The process:
Biocatalysis in non conventional media

How biocatalysts work under non-physiological conditions

Neat substrates + Native enzyme + Traces of solvent

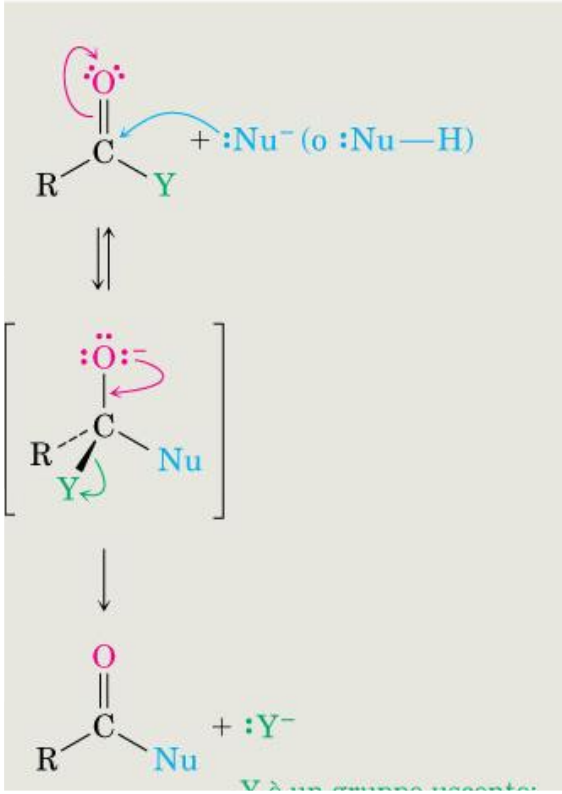
Neat substrates + Immobilized enzyme

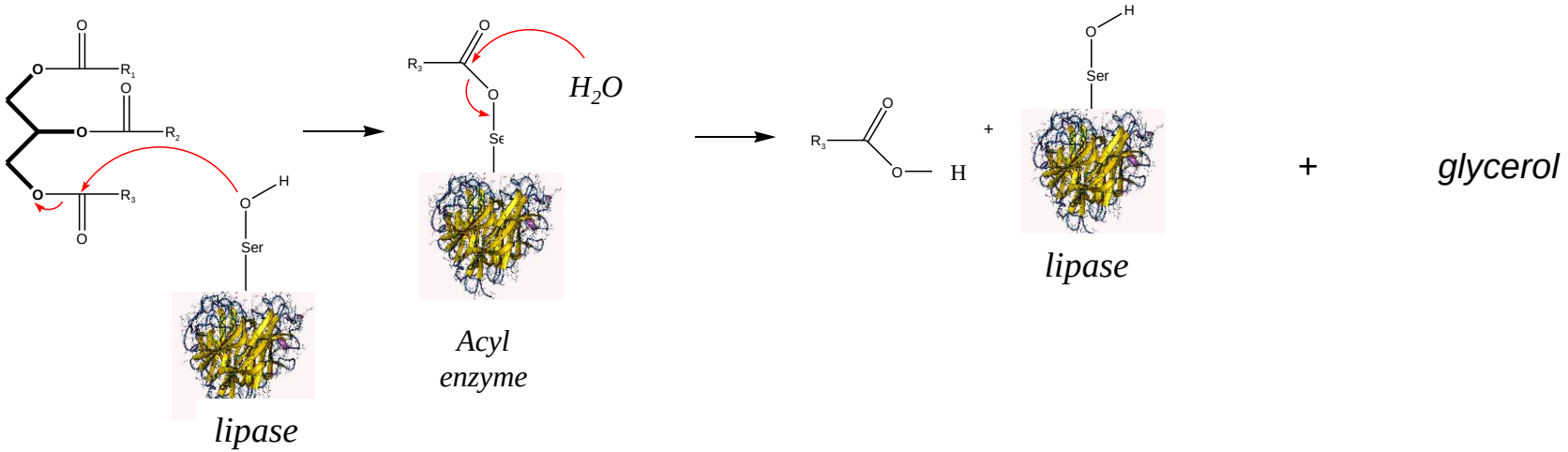
Low -water media: advantages

- 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

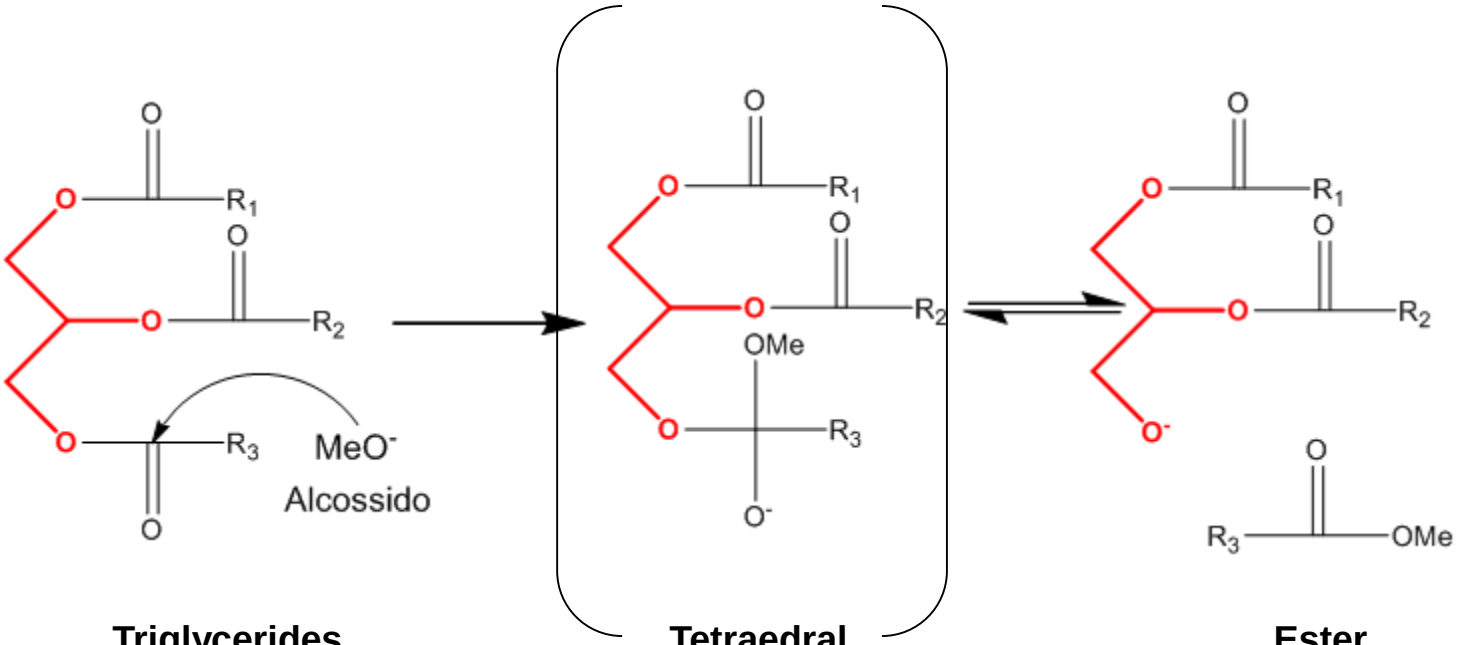
Esterases
Amidases
Lipases
Peptidases





Chemical synthesis of biodesel

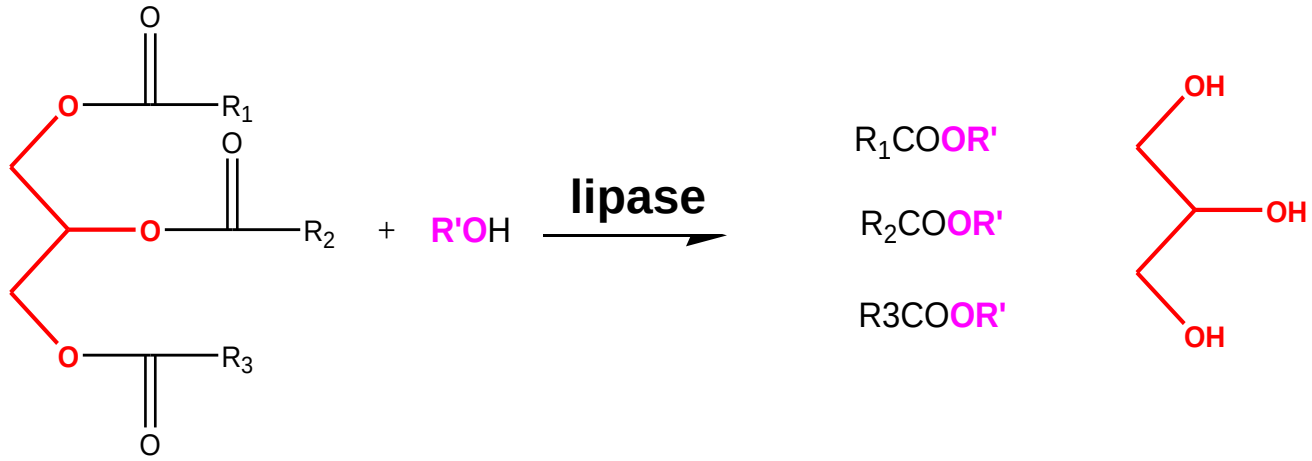
Alkaline or acid conditions



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

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

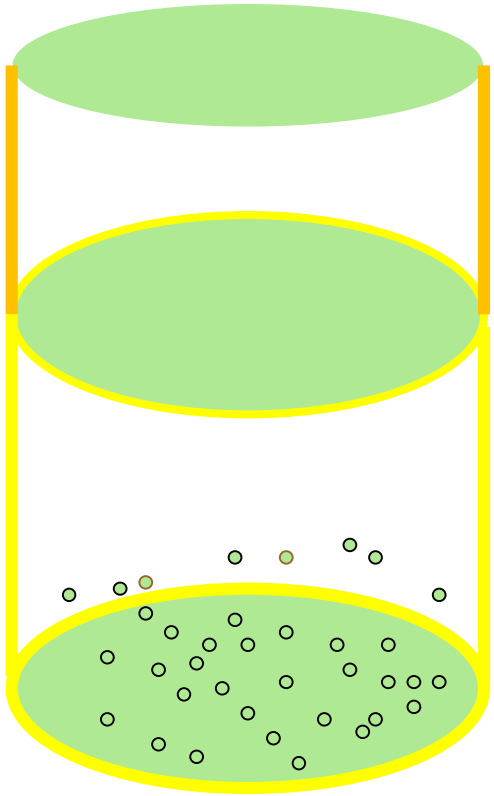
Industrial examples: enzymatic esterification/transesterification of fats and oils



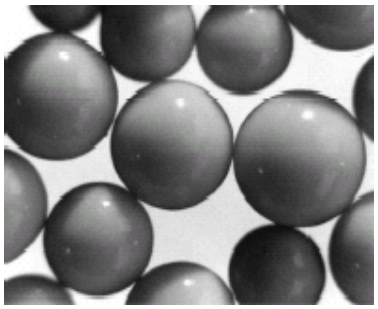
Trigliceride (TG) Alcol Esteri Glicerolo

Biodiesel

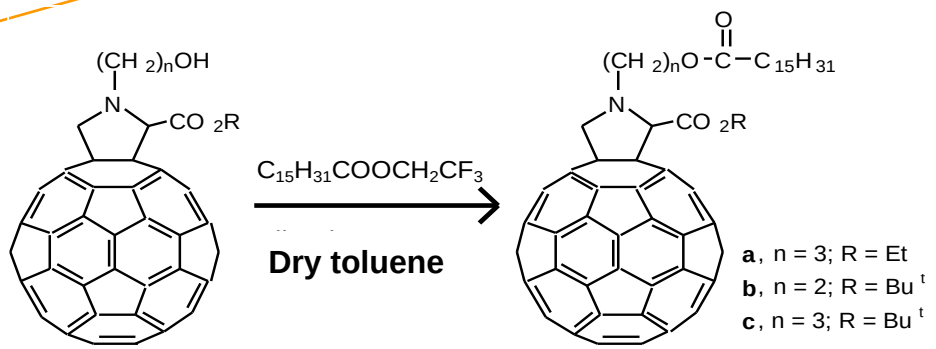
Low-water media: different systems



Immobilized



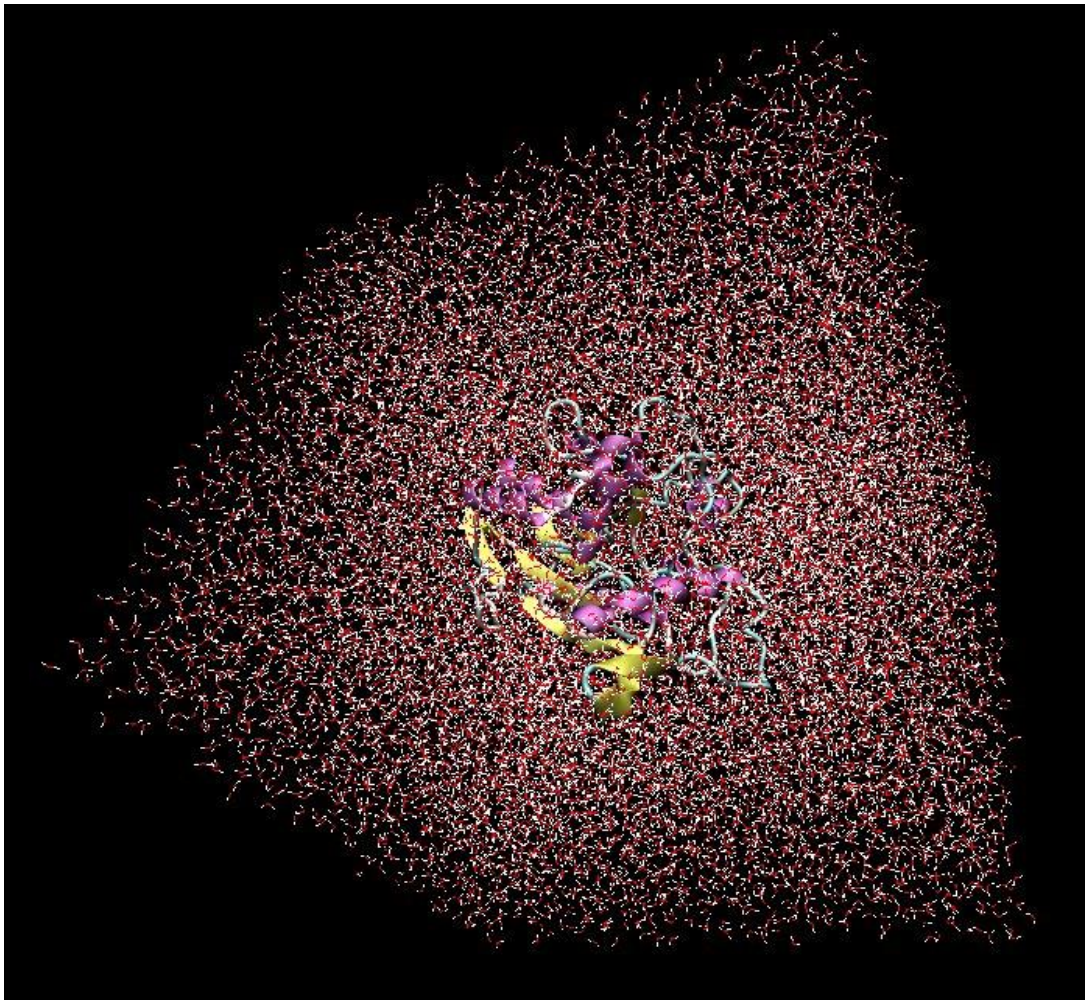
Enzymes suspended in organic solvent



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 .



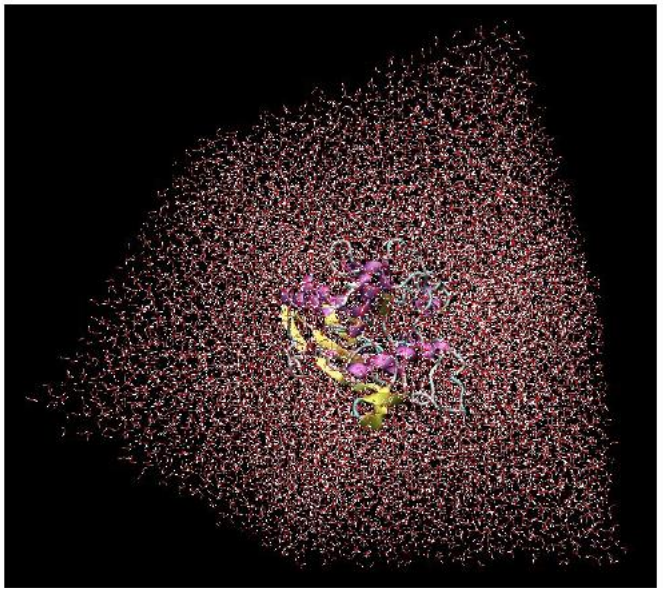
The contribution of water to protein structure

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.

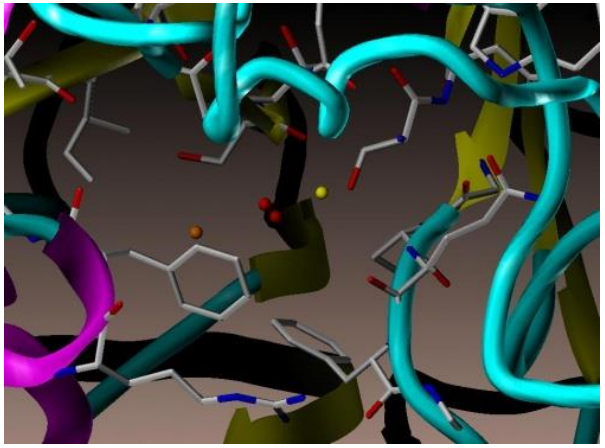
In solution they possess a **conformational flexibility with** a wide range of **hydration states**,

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.

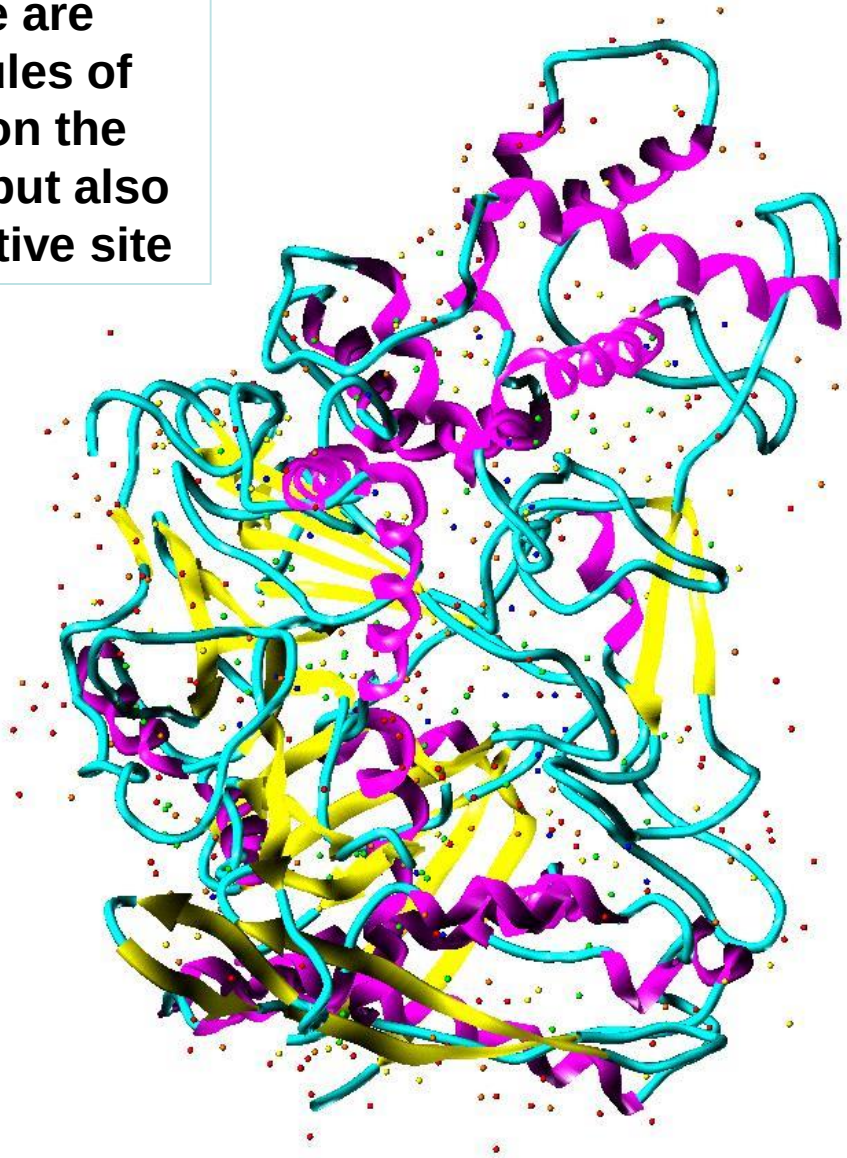


Biocatalysts in organic solvents: residual water in PGA

Active site



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

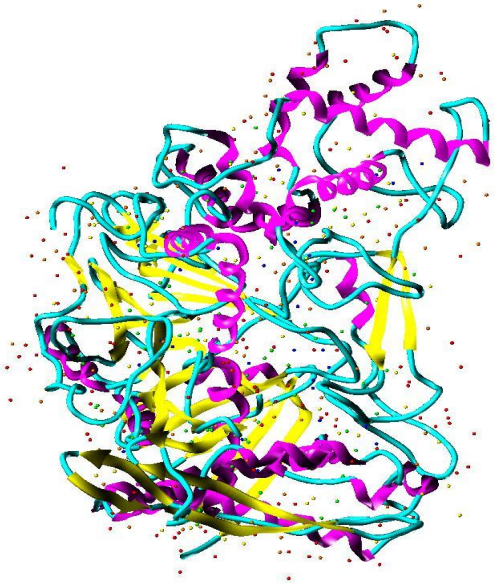


β

Violet
Blue
Green
Yellow
Orange
Red

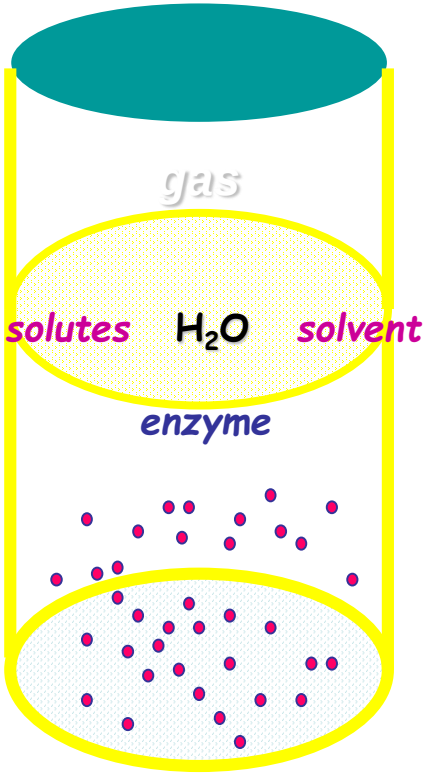
Strenght of bond

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



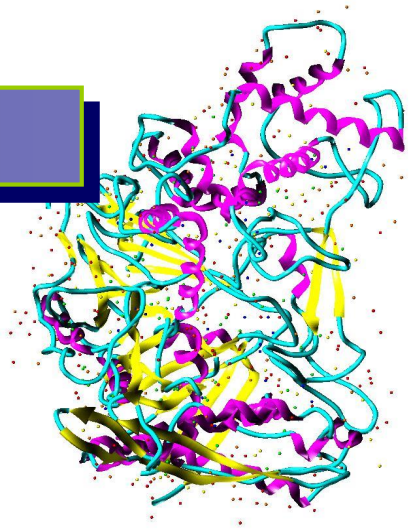
- It is not sufficient to state the amount of added water

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

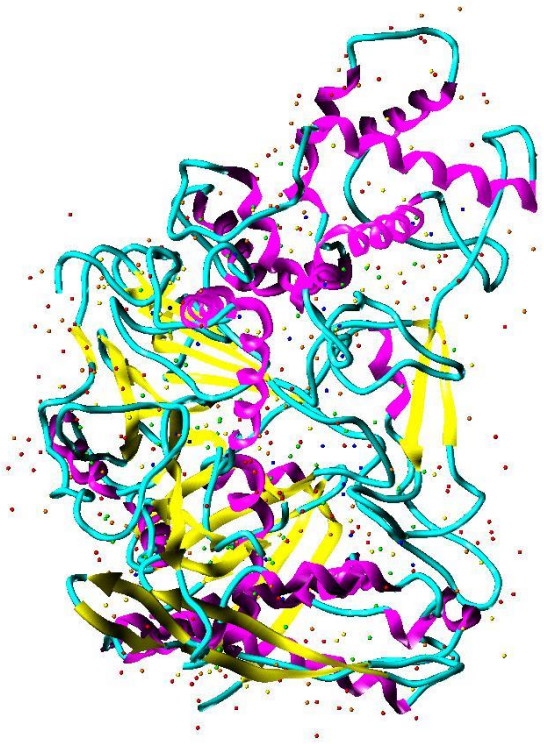
Log P of organic solvents and effect on enzymatic activity

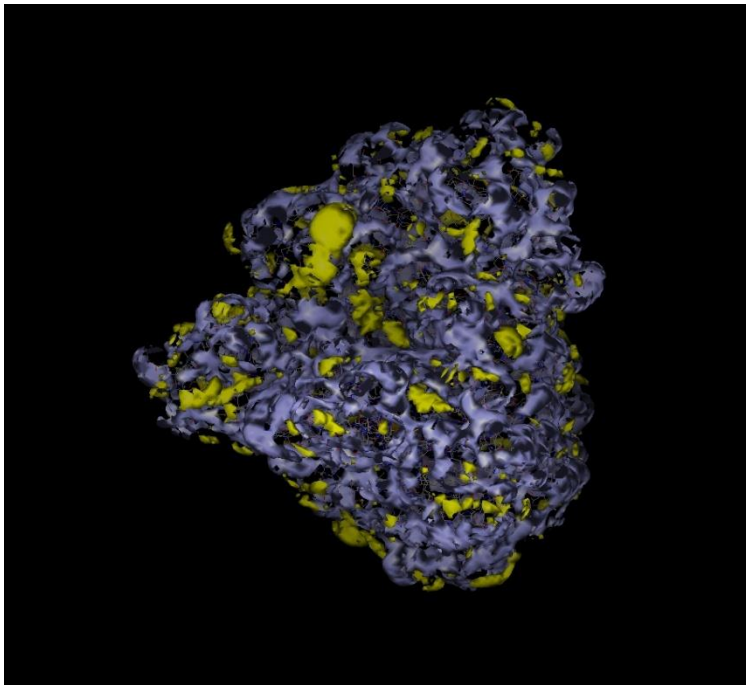


Reaction media
-can compete for water molecules on the surface thus inducing denaturation
-can remove water molecules essential for the mechanism of action: enzyme retains its conformation but looses its activity

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**

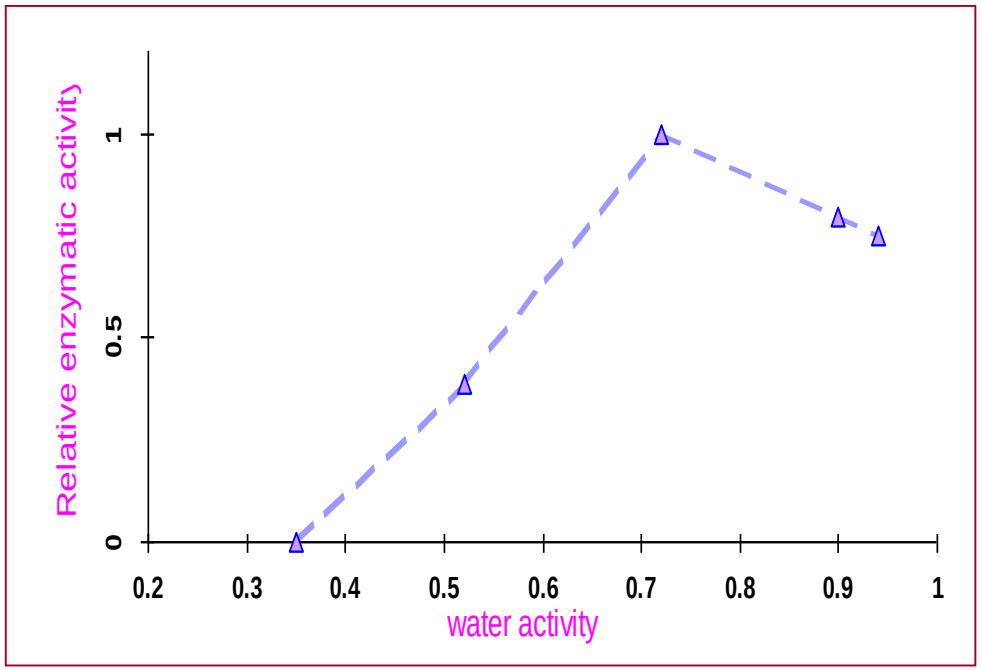
Penicillin G amidase in organic solvent: active when sufficiently hydrated

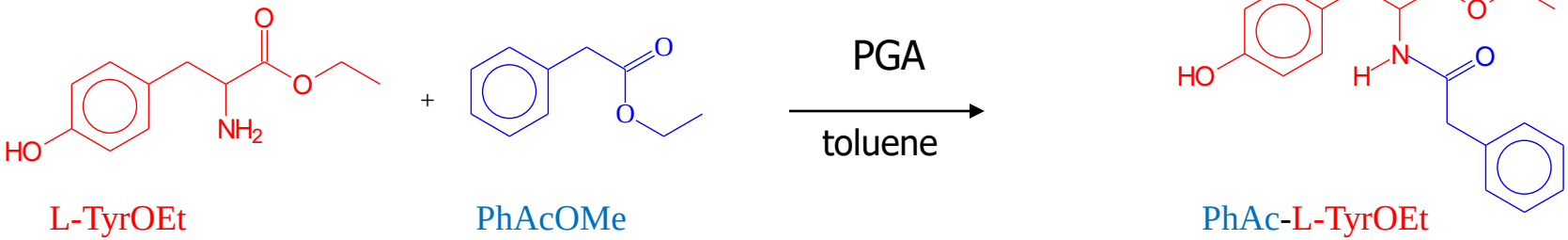




The hydration of the biocatalyst will depend on the amount of “free water” (i.e. water activity) rather then on the amount of total water present in the system.

a_w effect on synthetic activity of PGA in organic solvent



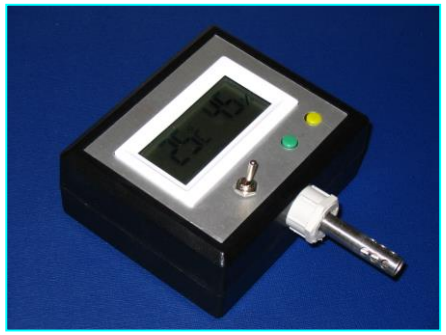


L-TyrOEt PhAcOMe PhAc-L-TyrOEt

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

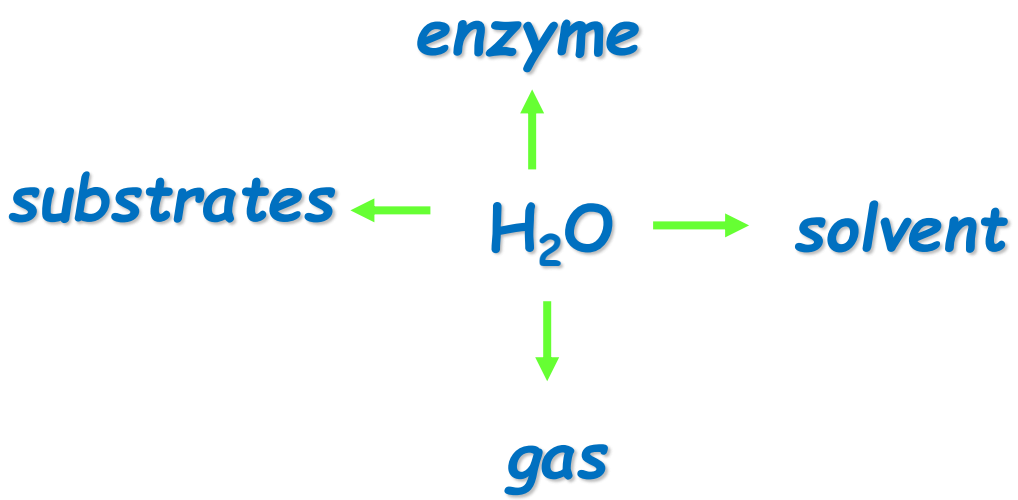
Ebert, C.; Gardossi, L.; Linda, P, *J. Mol. Catal. B*, 1998, *5*, 241-244.

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.

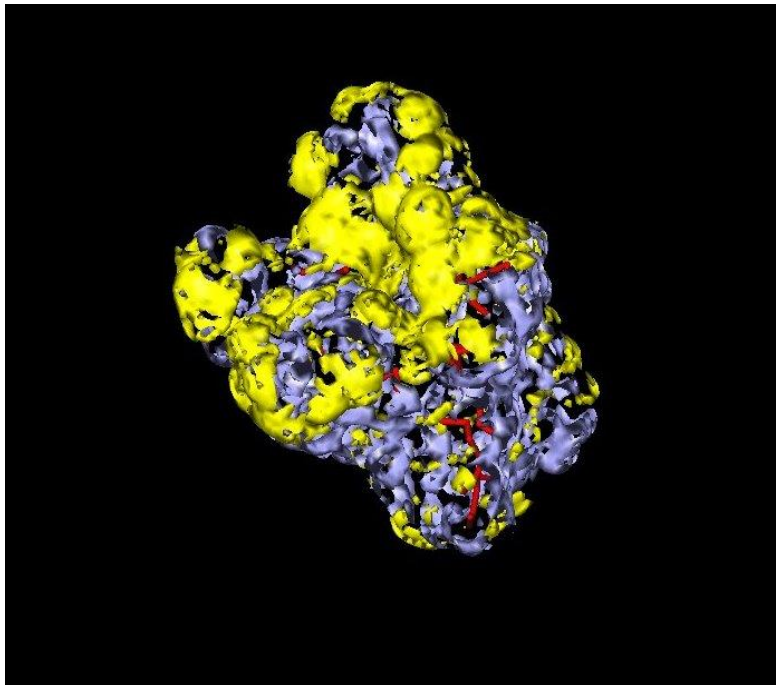


a) Evaluating water activity by measuring water pressure in the gas phase of the close system at the equilibrium
b) Exploiting the ability of porous materials/carriers to control water distribution and mobility
c) Drying or bring to a defined water content all ingredients/phases:
   By using pairs of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

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

hydrophobic

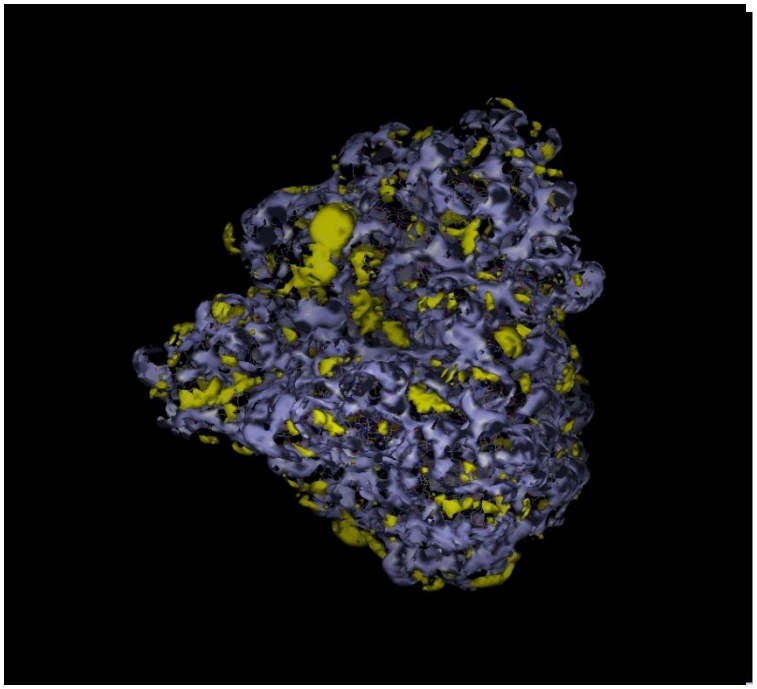
hydrophilic



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

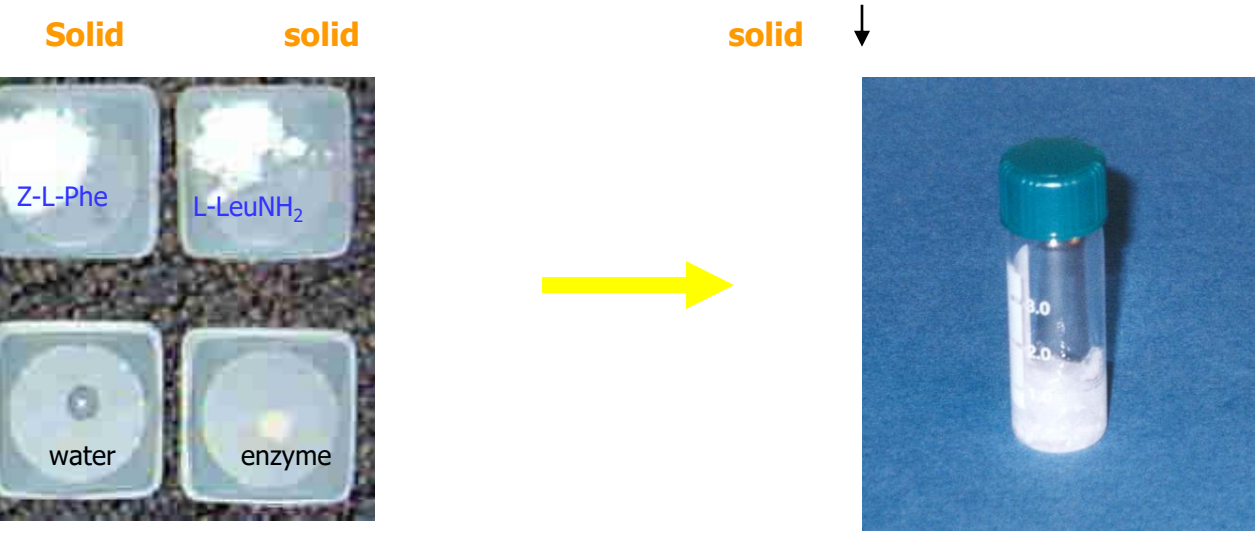
>93% isolated yield (48h)

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

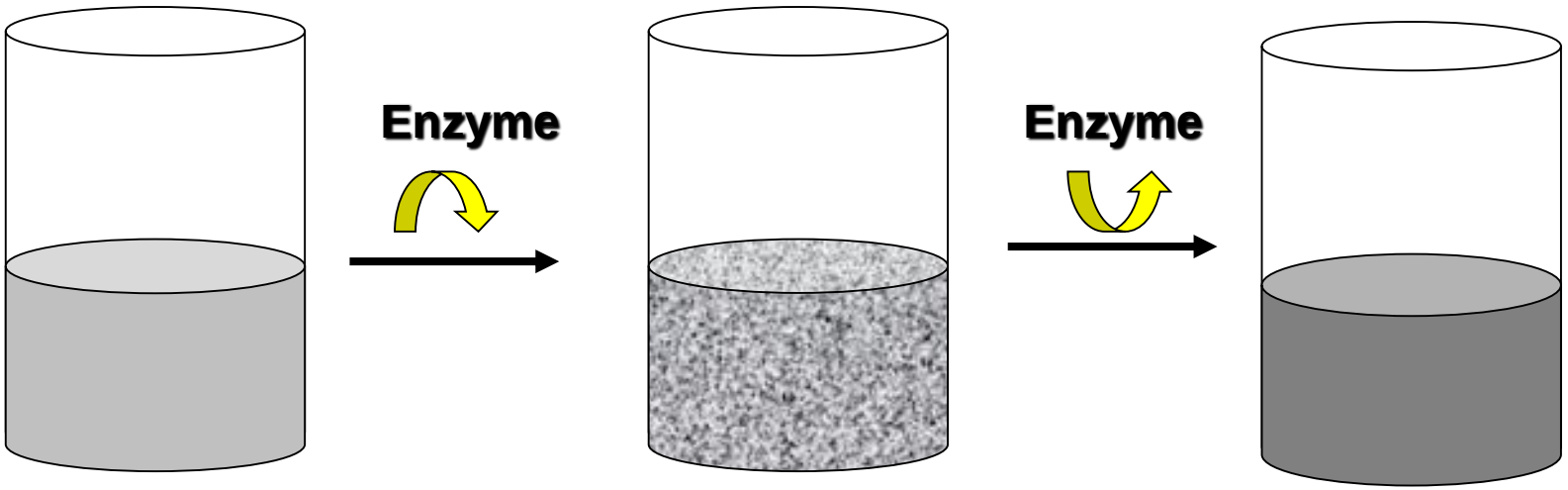
Precipitation driven "solid to solid" peptide synthesis: product solubility must be lower than substarte solubility



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

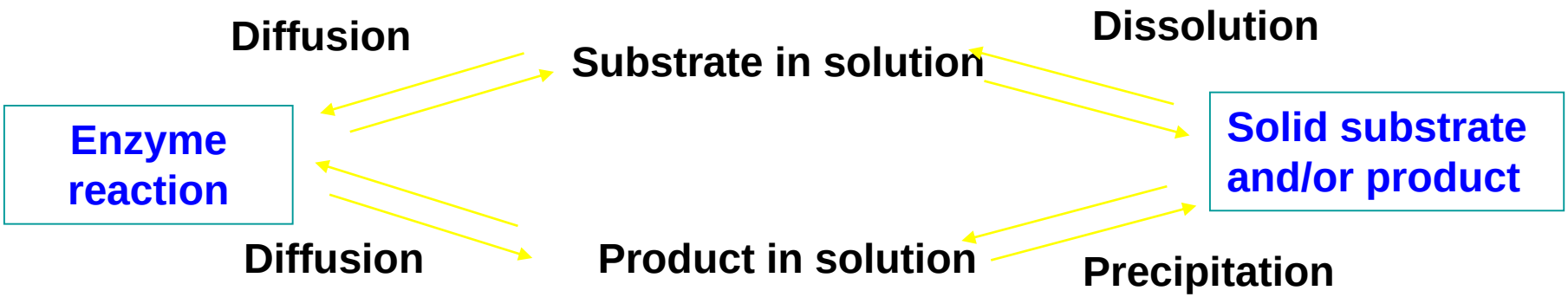
Solid Product + Liquid Phase

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.

What about kinetics?



The overall conversion rate is governed by 3 sub-processes all taking place at the same time

- **Substrate dissolution**
- **Enzyme catalysis**
- **Product crystallisation**

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

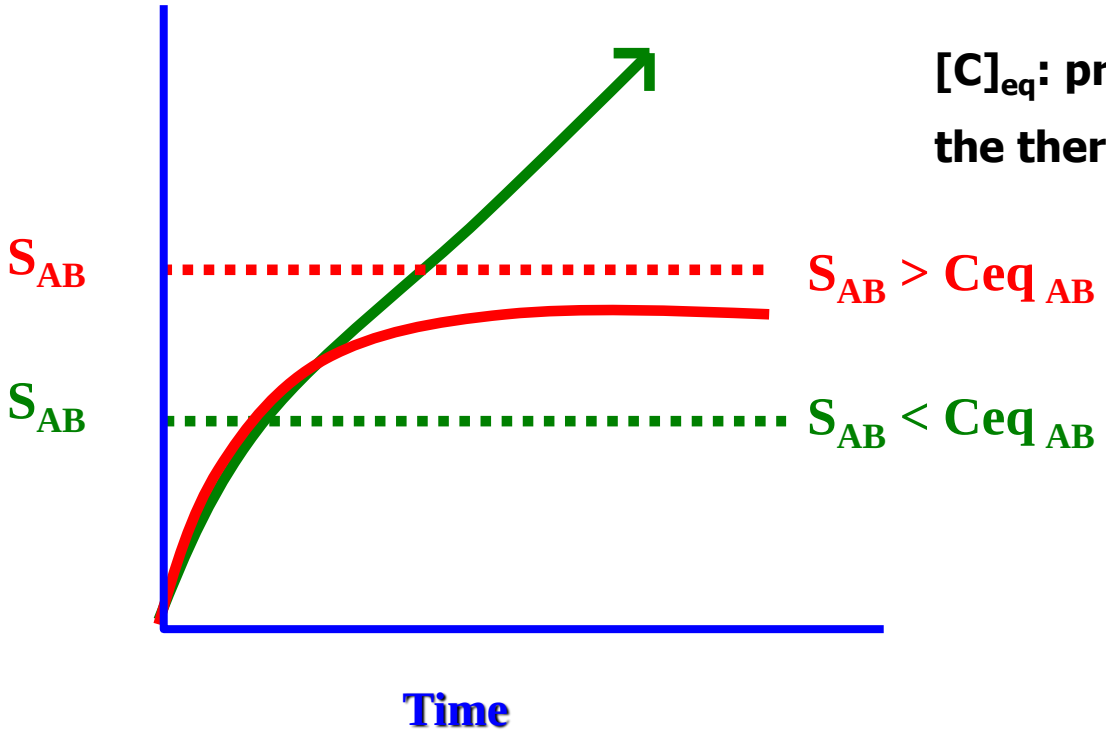
$$A + B \rightleftharpoons AB + H_2O$$

When:

- $S_{AB} < [C]_{eq}$ the product precipitates and thermodynamic 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

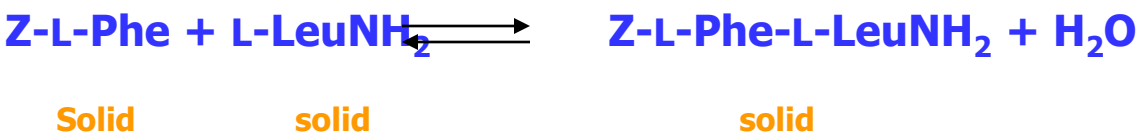
- The yield of crystalline product can be maximised by choosing a solvent where product solubility is lowest

- for hydrophobic targets water is generally a good choice

- in the synthesis of hydrophilic targets good yields are expected in hydrophobic solvents

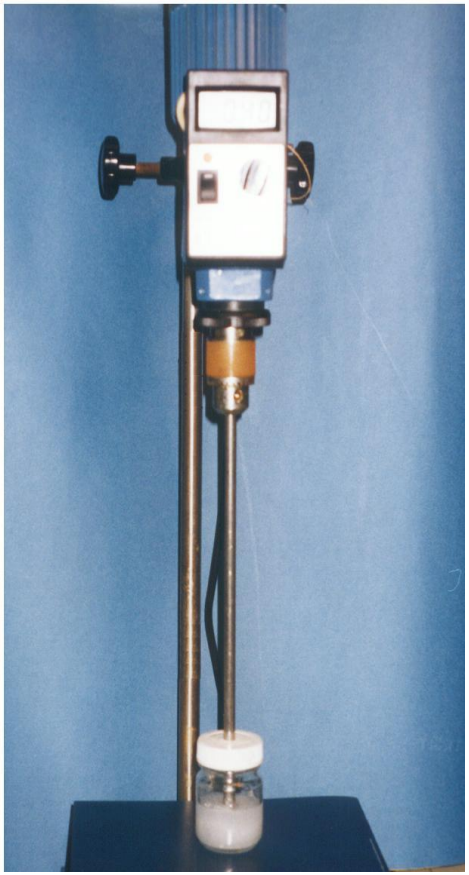
Always use an excess of the most soluble compound

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



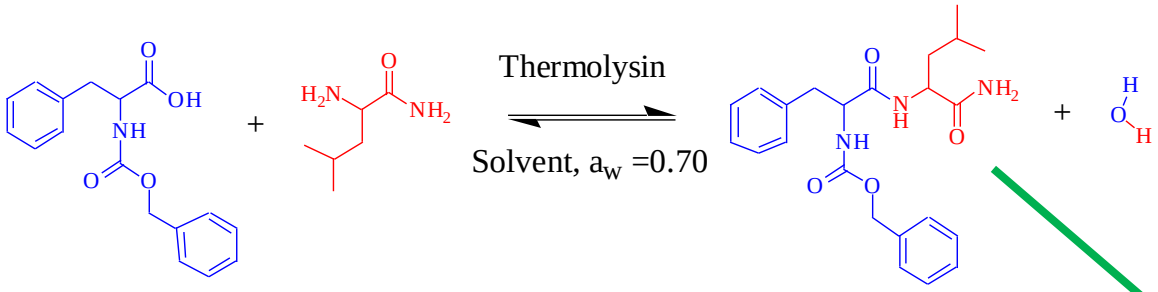
- 2 millimoles of Z-L-Phe and L-LeuNH$_2$ in 20mL toluene
- Conv. after 8h: > 99%
- 96% (1.92 millimoles) of pure solid product recovered
- Enzyme recycled 4 times

Mechanic stirrer

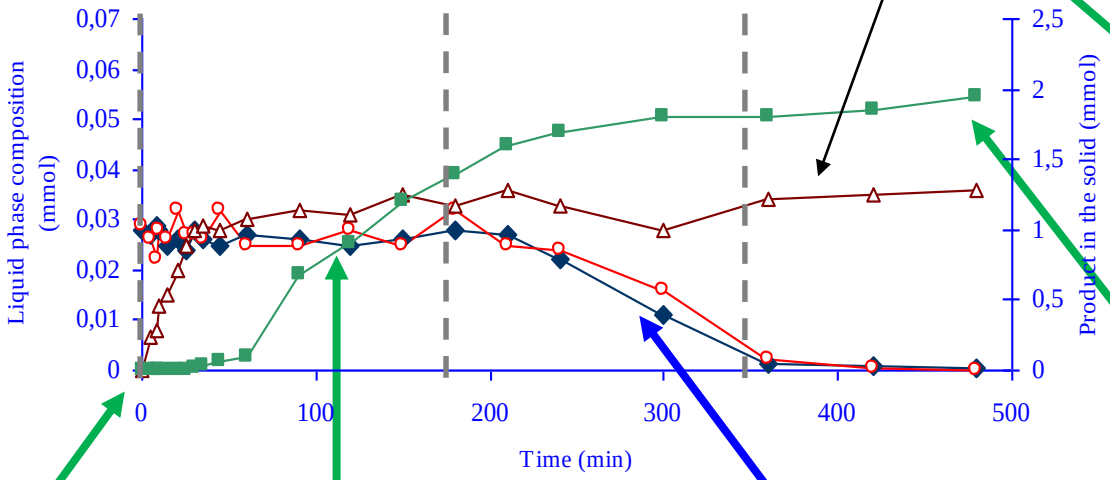


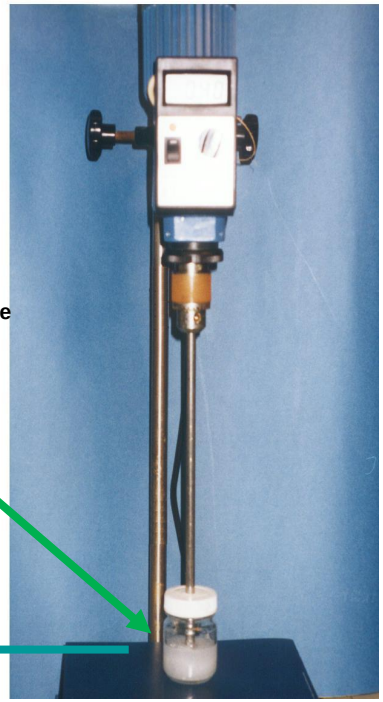
L. De Martin, PhD Thesis, University of Trieste, 2001.

How does it work?



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





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

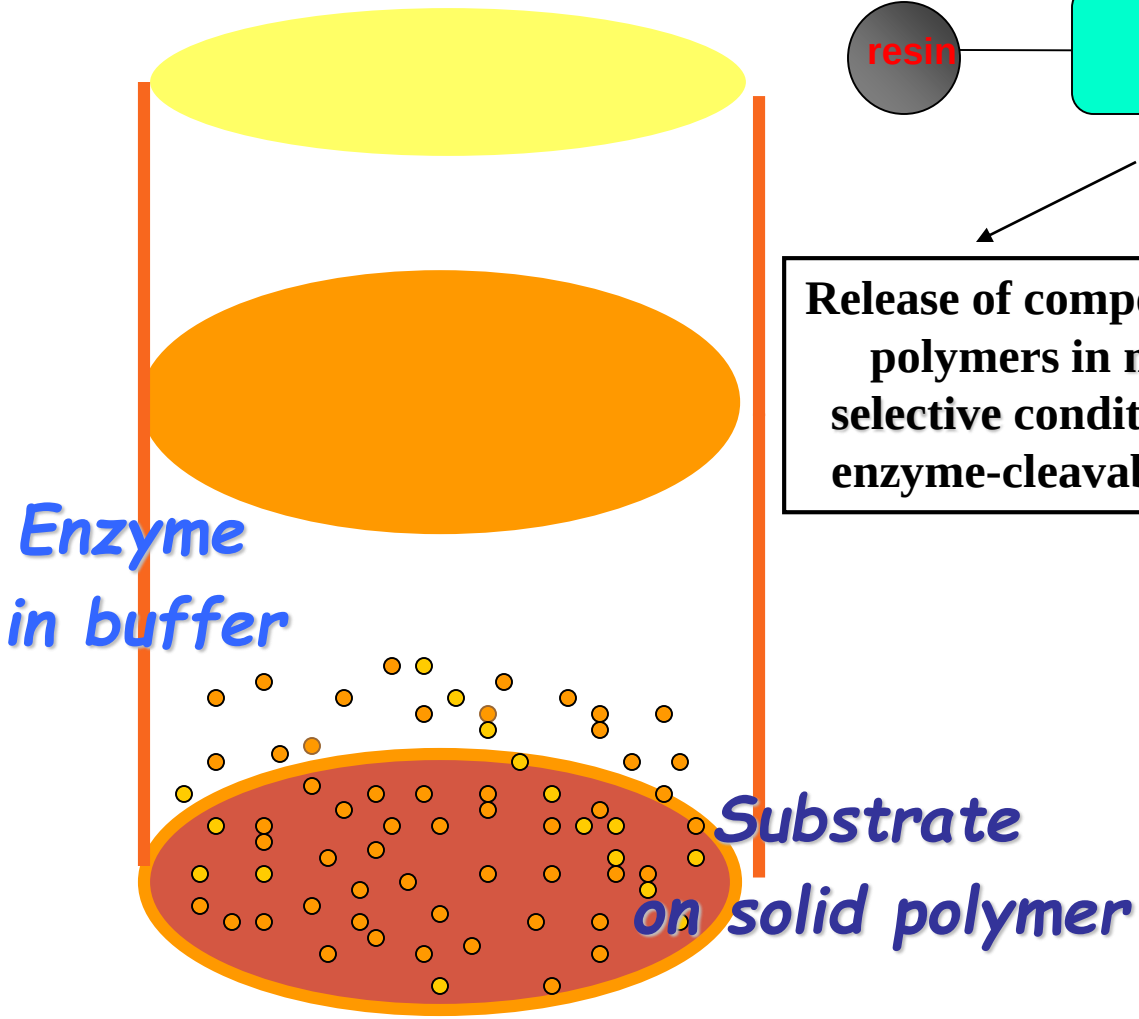
The solution phase reaches a steady state situation

-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

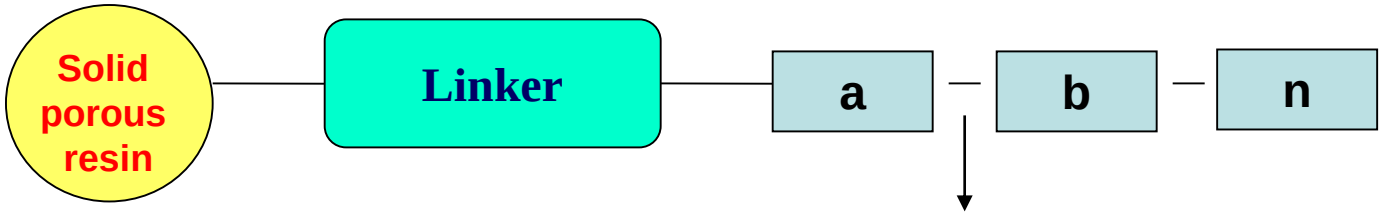
Solid phase biocatalysis



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

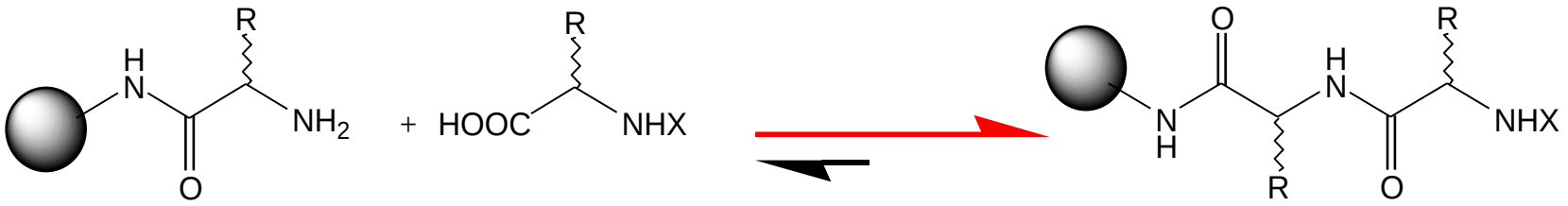
Advantages:

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

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

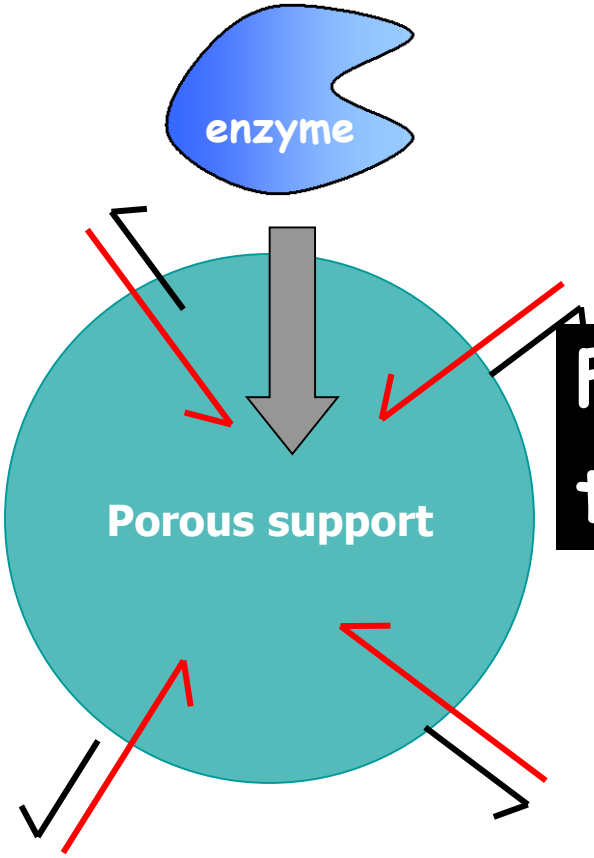
Solid phase biocatalysis: peptide synthesis

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



Problems:
-Diffusion barriers

Fully permeable
to protein up to 35KDa

Enzyme		yield on solid phase *(PEGA polymer)*
α-chymotrypsin	22K Da	98%
Subtilisin C.	27K Da	98%
PGA	88 K Da	15-20%

Meldal, Biopolymers 2002, 66, 93

Conclusions:

1. The availability of the right protein (enzyme) is not sufficient

Making an enzyme suitable for practical applications requires:
-Understanding and controlling the microenvironment
-Understanding interactions between all phases of the system (e.g. enzyme-solvent-solid supports, water, salts...)

Conclusions:

2. The selection and development of biocatalysts is often done on empirical basis (trial & error) and this translates into long and costly works