BIOCATALYTIC PROCESSES

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Summary

Enzymes form an abundant class of very effective and precise (bio)-catalysts that perform and regulate the processes in living matter. Biocatalysis exploits the ability of enzymes to transform also compounds that are not their natural substrates. Biocatalysis represents already an important tool for the production of fine chemicals and especially pharmaceuticals. Biocatalysts can as free or immobilized enzymes as well as microbial or plant cells.

The surge in practical utilization of biocatalysts is driven by their versatility, regio-, chemo-, enantioselectivity along with the necessity of chemical industry of translate to environmentally compatible catalysts and processes.

Novel methodologies for discovering industrial biocatalysts based on new techniques for microbial screening and molecular biology (including directed evolution and metabolic engineering) have led to the production of stable biocatalysts with customized activity and selectivity. Conversely, novel biocatalyst formulation based on innovative immobilization techniques has resulted in improved types of highly stable and efficient biocatalysts.

1. Biocatalysis: Definitions

1.1. Enzymes as Biocatalysts

Chemical transformations carried out by every living organism are enabled by thousands of proteins (enzymes) which have catalytic activity for conversion of a particular set of substrates to specific products. Biocatalysis is the general term for the transformation of natural and non-natural compounds by enzymes. Because of this, the term biocatalysis is also referred to the application of enzymes in chemistry (Bommarius and Riebel, 2004).

Over 3,000 enzymes have so far been identified, and this number will greatly increase thanks to the contribution of genomic and proteomic research. As catalysts, enzymes have remarkable specificities and sometimes phenomenal rate accelerations. A wide array of complex molecules is accepted by enzymes, including synthetic molecules with structures very different forms the substrates found in nature. Biocatalysts are also endowed with selectivity, catalyzing reactions with unique chiral (stereo-) and positional (regio-) selectivities. The basis for the action of all enzymes as chemo-, regio- and stereospecific catalysts lays in their structure. Out of 20 amino acid, 19 of them are enantiopure L-amino acids providing an asymmetric microenvironment for substrate binding and subsequent chemical transformation in the enzyme active site .

These features make biocatalysis attractive as a complementary tool for transformations both in organic chemistry and in industry (Liese et al., 2006)

1.2. Parameters Affecting the Efficiency of Biocatalysts

Enzymes are able to accelerate the rate of some reactions by a factor of over 1000, as, for instance, in the protease- catalyzed hydrolysis of peptide bonds. Enzymes accelerate reactions by lowering the free energy of the transition state of a given reaction. Firstly the enzyme recognizes the substrate and forms an enzyme-substrate complex (ES), which is then converted into the product (P) (Figure 1).

Several parameters affect the practicality of an enzymatic reaction. Of particular importance are the specific activity (quantified by k_{cat}), specificity (determined by k_{cat} / K_M). In addition, the degree of inhibition by substrate or product (often determined by their affinity to the enzyme, K_M and K_P) may be particularly important in the outcome of a reaction. Ideally, the enzyme should have high specific activity and stability, and should undergo minimal substrate and product inhibition. Furthermore, the extent of substrate specificity can determine whether a given enzyme will have general

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synthetic utility. Although enzymes with narrow substrate specificity are often efficient in catalyzing reactions using their natural substrate, this property becomes a limitation when developing catalysts for general purposes (Koeller and Wong, 2001).

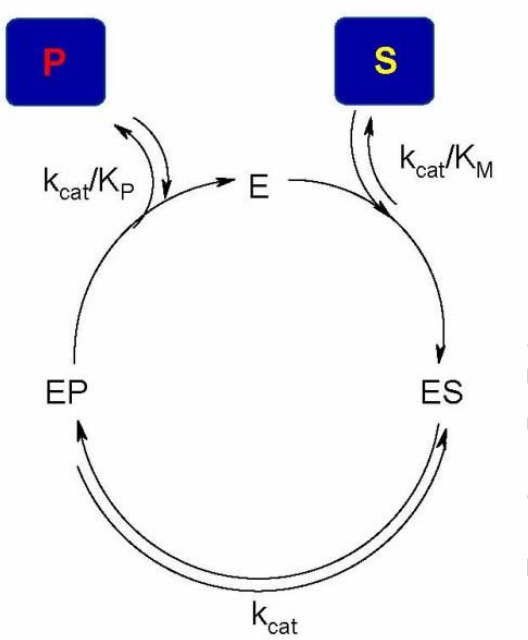

Figure 1. Parameters affecting enzymatic transformations

Finally, the stability of enzymes to environmental factors represents a further parameter of fundamental importance for the practical applications of biocatalysts.

2. Traditional and Modern Applications of Enzymes and Biocatalysts

Enzymes found in nature have been used since ancient times in the production of food products, such as cheese, sourdough, beer, wine and vinegar, and in the manufacture of commodities such as leather, indigo and linen. All of these processes relied on either enzymes produced by cells of spontaneously growing microorganisms or produced by plant and animal cells present in added preparations such as rumen of calf or papaya fruit. The development of fermentation processes and biochemical methods specifically aimed at the production of enzymes made it possible to manufacture enzymes as purified, well-characterized preparations even on a large scale. This development allowed the introduction of enzymes into true industrial products and processes, for example in the formulation of detergents and in textile and starch industries. 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 (see Section 7.3). 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.

Table 1 (adapted from F. Hasan et al.) illustrates the estimated value of the worldwide use of industrial enzymes and the market segmentation. The technical industries,

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dominated by the detergent, starch, textile and fuel alcohol industries, account for the major consumption of industrial enzymes.

	2002	2003	2004	2009
Technical enzymes	978.2	1009.2	1040.0	1222.0
Food enzyme	701.0	720.0	740.0	863.0
Animal feed enzyme	210.8	215.6	220.0	267.0
Total	1890.0	1945.0	2000.0	2352.0

Table 1. Global enzyme markets by application sectors, through 2009 ($ millions). Adapted from F. Hasan et al. / Enzyme and Microbial Technology 39 (2006) 235–251

Table 2 reports the more important applications of industrial enzymes. Most of the industrial enzymes are hydrolases and are used for the degradation of various natural substances (http://www.amano-enzyme.co.jp/english/index.html). Proteases are traditionally among the most widely employed enzymes, because of their extensive use in the detergent and dairy industries. Glycosidases (amylases and cellulases) are largely used in the textile, detergent and baking industries; in the last few years the production of amylases and cellulases has dramatically improved because of their use in the bioethanol industries as catalysts for the saccharification of starch and cellulose.

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipases	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch	Amylase	Starch liquefaction and saccharification
	Cellulase	Cellulose saccharification
	Xylanase	Viscosity reduction
	Pullulanase	Saccharification
	Glucosidase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor

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	Lactase (β-galactosidase)	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase and phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Leather	Protease	Unhearing, bating

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Personal care	Lipase	De-pickling
	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

Table 2. Examples of applications of enzymes in industries different from chemical and pharmaceutical (adapted from http://www.novozymes.com)

3. Advantages and Drawbacks Related to the Use of Biocatalysts in Chemistry

Many enzymes have been found to catalyze a variety of reactions that can be very different from the reaction and substrate with which the enzyme is associated in nature.

In many cases biocatalysts respond to the needs of technological solutions coming from the transformation industry, as many classical chemical transformation processes have inherent drawbacks from a commercial and environmental point of view. As a matter of fact, non-specific reactions may result in poor product yields. High temperatures and/or pressures needed to drive reactions leads to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures acidity or alkalinity need high capital investment and specially designed equipment and control systems. Unwanted by-products may prove difficult or costly to dispose of. High chemical and energy consumption as well as harmful by-products have a negative impact on the environment.

Compared with traditional methods, biocatalysis often offers a number of advantages such as:

- High stereo-, regio- and chemoselectivity
- Decreased requirements of tedious protection and de-protection schemes
- Lower incidence of by-products
- Mild reaction conditions
- Efficient catalysis of both simple and complex transformations
- Uncomplicated and cheap refining and purification
- Reduced impact of manufacturing on the environment by reducing the consumption of chemicals and energy, and the subsequent production of waste.

Finally, it must be underlined that only small amounts of enzymes are required to carry out chemical reactions even on an industrial scale.

Most of these advantages are potential; for example stereoselectivity is not often guaranteed, especially when synthetic substrates are employed.

In order to judge the environmental impact of a process, the *E* factor can be used (Margreth et al., 2001), which is the ratio between the mass of waste material and the mass of desired products.

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$$E\text{-}Factor = \frac{Mass\ of\ \ waste\ material}{Mass\ of\ \ products}$$

The *E* factor allows to compare processes and designate the amount of by-products (e.g. solvent losses, acids and bases used in work-up, process aids, waste from energy production), produced per kg of product. In this context, waste will include any reaction product that does not have any further use, and also reagents and solvents used during the course of manufacture that are not re-used or recycled. The *E*-factors vary enormously between the different sectors of the chemical industry as reported in Table 2.4: It increases substantially from bulk to fine chemicals and specialties. This is partly due to the fact that the production of fine chemicals generally involves multi-step syntheses and partly to the widespread use of stoichiometric rather than catalytic reagents, as in the case of biocatalysis.

Industry	**Product tonnage**	**Kg by-product / kg product**
Oil refining	10^6- 10^8	<<0.1
Bulk chemical	10^4- 10^6	< 1-5
Fine chemical	10^2- 10^4	5-50
Pharmaceuticals	10- 10^3	25->100

Table 3. Relation between the *E* factor and sectors of industry (Adapted from Wegman et al.).

A "good" *E*-factor would typically be around 0.1 so that 10 kg of desired product produces 1 kg of waste and by-product. At the other extreme, in pharmaceutical manufacturing when a high-purity is essential, the E-factor can be as high as 100, meaning that 1 kg of product produces 100 kg of waste. These figures indicate that new, more efficient synthetic methodologies for the production of pharmaceuticals are urgently required. As an example, the translation of the productive processes of β-lactam antibiotics to biocatalysis allowed a 5-fold reduction of the corresponding E-factor (see Section 12.2).

Besides the above mentioned advantages, some drawbacks coming form the application of biocatalysis in chemistry must be recognized, such as:

- Biocatalysts often show lower stability than conventional catalyst
- Development of industrial biocatalytic processes are usually much longer to establish
- Low number of commercially available biocatalysts
- Necessity of microbiological facilities if the biocatalyst is not a commercial enzyme

Most of these disadvantages might be overcome by modern techniques (screening, molecular biology, protein engineering, immobilization) able to furnish a much higher number of biocatalysts with improved performances.

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Relevant web-sites

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http://www.brenda-enzymes.info [A comprehensive enzyme information system with structural and functional data]
http://www.novozymes.com [The web-site of the major enzyme producer (Novozymes, Denmark), providing information on enzyme industrial applications]
http://www.amano-enzyme.co.jp/english/index.html [The web site of the major Japanese enzyme producer, with commercial and technical information on biocatalysts]

Biographical Sketches

Lucia Gardossi was born in Trieste, Italy in 1963 and graduated in Pharmaceutical Chemistry and Technology in 1988 at the University of Trieste. Since the beginning of her scientific activity she has always investigated the application of computational chemistry to the understanding and development of novel biocatalyzed systems. She got the PhD in Medicinal Chemistry at the University of Trieste working on enzymatic modification of peptides. From 1989 to 1991 she worked at the Massachusetts Institute of Technology (Cambridge, USA) in the group of Prof. A.M. Klibanov studying the use of enzymes in non-conventional media. From 1993 to 1995 she studied enzymatic modification of charbohydrates at POLY-TECH Soc. coop. (Trieste- Italy.

Currently she is associate professor of organic chemistry at the Faculty of Pharmacy of the University of Trieste. She is author of more than 50 publications in the field of biocatalysis and 3 patents. She is Italian delegate inside the European Section of Applied Biocatalysis (ESAB) of the European Federation of Biotechnology (EFB). She is member of the scientific board of the Italian Technology Platform on Sustainable Chemistry and of the Italian Association of Biocatalysis and Bioseparations (AIBB).

Her current research activity is focused on different aspects of biocatalysis: enzymes in non-conventional media, computational methods for the prediction of enzyme selectivity/stability, immobilization of enzymes, chemo-enzymatic synthesis on solid phase, development of novel supports for solid phase synthesis and for enzyme immobilization, modeling of thermodynamics of bio catalyzed reactions.

Francesco Molinari was born in 1961 in Milano, Italy. He studied chemistry and graduated from the University of Milano, Italy with a Ph.D. thesis about "Supramolecolar Chemistry: some applications to covalent bond cleavage and formation" in 1992 under the supervision of Prof. Cesare Gennari. During these years his fields of interest have been organic synthesis applied to the problems of molecular recognition, bifunctional catalysis and stereoselective carbon-carbon formation. He was a postdoctoral fellow at the Instituto Tecnico of Lisbon working on extractive bioconversions. He began his independent career at the Industrial Microbiology Section of the Department of Food Science and Microbiology, University of Milano in 1992. Since 2000 he has been Professor of Biotechnology and Chemistry of Fermentations at the University of Milano. Professor Molinari‘s research interests include (stereo)-selective biotransformations, production and isolation of microbial secondary metabolites and wine fermentations.

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