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FACULTATY OF CHEMISTRY

LITERATURE REPORT

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2020

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UNIVERSITATEA DIN BUCUREȘTI

VIRTUTE ET SAPIENTIA

FACULTATY OF CHEMISTRY

DEPARTAMENT OF ORGANIC CHEMISTRY, BIOCHEMISTRY AND CATALYSIS

LITERATURE REPORT

DESIGNING BIOCATALYSTS BASED ON ENZYMES CO-IMMOBILIZATION WITH APPLICATION FOR CASCADE REACTIONS

MASTER: CHEMISTRY OF ADVANCED MATERIALS

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February 2020

Introduction

Biocatalysis represents an alternative for industrial processes to obtain desired products. In the last period of time, biocatalysis gained a lot of attention due to the fact that biocatalysts have multiple selectivity; their capacity to perform a catalytic reaction is far greater than the one of a chemical catalyst. They are also environmentally friendly, being biodegradable; they work under mild conditions; they are not restricted by their natural role, and can catalyze a broad spectrum of reactions. In last period, the alternatives offered by biochemical engineering for enzyme modification is an important feature which have broaden the enzymes applicability.

Enzymes isolated from the biological cell or used in the natural environment (whole cell) can play the biocatalyst role in the reactions. Isolated enzymes are preferred for their high selectivity on the reaction products and specificity on the reaction pathway, with more advantages compared with the whole cell systems. Enzymes are very efficient catalysts (TON = 10^6 - 10^{12}) with largi possibility for catalysis, especially multi-reaction process (e.g. cascade process) for which two or more enzymes can act simultaneously for providing an efficient catalytic transformation.

Multi-enzymatic systems can offer a broad spectrum of reactions that cannot be performed by only an isolated enzyme. Generally, a bienzymatic system would be a useful design for a cascade reaction involving two steps, i.e. the conversion of the substrate will be catalyzed by one enzyme, and the resulted product will become a substrate for the next transformation assisted by the other enzyme, leading to the final product as target compound for the designed biocatalytic process.

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CHAPTER I

Enzyme

1.1. Enzymes as protein materials

Enzymes are proteins that catalyze reactions in a living organism. The structure of the enzyme is similar to other proteins, meaning that it is composed of one or more polypeptide chains. The sequence of the composing aminoacids is determined during the protein synthesis inside the living organism. As a protein, the enzyme is composed of a primary structure – the sequence of the composing aminoacids –, the secondary structure – the conformation of the aminoacid chain –, tertiary structure – the over-all 3D structure of the polypeptide chain –, and the quaternary structure – made by multiple proteins subunits (Figure.1.) [1].

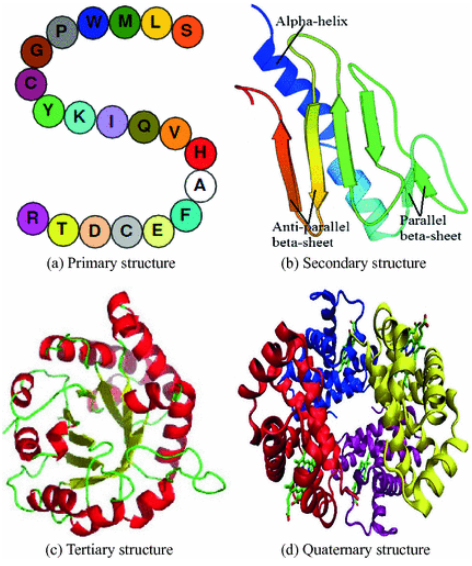


Figure. 1. Protein structures [a].

Enzymes are complex organic molecules with complex proprieties. Some misconceptions about the enzymes arise from their complicated structure and mechanism. Therefore, enzymes might be considered too sensitive, too expensive, can be active only on their natural substrates or only in their natural environment [2].

[a] https://link.springer.com/chapter/10.1007/978-3-319-74775-0_1

First of all, enzymes are usually considered too sensible due to the fact that they are sensible to extreme temperature because it affects their structure integrity. However, in the right environment enzymes can withstand temperatures greater than 100°C and pressures over hundreds of bars [3].

Secondly, if produced at a reasonable scale the cost of enzymes is not too high because if taking into account their higher catalytic power, the overall efficiency of an enzymatic process might be very cost efficient. Moreover, if they are immobilized enzymes they can be reused. The enzymatic activity has a complex mechanism, and for many years it was believed that enzymes react only with their natural substrate. However, the research of the past few decades had shown that substrate tolerance for many enzymes is higher than expected, and that the more complex mechanism the enzyme has, the narrower the limit for other substrates. In general, enzymes exhibit the highest catalytic activity in water. However, it was proven that biocatalysts can function in nonaqueous media, such as: organic solvents, ionic liquids, and even supercritical fluids, if certain guidelines are followed. Although the catalytic activity is lower in a nonaqueous media but by performing the catalytic process in a different solvent one can enable reactions impossible to effectuate in water and, therefore, making the process more efficient [4].

For identification purposes, every enzyme has a four-digit number in the general form [EC A.B.C.D], where EC stands for 'Enzyme Commission'. This identification number is given by the International Union of Biochemistry and Molecular Biology (IUBMB). The following properties are encoded:

A. denotes the main type of reaction (Table. 1.);

B. stands for the subtype, indicating the substrate class or the type of transferred molecule;

C. indicates the nature of the co-substrate;

D. is the individual enzyme number.

Enzymes have been classified into six categories according to the type of reaction they can catalyze [5].

At first glance, it would seem advantageous to use this classification when discussing the enzymes, unfortunately, this does not work in practice for the following reasons: due to the varying tolerance for non-natural substrates, the importance for practical applications is not at all

evenly distributed amongst the different enzyme classes. Furthermore, due to the widespread use of crude enzyme preparations - consisting of more than one active biocatalyst -, one often does not know which enzyme is actually responsible for the biotransformation. Last but not least, there are many useful reactions which are performed with whole microbial cells, for which it can only be speculated as to which of the numerous enzymes in the cell is actually involved in the transformation [6].

Table. 1. Enzymes classification [i].

Enzyme class	Number		Reaction type	Utility[a]
	Classified	Available		
1. Oxidoreductases	~700	~100	Oxidation–reduction: oxygenation of C–H, C–C, C=C bonds, or overall removal or addition of hydrogen atom equivalents	+++
2. Transferases	~750	~100	Transfer of groups: aldehydic, ketonic, acyl, sugar, phosphoryl, methyl, NH_3	++
3. Hydrolases	~650	~180	Hydrolysis formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, glycosides, organohalides	+++
4. Lyases	~300	~40	Addition–elimination of small molecules on C=C, C=N, C=O bonds	++
5. Isomerases	~150	6	Isomerizations such as racemization, epimerization, rearrangement	+
6. Ligases	~80	5	Formation-cleavage of C–O, C–S, C–N, C–C bonds with concomitant triphosphate cleavage	±

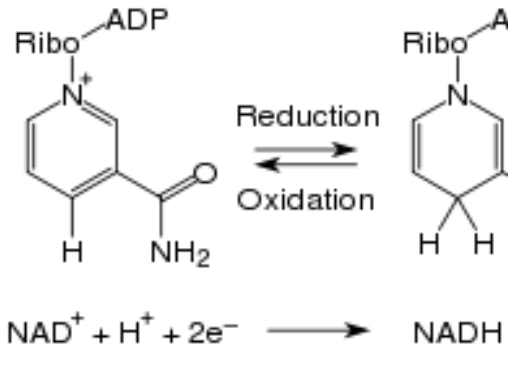
Catalytic activities are measured in several different systems. According to the SI system, catalytic activity is defined by the katal (1 kat = 1 mol s^{-1} of substrate transformed). Since its magnitude is far too big for practical application, it has not been widely accepted, and the 'International Unit' (1 I.U. = 1 µmol of substrate transformed per min) – has been defined. The catalytic power of a (bio)catalyst can be conveniently described by the so-called 'turnover frequency' (TOF), which has the dimension of [$time^{-1}$]. It indicates the number of substrate molecules which are converted by a single (bio)catalyst molecule in a given period of time. Since it is mass-independent, it allows to compare the performance of different (chemo- and bio-) catalytic systems. For the majority of enzymes used in biotransformations, TOFs are within the range of 10–1000 s^{-1}, whereas the respective values for chemical catalysts are one to two orders of magnitude lower [5].

[i] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 1. Introduction and Background Information: Enzyme Properties and Nomenclature, pgs. 11-27

1.2. Co-factor/coenzyme

Some enzymes necessitate having a cofactor bound to them or the presence of a coenzyme in order to exhibit catalytic activity. A cofactor represents a non-protein compound strongly bonded to the enzyme, and removing it would result in the denaturation of the enzyme. If the cofactor is loosely bonded to the enzyme and it is an organic molecule then it is called a coenzyme. Usually, the cofactor contains a metallic ion, such as: iron or copper, with a role in the catalytic process [7].

The coenzyme can also be freely diffusing around the enzyme, and participate actively in the catalytic mechanism in stoichiometric amounts, usually mole-for-mole. During the reaction, the coenzyme is modified, and would need another enzyme-catalyzed reaction in order to be restored to its initial shape. The coenzymes are sometimes referred as "co-substrates" because they do not possess catalytic activity by themselves, and not considered as being a part of the enzyme. Some examples of coenzymes include: adenosine triphosphate (ATP), which transfers phosphate groups, nicotinamide adenine dinucleotide (NADH, NAPH), which transfers electrons and hydrogen atoms (Scheme. 1.), and coenzyme A (Figure. 2.), which transfer acetyl groups [7].



Scheme. 1. Redox reaction of NADH [b]

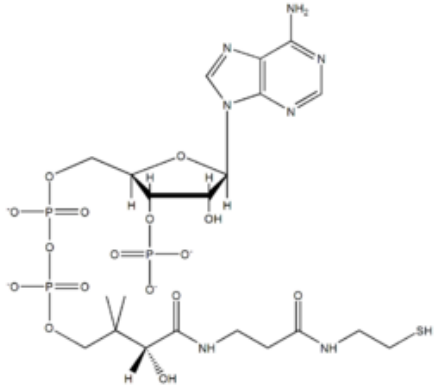


Figure. 2. Coenzyme A[b].

The most important part of an enzyme is the active site, which represents the area where the substrate binds and the catalytic reaction takes place. Since enzymes are proteins, their active site is consisting of sequences of aminoacids with different side chains. The position, sequence, structure, and proprieties of this side chain create a very specific environment that can bind with a very specific substrate [8].

[b] https://www.newworldencyclopedia.org/entry/coenzyme

CHAPTER II

Enzyme in biocatalysis

2.1 General aspects

Biocatalysis represents a chemical transformation inside/outside of the cell catalysed by biological components (e.g. enzyme, antibody, etc). High catalytic efficiency, substrate selectivity and also reaction specificity are properties of the biocatalyst making biocatalysis an attractive catalytic field. This propriety may be translated into the biocatalyst recognizing the substrate and the obtaining of the desired product without having secondary reactions. Biocatalysis is considered a part of green chemistry because of its eco-friendly nature [17].

2.2. Isolated enzymes vs. whole cell

A biocatalyst is a substance, usually enzymes or whole cells, that can improve or speed up a biochemical reaction. The most general example of biocatalysis is the yeast-mediated transformation of sugars into alcohol (fermentation process). Due to the nature of the enzymes, being usually proteins, they are very sensible to changes in the reaction medium. Any change in their structure could result in a change of the active site and, therefore, the enzyme can lose its catalytic activity. Also, in order to use an enzyme for a specific substrate, the enzyme needs to be purified, which is a time-consuming and very costly process. As a result, whole cells represent a convenient strategy because in this case the enzyme acts in its natural cellular environment, which provides protection against destabilization and degradation [18].

A disadvantage of using whole cells as catalysts would be the restriction of accessibility for the substrate to the catalytic site, and interaction with other enzymes. To overcome such restrictions, surface-displayed enzymes are usually used in whole cells reactions, as seen in Figure. 3. By making a compromise between the advantages and disadvantages, enzyme catalysis is still the most useful, especially in scientific research field [19].

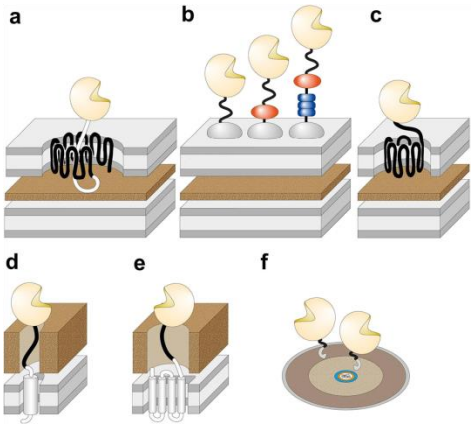


Figure. 3. Types of surface-displayed enzymes in whole cells catalysts [a1].

The most used biocatalysts are purified enzymes. The use of enzymes outside the cell provides both advantages and disadvantages. If in the whole cell catalysts there could appear accessibility restrictions for the substrate or interactions between products and other enzymes, by using purified enzymes these issues are overcome. However, other problems arise from the fact that most enzymes are proteins and, therefore, are sensible to the changes in the environment, such as changes in pH level or temperature. The most important part of the enzyme is the active site which is responsible for the catalytic proprieties. If the parameters of the reaction environment are too harsh this could affect the structural integrity of the protein, and by inducing a modification of the structure this would involve a modification of the structure of the catalytic site, which means that the enzyme would not be able to recognize the substrate anymore [20].

Enzymes are very efficient catalysts and have some major advantages, such as: typically the rates of enzyme-mediated processes are faster by a factor of 10^8–10^{10} than those of the corresponding noncatalyzed reactions, and are thus far above the values that chemical catalysts are capable of achieving. As a consequence, chemical catalysts are generally employed in concentrations of a mole percentage of 0.1–1%, whereas most enzymatic reactions can be performed at reasonable rates with a mole percentage of 10^{-3}–10^{-4}% of catalyst, which clearly makes them more effective by some orders of magnitude (Table. 2.). The high TON values for enzymatic catalysis is explained by the close contact between the enzyme and substrate, the substrate being located inside the protein structure, exactly on the catalytic site [21].

Moreover, biocatalysts are environmentally benign reagents since they are completely biodegradable. Enzymes act under mild conditions within a range of about pH 5–8 and in a temperature

[a1] https://www.creative-enzymes.com/service/whole-cell-biocatalysts_408.html

range of 20–40ºC. This minimizes problems of undesired side-reactions. Since enzymes generally function under the same or similar conditions, several biocatalytic reactions can be carried out in a reaction cascade in a single flask, thus, sequential reactions are feasible by using multienzyme systems in order to simplify reaction processes, in particular if the isolation of an unstable intermediate can be omitted. Furthermore, an unfavorable equilibrium can be shifted towards the desired product by linking consecutive enzymatic steps. Enzymes exhibit a high substrate tolerance by accepting a large variety of man-made non-natural substances and often they are required to work in water. There is an enzyme-catalyzed process equivalent to almost every type of organic reaction, for example: hydrolysis-synthesis of esters, amides, lactones, lactams, ethers, acid anhydrides, epoxides, and nitriles [22].

Table. 2. Catalytic efficiency of representative enzymes[a2].

Enzyme	Reaction catalyzed	TON
Carbonic anhydrase	Hydration of CO_2	600,000
Acetylcholine esterase	Ester hydrolysis	25,000
Penicillin acylase	Amide hydrolysis	2,000
Lactate dehydrogenase	Carbonyl reduction	1,000
Mandelate racemase	Racemisation	1,000
α-Chymotrypsin	Amide hydrolysis	100

TON = turnover number

Enzymes display three major types of selectivities. First of all, enzymes exhibit chemoselectivity because the purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, do survive unchanged. As a result, reactions generally tend to be ‘cleaner’ so that laborious removal of impurities, associated to side reactions, can largely be omitted [23].

Second of all, regioselectivity and diastereoselectivity are exhibited due to their complex three-dimensional structure, enzymes may distinguish between functional groups which are chemically identical but situated in different positions within the same substrate molecule. Last but not least, enantioselectivity is caused by the fact that all enzymes are made from L-amino acids and thus are chiral catalysts. Additionally, the enzyme-substrate interaction in minimum three points gives a reasonably reason for enatioselectivity of the enzymatic transformation. [22]

As a consequence, any type of chirality present in the substrate molecule is ‘recognized’ upon formation of the enzyme-substrate complex. Thus, a prochiral substrate may be transformed into an optically active product through a desymmetrization process and both enantiomers of a racemic substrate usually react at different rates, affording a kinetic resolution. Since the majority of them are

[a2] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 1. Introduction and Background Information: Advantages and Disadvantages of Biocatalysts, pgs. 3-9.

highly selective with respect to the chirality of a substrate, it is obvious that the enantiomers of a given bioactive compound such as a pharmaceutical or an agrochemical will cause different biological effects. Consequently, in a biological context, enantiomers must be regarded as two distinct species. The isomer with the highest activity is denoted as the 'eutomer', whereas its enantiomeric counterpart, possessing less or even undesired activities, is termed as the 'distomer'. Probably the most well-known and tragic example of a drug in which the distomer causes serious side effects is 'Thalidomide', which was administered as a racemate in the 1960s. As a consequence, racemates of pharmaceuticals and agrochemicals should be regarded with great caution [24].

There are certainly some drawbacks worthy of mention for the use of biocatalysts, for example, enzymes are provided by nature in only one enantiomeric form. Since there is no general way of creating mirror-image enzymes from D-amino acids, it is impossible to invert the chiral induction of a given enzymatic reaction. To gain access to the other enantiomeric product, one has to follow a long and uncertain path in searching for an enzyme with exactly the opposite stereochemical selectivity [21].

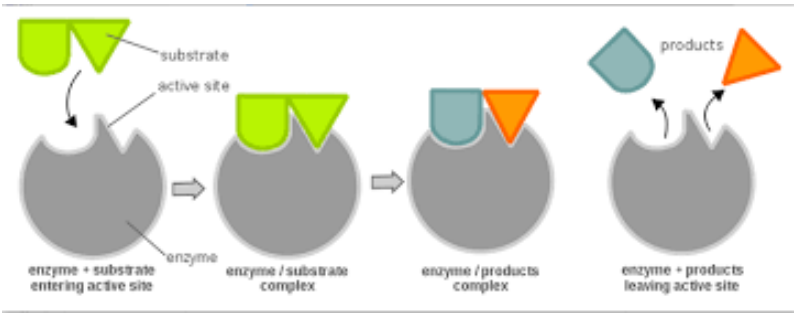
Enzymes require narrow operation parameters and if a reaction proceeds too slow under given parameters of temperature or pH, there is only a narrow operational window for alteration because elevated temperatures as well as extreme pH lead to deactivation of the protein, as do high salt concentrations [21].

Enzymes display their highest catalytic activity in water and due to its high boiling point and high heat of vaporization, water is usually the least suitable solvent for most organic reactions. Furthermore, the majority of organic compounds are only poorly soluble in aqueous media. Thus, shifting enzymatic reactions from an aqueous to an organic medium would be highly desired, but the unavoidable price one has to pay is usually some loss of catalytic activity, which is often in the order of one magnitude [21].

Enzymes are bound to their natural cofactors. It is a still unexplained paradox, that although enzymes are extremely flexible for accepting non-natural substrates, they are almost exclusively bound to their natural cofactors which serve as molecular shuttles of redox or as storage for chemical energy. Enzymes are prone to inhibition phenomena. Many enzymatic reactions are sensitive to to substrate and/or product inhibition, which causes a drop of reaction rate at higher substrate and/or product concentrations, a factor which limits the efficiency of the process. Whereas substrate inhibition can be circumvented comparatively easily by keeping the substrate concentration at a low level through continuous addition, product inhibition is a more complicated problem. Enzymes may cause allergies but this can be easy to contain if they are handled as reagents [22].

2.3. Enzimatic Kinetics

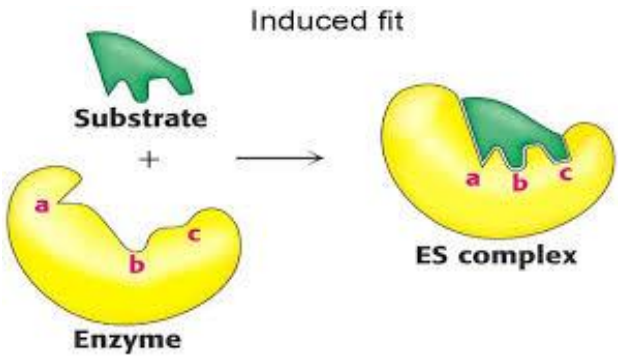
The unparalleled catalytic power of enzymes has sparked a lot of theories about their mechanism. One of the first mechanisms for enzymatic catalysis was the "lock – and – key" model, which assumes that the enzyme and its substrate interact in a lock and key fashion, although this assumption was quite sophisticated at the time, it assumes that the enzyme has a rigid structure, which fails to explain some aspects of the enzymatic catalysis, such as why enzymes can act on larger substrates while being inactive on the smaller ones [9]. Given the rationality of it, the enzyme should transform smaller substrates at a higher rate than the larger ones because they would have an easier access to the active site. However, the experimental data shows that larger substrates are transformed while the smaller ones have lower rates or no rates at all. Another fact failed to be explain by this theory is why enzymes are able to react with other substrates than their natural ones [10].



Scheme. 2. "Lock – and – key" mechanism [c].

The theory that followed the "lock – and – key" mechanism was the induce fit one, which takes into account the fact that enzymes are not entirely rigid, but rather soft and delicate structures [9]. Given the fact that enzymes are not rigid structures, the mechanism assumes that enzymes can change conformation during the formation of enzyme – substrate complex, in order to wrap itself around the guest. This "induced fit" is compared with the interaction of a hand, representing the substrate, and a glove, representing the enzyme [10].

[c] https://infograph.venngage.com/p/150383/lock-and-key-mechanism



Scheme. 3. Induced fit mechanism [d].

The most recent mechanism was developed by Dewar in order to explain the higher transformation rates of enzymes compared with non-enzymatic catalysts. The desolvation theory assumes that the kinetics of enzymatic reactions is similar with the kinetics of gas-phase reactions, therefore, if a substrate enters the active pocket of an enzyme it replaces all water molecules existent in the active site of the enzyme. Then, a formal gas-phase reaction takes place, meaning that the two partners interact without the presence of a disturbing solvent. According to this theory, in solution the water molecules impede the approach between the enzyme and the substrate. This mechanism explains why smaller substrates have slower reaction rates because they cannot replace all the water molecules existent in the active site of the enzyme [9].

The theory has been extended to a "solvation – substitution" theory because the enzyme would not be able to strip off all the water molecules surrounding the substrate, giving the fact that this effect would be energetically unfavorable. Instead of a desolvation, the solvent is displaced from the enzymatic active site. This replacement of water molecules from the active site during the substrate approach is characterized by a decrease of the dielectric constant of this area, which enhances the electrostatic interactions between the enzyme and its substrate. The latest cause proper substrate orientation, thereby an enhancement in the catalytic activity is seen [9].

Enzymatic kinetics is described using the Michaelis – Menten mechanism. The model is named after the German biochemist Leonor Michaelis and Canadian physician Maud Menten [11].

. The mechanism takes the form of an equation relating reaction velocity to substrate concentration for a system where a substrate *S* binds reversibly to an enzyme to form an enzyme-substrate complex, which then reacts irreversibly to generate a product and to regenerate the free enzyme. The Michaelis – Menten equation follows the equation for reaction velocity: $v=\frac{V\,\max*[S]}{K\,M+[S]}$,

[d] https://www.toppr.com/ask/question/the-characteristic-of-the-inducedfitmodel-is/

where V_{max} represents the maximum velocity achieved by the system, [S] is the substrate concentration, and K_M is the Michaelis – Menten constant and represents the substrate concentration for which the reaction velocity is 50% of V_{max}. The Michaelis – Menten kinetics shows how the concentration of substrate, or enzyme, affects the reaction rate. Giving the fact that in an enzymatic system, there is a given number of enzymes, each with one active site, by increasing the concentration of the substrate in the system, the reaction rate will continue to grow until it reaches a saturation, because at a high enough substrate concentration all the active sites would be occupied, and the enzymes would work at the maximum velocity, as it can be seen in Figure. 4. [12].

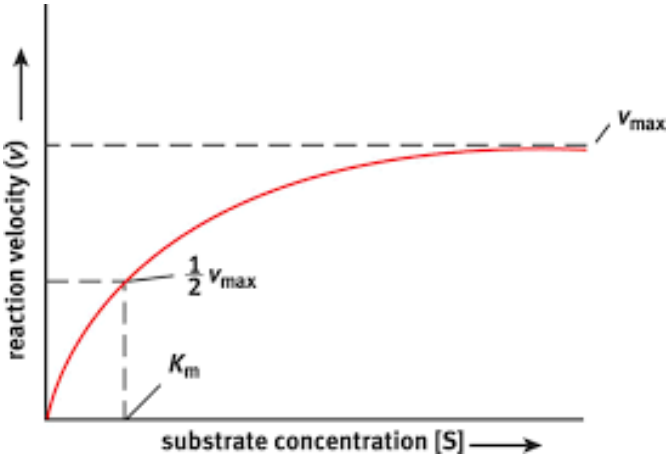


Figure. 4. Michaelis – Menten equation plot [e].

Enzymes (Enzy) are biocatalysts, thus they accelerate the reaction rate by lowering the energy barrier between the substrate (S) and products (P) – the activation energy (E_a). the origin of the enzymatic catalytic power has been attributed to the stabilization of the transition – state by the enzyme (Figure. 5.). This stabilization is attributed to the fact that the enzyme binds more strongly to the transition state than to the ground state by a factor approximately equal to the reaction rate. The dissociation constant for the enzyme-transition state complex $[EnzS]^{\neq}$ has been estimated to be in the range of 10^{-20} molar. The selectivity of all enzymes originates from the energy difference of enzyme – transition state complex. For example, for two enantiomers A and B that have the substrate role, each would have a dissociation constant for the transition – state complex, however, the active site of an enzyme is chiral, therefore there would be a difference between the dissociation constants noted as $\Delta\Delta G^{\neq}$, and one of the two enantiomers would react much faster. This process is referred as 'chiral recognition'. The value of this difference in free energy, expressed as $\Delta\Delta G^{\neq}$, is a direct measure for the selectivity of the reaction which in turn determines the ratio of the individual reaction rates of

[e] https://schoolbag.info/chemistry/mcat_biochemistry/11.html

enantiomeric substrates A and B. These values are of great importance since they determine the optical purity of the product. $\Delta\Delta G^{\neq}$ is composed of an enthalpy ($\Delta\Delta H^{\neq}$) and an entropy term ($\Delta\Delta S^{\neq}$). The enthalpy of activation is usually dominated by the breakage and formation of bonds when the substrate is transformed into the product. The entropy contribution includes the energy balance from the 'order' of the system, and various concentration and solvatation effects [13].

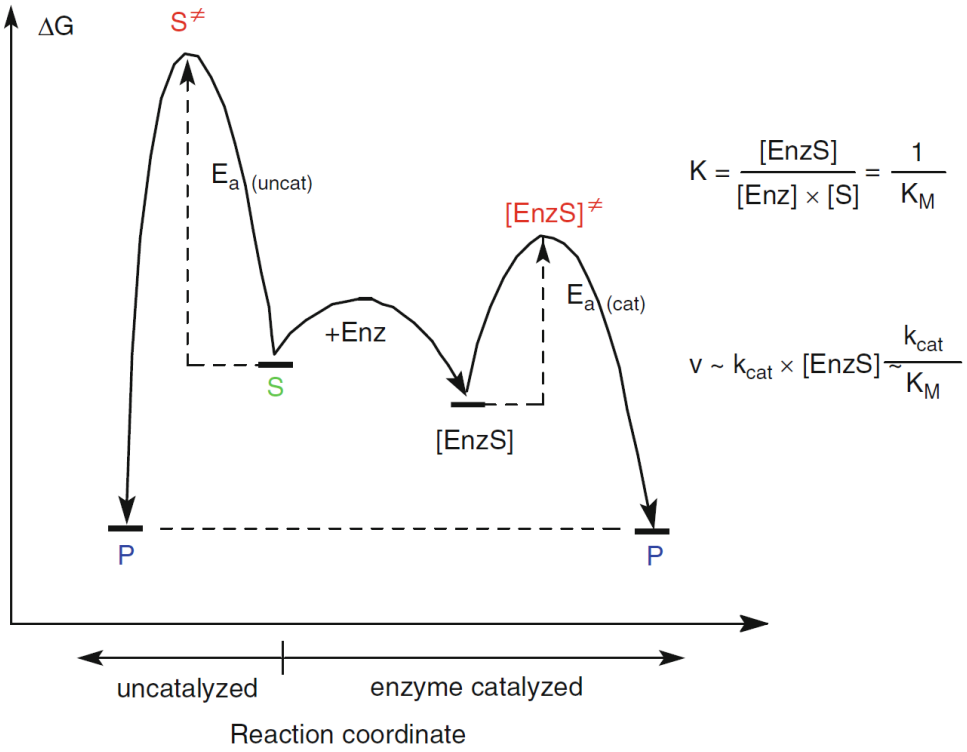


Figure. 5. Energy diagram of catalyzed vs. uncatalyzed reaction [f].

[f] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 1. Introduction and Background Information: Enzyme Properties and Nomenclature, pgs. 11-27

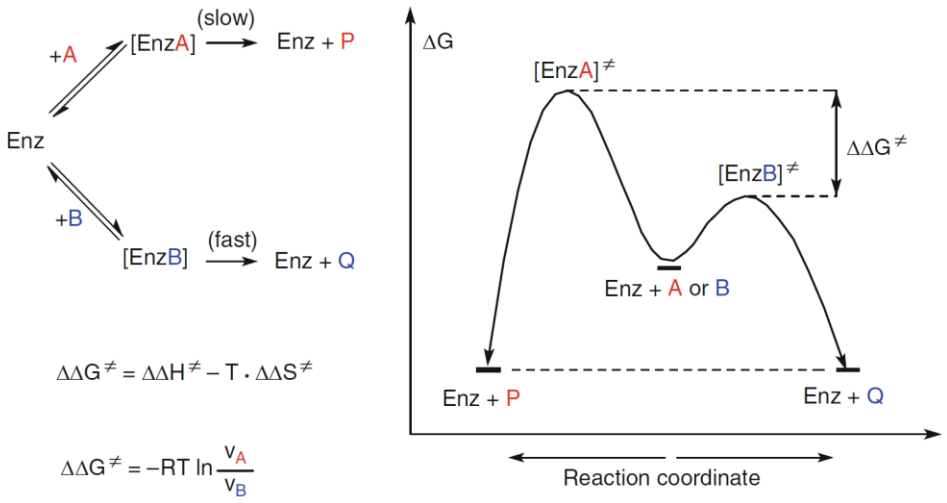


Figure. 6. Energy diagram for an enzyme-catalyzed enantioselective reaction [g].

Catalytic activities are measured in several different systems. According to the SI system, catalytic activity is defined by the katal (1 kat = 1 mol s^{-1} of substrate transformed). Since its magnitude is far too big for practical application, it has not been widely accepted, and the 'International Unit' (1 I.U. = 1 µmol of substrate transformed per min) – has been defined. The catalytic power of a (bio)catalyst can be conveniently described by the so-called 'turnover frequency' (TOF), which has the dimension of [$time^{-1}$]. It indicates the number of substrate molecules which are converted by a single (bio)catalyst molecule in a given period of time. Since it is mass-independent, it allows to compare the performance of different (chemo- and bio-) catalytic systems. For the majority of enzymes used in biotransformations, TOFs are within the range of 10–1000 s^{-1}, whereas the respective values for chemical catalysts are one to two orders of magnitude lower [14].

The productivity of a (bio)catalyst is characterized by the dimensionless 'turnover number' (TON). It denotes the number of substrate molecules converted per number of catalyst molecules used within a given time span. TONs for enzymes typically range from 10^3 to 10^6.

The comparison of TOFs and TONs of different (bio)catalysts is not very relevant because these numbers only indicate how fast the catalysts act at the onset of the reaction within a limited time span, but they do not tell whether the activity remains at a constant level or if it dropped due to catalyst deactivation [15].

In every catalytic process, the operational stability of the catalyst under process conditions is a key parameter. It is described by the dimensionless 'total turnover number' (TTN), which is determined by the moles of product formed by the amount of catalyst spent and – in other words – it stands for the amount of product which is produced by a given amount of catalyst during its whole

[g] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 1. Introduction and Background Information: Enzyme Properties and Nomenclature, pgs. 11-27

lifetime. If the TONs of repetitive batches of a reaction are measured until the catalyst is dead, the sum of all TONs would equal to the TTN. TTNs are also commonly used to describe the efficiency of cofactor recycling systems. The efficiency of microbial transformations (where the catalytic activity of enzymes involved cannot be measured) is characterized by the so-called ‘productivity number’ (PN) the amount of product formed by a given amount of whole cells (dry weight) within a certain period of time. This number resembles the specific activity as defined for pure enzymes, but also includes several other important factors such as inhibition, transport phenomena, and concentration [5].

The large majority of enzymes used for biotransformations in organic chemistry are employed in a crude form and are relatively inexpensive. The preparations typically contain only about 1–30% of actual enzyme, the remainder being inactive proteins, stabilizers, buffer salts, or carbohydrates from the fermentation broth from which they have been isolated. Crude preparations are often more stable than purified enzymes. The main sources of enzymes for biotransformations are as follows: additives for detergents to effect the hydrolysis of proteinogenic and fatty impurities in the laundry process, proteases and lipases for meat and cheese processing and for the amelioration of fats and oils, glycosidases and decarboxylases employed in the brewing and baking industries [Schmid, A., [16].

CHAPTER III

Multi-enzymatic catalysis

3.1. General aspects

Multi-enzymatic reactions represent the combination of several enzymatic transformations in concurrent processes, offering considerable advantages: the demand of time, costs and chemicals for product recovery may be reduced, reversible reactions can be driven to completion and the concentration of harmful or unstable compounds can be kept to a minimum. A particular case of multi-enzymatic reactions are the bienzymatic ones, which refer to a series of reactions which use two different enzymes with specificity for different substrates [25].

Multi-enzymatic reactions are a most important technology to succeed in industrial process development, such as synthesis of pharmaceutical, cosmetic, and nutritional compounds. Different strategies to construct multienzyme structures have been widely reported. Enzymes complexes are designed by three types of routes: fusion proteins, enzyme scaffolds, or immobilization. As a result, enzyme complexes can enhance cascade enzymatic activity through substrate channeling. In particular, recent advances in materials science have led to syntheses of various materials applicable for enzyme immobilization [26].

Fused proteins, also named chimeric proteins, are produced by genetic engineering, and are currently made from effector peptides, using a ligand-binding portion of a cytokine, extracellular domains of lymphocyte antigens, or a toxin linked to a suitable fusion partner [27]. An example of chimeric proteins is Cytochrome P450 monooxygenases, which is a versatile enzyme able to catalyze a broad range of reactions, from hydroxylations to dehalogenations [28].

Enzyme scaffolds are enzymes attached to scaffold proteins, that provide a quick communication along partners within the same enzyme networks. This method has some advantages compared to the fused enzymes because the nanoscale organization of scaffold enzymes has been shown to increase local concentration of enzymes and their substrate, and to improve intermediate channeling between consecutive enzymes. In organisms, these types of enzyme complexes that can form are metabolons (simple enzyme clusters), or bimolecular scaffolds [29].

An immobilized enzyme is an enzyme attached to a material. Compared to free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes. More importantly, the heterogeneity of the immobilized enzyme systems allows an easy recovery of both enzymes and products, multiple re-use of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bioreactor designs. For example, by immobilizing an enzyme with ferromagnetic nanoparticles, they can be separated from the reaction using a magnet [30].

Biocatalysis has made tremendous advances in the field of synthesis of industrially important products and intermediates. For enzymes, cofactors are an important part which are involved in biocatalysis. These cofactors are expensive and stoichiometric additions are not economically feasible. This necessitates the in situ cofactor regeneration in biocatalytic processes. [31].

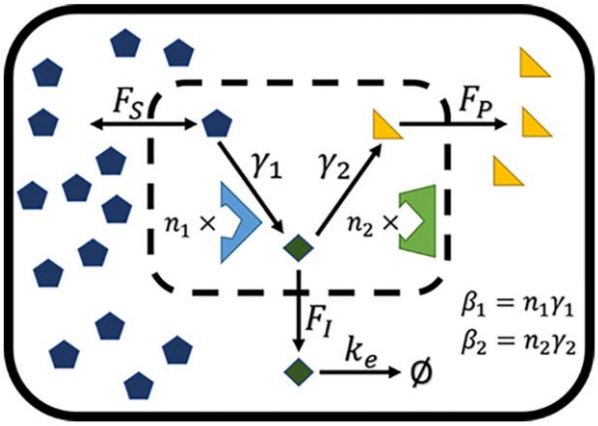
As example, for NADH regeneration, in an enzyme – membrane reactor, the enzymes are retained using an ultrafiltration membrane, and NADH has attached a water – soluble polymer in order to be separated [32].

3.2. Cascade Reactions

Bienzymatic reactions can be classified in cascade reactions and simultaneous reactions. Cascade reactions represent a chemical process that comprises at least two consecutive reactions coupled in such a way that each subsequent reaction occurs only in virtue of the chemical functionality formed in the previous step. In cascade reactions, isolation of intermediates is not required because each reaction composing the sequence occurs spontaneously. In the strictest definition of the term, the reaction conditions do not change among the consecutive steps of a cascade and no new reagents are added after the initial step. By contrast, one-pot procedures similarly allow at least two reactions to be carried out consecutively without any isolation of intermediates, but do not preclude the addition of new reagents or the change of conditions after the first reaction. Thus, any cascade reaction is also a one-pot procedure. Although often composed solely of intramolecular transformations, cascade reactions can also occur intermolecularly [33].

Compartmentalization of enzyme cascade reactions can both create a safe space for volatile reaction intermediates, and a quarantine for toxic intermediates. Therefore, by compartmentalization of an enzymatic reaction one can create an environment where volatile reaction intermediates are protected from cellular degradation mechanisms, and where they are prevented from impeding cellular

function. Most existing models for studying compartmentalized cascades focus on a specific biological system and are highly detailed. Compartmentalization is vital to the functioning of cells and a topic of tremendous interest in biocatalysis and synthetic biology. Despite its intuitive usefulness in containing toxic reactants and enhancing the concentration of unstable intermediates, quantitative models have only recently been published and they have revealed that compartmentalization reduced the number of inhibitory enzymatic interactions in the cell. This basic compartmentalization model describes a two-enzyme cascade and makes the generally valid assumption of a well-mixed interior and exterior of the compartment. Substrate, intermediate, and product molecules can enter the compartment through openings in the compartment boundary. It is assumed that the total number of molecules moving through all these openings per unit of time is proportional to the concentration difference across the opening [34].

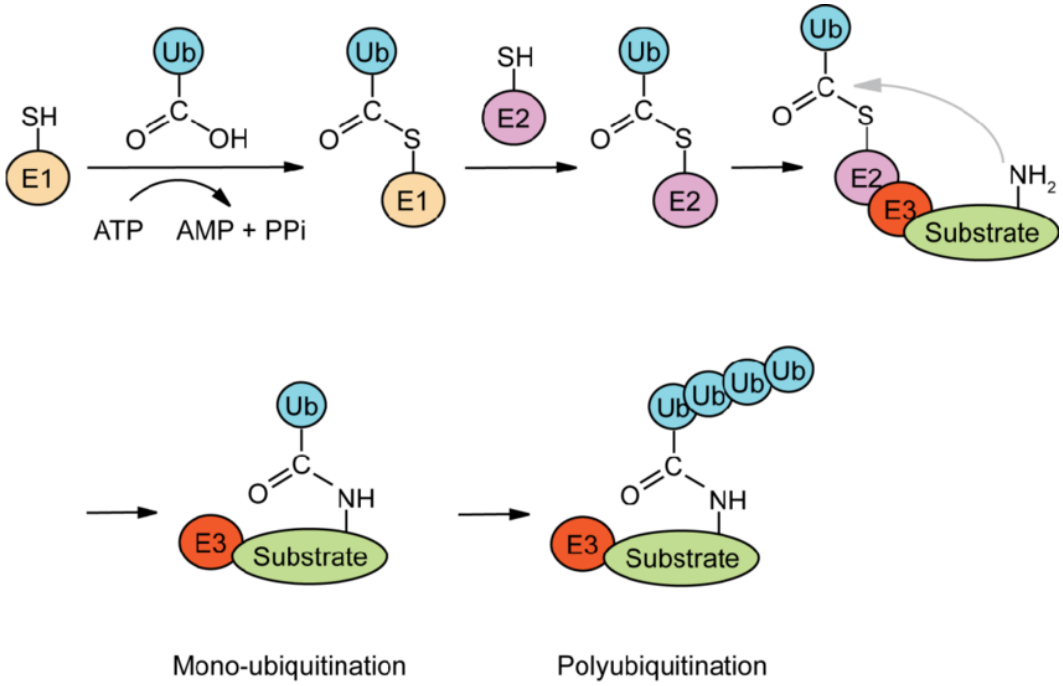


Scheme. 4. Compartmentalized cascade reaction [j]

Cascade reactions in synthetic chemistry, particularly in assembly of the complex frameworks of many classes of bioactive natural products, have garnered attention for an easier control of developing molecular architecture. The efficiency of enzymatic complexity generation through short metabolic pathways is illustrated in a number of cascade examples, particularly in carbacyclic and heterocyclic framework constructions and rearrangements. Enzymes have access to a limited array of accessible carbon nucleophilic and carbon electrophile building blocks.The concept of performing multi-step syntheses in one-pot has gained some attention in the past period because from an environmental point of view, cascades represent a very promising approach, mainly due to the avoidance of intermediate extraction and purification steps, resulting in a significant reduction of both waste and production costs on industrial scale. There are, however, some technological and scientific challenges to be overcome in order to reach industrial scale implementation of cascades. One of the

[j] Tsitkov, S., Henry Hess, H., Design Principles for a Compartmentalized Enzyme Cascade Reaction. ACS Catal., 9 (2019) 2432:2439.

most common challenges for the practicability of a cascade reaction is the combination of biocatalysts from different sources, which often have different optimal reaction conditions and show undesired side reactions [33].



Scheme. 5. Scheme of an enzymatic cascade reaction [k]

Multi-enzymatic cascade reactions offer considerable advantages: the demand of time, costs and chemicals for product recovery may be reduced, reversible reactions can be driven to completion and the concentration of harmful or unstable compounds can be kept to a minimum. They can be employed for the asymmetric synthesis of chiral alcohols, amines and amino acids, as well as for C-C bond formation [35]. As for example, the stereoselective three-enzyme cascade synthesis of diasteromerically pure γ-oxyfunctionalized α-amino acids [33].

3.3. Simultaneous Reactions

On the other hand, a simultaneous reaction represents any of two or more chemical reactions occurring at the same time in the same system. Simulation studies in industrial processes are usually used to adjust the enzyme feed rate in order to maximize the value of the product less the cost of the enzymes. However, in a multi - enzymatic system simultaneous reactions are not preferred because some factors may intervene during the reaction, as for example, competition for the active site for

[k] https://www.researchgate.net/figure/Ubiquitination-reaction-mediated-by-an-enzymatic-cascade-involving-E1-E2-and-E3_fig3_272791801

different substrates, or a product from a reaction may act as an inhibitor for another enzyme in the system, the competitive inhibition phenomenon being present [36].

An example of a simultaneous enzymatic reaction is the simultaneous enzymatic hydrolysis and lactic fermentation in order to obtain low lactose yogurt in a single stage process, by adding β-galactosidase and lactic culture [37].

CHAPTER IV

Biocatalyst design

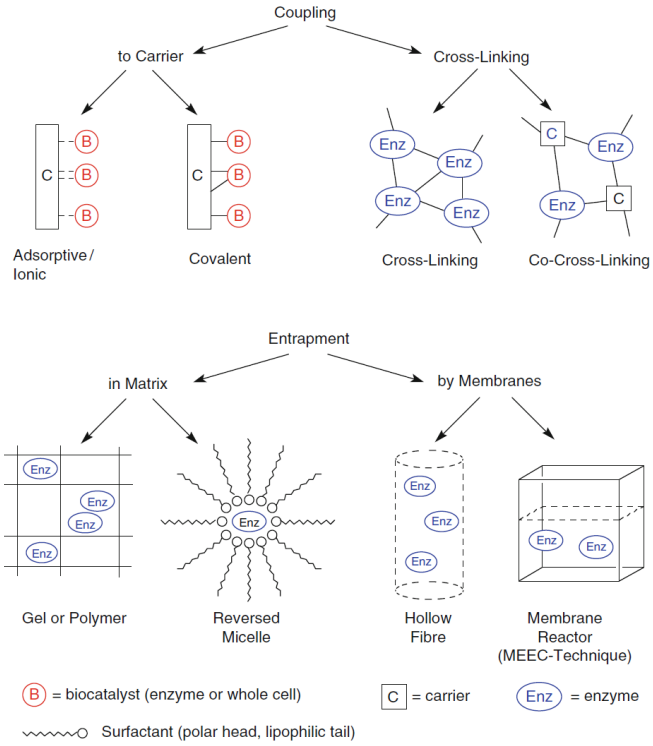
4.1. General aspects

In practice, three significant drawbacks are often encountered in enzyme-catalyzed reactions. Firstly, many enzymes are not sufficiently stable under the operational conditions and they may lose catalytic activity due to auto-oxidation, self-digestion or denaturation by the solvent, the solutes or due to mechanical shear forces. Secondly, enzymes are water-soluble molecules and their repeated use, which is important to ensure their economic application, is problematic due to the fact that they are difficult to recover from aqueous systems and to separate from substrates and products. Thirdly, the productivity of industrial processes, measured as the space-time yield is often low due to the limited tolerance of enzymes to high concentrations of substrate and product [38].

These problems may be overcome by 'immobilization' of the enzyme. This technique involves either the attachment of an enzyme onto a solid support via coupling onto a carrier or linkage of the enzyme molecules to each other via cross-linking. Alternatively, the biocatalyst may be confined to a restricted area from which it cannot leave but where it remains catalytically active by entrapment into a solid matrix or a membrane-restricted compartment. Thus, homogeneous catalysis using a native enzyme turns into heterogeneous catalysis when immobilized biocatalysts are employed [32].

4.2. Enzyme immobilization

Enzyme immobilization is an important aspect of the biocatalytic process especially for industrial biotechnology. Immobilized enzymes offer many advantages such as robust biocatalyst, economic process, stability and recyclability of the enzyme. Sometimes, as it is the case of lipase enzyme, the enzyme is apparently activated after the immobilization step. Depending on the immobilization technique, the properties of the biocatalyst such as stability, selectivity, rate constant and K_M value, pH and temperature characteristics, may be significantly altered. The immobilization approaches developed during the time comprises at least four different strategies: adsorption, covalent attachment, cross-linker and entrapment [32].



Scheme. 6. Principles of immobilization techniques [1].

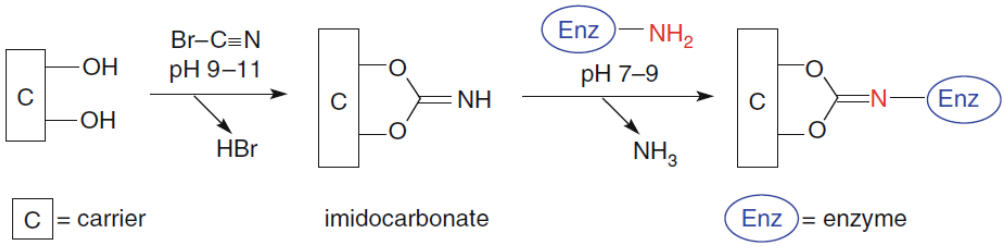
4.2.1. Immobilization on Support

Adsorption of a biocatalyst onto a water-insoluble macroscopic carrier is the easiest and oldest method of immobilization. It may be equally well applied to isolated enzymes as well as to whole viable cells. Adsorbing forces are of different types, such as van der Waals (London) forces, ionic interactions, and hydrogen bonding, and are all relatively weak. The appealing feature of immobilization by adsorption is the simplicity of the procedure. As a result of the weak binding forces, losses in enzyme activity are usually low, but desorption from the carrier may be caused by even minor changes in the reaction parameters, such as a variation of substrate concentration, the solvent, temperature, or pH. Examples of materials that have been used as carriers: activated charcoal, alumina, silica, diatomaceous earth (Celite), cellulose, controlled-pore glass, and synthetic resins. In contrast to the majority of enzymes, which preferably adsorb to materials having a polar surface, lipases are better adsorbed onto lipophilic carriers due to their peculiar physicochemical character. Adsorption is the method of choice when enzymes are used in lipophilic organic solvents, where desorption cannot occur due to their insolubility in these media [39].

[1] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

Due to their polar surfaces, ion exchange resins can adsorb proteins, thus, they have been widely employed for enzyme immobilization. Both cation exchange resins, and anion exchange resins, or sephadex, are used industrially. Although the binding forces are stronger than the forces involved in simple physical adsorption, ionic binding is particularly susceptible to the presence of other ions, and as a consequence, proper maintenance of ion concentrations and pH is important for continued immobilization by ionic binding and for prevention of desorption of the enzyme [40].

Covalent binding of an enzyme onto a macroscopic carrier leads to the irreversible formation of stable chemical bonds, thus inhibiting leakage completely. A disadvantage of this method is that rather harsh conditions are required since the biocatalyst must undergo a chemical reaction. Consequently, some loss of activity is always observed. As a rule of thumb, each bond attached to an enzyme decreases its native activity by about one fifth. Consequently, residual activities generally do not exceed 60–80% of the activity of the native enzyme, and values of around 50% are normal. The functional groups of the enzyme which are commonly involved in covalent binding are nucleophilic. In general, covalent immobilization involves two steps: activation of the carrier with a reactive 'spacer' group, and enzyme attachment. Since viable cells usually do not survive the drastic reaction conditions required for the formation of covalent bonds, this type of immobilization is only recommended for isolated enzymes. Porous glass is a popular inorganic carrier for covalent immobilization. A novel approach to immobilization of enzymes via covalent attachment is the use of stimulus-responsive 'smart' polymers, which undergo dramatic conformational changes in response to small alterations in the environment, such as temperature, pH, and ionic strength [41].

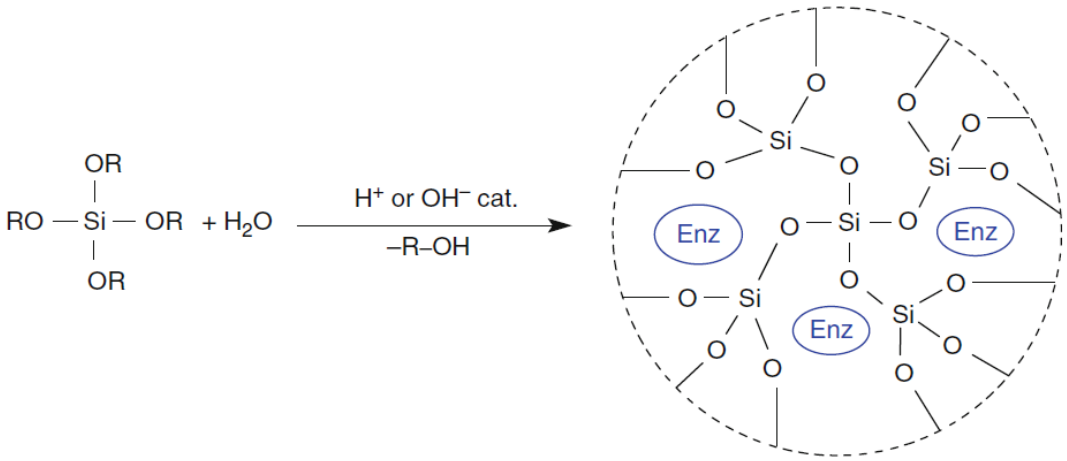


Scheme. 7. Covalent immobilization of enzymes onto natural polymers[m]

[m] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

4.2.2. Entrapment

In case an enzyme does not tolerate direct binding, it may be physically 'encaged' in a macroscopic matrix. To ensure catalytic activity, it is necessary that substrate and product molecules can freely pass into and out of the macroscopic structure [38]. Due to the lack of covalent binding, entrapment is a mild immobilization method which is also applicable to the immobilization of viable cells. Entrapment into a biological matrix such as agar gels, alginate gels, or k-carrageenan is frequently used for viable cells. In order to circumvent the disadvantages of gels based on biological materials, more stable inorganic silica matrices formed by hydrolytic polymerization of metal Si-alkoxides became popular. This so-called sol-gel process is initiated by hydrolysis of a tetraalkoxysilane of type Si(OR)4, with R being a short-chain alkyl group such as n-propyl or n-butyl, in the presence of the enzyme. A tight network which is able to contain isolated enzyme molecules may be obtained by polymerization of synthetic monomers such as polyacrylamide in the presence of the enzyme. It is obvious that the harsh conditions required for the polymerization makes this method inapplicable to whole cells [42].

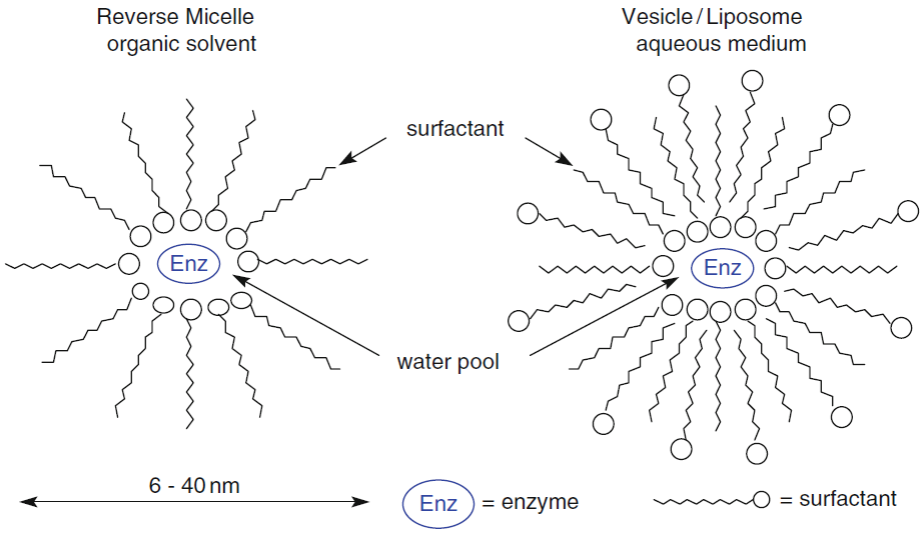


Scheme. 8. Entrapment of enzymes in silica sol-gels[n]

Enzymes may be enclosed in a restricted compartment bordered by a membrane. Although this does not lead to 'immobilization', it provides a restricted space for the enzyme, which is separated from the rest of the reaction vessel. Small substrate and/or product molecules can freely diffuse through the pores of the membrane, but the large enzyme cannot. The separation of a reaction volume into compartments by membranes is a close imitation of 'biological immobilization' within a living cell. Many enzymes are membrane-bound in order to provide a safe micro-environment for them within the cell. Two general methods exist for the entrapment of enzymes into membrane-restricted compartments [38].

[n] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

Firstly, micelles and vesicles in which mixtures of certain compositions containing water, an organic solvent and a detergent give transparent solutions in which the organic solvent is the continuous phase. The water is present in microscopic droplets, and are surrounded by the surfactant. The whole structure is embedded in the organic solvent and represents a micelle which is turned inside out. It is therefore termed a 'reverse micelle'. The latter are mimics for the micro-environment of the cell and can be regarded as artificial micro-cells. Thus, they provide high enzyme activity. On the other hand, when water constitutes the bulk phase, micelles may be formed by a symmetrical double layer of surfactant. The latter structure constitutes a 'vesicle' (liposome) [38].

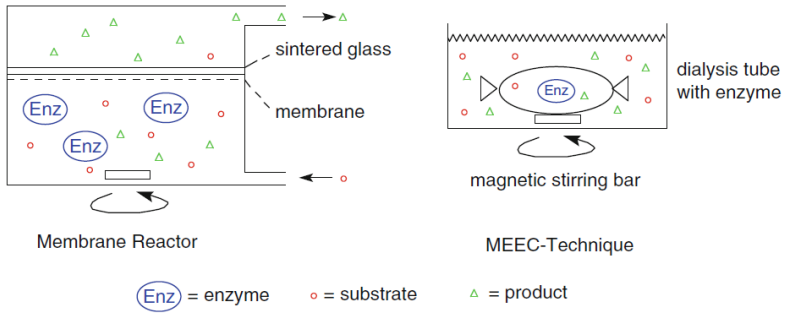


Scheme. 9. Entrapment of enzymes in reversed micelles and vesicles[o]

Secondly, synthetic membranes are a practical alternative to the use of sensitive biological matrices is the use of synthetic membranes based on polyamide or polyethersulfone. They have long been employed for the purification of enzymes by ultrafiltration, which makes use of the large difference in size between high-molecular biocatalysts and small substrate/products molecules. Synthetic membranes of defined pore size, covering the range between 500 and 300,000 Da, are commercially available at reasonable cost. The biocatalyst is detained in the reaction compartment by the membrane, but small substrate/product molecules can freely diffuse through the pores of the barrier. This principle allows biocatalytic reactions to be performed in highly desirable continuous processes. Furthermore, disadvantages caused by heterogeneous catalysis, such as mass-transfer limitations and alteration of catalytic properties, are largely avoided. A variety of synthetic membranes are available in various shapes such as foils or hollow fibers. A simplified form of a membrane reactor which does not require any special equipment may be obtained by using an enzyme solution enclosed

[o] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

in dialysis tubing like a tea bag. In some enzyme-catalyzed processes it is of an advantage to couple an additional reaction onto the process in order to drive an unfavorable equilibrium in the desired direction. Very often, however, the harsh reaction conditions required for the auxiliary step are incompatible with the enzyme. In such cases, a membrane may be used to separate the enzyme-catalyzed reaction from the auxiliary process, while the chemical intermediates can pass freely through the barrier [43].



Scheme. 10. Principle of a membrane reactor and the membrane-enclosed enzyme catalysis (MEEC)-Technique [p].

4.2.3. Cross-linking

The use of a carrier inevitably leads to ‘dilution’ of catalytic activity owing to the introduction of a large proportion of inactive ballast, ranging from 90 to > 99%, which leads to reduced productivities. This can be avoided via attachment of enzymes onto each other via ‘cross-linking’ through covalent bonds. [38].

By cross - linking, insoluble high-molecular aggregates are obtained. R. A. Sheldon was the one who proposed this structure. The enzyme molecules may be cross-linked either with themselves or may be co-crosslinked with other inactive ‘filler’ proteins such as albumins. The most widely used bifunctional reagents used for this type of immobilization are a,o-glutardialdehyde, dextran polyaldehyde dimethyl adipimidate, dimethyl suberimidate, and hexamethylenediisocyanate or -isothiocyanate. The advantage of this method is its simplicity, but it is not without drawbacks. The soft aggregates are often of a gelatine-like nature, which prevents their use in packed-bed reactors [44].

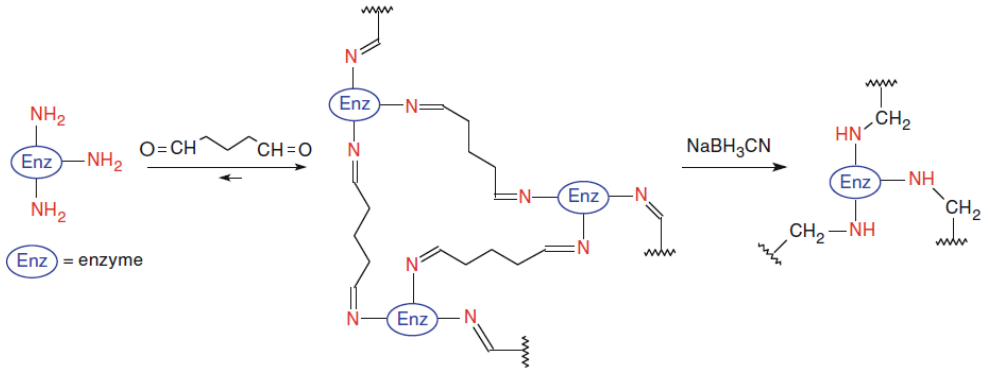
Furthermore, the activities achieved are often limited due to diffusional problems, since many of the biocatalyst molecules are buried inside the complex structure which impedes their access by the substrate. The reactive groups involved in the crosslinking of an enzyme are not only free amino functions but also sulfhydryl- and hydroxyl groups. Crosslinking can be performed with dissolved

[p] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

(monomeric) proteins, and also by using them in microcrystalline or amorphous form. Due to the close vicinity of the individual enzyme molecules, cross-linking is particularly effective for oligomeric proteins [41].

The first cross-linking of a crystalline enzyme by glutaraldehyde had the main objective to stabilize enzyme crystals for X-ray diffraction studies but they also showed that catalytic activity was retained. The use of cross-linked enzyme crystals (CLECs). CLECs are robust, highly active immobilized nzymes of controllable particle size. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, renders them ideally suited for industrial biotransformations [44].

However, an inherent disadvantage of CLECs is the need to crystallize the enzyme, which is often a laborious procedure requiring enzyme of high purity. There was also reasoned that subsequent cross-linking of these physical aggregates would render them permanently insoluble while maintaining their pre-organized superstructure, and, hence their catalytic activity. This indeed proved to be the case and led to the development of a new family of immobilized enzymes: cross-linked enzyme aggregates (CLEA). The CLEA methodology essentially combines purification and immobilization into a single unit operation that does not require a highly pure enzyme [45].



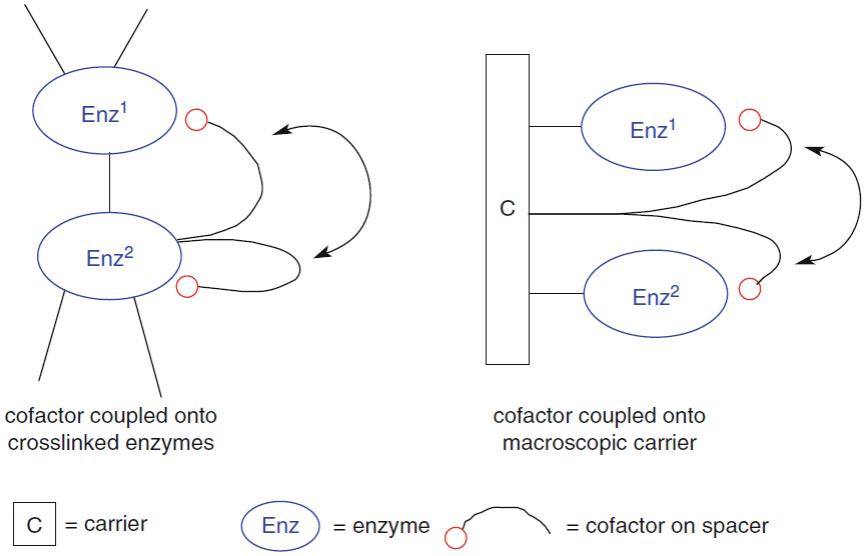
Scheme. 11. Crosslinking of enzymes by glutaraldehyde [r].

4.2.3. Co-factor immobilization

All of the above-mentioned immobilization techniques can readily be used for enzymes which are independent of cofactors and for those in which the cofactors are tightly bound. For enzymes, which depend on charged cofactors, such as NAD(P)H or ATP, which readily dissociate into the medium, co-immobilization of the cofactor is often required to ensure a proper functioning of the overall system. Two solutions to this problem have been put forward. Firstly, the cofactor may be

[r] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

bound onto the surface of a crosslinked enzyme or it may be attached to a macroscopic carrier. In either case it is essential that the spacer arm is long enough so that the cofactor can freely swing back and forth between both enzymes. However, these requirements are very difficult to meet in practice. Secondly, in membrane reactors a more promising approach has been developed in which the molecular weight of the cofactor is artificially increased by covalent attachment of large groups such as polyethylene glycol, polyethylenimine, or dextran moieties. Although the cofactor is freely dissolved, it cannot pass the membrane barrier due to its high molecular weight [41].



Scheme. 12. Co-immobilization of cofactors [q].

[q] Faber, K., Biotransformations in Organic Chemistry. A Textbook. Sixth revised and corrected edition, Springer, 2011, Chapter 3. Special Techniques: Immobilization, pgs. 356-367.

Aim of The Dissertation Thesis

We propose a detailed study for designing multi-enzyme biocatalyst for cascade biocatalytic process. Using classic approaches for the enzyme immobilization, the biocatalytic molecules will be coupled in the biocatalyst design based on specific approach. In this way, we expected to control the enzyme immobilization separately for each enzymatic component.

The biocatalyst design will be used for different enzymatic cocktails such as enzyme with the coenzymatic component providing the natural co-factor or the co-factor regeneration, and also multi-enzyme for cascade process.

Our intention is to construct multi-enzyme biocatalyst for enzymatic cascade processes dedicated to monoterpenoid transformation leading to flavors and fragrances products. Terpenes are a large class of compounds produced by plants, and they are very abundent raw materials. Therefore, it wold be a very useful study in order to designe a catalyst capable to transform this abundent substrate in diols, which can be used as intermediates in pharmaceutical industry.

The biocatalyst design comprises lipase and hydrolyze as the biological components. Lipase will catalyze the conversion of the substrate into epoxide derivatives while hydrolysed will open the epoxide cycle through enantioseletive way in order to generate the diol derivatives enantiopure.

Co-immobilization of both proposed enzymes will be performed using cross-linking as well as entrapment approaches. In this way, we suppose to have a minimum effect of the immobilization approach on the enzymatic catalytic activity.

The biocatalyst developed in this way will be tested for alpha/betha-pinene, L/D-limonene and alpha/betha-phellandrene substrates.

Conclusions

In conclusion, enzyme biocatalysts have more advantages compared to the chemical catalysts. The most notable ones are the selectivity and specificity, environmentally friendly nature of biocatalyst, by being biodegradable, working under mild conditions, and producing no toxic byproducts.

Enzymatic catalysis with purified enzyme is the best choice of biocatalysis, improving the process selectivity and limiting the interferences of the sample matrix more complex in the case of the content of the biological cell.

Also, the immobilization of enzyme allows to solve some specific issues, such as the possibility of enzyme deactivation during the reaction conditions, or the difficulty of recovering the enzymes from aqueous environment, are resolved. Immobilization provides enzymes with a higher stability and a longer lifespan.

Because enzymes are compatible with each other, multi-enzymatic systems can be created to perform a high number of reactions, and in this way a multi-enzymatic system can be designed to appeal a desired pathway. In this context, co-immobilization of enzymes is also a promising perspective.

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