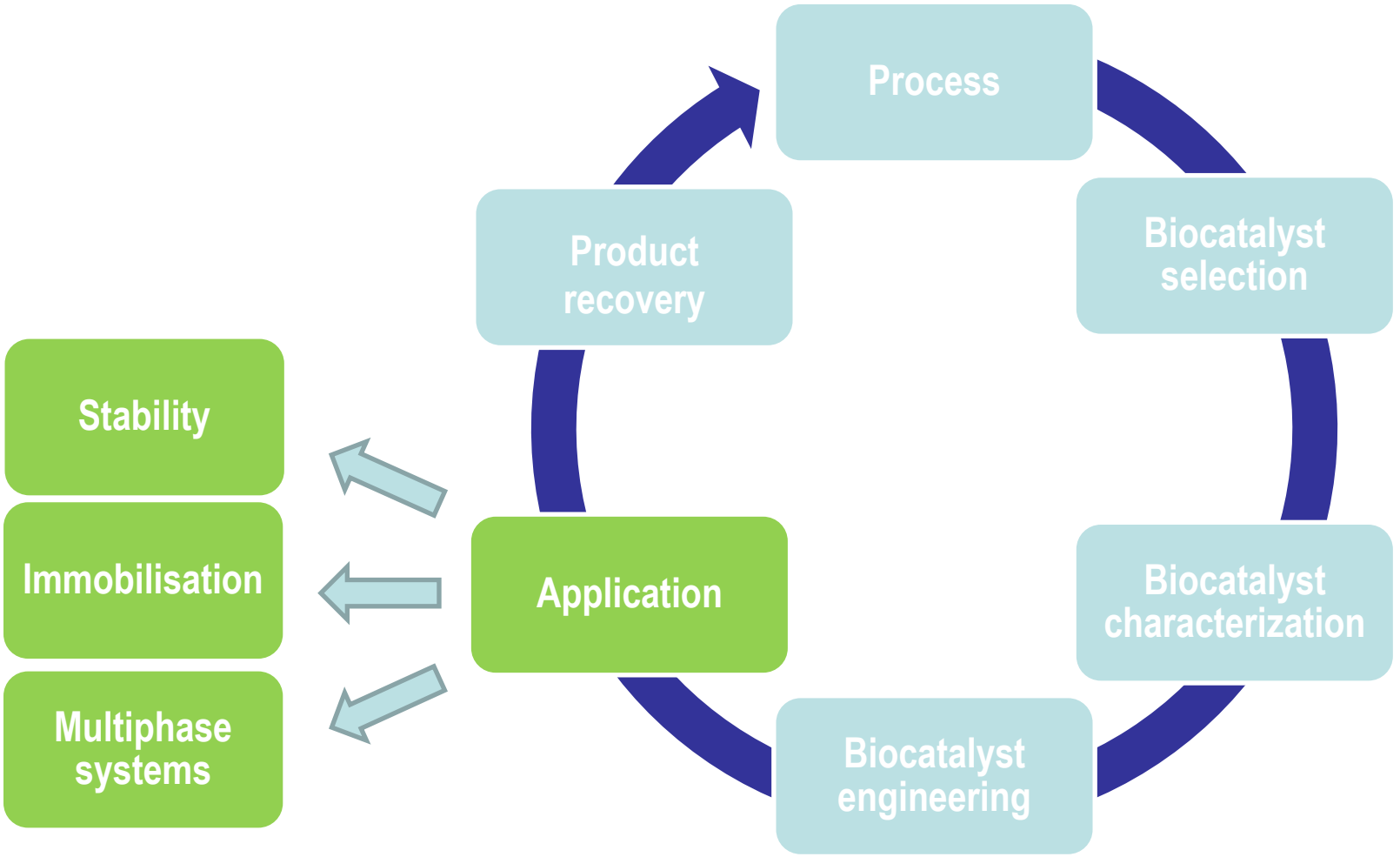
# Formulation, stabilization and immobilization of enzymes for biotransformations

# Biocatalysis for industrial synthesis




**Trasform a good enzyme**
**into an efficient industrial biocatalyst**

Chem Soc Rev

RSC Publishing

TUTORIAL REVIEW

View Article Online
View Journal

Cite this: DOI: 10.1039/c3cs35464d

# Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of organic polymeric carriers and immobilisation methods†

Sara Cantone,[a] Valerio Ferrario,[b] Livia Corici,[a] Cynthia Ebert,[b] Diana Fattor,[a] Patrizia Spizzo[a] and Lucia Gardossi*[b]

*Adv. Synth. Catal.* **2007**, *349*, 1289–1307

**DOI: 10.1002/adsc.200700082**

# Enzyme Immobilization: The Quest for Optimum Performance

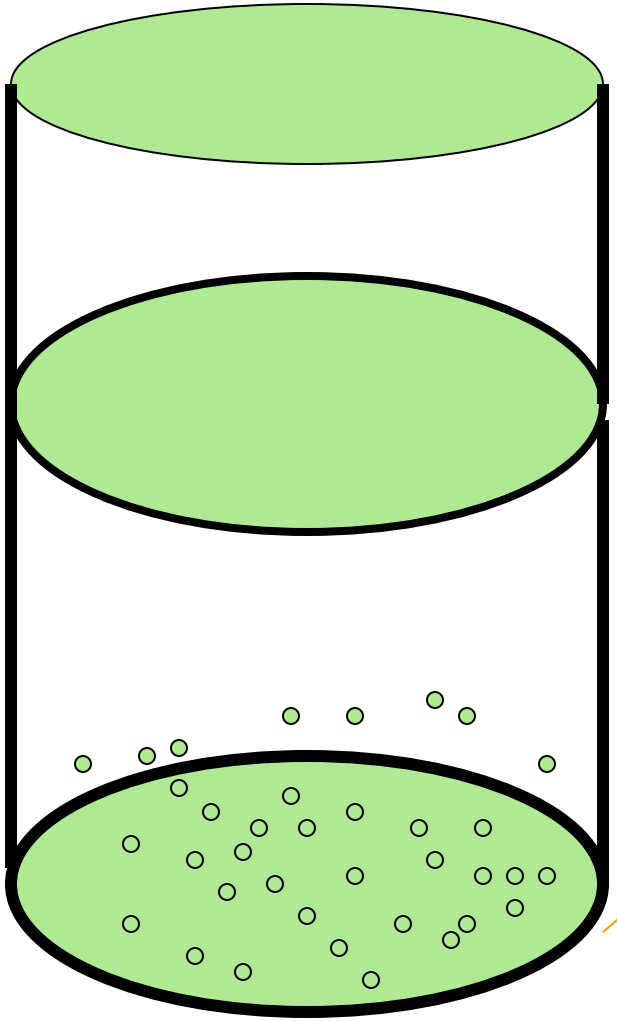
Roger A. Sheldon[a,*]

# Understanding enzyme immobilisation

**Ulf Hanefeld,*[a] Lucia Gardossi[b] and Edmond Magner[c]**

*Chem. Soc. Rev.*, 2009, **38**, 453–468

# Immobilized enzymes: heterogeneous systems



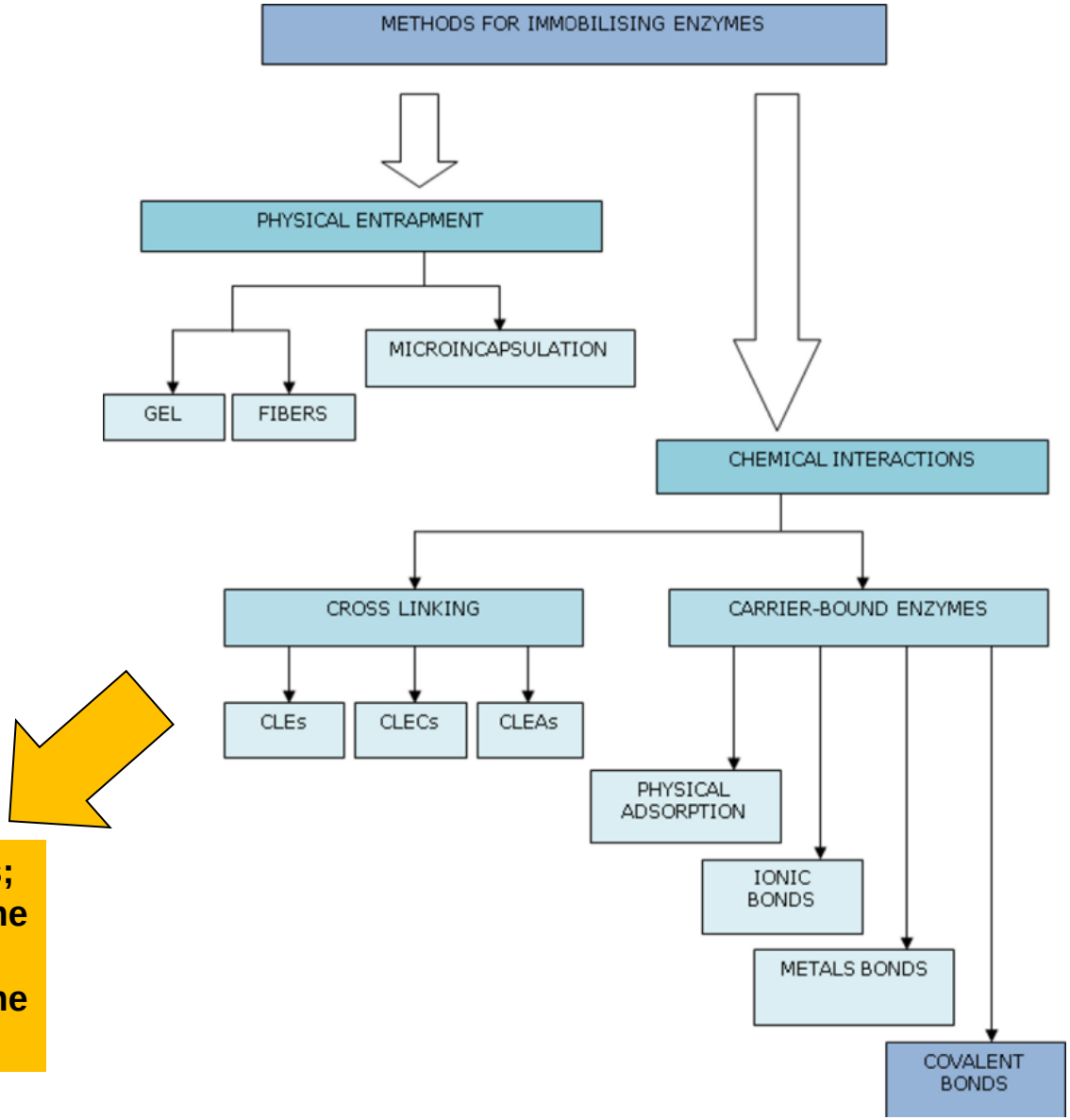
# Immobilized enzymes: potential advantages

- enhanced stability,
- repeated or continuous use,
- easy separation from the reaction mixture,
- possible modulation of the catalytic properties,
- prevention of protein contamination in the product,
- easier prevention of microbial contaminations.

↓

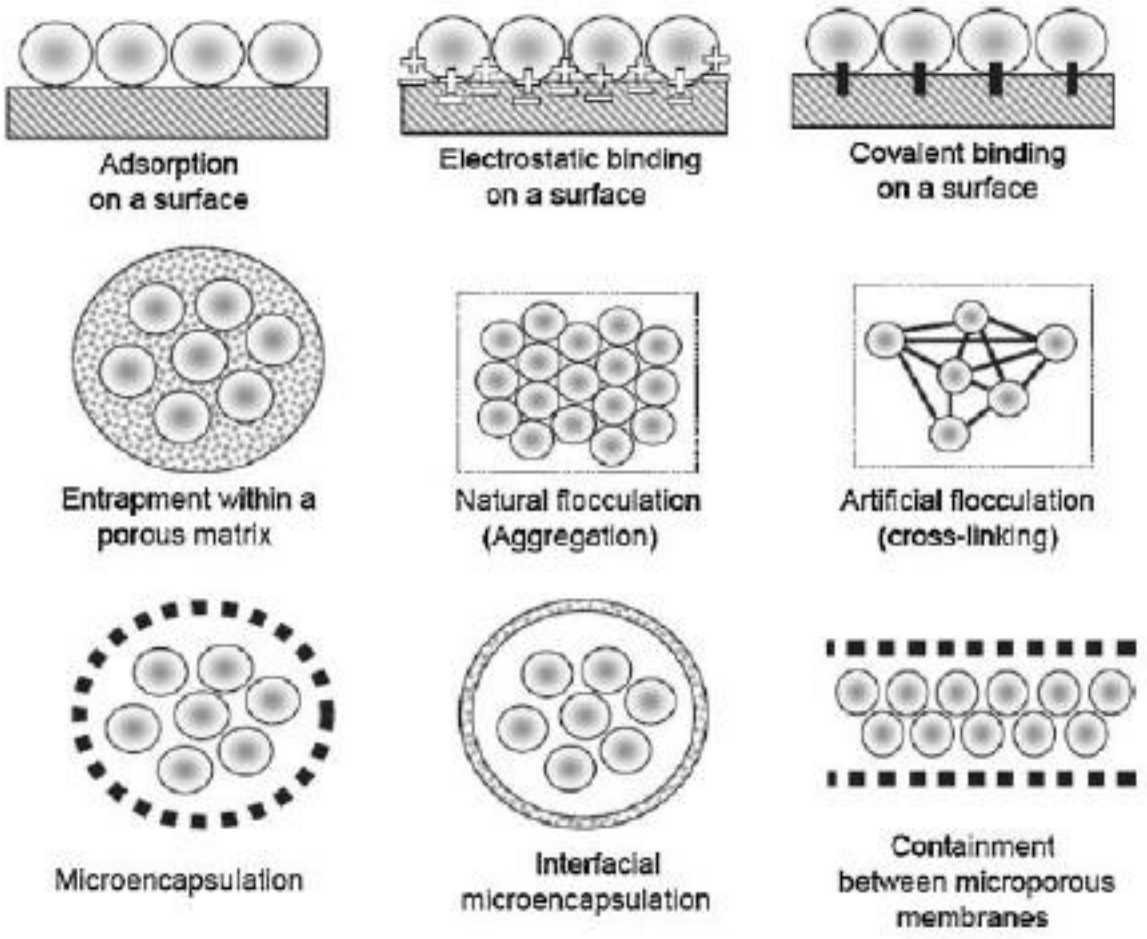
**Most often a compromise is reached between stabilization and % of retained activity**

# Enzyme immobilization: selecting the method




**CLEs:Cross-Linked Enzymes;**
**CLECs:Cross-Linked Enzyme Crystals;**
**CLEAs:Cross-Linked Enzyme Aggregates.**

# Most common immobilization methods



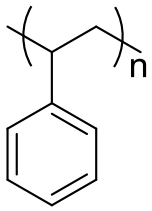
# Immobilization on solid carriers


**Dott. Simone Lotteria, Thesis 2016**

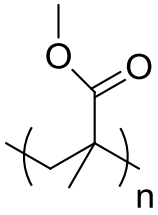
# Carrier

- Either organic or inorganic (e.g. silicates)
- Must be chemically and mechanically stable under operational conditions
- When immobilization occurs via covalent linking the carrier must present suitablefunctional groups on the surface

**Synthetic polymers**

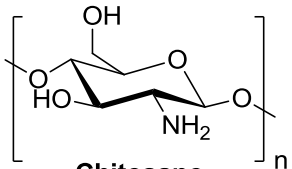


Polistirene

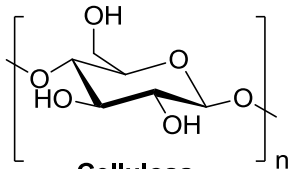


Poliacrilato

**Biopolymers**




**Chitosano**




**Cellulosa**

**Inorganic**

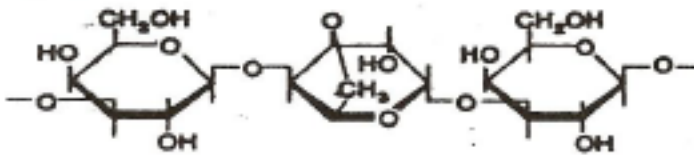
**Silicates, celite**

# Organic polymers used as carriers

| | |
|---|---|
| Polysaccharides: | Cellulose |
| | Agar/agarose |
| | Chitosan |
| | Dextran |
| | Carrageenan |
| | Alginate |
| | Pectate |
| | Xanthan gum |
| Proteins: | Collagen |
| | Gelatin |
| | Albumin |
| | Fibrin |
| Synthetic Polymers: | Polyacrylamide |
| | Methacrylate |
| | Polyurethane |
| | Epoxy resin |
| | Polystyrene |
| | Polyester |
| | Polypropylene |
| | Polyphenylene oxide |
| | Polyvinyl alcohol |
| | Polyvinyl chloride |

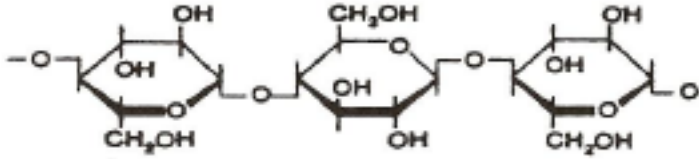
**Polysaccharides and polyamides frequently serve as matrices**

**Agarose**

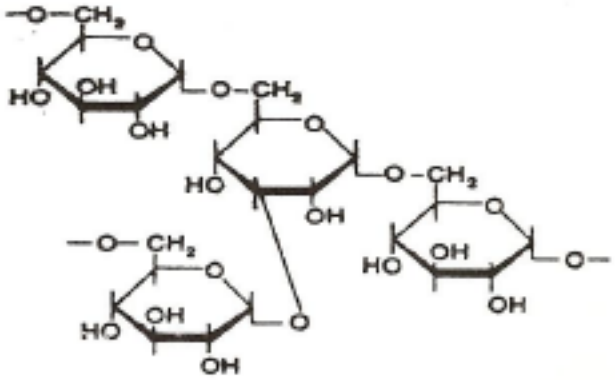


—o-galactose-3, 6-anhydro-L-galactose-o-galactose—

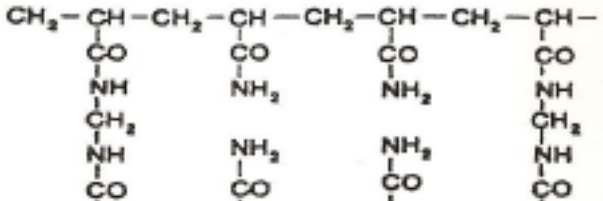
**Cellulose**



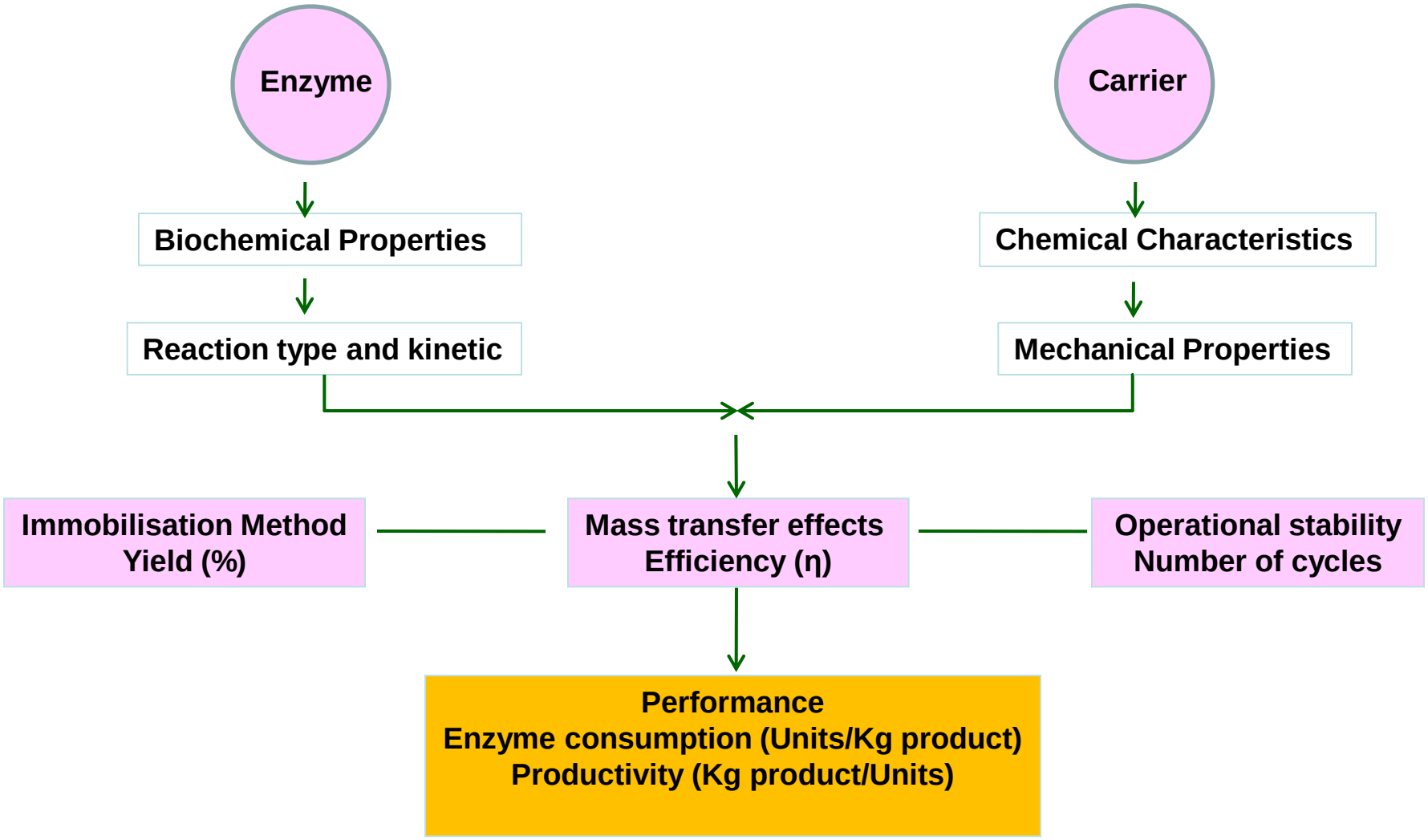
**Crosslinked dextran (Sephadex)**



**Crosslinked polyacrylamide**

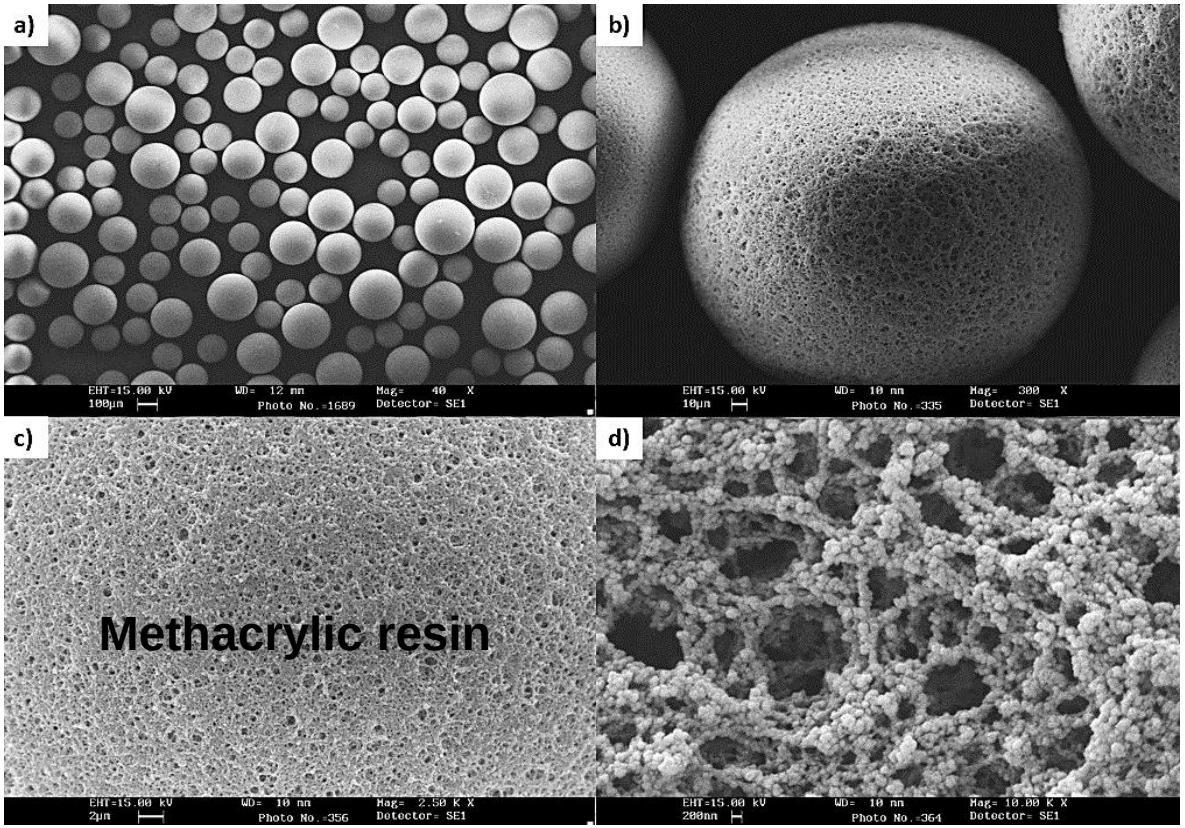


# Immobilization on solid carriers



The physical characteristics of the supports (such as **particle diameter**, **swelling behavior, mechanical stability, and compression** behavior) are of paramount importance for the performance of the immobilised systems and will determine the type of reactor used under technical conditions.

In particular, **pore volume, pore diameter** and particle size determine the total surface area and thus critically affect the **loading capacity** of the resin.



In general, an essential requirement for any carrier is to have a **large surface area** (>100 $m^2$ $g^{-1}$) to promote the contact between the enzyme and the substrate.

The **pore size** of the selected carriers should meet three requirements:
(i) suitable to enable the adsorption of the enzyme molecule in the interior of the carriers;

(ii) larger than the size of the enzyme molecules thus preventing the decrease of enzyme-conformation mobility; and

(iii) diffusion constraints should be mitigated to ensure the accessibility of the substrate to the catalytic site of the enzyme.

**Very large substrates might require lower porosity** to avoid the immobilisation of the enzyme in the inner pores that would be hardly accessible by bulky substrates.

Maintaining the catalyst on the external layer of the support can be preferable when scarcely soluble substrates are used, which might precipitate inside narrow pores.

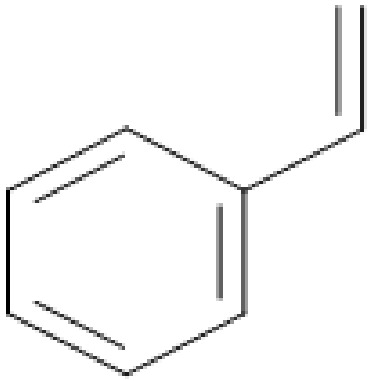
# Organic synthetic polymers and resins

A range of **hydrophobic carriers**, such as polypropylene, acrylic or styrene, with different degrees of hydrophobicity and porosity are available on the market.

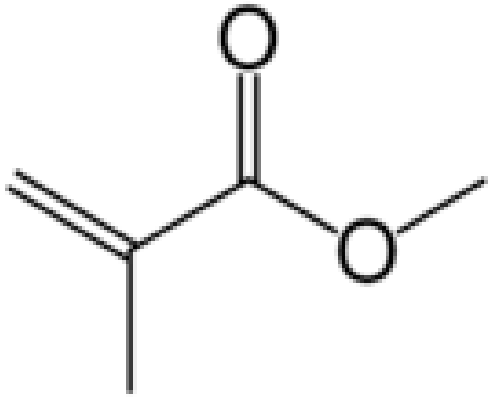
They are synthetized via radicalic polymerization

Indeed, **styrenic polymers** are widely used in refining of pharmaceuticals and natural extracts, since these are suitable for adsorbing large molecules because of their relatively large pore sizes and adsorption–desorption capacity.

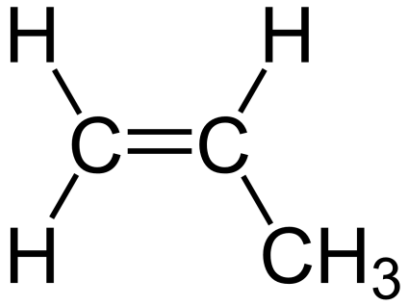
As a consequence, a considerable number of **acrylic or styrenic resins**, with different degrees of hydrophobicity, are available



**Styrene**

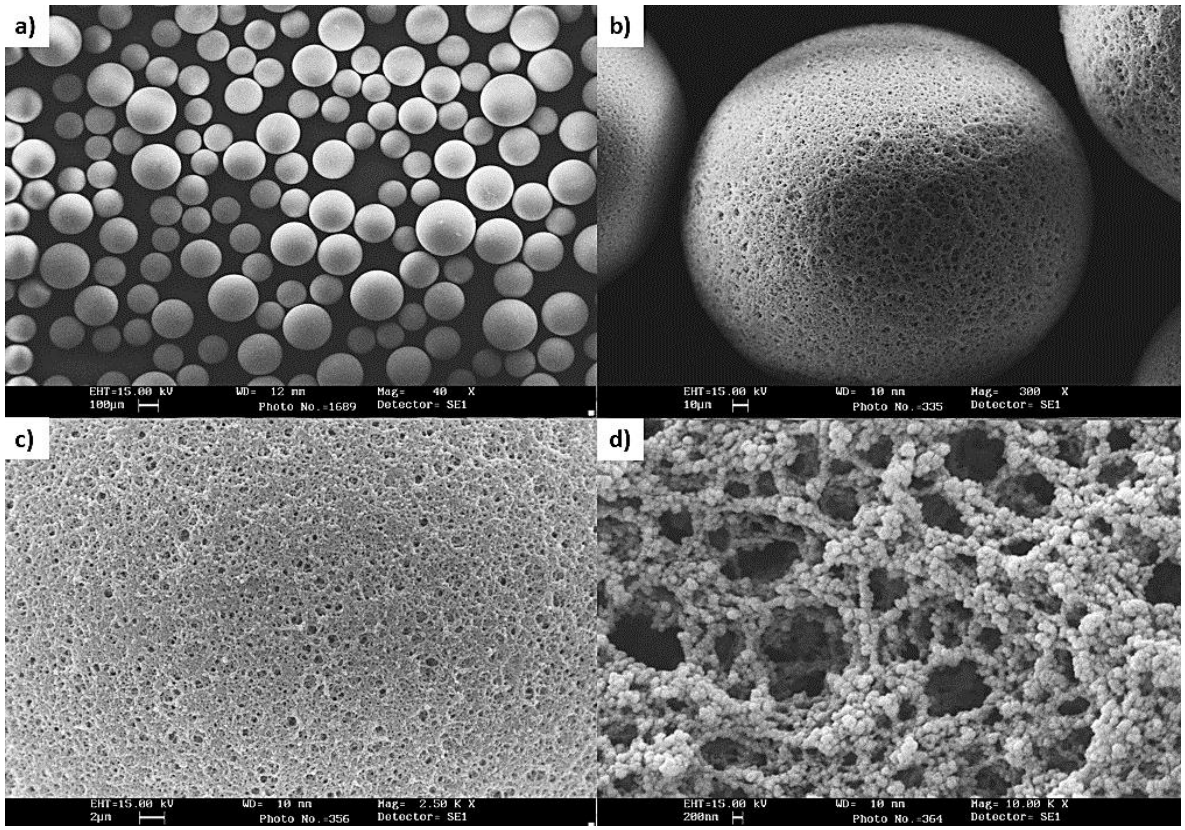



**methyl acrylate**




**propilene**

**acrylic or styrenic resins**,
with different degrees of hydrophobicity, are available and they usually have a surface area **>40 $m^2$ $g^{-1}$**

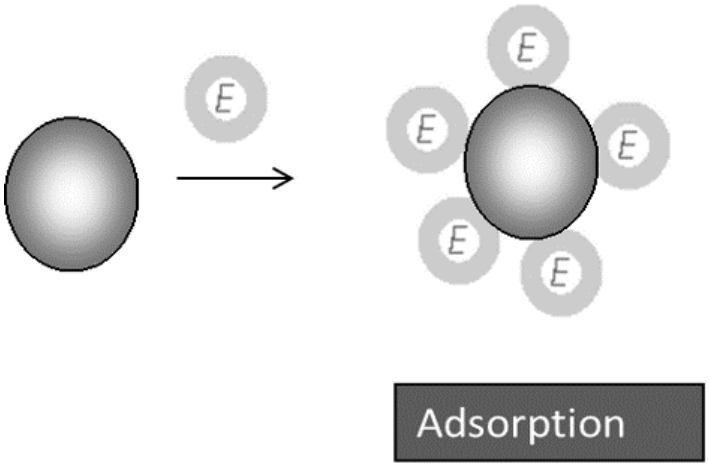



**Porosity :**
several materials used for enzyme adsorption can be considered **macroporous,** since pore diameters are higher than 50 nm.

**Macroporous greater than 50 nm;**
**Mesoporous diameters between 2 and 50 nm.**
**Microporous less than 2 nm**

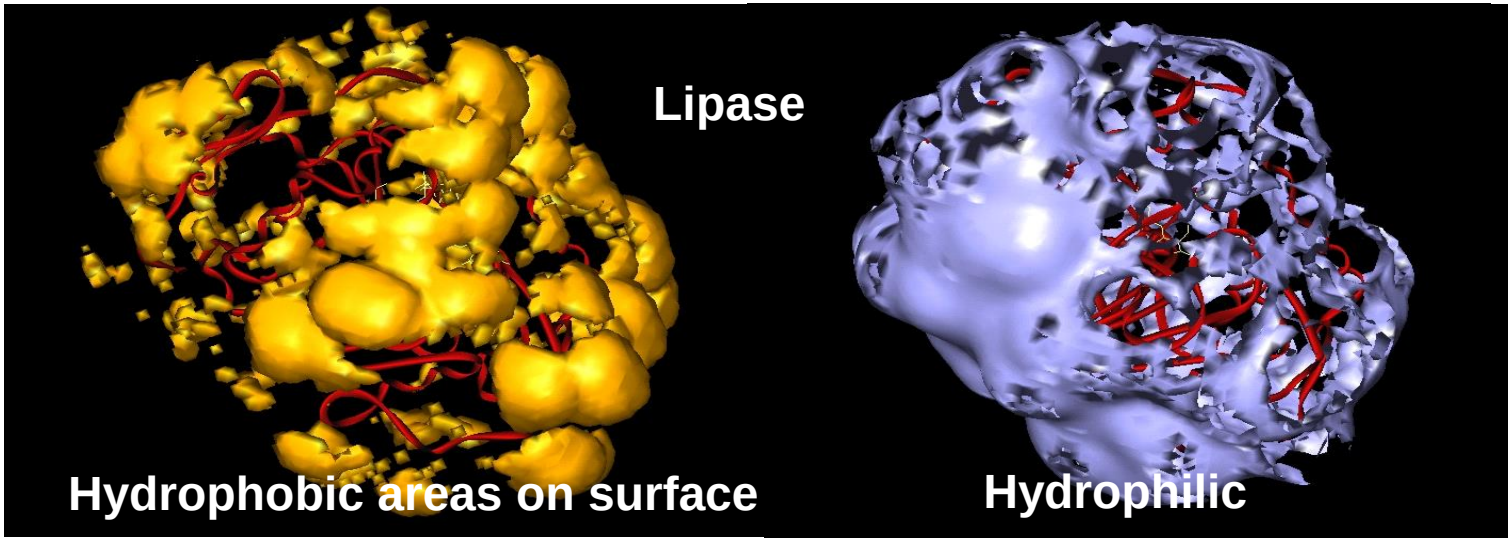
# Enzyme immobilization on solid carriers: adsorption

**Support binding can simply exploit weak interactions**



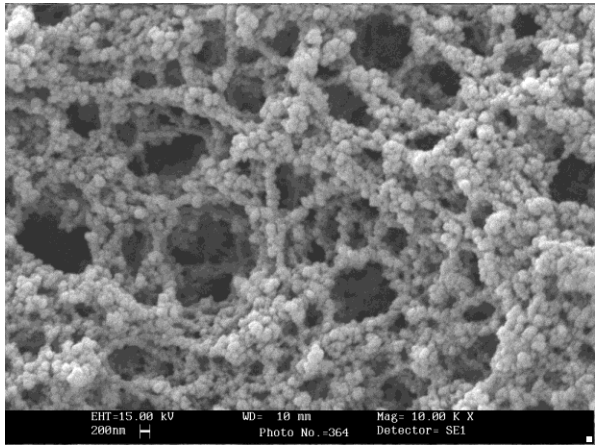

One of the most successful examples of lipase adsorption on organic resins is the widely used enzyme Candida antarctica lipase B commercially available in the immobilised form as Novozym 435$_s$ which consists of the enzyme adsorbed on a macroporous polymethyl/butylmethacrylate-divinylbenzene) resin.

# Hydrophobic, van der Waals interactions




## Methacrylic and styrenic porous polymers

**High porosity and large internal surface**

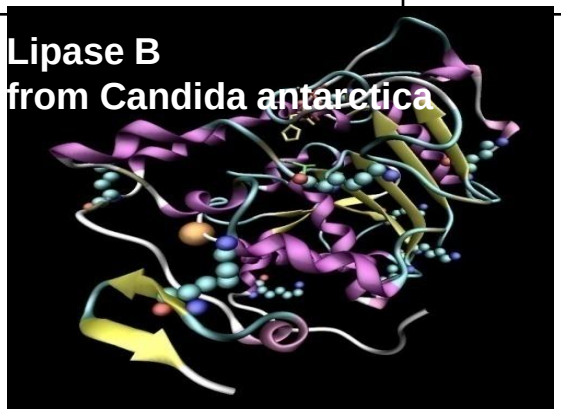



→

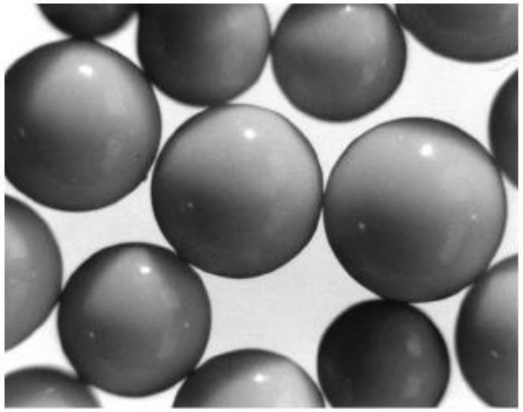
**Increasing the ionic stenght for forcing the partition of enzyme onto the polymer**

# Important!!!
# Commercial enzymes contain impurities and additives

| Product | Appearance | Protein content (from the manufacturer) | Specific hydrolytic activity |
|---|---|---|---|
| **Lipozyme CALB L** | Viscous brown-yellowish liquid<br>Small proteic impurities ì | 6%<br><br>Declared content for 1g solution:<br>440 mg water<br>250 mg sorbitol<br>250 mg glycerol<br>60 mg protein<br>2 mg sodium benzoate<br>1 mg potassium sorbate | 4800-5200 U/ml |
| **Chyrazyme L-2 Roche** | White powder.<br>Lyophilized | 43% | 60-70 $U/mg_{prep}$ |

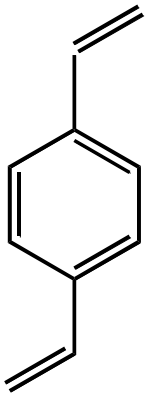


# Acrylic + DVB



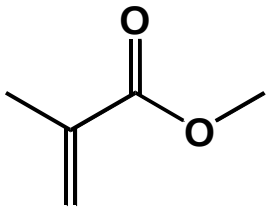
**Novozym® 435**

**CAL-B immobilised (adsorption) on a hydrophobic polymer**



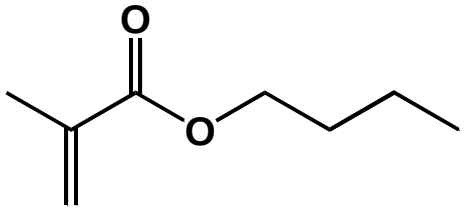
**DVB (Divinylbenzene)**

+



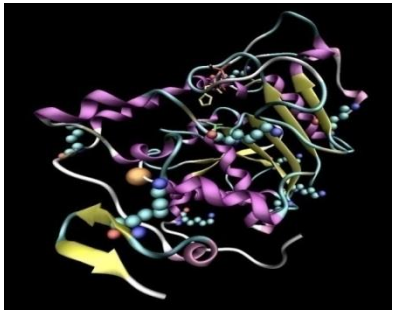

**MA (Methylacrylate)**

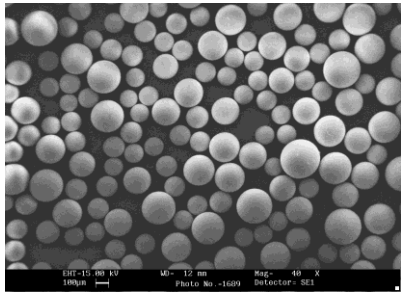
+




**BA (Buthylacrylate)**

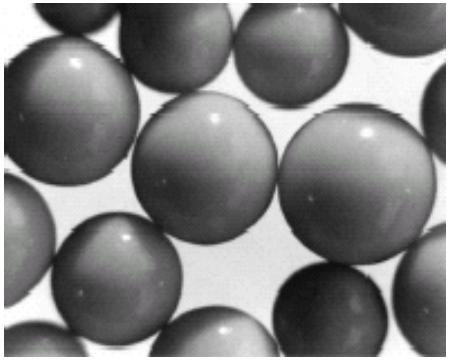
# Lipase B from *C. antarctica*





**Enzyme** **Solid support** **Immobilised bio-catalyst**

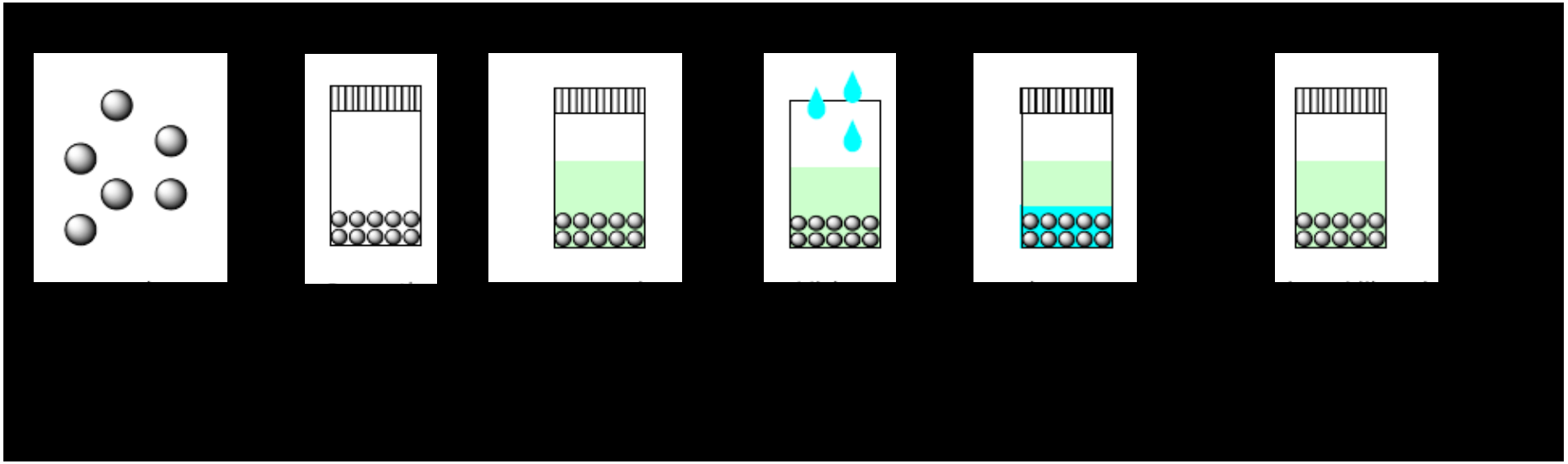
**Adsorption** **Immobilisation**

**Novozyme 435**

Lipase B from Candida antarctica, adsorbed on acrylic resin

# Enzyme immobilization: enzyme solubilized in buffer is adsorbed on solid supports



**Support binding can simply exploit weak interactions**

**More appropriate for industrial applications is the covalent binding of the enzyme to the support since it has the advantage that the enzyme cannot be leached from the solid support.**

# Inorganic Supports

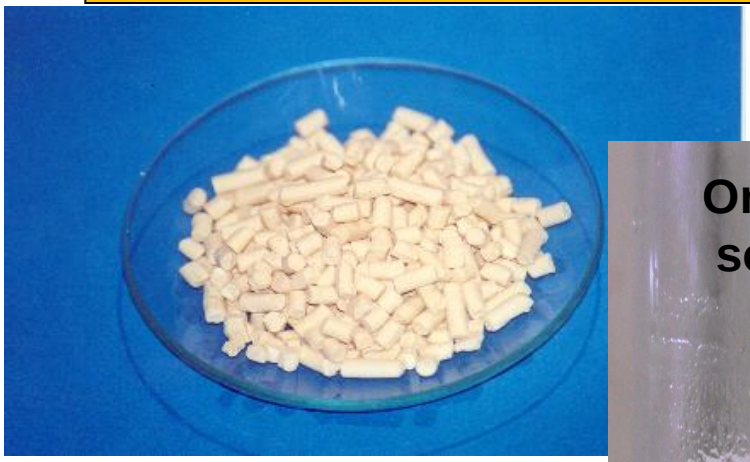
A variety of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica, zeolites and mesoporous silicas.

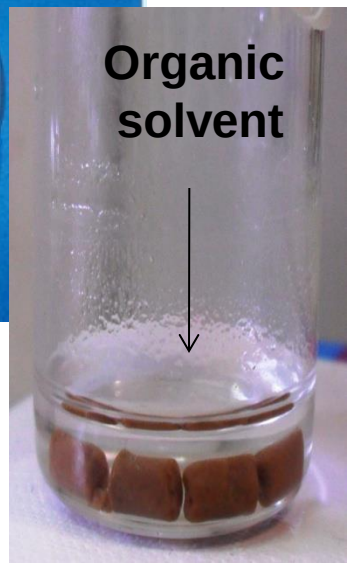
# Silicates

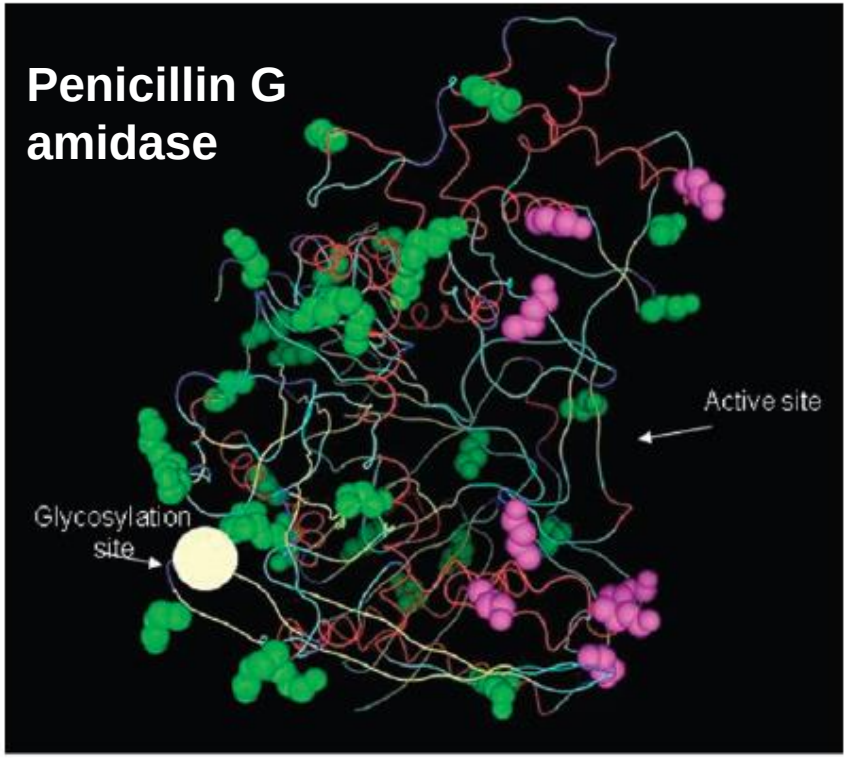
- Chemically inert, stable shape, do not swell

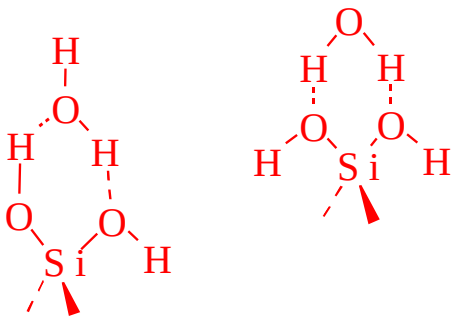
**Celite derives from Diatomaceous earth (powder)**

# Immobilization on Celite: Hydrogen bonds and hydrophilic interactions. Suitable for applications in non aqueous media





**in most cases hydrophilic amino acid residues prevail on the surface of enzymes.**

**In addition, enzymes may be glycosylated, further increasing the hydrophilicity of the protein. Therefore they can easily form hydrogen bonds and thus can be immobilised on hydrophilic carriers (cellulose, Celite, porous glass, clay, silica gel). A particularly popular carrier is Celite (diatomaceous earth), the silicate skeletons of diatoms. Powder are calcinated at high temperature.**

**Inorganic Supports**

One of the simplest and most inexpensive methods to immobilize an enzyme is by **silica Granulation**.

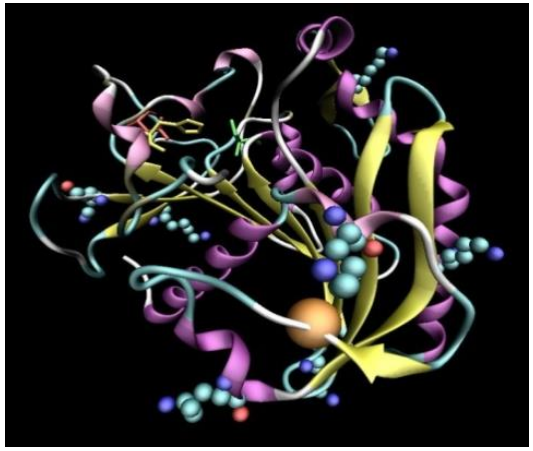
It is used, for example, to formulate enzymes for detergent powders which release the enzyme into the washing liquid during washing. **Granulation technology was used to immobilize lipase on silica granules, by first adsorbing the lipase on silica powder followed by agglomeration.**

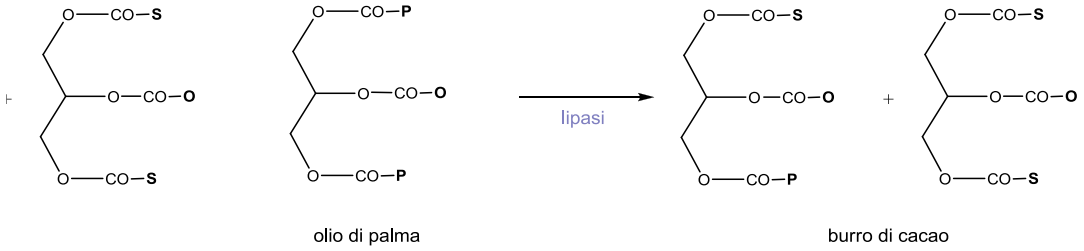
Owing to the composition of the granulates, they are intended for use only in organic media.
In an aqueous medium the lipase is desorbed and the particle slowly disintegrates.

# Granulation on silica: industrial scale

**Lipozyme TL IM is a kosher- and halal-certified, food-grade lipase from *Termomyces lanuginosa*. In its non-immobilised form, it is a 1,3-specific lipase. As an immobilised enzyme it preferentially rearranges the fatty acids in the 1- and 3-positions on the fats (the 2-position is partly preserved).**

**The lipase is immobilized onto porous silica granulates which are insoluble in oil. Lipozyme TL IM is intended for use with interesterification of bulk fats for frying fats, shortenings & margarine hardstock.**





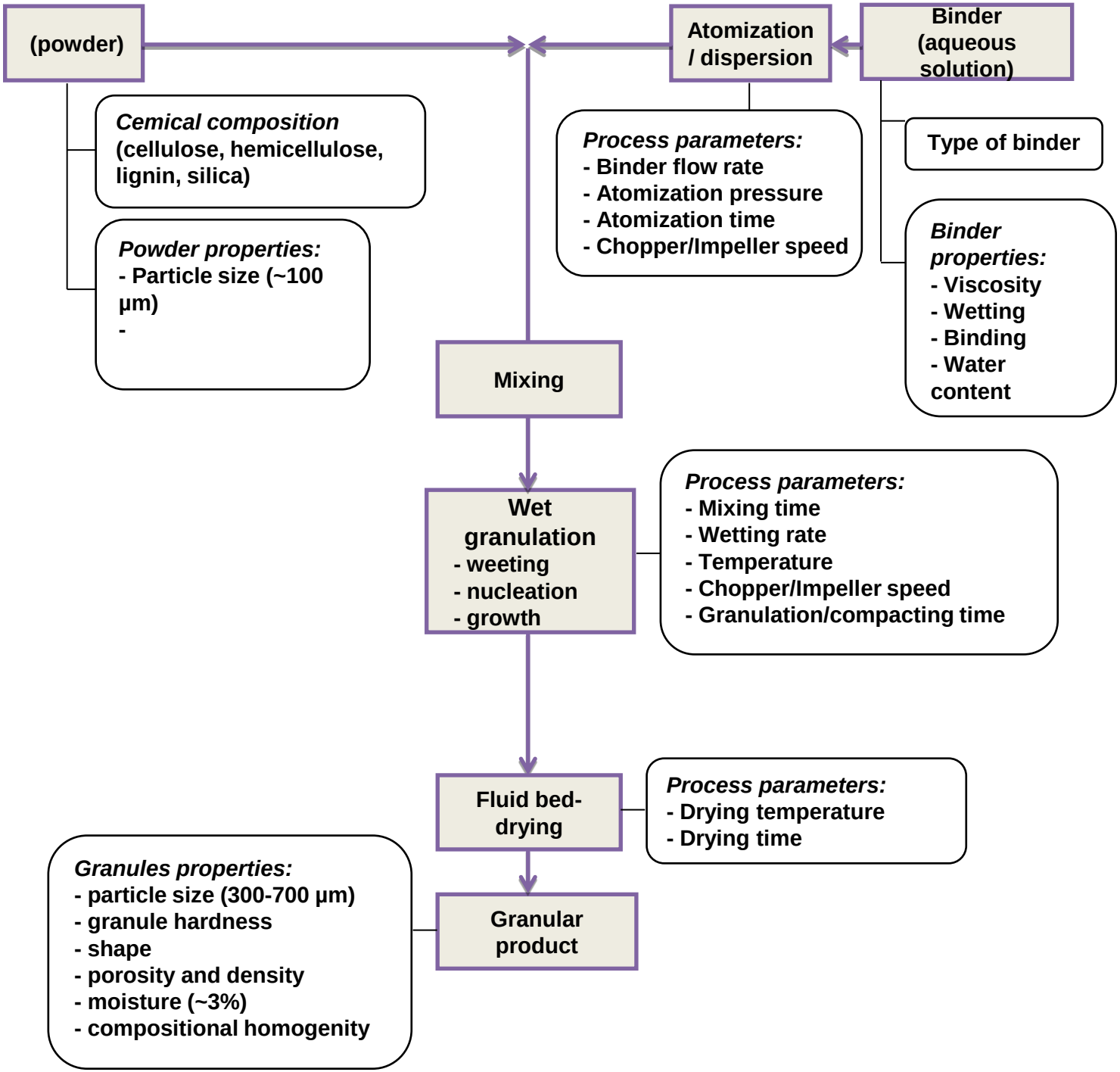
**The enzyme and a liquid binder (gum, PVA,…) are sprayed by atomization onto a silica carrier with a particle size below 100 μm.**

**During the granulation, the silica particles become agglomerated into larger, porous particles with the enzyme distributed evenly over the whole surface area of the silica.**

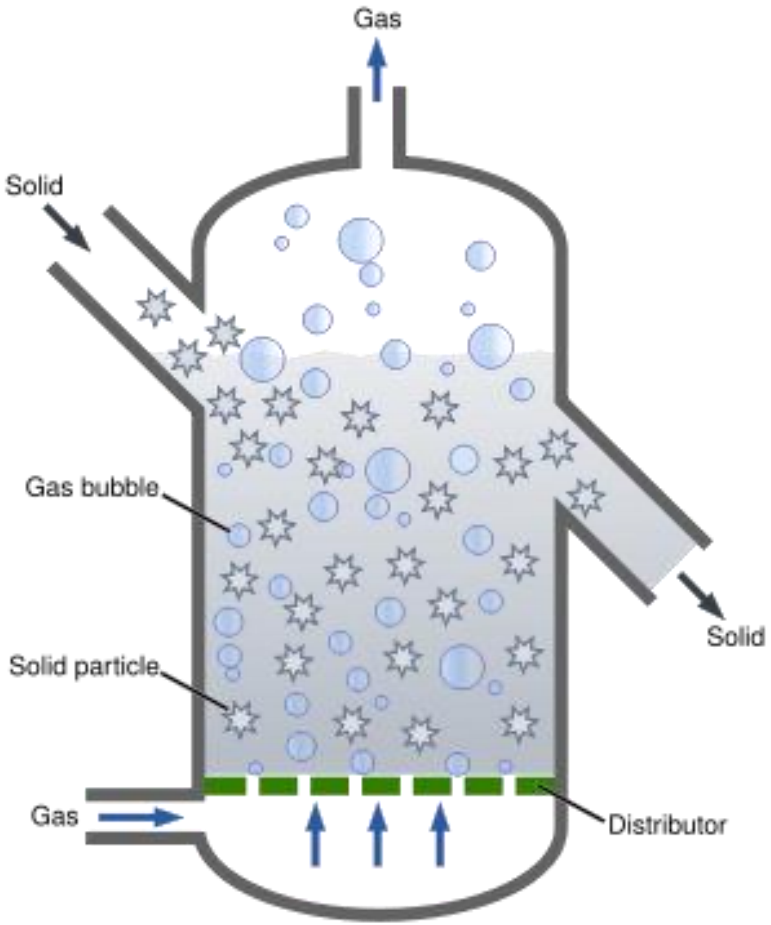
**The mean diameter of the particles is around 600 μm and the surface area is around 50 $m^2$ per gram.**

**This gives a large area where the substrate can come into contact with the enzyme. Even though the silica granules are porous, they are mechanically stable both for batch and fixed bed column operation.**
**Furthermore, all the granulation components are of food-grade quality**

The immobilized lipase Lipozyme® TL IM viewed through a light microscope. The enzyme is bound to a silica carrier


(powder)
Atomization / dispersion
Binder (aqueous solution)
Cemical composition (cellulose, hemicellulose, lignin, silica)
Powder properties:
- Particle size (~100 µm)
-
Process parameters:
- Binder flow rate
- Atomization pressure
- Atomization time
- Chopper/Impeller speed
Type of binder
Binder properties:
- Viscosity
- Wetting
- Binding
- Water content
Mixing
Wet granulation
- weeting
- nucleation
- growth
Process parameters:
- Mixing time
- Wetting rate
- Temperature
- Chopper/Impeller speed
- Granulation/compacting time
Fluid bed-drying
Process parameters:
- Drying temperature
- Drying time
Granular product
Granules properties:
- particle size (300-700 µm)
- granule hardness
- shape
- porosity and density
- moisture (~3%)
- compositional homogenity

# Fluidized bed

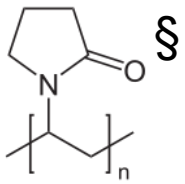


## Type of Binders

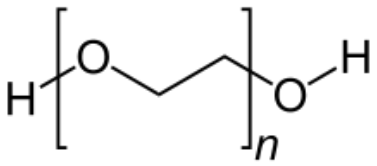
### Classification of organic binders

| Sugars | Natural Binders | Synthetic/Semisynthetic Polymer |
|---|---|---|
| **Sucrose** | Acacia | Methyl cellulose |
| **Liquid glucose** | Tragacanth* | Ethyl cellulose |
| **Maltodextrin** | Gelatin | Hydroxy propyl methyl cellulose ( HPMC) |
| | Starch paste, corn starch | Hydroxy propyl cellulose |
| | Pregelatinized starch | Sodium carboxy methyl cellulose |
| | Alginic acid | Polyvinyl pyrolidone (PVP)§ |
| | Cellulose | Polyethylene glycol (PEG)** |
| | Collagen | Polyvinyl alcohols (PVA)*** |
| | Spirulina | Polymethacrylates |
| | | Glycerol |
| | | Calcium lignosulfonate |

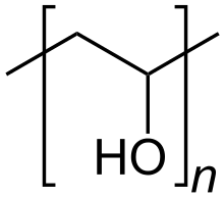
*Tragacanth is a natural gum obtained from the dried sap of several species of Middle Eastern legumes



§



**



***

# Immobilization on functionalized solid carriers

## Immobilization on functionalized solid carriers

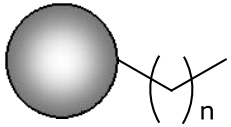
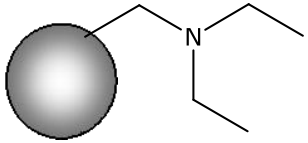
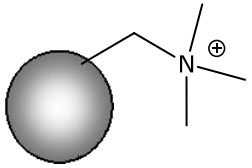
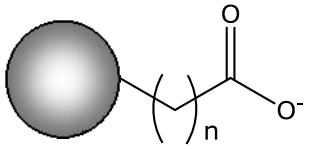
It is related to the presence of **specific chemical functionalities** on the surface of the carriers.

These functional groups can be part of the inherent **structure of the monomers comprising the carrier** (e.g. –OH groups of carbohydrates) or can be deliberately **introduced during the polymerization process**

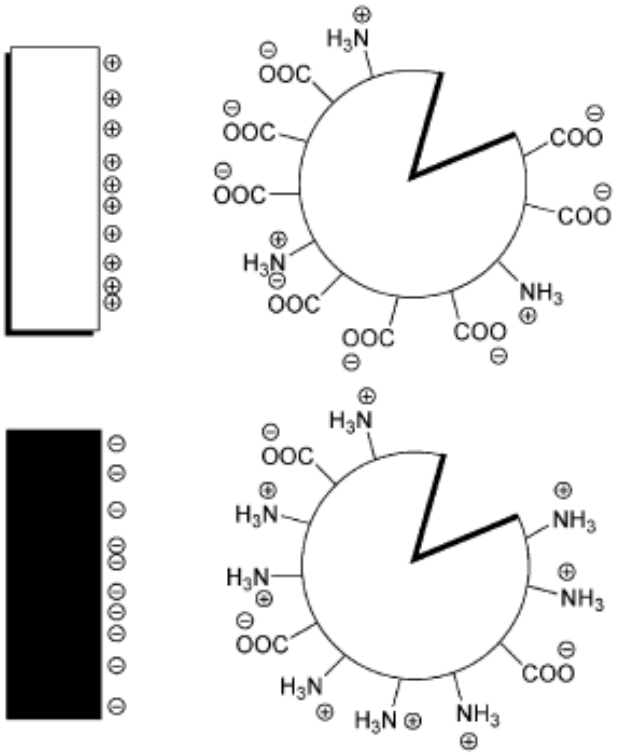
Finally, they can also be **introduced by chemical modification** of the surface of the carrier through a "pre-activation" treatment.

**polymer–enzyme spacers:**
Longer spacers are expected to allow a wider conformational flexibility to the protein.
Shorter spacers can confer higher thermal stability since they restrict the enzyme mobility and prevent unfolding. They can also cause steric hindrance and lower accessibility to the active site.

# Organic polymeric resins with functional groups: different types of interactions

| Method of immobilisation | Functional group | Structure | Binding | Reactive group on enzyme |
|---|---|---|---|---|
| van der Waals and hydrophobic interactions | alkyl |   | maximizes hydrophobic interactions (adsorption) | Hydrophobic areas on surface of lipases |
| | | | | |
| Ionic interactions | Trialkyl ammine |   | Ionic adsorption | Negatively charged a.a. |
| | Tetra alkyl ammonium |   | Ionic adsorption | Negatively charged a.a. |
| | Carboxylate |   | Ionic adsorption | Positively charged a.a. |
| | | | | |
| | | | | |

# Immobilization *via* ionic interactions



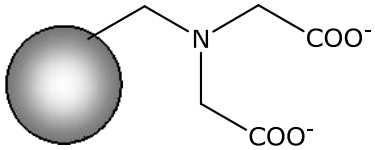

**Depending on the pH of the solution and the isoelectric point the surface of the enzyme may bear charges. Using widely available modelling systems, the surface charge and charge distribution of an enzyme can be readily calculated and displayed. Essentially any ion exchanger can act as carrier in immobilisation via ionic and strongly polar interactions. Depending on the predominant charge on the enzyme, the ion exchanger needs to be negatively (for instance carboxylate) or positively charged (for instance protonated amino groups).**

**Chem. Soc. Rev., 2009, 38, 453–468**

**Table S2:** A number of synthetic organic polymers employed for ionic immobilisation available on the market. Legend: a) Resindion S.r.l. (Mitsubishi Chemical Corporation); b) ChiralVision

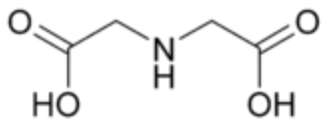
| Product Name | Chemical Matrix | Functional group | Pore diameter (Å) |
|---|---|---|---|
| [a]Sepabeads EC-EA | poly(methacrylate) | ethylamino | 100-200 |
| [a]Sepabeads EC-HA | poly(methacrylate) | hexamethylamino | 100-200 |
| [b]IB-D152 | polyacrylic | carboxylic acid | N/A |
| [b]IB-C435 | polyacrylic | carboxylic acid | N/A |
| [b]IB-A161 | polystyrene | quaternary ammonium | N/A |
| [b]IB-A171 | polystyrene | quaternary ammonium | N/A |
| [b]IB-A369 | polystyrene | quaternary ammonium | N/A |

# Organic polymeric resins fo metal binding

| Method of immobilisation | Functional group | Structure | Binding | Reactive group on enzyme |
|---|---|---|---|---|
| | | | | |
| Metal affinity | Iminodiacetic |  | Loading metals such as $Ni^{2+}$, $Zn^{2+}$, $Cu^{2+}$ | His-tag |
| | | | | |



**Iminodiacetic acid**, $HN(CH_2CO_2H)_2$, often abbreviated to **IDA**, is a dicarboxylic acid amine (the nitrogen atom forms an amino group, not an imino group as the name suggests).





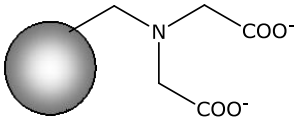
Metal chelated supports are used in protein chromatography (IMA – Immobilised Metal-Ion Affinity).

# Metal binding.

The **DNA sequence specifying a string of six to nine histidine** residues is frequently used in vectors for production of recombinant proteins.

The result is expression of a **recombinant protein with a 6x His** fused to its N- or C-terminus. Expressed His-tagged proteins can be purified and detected easily because the string **of histidine residues binds to several types of immobilised metal** ions, including nickel, cobalt and copper, under specific buffer conditions.

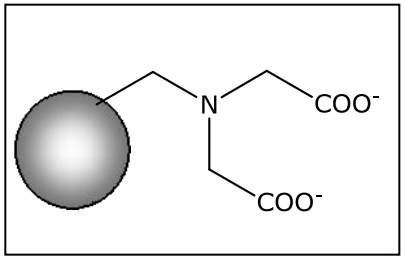
a) **Chelator ligands** can be immobilised on the solid supports by means of stable covalent bonds and




B ) the metal ions are then bound by **coordination**.

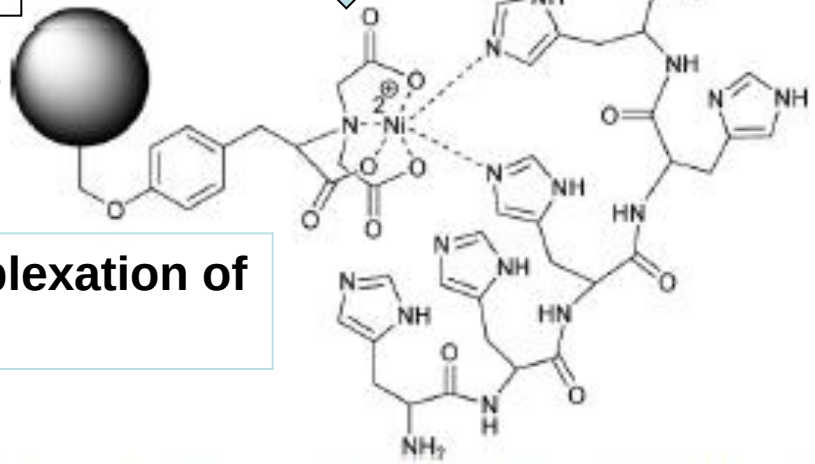
The chelators most commonly used as ligands for IMAC is **iminodiacetic acid (IDA).**

The stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is **subsequently regenerated** by washing with a **stronger chelator** such as ethylene diamine tetraacetic acid (**EDTA**) when desired.




**IDA**

**Polyvinylpirrolidone**




**Imidazole complexation of Ni $^{2+}$**

**Fig. 5** $Ni^{2+}$ attached to a carrier anchors an enzyme with a His tag to the carrier.

# Covalent immobilization of enzymes

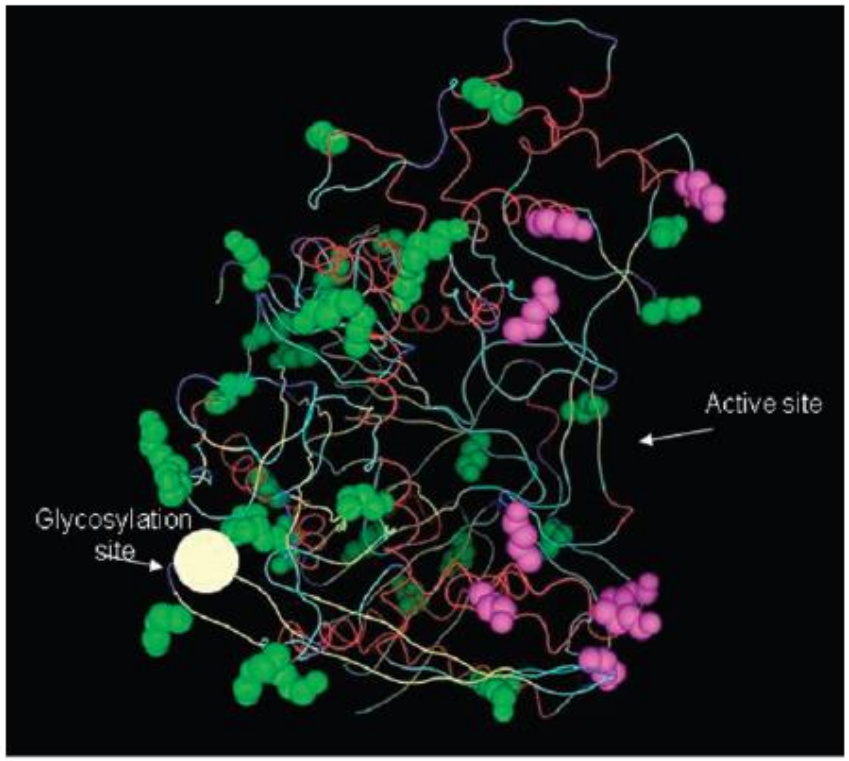
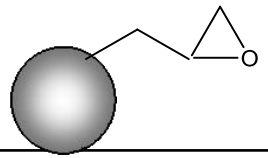
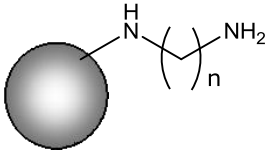
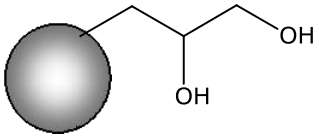



**Fig. 13** Model of the structure of the PGA from *E. coli*. The residues of lysine are pointed out in the space-filling modality. The violet residues correspond to those lysines closer to the opening of the active site. On the opposite side there is the glycosylation site (yellow).

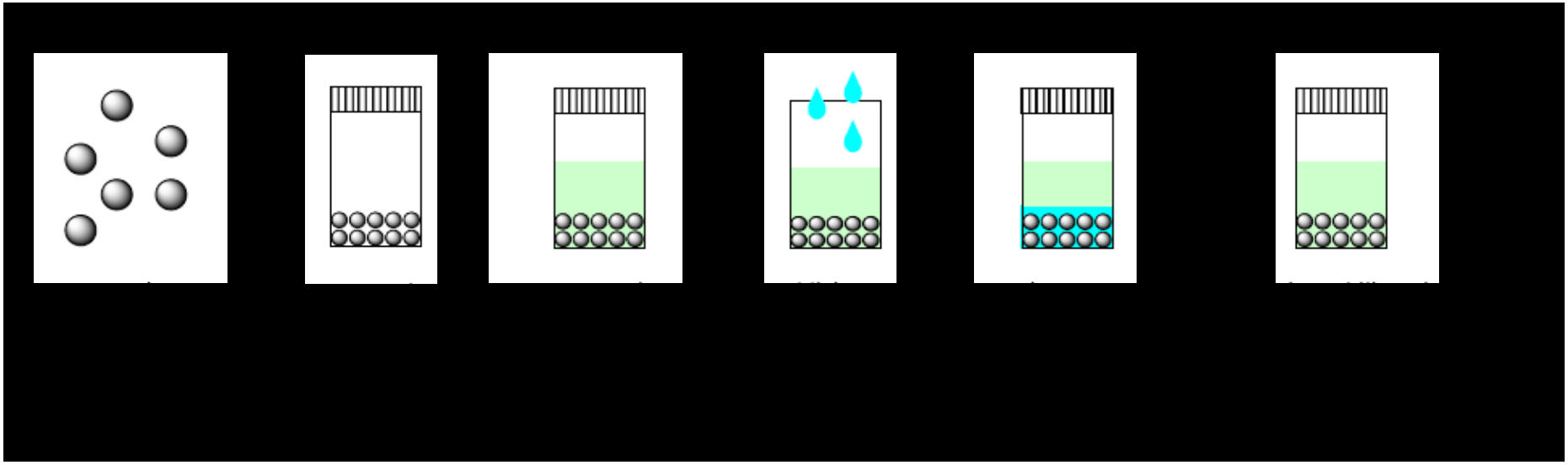
**Most often protocols exploit the nucleophilic reactivity of amino groups on Lys side chain on the surface of enzyme**

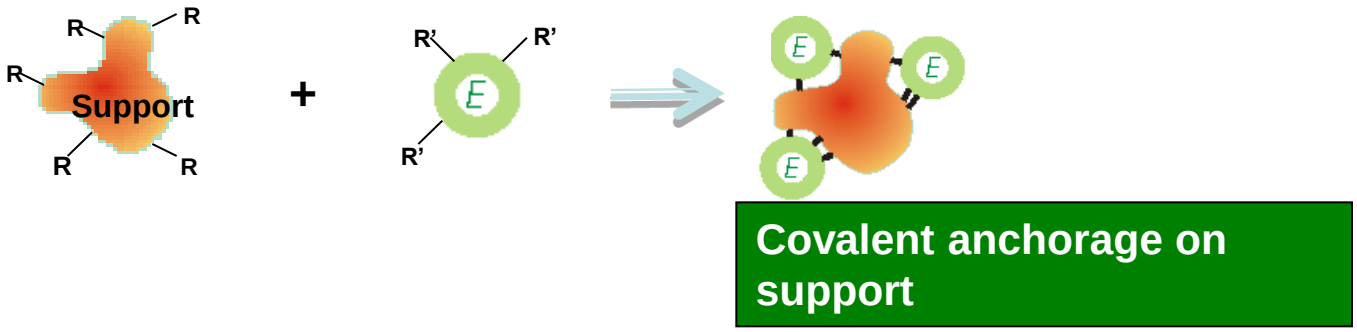
# Covalent immobilization of enzymes on commercially available organic resins with functionalized surface

# Organic polymeric resins: functional groups for covalent immobilization

| Method of immobilisation | Functional group | Structure | Binding | Reactive group on enzyme |
|---|---|---|---|---|
| Covalent bonds | Epoxy |   | Formation of covalent bonds via nucleophilic attack and opening of epoxy ring | Nucleophilic groups (mainly – $NH_2$ and -SH ) |
| | Amino |   | Pre-activation with glutaraldehyde and formation of imino bond with a primary amine | Primary amines (terminal amine and Lys side chains) |
| | Diol |   | Activation with BrCN to imido-carbonate. Oxidation of adjacent cis-diols with $NaIO_4$ to give dialdehydes. | Primary amines (terminal amine and Lys side chains) |

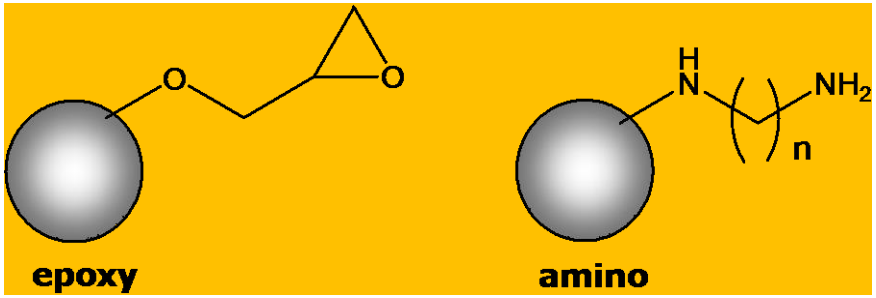
# Enzyme immobilization: covalent binding on organic polymeric resins



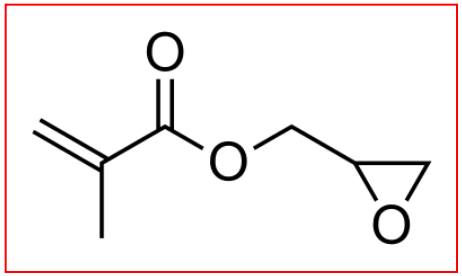



**Covalent anchorage on support**

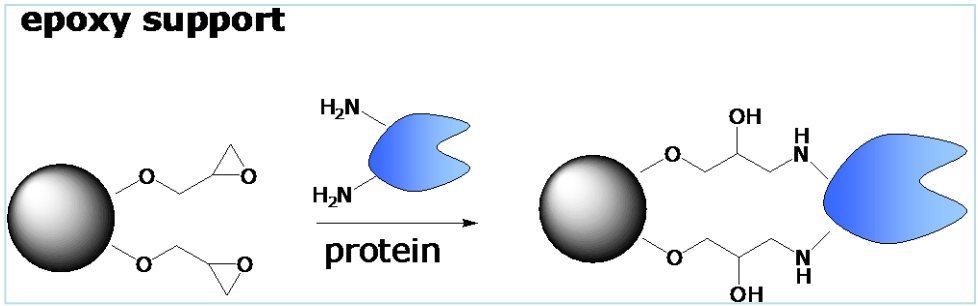
# Covalent immoblization on functionalized supports

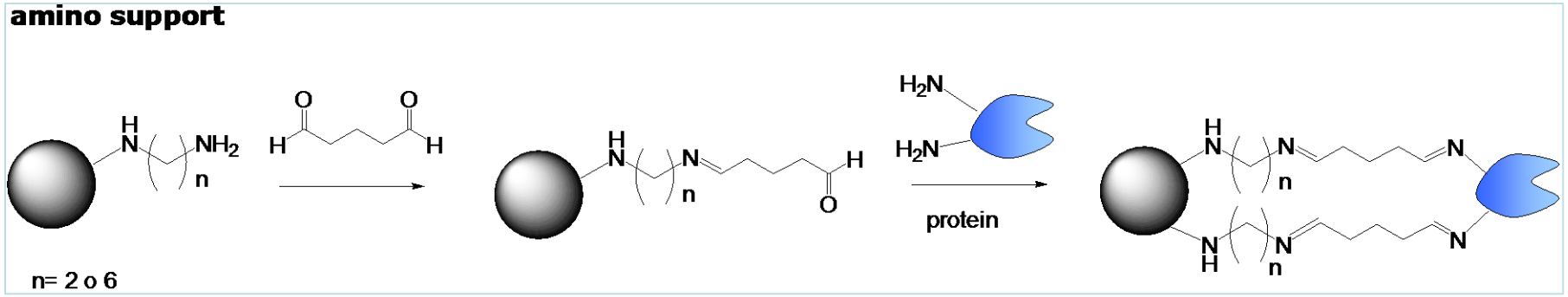



a certain percentage
of the glycidyl methacrylate monomer
is added in the synthesis
of methacrylic polymers



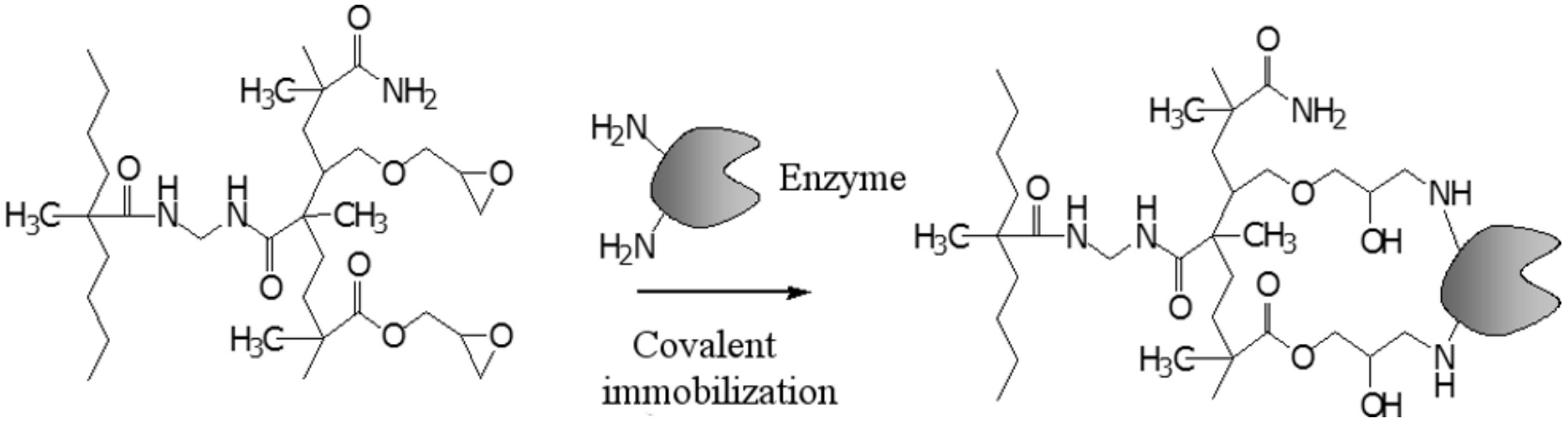
**Glycidyl methacrylate**

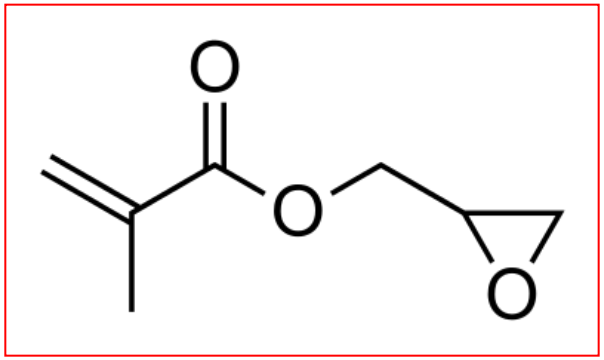



Serenovic et al., Biotechnol. Bioeng. 2006.
Basso et al, Adv. Synth. Catal. 2007.

# Structure of methacrylic epoxy supports

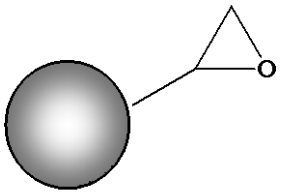


**Glycidyl methacrylate**

# Enzyme immobilisation on epoxy and amino carriers

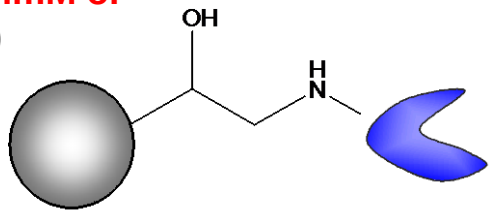
**Higher ionic strenght to promote the partition of enzymes onto the hydrophobic resins**




**Kpi buffer**

**(pH 8.0, 50mmM or 1M)**

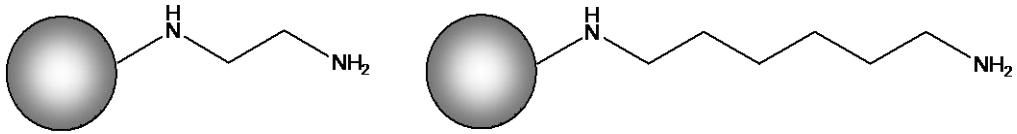
**Enzyme**

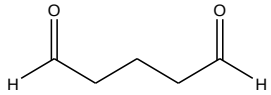



**Covalent immobilisation**

**Sometimes followed by post-blocking of unreacted epoxydes with Gly**

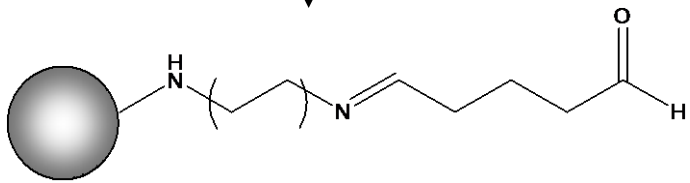
**Lower ionic strenght to promote the partition of enzymes onto the less hydrophobic resins**




**Support pre-activation**

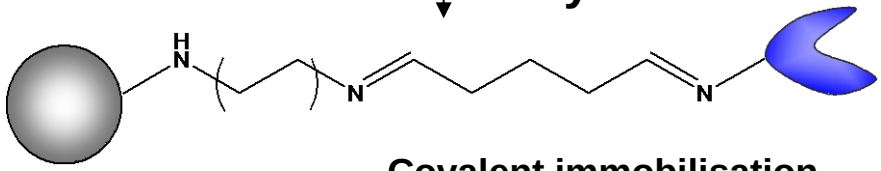
**(2% glutaraldehyde)**




**Kpi buffer**

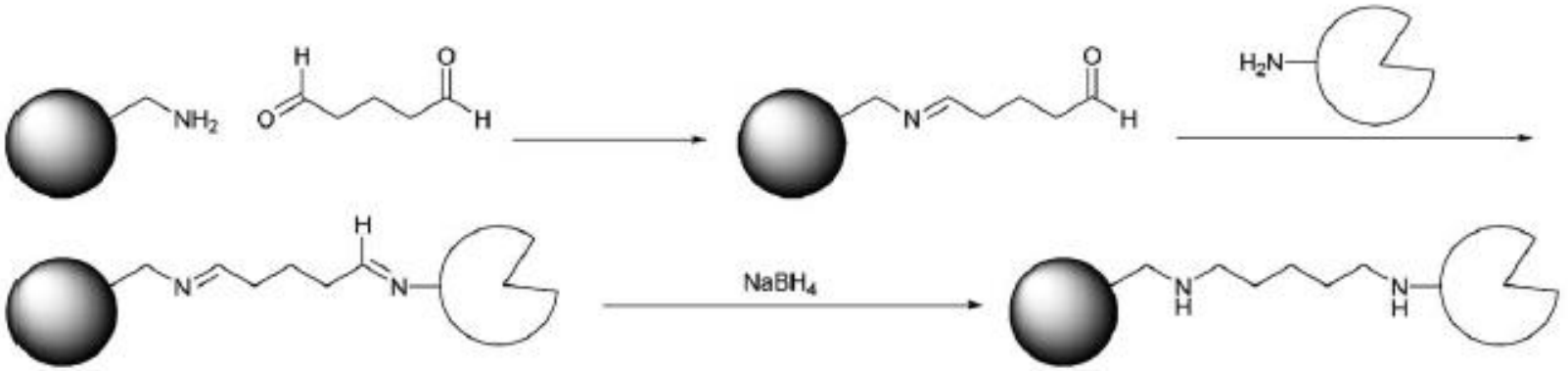
**(pH 8.0, 0.02M)**

**Enzyme**



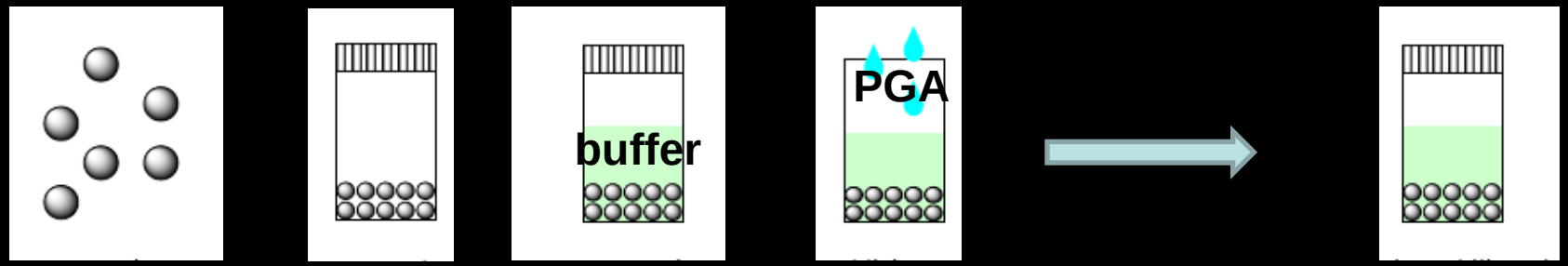

**Covalent immobilisation**

**(Shiff base formation)**

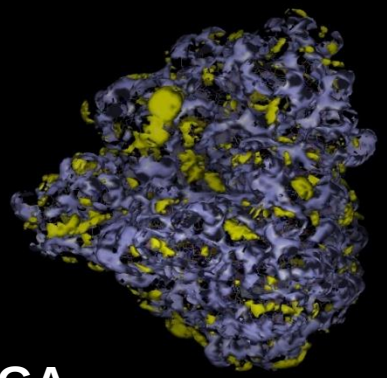



**Imine bond is unstable at low pH: reduction with NaBH4 makes the anchorage more stable but enzyme activity can be lost**

# No general protocol for enzyme immobilization

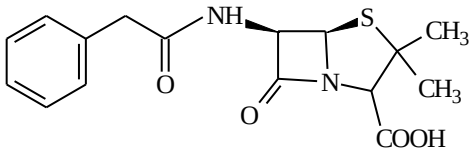



**Enzyme immobilization on carriers in aqueous buffer**

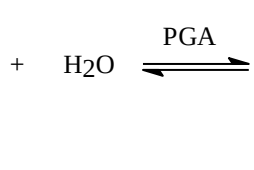


PGA

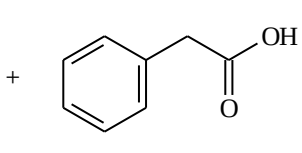
**Tons of immobilized PGA consumed per year**
**At industrial level used for >600 cycles**



penicillin G



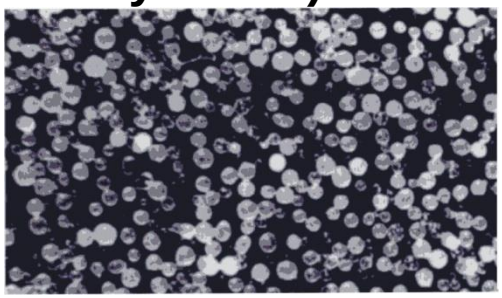

6-APA

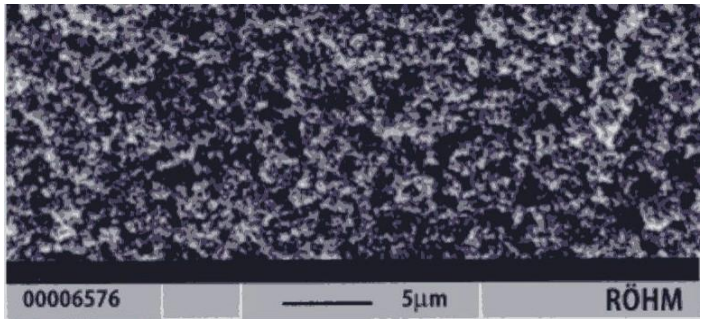


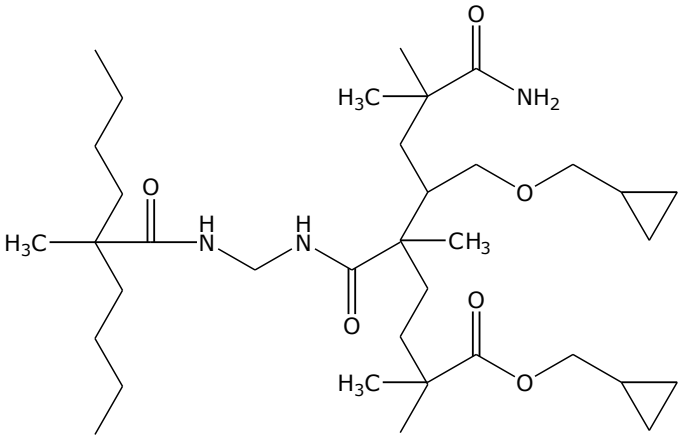
phenylacetic acid

# Acrylic polymers - Eupergit

**Eupergit C is a macroporous copolymer of methacrylamide, glycidyl methacrylate and allyl glycidyl ether, cross-linked with N,N'-methylene-bis(methacrylamide)**



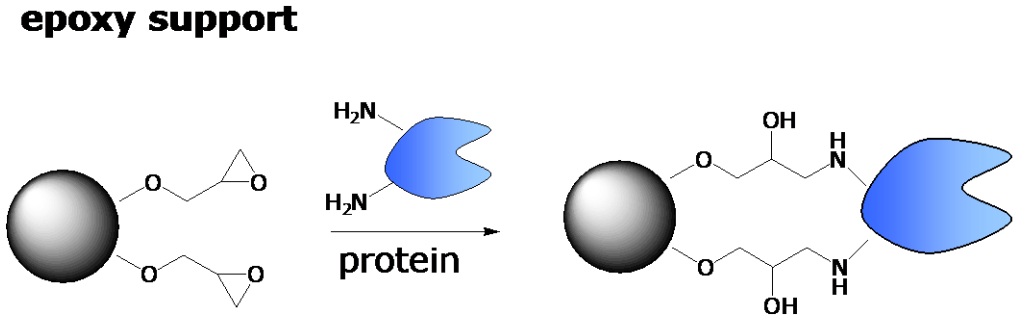



**particle size 170 m and pore diameter 25 nm**

*T. Boller, C. Meier, S. Menzler, "Eupergit Oxirane Acrylic Beads: How to make enzymes fit for Biocatalysis", Org. Proc. Devel. 2002, 6, 509-519*

# Acrylic polymers - Eupergit

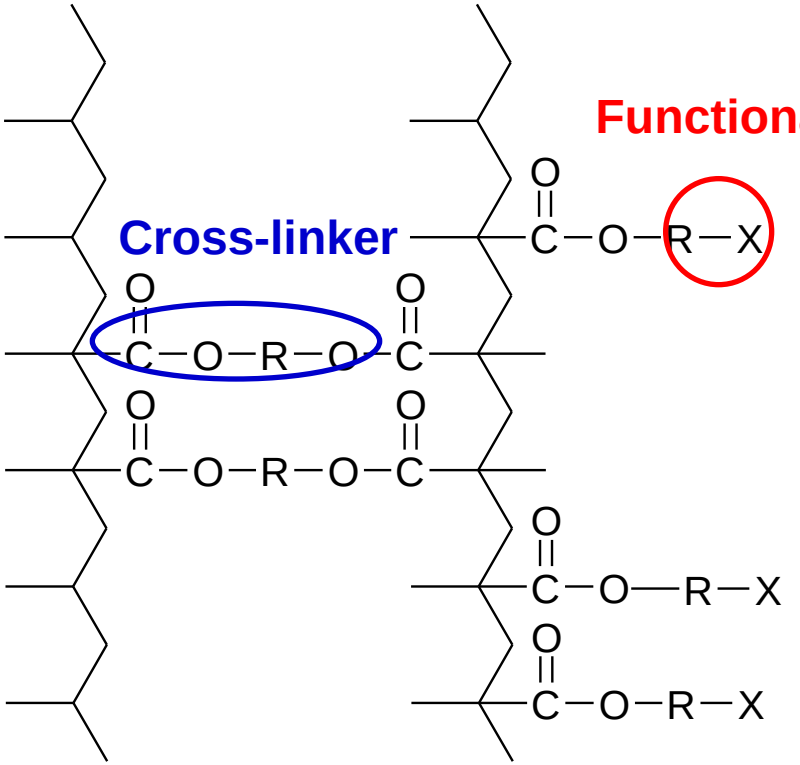



**Beads dimension: 100 - 250µm**

**Multipoint attachment**

| | |
|---|---|
| 1. oxidoreductases | |
| alcohol dehydrogenase | E.C. 1.1.1.2 |
| lactate dehydrogenase | E.C. 1.1.1.27 |
| α-hydroxysteroid dehydrogenase | E.C. 1.1.1.50 |
| β-hydroxysteroid dehydrogenase | E.C. 1.1.1.51 |
| pyranose oxidase | E.C. 1.1.3.10 |
| nucleoside oxidase | E.C. 1.1.3.28 |
| phenylalanine dehydrogenase | E.C. 1.4.1.20 |
| D-amino acid oxidase | E.C. 1.4.3.3 |
| formate dehydrogenase | E.C. 1.2.1.2 |
| 2. transferases | |
| transketolase | E.C. 2.2.1.1 |
| 3. hydrolases | |
| carboxylesterase | E.C. 3.1.1.1 |
| triacylglycerol lipase | E.C. 3.1.1.3 |
| β-glucosidase | E.C. 3.2.1.21 |
| β-galactosidase | E.C. 3.2.1.23 |
| trypsin | E.C. 3.4.21.4 |
| thermolysin | E.C. 3.4.24.27 |
| glutaryl-7-ACA acylase | E.C. 3.5.1.4 |
| penicillin amidase | E.C. 3.5.1.11 |
| aminoacylase | E.C. 3.5.1.14 |
| cytidine deaminase | E.C. 3.5.4.5 |
| 2-haloacid dehalogenase | E.C. 3.8.1.2 |
| 4. lyases | |
| oxynitrilase | E.C. 4.1.2.10 |
| Neu5ac aldolase | E.C. 4.1.3.3 |

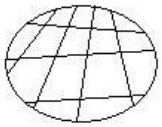
# Acrylic polymers - Sepabeads



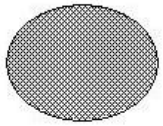

X = Active Functional Group

**Sepabeads®**

- methacrylic copolymers
- prepared through suspension polymerisation
- high mechanical stability



Conventional macro porous support



Conventional micro porous support

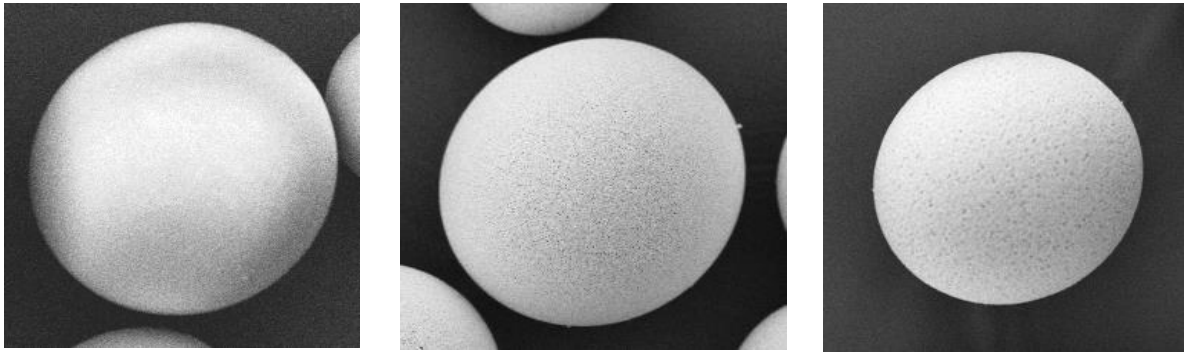
**Sepabeads®**

Non conventional Highly porous support

***Biotechnol Prog. 2002, 18, 629-634.***

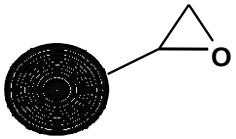
# Carrier: criteria for the selection

**Particle diameter (μm):**
**150-300**
**200-600**

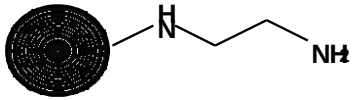


**Porosity (Ø)**

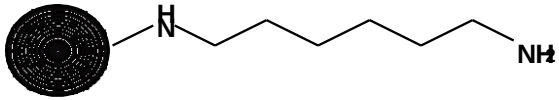
**300 Å** → **> 2000 Å**




**EC-EP**

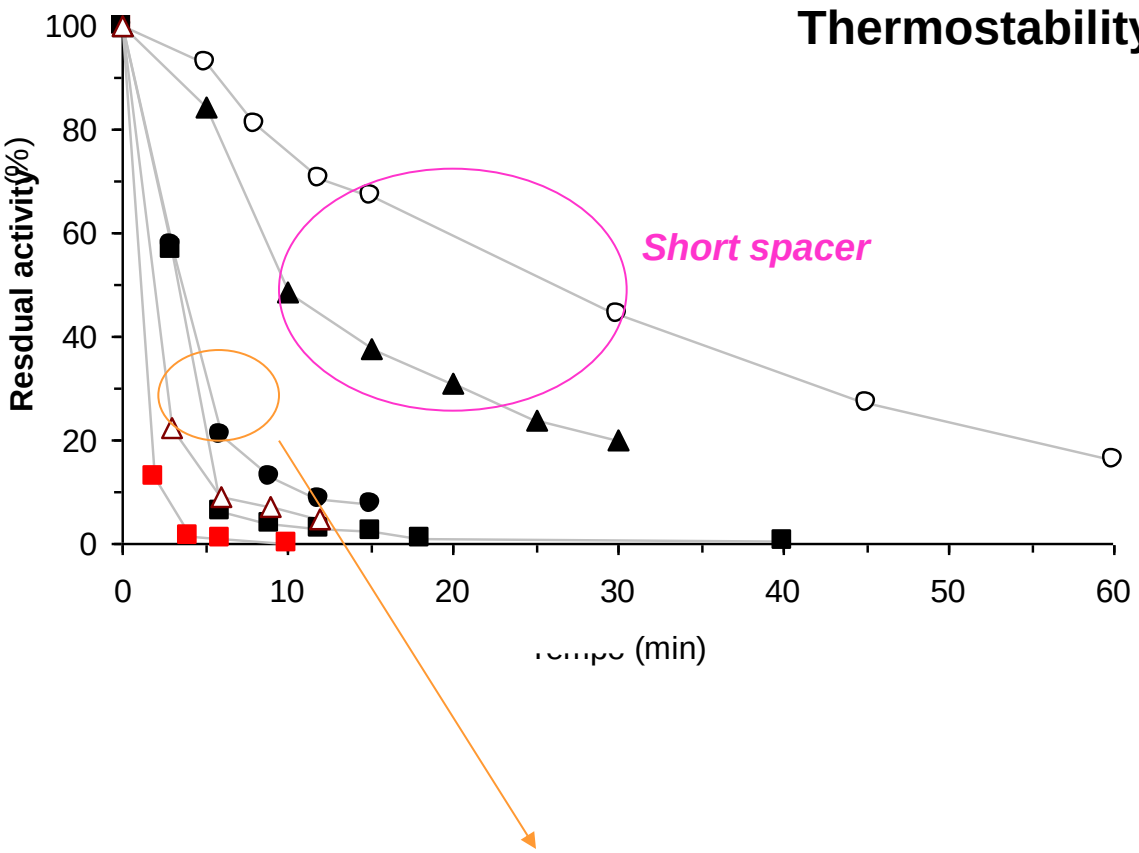



**EC-EA**




**EC-HA**

# Penicillin G amidase: higher stabilization on supports having short spacers



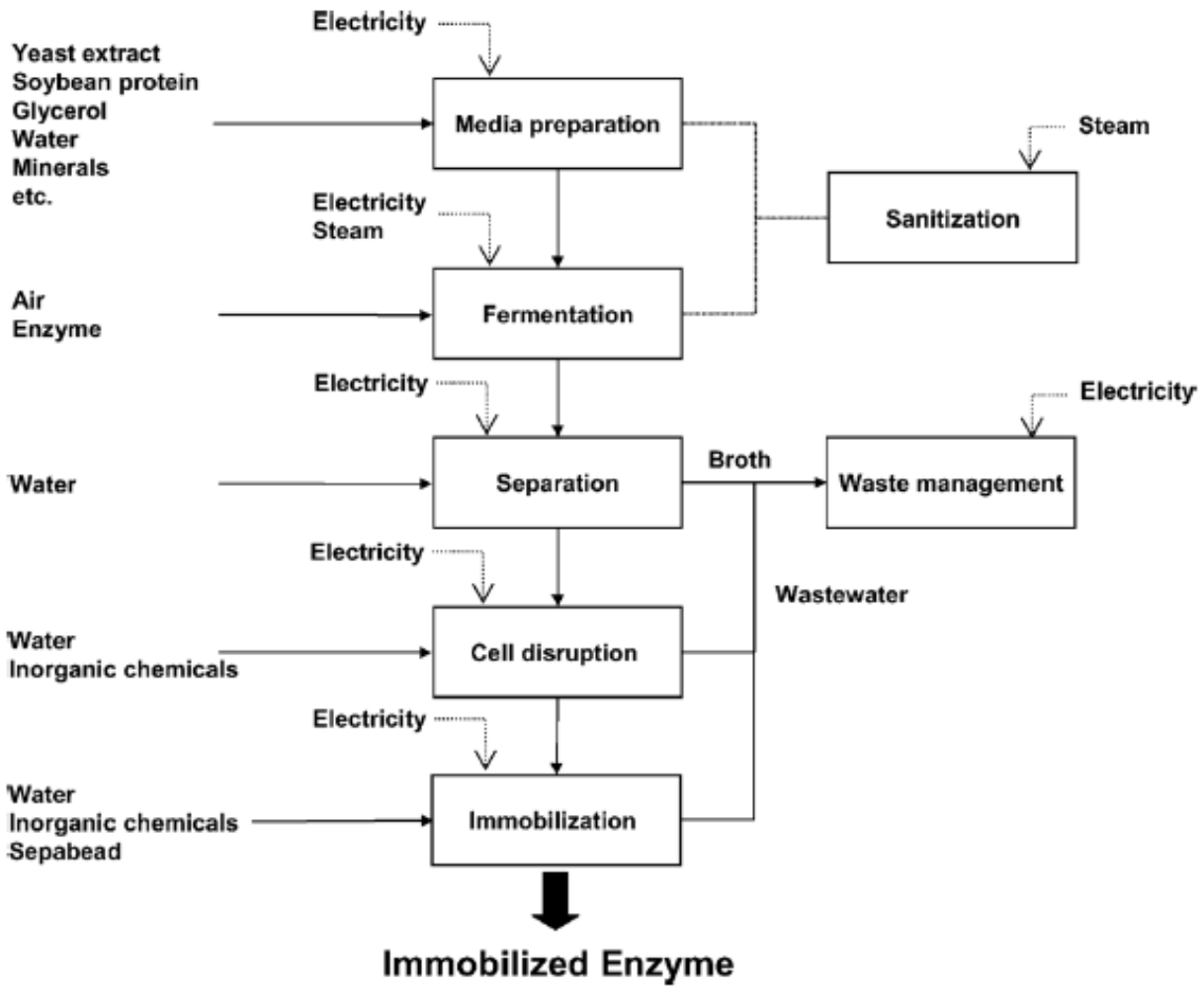

**Long spacers:**
**higher conformational freedom → lower stability**

# Life Cycle Analysis (LCA): how sustainable are immobilized enzymes?

Microorganism growth and fermentation

Enzyme separation

Enzyme immobilization



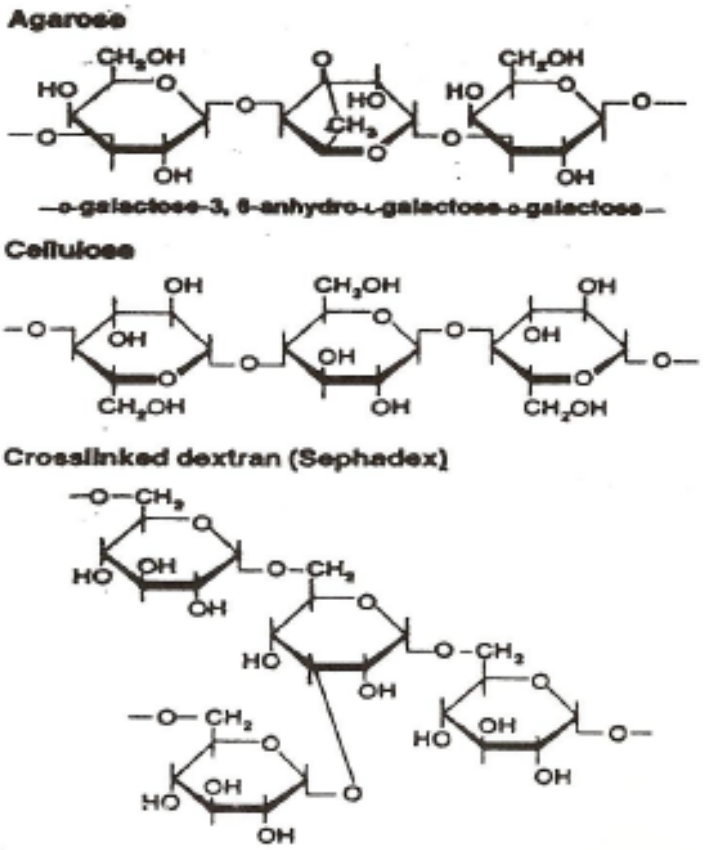
**LCA studies demonstrated that epoxy activated methacrylic resins represents the primary greenhouse gas emission source for immobilized enzymes because of the fossil based raw materials (glycidyl methacrylate, ethylene dimethyl acrylate)**

# CARRIERS MADE BY NATURAL RENEWABLE POLYMERS AND BIOPOLYMERS REPRESENT A SUSTAINABLE ALTERNATIVE

| | |
|---|---|
| Polysaccharides: | Cellulose |
| | Agar/agarose |
| | Chitosan |
| | Dextran |
| | Carrageenan |
| | Alginate |
| | Pectate |
| | Xanthan gum |
| Proteins: | Collagen |
| | Gelatin |
| | Albumin |
| | Fibrin |

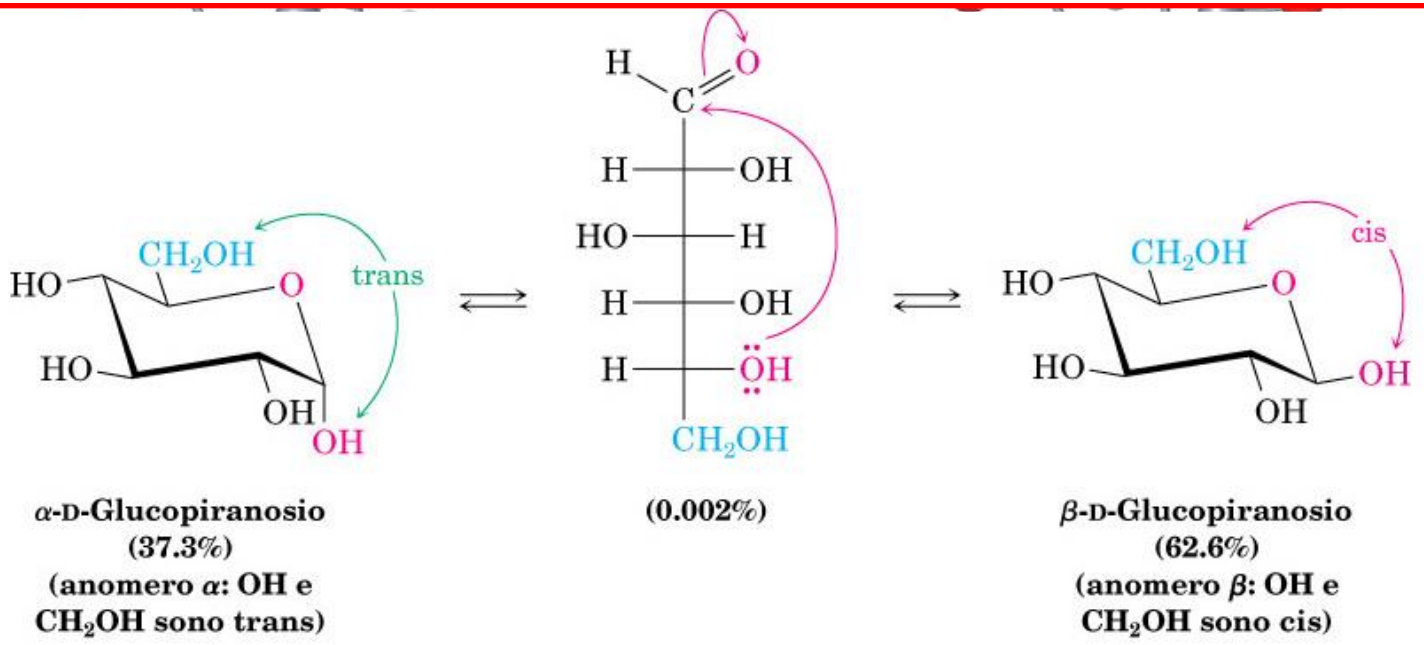
**Natural biopolymers** may represent an attractive alternative also from the economic point of view. Water-insoluble **carbohydrates** such as **cellulose**, starch, dextran, **agarose** and **chitosan** and proteins such as albumin and gelatin have been widely used as supports for immobilising enzymes.

From this group, polysaccharides are of special interest, since they do not suffer from **biological safety** aspects like protein matrices isolated from animal sources and they are highly hydrophilic, which provides a desirable microenvironment for many enzymes.



# Breve richiamo alla chimica del glucosio

**Equilibrio tra la forma lineare e la forma ciclica**




α-D-Glucopiranosio (37.3%) (anomero α: OH e $CH_2OH$ sono trans)

(0.002%)

β-D-Glucopiranosio (62.6%) (anomero β: OH e $CH_2OH$ sono cis)

**I due differenti stereo-isomeri vengono in questo caso definiti anomeri**

# Proiezioni di Haworth della forma ciclica

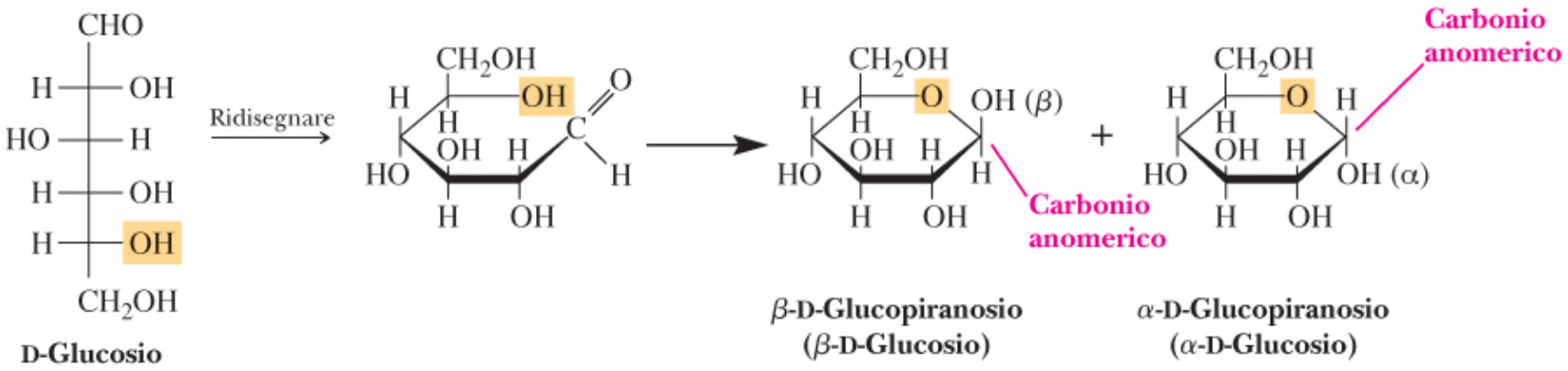
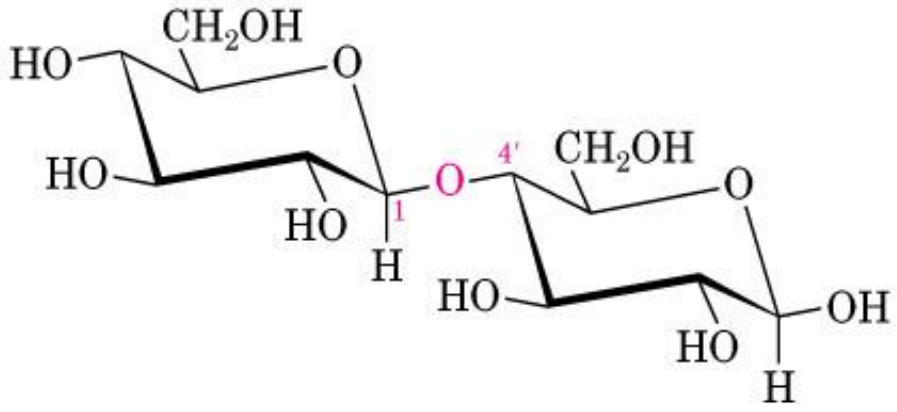



**Figura 25.1**
Proiezioni di Haworth dell'α-D-glucopiranosio e del β-D-glucopiranosio.

**Il nuovo gruppo –OH può stare sopra o sotto il piano**

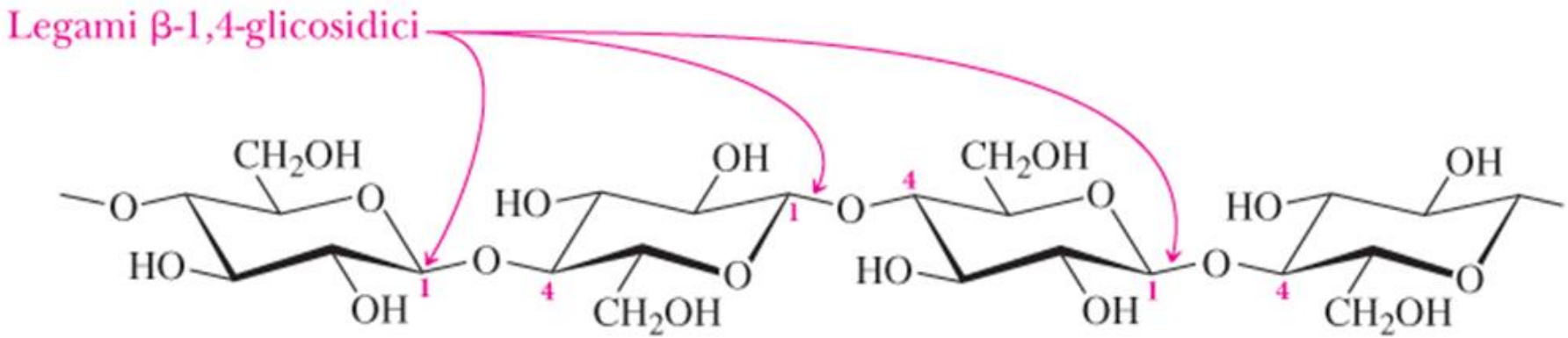
# Legami glicosidici tra unità zuccherine
# Disaccaridi: cellobiosio




**Cellobiosio, un 1,4′-β-glicoside**
**[4-*O*-(β-D-glucopiranosil)-β-D-glucopiranosio]**

**Legame glicosidico «β» idrolizzato dagli enzimi β -amilasi (assenti nell'uomo)**

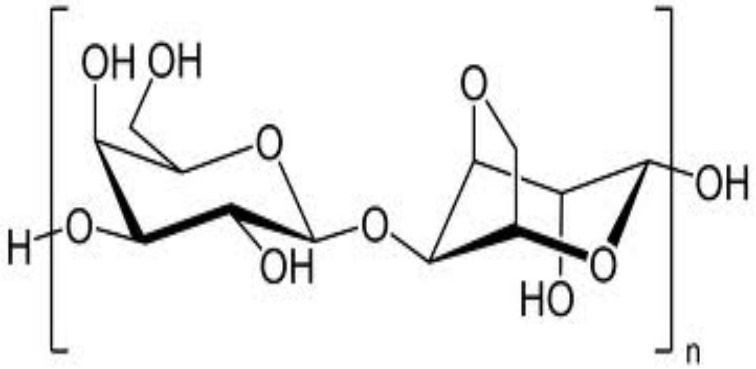
# Polisaccaride: cellulosa




La cellulosa è un polimero lineare contenente fino a 2200 unità di D-glucosio legate da legami $\beta$-1,4-glicosidici.

**Sugar based bio-polymers as carriers.**
The hydroxyl groups of polysaccharides can be exploited for covalent immobilisation of proteins after **ACTIVATION**.

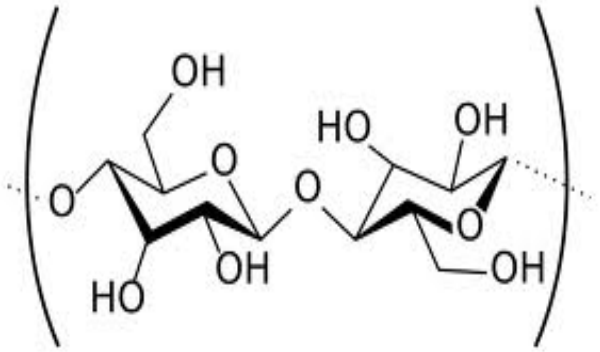
- **Agarose**




Chemically it is a (poly-{β-1,3-$_D$-galactose-α-1,4-(3,6-anhydro)-$_L$-galactose}) gel.

**Agarose** is generally extracted from red seaweed

- **Cellulose**



**Agar** is extracted from the cell walls of some red seaweeds, belonging to Rhodophyceae class,widespread all along the world (including Japan, Korea, Spain, Portugal, some African countries, Mexico, Chile, and India). The genera Gelidium and Gracilaria in particular are the sources of a great
part of the commercialized agar.

molecules

MDPI

*Review*

# Agarose and Its Derivatives as Supports for Enzyme Immobilization

**Paolo Zucca [1], Roberto Fernandez-Lafuente [2] and Enrico Sanjust [1,*]**

[1] Dipartimento di Scienze Biomediche, Università di Cagliari, 09042 Monserrato (CA), Italy; pzucca@unica.it
[2] Departamento de Biocatalisis, ICP-CSIC; C/Marie Curie 2, Campus UAM-CSIC, Madrid 28049, Spain; rfl@icp.csic.es
* Correspondence: sanjust@unica.it; Tel.: +39-070-675-4518

Academic Editor: Derek J. McPhee
Received: 27 September 2016; Accepted: 16 November 2016; Published: 19 November 2016

**Table 1.** Main agarophytes used for agar production [1].

| Species | Location |
|---|---|
| *Gelidium amansii* | Japan, China |
| *Gelidium cartilagineum* | USA, Mexico, South Africa |
| *Gelidium corneum* | South Africa, Portugal, Spain, Morocco |
| *Gelidium liatulum* | Japan |
| *Gelidium lingulatam* | Chile |
| *Gelidium pacificum* | Japan |
| *Gelidium sesquipedale* | Portugal, Morocco |
| *Gelidiella acerosa* | Japan, India, China |
| *Gracilaria verrucosa* | Turkey |
| *Gracilaria dura* | France |
| *Gracilaria tenuistipitata* | Philippines |
| *Pterocladia lucida* | New Zealand, Azores |
| *Pterocladia capilacea* | Egypt, Japan, New Zealand |
| *Ahnfeltia plicata* | Russia |

# Two main components can be identified in agar: agarose and agaropectin

Agarose is a neutral gelling eteropolysaccharide, accounting for the major fraction of agar. It is a linear polymer with the repeating unit containing both alfa- and beta-glycosidic bonds (unlike the majority of the most common polysaccharides)

The two monosaccharide present are D-galactose and 3,6-anhydro L-galactose, linked by glycosidic bonds β (1–4) (between D-galactose and 3,6-anhydro L-galactose, giving the disaccharide basic unit called neoagarobiose) and (1–3) (between 3,6-anhydro L-galactose and D-galactose, giving the disaccharide basic unit called agarobiose).

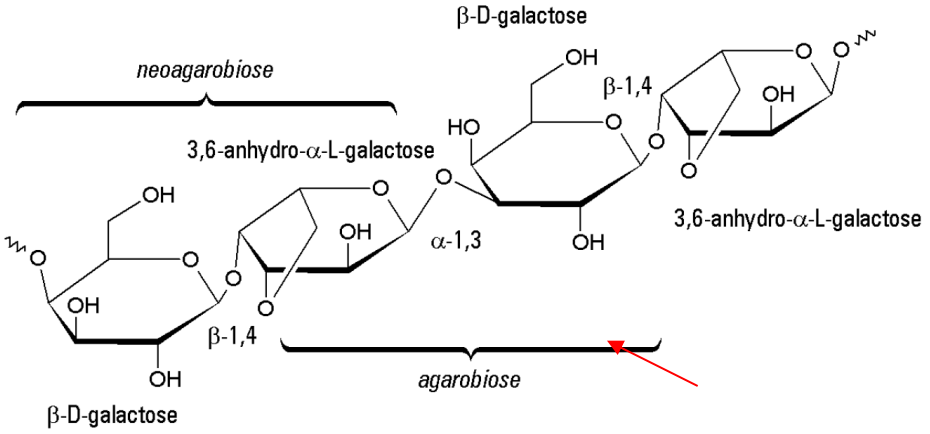


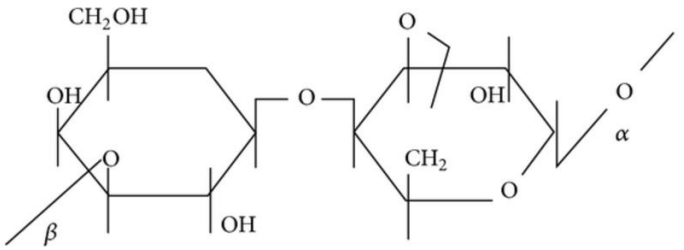

**Figure 3.** Backbone structure of agarose. The repeating disaccharide units are called agarobiose and neoagarobiose. In the case of agaropectin, 2 or 6 positions of 3,6-anhydro-α-L-galactose residues can be substituted by $-OSO_3^-$, $-OCH_3$, glucuronate, or pyruvate residues [79,80].

Each year about 35,000–40,000 tons dry weight of Gracilaria are produced, and whereas the production of Gelidium spp. is below 20,000 tons. The total annual production of agar from these starting materials is about 7500 tons.
The two agar sources sharply differ regarding the required pre-treatment.

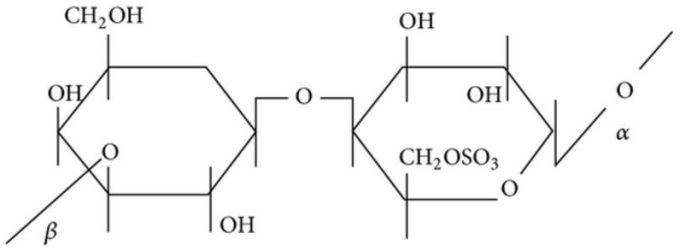
In the case of Gelidium the plant sample is diluted in a mild acid solution to improve the efficiency of the extraction.

Gracilaria samples require a strong alkali solution treatment (2%–5% NaOH for 1 h at 90 C) to convert sulfate groups to 3,6-anhydrogalactose.

In the absence of this treatment the mechanical properties of the obtained gels are too poor for practical applications.

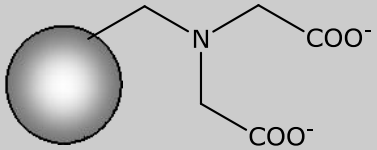
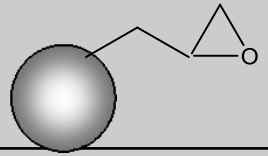
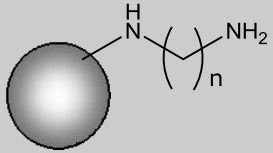
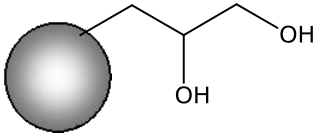


Agarose



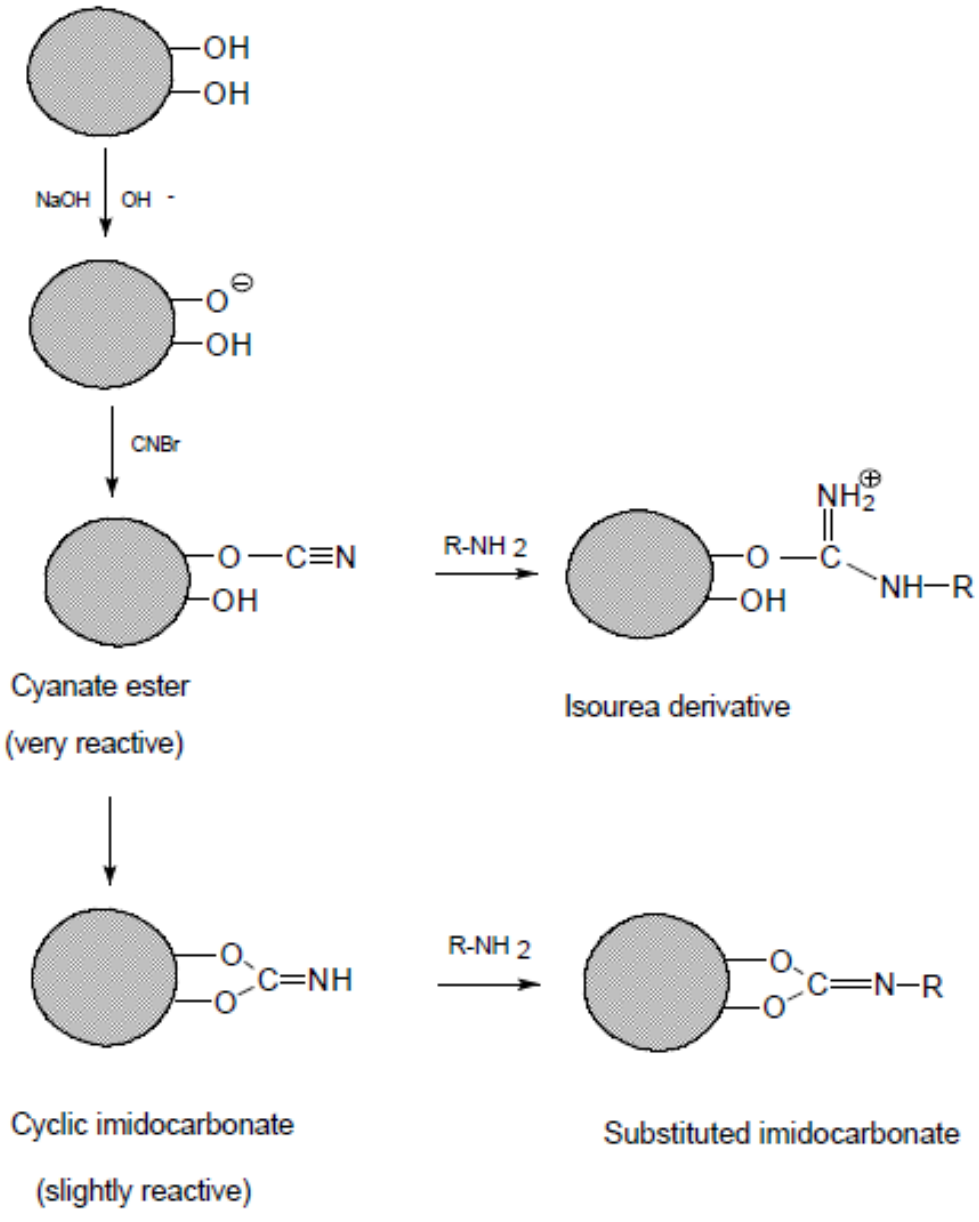
Agaropectin

# Functional groups of carriers

| Method of immobilisation | Functional group | Structure | Binding | Reactive group on enzyme |
|---|---|---|---|---|
| | | | | |
| Metal affinity | Iminodiacetic |   | Loading metals such as $Ni^{2+}$, $Zn^{2+}$, $Cu^{2+}$ | His-tag |
| | | | | |
| Covalent bonds | Epoxy |   | Formation of covalent bonds via nucleophilic attack and opening of epoxy ring | Nucleophilic groups (mainly – $NH_2$ and -SH ) |
| | Amino |   | Pre-activation with glutaraldehyde and formation of imino bond with a primary amine | Primary amines (terminal amine and Lys side chains) |
| | Diol |   | 1)Activation with BrCN to imido-carbonate. 2)Oxidation of adjacent cis-diols with $NaIO_4$ to give dialdehydes. | Primary amines (terminal amine and Lys side chains) |

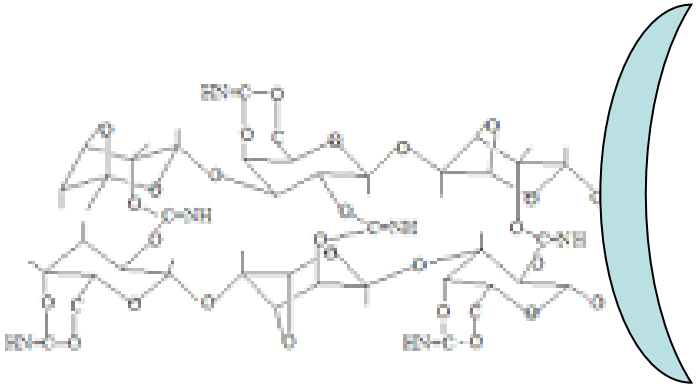
**cyanogen bromide!**

# Activation of agarose




The hydroxyl groups of this polysaccharide combine with **cyanogen bromide** to give the reactive cyclic imido-carbonate. This reacts with primary amino groups (i.e. mainly lysine residues) on the enzyme under basic conditions (pH 9–11.5).

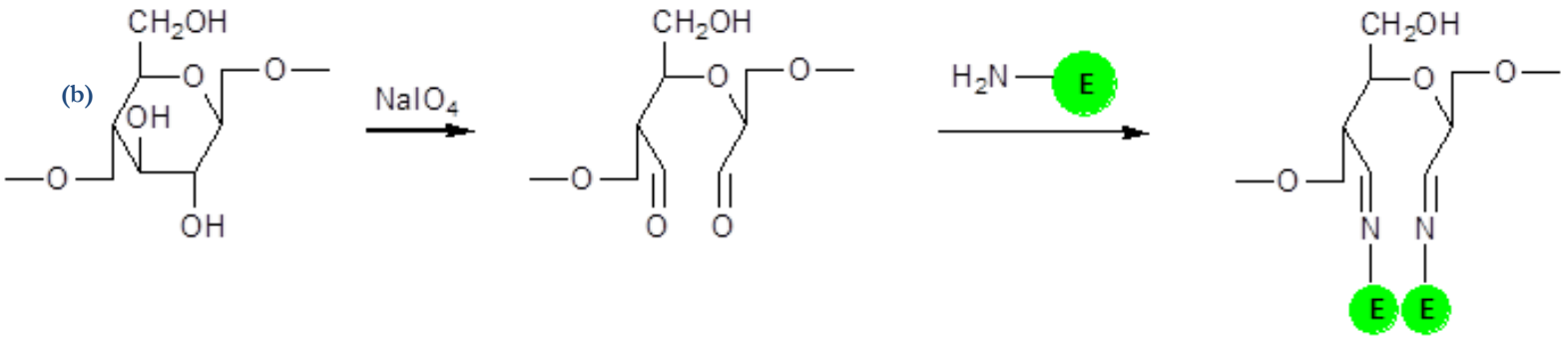
This is a simple, often successful method but the high toxicity of cyanogen bromide confined its use to the laboratory scale.



**Activated agarose**

**Immobilization by covalent coupling of enzyme on oxidized CELLULOSE support.**

**The carrier is activated by a process involving oxidation of cellulose to provide aldehyde groups, and covalent binding of enzyme molecules on aldehyde activated support.**




The binding capacity of cellulose is generally lower as compared to agarose but it is inexpensive and commercially available in fibrous and granular forms. Some drawbacks are the low particle sizes, which prevent their use in high pressure processes.

# Chitin and chitosan

Every year, molluscs, crustaceans, insects, fungus, algae, and related organisms approximately produce 10 billion t of chitin. **Chitin** is biorenewable, environmentally friendly, biocompatible, biodegradable and biofunctional, and is beneficial as a chelating agent, water treatment additive, drug carrier, biodegradable pressure-sensitive adhesive tape, wound-healing agents, in membranes and has other advantages for several important applications.

ChemBioEng Reviews
Wiley Online Library

**A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications**

Vida Zargar[1], Morteza Asghari[1]*, Amir Dashti[1]

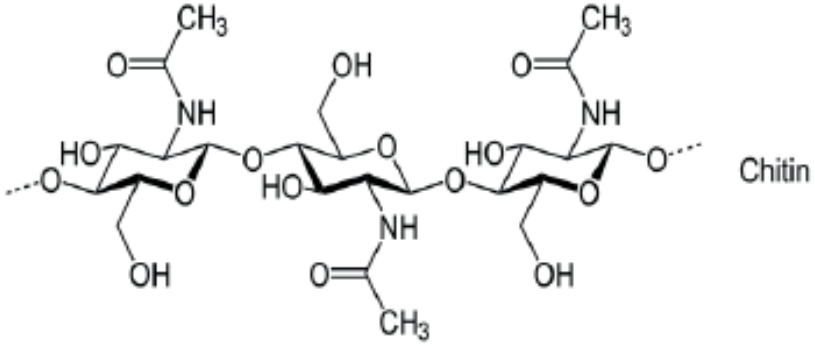
**Abstract**

Chitin and chitosan are considerably versatile and promising biomaterials. The deacetylated chitin derivative chitosan is a useful and interesting bioactive polymer. Despite its biodegradability, it has many reactive amino side groups, which offer possibilities of chemical modifications, formation of a large variety of beneficial derivatives, which are commercially available or can be made available via graft reactions and ionic interactions. This study looks at the contemporary research in chitin and chitosan towards structure, properties, and applications in various industrial and biomedical fields.

**Keywords:** Chitin, Chitosan, Deacetylation, Membranes, Organic materials, Polymers

*Received:* August 21, 2014; *revised:* December 12, 2014; *accepted:* December 19, 2014

**DOI:** 10.1002/cben.201400025



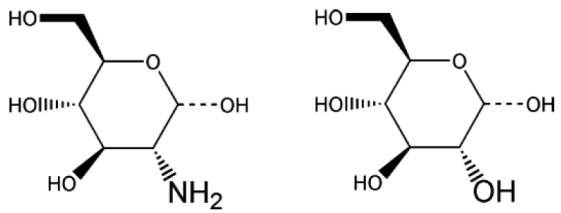
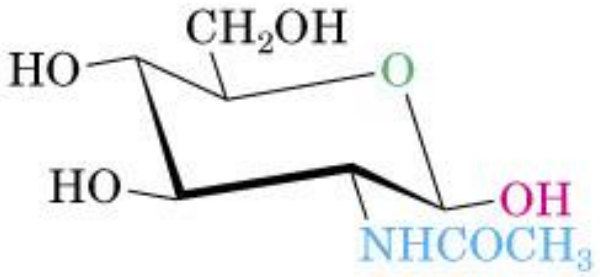



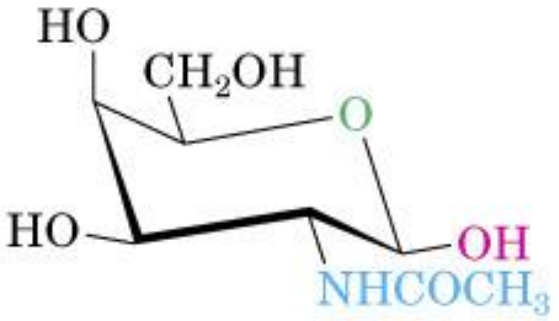

**Figure 1.** Structure of glucosamine (monomer of chitosan) and glucose (monomer of cellulose).

Chitin, a linear polysaccharide composed of (1-4)-linked 2-acetamido-2-deoxy-b-D-glucopyranose units , is the second prevalent form of polymerized carbon in nature. It is categorized as a cellulose derivative, in spite of the fact that it does not appear in organisms producing cellulose. Its structure is similar to cellulose, but at the C2 position, it has an acetamide group (-NHCOCH3).

# Amminozuccheri




**β-D-*N*-Acetilglucosammina**
**(D-2-acetammino-2-deossiglucosio)**




**β-D-*N*-Acetilgalattosammina**
**(D-2-acetammino-2-deossigalattosio**

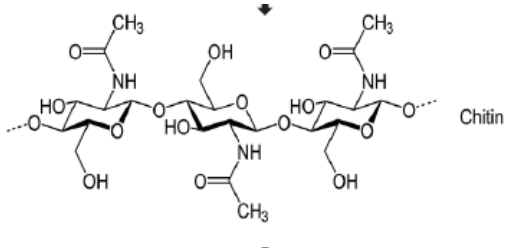
**Chitin** is a white, inelastic, rigid, nitrogenous polysaccharide that is present in the exoskeleton and internal structure of invertebrates.

The wastes of these natural polymers cause surface pollution in coastal regions.

The waste of the food industry is a suitable source for production of chitosan from crustacean shells and economically feasible.

**Table 1.** Sources of chitin and chitosan [22].

| Sea animals | Insects | Microorganisms |
|---|---|---|
| Crustaceans | Scorpions | Green algae |
| Coelenterata | Brachiopods | Yeast ($\beta$-Type) |
| Annelida | Cockroaches | Fungi (cell walls) |
| Mollusca | Spiders | Mycelia penicillium |
| Lobster | Beetles | Brown algae |
| Shrimp | Ants | Chytridiaceae |
| Prawn | | Ascomydes |
| Krill | | Blastocladiaceae |
| Crab | | Spores |




However, nowadays, chitin is not vastly employed by the pharmaceutical industry. Because of its weak solubility, it has unique applications. Chitin is insoluble in common organic solvents and diluted aqueous solvents because of the highly expanded hydrogen-bonded semicrystalline structure of chitin.

# Chitin and chitosan

Shrimp Crab Squid

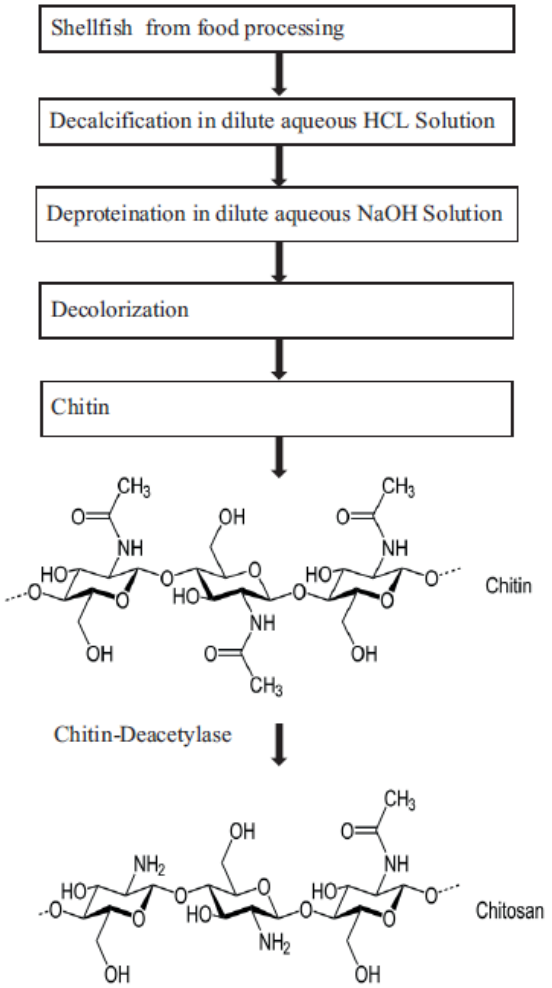
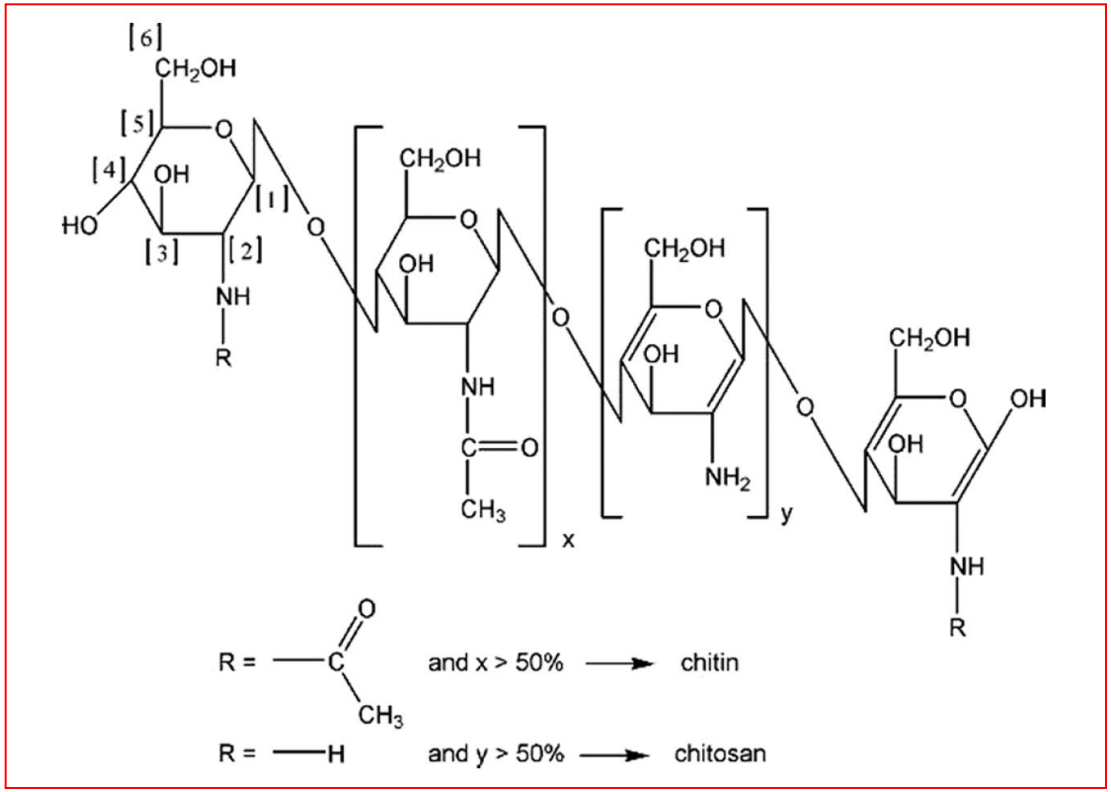



**Figure 3.** Chitin and chitosan processing.

Its derivative, **chitosan**, is prepared by deacetylation and depolymerization of native chitin, (partial) deacetylation of chitin in the solid state under alkaline conditions (concentrated NaOH), or enzymatic hydrolysis in the presence of a chitin deacetylase.

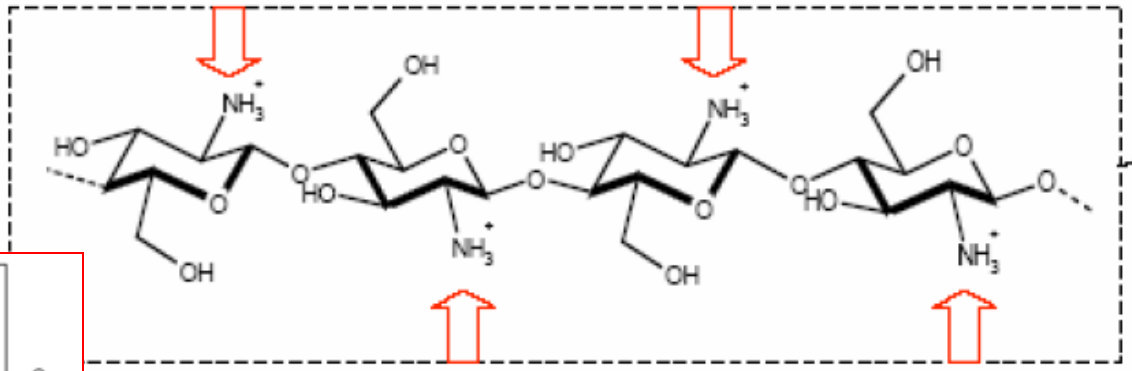


# Chitosan

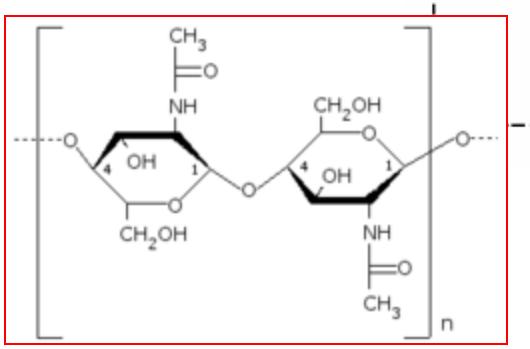
**It derives from deacetilated chitin, a polysaccharide of animal origin (crustaceans' exoskeleton)**

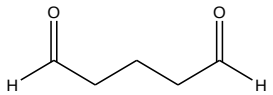
**Chitosan is structurally similar to cellulose but with amino groups on C2**
**Monomer: 2-amino-2-deoxy-D-glucopyranose**
**Glycosidic bonds: (1-4)β**
**Chitosan is insoluble in $H_2O$, but more soluble in diluted acidic aqueous solutions.**
**Amino groups can be exploited for covalent binding via cross linking with glutaraldehyde**

**chitosan**



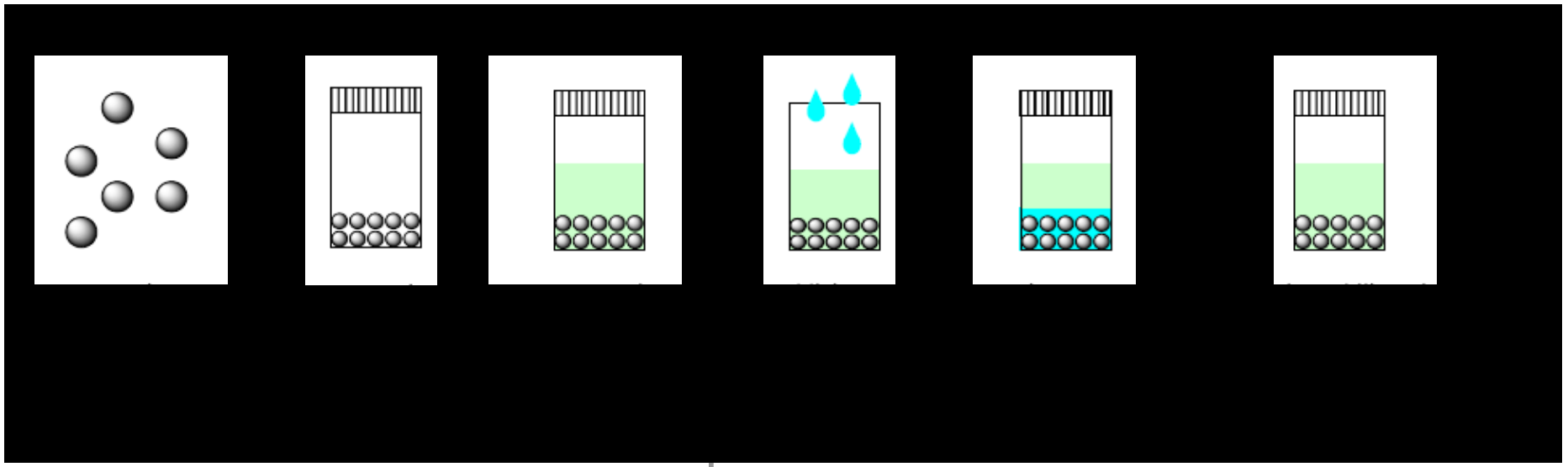
**chitin**

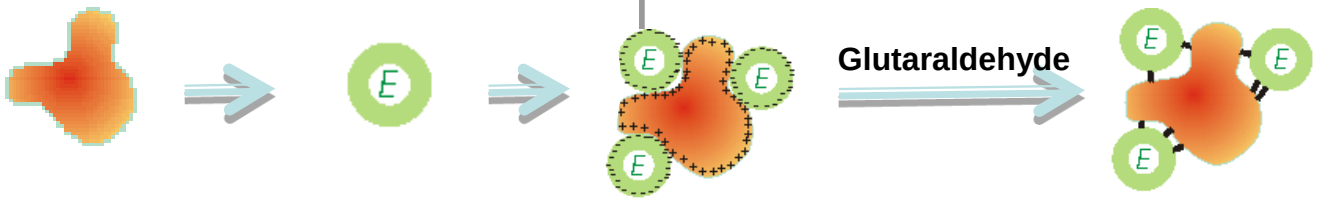




**glutaraldehyde**

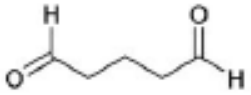
# Covalent Enzyme immobilization via adsorption + crossInking






**Adsorption**

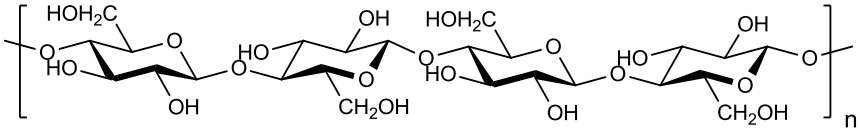
**Adsorption + cross-linking**



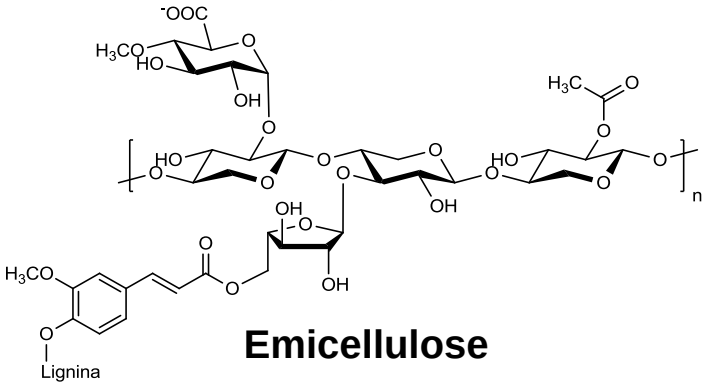

**Use of a difunctional chemica reagent: imino group formation**

# Lignocellulosic natural biomass as carriers

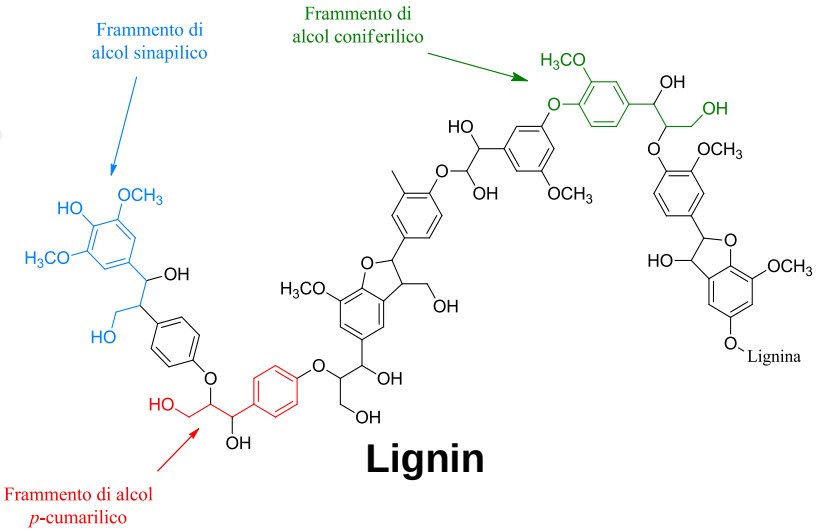
**lignocellulosic**




**Cellulose**




**Emicellulose**




**Lignin**

# Rice husk as enzyme carrier

**120 M tons year , inexpensive**

**Corici, L.; Ferrario, V.; Pellis, A.; Ebert, C.; Lotteria, S.; Cantone, S.; Voinovich, D.; Gardossi, L. *RSC Advances* 2016, 6, 63256-63270.**

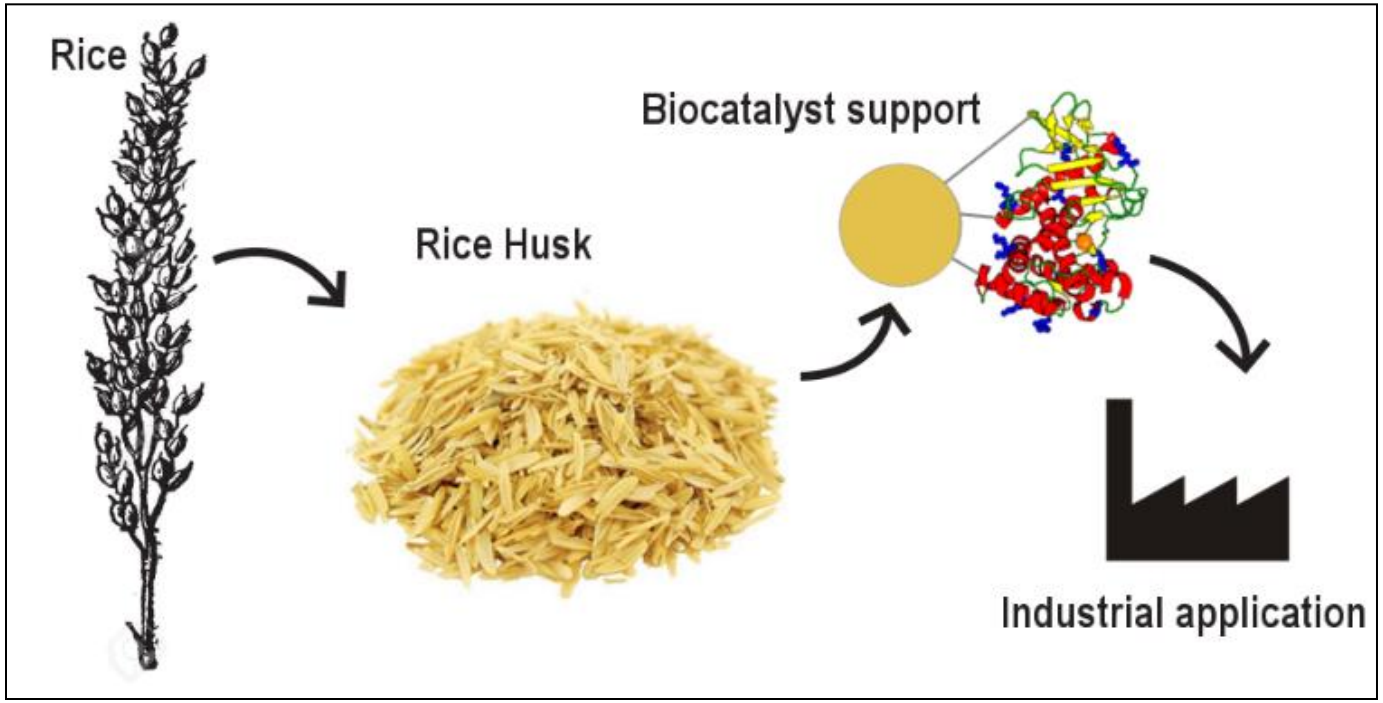
## Composition

- 20-25 % $SiO_2$ ( and traces of $Al_2O_3$, MgO, CaO,…)
- 75-80 % organic

| | ($\%_{w/w}$) |
|---|---|
| Lignin | 32 |
| Cellulos | 47 |
| Pentosans (Emicellulos) | 22 |
| water | 8 |

Corici, L.; Ferrario, V.; Pellis, A.; Ebert, C.; Lotteria, S.; Cantone, S.; Voinovich, D.; Gardossi, L. *RSC Advances* 2016, 6, 63256-63270.

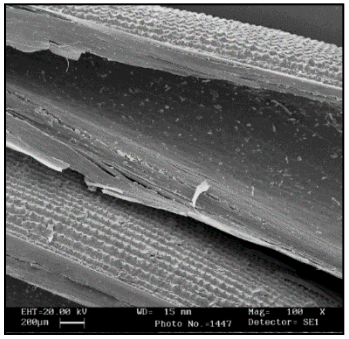
# Rice husk as enzyme carrier

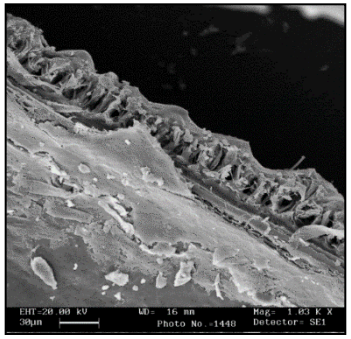


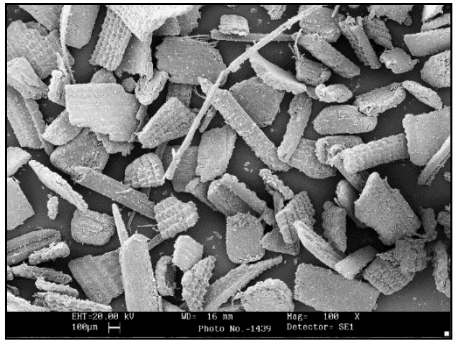

Corici, L.; Ferrario, V.; Pellis, A.; Ebert, C.; Lotteria, S.; Cantone, S.; Voinovich, D.; Gardossi, L. *RSC Advances* 2016, 6, 63256-63270.

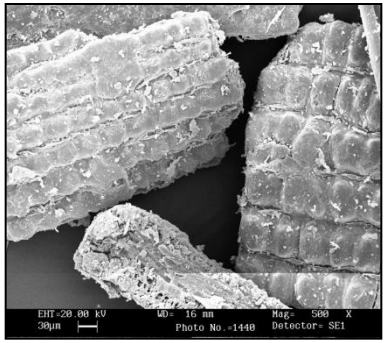
# Morphology - SEM

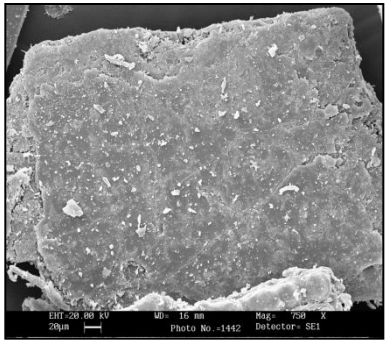
- **Caratterizzazione della morfologia tramite microscopia elettronica a scansione**



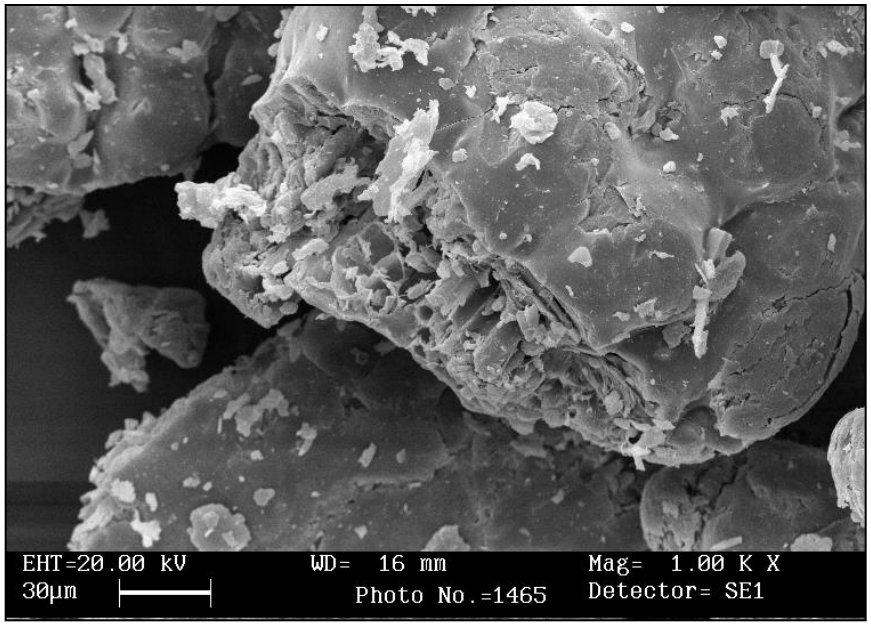



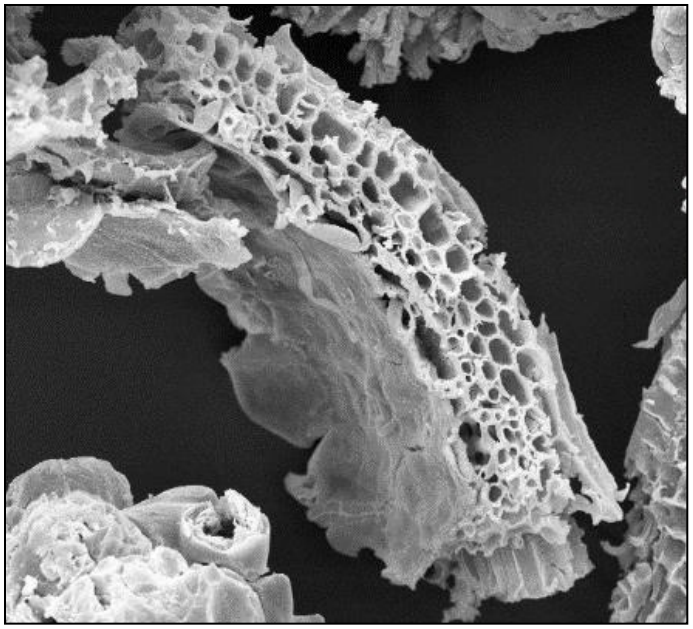




Thesis 2016 S. Lotteria, Photos by F. Vita

**Corici, L.; Ferrario, V.; Pellis, A.; Ebert, C.; Lotteria, S.; Cantone, S.; Voinovich, D.; Gardossi, L. *RSC Advances* 2016, 6, 63256-63270.**

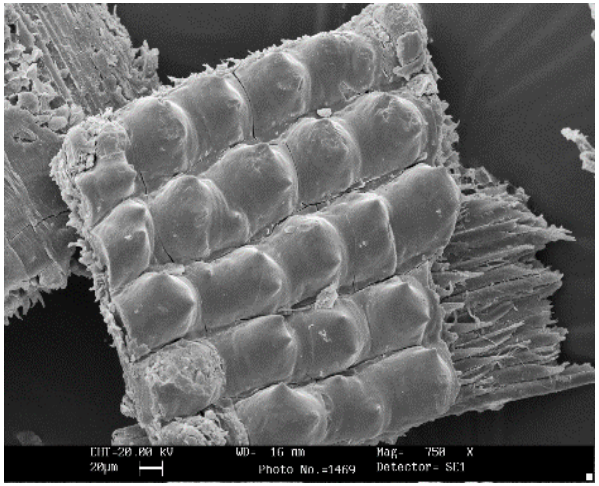
# SEM, tracheids and surface in detail details



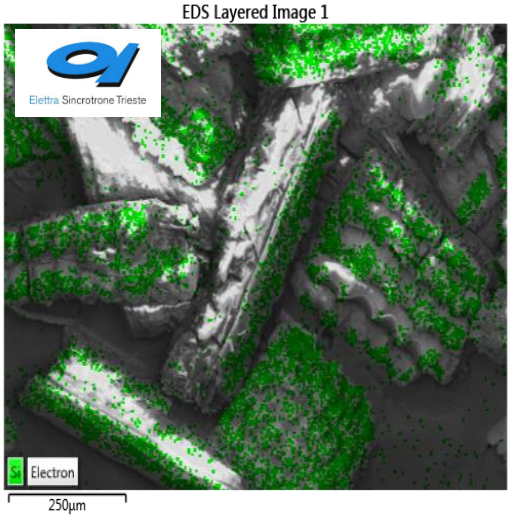



Thesis 2016 S. Lotteria, Photos by F. Vita

**(100-200 µm)**

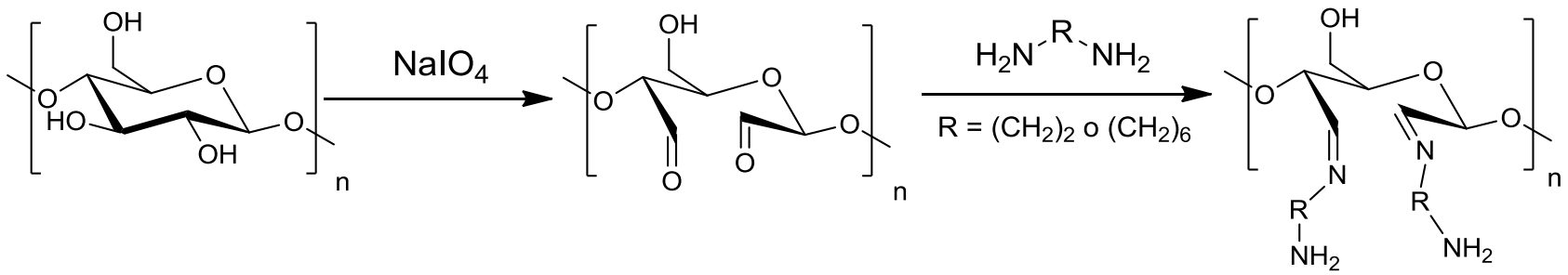


## Energy Dispersive X-ray Spectrometry (EDS)



# Functionalization by oxidation of cellulose

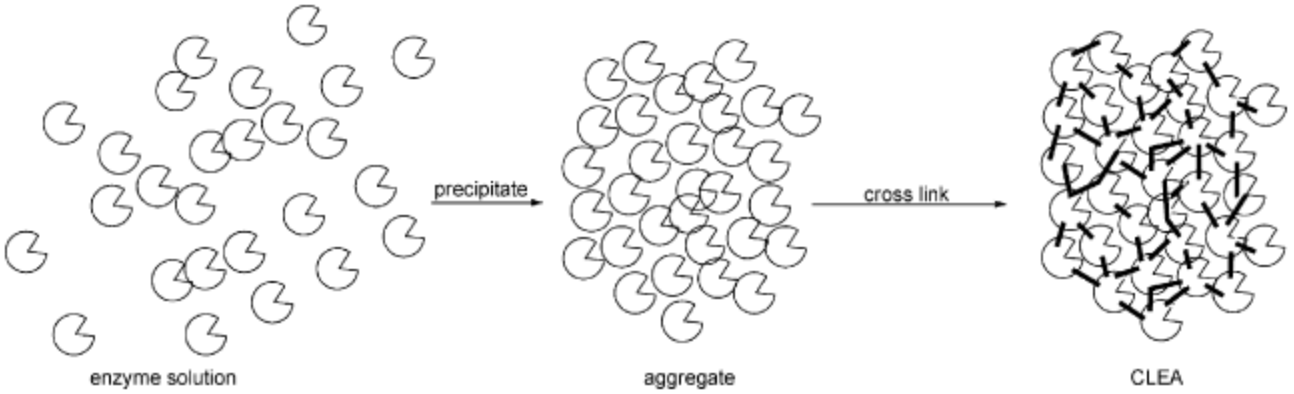
**c)**




**Corici, L.; Ferrario, V.; Pellis, A.; Ebert, C.; Lotteria, S.; Cantone, S.; Voinovich, D.; Gardossi, L. *RSC Advances* 2016, 6, 63256-63270.**

# Carrier-free immobilised enzymes

**Carrier-free immobilized enzymes are prepared by the cross-linking of enzyme aggregates or crystals, using a bifunctional reagent. This procedures lead to macroparticles, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs). This approach offers the advantage of highly concentrated enzyme activity in the catalyst and low production costs owing to the exclusion of an additional carrier. However, their mechanical stability in some cases may not match industrial requests.**



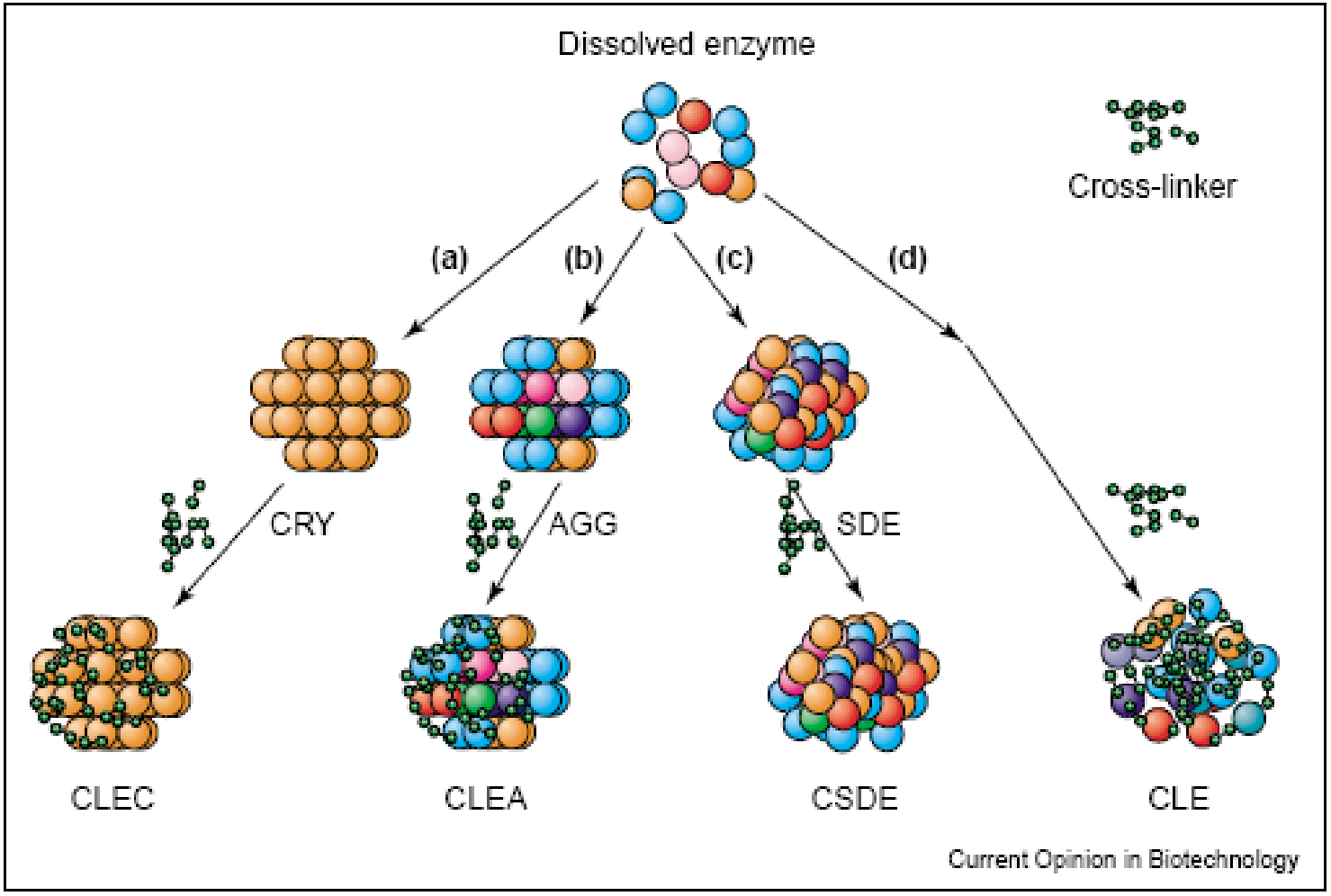

**Chem. Soc. Rev., 2009, 38, 453–468**

# CLEs

- CLEs Cross-linked enzyme
- CLECs Cross-linked enzyme crystals
- CLEAs Cross-linked enzyme aggregates
- CLSDs Cross-linked spray-dried

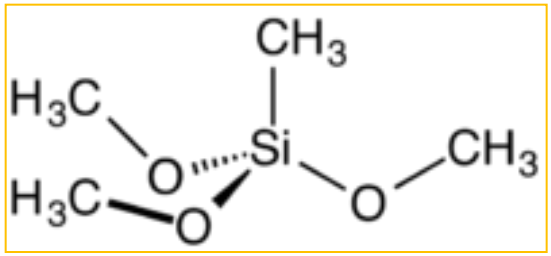
***Amotz S: Method for production of an immobilized enzyme preparation by means of a crosslinking agent. (Novo Industri A/S) 1987; US 4,665,028.***

# Carrier-free immobilised enzymes

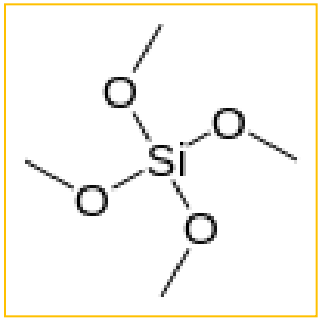


# Immobilization by entrapment in silica sol-gel

The **sol–gel** process is a method for producing solid materials made by metal oxides, especially the oxides of silicon and titanium.
The process involves conversion of monomers into a colloidal solution (*sol*) that acts as the precursor for an integrated network (or *gel*) of either discrete particles or network polymers.
Typical precursors are metal alkoxides.




**Methyltrimethoxysilane**



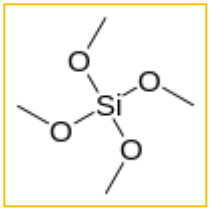

**Tetramethyl orthosilicate**

**Un colloide è una particolare miscela in cui una sostanza si trova in uno stato finemente disperso, intermedio tra la soluzione e la dispersione.**

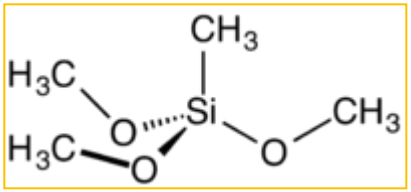
**Questo stato "microeterogeneo" consiste quindi di due fasi: una fase costituita da una sostanza di dimensioni microscopiche e una fase continua disperdente.**

# Immobilization by entrapment in sol-gel

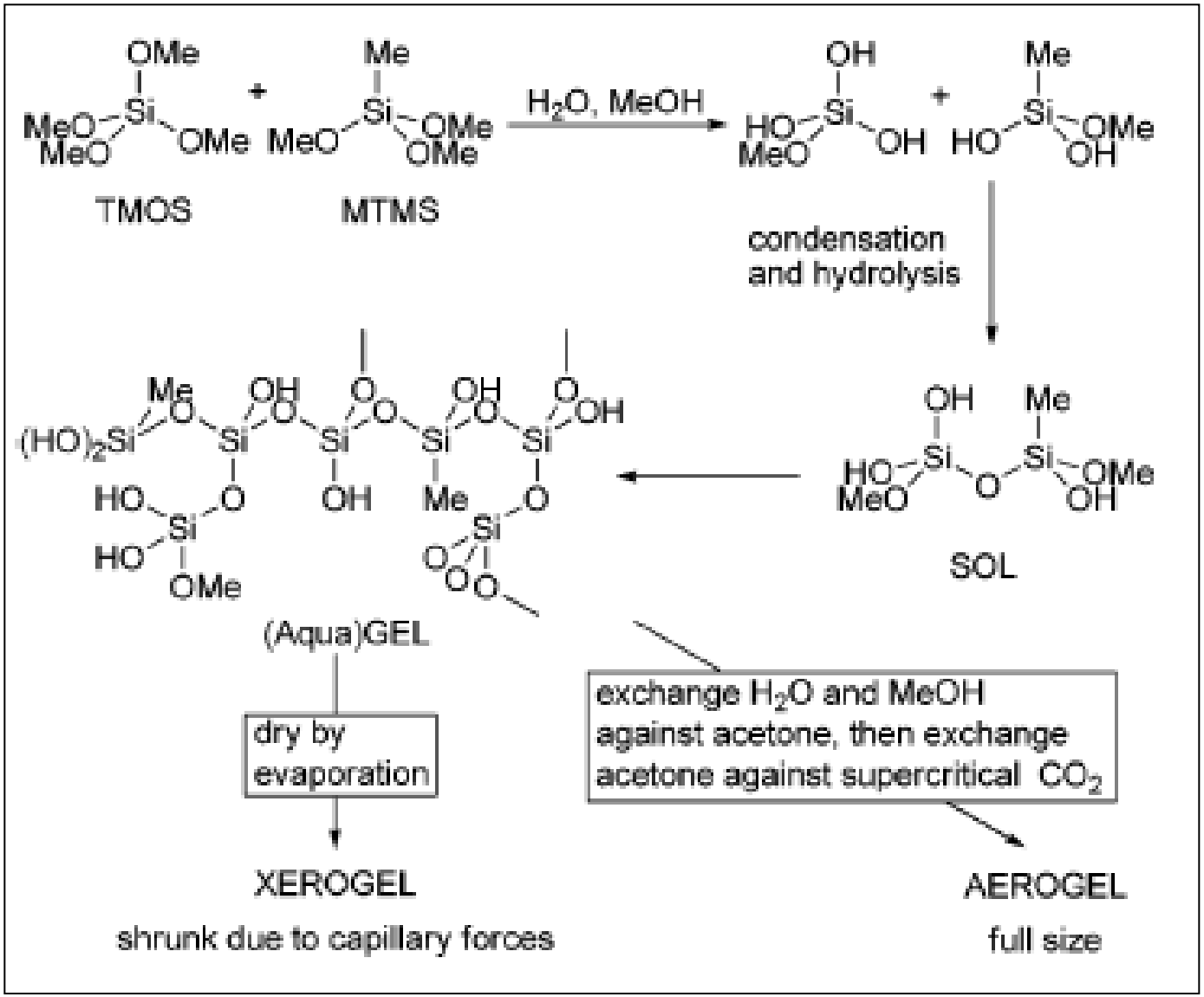
**Synthesis of sol-gels**



Tetramethyl orthosilicate



Methyltrimethoxysilane



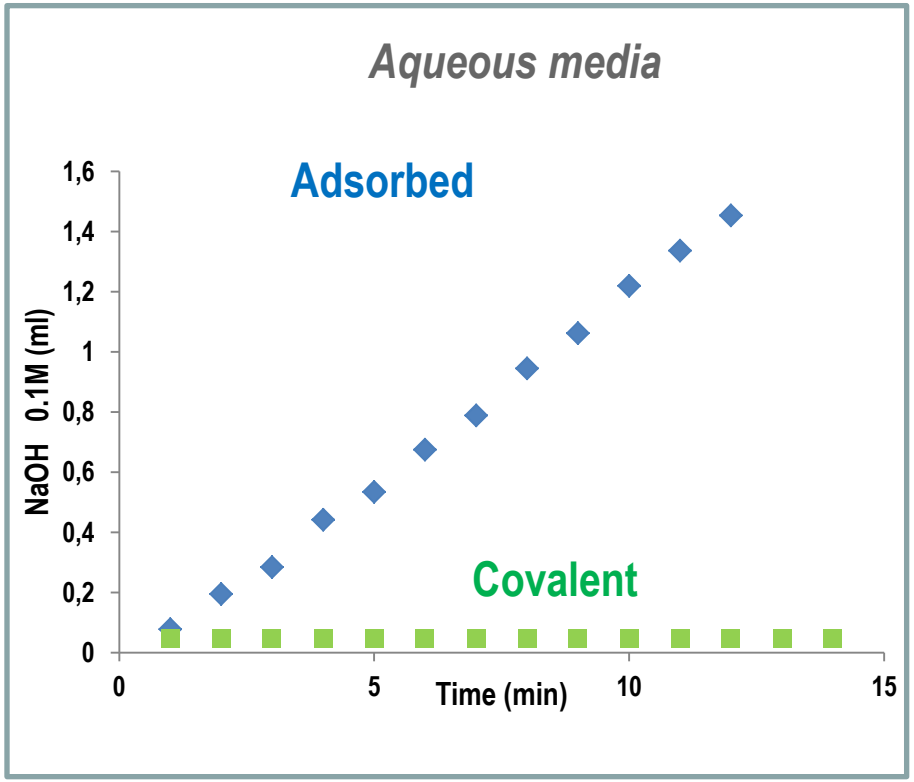

Chem. Soc. Rev., 2009, 38, 453–468

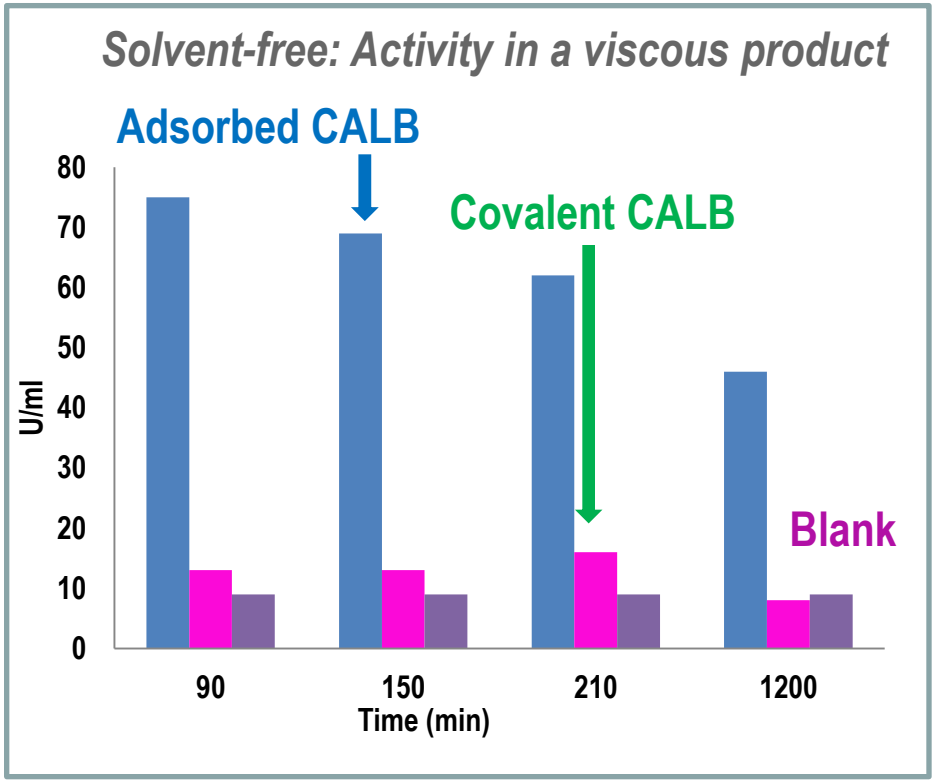
# A different immobilization technique for each system

| | Aqueous | Aqueous/solvent | Highly viscous | Hydrophobic solvent |
|---|---|---|---|---|
| Covalent | X | X | X | X |
| Adsorption | | | | X |
| Adsorption + cross-linking | X | X | X | X |

# Comparison between adsorbed and covalently linked enzymes

## Residual hydrolytic activity detected in the final product after filtration of the biocatalyst



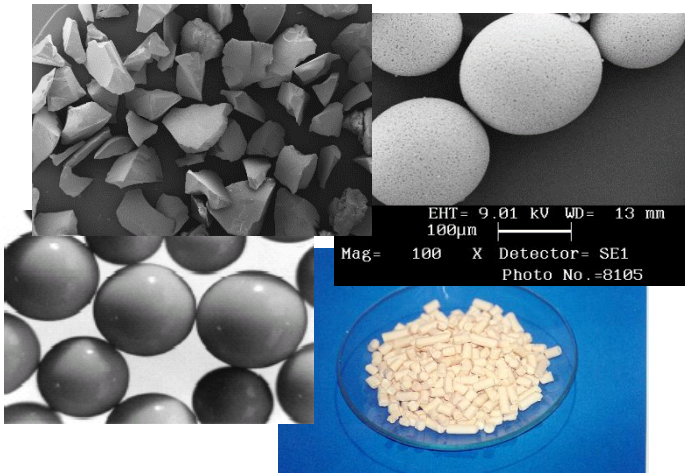

**Leaching phenomena affect kinetic studies when adsorbed preparations are employed**

# Criteria for selecting immobilization methods

# Formulating active and stable immobilized enzymes for industry

## *The support*

- Hydrophobic/hydrophylic
- Porosity
- Chemical functionality
- Polymer-enzyme spacer
- Parcicle size




## *The enzyme*

- Additives in the enzyme formulation?
- Covalent linking possible?
- Conformational flexibility required?
- Hydration required?
- Enzyme glycosylated?

## *The process*

- Reaction medium?
- Heterogeneous system?
- Diffusion limitations?
- Solutes adsorbtion/partition?
- Thermodynamics to be controlled?

**COST?**

# When industry uses immobilized enzymes

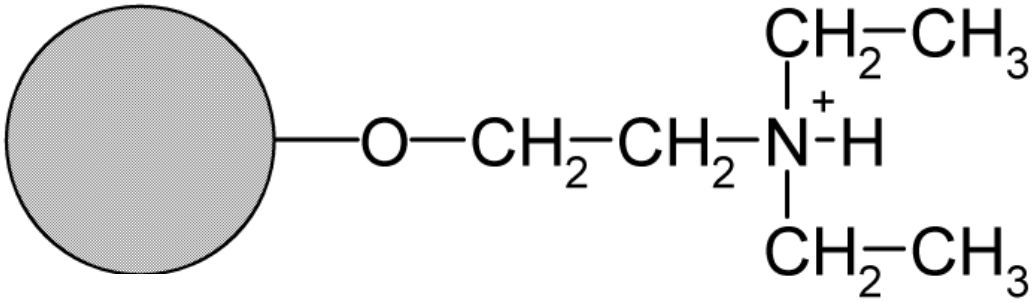
**Table 1** Attributes of immobilized biocatalysts

| Advantages | Disadvantages |
|---|---|
| Amenable to continuous and batch formats | Loss of enzyme activity upon immobilization |
| Reuse over multiple cycles possible | Unfavorable alterations in kinetic properties |
| Improved stability over soluble enzyme forms | Cost of carrier and fixing agents |
| Favorable alterations in pH and temperature optima | Cost of immobilization process |
| Sequester enzyme from product stream | Mass transfer limitations |
| Co-immobilization with other enzymes possible | Subject to fouling |

In reality the cost of most industrial enzymes is in the $50 to $500 per kg enzyme protein range, and they are often only a minor component in overall process economics.

The **first full scale industrial use of an immobilised enzyme** was the production of L-amino acids by resolution of racemic acylamino acids using an aminoacylase from *Aspergillus oryzae* immobilised by adsorption on **DEAE-Sephadex**, which consists of cross-linked dextran functionalized with **diethylaminoethyl** groups.

The process was performed in continuous operation in a fixed-bed reactor (Tanabe process – 1960)



Chem Soc Rev

RSC Publishing

REVIEW ARTICLE

View Article Online
View Journal | View Issue

# Industrial use of immobilized enzymes

Cite this: *Chem. Soc. Rev.*, 2013, **42**, 6437

Robert DiCosimo,*[a] Joseph McAuliffe,[b] Ayrookaran J. Poulose[b] and Gregory Bohlmann[b]

Received 10th December 2012

DOI: 10.1039/c3cs35506c

www.rsc.org/csr

Although many methods for enzyme immobilization have been described in patents and publications, relatively few processes employing immobilized enzymes have been successfully commercialized. The cost of most industrial enzymes is often only a minor component in overall process economics, and in these instances, the additional costs associated with enzyme immobilization are often not justified. More commonly the benefit realized from enzyme immobilization relates to the process advantages that an immobilized catalyst offers, for example, enabling continuous production, improved stability and the absence of the biocatalyst in the product stream. The development and attributes of several established and emerging industrial applications for immobilized enzymes, including high-fructose corn syrup production, pectin hydrolysis, debittering of fruit juices, interesterification of food fats and oils, biodiesel production, and carbon dioxide capture are reviewed herein, highlighting factors that define the advantages of enzyme immobilization.

The **cost contribution** from an immobilized enzyme is dependent on the number of times the enzyme is reused, an indirect measure of total productivity on a kg product per kg biocatalyst basis.

**This amount varies between a few hundred $ per kg for specialty chemicals, down to a few cents per kg for bulk chemicals, and is often in the range of $0.1 to $10 per kg**

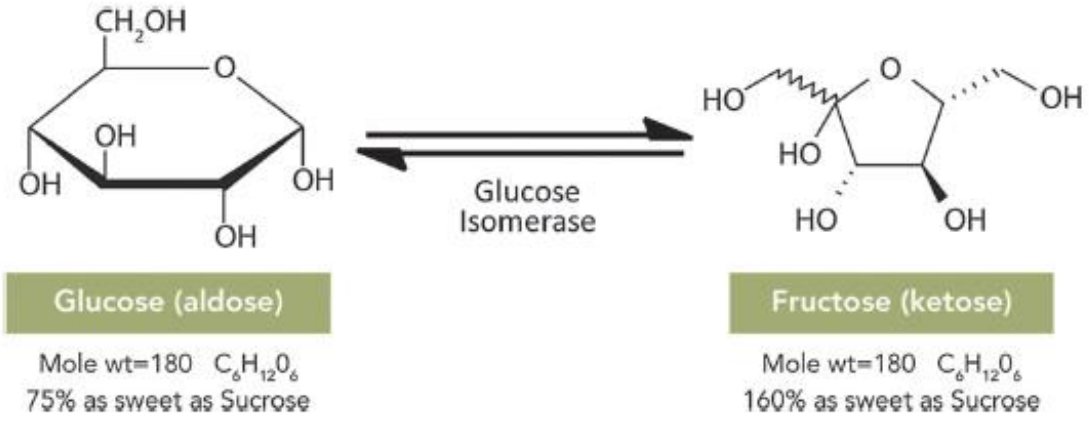
**Table 2** Large scale industrial processes utilizing immobilized biocatalysts

| Enzyme | Form[a] | Process | Product scale (ton per year) | Ref. |
|---|---|---|---|---|
| Glucose isomerase | CWC, IME, CIE | High fructose corn syrup from corn syrup | $10^7$ | 7,19,21–23 |
| Nitrile hydratase | CWC | Acrylamide from acrylonitrile | $10^5$ | 334–336 |
| Lipase | IME | Transesterification of food oils | $10^5$ | 205–209 |
| Lactase | IME | Lactose hydrolysis, GOS synthesis | $10^5$ | 337–339 |
| Lipase | IME | Biodiesel from triglycerides | $10^4$ | 269,271–277 |
| Penicillin G acylase | CIE | Antibiotic modification | $10^4$ | 340–342 |
| Aspartase | CWC, IME | L-Aspartic acid from Fumaric acid | $10^4$ | 343–345 |
| Thermolysin | IME | Aspartame synthesis | $10^4$ | 346–348 |
| Lipase | IME, CIE | Chiral resolution of alcohols and amines | $10^3$ | 349–351 |

[a] CWC = cross-linked whole cell; IME = immobilized enzyme; CIE = covalently immobilized enzyme.

**Glucose isomerase (GI), also known as xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5), is one of the most important industrial enzymes in commerce today, driven primarily by the rise of D-fructose as a sweetener for beverages and foodstuffs.**

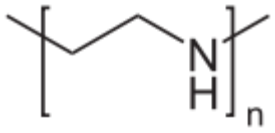
**Although D-xylose is the native substrate, the enzyme has broad substrate specificity and efficiently converts D-glucose to D-fructose (Scheme 1).**



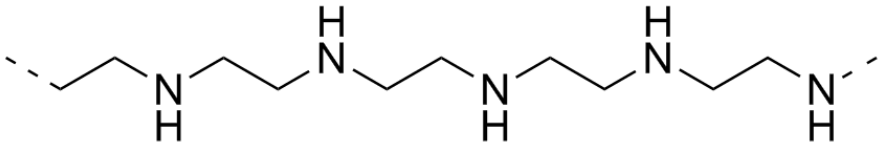

**Scheme 1** Isomerization of D-glucose to D-fructose.

**Table 3** Examples of commercial immobilized glucose isomerase products[22,23,44]

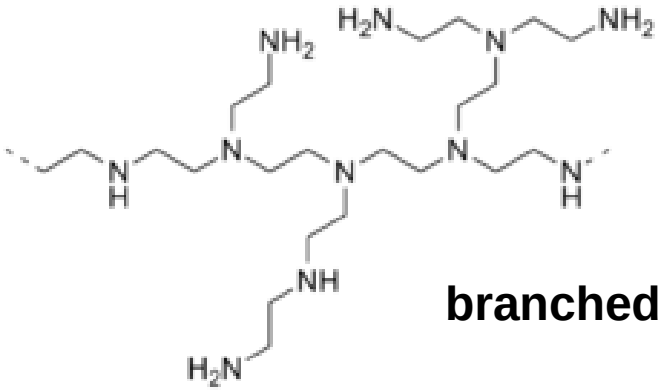
| Product | Producer | GI source | Description | Currently sold? |
|---|---|---|---|---|
| Optisweet® 22 | Miles-Kali/Solvay | *S. rubiginosus* | Adsorption of GI on to $SiO_2$ followed by crosslinking with glutaraldehyde | N |
| TakaSweet® | Miles Labs/Solvay | *Flavobacterium arborescens* | Polyamine/glutaraldehyde crosslinked cells extruded and spheronized | N |
| Maxazyme® GI | Gist-Brocades | *A. missouriensis* | Crosslinked cells entrapped within gelatin beads | N |
| Ketomax GI-100 | UOP | *S. olivochromogenes* | Glutaraldehyde crosslinked GI adsorbed to PEI-treated alumina | N |
| Spezyme® | Genencor | *S. rubiginosis* | Crystallized crosslinked GI adsorbed to granular DEAE-cellulose | N |
| Sweetase® | Denki Kagku-Nagase | *S. phaeochromogenes* | Heat-treated cells entrapped within polymer beads | N |
| Sweetzyme® T | Novozymes A/S | *B. coagulans* *S. murinus* | Gluteraldehyde crosslinked whole cell homogenate containing inorganic carrier | Y |
| GENSWEET®SGI | Genencor/DuPont | *S. rubiginosis* | Soluble GI product for adsorption to DEAE-cellulose anionic resin | Y |
| GENSWEET® IGI | Genencor/DuPont | *S. rubiginosis* | PEI/glutaraldehyde crosslinked cells, mixed with inorganics (clay, DE) | Y |



**PEI: polyethyleneimine**



**Linear**



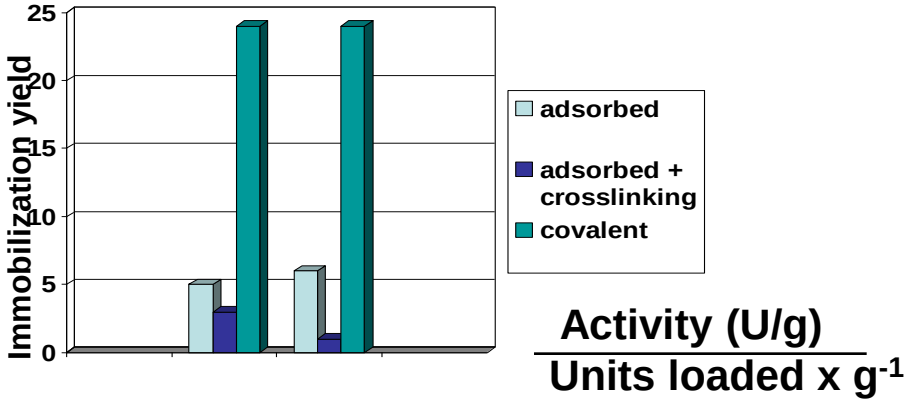
**branched**

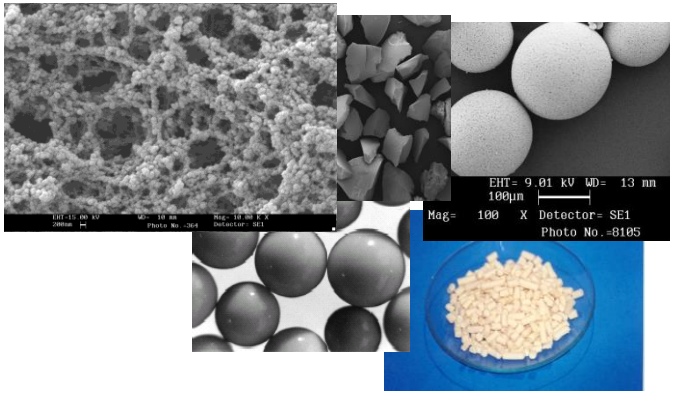
# How to describe a biocatalysed process and report a biocatalysed experiment

# Immobilized biocatalysts: data to be reported in protocols

**Clear protocol of immobilization with characterization:**

- **Amount of support and enzyme / cells**
- **amount immobilised, (e.g. difference method)**
- **activity of immobilised preparation**
- **residual water content**
- **data on support (when available)**
- **distribution within particles (when feasible)**




Proteomic Methods Applied to The Analysis of Immobilized Biocatalysts, I. Petry, et al. Biotechnol. Bioeng., 95, 984, 2006

**Factors to be considered when planning enzyme immobilization**

**Enzyme**
Size of the enzyme
Conformational flexibility required by the mechanism
Isoelectric point
Surface functional groups/charge density
Glycosylation
Stability under immobilisation conditions
Presence of hydrophobic regions
Presence of hydrophilic regions
Additives in the enzymatic preparation

**Carrier**
Organic or inorganic
Hydrophobic or hydrophilic
Surface charges
Surface functionalisation
Chemical and mechanical stability
Surface area
Porosity
Particle size

**Specific factors related to the reaction system**
Reaction medium
Diffusion limitations
Enzyme inhibition
Precipitation of products
Viscosity of the mixture
Reaction thermodynamics
Non-specific solute-support interactions

| Method of immobilization | Relevant Factors |
|---|---|
| **General** | Additives in the enzyme preparation that might interfere<br>Stability of the enzyme under immobilisation conditions<br>Stability of the carrier under operative conditions<br>Protein leaching under operative conditions<br>Non-specific carrier-substrates interactions<br>Cost and availability of the carrier |
| **Adsorption/deposition** | |
| Hydrophobic organic carrier | Presence of hydrophobic regions on enzyme<br>Ionic strength of the immobilisation buffer to favour protein adsorption |
| Hydrophilic organic carrier | Presence of hydrophilic regions on enzyme/glycosylation |

Chem. Soc. Rev., 2009, 38, 453–468

| Method of immobilization | Relevant Factors |
| --- | --- |
| **Ionic interactions** | pI of the enzyme<br>Charged residues (type and density) on the enzyme surface<br>pH and ionic strength of immobilisation buffer |
| **Covalent binding/crosslinking** | Location of the residues necessary for linking<br>pH of immobilisation suitable for nucleophilic attack<br>Conformational flexibility required by the catalytic mechanism |
| **Encapsulation** | Size of the enzyme<br>Synthesis conditions for the polymer |

Chem. Soc. Rev., 2009, 38, 453–468

| Reaction system | Method of immobilisation |
|---|---|
| Dilute aqueous solution | Covalent<br>Crosslinking<br>Encapsulation |
| Dilute organic solution | Any |
| Concentrated, viscous organic/ inorganic mixtures | Covalent, crosslinking |