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 immobilisation techniques have 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, catalysing 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 of enzymes.
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-catalysed 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).

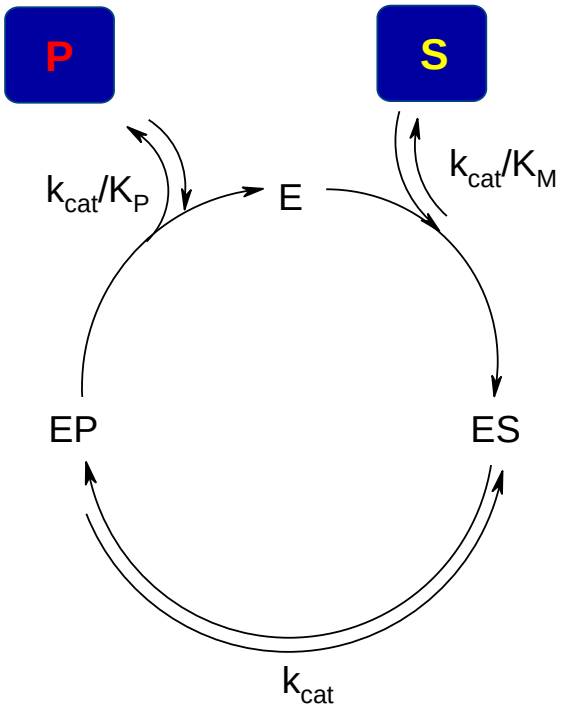


Figure 1: Parameters affecting enzymatic transformations

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 synthetic utility. Although enzymes with narrow substrate specificity are often efficient in catalysing reactions using their natural substrate, this property becomes a limitation when developing catalysts for general purposes (Koeller and Wong, 2001).
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, dominated by the detergent, starch, textile and fuel alcohol industries, account for the major consumption of industrial enzymes.

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

	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 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.

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

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

3. Advantages and drawbacks related to the use of biocatalysts in chemistry

Many enzymes have been found to catalyse 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.

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

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 specialities. 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.

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

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

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.

4. Classification of enzymes used in organic synthesis.

Table 4 presents a list of the enzymes most commonly used in organic synthesis, stemming on their classification into six classes that account for the different type of reaction they catalyze (http://www.brenda-enzymes.info).

Table 4: Enzymatic classes and some examples of application in organic synthes

Class	**Enzyme**	**Common reaction**
1. Oxidoreductases	Dehydrogenases	Oxidation of alcohols and aldehydes, reduction of aldehydes and ketones; oxidation of C-C single bonds, reduction of C-C double bonds
	Oxidases	Oxidation of alcohols and amines
	Mono- and dioxygenases	Hydroxylation, sulphoxidation, epoxidation, Baeyer-Villiger oxidation, dihydroxylation)
	Peroxidases	Oxidation, epoxidation, halohydration
2. Transferases	Kinases	Phosphorylation (ATP-dependent)
	Sulphotransferases	Formation of sulphate esters

	Glycosyltransferases	Glycosidic bond formation
	Transketolases	Ketol (α-hydroxyketones) group transfer
3. Hydrolases	Esterase, lipases	Ester hydrolysis / synthesis
	Amidohydrolases (amidases or acylases)	Amide hydrolysis / synthesis
	Proteases	Peptide bond hydrolysis / synthesis
	Glycosidases	Glycosidic bond formation/hydrolysis
	Nitrilase (nitrile aminohydrolase)	Hydrolysis of nitrile to carboxylate
	Epoxide hydrolases	Hydrolysis of epoxides
	Phosphatases	Hydrolysis of phosphate esters
	Dehalogenases	C-halide hydrolysis
4. Lyases	Aldolases	Aldol reaction (C–C bond)
	Oxynitrilase	Cyanohydrine formation
5. Isomerases	Glucose isomerase	Isomerisation of carbohydrates,
	Mandelate racemase	Racemisation
6. Ligases		Not used at present for practical applications

A number of synthetically practical enzymatic reactions require expensive organic coenzymes (e.g. oxidoreductases and aldolases), which precludes their use as stoichiometric (non-regenerated) reagents. Thus, regeneration of the cofactor (see paragraph on oxidoreductases) is necessary for the process to be economically and industrially feasible. Some coenzymes/cofactors are tightly bound to their respective enzymes and, consequently, external recycling is not required. This is, for instance, the case of enzymes requiring coordinated metals as cofactors.

Table 5: Main coenzymes present in enzymes used in biocatalysis

Coenzyme	**Reaction type**
Flavines	Oxygenation
Thiamine pyrophosphat	Decarboxylation, transchetolization
$NAD(P)^+$ /NAD(P)H	Hydrogenation/dehydrogenation
$NAD(P)^+$ /NAD(P)H	Oxygenation
ATP	Phosphorylation
Pyridoxal- phosphate	Modification of aminoacids
Metal-phorphyrin complexes	Peroxidation, oxygenation

When required, coenzyme regeneration can be also synthetically advantageous, as it drives the reaction to completion, prevents the accumulation of inhibitory cofactor by-products, simplifies the reaction work-up, and increases enantioselectivity. Several cofactors can be recycled effectively, including nucleoside triphosphates such as ATP in phosphoryl transfer reactions, nicotinamide

adenine dinucleotide and its phosphate (NAD^+/NADH and $NADP^+$/NADPH) in oxidoreductions, acetylCoA in acyl transfer reactions, and sugar nucleotides in glycosyl transfer reactions. Many cofactor dependent reactions have been applied on preparative or industrial scales.
Nevertheless, hydrolases still remain the most widely employed enzymes due to their large availability and to the fact that they do not need organic coenzymes.

5. Enzymes most commonly employed in organic synthesis

5.1 Lipases and phospholipases (hydrolases)

The reasons for the enormous biotechnological potential of microbial lipases include the facts that they are stable in organic solvents, (see section 9.1) do not require organic cofactors, possess a broad substrate specificity and exhibit a high enantioselectivity in the hydrolysis/formation of *sec*-alcohols esters. Moreover, a number of lipases are commercially available, with the majority of them originating from bacteria (i.e. from different *Bacillus* and *Pseudomonas* species), yeasts (i.e. from *Candida* species) and moulds (i.e. *Aspergillus* and *Rhizopus* species) (Hasan et al., 2006).
Lipases belong to a large class of enzymes that hydrolyse the ester bond between the fatty-acyl side chains and the lipid backbone. Understanding the catalytic cycle of lipases has been of significant importance to their widespread use in different biotechnological applications. The lipase active site is composed of three different residues: serine, hystidine and aspartate or glutamate and similar motifs can be found in other classes of hydrolases, as, for instance, some proteases.
Figure 2 illustrates the mechanism of the hydrolysis of an ester.

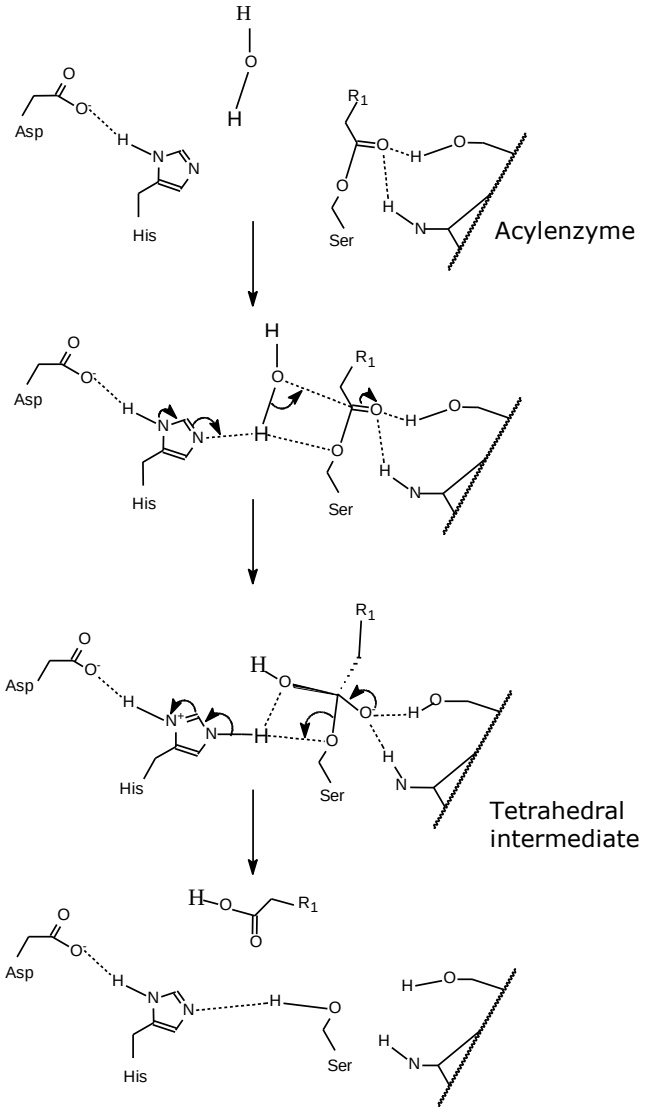


Figure 2: A schematic representation of the mechanism of the lipase catalyzed hydrolysis of an ester.

The catalytic cycle starts by nucleophilic attack of the hydroxyl group of the side chain of a serine (Ser) on the carbonyl carbon atom of the ester bond, with the consequent formation of the acylenzyme. The complex undergoes the nucleophilic attack of water. A tetrahedral intermediate is thus formed, which then liberates the fatty acid and the regenerated enzyme. As appears from Figure 2, residues of aspartic acid (Asp) and histidine (His) contribute to the mechanism by acting as acid and base catalysts.

As lipases are active in organic solvents, water can be replaced by other nucleophiles such as alcohols or amines. The result of this reaction is a transesterification and aminolysis respectively. Lipolytic reactions occur at the lipid–water interface, implying that the kinetics cannot be described by Michaelis–Menten equations, as these are valid only if the catalytic reaction takes place in one homogenous phase.

Lipases, in contrast to esterases, were therefore defined as carboxylesterases acting on emulsified substrates and the activity of lipases is enhanced towards insoluble substrates that form an emulsion. This phenomenon is generally defined 'interfacial activation'. The determination of 3D structures of several lipases indicated that the active site is covered by a surface loop, which was called the lid. Upon binding to the interface, this lid moves away, turning the 'closed' form of the enzyme into an 'open' form, with the active site now accessible to the solvent. At the same time, a

large hydrophobic surface is exposed, which facilitates the approach and biding of the hydrophobic substrate.

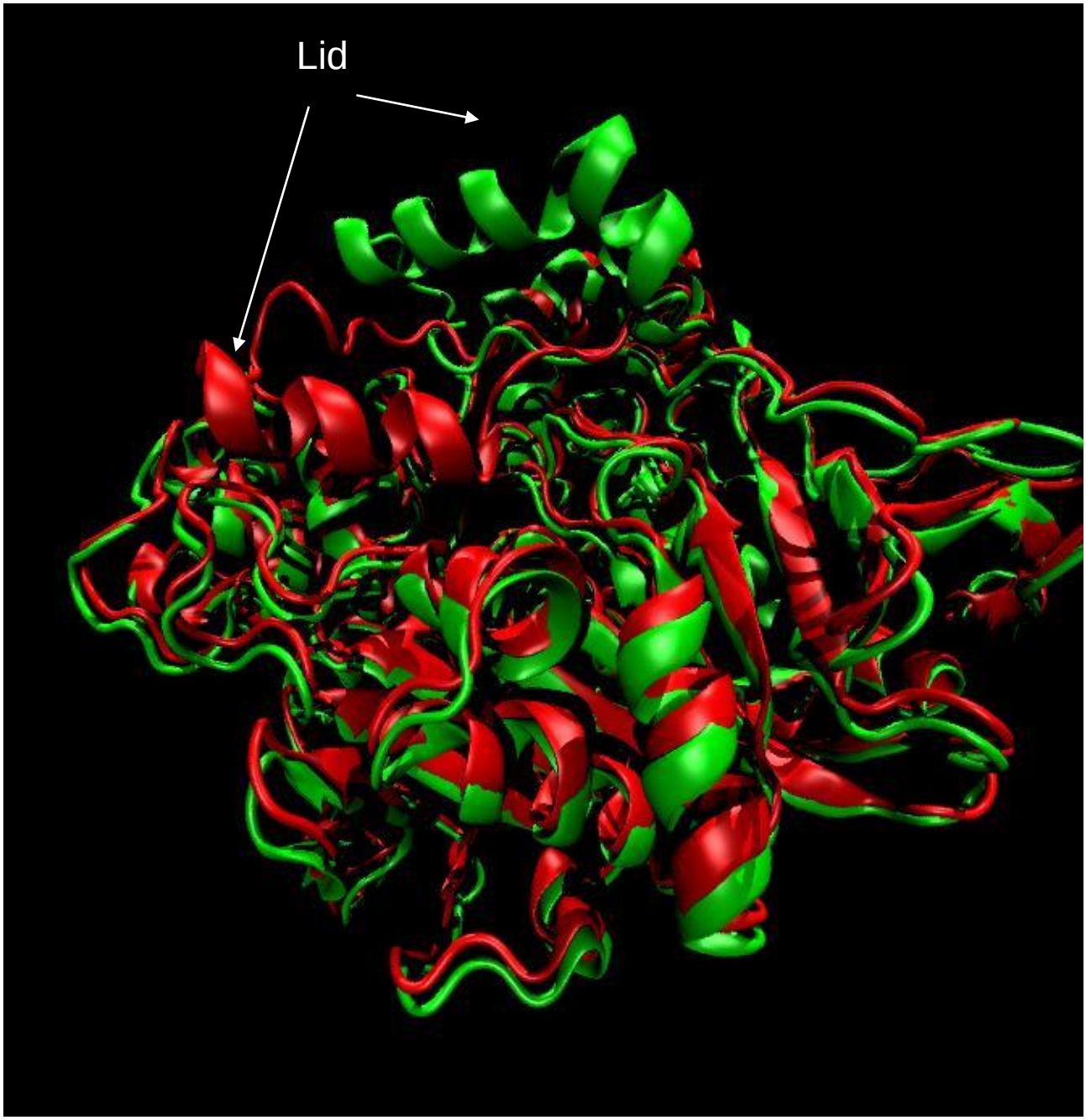


Figure 3: Molecular simulation of the superimposition of the two different forms of the lipase from *Candida rugosa*. In the open form (green) the lid moves upward, thus leaving the active site freely accessible whereas in the closed form (red) the lid covers the active site (*corteously by Valerio Ferrario, University of Trieste*).

Phospolipases, which modify phospholipids in natural membranes, are available from different sources for application in biocatalysis. All sites of phospholipids can be selectively modified by enzymatic catalysis. By coupling the phospholipase action with well-established chemical

transformations, natural and unnatural phospholipids can be prepared. Phospholipids find applications as pharmaceuticals, food additives, cosmetics, in liposome technology and in gene transfer therapy.

5.2 Proteases (hydrolases)

Proteases and peptidases constitute one of the most important groups of industrial enzymes, accounting for at least a quarter of the total global enzyme production sales and they are used in detergent, protein, brewing, meat, photographic, leather and pharmaceutical industry (Table 2). These enzymes act both extracellularly (e.g. in the intestine of animals) and intracellularly.

Proteases can be distinguished on the basis of their mechanism in serine-proteases, cysteine-proteases, aspartic-proteases, metallo-proteases. Generally, the four classes of peptidases differ in the groups that perform nucleophilic attack, general base catalysis and electrophilic assistance.

The serine proteases is the class of proteases most widely studied. Similarly to lipases (Figure 2), they have a reactive serine (Ser) residue and the hydrolysis of a peptide substrate involves an acyl-enzyme intermediate in which the hydroxyl group of Ser is acylated by the acyl moiety of the substrate releasing the amine fragment of the substrate as the first product.

The acyl-enzyme thus formed will be the same for a series of substrates which differ in their leaving group. The attack of Ser on the peptide bond of the substrate forms a high energy tetrahedral intermediate. Finally, a water molecule binds to the carbon, once again forming a tetrahedral intermediate which collapses to expel Ser and the remaining fragment of the substrate.

Although proteases hydrolyse peptide bonds *in vivo*, these enzymes can be induced to catalyze esters and amide bonds under specific conditions *in vitro* (Lombard et al., 2005). However, it is not possible to accomplish peptide bond formation by simple reversal of hydrolysis, even using high concentrations of amino acid as long as they are present as zwitterions. Therefore there are two basic strategies to approach peptidase-catalyzed peptide bond formation: *i*) thermodynamic controlled synthesis, in which the carboxyl component bears a free carboxyl group and *ii*) kinetically controlled synthesis, in which the carboxyl group is employed in an activated form.

In the thermodynamic controlled synthesis the final yield of the reaction will be determined and limited by the position of the thermodynamic equilibrium in the particular reaction conditions used in the experiments.

In the “kinetic” approach (aminolysis of esters) two nucleophiles (water and an activate substrate, e.g. amino acid ester) are competing for the acyl-enzyme intermediate.

Engineered proteases for protein synthesis provide facile access to proteins that contain unnatural amino acids or functionality, yielding new structures that cannot be obtained through normal biosynthetic pathways. Proteases can be used in non aqueous media, although most of them require to maintain a degree of hydration which is generally higher as compared to lipases (see section 9.1).

5.2 Glycosidases (hydrolases)

Glycosyl hydrolases (glycosidases) are a wide group of asymmetric biocatalysts widespread in nature. Some of them have been also extensively used for practical purposes. The enzymatic hydrolysis of poli- and oligosaccharides is not only mild and regioselective, but also highly enantioselective. The hydrolysis of glycosides occurs by retaining or inverting the stereocenter involved in the hydrolysis, but always with high stereoselectivity (Davies and Henrissat, 1995). Figure 4 outlines the mechanism: the glycosidic oxygen is protonated by the acid catalyst (i.e. the carboxylic function of a glutamic residue occurring on the glycosidase) and nucleophilic assistance to the departing aglycone is provided by a base (i.e. the charged carboxylate function of an aspartic residue); the resulting glycoside-enzyme is finally hydrolysed by water generating a stereocenter with the same configuration.

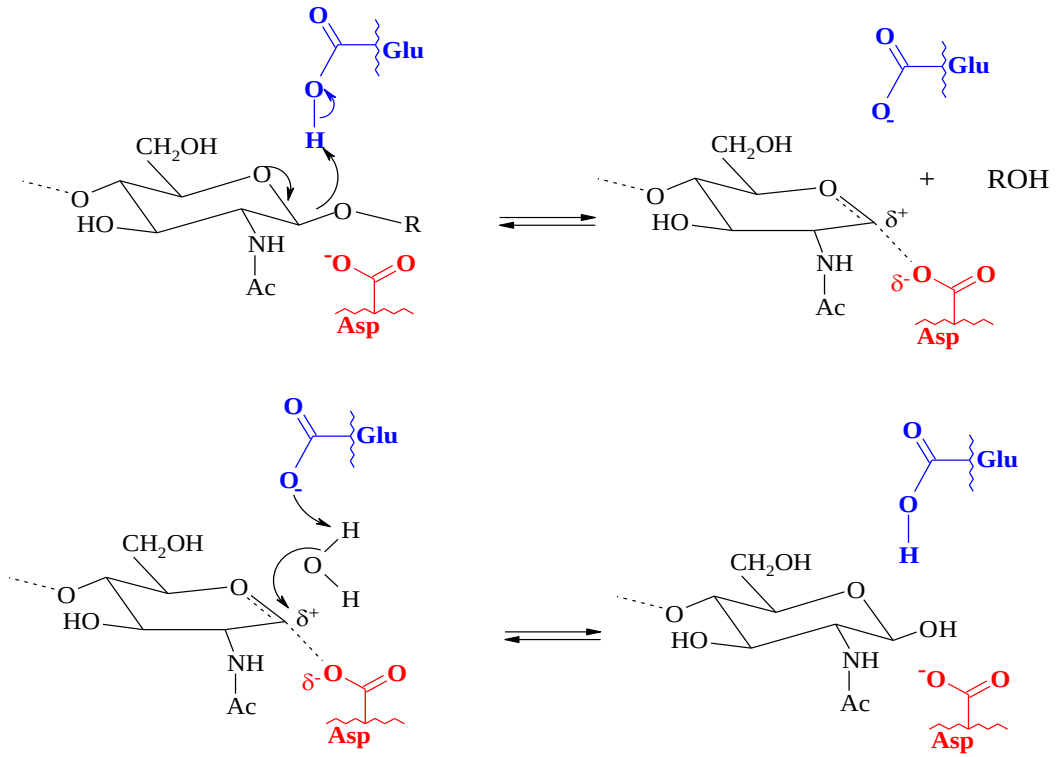


Figure 4: General mechanism of glycosidase with retention of the stereocenter involved in the hydrolysis.

Glycosidases normally cleave glycosidic linkages in oligosaccharide chain, but can be used as synthetic catalysts under kinetically controlled conditions (see section 5.2). Unlike glycosyltransferases, which also catalyze the formation of glycosidic linkages, glycosidases do not require co-factors.

The hydrolysis of starch and cellulose are among the most important biotransformations; a mixture of glycosidases (α- and β-amylases, glucosidases, glucoamylases) is used to convert starch into glucose, maltose, maltodextrins and low- molecular weight dextrins.

Enzymatic hydrolysis of starch is used in the fermentation industry for making available more easily fermentable sugars and for the production of glucose, mostly used as substrate for the production of high fructose corn syrup (HFCS). In this process the glucose is converted to the sweeter fructose by enzymatic isomerization.

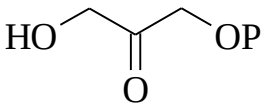
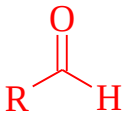
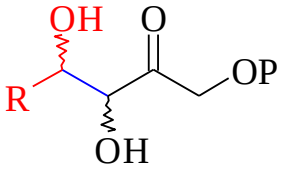
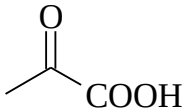
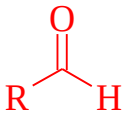
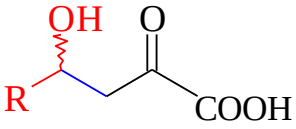
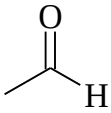
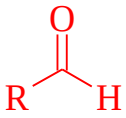
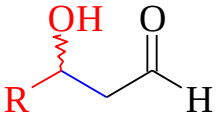
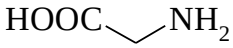
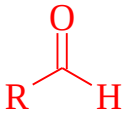
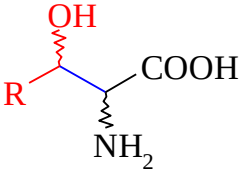
5.4. Enzymes catalyzing carbon–carbon bond formation

The construction of C–C bonds with complete stereochemical control is of utmost importance in organic synthesis. Aldolases are enzymes able to catalyze reverisble aldol condensation with high degree of stereoselectivity. These enzymes are ubiquitous in the microbial and plant world, but unfortuantely the range of substrates accepted by the aldolases known until now is quite restricted.

Aldolases generally controls configuration of newly formed stereogenic centres and are also highly specific for the donor substrate (that is, the nucleophilic enolate) but relatively flexible with respect to the acceptor (electrophilic) group (Dean et al., 2007).

Table 6: Examples of stereoselective aldol condensations catalyzed by aldolases..

Donor (nucleophile)	Acceptor (electrophile)	Product	Aldolase

			Dihydroxyacetone aldolase
			Pyruvate aldolase
			2-Deoxyribose-5-phosphate aldolase
			L-threonin aldolase

By choosing the suitable acceptor substrate numerous carbohydrates and their analogues can be prepared. These molecules have further served as intermediates in the synthesis of complex bioactive molecules, such as inhibitors of glycosyltransferase and glycosidase.
Other synthetically useful enzymes catalysing C–C bond formation include transaldolases, transketolases, cyanohydrin synthetases (also called oxynitrilase).
Although carbon-carbon bond formations are pivotal processes in organic chemistry, on an industrial scale relatively few enzymatic processes are known. The formation of enantiopure (S)-meta-phenoxybenzaldehyde cyanohydrin using the recombinant (S)-hydroxynitrile lyase (HNL) from the rubber tree Hevea brasiliensis has been implemented on an industrial scale for the synthesis of pyrethroid insecticides. One of the oldest examples of enzyme-catalyzed C-C bond formation is the synthesis of enantiopure hydroxynitriles using (R)-HNL from almonds (*Prunus amygdalus*).
Decarboxylases can also be used for C-C formation. One of the classical and older examples of stereoslective biocatalysis concerns the production of (*R*)-phenylacetylcarbinol (PAC), employed for the synthesis of ephedrine and pseudoephedrine. The reaction takes place by simply adding benzaldehyde to fermenting *Saccharomyces cerevisiae*.

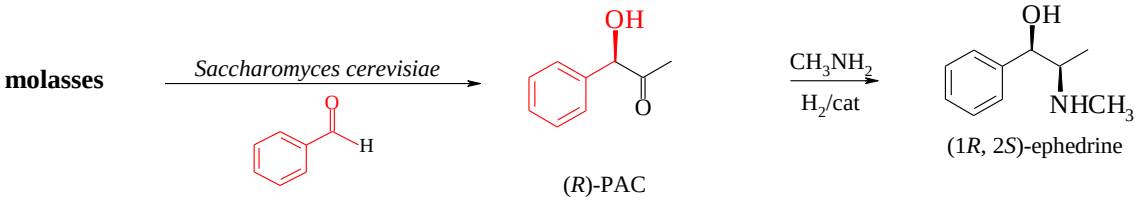


Figure 5: Chemo-enzymatic synthesis of ephedrine

The bioconversion occurs with complete enantioselectivity and can be performed with other microorganisms having a high expression of pyruvate decarboxylase (PDC), such as bacteria (i.e. *Zymomonas mobilis*), yeasts (i.e. *Candida utilis* and *Torulopsis glabrata*) and moulds (i.e. *Rhizopus javanicus*).

5.5 Oxidoreductases

Enzymes employed in redox reactions are classified into three categories: dehydrogenases, oxidases and oxygenases (mono- and di-oxygenases) .

Table 7: Oxidoreductases and the role of oxygen in their mechanism.

Enzyme	**Role of O_2**
Dehydrogenase	Oxygen is not directly involved in the enzymatic activity. Electron donors and acceptors are organic substrates
Oxidase	O_2 is the electron acceptor being reduce to H_2O_2
Monooxygenase	O_2 is the electron acceptor: one oxygen atom is introduced into an organic substrate, while the second one is used to H_2O
Diooxygenase	O_2 is the electron acceptor and both the oxygen atoms are introduced into an organic substrate

Dehydrogenases oxidize mostly alcohols (primary and secondary) (Molinari, 2006) and C-C single bonds by removing hydrogen, while they reduce (dehydrogenate) mostly carbonyls, C-C double bonds and C-N double bonds (Figure 6) (Nakamura and Matsuda, 2006).

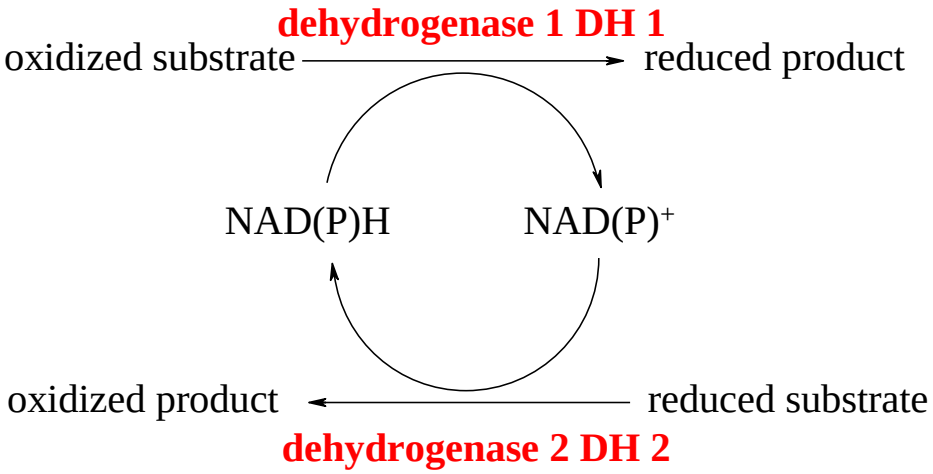


Figure 6: A schematic representation of the reductive and oxidative actions of dehydrogenases with the involvement of NAD(P)H cofactors.

A broad range of prochiral ketones can be reduced stereoselectively using dehydrogenases to give chiral secondary alcohols. The interconversion of a ketone to the corresponding alcohol and vice versa represents one of the most common redox-reactions in organic chemistry. Biotransformations offer some significant advantages as compared to traditional synthetic methods that predominantly use toxic metals and expensive complex hydrides, The vast majority of dehydrogenases and reductases used for ketone reduction and alcohol oxidation require nicotinamide cofactors, such as NADH and NADPH. To improve the efficiency and applicability of biocatalytic redox-reactions for asymmetric ketone-reduction and enantioselective alcohol-oxidation catalyzed by nicotinamidedependent dehydrogenases/reductases, several achievements for cofactor-recycling have been made during the last years. Because of the high price of commercial alcohol dehydrogenases and their cofactors, cheap alternatives, such as whole cells of bacteria and yeasts

(including *Saccharomyces cerevisiae*) is still a frequently used option for small-scale reductions on a laboratory-scale.
Oxygenases are often used to synthesize compounds that are not accessible by chemical routes, as is the case for many hydroxylated steroids. Following an extensive screening and structure analysis effort in many laboratories, steroids can now be oxidized at virtually any carbon (see section 12.3). Commercial oxygenase applications include the production of compounds such as pravastatin, cis-muconic acid and 4-hydroxyproline. Small scale industrial applications of oxygenases are numerous (Urlacher et al.,2004). For example, mammalian hydroxylases (mainly cytochrome P450 enzymes) and their bacterial or fungal equivalents are used to synthesize a wide range of intermediates of human drug metabolism.
Oxygenases typically have relatively low k_{cat} values compared with hydrolytic enzymes, for example. Therefore oxygenases have to be expressed to high levels to obtain significant whole cell oxygenase activities. Oxygenases are often unstable, consist of multiple components some of which might be membrane-bound and require expensive co-factors such as NAD(P)H. In addition, oxygenase-based bioprocesses are frequently carried out in two-liquid phase bioconversion media because oxygenase substrates and products are often hydrocarbons and thus not water-soluble. However, if suitable oxygenase biocatalysts can be developed, expensive multistep synthesis routes towards (chiral) epoxides, alcohols, diols, aldehydes, sulfoxides, carboxylic acids or their derivatives can be replaced by much shorter routes.

6. Biocatalysts in enantiotransformations

The rapid expansion of biocatalysis in organic synthesis has been boosted primarily by the increasing demand for enantiomerically pure fine chemicals (i.e. agrochemicals and pharmaceuticals) and novel materials (i.e. liquid crystals and polymers) together with the need for environmentally more benign chemistry (Trost, 2004).
Biotransformations are particularly valuable in the fine-chemical sector and enantioselective catalysis for the industrial production of enantiomerically enriched chiral fine chemicals has a pivotal role: worldwide sales of chiral drugs in 2001 corresponded to 150 billion US$, and the market for chiral building blocks can be estimated to be 5 billion US$. The increasing size and complexity of these molecules frequently results in multiple chiral centres. In 2000, 35% of intermediates in pharmaceutical industry were chiral and this number is expected to increase to 70% by 2010. 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 (Koeller and Wong C.H. 2001; Patel, 2006).
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 enantiocomplementary reverse hydrolysis (esterification, transesterification, aminolysis or amidation), providing access to both enantiomers of a desired product (see section 5.1). A particularly practical development in this respect is the use of enol esters as transesterification reagents, which irreversibly force the enzymatic process in the forward direction. This prevents loss of enantioselectivity resulting from the reverse reaction, and eliminates product-inhibition problems.
Molecular modelling techniques can aid in prediction of the stereochemical course of the reaction, and also give insight into potential substrate or enzyme modifications that may increase selectivity.
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 as “deracemization”, have been developed that allow the transformation of both enantiomers of a racemate into a single enantiomer of the product.
The first approach to “deracemization” is realized when the R and S enantiomers that react at different rates, are in equilibrium with one another and therefore the product can be obtained

optically pure in 100% yield (Gadler et al., 2006). As an example, in situ racemization of substrate combined with kinetic resolution leads to the concept of "dynamic kinetic resolution" (DKR) (Figure 7).

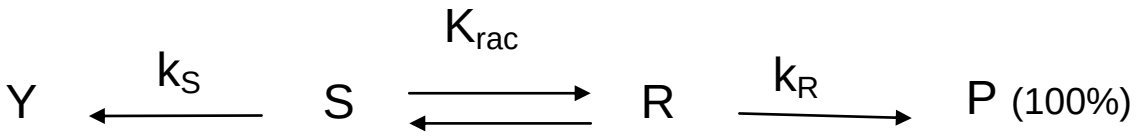


Figure 7: The schematic description of an ideal dynamic kinetic resolution (DKR) where the two enantiomers (R and S) are in a equilibrium defined by the K_{rac} constant. The R enantiomer reacts faster according to a kinetic constant k_R. and one single product (P) is produced in 100% yield.

The obvious basic requirements for an efficient DKR are that an efficient kinetic resolution and an efficient racemization method have to be chosen. Moreover, the compatibility between both processes is crucial for the success of the DKR process. The combination of enzyme catalysis (for the resolution of a racemate) and chemo catalysis (for the racemization of the slow-reacting enantiomer) has been successfully used in dynamic lipase resolutions processes. In view of the fact that approximately two-thirds of all racemization methods reported to date require harsh reaction conditions which are incompatible with the presence of a stereoselective (bio)catalyst, enzymatic racemization holds great potential. One of the best studied racemases so far is mandelate racemase (see Table 4) from *Pseudomonas putida*, a cofactor-independent enzyme acts through deprotonation–protonation of the α-H of both substrate enantiomers.

The second approach to "deracemization" is based on the independent stereo-convergent transformation of both enantiomers into a single enantiomeric product. In this case the two transformation pathways must proceed through inversion and retention of configuration respectively.

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 prochira carbonyls has been thoroughly investigated using whole cells of bacteria and yeasts (see section 5.5). The use of whole cells is cheap and easy, but the occurrence of different dehydrogenases may affect the overall stereoselectivity. To overcome this limitation, genes of stereoselective dehydrogenases and cofactor-regenerating enzymes have been co-expressed in cells of *E. coli* with no activity towards prochiral carbonyls.

7. Selecting and designing biocatalysts for practical applications

7.1 Isolated enzymes versus whole cell biocatalysts

One of the most important decisions when planning and designing a process using biocatalysis is whether the conversion is carried out using one or more enzymes contained within a whole-cell extract or an isolated enzyme. From the industrial point of view, the first choice for the development of the process will involve the use of an isolated enzyme, whereas, to keep the upstream costs low, whole cells (often recombinant) will often be used for the final manufacturing process. Whole-cell processes are less expensive because there is no need for enzyme isolation although the need for fermentation facilities can preclude some chemical companies from using this

option. Ideally, the process devoted to fermentation (growth of the cells and expression of the enzyme) should be separated from conversion (enzyme catalysis). Separation enables media and catalyst concentration to be modified between operations with a range of advantages. Whole-cells can provide an efficient way to regenerate cofactors *in situ* and contain multiple enzymes enabling multi-step chemistry. However, they generally will not tolerate high concentrations of substrate or product (>1 g/l), have limited use with organic solvents and can generate unwanted side products as a result of the presence of multiple enzymes. Isolated enzyme processes are simple to implement because they are amenable to rapid process development and there are few side reactions or other metabolites that require to be separated from the reaction product. The general industrial aim is to minimize the enzyme purification and operate the process with the crudest form of catalyst possible (usually a lysate). 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.

7.2 Availability of the biocatalysts

The feasibility of new biocatalytic processes is often determined by the availability of the biocatalyst. Nowadays, a wide array of enzymes catalyzing various reactions are commercially available either as crude extracts, isolated protein or immobilized biocatalysts .

Dedicated efforts to explore the worldwide biodiversity for novel enzymes at a genetic and functional level have further expanded the array of biocatalysts available for industrial application. Even though the unique potential of enzymes is still far from being fully explored (Pollard and Woodley, 2007). Estimates generally agree that <1% of the microorganisms in the environment have been cultivated to date.

Traditionally, active biocatalysts have been obtained by screening a broad variety of microorganisms, ranging from archaea to fungal systems, frequently isolated from extreme environments. The discovery of life in seemingly prohibitive environments continues to challenge conventional concepts of the growth-limiting conditions of many cellular organisms. The diversity of extremophiles provide remarkable opportunities that these uncommon organisms present for biotechnological applications (Bouzas et al., 2006). However, only few examples can be reported for their exploitation. This lack of progress from the research findings at a laboratory-scale to the actual development of pilot and large-scale production is correlated with the difficulties encountered in extremophile cultivations. Nevertheless, the production of extremophilic biomass is very important to provide sufficient material for enzyme and biomolecule isolation and characterization. Hence, special equipment and custom-tailored processes have been developed and are currently under evaluation for the improvement of fermentation productivity.

Successful efforts to clone and express the genes encoding hyperthermophilic enzymes in mesophilic hosts have improved the availability of high temperature biocatalysts. Moreover, recent developments have enhanced the prospects for the discovery of hyperthermophilic enzymes, This is crucial for the understanding of the intrinsic basis of their extraordinary stability and, ultimately, for engineering this characteristic into less thermophilic enzymes.

Metagenome technology uses culture-independent approaches to overcome problems related to microorganisms cultivation from environmental samples, which is often difficult or impossible. Metagenome-based approaches allows the accumulation of an increasing number of DNA sequences, coming from uncultured microbes and the. cloning of metagenome of non-culturable microorganisms. The cloning of these genomic sequences have increased the availability of biocatalysts and are also exploited to increase the knowledge on microbial ecology and physiology of these microbes.

7.3 Tailoring the catalytic properties of biocatalysts

Modern molecular biology techniques in combination with high-throughput screening and a tremendous progress in genomics and bioinformatics, have led to a substantial increase in the availability of enzymes with requested properties (Leisola and Turunen, 2007). Recombinant

systems can be developed in which the genes encoding the desired enzyme are over-expressed in industrial host microorganisms. The resulting microorganisms have an elevated level of the desired enzyme, whereas the levels of enzymes catalyzing unwanted side reactions are kept low since the genes coding for the latter enzymes are not transferred from the source microorganism.

Over the past twenty years, protein engineering based on site-directed mutagenesis has contributed significantly to our understanding of enzyme catalysis, and has led to the development of enzyme variants with modified properties for synthetic transformations.

Molecular modelling was used as a complementary to understand and to optimize enzyme-substrate interactions, as well as the enzymatic catalytic machinery (Braiuca et al., 2006). Recent studies illustrated the use of molecular simulation methods in the induction of catalytic promiscuity (i.e. different catalytic activity from the natural one) of enzymes. However, computational design in general is far less efficient in the identification of the effect of mutations that are distant from the active site. Such mutations might still subtly alter the protein structure and modify the catalytic behaviour or the enzyme stability and in this contest "directed evolution" can offer a solution to the problem (Arnold, 2001).

In vitro-directed evolution of enzymes uses random genetic mutation and recombination, followed by screening or selection for a desired enzymatic activity. This technique has the advantage that it does not require *a priori* knowledge of the relationship between protein structure and function for experimental design. Current progress has shown that directed evolution can yield new enzymes with altered substrate specificity, enantioselectivity, protein topology, thermal stability and tolerance to organic solvents. For example, lipase from the ubiquitous environmental bacterium *Pseudomonas aeruginosa* was evolved to catalyse the hydrolysis of a model ester with >90% enantiomeric excess, compared with 2% enantiomeric excess for the wild-type enzyme.

Notably, in many cases the mutations found in the variants obtained through directed evolution are not in the active site, underscoring the power of directed evolution and the unpredictable factors that influence enzyme specificity. Therefore, nowadays, to select an enzyme for a given reaction, one can start with one enzyme capable of catalysing that specific type of reaction, optimize the reaction conditions, and further improve the catalyst through directed evolution and the protein engineering cycle.

In the attempt of tailoring specific catalytic activity, catalytic antibodies have been explored as possible biocatalysts. To prepare these antibodies, ligands are synthesized that typically mimic transition states of particular chemical transformations, such as ester hydrolysis, amide synthetase and Claisen condensation. Monoclonal antibodies are then selected that display high-affinity binding to the ligands, thus enriching for antibody proteins with a binding-site geometry complementary to the shape of the true transition state. Some of the antibodies selected in this way displayed catalytic activity towards the desired reactions. However, the low catalytic turnover numbers have so far limited the use of catalytic antibodies in chemical synthesis or processes. The low efficiency of these new enzymes reflects the fact that transition-state binding is only one aspect of the catalytic process.

8. Immobilization of biocatalysts

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:

- enhanced stability,
- repeated or continuous use,
- easy separation from the reaction mixture,
- 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 (Cao, 2006; Wijffels, 2001).
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. Recent trends are based on the use of new reagents and/or carriers, as well as on the increasing knowledge of enzyme structure and mechanism.
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.
Enzymes can be also entrapped in polymers network such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber 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 procedures lead to macroparticles, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs). This approach offers the advantage of highly concentrated enzyme activity in the catalyst and low production costs owing to the exclusion of an additional carrier. However, their mechanical stability in some cases may not match industrial requests.
Entrapment is more suited for the immobilization of whole cells; immobilized cells have been studied widely during the last decades since in many processes it is more efficient to use immobilized cells than freely suspended cells. Microencapsulation of microbial cells is based on two main steps: droplet formation (suspension of cells in the presence of soluble polymer) followed by solidification/gelation of the polymer with formation of beads which entrap the cells. Gelation of polymers can be achieved by different means: ionic gelation (i.e. alginate beads), thermal gelation (i.e. k-carrageenan beads), chemical polymerization (i.e. gel of polyacrylamide)
The increasing knowledge of enzyme structures and mechanism should also enable more controlled immobilizations. In the future, information derived from protein sequences, 3D-structures, and reaction mechanism should be further combined with the properties of carriers (functional groups, hydrophobicity, magnetic properties) and physical/chemical methods in order to produce a directed immobilization strategy.

9 Reaction media for biocatalysis

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 diluted aqueous solution of an enzyme.
During the last decades much attention has been paid, by both academia and industry, to the development of new solvents that are environmentally friendly. Following a series of green principles, they should be environmentally benign, less hazardous and help to improve industrial processes. Two new classes of solvents seem to fulfil these criteria. These non-conventional solvents are ionic liquids (ILs) and supercritical fluids (sc-fluids). They would both exactly fill the gap in solubility for enzyme-catalysed reactions that is left open between conventional solvents and

water (Cantone et al., 2006). The following paragraphs illustrate some basic and practical issues related to the use of biocatalysts in different reaction media.

9.1: Organic solvents

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

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

First examples of biocatalysis in organic solvents actually date back to the end of the nineteenth century, when the experiments were done in the presence of non-water miscible solvents as toluene, in order to prove the reversibility of the catalytic reaction.

In 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. However, despite the rational and modern approach of his studies it is surprising that biocatalysis in organic solvent did not "take off" until 1980s when the application of enzymes in monophasic organic solvents was finally studied by A.M. Klibanov at the Massachusetts Institute of Technology (U.S.A.). His pioneering work demonstrated how the use of organic solvent is not only feasible, but also that in such seemingly hostile environments enzymes become more stable. In addition, the use of solvents allow improved solubility of water insoluble organic compounds, suppresses unwanted hydrolytic side reactions, eases product recovery and shifts the unfavoured thermodynamic equilibria of aqueous reaction media (Klibanov, 2001).

This system 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 in a macroscopic view, it must have necessary residual bound water to remain catalytically active. The enzyme may be crystalline, lyophilized or precipitated. Enzymatic preparations can be recovered by simple filtration after the reaction. Nevertheless, simple adsorption onto the surface of cheap macroscopic carriers is possible and often suggested, since desorption from the carrier into the medium cannot occur in a lipophilic environment.

An essential understanding of the behaviour of enzymes in organic solvent came from the studies of P.J. Halling (at Strathclyde University, Glasgow) on the water activity (a_w), a crucial parameter for determining the correct degree of hydration of enzymes in non-aqueous media (Halling, 2000). The concept of water activity can be assimilated to the "free" water present in the system, which is available to react or hydrate other molecules. Figure 8 illustrates how water partition between the different phases of a closed reaction system. After a certain period of time the system will reach the equilibrium, so that the water activity (or the "free water") will be the same in all phases. Therefore, the reaction and the enzyme activity will be affected by the a_w rather than by the water concentration in the system.

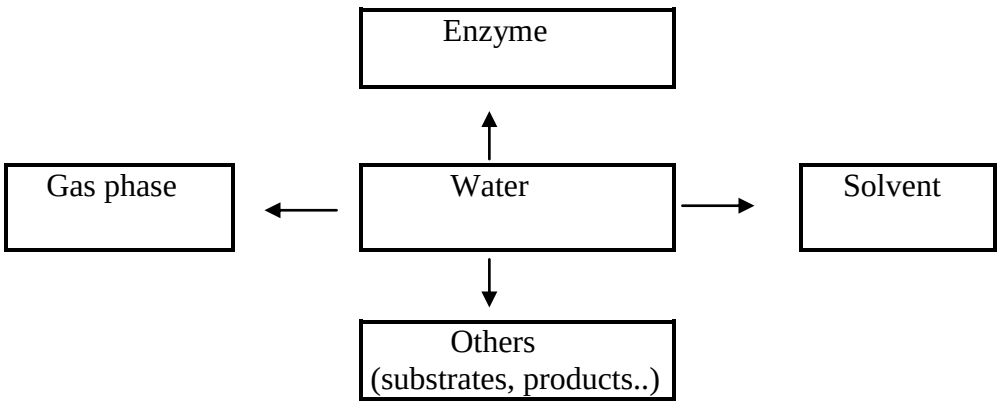


Figure 8: Distribution of water among the different phases of a closed reaction system. When all phases are in equilibrium a_w is equal in all the phases. Therefore it is more conveniently measured in the gas phase with a proper sensor (e.g. a hygrometer). A completely dry system has an a_w of 0, while 1 is the value for pure water.

Lipases, proteases, hydrolases and many other enzymes work efficiently in organic solvent containing little or no 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). However, the control of water activity is not the only parameter determining the activity of enzymes in organic solvents. Enzymes work well in water and in relatively non-polar organic solvents (log P > 2), those that do not mix with water. These hydrophobic solvents do not strip the crucial water from the enzyme surface, while hydrophilic organic solvents (log P < 2) remove this water leading to protein unfolding (Table 7). Consequently the catalytic activity of enzymes in water/water miscible solvent systems is often much lower than activities in aqueous solutions.

Table 7: Log P (logarithm of the partition coefficient) of organic solvents and their effect on enzymatic activity

Log P	**Water-Miscibility**	**Effects on enzyme activity**
-2.5 to 0	Completely miscible	Used to solubilise lipophilic substrates in concentrations of 20-50% v/v without deactivating the enzyme
0 to 2	Partially miscible	Limited use due to rapid enzyme deactivation
2 to 4	Low miscibility	Causes weak enzyme distortion, may be used with caution, since enzyme activity is often unpredictable
> 4	Immiscible	Causes no enzyme distortion and ensures high retention of activity

This means that enzymes work well in relatively apolar solvents and in water, but not in polar and protic solvents. Consequently a large gap in the solubility for enzyme-catalysed reactions is left.
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 short chain alcohol. If the portion of the solvent exceeds a certain threshold, the essential bound water (structural water) is stripped from the enzyme surface leading to deactivation.
Usually solvents can be applied in concentrations up to about 10% of the total volume but in some cases even 50-70% of co-solvent may be used.

9.2 Ionic liquids (ILs)
An ionic liquid usually consists of an organic cation, often containing a nitrogen heterocycle and an inorganic anion. The 1-alkyl-3-methylimidazolium ionic liquids are the most widely used for

biocatalysis, although also 1,4-dialkylpyridinium ionic liquids have been used for some biocatalytic processes. Anions can be fluorinated (PF_6^- or BF_4^-), alkylsulfates or alkylsulfonates (RSO_4^- or RSO_3^-) and triflates (Tf_2N^-; see Table 1). Their characteristics can be tuned by modifying anion and cation properties. For instance, the hydrophilicity and lipophilicity are determined mainly by the anions of an ionic liquid. Therefore ILs can be considered as the ideal tailor-made solvents due to the possibility to modify the anions or the cations that compose these liquid salts.

Due to their virtually absent vapour pressure, ILs have been considered, in the last years, as "green" (i.e. environmentally compatible) solvents that, in the future, could substitute the toxic and flammable organic solvents. In the light of preliminary studies, the toxicology of ILs remains unclear and further studies are necessary to assess their sustainability. With a green vision of the overall process it is essential to recycle ILs and to avoid the use of organic solvents to recover products from the IL at the end of it.

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. Starting from these preliminary studies the number of papers in this field has increased spectacularly and several reviews on the topic are now available. However, so far most studies are of an exploratory nature. The relationships between structure of 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 (see section 91). It 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.

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, it is possible to couple (bio)transformations in ILs with extraction by supercritical fluids.

9.3 Supercritical fluids (SFs)

The use of supercritical fluids as non-aqueous solvents for enzyme-catalysed reactions was reported for the first time almost twenty years ago. 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 are non flammable and at the end of enzymatic processes, traces of sc-fluids can be removed by depressurisation.

The most used SF for biocatalysed processes is supercritical-CO_2, but also other supercritical fluids as 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). Since the changes in properties are predictable, supercritical fluid properties can be rationally controlled.

Super critical-CO_2 has been used in enzymatic reactions also catalysed by whole cells. Cells of Bacillus megaterium were employed in supercritical-CO_2 for the fixation of CO_2 from pyrrole to pyrrole-2-carboxylate catalysed by decarboxylase obtaining much higher yields (55%) than at atmospheric pressure (7 %).

Enzyme activity is often reduced in supercritical carbon dioxide, due to the formation of carbonic acid in the presence of water and carbamates by reaction with amine groups on the enzyme. This is not the case for the supercritical ethane. Analogously to the behaviour of enzymes in organic solvents and in ionic liquids, the degree of enzyme hydration plays a key role in the exhibited specific activity also in the supercritical fluids. As an example, cholesterol oxidase in sc-CO_2 was less active in dry CO_2 than in the same system containing water. The effect was found to be reversible, since the enzyme recovered its activity upon addition of water.

10. Special techniques for the use of biocatalysts

10.1 Solid-gas biocatalysis

Solid-gas biocatalysis today is 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.

Solid-gas biocatalysis presents many advantages compared to other systems (mono- or biphasic liquids): very high conversion yields compatible with a high production rate for a minimal plant scale, more efficient mass transfer, reduced diffusion limitations due to low gas viscosity and better stability of enzymes and cofactors. In addition, downstream processing is simplified due to the absence of a solvent phase and the scale-up is simpler due to the use of a gaseous circulating phase.

Many enzymes have been explored in solid-gas systems such as alcohol oxidase in the ethanol oxidation,, alcohol dehydrogenase for alcohol and aldehyde production.

Solid-gas biocatalysis has not been restricted to the use of isolated enzymes. Lyophilised Saccharomyces cerevisiae cells catalysed, in a solid-gas system at controlled a_w, the reduction of an aldehyde to the corresponding primary alcohol, with an efficient in situ regeneration of the expensive nicotinamide cofactor. Similar results were also found for haloalkane dehalogenases, which are attractive biocatalysts for gas-phase bioremediation of pollutants. Due to their ability to convert short-chain aliphatic halogenated hydrocarbons to the corresponding alcohols, they can detoxify halogenated vapour emissions.

Systems consisting of several enzyme-catalysed reactions have been described. Two enzymatic systems, consisting of alcohol oxidase from cells of *Pichia pastoris* and isolated catalase (or peroxidases) were used in the same reactor. Thus, after oxidaxion of primary alcohols by alcohol oxidase, the isolated enzymes catalysed the conversion of produced by-products. A further example is the complete degradation of trichloroethylene, in a continuous way, by cells of *Methylosinus trichosporium*, with the combination of methane monooxygenase, dehydrogenases and other monooxygenases in cascade.

10.2 Biocatalysis with undissolved solid substrates and products

Enzymatic synthesis in reaction mixtures with mainly undissolved substrates and/or products is a synthetic strategy in which the compounds are present mostly as pure solids (Ulijn et al. 2003).

Several different approaches, which involve suspended substrates or products with aqueous, organic or no separate solvent added, are reported in the literature of the last decade.

Although these reaction mixtures usually consist largely of solids, it has been recognised 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. A small liquid phase may also be formed from two solid. 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. 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 and esters starting from suspended substrates has also been reported.

10.3 Solid phase biocatalysis

Enzymes are able to recognize and transform substrates even when these molecules are anchored on solid supports (Halling et al, 2005). 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 condition at the end of the transformation in order to recover the product (Figure 9).

Figure 9: A schematic representation of a substrate immobilized on a resin through a chemical linker.

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 the microarray technology. This technology has found a promising route in the use of biocatalyst for the development of highly selective assays under mild operative conditions. For example, methods for screening protease selectivity have been developed. Protein chips are disposable arrays of micro-wells in silicone elastomer sheets placed on top of microscope slides. The high density and small size of the wells allows for high-throughput batch processing and simultaneous analysis of many individual samples and only small amounts of protein are required. With the increased understanding of enzyme reactions on immobilised substrates it is likely that these systems will find new applications beyond those in screening and solid phase chemistry described above.

11. Industrial applications of biocatalysts: economic issues

Biocatalysis are used by fine chemicals companies and bulk chemicals manufacturers in several large-scale processes to produce commercial quantities of intermediates and chemicals.
In few cases, such as high-fructose syrup production by isomerisation of glucose (see section 5.3), biocatalysis is a forced option, since no other technology is available. In other cases, such as acrylamide (see section 12.1) production or antibiotics modifications (see section 12.2), biocatalysis is becoming the first choice as production technology. In the remaining situations, biocatalysis is just a possible solution and therefore economic and environmental benefits must be carefully considered.
The economic feasibility of a biocatalytic process 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 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 of biocatalytic industrial processes still operate in aqueous environments that generally correspond to low product concentrations because of the scarce solubility of most organic molecules in water. This necessitates the development of special methods for product extraction. The major waste products of aqueous bioprocesses are waste water, salts and biomass. Organic solvents represent minor waste products if used in emulsion processes or reactions in pure organic solvents and they can be recycled, apart from a minor fraction obtained as insoluble emulsion. The main waste products are treated in conventional industrial waste-water treatment systems. For processes based on recombinant microorganisms, the treatment of biomass includes inactivation of the genetically engineered cells.
A crucial factor in the development of a biocatalytic process at industrial level is represented by the cost of the biocatalyst. Production of a biocatalysts (isolated enzyme or whole cells) is obtained by fermentation and can be expensive; therefore, in most of the cases the biocatalyst must have a high turnover number. Turnover number has generally two slightly different meanings. In enzymology, turnover number is defined as the maximum number of molecules of substrate that an enzyme can convert to product per catalytic site per unit time (also termed $k_{cat,}$ see section 1.2). In the field of chemical transformation, turnover number (abbreviated *TON*) is the number of moles of substrate that a mole of catalyst can convert before becoming inactivated.
In analogy to chemical processes, most biocatalysts are used in immobilized form (see section 8) as heterogeneous catalysts that can be recovered and reused. However this represents a further cost to be considered. There are also processes based on homogeneously suspended cells or enzymes, that are sufficiently inexpensive to permit single use, without recovery or reuse. Several speciality chemical companies use living cells as catalysts for reactions such as specific coenzyme-dependent. Depending on the type of biocatalyst to be used, specific reactor and hardware configurations are needed .

In the case of enantiomeric transformations, enantiomeric excess (ee) of at least 98% are expected in order to consider the process economically competitive. Multiple enrichment steps are possible in some cases where enrichment cannot be achieved in a single stage.

12. Examples of large scale productive processes employing biocatalysts in chemical and pharmaceutical industry

12.1 Nitrile hydratase in the production of acrylamide

Acrylamide is an important monomer needed for the production of a range of economically useful polymeric materials. Nitto Chemical (now Mitsubishi Rayon, Japan) has developed a process where the addition of water to acrylonitrile is catalysed by bacterial nitrile hydratases (previously from *Pseudomonas chloraphis* and more recently from *Rhodococcus rhodochrous*).

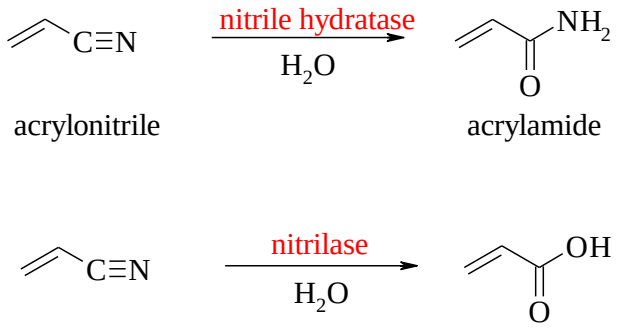


Figure 10: Enzymatic transformation of acrylonitrile into acrylamide catalyzed by microbial nitrile hydratase. Complete hydrolysis of acrylonitrile to acrylic acid can be obtained by means of nitrilases.

This process may be achieved by the use of a reduced copper catalyst; however, the yield is poor, since unwanted polymerisation or conversion to acrylic acid (CH_2=CHCOOH) may occur at the relatively high temperatures involved (80-140°C) and the catalyst is difficult to regenerate. The biotransformation is carried out by using immobilized whole cells (cells entrapped in a cross-linked 10% (w/v) polyacrylamide/dimethylaminoethylmethacrylate gel) on 30000 tons/year scale; fed-batch addition of the substrate allows for product concentrations of up to 20% (w/v), containing negligible substrate and less than 0.02% (w/w) acrylic acid. Using 1% (w/v) immobilised-enzyme concentration the process takes about a day.

A similar approach is used by DuPont for the regioselective hydration of adiponitrile to 5-cyanovaleramide, an early intermediate in the manufacture of a herbicide. Cells containing nitrile hydratase are employed after entrapment in calcium alginate and are recycled multiple times, generating close to 3150 kg of product per kg of catalyst.

12.2 Amidases in the production of antibiotics

Beta-lactam antibiotics represents the largest segment of the antibiotic market in the world. A successful example of industrial enzymatic catalysis is the application of penicillin G amidase in the production of 6-aminopenicillanic acid (6-APA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA), intermediates for a wide range of β-lactam antibiotics, such as amoxicillin (>15000 tons/year), ampicillin (6000 tons/year), cephalexin (3000 tons/year), cephradine (800 tons/year) and cefadroxil (600 tons/year).

Penicillin G and V are produced by fermentation. Current processes use penicillin acylases to remove the side chain and to convert penicillin G/V to 6-APA, which serves as a backbone for the

synthesis of variants of semisynthetic penicillins differing for antibiotic characteristics. This process was discovered in the early 1960s and is now practiced on a multi-ton scale.

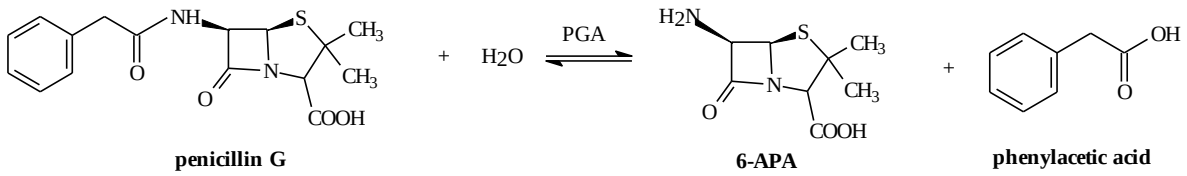


Figure 11: Enzymatic hydrolysis of penicillin G to 6-aminopenicillanic acid (6-APA) and phenylacetic acid catalyzed by penicillin G amidase.

The beta-lactam ring of penicillin G can be converted chemically or enzymatically to phenylacetyl-7-aminodesacetoxycephalosporanic acid (phenylacetyl-7-ADCA), which is also accepted by the acylase. This allows the production of the nucleus (7-ADCA) for the synthesis of semisynthetic cephalosporins.

A biocatalytic process employing penicillin acylase is used for the coupling of the core structures and the side chains to generate derivatives of penicillins and cephalosporins that previously have traditionally been produced using complex solvent-based chemistry, typically conducted at temperatures as low as -80 °C to preserve the labile beta-lactam ring.

Finally, the side chains (non proteogenic aminoacids like D-phenylglycine) that are attached to the 6-APA or 7-ADCA back-bone in the semisynthetic antibiotics ampicillin, amoxycillin, cephalexine and cephadroxyl, are also produced industrially (by the DSM company, NL) in enantiopure form thanks to the use of biocatalysts (Figure 12). A series of non-proteinogenic L-aminoacids are produced by DSM thanks to the resolution of racemic amides of amino-acids, which are chemically synthesized. Resolution is achieved by means of hydrolases (amidases). Although the enzymes are strictly L-selective, the undesired enantiomer can be recycled (see section 6).

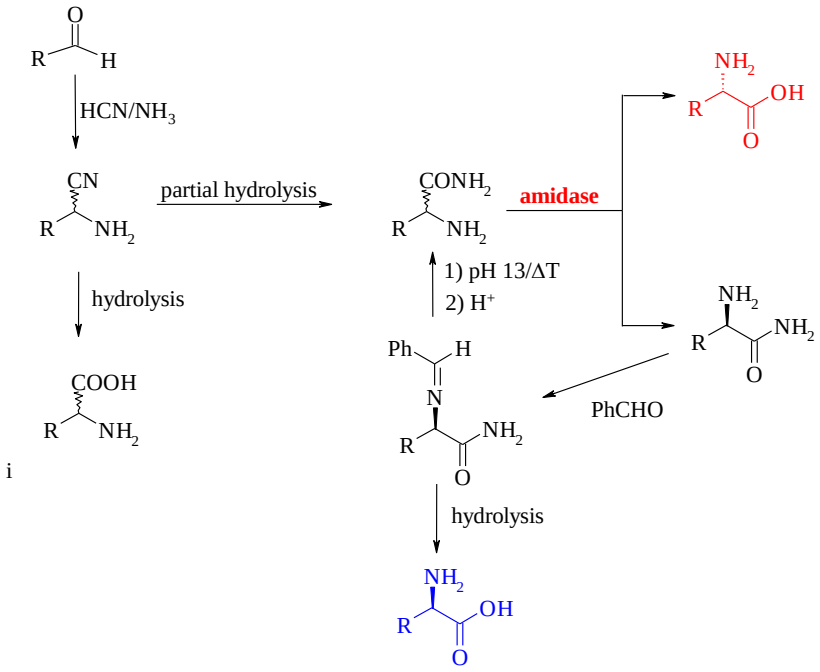


Figure 12: Production of enantiomerically pure non proteinogenic L-aminoacids via amidase resolution of amides.

It must be underlined that non-proteinogenic L-aminoacid are fundamental building blocks for the synthesis of pharmaceutical compounds and they are not producible by fermentation, in contrast to most of the 20 proteinogenic amino acids that are incorporated into polypeptides.

12.3 Steroids modification

Steroids world market exceeds 1,000 tons per year. These steroids are used as basic substrates for subsequent chemical and biotechnological syntheses of drugs. Biocatalytic processes with economic significance are hydroxylations, dehydrogenations, reduction and sterol side-chain cleavage.

The complexity of steroid molecules renders the use of biocatalysis particularly appealing. As a result of the massive screening programs performed since the 50's, a number of biocatalysts are available for steroid modifications, such as hydroxylation. Virtually all positions of the steroid nucleus may be hydroxylated (see section 5.5). These hydroxylations are carried out with whole cells (usually bacteria or moulds) and often involve cytochrome P450 enzymes which are $NADP^+$ dependant. The use of mutants and suited conditions of biotransformation (pH, temperature, substrate concentration, use of specific inhibitors, immobilization, use of co-solvents and solubilising agents) have allowed for the set-up of processes at industrial level. Some of these processes are run industrially (by Schering, U.S.A.) at a scale around 100 tons per year. The major limitation of using whole cells is the obtainment of products with multiple hydroxylation sites. To avoid this problem, the gene of region- and stereospecific hydroxylases may be overexpressed in suited hosts. For instance, recombinant cytochrome P450 was expressed in *Escherichia coli* and the transformed cells were used for the hydroxylation of progesterone to dehydroepiandrosterone (DHEA).

12.4 Applications of lipases in oleochemical industry

The scope for the application of lipases in the oleochemical industry is enormous. Average annual world oil production is around 105-110 x 10^6 tons and it has been estimated that it will increase in the years 2016-2020 to 184-185 x 10^6 tons. Lipids are mostly available from vegetables (80%) and they are currently used especially for food (80-81%) and feed (5-6%), while the rest is used by chemical industry for further modifications. The conditions for steam fat splitting and conventional glycerolysis of oils involve high temperatures of 240–260°C and high pressures. The resulting products are often unstable as obtained and require re-distillation to remove impurities and products of degradation. Moreover, highly unsaturated oils cannot be used in this process without prior hydrogenation because they are heat sensitive. Minimization of thermal degradation and energy saving are probably the major attractions in replacing the current chemical technologies with biocatalysed ones.

Lipases have been extensively used for the hydrolysis and transesterification of triacylglycerols. Chemo- and regio-selective hydrolysis of triacylglycerols has been exploited for enrichment of specific fatty acids, such as polyunsaturated fatty acids (PUFA) from fish oils. The possibility of using lipases in low-water environments has made possible their application for the production of structured triacylglycerols by enzymatic inter- and transesterifications. This mild and selective approach has been utilized for producing triacylglycerols containing polyunsaturated fatty acids (PUFA), cocoa-butter equivalent and infant-formula substitutes.

Cocoa butter is mainly composed of SOP and SOS triacylglycerols (where S = stearic and O = oleic). Palm oil mid-fraction is rich of POP triacylglycerol (where P = palmitic acid) and can be converted into cocoa butter by chemo- and regiospecific lipases able to introduce stearic acid in position *sn*1 and *sn*3 (Figure 13). The biotransformation can be carried out in solvent-free medium, where palm oil is used in large excess.

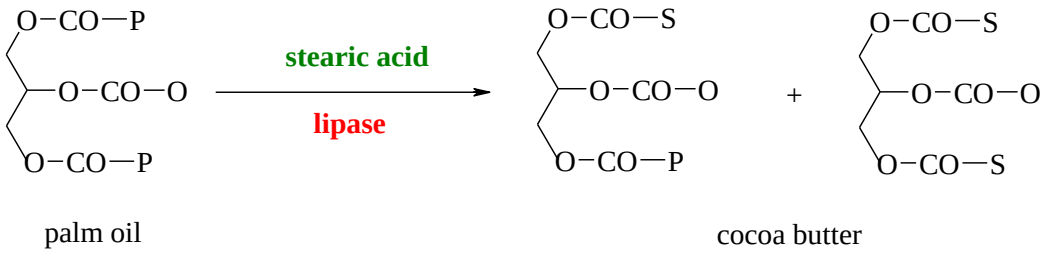


Figure 13: Lipase-catalyzed production of cocoa butter for palm oil.

The observation that lipases selectively catalyze both hydrolysis and esterifications has been further exploited for the obtainment of Betapol® from tripalmitin. Betapol® consists of triglyceride fatty acids commonly found in vegetable and animal fats. A similarity to human milk fat indicated a potential use in infant formulae as well as for food use in general. Tripalmitin is firstly hydrolysed with a lipase from *Rhizopus oryzae* able to remove only the fatty acids in position 1 and 3. The resulting 2-mono-palmitin has been esterified with oleic acid using the *Rhizomucor miehei* lipase in organic solvent (Figure 14).

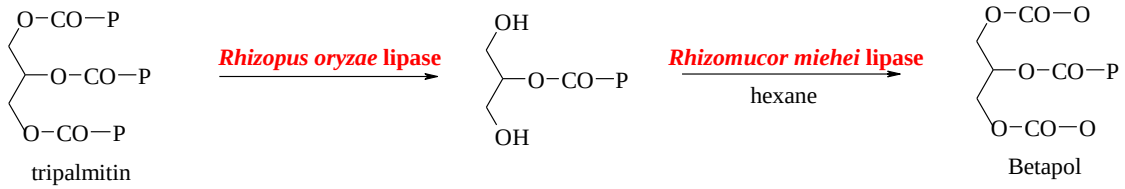


Figure 14: Enzymatic transformations of tripalmitin for the production of Betapol®.

Finally, lipases (i.e. from *Pseudomonas cepacia* and *Candida antarctica*) have been also employed for the production of biodiesel by interesterification of triacylglycerols (mostly from soy oil) with different alcohols.

13. Conclusions:

Modern chemical and pharmaceutical industries face novel challenges coming from the urgent need of novel sustainable processes. Although biocatalysts already represent a consolidated tool for industrial (commodity) and specialty (low volume) applications, their potential is far from being fully exploited. Major efforts of the scientific research in this area are focused on the exploitation of new enzymes to selectively convert non-natural compounds and the application of known biocatalysts in novel processes by making use of their catalytic plasticity. The integrated use of modern techniques such as directed-evolution, protein engineering, computational chemistry together with the ever-increasing genetic information will likely boost the rising of more efficient and sustainable production processes.

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Web-sites

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]

Glossary

Archea: a major division of microorganisms. Like bacteria, archaea are single-celled organisms lacking nuclei and are therefore prokaryotes, classified as belonging to kingdom Monera in the traditional five-kingdom taxonomy.

Biodiesel: a renewable fuel for diesel engines derived from natural oils like soybean oil.

Bioinformatics: bioinformatics and computational biology involve the use of techniques including applied mathematics, informatics, statistics, computer science, artificial intelligence, chemistry, and biochemistry to solve biological problems usually on the molecular level.

Catalytic antibody: an abzyme (from antibody and enzyme), also called *catmab* (from *catalytic monoclonal antibody*), is a monoclonal antibody with catalytic activity. Molecules which are modified to gain new catalytic activity are called synzymes. Abzymes are usually artificial constructs, but are also found in normal humans (anti-vasoactive intestinal peptide autoantibodies) and in patients with the autoimmune disease systemic lupus erythematosus, where they can bind and hydrolyze DNA.

Chiral: geometrical attribute of a compound, describing a molecule that cannot be super-imposed on its mirror image.

Co-factor: substance that needs to be present in addition to an enzyme for a certain reaction to take place.

Configuration: the spatial array of atoms that distinguishes stereoisomers other than distinctions due to differences in conformation.

Cytochrome P450: diverse superfamily of hemoproteins found in bacteria, archaea and eukaryotes.

Dextrins: mixtures of linear α-(1,4)-linked D-glucose polymers produced by the hydrolysis of starch.

Diastereomeric compounds: stereoisomers not related as mirror images and which usually differ in chemical and physical properties

Directed evolution: the use of random mutagenesis or gene casual mutation (such as DNA shuffling) applied to a protein for picking out variants that have the desired qualities. Further rounds of mutation and selection are then applied.

Enantiomer excess (ee%): the percentage excess of one enantiomer over the other in a mixture of two enantiomers

Enantiomer: one of a pair of molecules that are mirror images of each other and not super-imposable.

Enantioselective reaction: a chemical reaction or synthesis that produces the two enantiomers of a chiral product in unequal amounts.

Enzyme inhibitor: a molecule that binds to enzymes and decrease their activity.

Fermentation: an energy-yielding metabolism in which an organic substrate (and not oxygen) is involved as final electron acceptor.

Flavin: tricyclic heteronuclear organic ring based on pteridine whose biochemical source is the vitamin riboflavin.

Genomic: the study of the entire genomean of an organism.

High-throughput screening (HTS): a combination of modern robotics, data processing and control software, liquid handling devices, and sensitive detectors. HTS allows a researcher to effectively conduct millions of biochemical, genetic or pharmacological tests in a short period of time. Through this process one can rapidly identify active compounds, antibodies or genes.

Hyperthermophilic enzymes: enzymes coming from thermophiles, organisms – a type of extremophile - which thrive at relatively high temperatures, above 45 °C. Many thermophiles are archaea.

Ionic liquids: a liquid that contains essentially only ions.

Kinetic resolution: partial or complete resolution of a racemate by virtue of unequal reaction rates of the enantiomers with a chiral agent, such as a biocatalyst.

Meso compounds: chemical compound with molecules that contain 2 or more asymmetric atoms (stereocenters) but which is optically inactive (or achiral) because it contains an internal plane of symmetry.

Mesophilic enzymes: enzymes whose optimum temperature for activity lies within a range generally accepted as ca. 20-40°C.

Metagenome: the study of genomes recovered from environmental samples, as opposed to genomes extracted from clonal cultures.

Molecular modelling: a collective term that refers to theoretical methods and computational techniques to model or mimic the behaviour of molecules.

Peptide bond: a chemical bond formed between two molecules when the carboxyl group of one molecule reacts with the amino group of the other molecule, releasing a molecule of water (H_2O).

Phage display: a test to screen for protein interactions by integrating multiple genes from a gene bank into phage.

Protease: any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain.

Protein engineering: application of complementary methods for developing useful or valuable proteins. There are two general strategies for protein engineering: *rational design* and *directed evolution.*

Proteomic: the large-scale study of proteins, particularly their structures and functions.

R (*rectus*), S (*sinister*): stereochemical descriptors describing the configuration of a given chiral molecule following the rules developed by Cahn, Ingold and Prelog (the CIP convention)
Racemate: an equimolar mixture of two enantiomeric species.
Rational design: in protein engineering it is considered the use of detailed knowledge of the structure and function of the protein to make desired changes.
Regioselective reaction: the preference of one direction of chemical bond making or breaking over all other possible directions.
Stereoisomers: isomers of identical constitution but differing in the arrangement of their atom in space. Subclasses are enantiomers and diastereomers.
Steroid: terpenoid lipid characterized by a carbon skeleton with four fused rings
Strain: an organism distinguishable from at least some of the other organisms within a given named taxon.
Supercritical fluids: any substance at a temperature and pressure above its thermodynamic critical point.
Turnover number: the maximum number of moles of substrate that an enzyme can convert to product per catalytic site per unit time

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