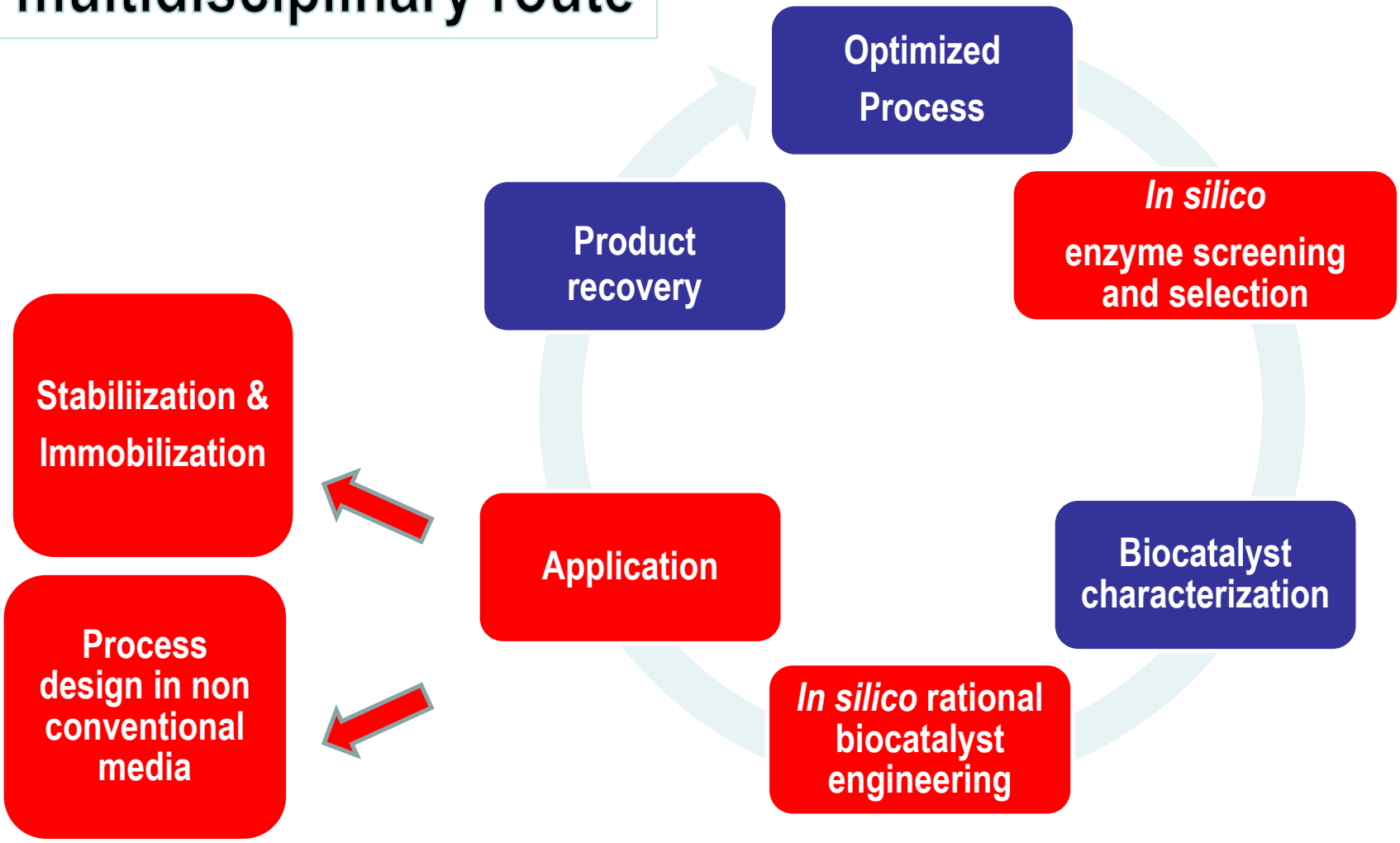
Biocatalysts in chemical reactions and organic synthesis

Strategies for planning rationally biocatalyzed reactions

Developing a biocatalyzed process: a multidisciplinary route

Contribution from chemists?



The reaction: identifying the most suitable enzyme and the reaction conditions to carry out the biotransformation at lab-scale (proof of concept)

The protein: Trying to predict enzyme properties (stability, ractivity, selectivity) on a rational basis in order to preserve such features as long as possible throughout the biotrasformation

The process: how making the enzyme and the biotransformayion effective at large (industrial) scale, the process ecomically viable and environmentally sustainable

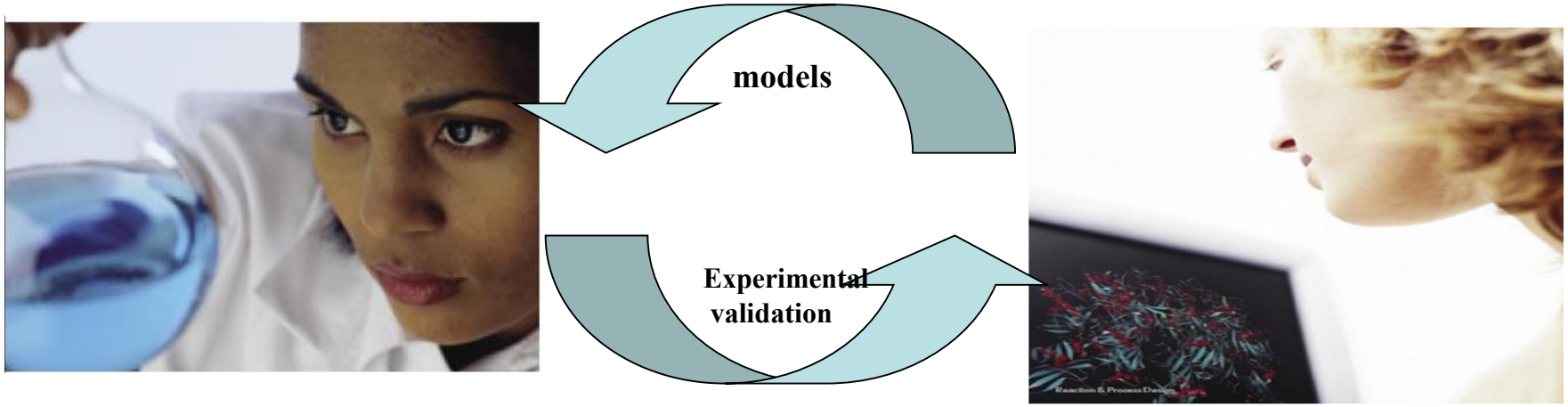
Aim:
making enzymes working under unconventional «desperate» conditions while keeping most of their catalytic activity

Solid to solid:

Organic solvent

Bulk –solventless - transformation

Integrating experimental and computational methods



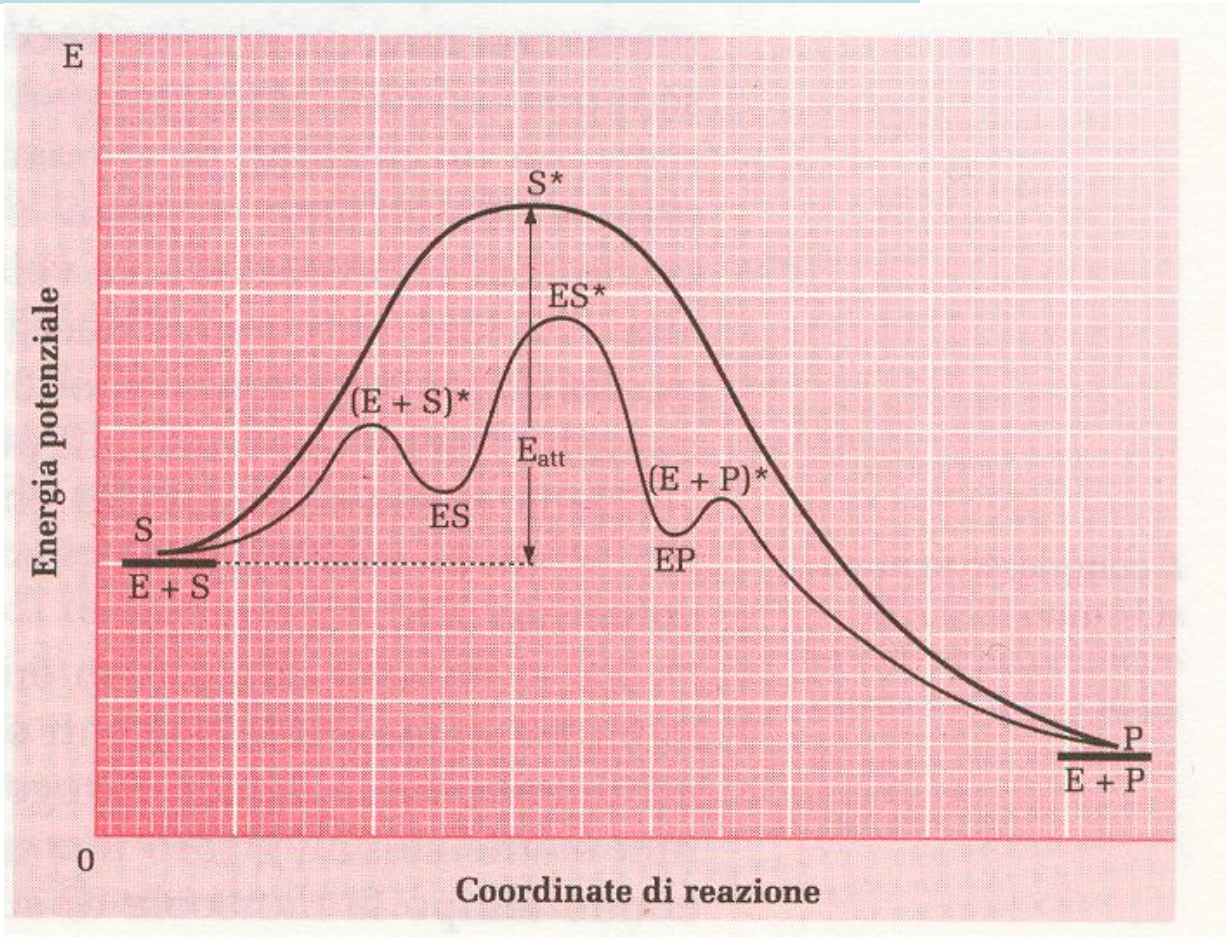
Computational simulations and predictions must be competitive with experimental approaches & time scale

Identifying a suitable enzyme and predict its ability to transform a certain substrate with the desidered chemo-regio-stereo-selectivity (when required)

How can we study and predict enzyme properties?

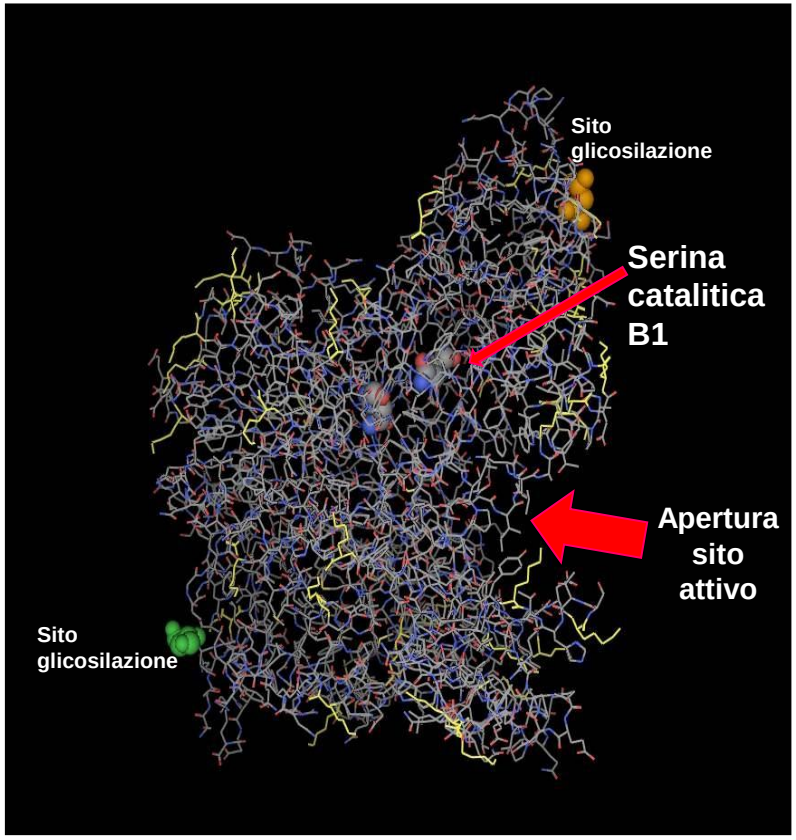
The theory:

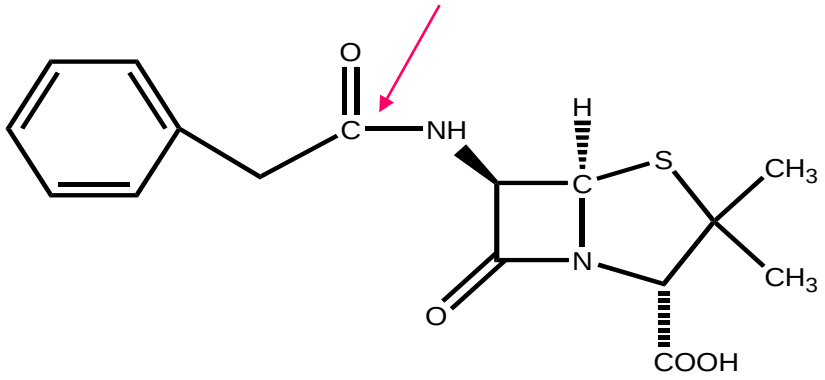
1) **Enzyme-substrate recognition**
2) **Catalysis and Transformation**



$$E + S \rightleftharpoons ES \longrightarrow ES^* \longrightarrow EP \rightleftharpoons E + P$$

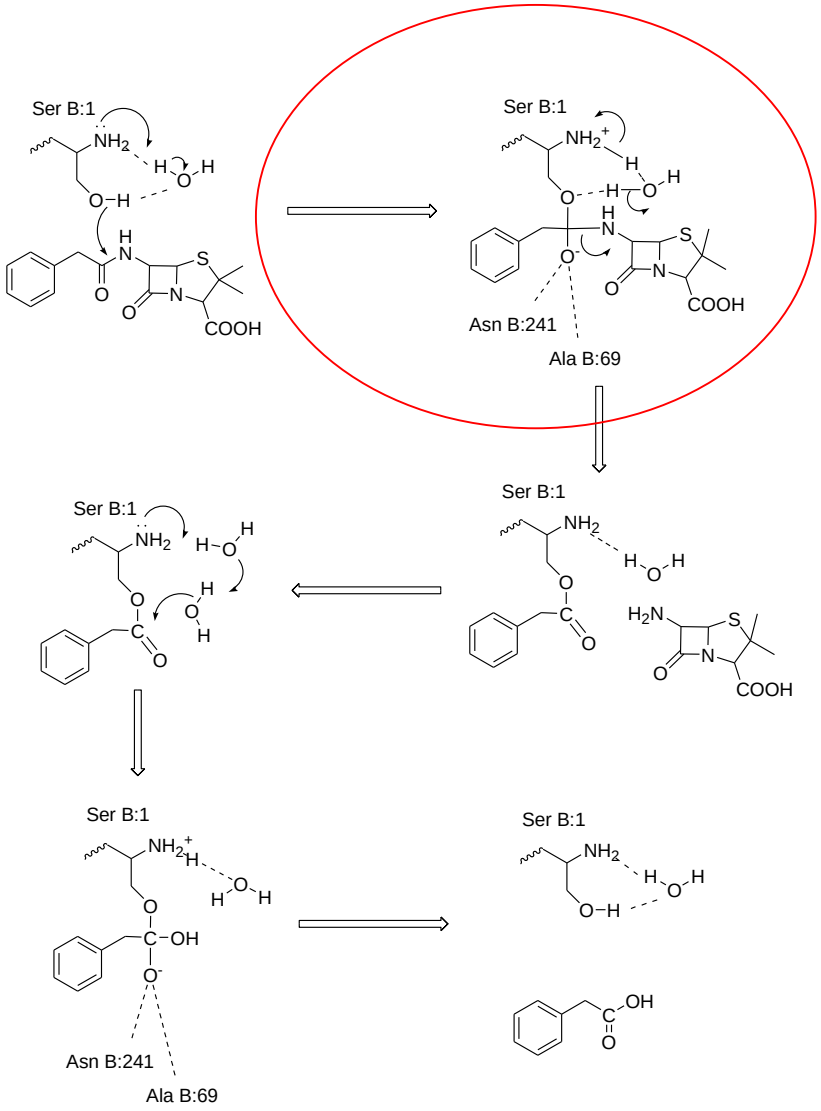
Molecular simulation for the study of enzyme-substrate recognition: penicillin G inside penicillin G amidase (PGA), a serine hydrolase (dimer)





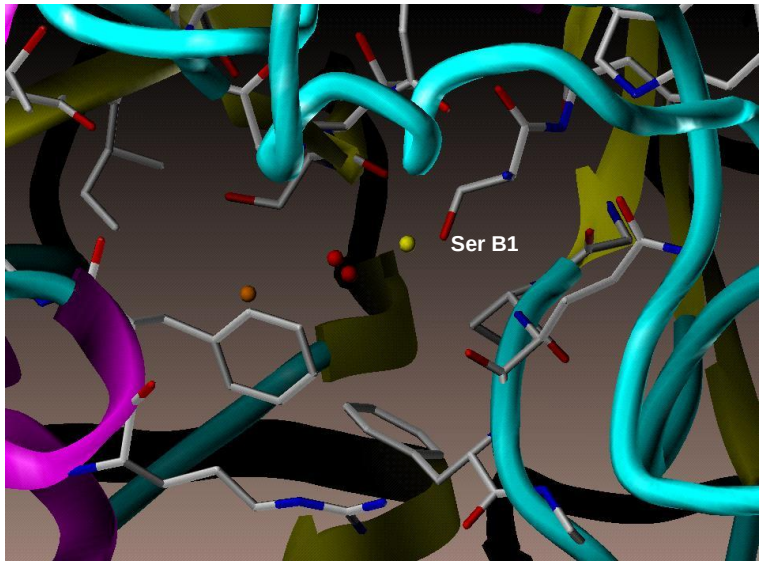
Penicillin G

Mechanism of action of PGA



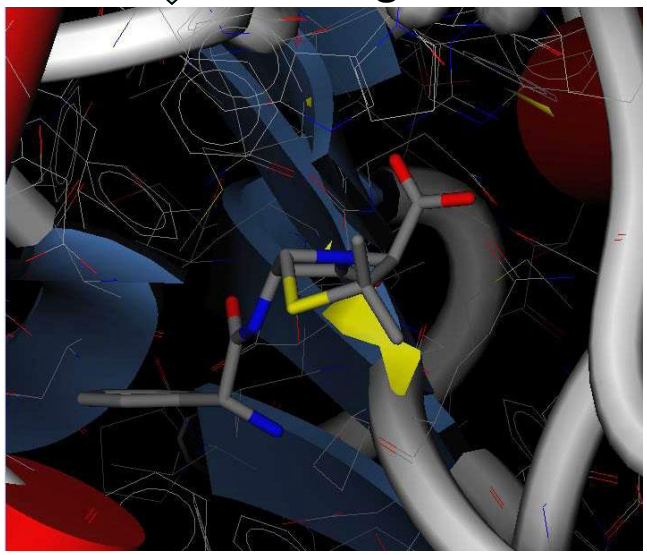
Tetrahedral intermediate

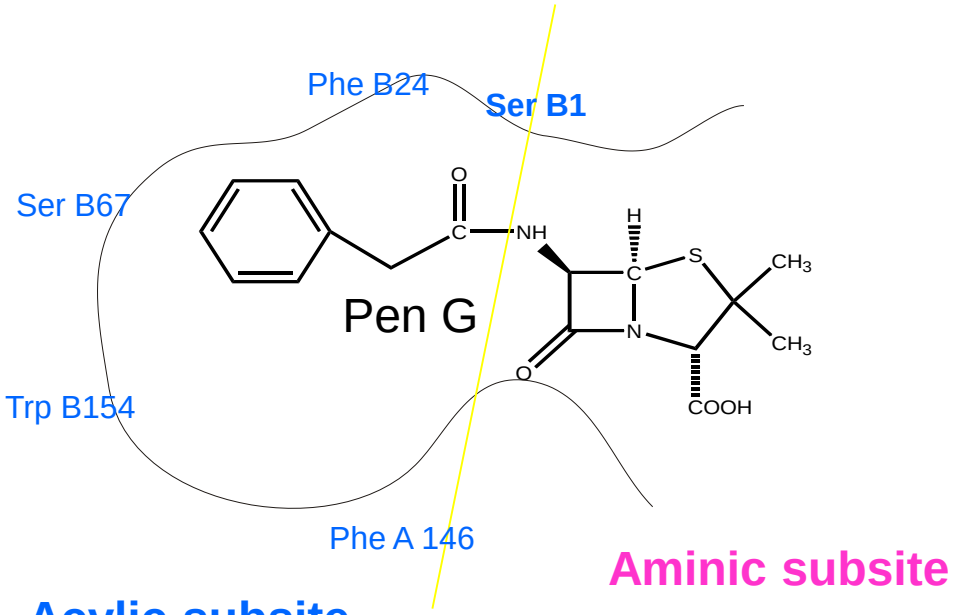
Penicillin G Amidase (PGA) recognizes substrates that are derivatives of phenylacetic acid



active site 3D structure with water molecules

Docking the substrate





Docking simulates enzyme-substrate recognition: affinity, Km

What is not accounted by docking?

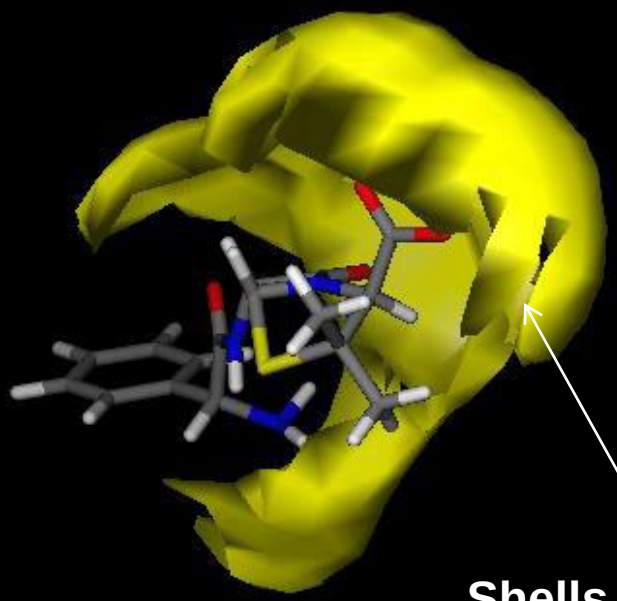
Physical chemical factors mostly related to solvation, desolvation, partition, diffusion

-***In the active site of the enzyme***
-***On the surface of the enzymes***

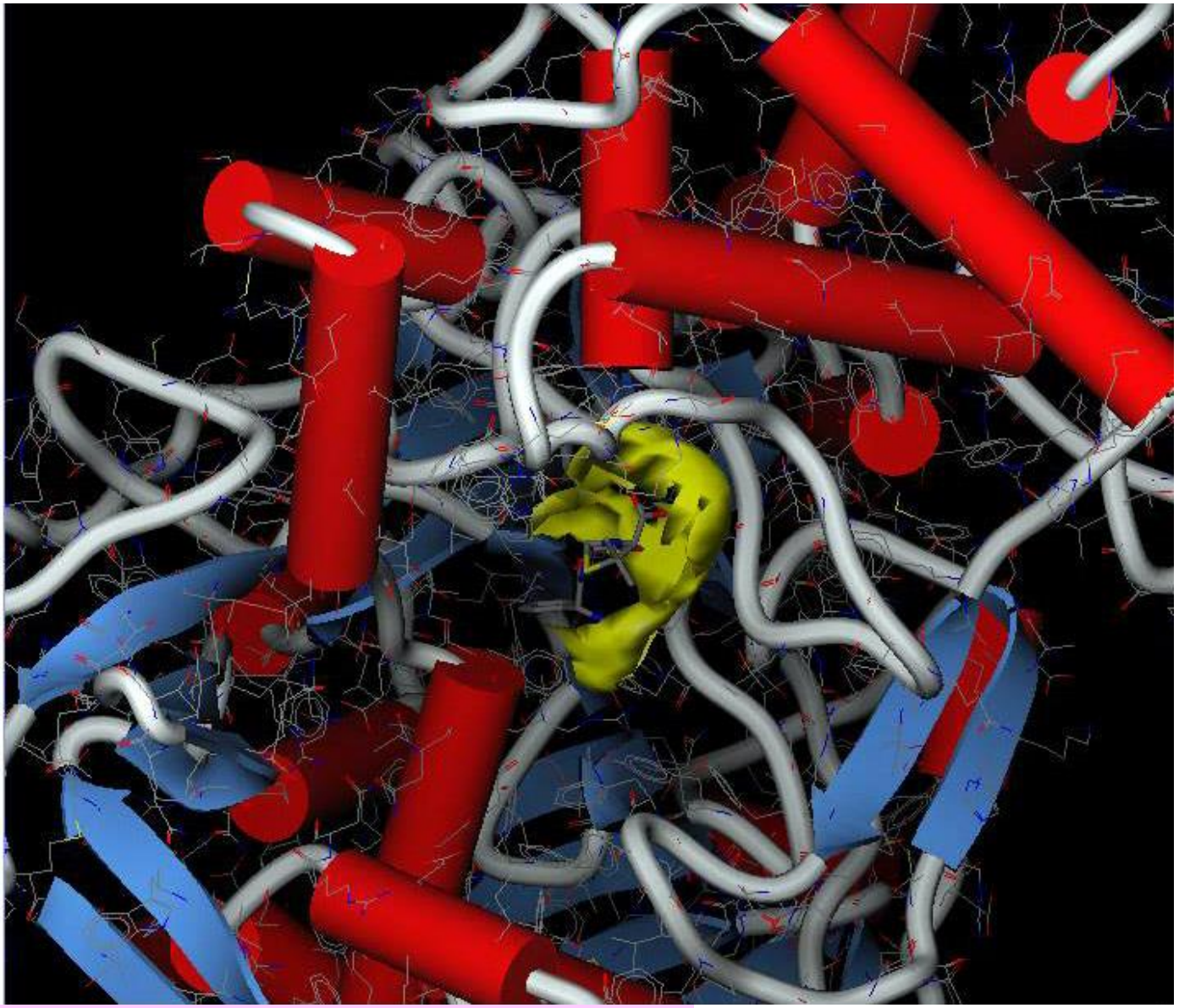
This is particularly relevant when enzymes work as heterogeneous catalysts, suspended in the medium

1.

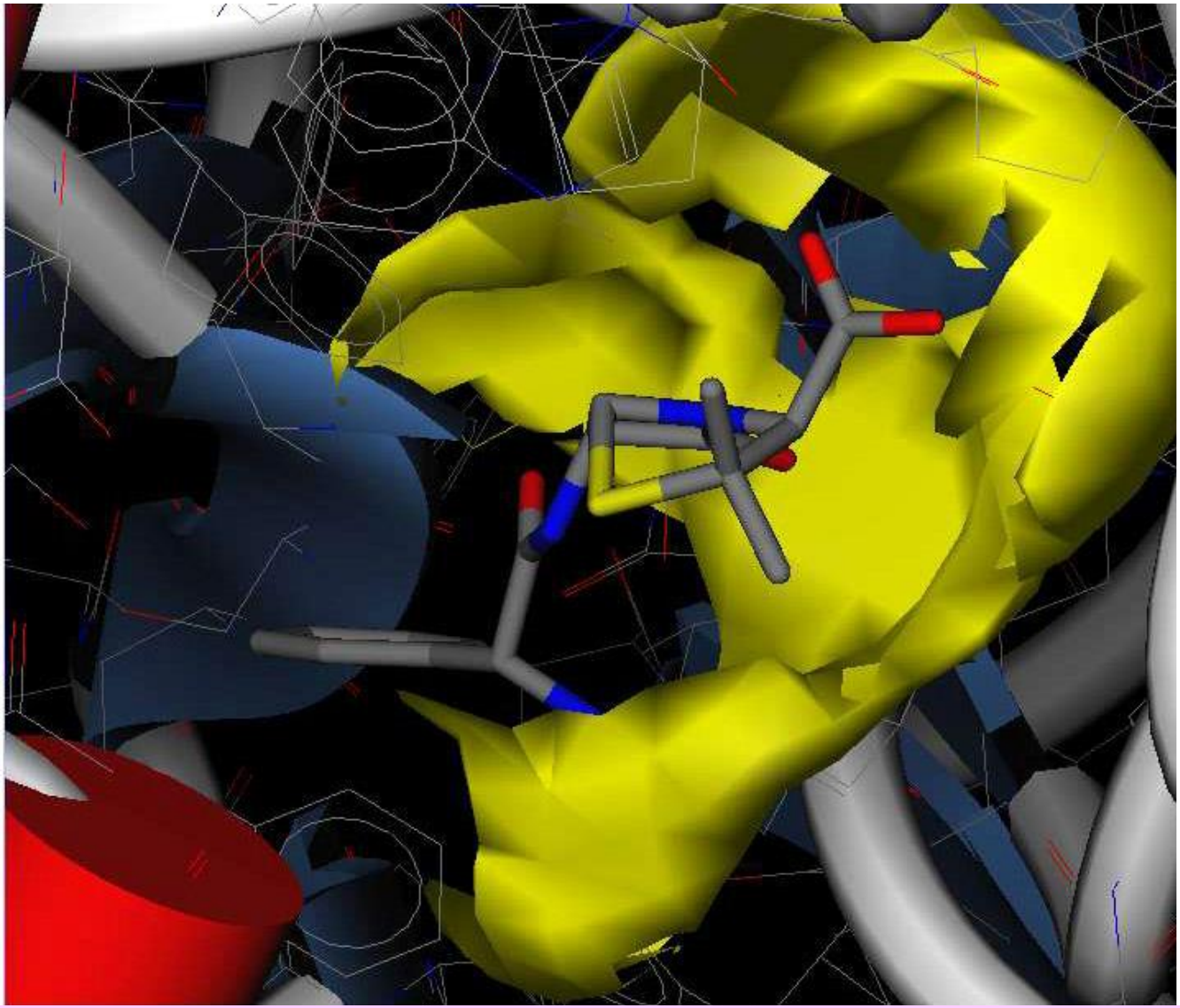
Penicillin before enzyme-substrate recognition



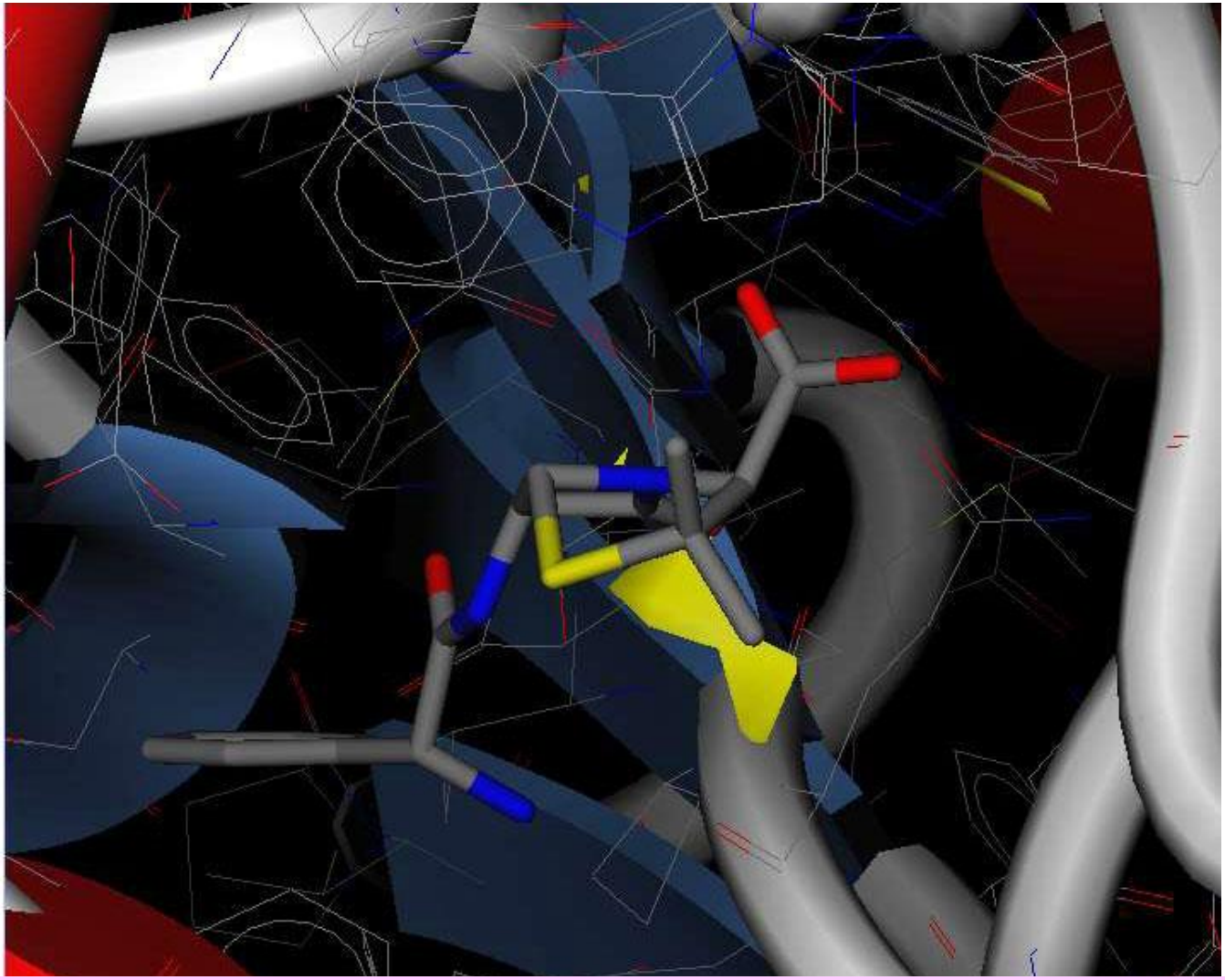
Shells of water solvating the substrate in the aqueous bulk medium



2. The substrate enters the active site

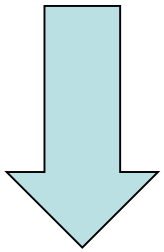


3. To enable enzyme substrate interactions/recognition, part of the water molecules must be displaced (desolvation)

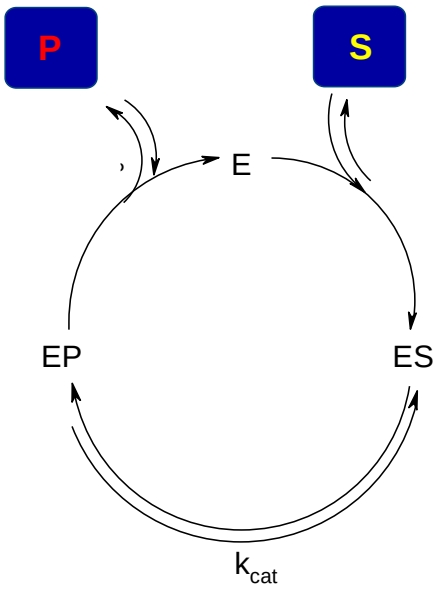


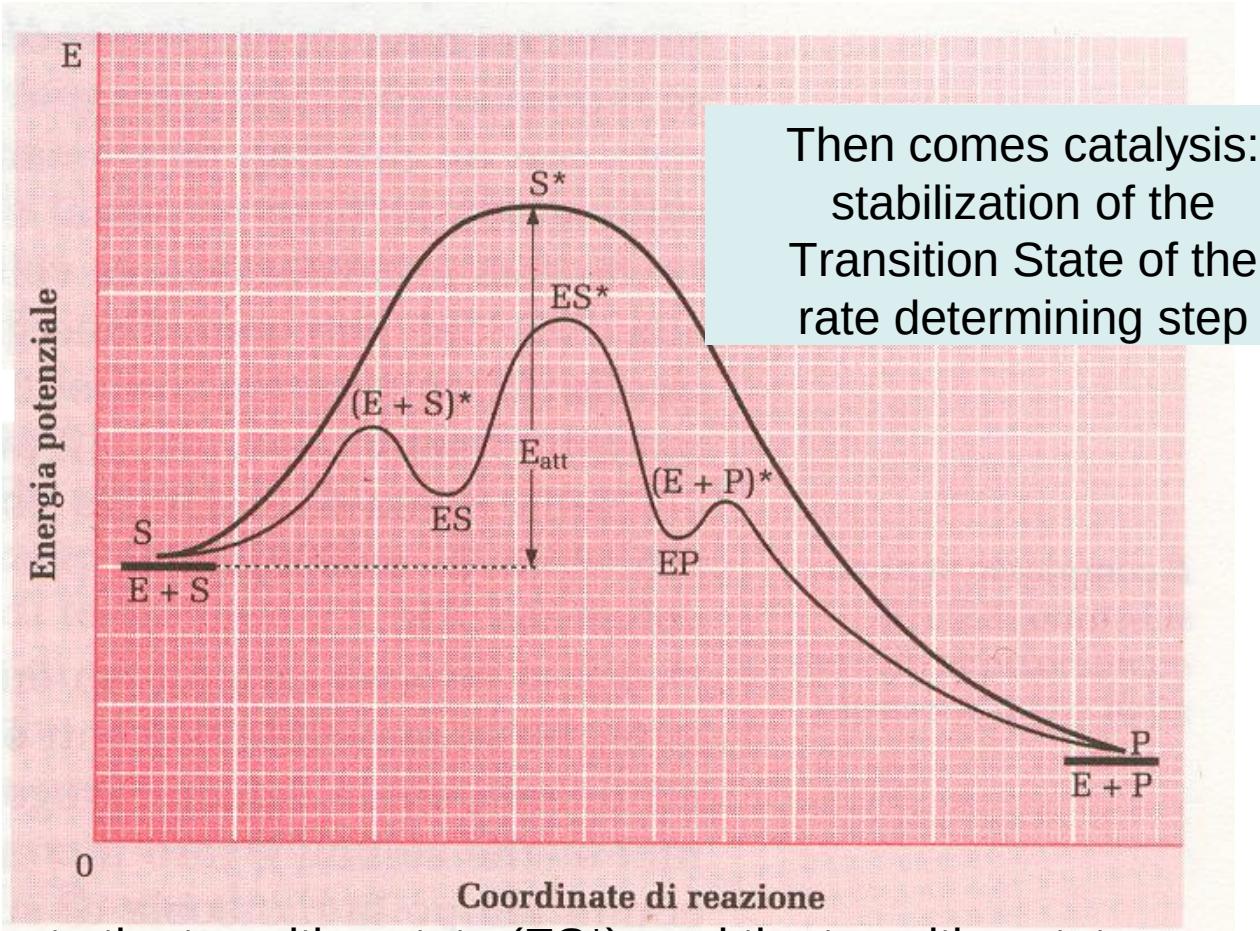
4. After displacement of water molecules, the substrate establish new electrostatic interactions with residues in the active site

Then comes catalysis: stabilization of the Transition State of the rate determining step



Kinetics !!!!



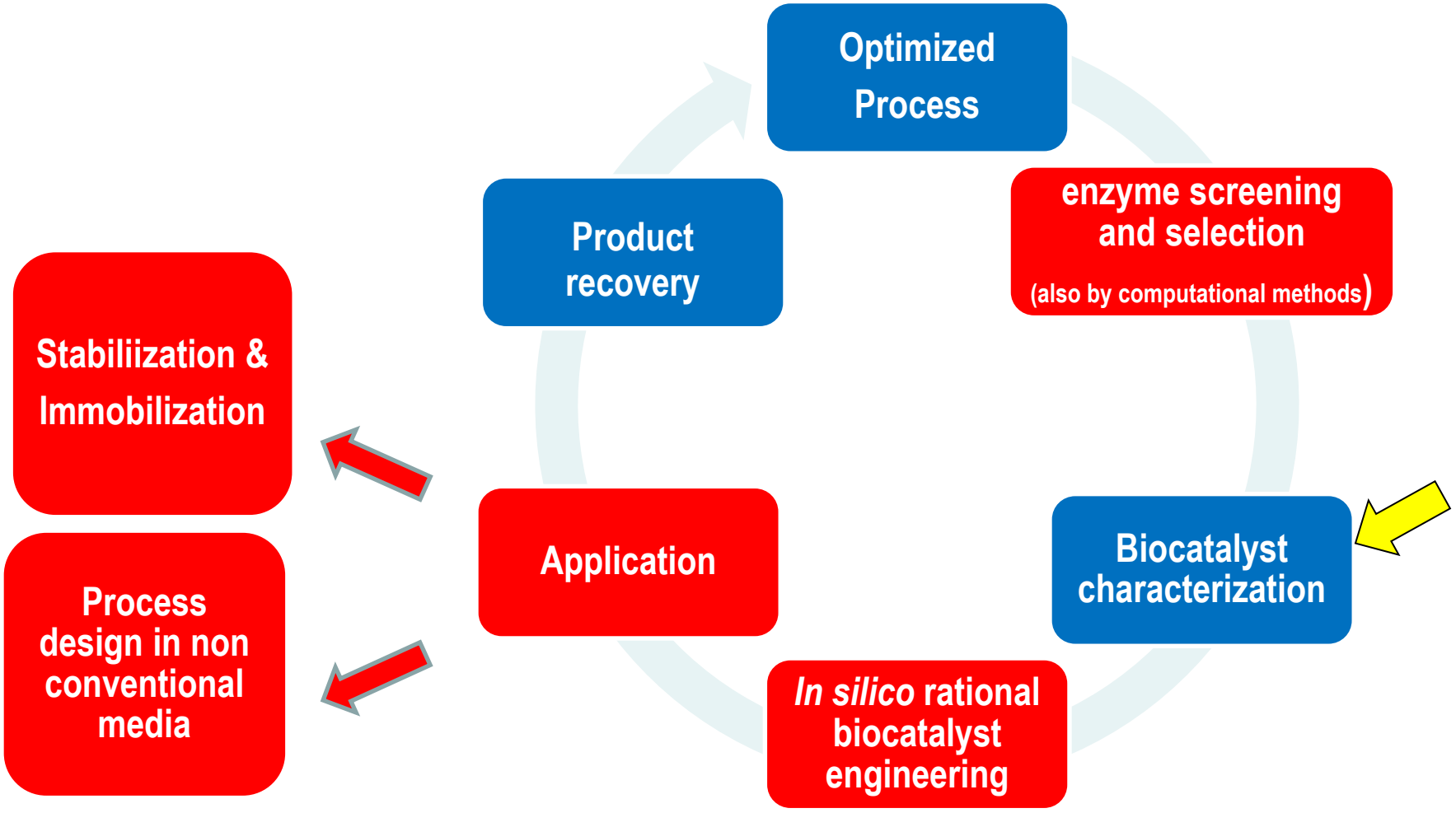


Then comes catalysis: stabilization of the Transition State of the rate determining step

ES complex must pass to the transition state (ES*); and the transition state complex must advance to an enzyme product complex (EP). The latter is finally competent to dissociate to product and free enzyme. The series of events can be shown as:

$$E + S \rightleftharpoons ES \longrightarrow ES^* \rightarrow EP \rightleftharpoons E + P$$

Enzymes are characterized by enzymologists in diluted buffers, therefore in conditions quite distant from the operational conditions in industry



Most often biocatalysts are used under non phisiological conditions, the so called:

non conventional media

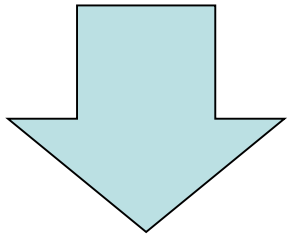
……anything different from a dilute aqueous solution.

*Most often a **multi-phase system**, some examples:*

Neat substrates + Native enzyme + Traces of solvent

Neat substrates + Immobilized enzyme

The point of view of kinetic studies in fundamental enzymology: enzyme solubilized in buffer



Most often models developed by enzymologists cannot be used in applied biocatalysis

Mass transfer and diffusion limitations are most often rate determining

Parameters and equation refer to simplified ideal conditions

Equazione di Michaelis – Menten (1913)

Leonor Michaelis
(1875–1949)

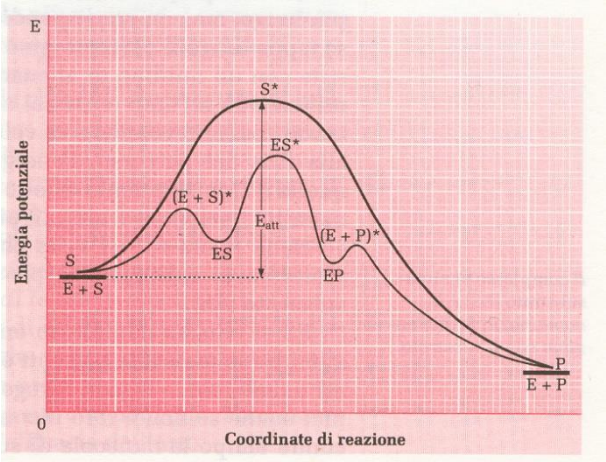
Maud Leonora Menten
(1879–1960)

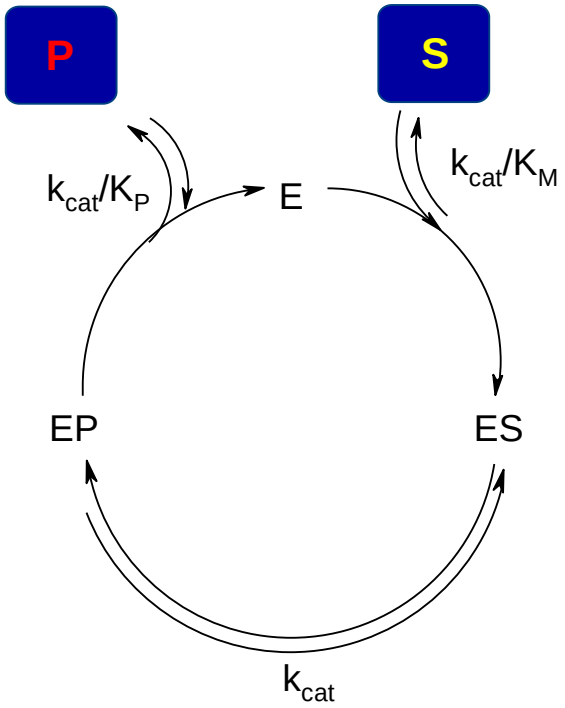
$$V = k_{cat}[E]_T \frac{[S]}{K_S + [S]}$$

oppure

$$v = V_{max} \frac{[S]}{K_M + [S]}$$

Parameters that describe enzyme efficiency in biocatalysis





$E + S \rightleftharpoons ES \longrightarrow ES^* \longrightarrow EP \rightleftharpoons E + P$

Specific activity: k_{cat}

k_{cat} (time^{-1})

First order rate constant for the conversion of ES, ES*, EP

All species involving E

What is really relevant in biocatalysis? Turnover number: biocatalyst productivity

In catalysis it refers to:

the number of moles of product formed per mole of catalyst over the reaction period.

dimensionless ratio:

(mol product) / (mol enzyme)

Specificity

k_{cat}/k_m specificity constant

Specific activity

affinity

K_{cat}/K_M must be calculated at low concentration of Substrate, when the enzyme is not saturated

Penicillin G amidase from different microorganisms display different specificity towards pen G

Enzima	Substrato	T(°C)	pH	k_{cat}/K_M ($M^{-1}s^{-1}$)
Penicillina amidasi				
E. coli	Penicillina G	25.0	7.8	4 800 000
A. faecalis	Penicillina G	25.0	7.8	10 000 000
K. citrofila	Penicillina G	25.0	7.8	3 000 000

Selectivity

k_{cat}/k_m specificity constant

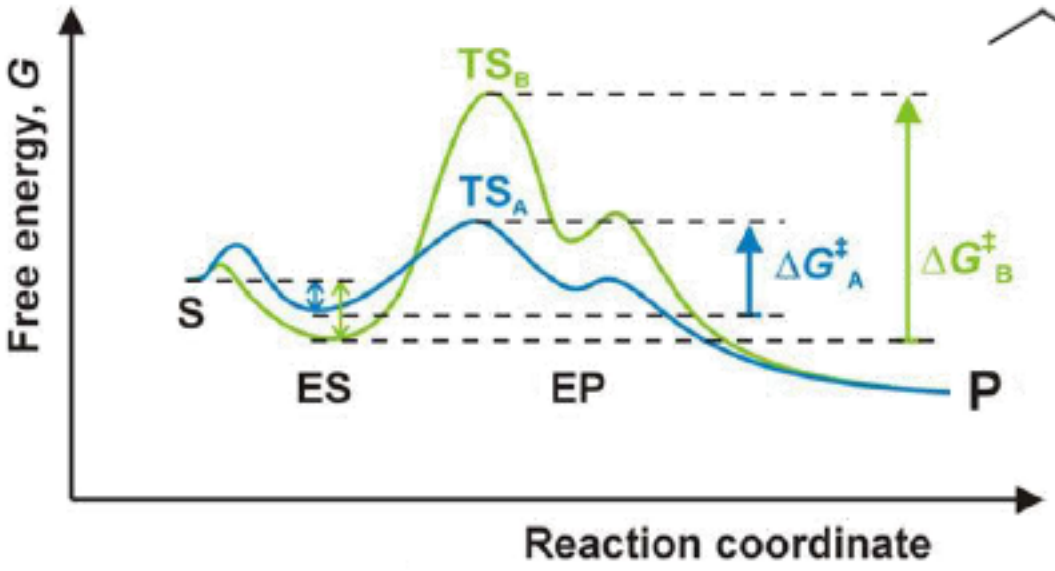
$$\text{selectivity} \quad \frac{(k_{cat}/k_m)_A}{(k_{cat}/k_m)_B}$$

Ratio between the specificity constants

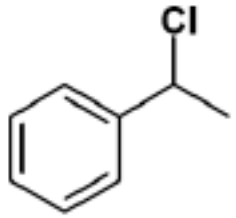
How the enzyme is able to discriminate between A and B

(chemo-regio-enantio-selectivity)

Enzyme selectivity: substrate A *vs* B



A



B

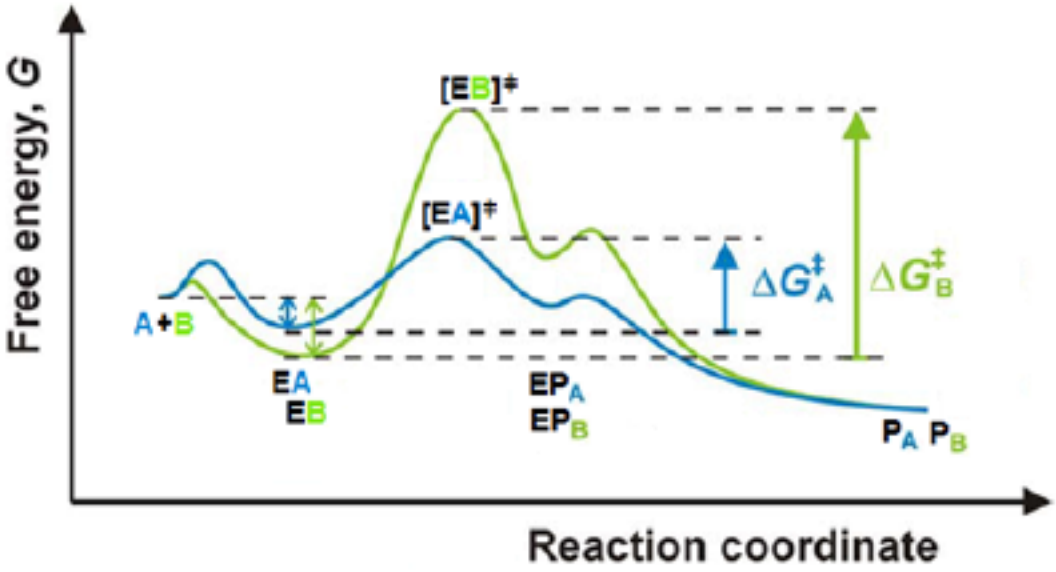
selettività: $\dfrac{(k_{cat}/k_m)_A}{(k_{cat}/k_m)_B}$

Selectivity depends on the ΔΔG activation of the RATE DETERMING STEP!

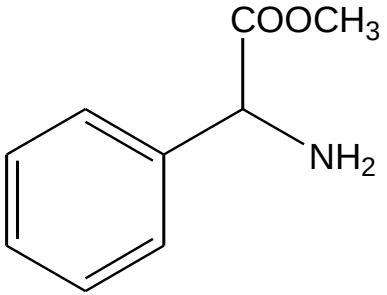
$\Delta\Delta G^{\#}$ di 5,71 kJmol^{-1} *v* x 10

$\Delta\Delta G^{\#}$ di 34 kJmol^{-1} *v* x 10^6

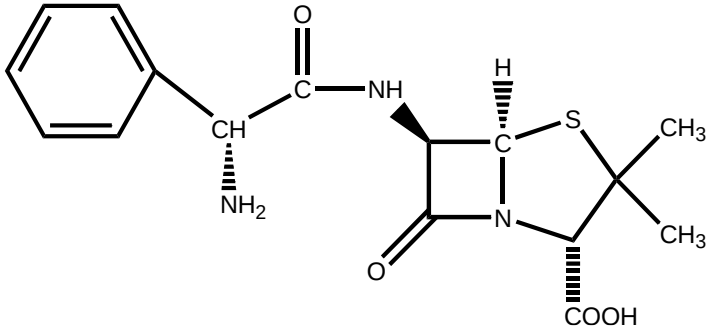
Enzyme enantioselectivity: enantiomer S *vs* R



$\Delta\Delta G^{\neq}$ [kcal/mol]	v_A/v_B	e.e. [%]
0.118	1.2	10
0.651	3	50
1.74	19	90
2.17	39	95
3.14	199	99
4.50	1,999	99.9



Phenylglycine



Only the R-enantiomer used for ampicillin semi-synthesis

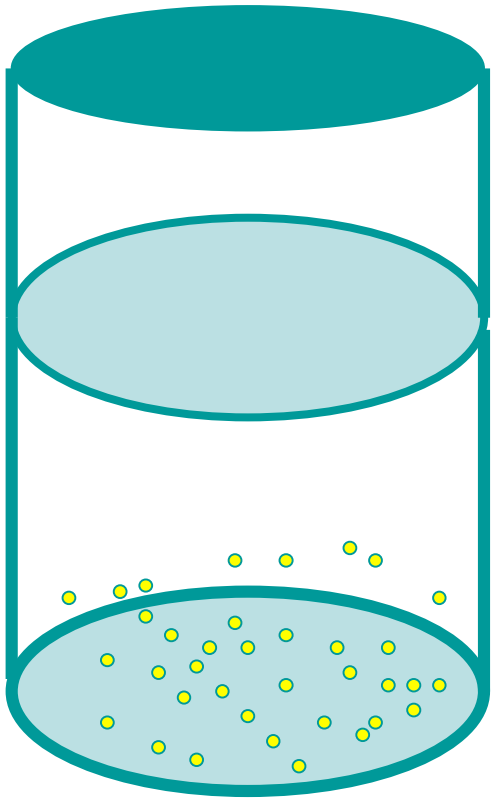
Substrato	Enzima	k_{cat}/K_M ($M^{-1}s^{-1}$)	Stereoselettività $(k_{cat}/K_M)_S/(k_{cat}/K_M)_R$
(*S*)-fenilglicil↓OMe (*R*)-fenilglici↓OMe	Penicillina amidasi da *E. coli*	550 1100	0.5
(*S*)-fenilglicil↓OMe (*R*)-fenilglicil↓OMe	α-Chimotripsina (bovino)	9.2 0.57	14
(*S*)-fenilglicil↓OMe (*R*)-fenilglicil↓OMe	Proteinasi K da *Tritirachium album*	0.6 0.3	2

Different hydrolases are able to hydrolyze the methyl ester of (R) and (S) phenylglicine but with different enantioselectivity

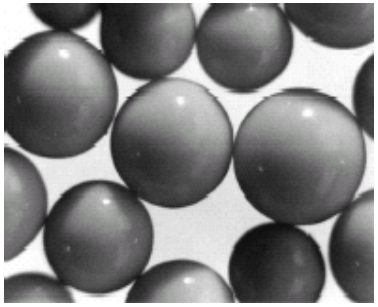
The process:
Biocatalysis in non conventional media

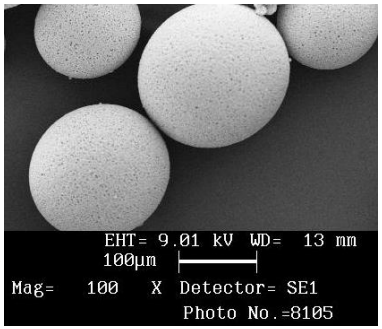
Biocatalysts in non-conventional media: multiphase systems

Anything different from a diluted aqueous solution



Immobilized Enzymes working in buffer or org. solvent





Enzymes active on substrates anchored on solid phases

Enzymes (or cells) in low water media:

Organic solvent
Neat substrates
Water/organic solvent
Ionic liquids
Supercritical fluids
Gases

Halling P.J., Current Opinion in Chemical Biology **4**, 74-80

How to control, investigate and understand the physical-chemical phenomena in biocatalyzed systems

How biocatalysts work under non-physiological conditions

Neat substrates + Native enzyme + Traces of solvent

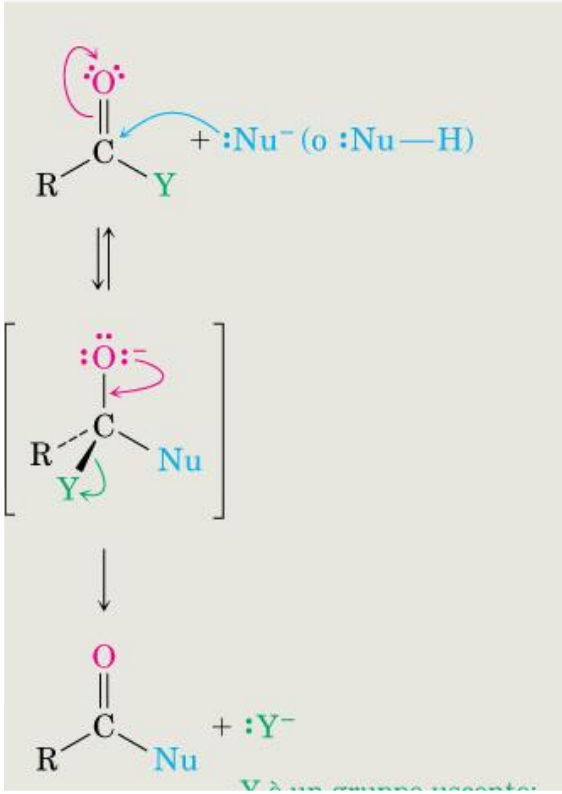
Neat substrates + Immobilized enzyme

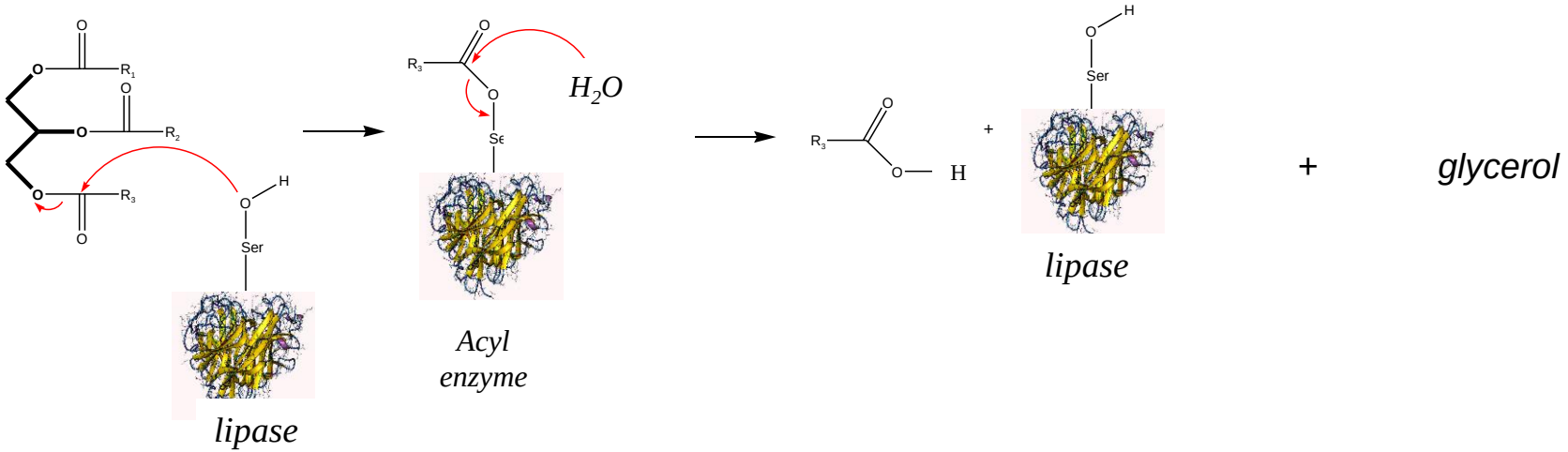
Low -water media: advantages

- Shift of thermodynamic equilibrium
- Recovery of products
- Solubility of hydrophobic substrates
- Microbial contamination negligeble
- Side-reactions reduced
- Simple recycling of the catalyst

Shifting the equilibrium towards the synthesis of acyl bond

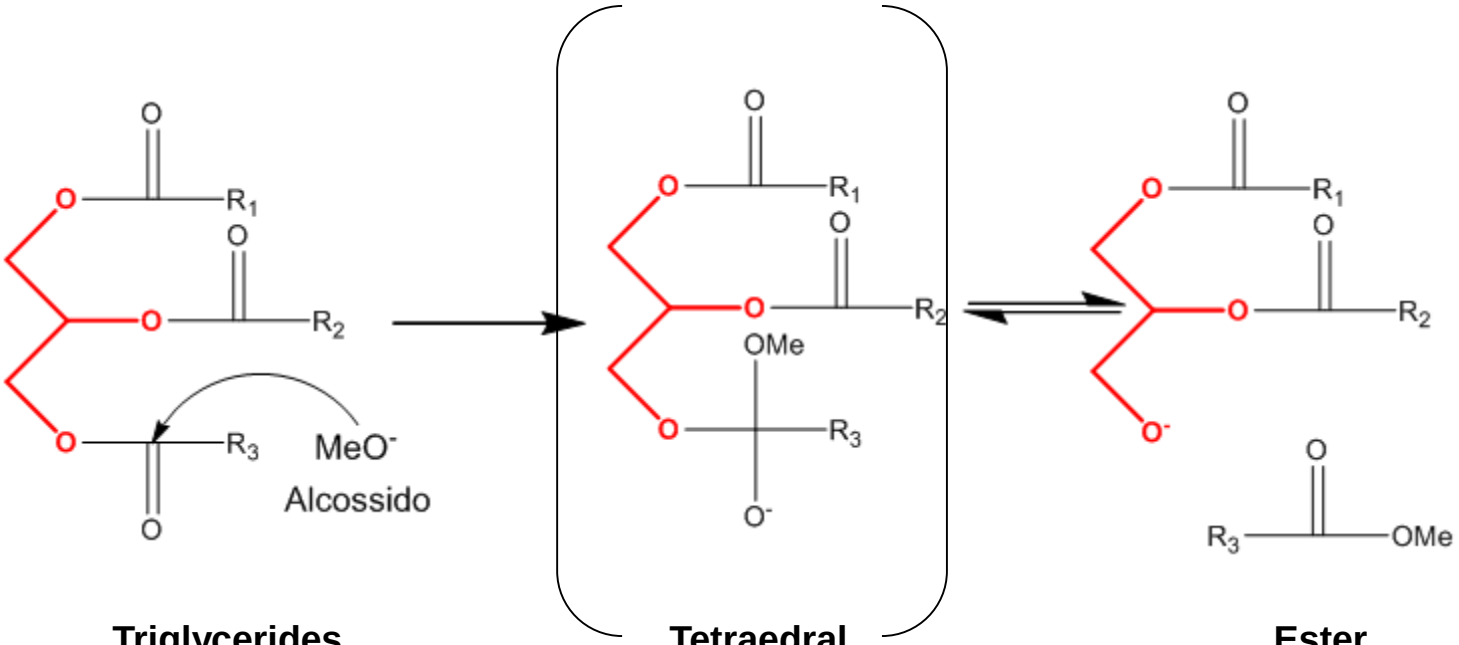
Esterases
Amidases
Lipases
Peptidases





Chemical synthesis of biodesel

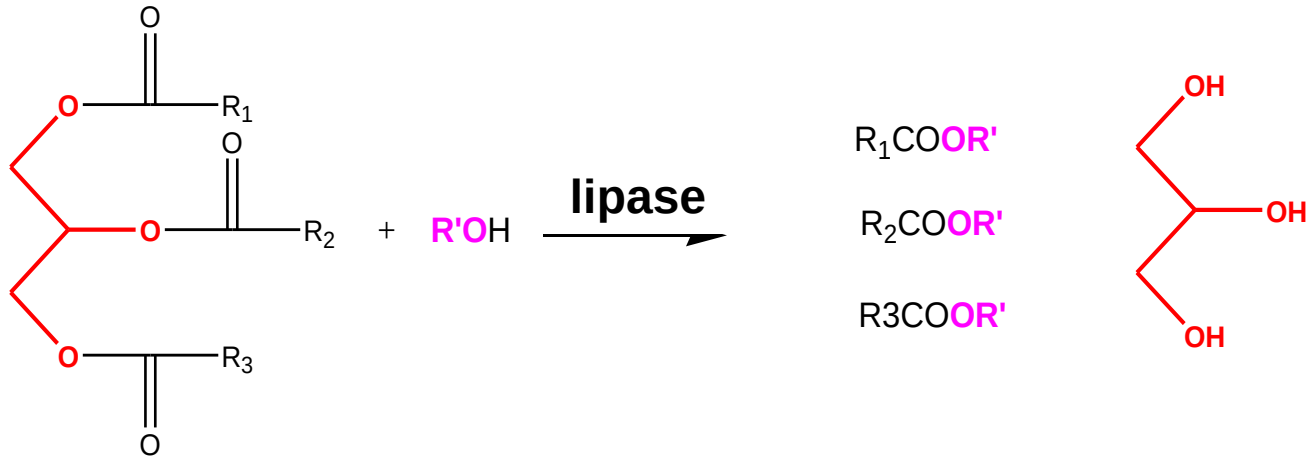
Alkaline or acid conditions



Triglycerides **Tetraedral Intermediate** **Ester**

High temperatures
Separation of by product at the end of the processes (distillation)
Catalysts disposal
Low quality of glicerol

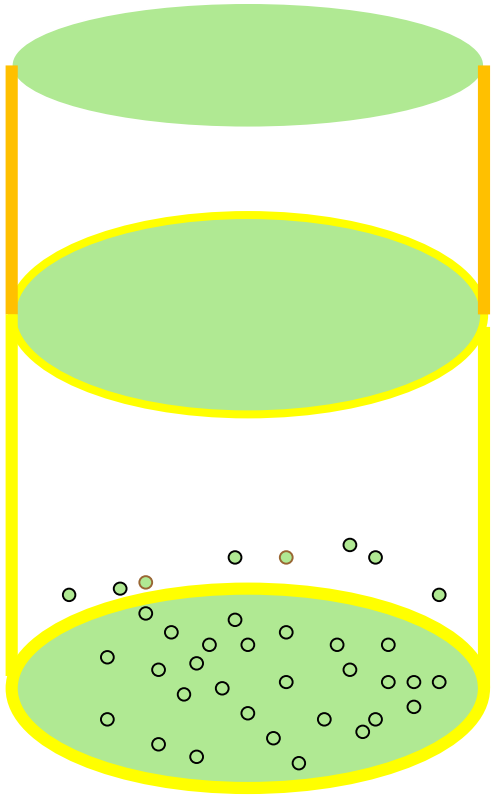
Industrial examples: enzymatic esterification/transesterification of fats and oils



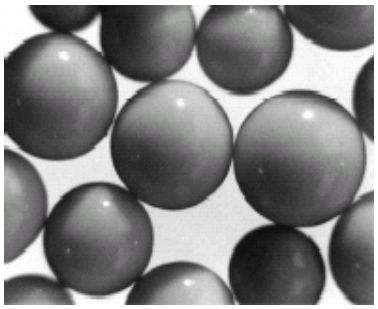
Trigliceride (TG) Alcol Esteri Glicerolo

Biodiesel

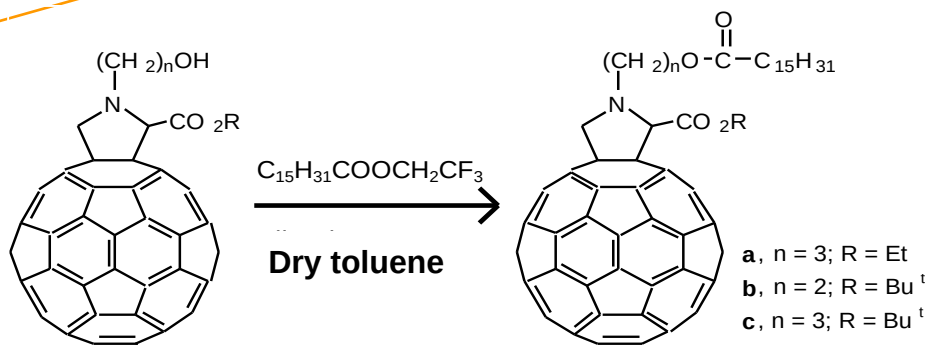
Low-water media: different systems



Immobilized



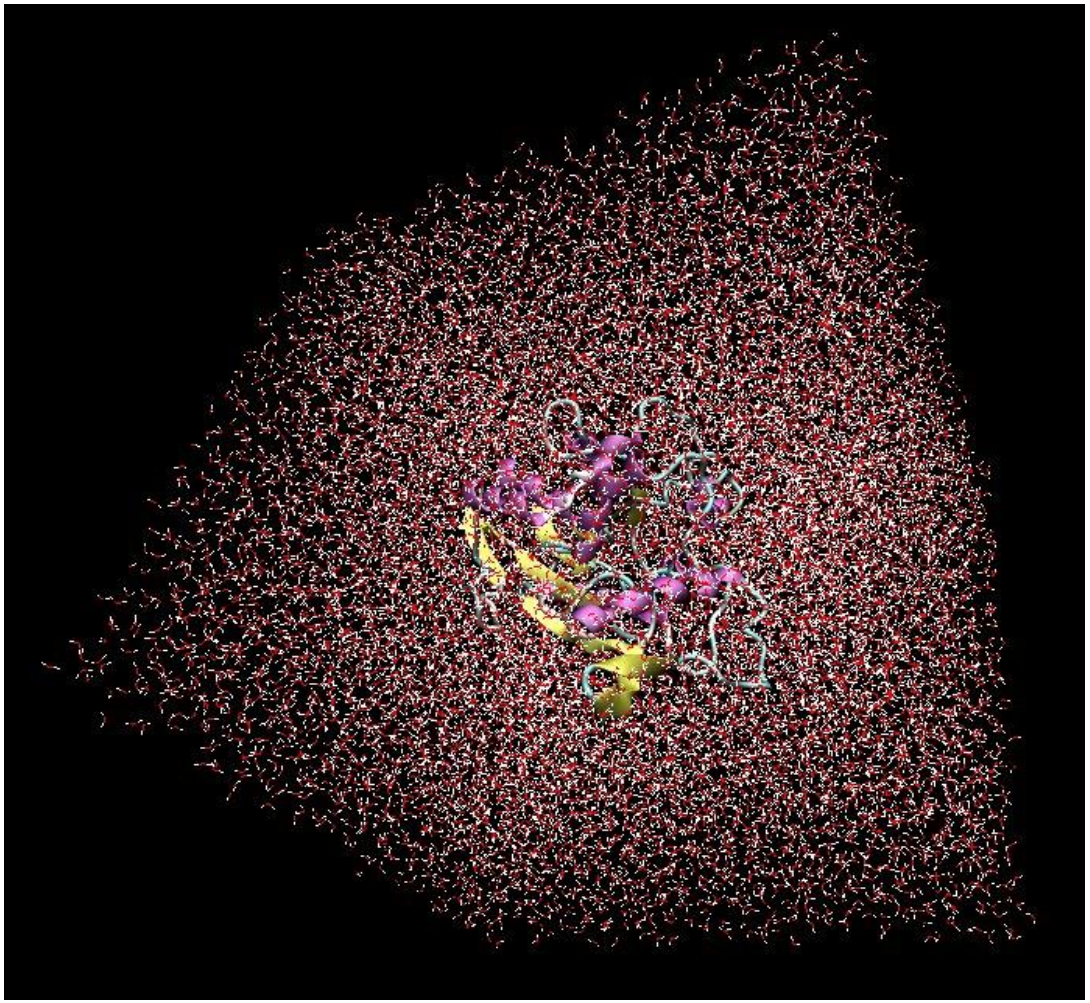
Enzymes suspended in organic solvent



Tetrahedron Lett., 1998, 39, 7791-7794.

Enzymes in aqueous medium: «close» to physiological environment

Surface water molecules are held to each other most strongly by the positively-charged basic amino acids. The exchange of surface water is controlled by the exposure of the groups to the bulk solvent .



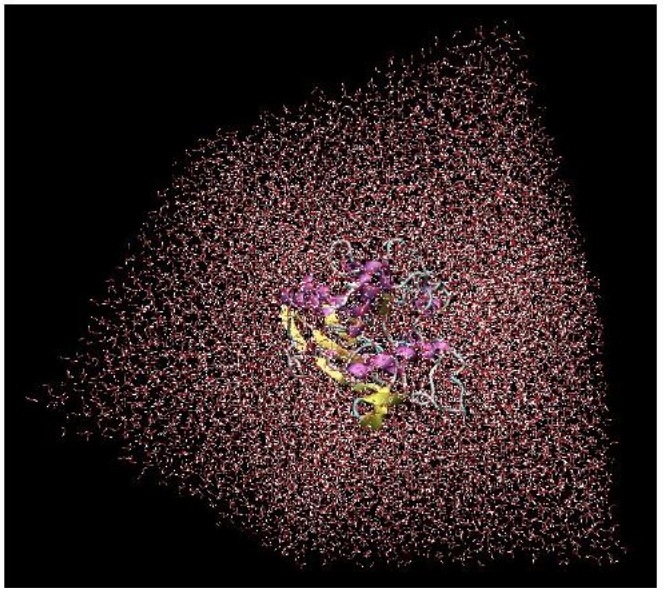
The contribution of water to protein structure

Protein hydration is very important for their three-dimensional structure and activity. Indeed, **proteins lack activity in the absence of hydrating water.**

The aqueous structuring around proteins is affected out to at least **1 nanometer** from its surface.

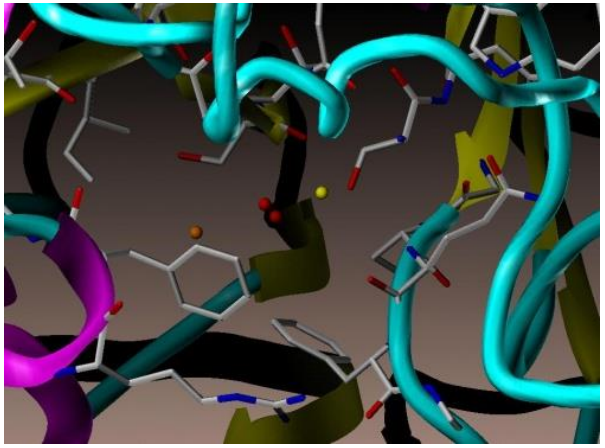
In solution they possess a **conformational flexibility with** a wide range of **hydration states**,

Equilibrium between these states will depend on the **activity of the water** (a_w); that is, the freedom that the water has to hydrate the protein.

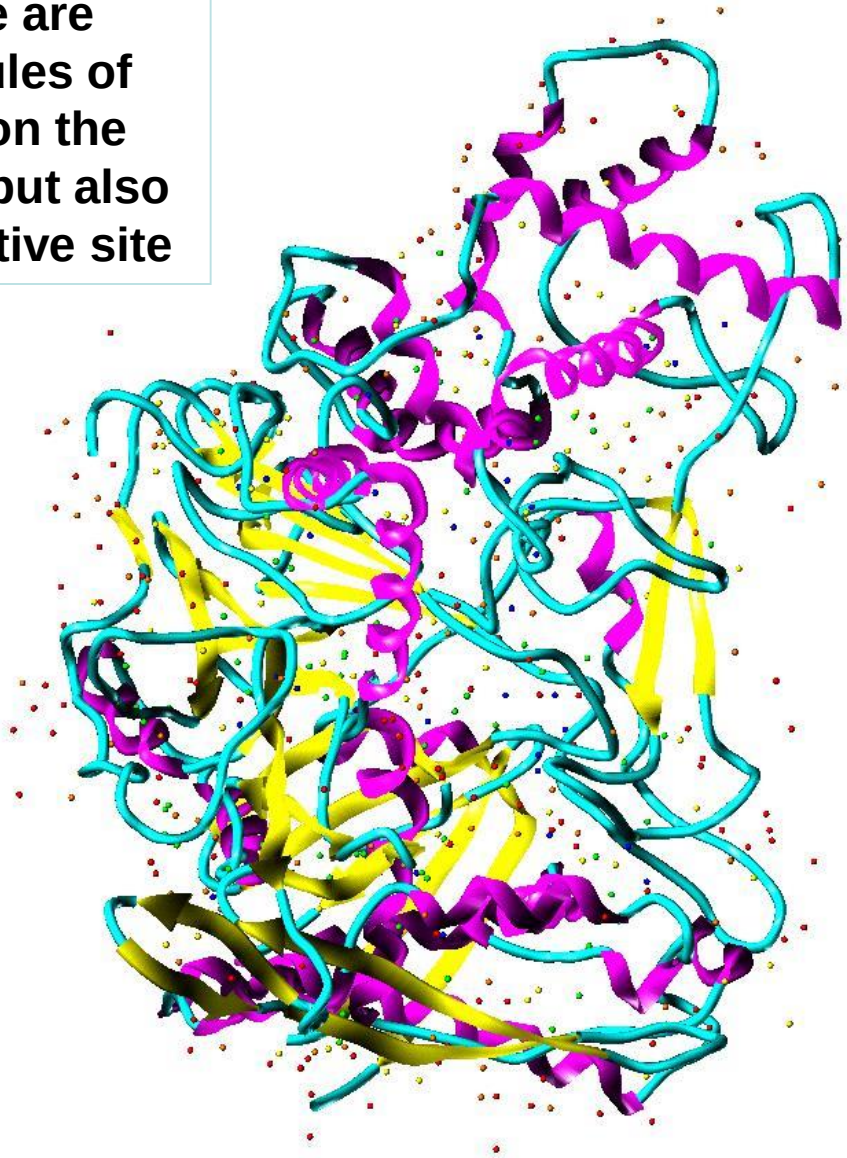


Biocatalysts in organic solvents: residual water in PGA

Active site



There are molecules of water on the surface but also in the active site

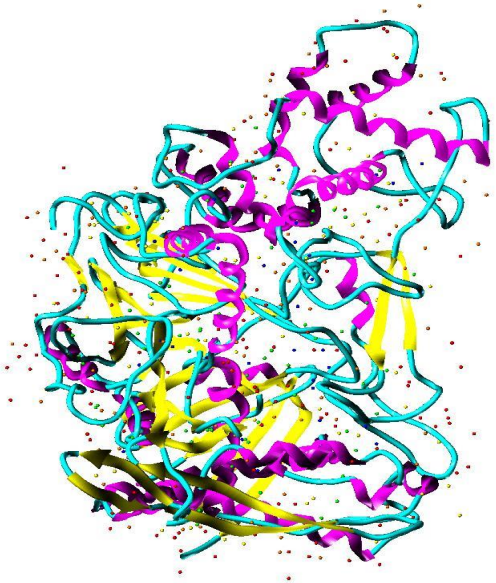


β

Violet
Blue
Green
Yellow
Orange
Red

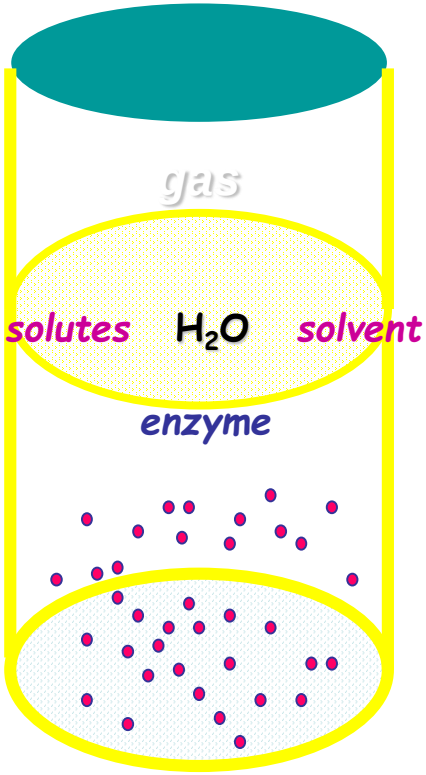
Strenght of bond

Low water media: residual water and water activity (a_w)



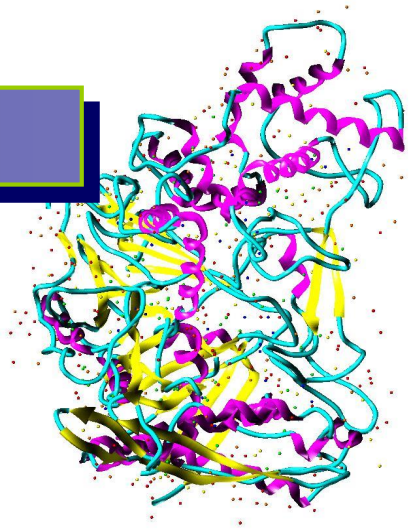
- It is not sufficient to state the amount of added water

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. When a system reaches the equilibrium, 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 solvent.



a_w

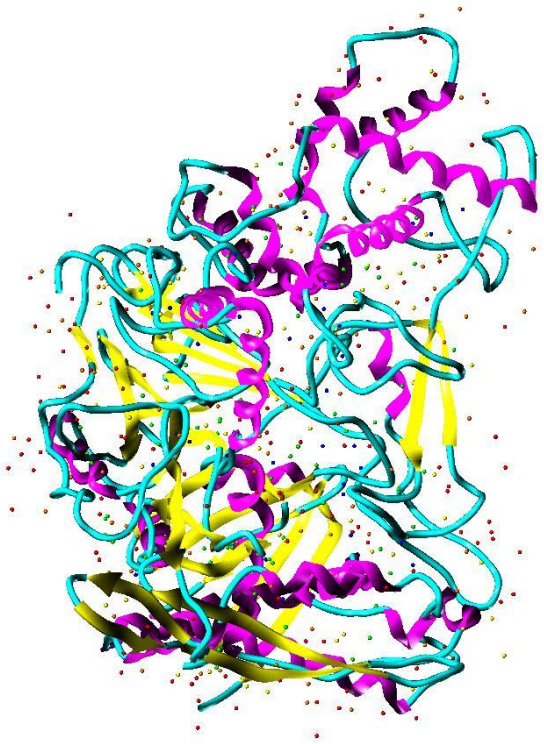
Log P of organic solvents and effect on enzymatic activity

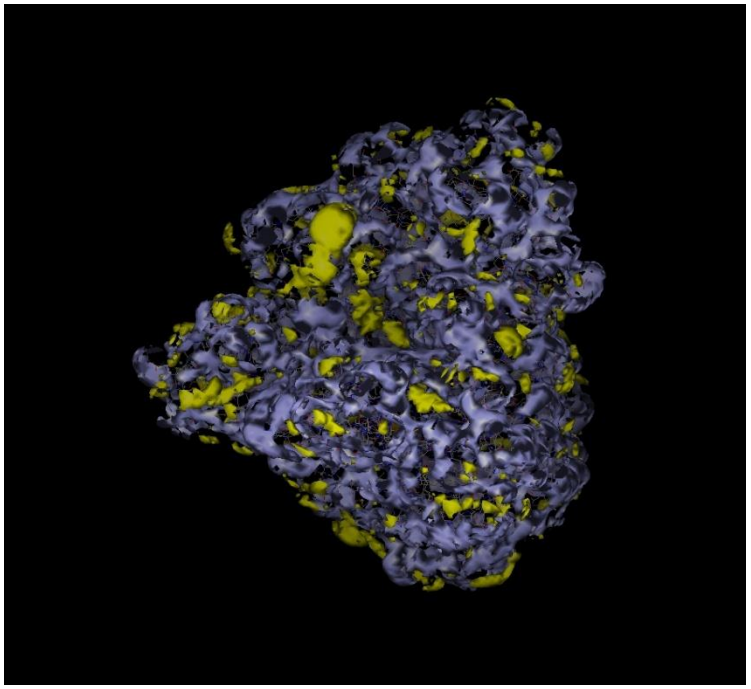


Reaction media
-can compete for water molecules on the surface thus inducing denaturation
-can remove water molecules essential for the mechanism of action: enzyme retains its conformation but looses its activity

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	May be used with caution
> 4	Immiscible	**Ensures high retention of activity**

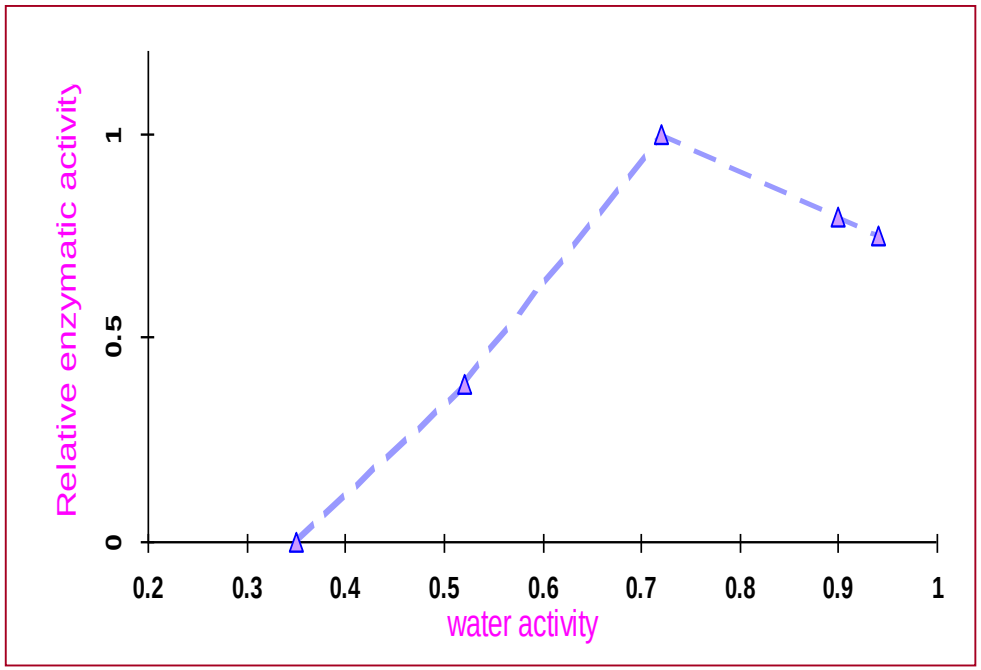
Penicillin G amidase in organic solvent: active when sufficiently hydrated

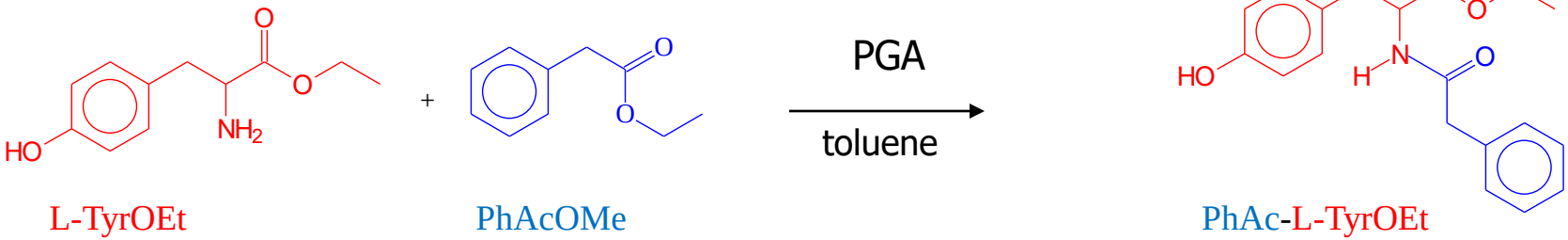




The hydration of the biocatalyst will depend on the amount of “free water” (i.e. water activity) rather then on the amount of total water present in the system.

a_w effect on synthetic activity of PGA in organic solvent



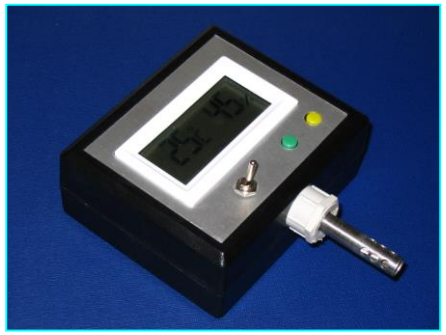


L-TyrOEt PhAcOMe PhAc-L-TyrOEt

Ebert, C.; Gardossi, L.; Linda, P., *Tetrahedron Lett.*, 1996, *37*, 9377-9380

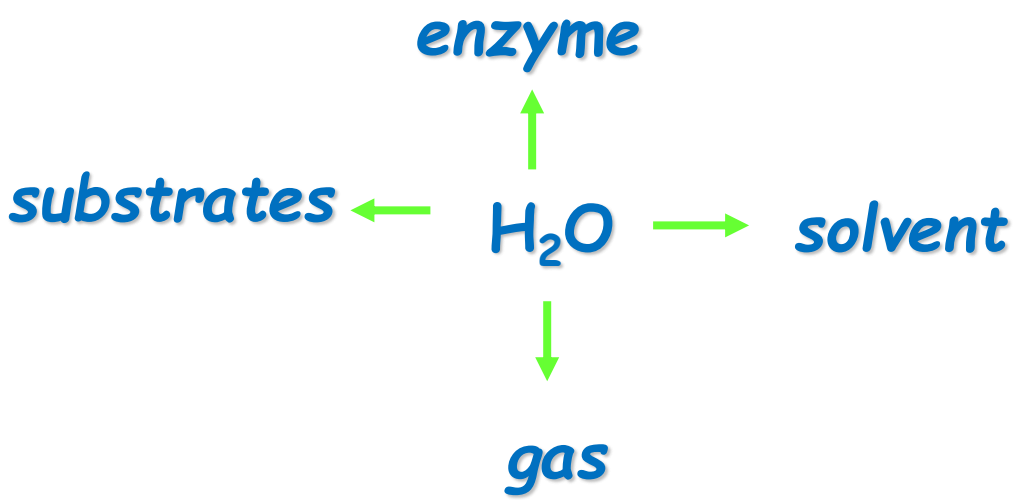
Ebert, C.; Gardossi, L.; Linda, P, *J. Mol. Catal. B*, 1998, *5*, 241-244.

Basso A., De Martin L., Ebert C., Gardossi L., Linda P., Zlatev, V., *J. Mol. Catal. B*, 2001, *11*, 851-855.



How measuring or controlling the water activity

Since at the equilibrium the "free water" will be the same in all phases, it can be measured in the most accessible one, generally the gas phase *via* the measurement of vapour pressure of water.

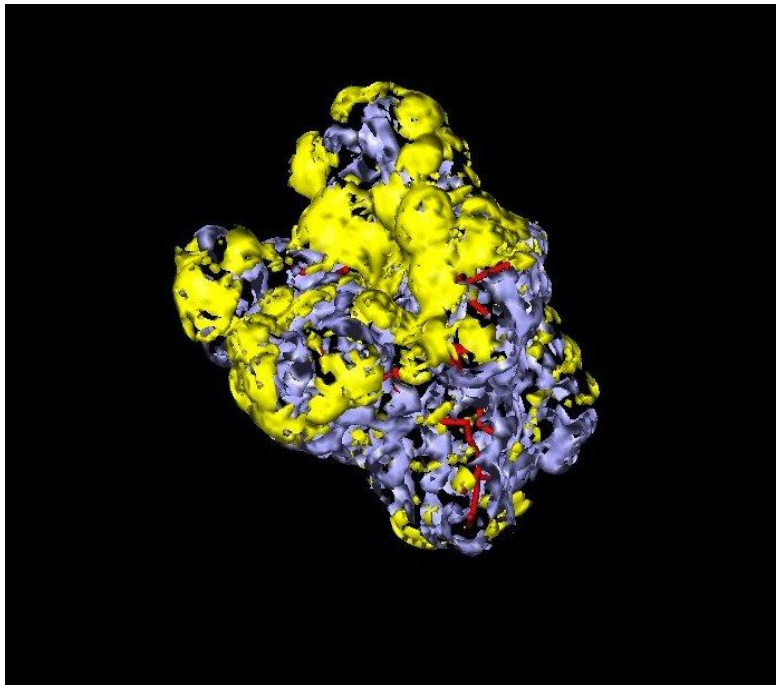


a) Evaluating water activity by measuring water pressure in the gas phase of the close system at the equilibrium
b) Exploiting the ability of porous materials/carriers to control water distribution and mobility
c) Drying or bring to a defined water content all ingredients/phases:
   By using pairs of hydrated salts "buffering" the a_w

Not all enzymes need the same amount of «free water»

hydrophobic

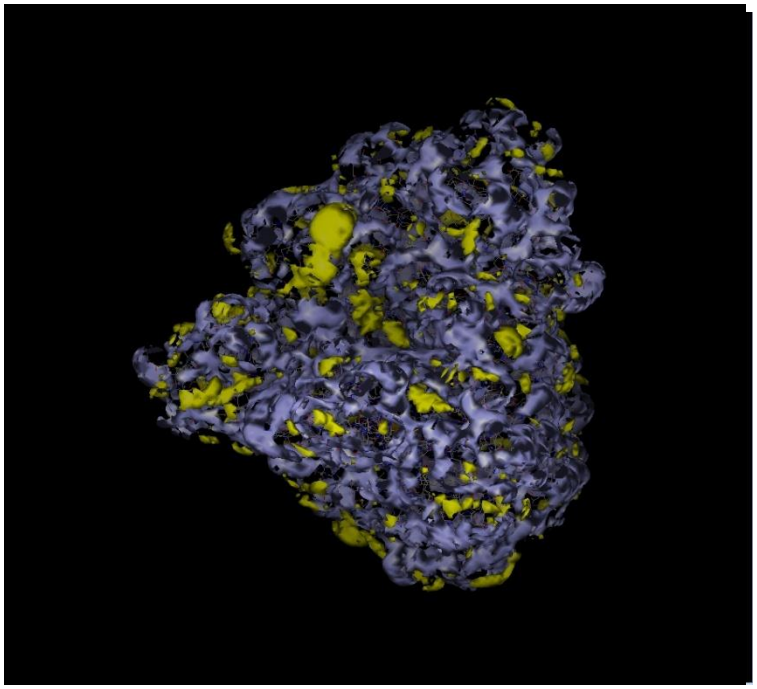
hydrophilic



Lipase from *P. cepacia*

↓

Very active even at low water activity (<0.2)



(PGA) Amidase from *E. coli*

↓

Active only at water activity > 0.4

Adv. Synth. Catal, 2007, 349, 877-886.

Thermodynamically controlled synthesis with substrate suspension in toluene at controlled a_w

toluene (1mL), a_w=0.73

Z-L-Phe-COOH + L-PheOEt → **Z-L-Phe-L-PheOEt** ↓

80 μmol *80 μmol*

Solid *liquid*

H_2O

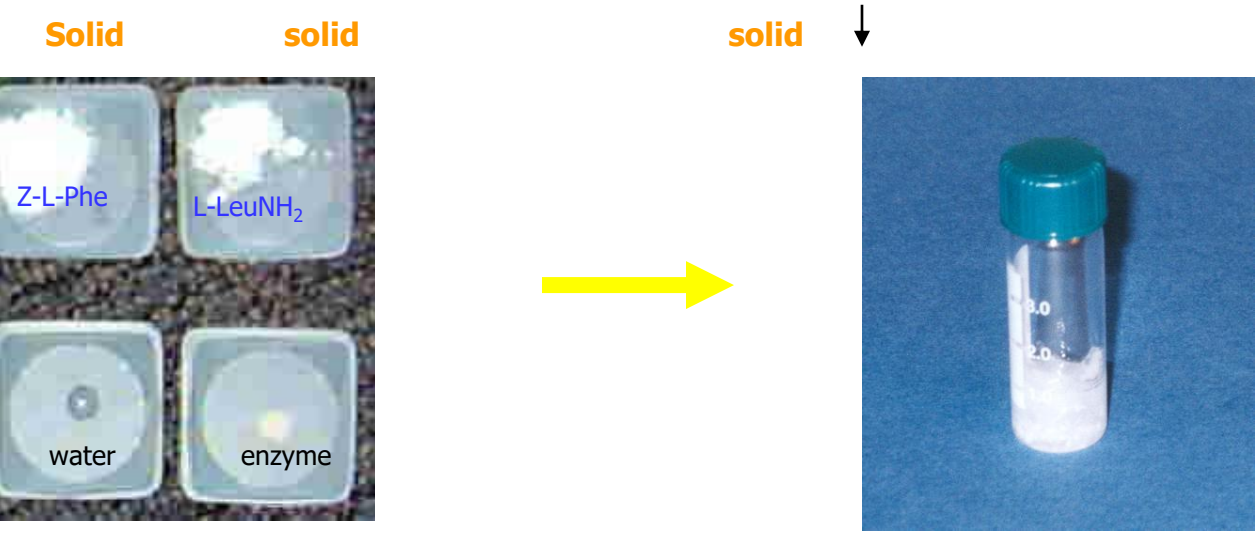
>93% isolated yield (48h)

Enzyme	Acyl donor	Nucleophile	Conv. (%)	Time (h)
Thermolysin	Z-L-Phe-COOH	L-Phe-OEt (s)	**98**↓	48
Thermolysin	Z-L-Phe-COOH	L-Tyr-OEt (s)	**97**↓	144
Thermolysin	Z-L-Phe-COOH	L-Leu-NH_2 (s)	**95**↓	96

Basso A., De Martin L., Ebert C., Gardossi L., Linda P., *Chem. Comm.*, **2000**, 467-468.

Some examples of even “more desperate “ experimental conditions

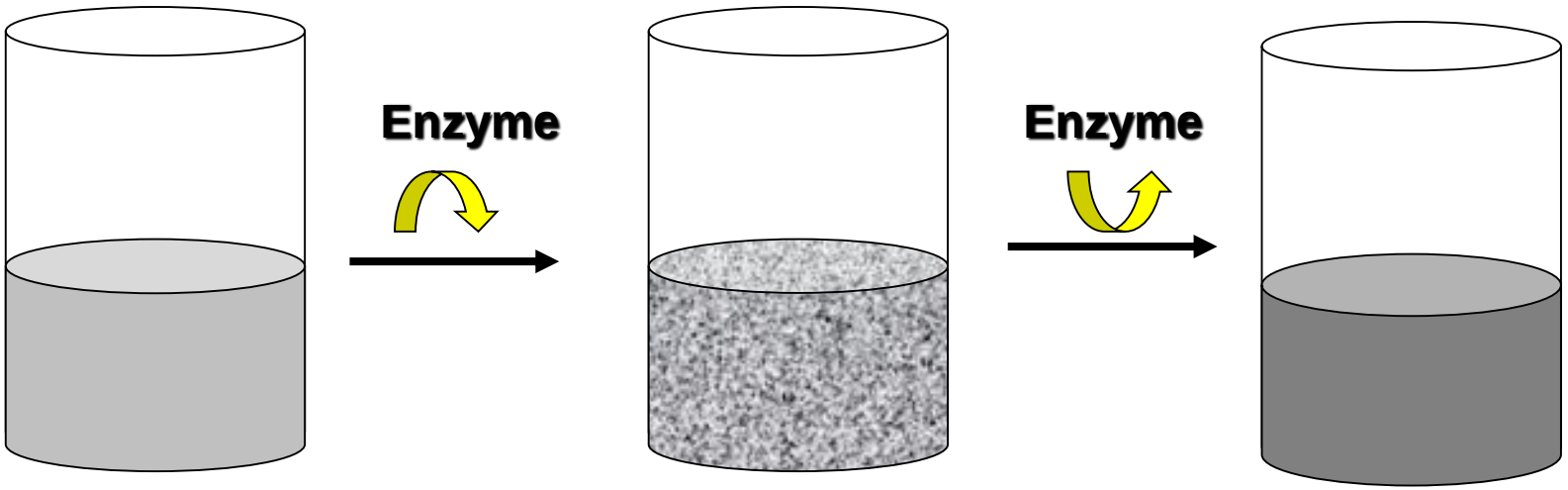
Precipitation driven "solid to solid" peptide synthesis: product solubility must be lower than substarte solubility



Ulijn R. V., De Martin L., Halling P. J., Janssen A.E.M., Gardossi L., Moore B. D., Biotech. Bioeng., **2002**, *80*, 509-515.

Ulijn R. V., De Martin L., Gardossi L., Halling P.J. , Current Org. Chem, **2003**, *7*, 1333-1346.

What is precipitation driven biocatalysis?



Solid Substrate + minimum Liquid Phase

Solid Substrate and Product + Liquid Phase

Solid Product + Liquid Phase

High volumetric productivity

The equilibrium lies completely either to the side of the solid substrate or to the side of the solid product

Ulijn R. V., De Martin L., Gardossi L., Halling P.J. "Biocatalysis in reaction mixtures with undissolved solid substrates and products." Current Org. Chem, in press.

When is precipitation driven synthes feasible?
It depends on the thermodynamics of the reaction

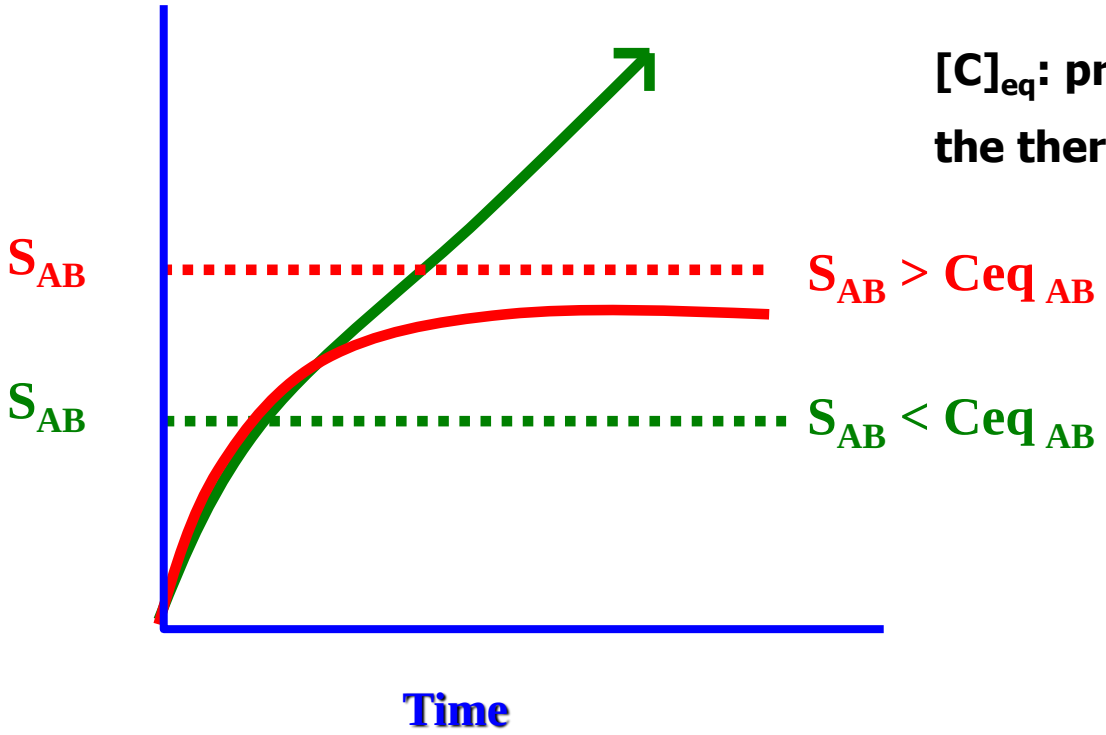
$$A + B \rightleftharpoons AB + H_2O$$

When:

- $S_{AB} < [C]_{eq}$ the product precipitates and th. equilibrium is reached only when the substrate excess is completely consumed.

S_{AB}: product solubility in the solvent;

$[C]_{eq}$: product concentration in solution at the thermodynamic equilibrium



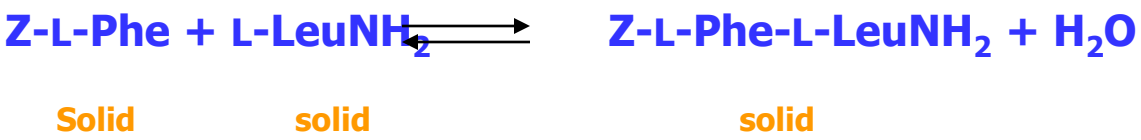
The feasibility is solvent INDEPENDENT

Rules for Solvent Selection

- The yield of crystalline product can be maximised by choosing a solvent where product solubility is lowest
- for hydrophobic targets water is generally a good choice
- in the synthesis of hydrophilic targets good yields are expected in hydrophobic solvents

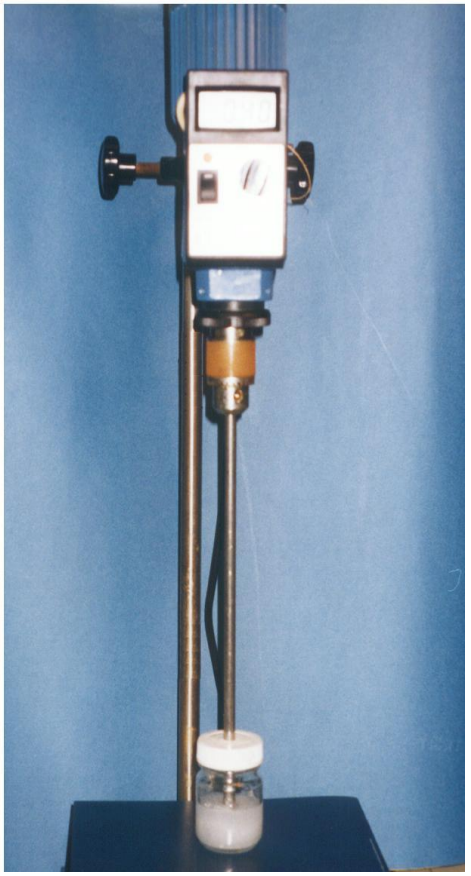
Always use an excess of the most soluble compound

Thermodynamically controlled synthesis of Z-L-Phe-L-LeuNH$_2$ catalysed by Thermolysin in toluene



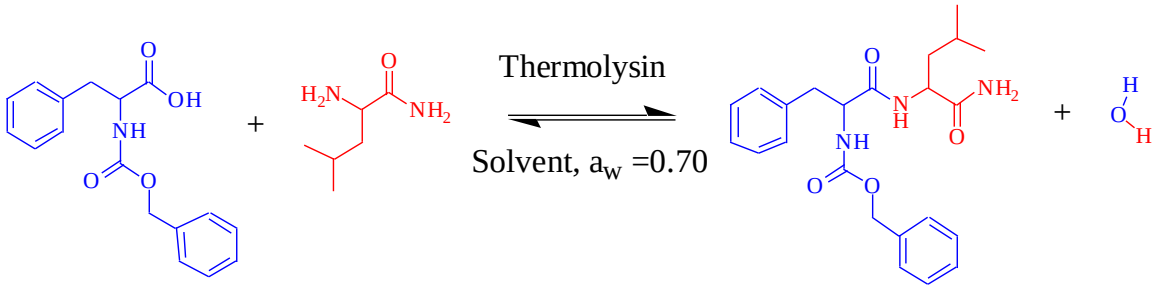
- 2 millimoles of Z-L-Phe and L-LeuNH$_2$ in 20mL toluene
- Conv. after 8h: > 99%
- 96% (1.92 millimoles) of pure solid product recovered by rinsing the enzymel
- Enzyme recycled 4 times

Mechanic stirrer

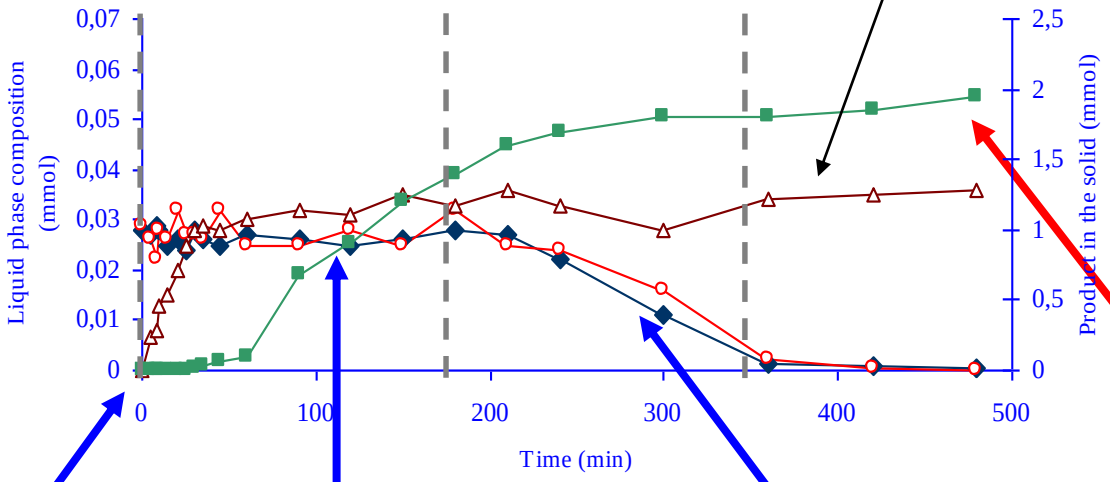


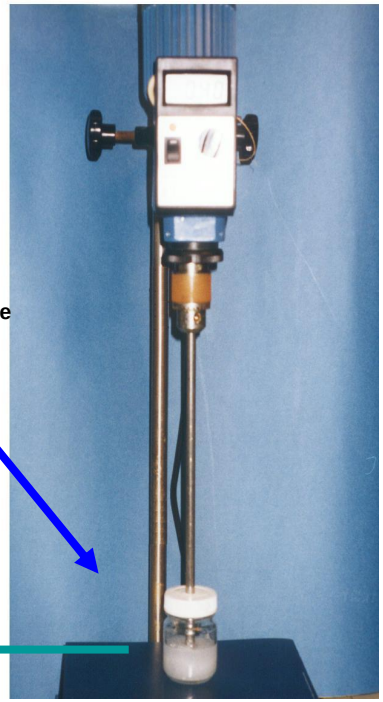
L. De Martin, PhD Thesis, University of Trieste, 2001.

How does it work?



Z-L-Phe (2 mmol) **L-LeuNH$_2$ (2 mmol)**





Product precipitation occurs when [ZPheLeuNH2] equals $S_{ZPheLeuNH2}$

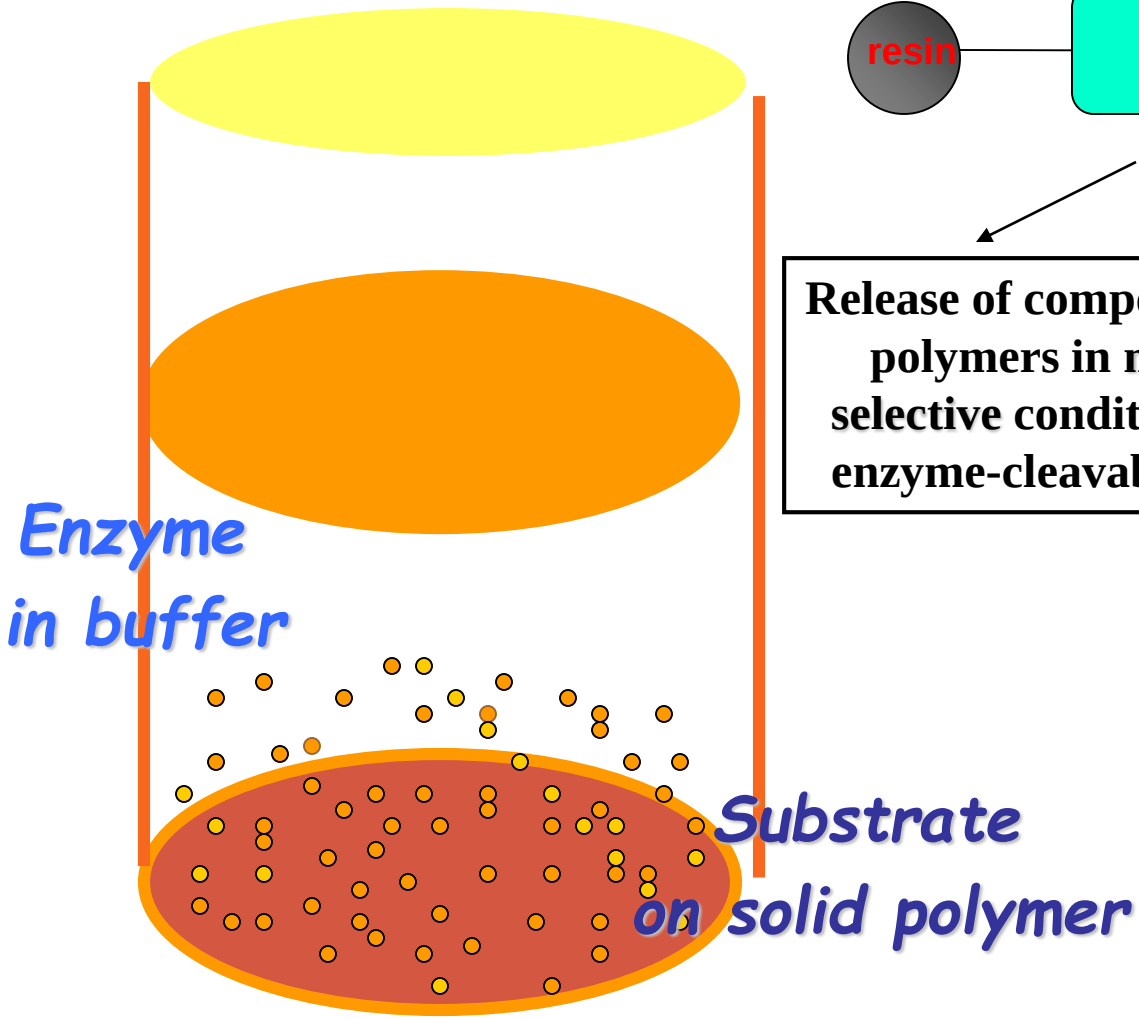
The solution phase reaches a steady state situation

-Substrates in solution reach the equilibrium concentration
-No more excess solid substrate is present

- Conv. after 8 hours > 99%
- 96% (1.92 millimol) of pure solid product at the equilibrium

Application of enzymes on solid substrates

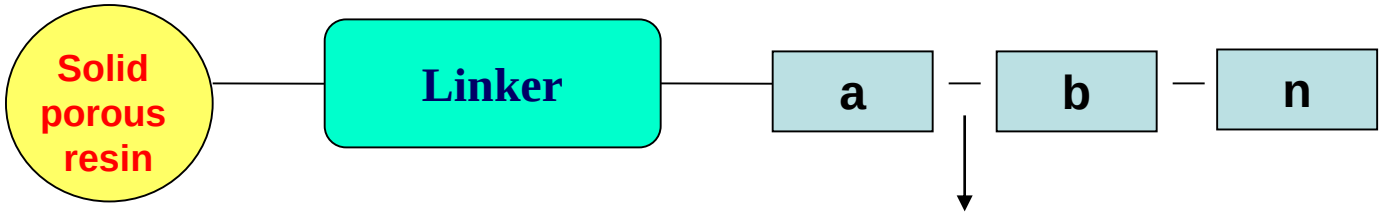
Solid phase biocatalysis



Release of compounds from polymers in mild and selective conditions using enzyme-cleavable linkers

- **Synthesis of libraries of compounds**
- **Solid phase peptide synthesis**
- **Activity assays**

Solid phase biocatalysis: peptide synthesis



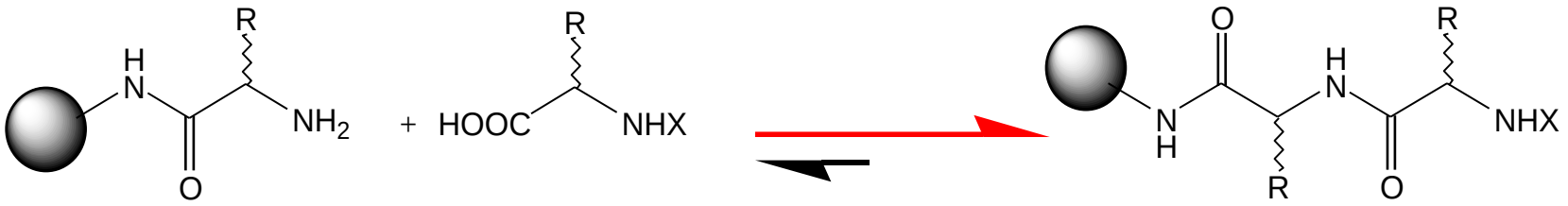
Peptidases: enzymatic coupling

Advantages:
- **Minimal protection**
- **No racemization**
- **Equilibrium shifted to synthesis even in aqueous media**

Basso et al., Journal of Chemical Technology & Biotechnology, 2006, 81, 1626-1640.

Solid phase biocatalysis: peptide synthesis

Taking the advantages of organic solvent… *in buffer*



- Suppressed ionisation of amino groups
- Solvation of hydrophobic substrates

R. V. Ulijn et al. *JACS* **2002**, *124*, 10988

Annexes

Equazione di Michaelis – Menten (1913)

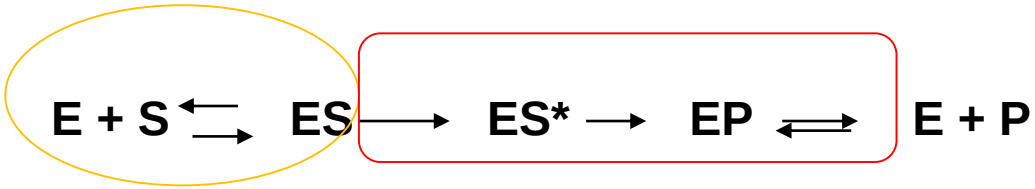
Leonor Michaelis
(1875–1949)

Maud Leonora Menten
(1879–1960)

$$V = k_{cat}[E]_T \frac{[S]}{K_S + [S]}$$

oppure

$$v = V_{max} \frac{[S]}{K_M + [S]}$$



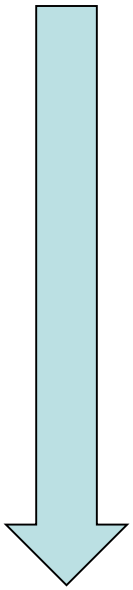
Cinetica semplificata

- Un enzima catalizza la reazione che dal substrato **S** porta al prodotto **P** attraverso il complesso enzima-substrato **ES** e il complesso enzima-prodotto **EP**:

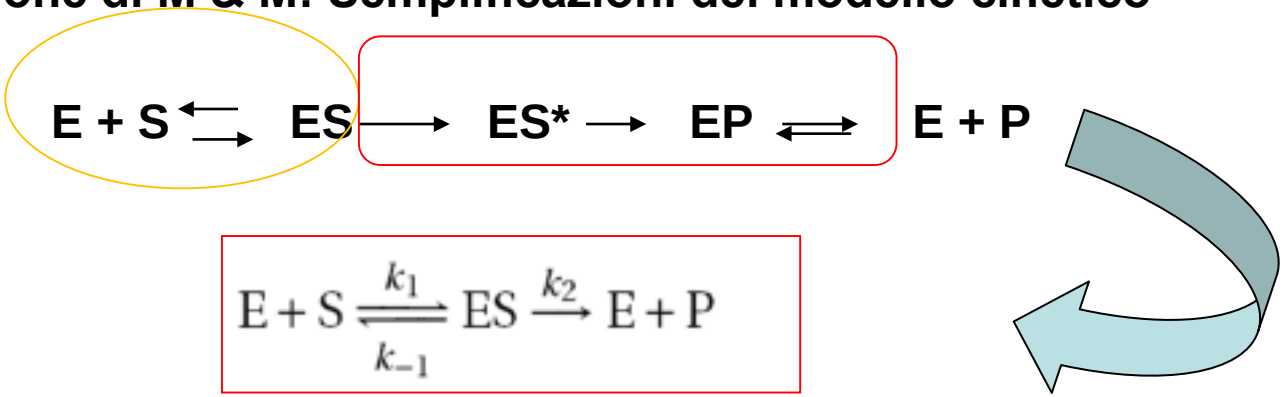
$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \rightleftharpoons \mathrm{EP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}$$

- Si consideri la dissociazione di EP molto rapida e irreversibile; lo stadio successivo:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}$$



L'equazione di M & M: Semplificazioni del modello cinetico



1. il primo stadio è rapido e reversibile, si forma il complesso enzima-substrato ES, la cui costante di dissociazione è K_S.

$$K_S = \frac{[E][S]}{[ES]}$$

2. Nel secondo stadio hanno luogo i processi chimici governati da una costante di velocità $k_2 = k_{cat}$

$$V = k_2[ES] = k_{cat}[ES] = \frac{d[P]}{dt} = -\frac{dS}{dt}$$

Come ricavare l'equazione di M & M

Scrivere il bilancio delle masse per ogni specie. Per l'enzima è:

$$[E]_T = [E]_{lib} + [ES]$$

$$[E]_{lib} = [E]_T - [ES]$$

$$K_S = \frac{[E][S]}{[ES]} = \frac{\{[E]_T - [ES]\}[S]}{[ES]}$$

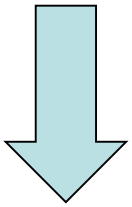
$$[ES]K_S = [E]_T[S] - [ES][S]$$

$$[ES]\{K_S + [S]\} = [E]_T[S]$$

$$[ES] = \frac{[E]_T[S]}{K_S + [S]}$$

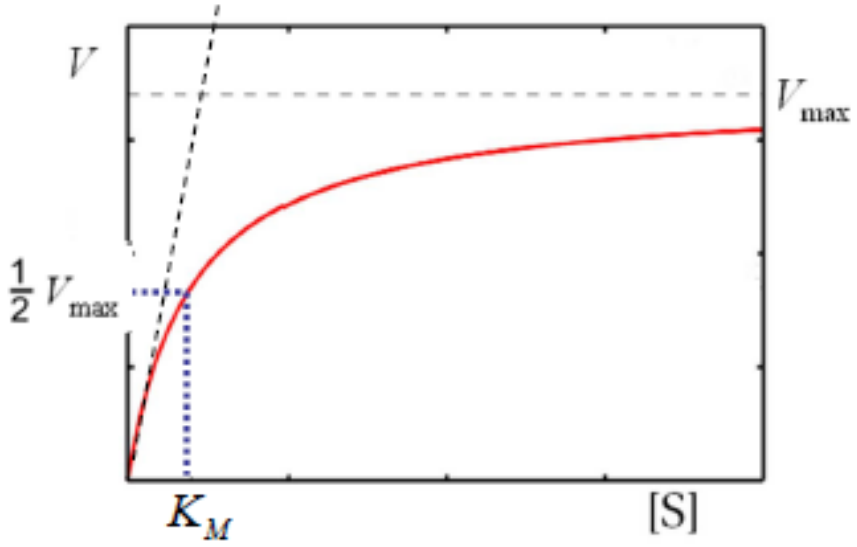
$$V = k_2[ES] = k_{cat}[E]_T \frac{[S]}{K_S + [S]}$$

L'enzima non deve essere saturato dal substrato



- Misurando V a diverse concentrazioni di S si trova la curva seguente:

$$V = k_{cat}[\mathrm{E}]_T \frac{[\mathrm{S}]}{K_S + [\mathrm{S}]}$$



- Ad alte concentrazioni di S, K_S diventa trascurabile e V diventa:

$$V = V_{max} = k_{cat}[\mathrm{E}]_T \quad \Rightarrow \quad V = V_{max} \frac{[\mathrm{S}]}{K_S + [\mathrm{S}]}$$

- Alla condizione nella quale $V = \frac{V_{max}}{2}$ $\Rightarrow$ $V = \frac{V_{max}}{2} = \frac{V_{max}[S]}{K_S + [S]}$

$$[\mathrm{S}] = K_S = K_M$$

costante di Michaelis – concentrazione del substrato necessaria a raggiungere una velocità pari a V = ½ V_{max}

$$V = V_{max} \frac{[\mathrm{S}]}{K_M + [\mathrm{S}]}$$

equazione di Michaelis-Menten

Significati di K_M

$$v = V_{max} \frac{[S]}{K_M + [S]}$$

velocità è indipendente da [S].

- [S] = K_M $V = V_{max}/2$, ossia
  - K_M *rappresenta la concentrazione di substrato che determina metà della velocità massima*.
  - K_M rappresenta pure *la concentrazione di substrato alla quale metà dei siti attivi sono occupati*, dando una misura della [S] richiesta affinché la catalisi avvenga in modo significativo
  - K_M è una misura dell'energia di binding ΔG_b

L'enzima non è saturato

k_{cat}/k_m : **specificity constant**

$$v = V_{max}\frac{[S]}{K_M + [S]}$$

- $[S] \ll K_M$ A concentrazioni molto basse di substrato, la velocità è direttamente proporzionale a [S], infatti vale la relazione $V = \frac{V_{max}}{K_M}[S]$
- $[S] \gg K_M$ A concentrazioni elevate di substrato, $V = V_{max}$, ossia la velocità è indipendente da [S].

L'enzima è saturato

N.B. La velocità che si misura è quella iniziale, quella relativa alla scomparsa del substrato o alla formazione del prodotto

$$V = \frac{d[P]}{dt} = -\frac{dS}{dt}$$

K_{cat}/K_M va calcolata a basse concentrazioni di S, quando l'enzima non è saturo