Description of the System and Monitoring of the Reactions

It must be underlined that in most cases biocatalyzed reaction systems are constituted by separated phases. This can result, for instance, from the employment of immobilized enzymes, enzymes suspended in low-water media, or from the presence of solid particles of substrates or products. In these cases, obtaining meaningful samples for monitoring reaction progress becomes difficult. Although the removal of immobilized enzymes by filtration or centrifugation is a common procedure generally accepted, the most reliable method consists in terminating and extracting the entire reaction mixture, using separate reaction vessels to explore different time-points, since the behaviour of the reaction mixture as a whole will be perturbed by sample removal [20].

If samples of a multi-phase reaction mixture are withdrawn under vigorous agitation, this always requires careful checks that the samples really are representative, with determination of the relative volumes or masses of different phases by a suitable approach. When a sample of one liquid phase can be removed uncontaminated by others (*e.g.* after briefly stopping agitation), analysis should give reproducible and meaningful concentrations. However, samples removed according to this procedure will not give complete information about the reaction progress, particularly for compounds mainly distributed into other phases. Furthermore, the total volume removed for analysis must account only for a small fraction of the total volume of this liquid phase.

The procedure for mixing the reaction system must also be described in detail, because in multi-phase systems the apparent reaction rates are often determined by mass transfer limitations. In relation to this, the diameter and height of the vessels are also parameters affecting the reaction progress and they must be specified.

Some biocatalyzed methods involve the use of enzymes in multiphase systems and under rather ''extreme'' conditions. An example is enzymatic synthesis carried out in reaction mixtures with mainly undissolved substrates and/or products. In this synthetic strategy the compounds are present mostly as pure solids [21]. Although these reaction mixtures usually consist largely of solids, it has been recognized that a liquid phase is essential for enzymatic activity. In a reaction with two solid substrates, this usually means the addition of a solvent (sometimes referred to as ''adjuvant'') to the mixture. One of the two substrates can be a liquid at the reaction temperature, so that it can then be used as the ''solvent'' to partially dissolve the other substrate. In some cases a liquid phase can be formed from two solid substrates by eutectic melting, when the reaction temperature lies below the melting points of the pure substrates, but above their eutectic temperature. The physical appearance of such reaction mixtures can vary widely depending on the ratio of the different components and on the nature of the liquid phase used. Thus, there are mainly solid systems or dilute suspensions in a large liquid phase in which a product can precipitate because its solubility in the solvent used is extremely low. When product precipitates the reaction yields are improved so that the necessity to use organic solvents to shift the thermodynamic equilibrium toward synthesis is reduced and synthesis is made favourable even in water. Although substrates are usually largely undissolved in such systems, very high conversion yields were observed in many of the reactions studied in the literature. The thermodynamics of these reaction systems have been investigated, resulting in methods to predict the direction of a typical reaction a priori. Furthermore, studies on kinetics, enzyme concentration, pH/temperature effects, mixing and solvent selection have opened new perspectives for the understanding, modelling, optimisation and the possible large scale application of such a strategy [22]. It is clear that several aspects of reaction systems with suspended substrates are significantly different from those in solution. The presence of solid substrates has important consequences for the reaction kinetics and thermodynamics and it requires different strategies for reaction engineering. The majority of the published work on this type of reaction was related to the synthesis of peptides, but the synthesis of beta-lactam antibiotics, glycosides, glycamides, esters and polyesters has also been reported.

Enzymes are able to recognize and transform substrates even when these molecules are anchored on solid supports [23]. When employed for solid phase synthesis, enzymes are generally dissolved in an aqueous buffer and react with the substrate anchored on a water-insoluble resin. The substrate is generally separated from the resin by a chemical linker that must be cleaved in selective and mild conditions at the end of the transformation in order to recover the product.

Figure 3. Enzymes, dissolved in buffer, can accept substrates anchored on solid supports. Enzymes can be used either for chemical transformation of target molecules or for the selective cleavage of linkers.

Examples of applications of enzymes on immobilized substrates are chemo-enzymatic synthesis of compound libraries ''on-bead'', peptide synthesis, screening for enzyme substrates or inhibitors in combinatorial libraries, applications in micro-array technologies and enzymatic optical resolution.

Many hydrolytic enzymes (proteases, esterases, glycosidases and amidases) have also been investigated for their ability to selectively cleave enzyme-scissile linker groups.

Evaluation of libraries of compounds generated by combinatorial chemistry has been appreciated during the last decade as an efficient and rapid approach to synthesise and screen arrays of compounds on a nanoscale. The application of enzymes in combinatorial chemistry has attracted significant attention and enzymatic methods have opened up advantageous alternatives to classical chemical techniques, since enzyme-catalysed transformations often proceed under very mild conditions and are highly selective. The ability of the enzyme to catalyse reactions on solid phase strongly depends on the dimension of the protein compared to the pore size of the resin. The permeability of enzymes into the resin can be improved in two ways: *i*) by creating in the polymer porosities of such dimensions that firstly the enzyme can freely approach the linked substrate and then undergo the conformational modification necessary to recognise and transform it; and *ii*) by inducing enlargement of the cavities inside the resin through efficient solvation and swelling of the polymer in the solvent, that in most cases consists of a buffered aqueous solution, due to the necessity of dissolving the molecules of enzyme.

The use of enzyme in solid phase has found application also in microarray technology. This technology has found a promising route in the use of biocatalysts for the development of highly selective assays under mild operating conditions.

The final example of a ''non-conventional'' multi-phase enzymatic system is represented by solid-gas biocatalysis, a promising technology for the development of new clean industrial processes. The use of enzymes or whole cells at the solid-gas interface offers some very interesting features since total thermodynamic control of the system can easily be achieved

[24]. Solid-gas biocatalysis presents many advantages compared to other systems (mono- or bi-phasic liquids): very high conversion yields compatible with a high production rate at a minimal plant scale, more efficient mass transfer, reduced diffusion limitations due to low gas viscosity and better stability of enzymes and cofactors. Many enzymes have been explored in solid-gas systems such as alcohol oxidase in ethanol oxidation, alcohol dehydrogenase for alcohol and aldehyde production.

Topics of Particular Relevance for Applied Biocatalysis: Specificity and Selectivity

The application of enzymes is particularly valuable in the fine-chemical sector because of their specificity and selectivity. Enzymes may be making a 'distinction' between two or more possible substrates, or between two or more possible reactions on a single substrate. In any statement about specificity or selectivity, it should be made absolutely clear what comparison is being made. Since the words 'selectivity' and 'specificity' are often used with different meanings, it is recommended to use the prefixes 'chemo-', 'regio-' and 'enantio-' because they do have clearly defined meanings, and their use might enable the comparison to be made clear.

Biocatalysts have a pivotal role in the industrial production of enantiomerically enriched chiral fine chemicals. The increasing size and complexity of fine chemicals (agrochemical, pharmaceuticals) along with the development of new materials (*e.g.* liquid crystals and polymers) imply that these molecules frequently contain multiple chiral centres. Moreover, due to the fact that the current USA Food and Drug Administration (FDA) regulations demand proof that the non-therapeutic isomer be nonteratogenic, compounds with a chiral centre are usually manufactured in single isomeric form. As chiral molecules, enzymes may be able to discriminate between a pair of enantiomers, or catalyze a chemical transformation resulting in the introduction of a new chiral centre in an enantiopure form [25].

Generally, the optical purity of a chiral compound is expressed by the enantiomeric excess (ee). For asymmetric syntheses generating a new chiral centre, product ee values will normally be constant as the reaction proceeds, and do directly characterise the biocatalyst [26]. However, in reactions involving the resolution of a racemate, where the two enantiomer substrates are bio-transformed at different rates (i.e. kinetic resolutions) the ee value of substrates and products will vary throughout the progress of the reaction [27]. When an enantio-resolution must be described and the enantioselectivity of an enzyme characterized quantitatively, some parameter expressing the differences of activation energies for the two competing reactions should be used.

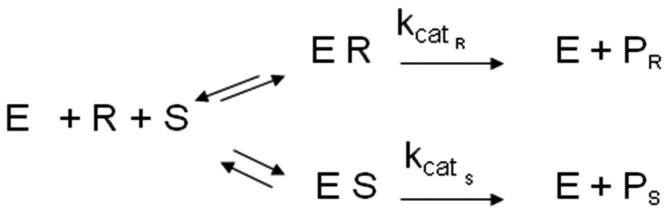

Figure 4. Scheme of the enzymatic resolution of a couple of enantiomers.

In this respect, the enantioselectivity can also refer to a ratio of the specificity constants (k_{cat}/K_m) for the two enantiomers, and can be treated quantitatively, provided it is clear which reactions are being compared [28]. However, since the experimental evaluation of k_{cat}/K_m is quite laborious, generally it is preferred to calculate the "E value", which expresses the ration of specificity constants in terms of relationship between the conversion (c) and the ee of the recovered substrate fraction (ee(S)) or the enantiomeric excess of the product (ee(P)).

$$E = \frac{\ln[(1-c)(1-ee(S))]}{\ln[(1-c)(1+ee(S))]} = \frac{\ln[1-c(1+ee(P))]}{\ln[1-c(1-ee(P))]}$$

Figure 5. Equation used for the experimental calculation of the "E value" (ratio of specificity constants).

Therefore, only the "E value" can sensibly be compared in order to evaluate the performance of the biocatalyst under different conditions [29, 30].

Hydrolases are used in enzymatic resolution because of their ability to preferentially hydrolyse one enantiomer of a racemic substrate, thus providing a means of separation. Hydrolytic enzymes also effectively catalyse enantio-complementary reverse hydrolysis (esterification, transesterification, aminolysis or amidation), providing access to both enantiomers of a desired product. The drawback to the usual strategy of enzymatic resolution is that the desired enantiomer is obtained in a maximal 50% yield, which is too low to allow a positive economic and environmental balance for such transformations. To overcome this limitation different strategies, generally referred to as "deracemization", have been developed that allow the transformation of both enantiomers of a racemate into a single enantiomer of the product. As an example, in situ racemization of substrate combined with kinetic resolution leads to the concept of "dynamic kinetic resolution" (DKR) [31].

Enzymatic reduction of carbonyls is a powerful tool for the production of optically pure chiral alcohols from prochiral compounds. Dehydrogenases can act as asymmetric catalysts and the theoretical yield of a single enantiomer of the chiral alcohol is 100%. Production of chiral alcohols through the asymmetric reduction of prochiral carbonyls has been thoroughly investigated using whole cells of bacteria and yeasts.

Conclusions

The previous paragraphs highlight the outstanding potential of enzymes as catalysts and the advances of applied biocatalysis towards more and more sophisticated experimental systems. This implies that controlling the properties of the biocatalytic system through rigorous experimental procedures is rather challenging.

Fundamental enzymology is expected to provide an essential support to applied biocatalysis by sustaining the necessary methodological evolution of this discipline and thus promoting the full exploitation of enzyme catalytic potential.

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