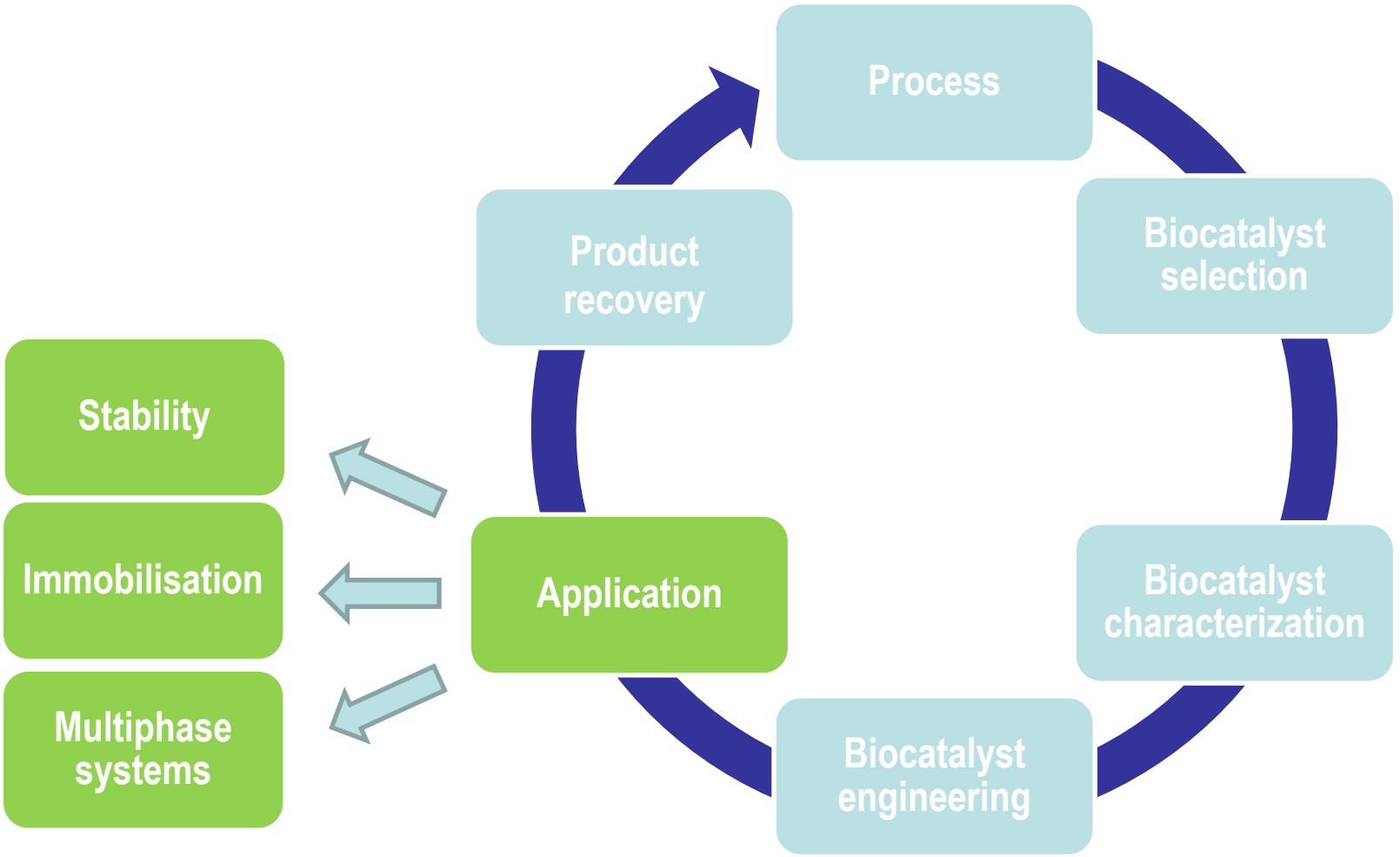
Formulation, stabilization and immobilization of enzymes for biotransformations

Biocatalysis for industrial synthesis



Trasform a good enzyme
into an efficient industrial biocatalyst

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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

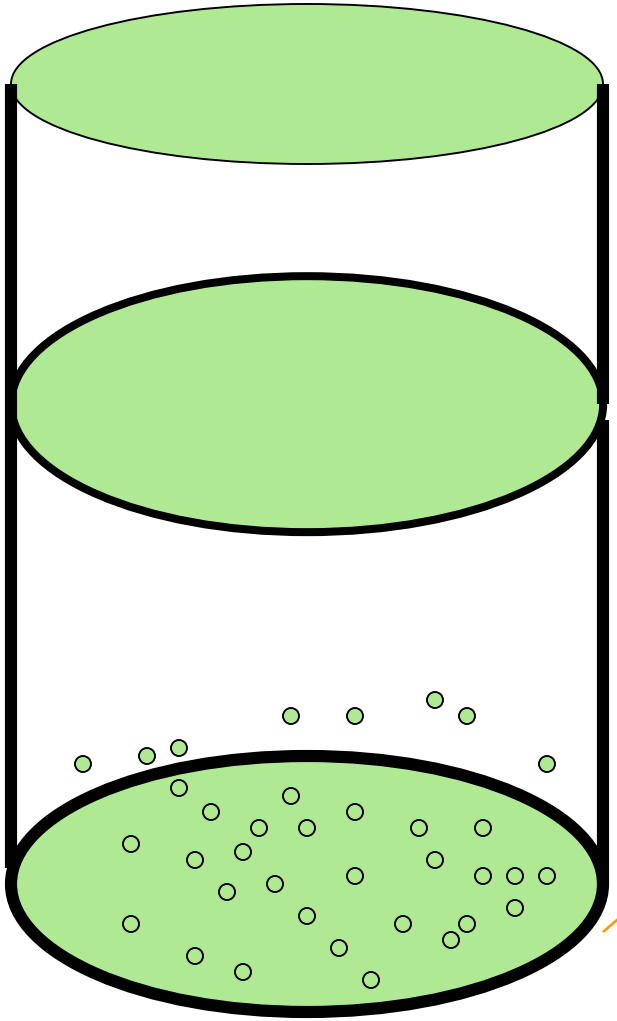
Roger A. Sheldon[a,*]

Understanding enzyme immobilisation

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

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

Immobilized enzymes: heterogeneous systems



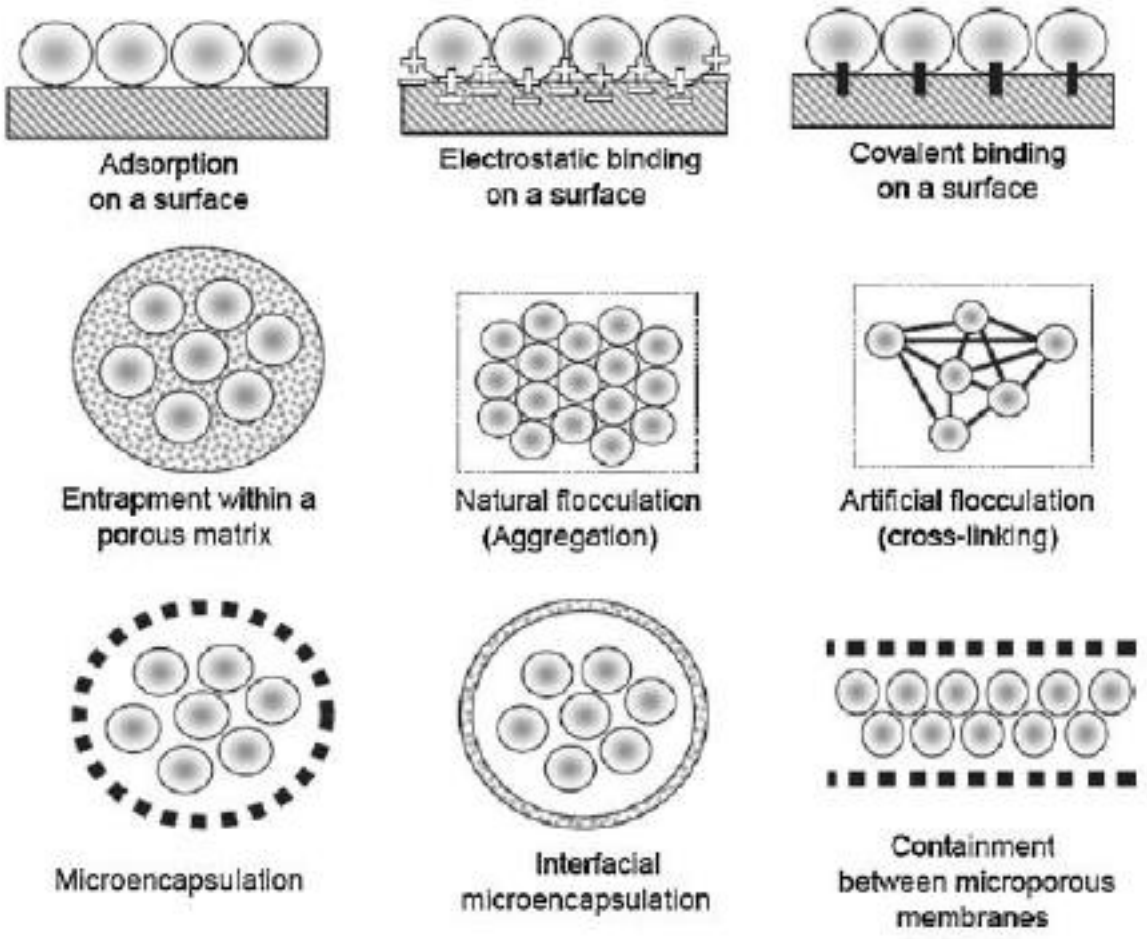
Immobilized enzymes: potential advantages

- **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.**

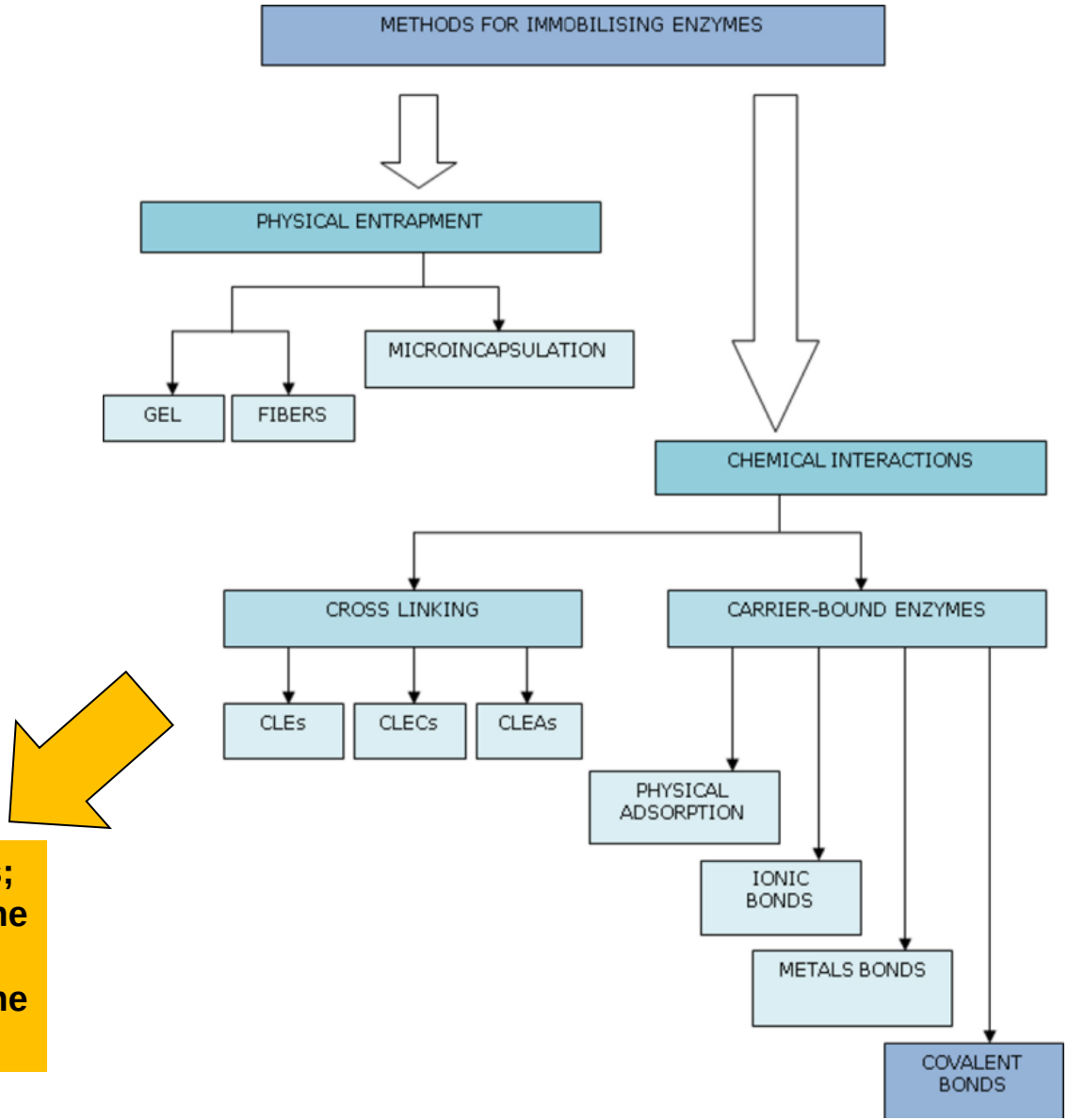
↓

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

Most common immobilization methods



Enzyme immobilization: selecting the method



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

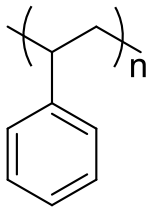
Immobilization on solid carriers

Dott. Simone Lotteria, Thesis 2016

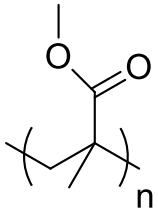
Carrier

- 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

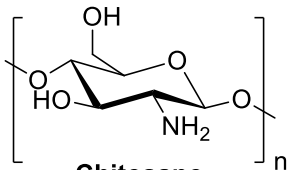


Polistirene

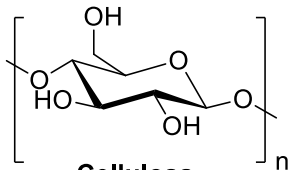


Poliacrilato

Biopolymers



Chitosano

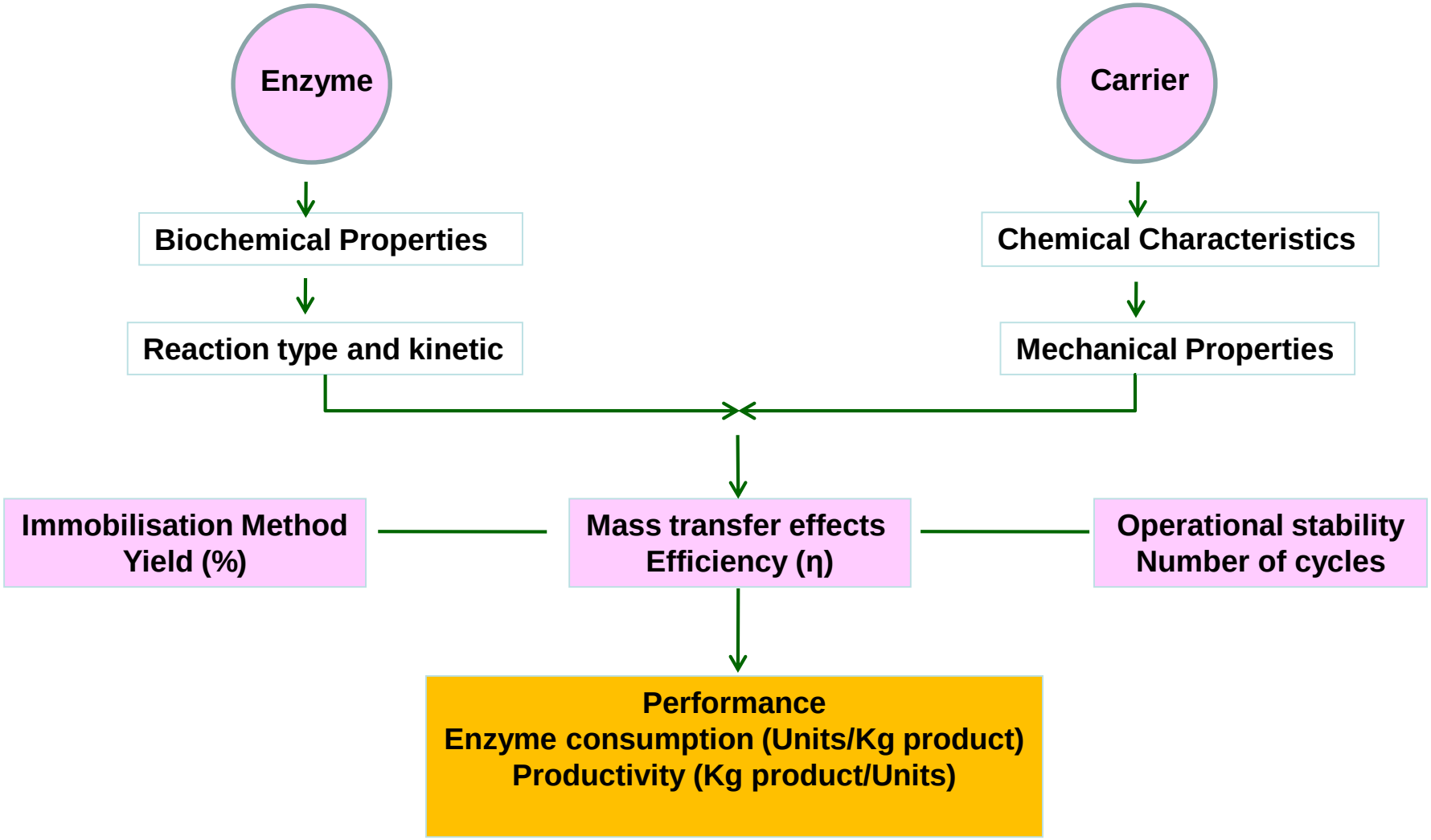


Cellulosa

Inorganic

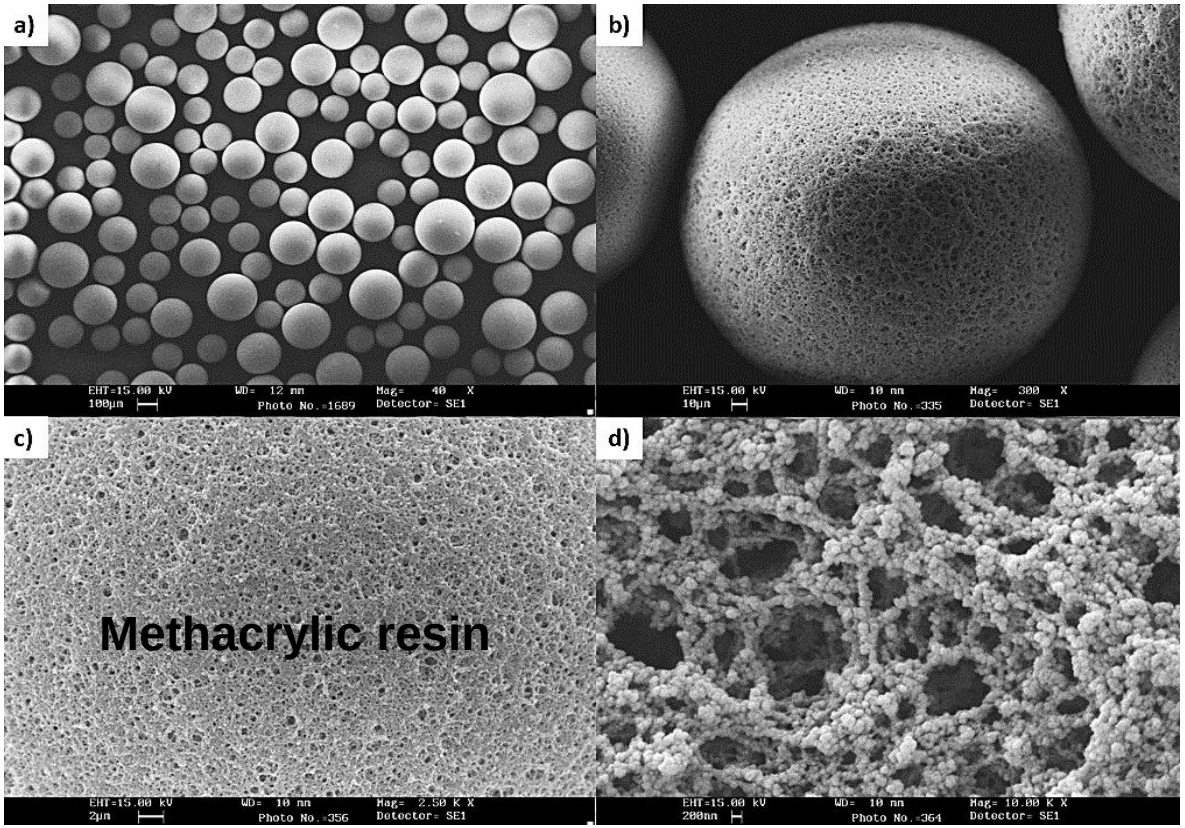
Silicates, celite

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.



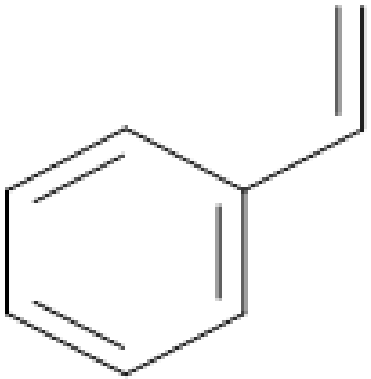
Organic synthetic polymers and resins

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

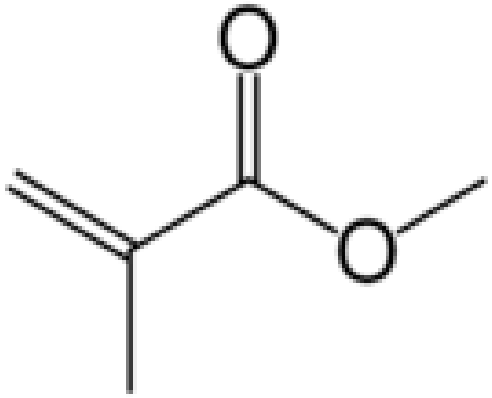
They are synthetized via radicalic polymerization

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.

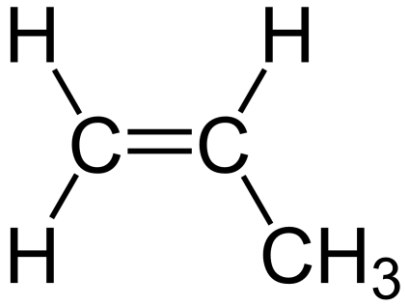
As a consequence, a considerable number of **acrylic or styrenic resins**, with different degrees of hydrophobicity, are available



Styrene

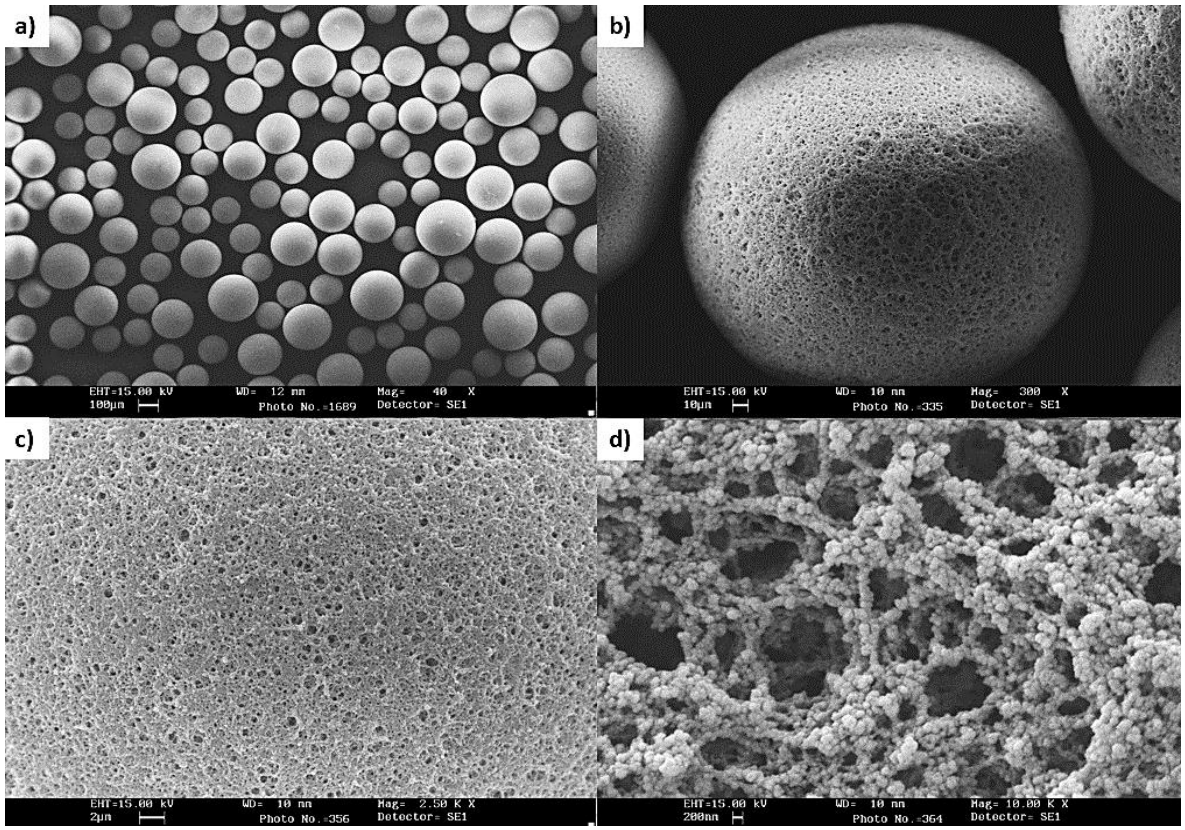


methyl acrylate



propilene

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

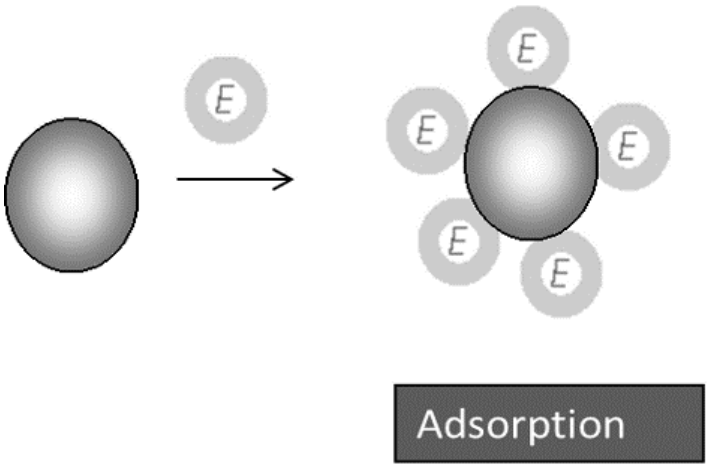


Porosity :
several materials used for enzyme adsorption can be considered **macroporous,** since pore diameters are higher than 50 nm.

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

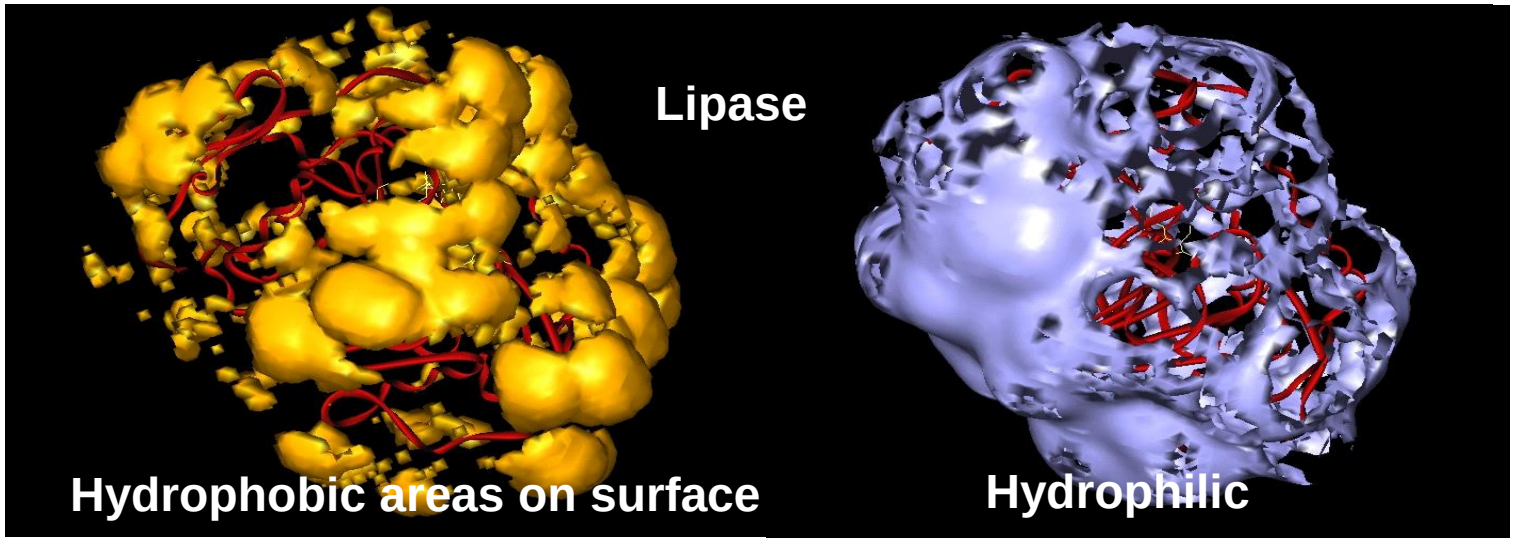
Enzyme immobilization on solid carriers: adsorption

Support binding can simply exploit weak interactions



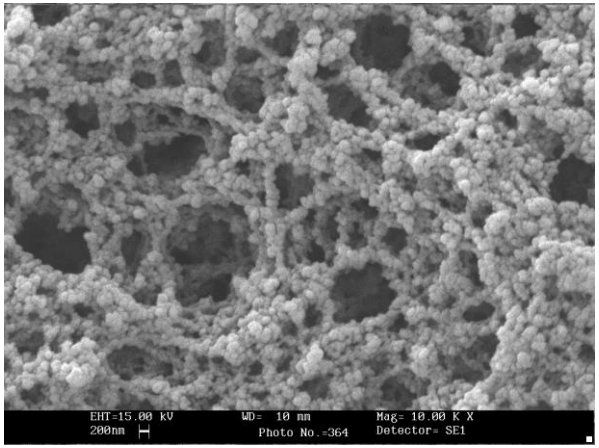
One of the most successful examples of lipase adsorption on organic resins is the widely used enzyme Candida antarctica
lipase B commercially available in the immobilised form as Novozym 435$_s$ which consists of the enzyme adsorbed on
a macroporous polymethyl/butylmethacrylate-divinylbenzene) resin.

Hydrophobic, van der Waals interactions



Methacrylic and styrenic porous polymers

High porosity and large internal surface

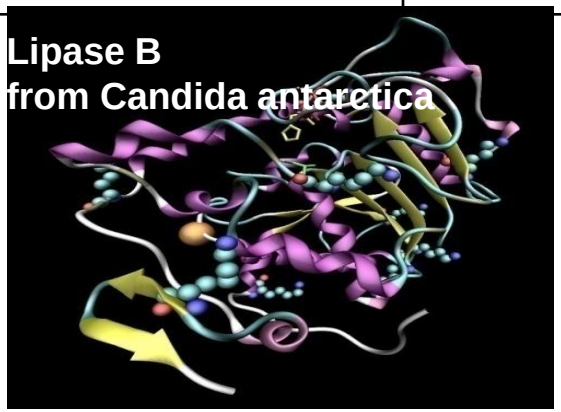


→

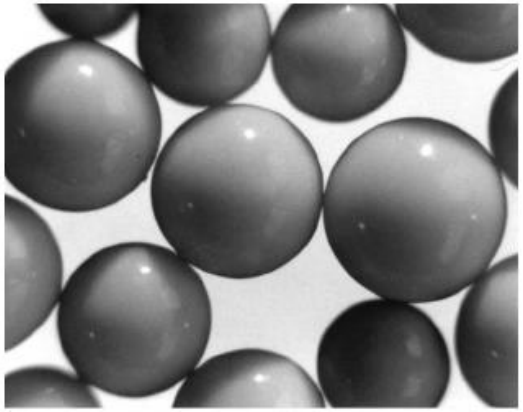
Increasing the ionic stenght for forcing the partition of enzyme onto the polymer

Important!!!
Commercial enzymes contain impurities and additives

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

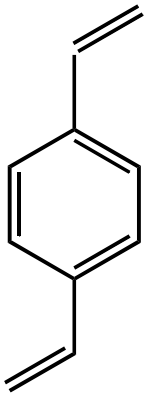


Acrylic + DVB



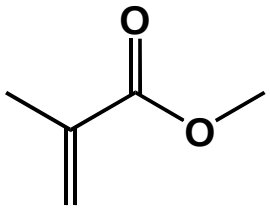
Novozym® 435

CAL-B immobilised (adsorption) on a hydrophobic polymer



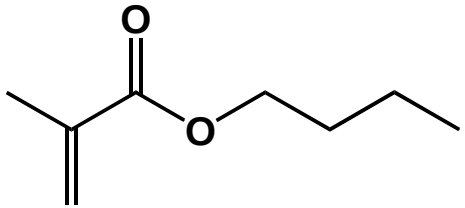
DVB (Divinylbenzene)

+



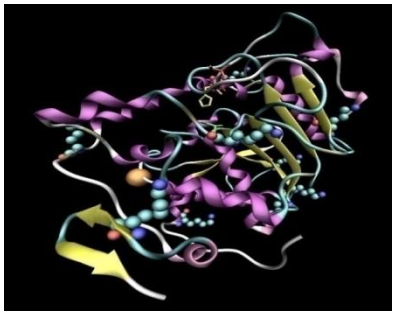
MA (Methylacrylate)

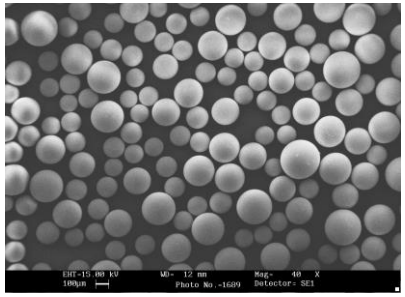
+



BA (Buthylacrylate)

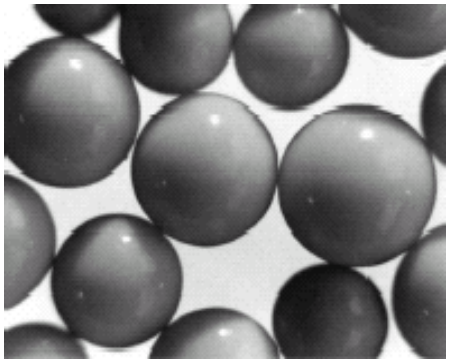
Lipase B from *C. antarctica*





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

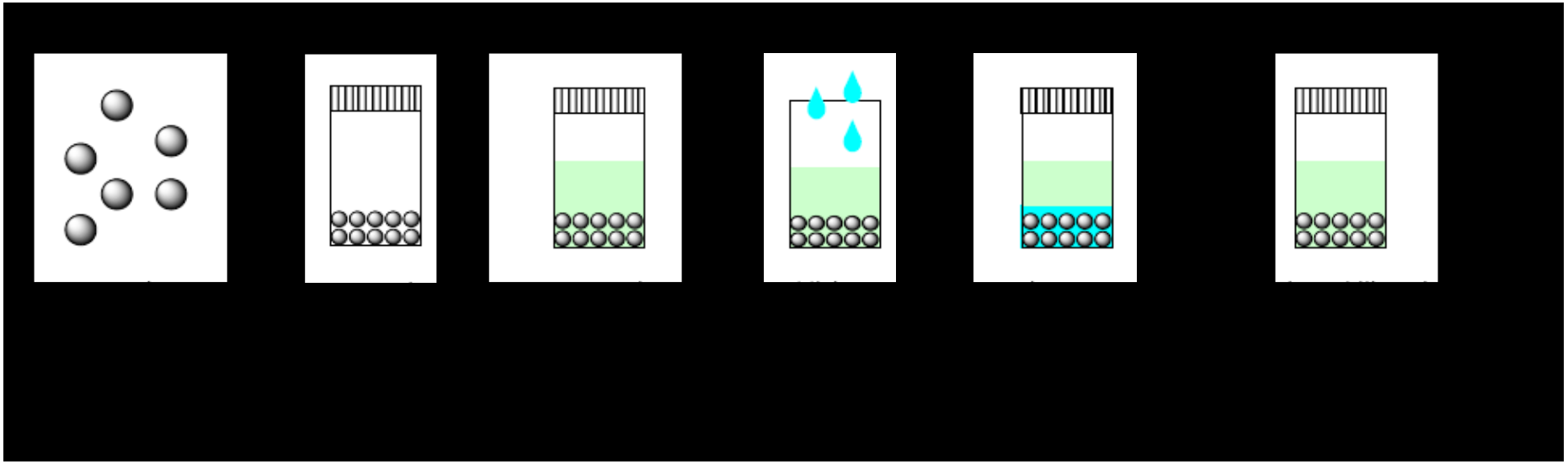
Adsorption **Immobilisation**



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

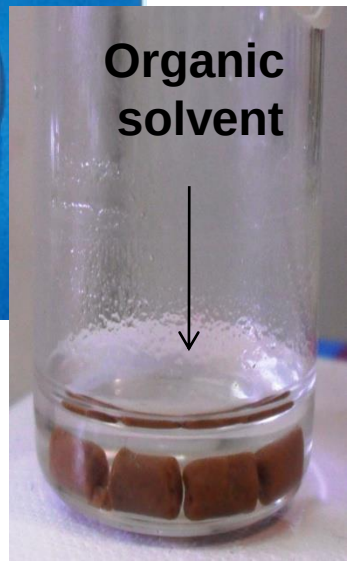
A variety of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica, zeolites and mesoporous silicas.

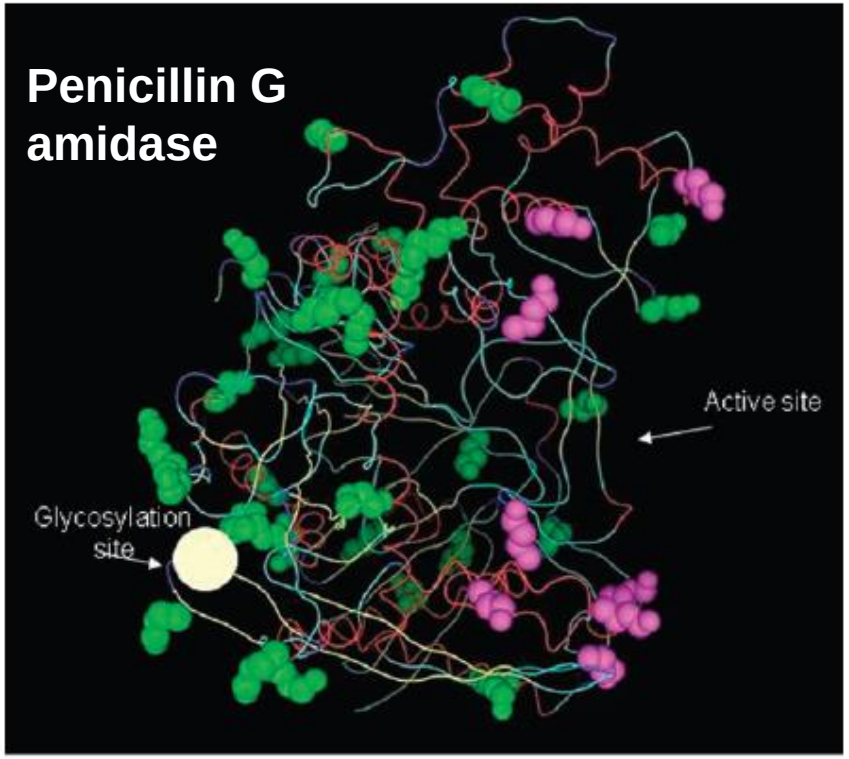
Silicates

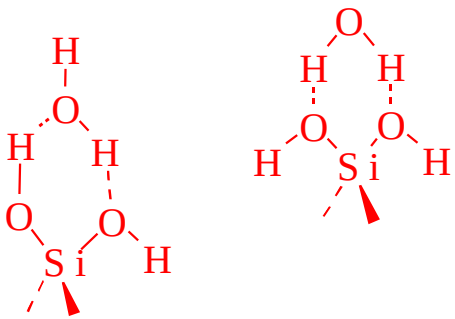
- Chemically inert, stable shape, do not swell

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**.

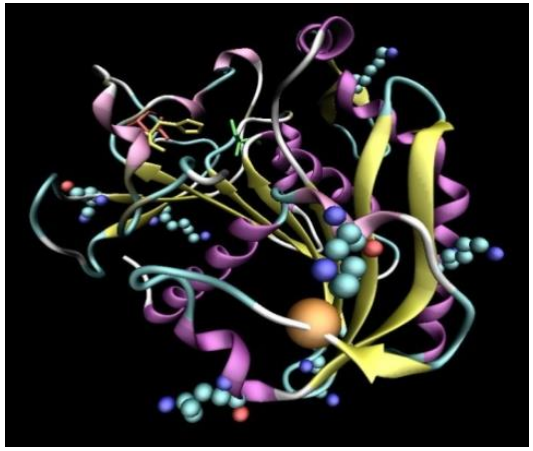
It is used, for example, to formulate enzymes for detergent powders which release the enzyme into the washing liquid during washing. **Granulation technology was used to immobilize lipase on silica granules, by first adsorbing the lipase on silica powder followed by agglomeration.**

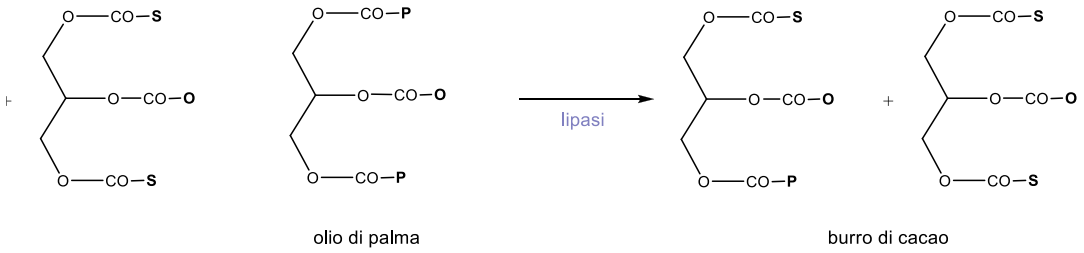
Owing to the composition of the granulates, they are intended for use only in organic media.
In an aqueous medium the lipase is desorbed and the particle slowly disintegrates.

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)

Cemical composition
(cellulose, hemicellulose, lignin, silica)

Powder properties:
- Particle size (~100 µm)
-

Atomization / dispersion

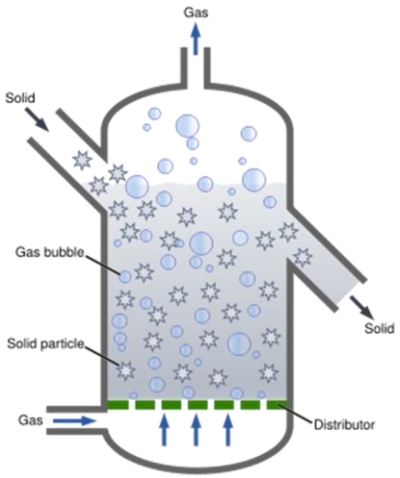
Process parameters:
- Binder flow rate
- Atomization pressure
- Atomization time
- Chopper/Impeller speed

Binder (aqueous solution)

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

Immobilization on functionalized solid carriers

Immobilization on functionalized solid carriers

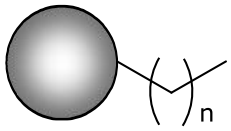
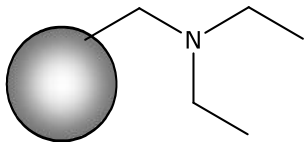
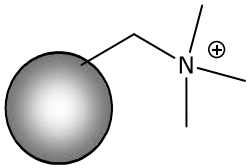
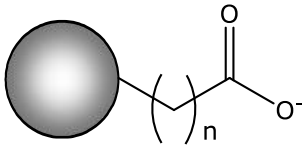
It is related to the presence of **specific chemical functionalities** on the surface of the carriers.

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**

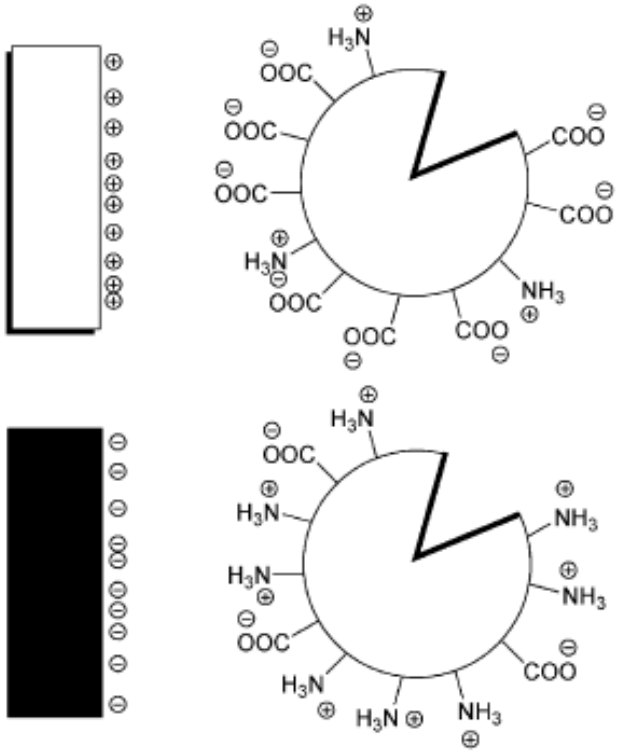
Finally, they can also be **introduced by chemical modification** of the surface of the carrier through a ‘‘pre-activation’’ treatment.

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

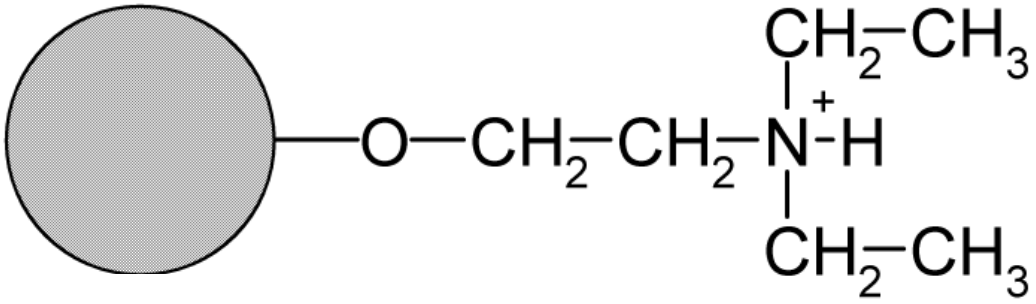


Depending on the pH of the solution and the isoelectric point the surface of the enzyme may bear charges. Using widely available modelling systems, the surface charge and charge distribution of an enzyme can be readily calculated and displayed. Essentially any ion exchanger can act as carrier in immobilisation 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).

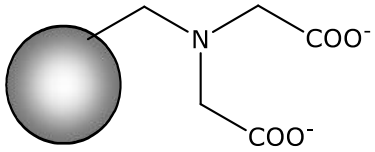
Chem. Soc. Rev., 2009, 38, 453–468

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.

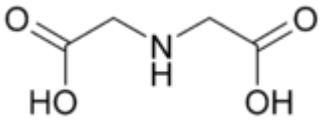
The process was performed in continuous operation in a fixed-bed reactor (Tanabe process – 1960)



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).

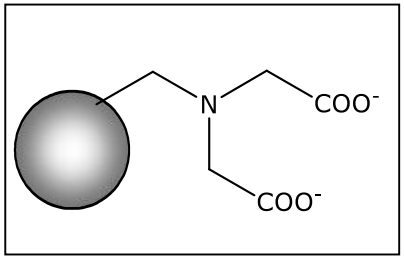


Metal chelated supports are used in protein chromatography (IMA – Immobilised Metal-Ion Affinity).

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.

Chelator ligands



IDA

Imidazole complexation of Ni^{2+}

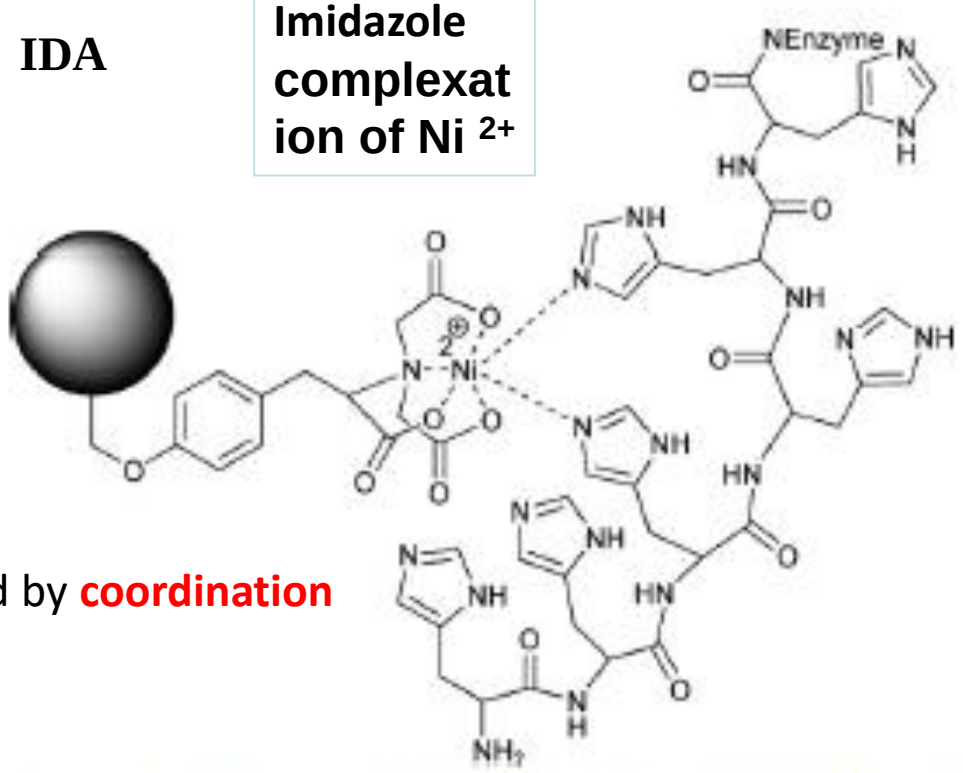


Fig. 5 Ni^{2+} attached to a carrier anchors an enzyme with a His tag to the carrier.

the metal ions are then bound by **coordination**

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.

Covalent immobilization of enzymes

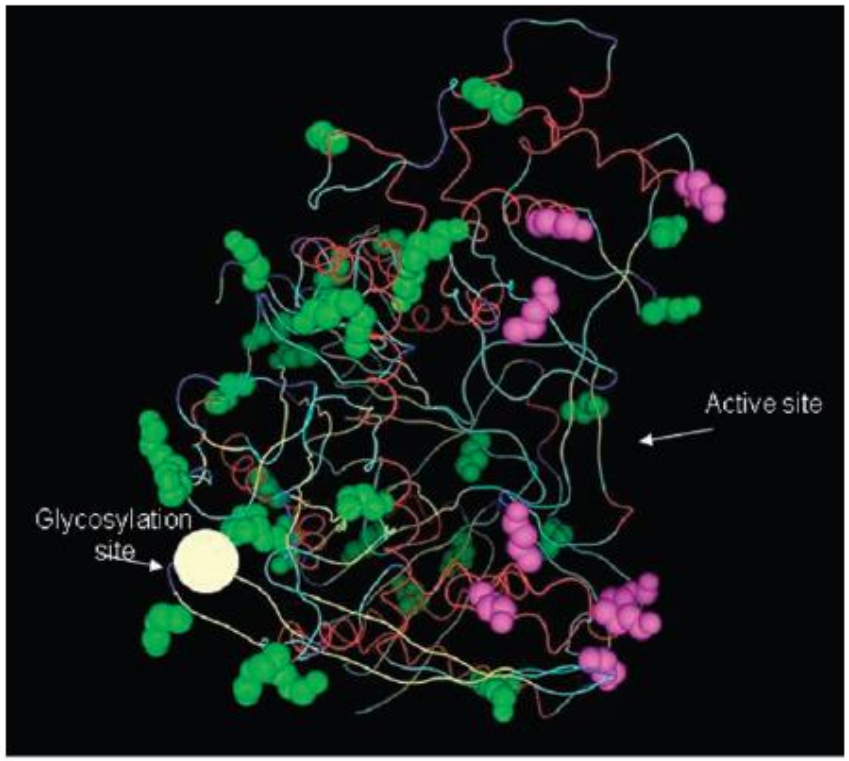
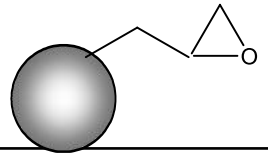
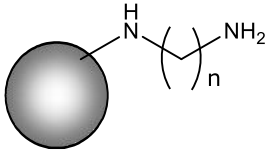


Fig. 13 Model of the structure of the PGA from *E. coli*. The residues of lysine are pointed out in the space-filling modality. The violet residues correspond to those lysines closer to the opening of the active site. On the opposite side there is the glycosylation site (yellow).

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

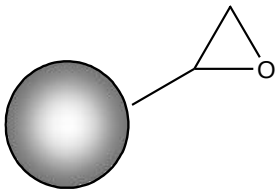
Covalent immobilization of enzymes on commercially available organic resins with functionalized surface

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)

Enzyme immobilisation on epoxy and amino carriers

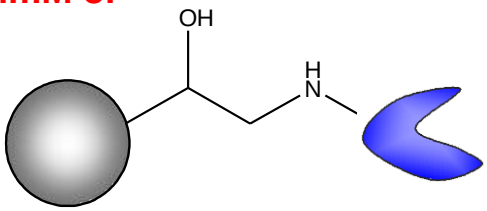
Higher ionic strenght to promote the partition of enzymes onto the hydrophobic resins



Kpi buffer

(pH 8.0, 50mmM or 1M)

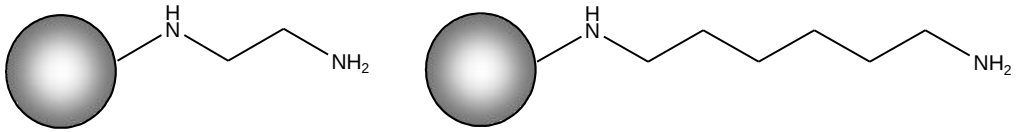
Enzyme

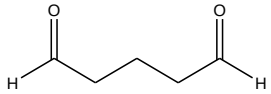


Covalent immobilisation

Sometimes followed by post-blocking of unreacted epoxydes with Gly

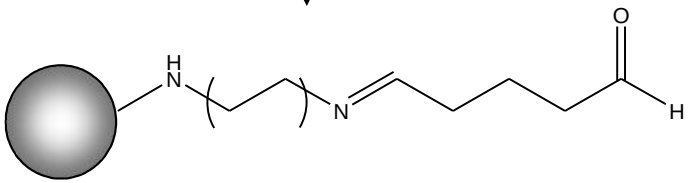
Lower ionic strenght to promote the partition of enzymes onto the less hydrophobic resins





Support pre-activation

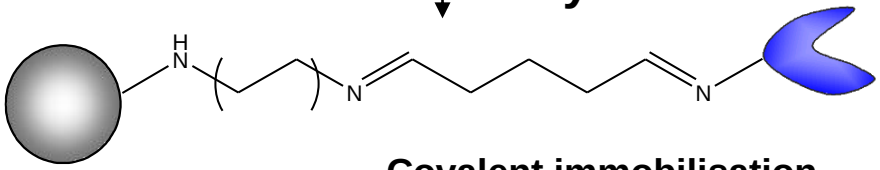
(2% glutaraldehyde)



Kpi buffer

(pH 8.0, 0.02M)

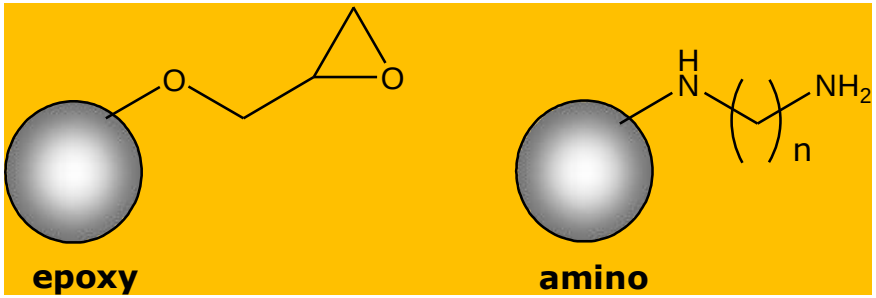
Enzyme



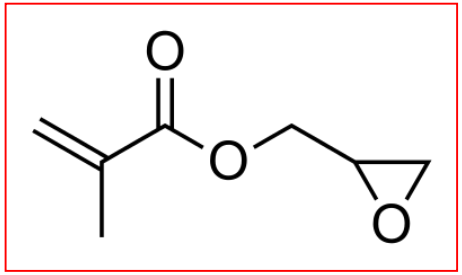
Covalent immobilisation

(Shiff base formation)

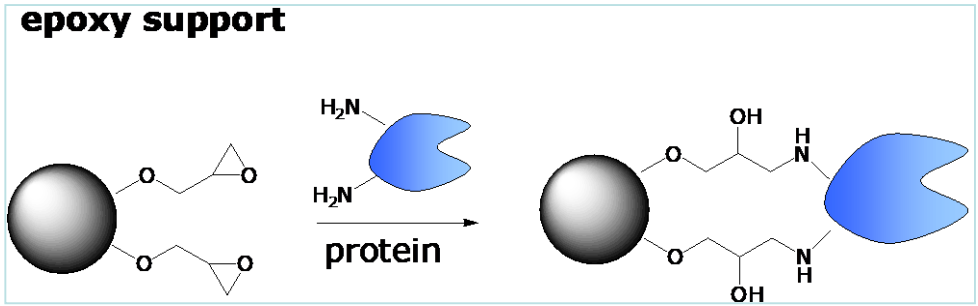
Covalent immoblization on functionalized supports

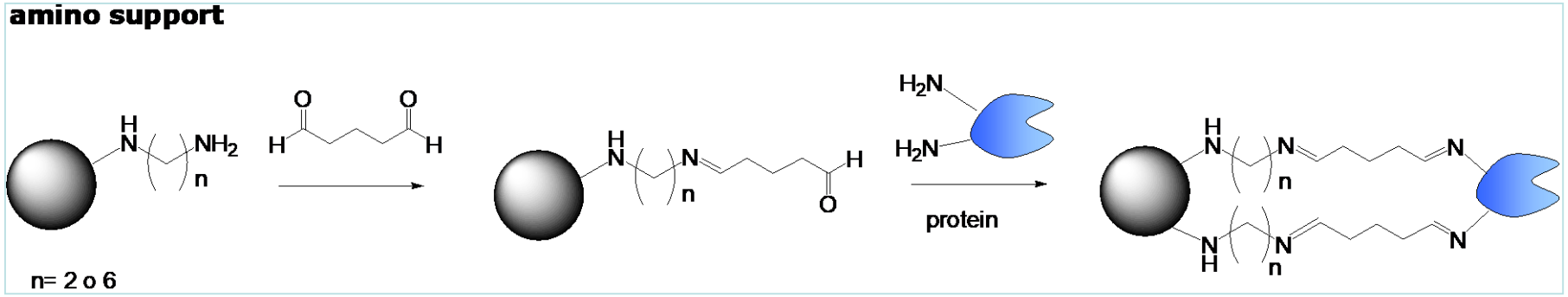


a certain percentage
of the glycidyl methacrylate monomer is
added in the synthesis
of methacrylic polymers



Glycidyl methacrylate





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

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

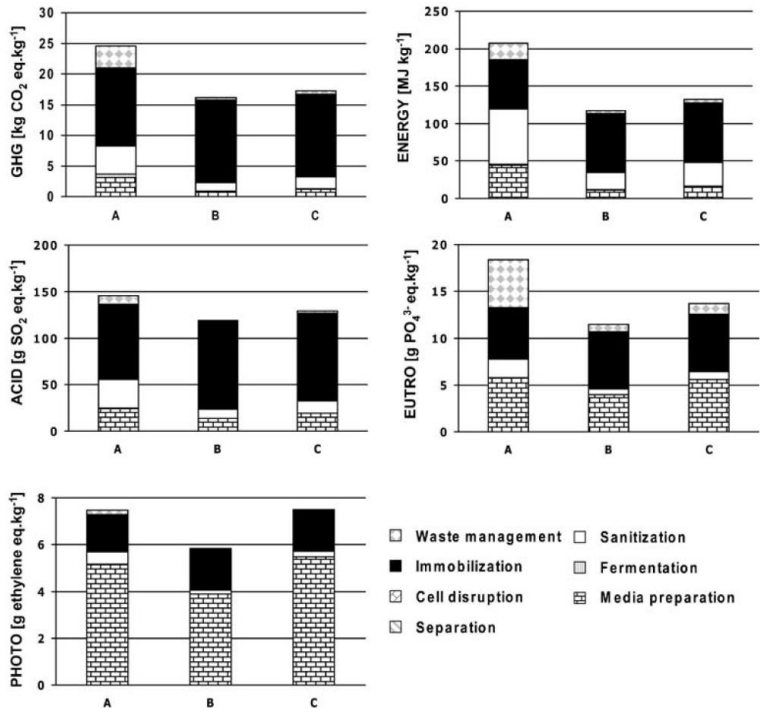


Fig. 2 Selected environmental impacts associated with the immobilized enzyme production systems *A*, *B*, and *C* [*GHG* global warming, *ACID* air acidification, *EUTRO* eutrophication, *PHOTO* photochemical smog formation, *ENERGY* nonrenewable energy consumption]

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)

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.

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

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

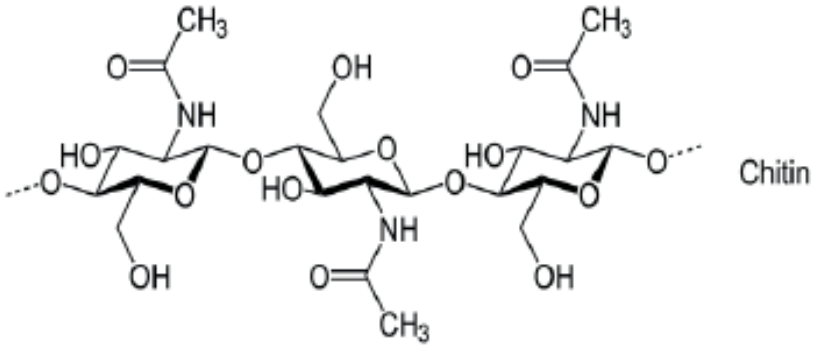
Abstract

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



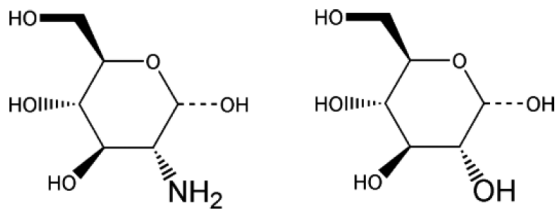


Figure 1. Structure of glucosamine (monomer of chitosan) and glucose (monomer of cellulose).

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).

Chitin and chitosan

Shrimp Crab Squid

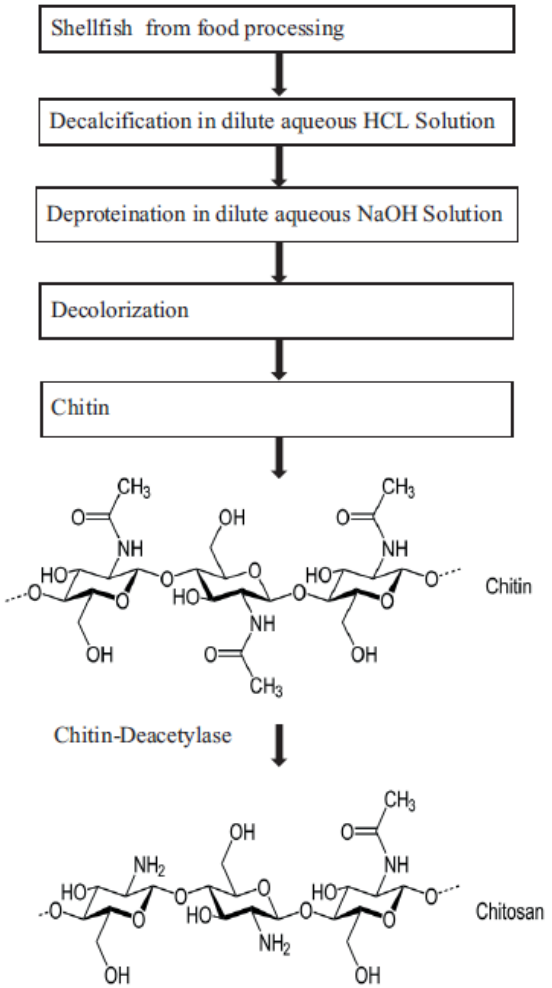
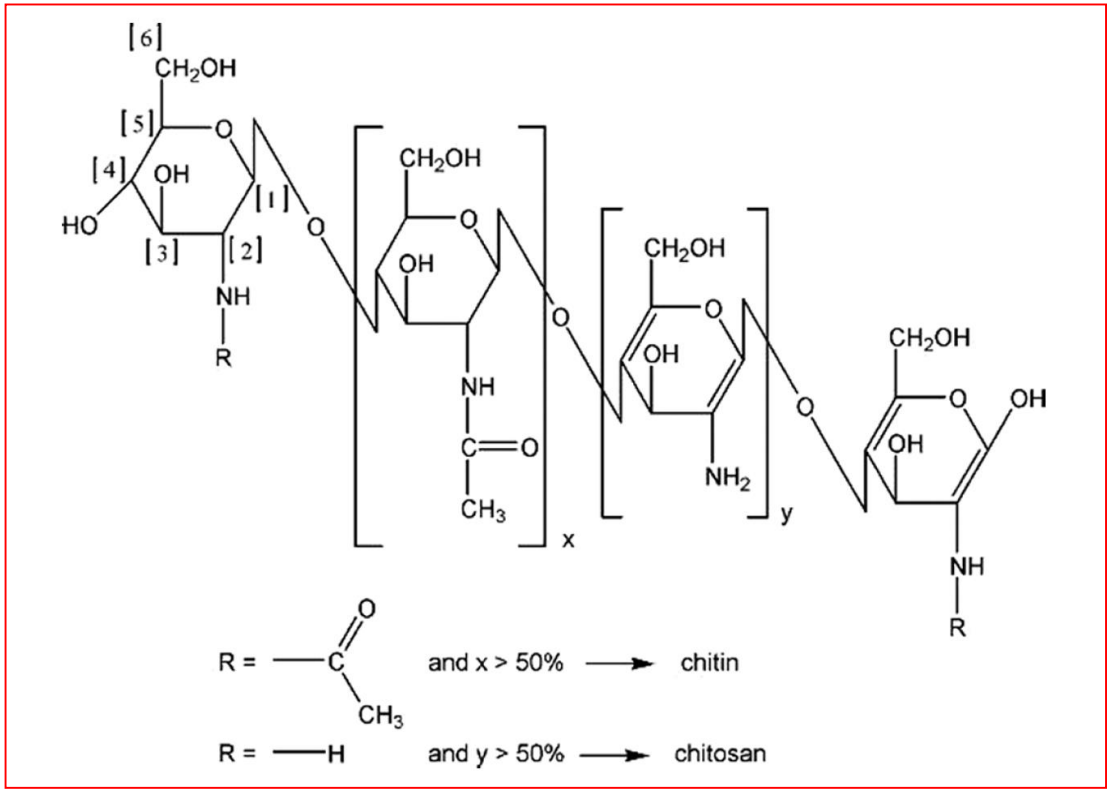


Figure 3. Chitin and chitosan processing.

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.

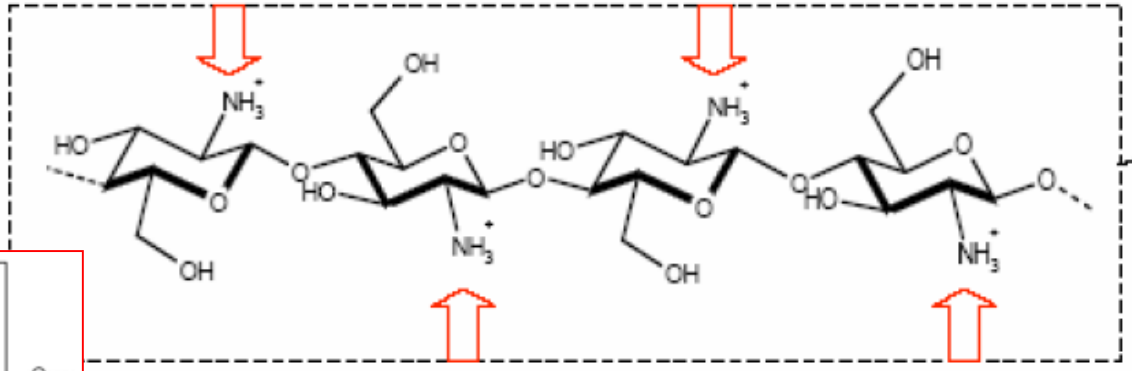


Chitosan

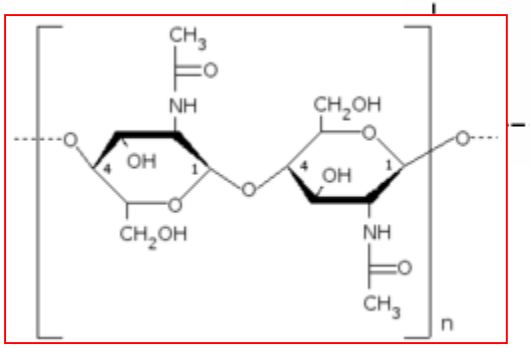
It derives from deacetilated chitin, a polysaccharide of animal origin (crustaceans' exoskeleton)

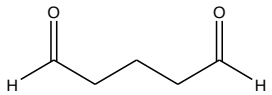
Chitosan is structurally similar to cellulose but with amino groups on C2
Monomer: 2-amino-2-deoxy-D-glucopyranose
Glycosidic bonds: (1-4)β
Chitosan is insoluble in H_2O, but more soluble in diluted acidic aqueous solutions.
Amino groups can be exploited for covalent binding via cross linking with glutaraldehyde

chitosan



chitin

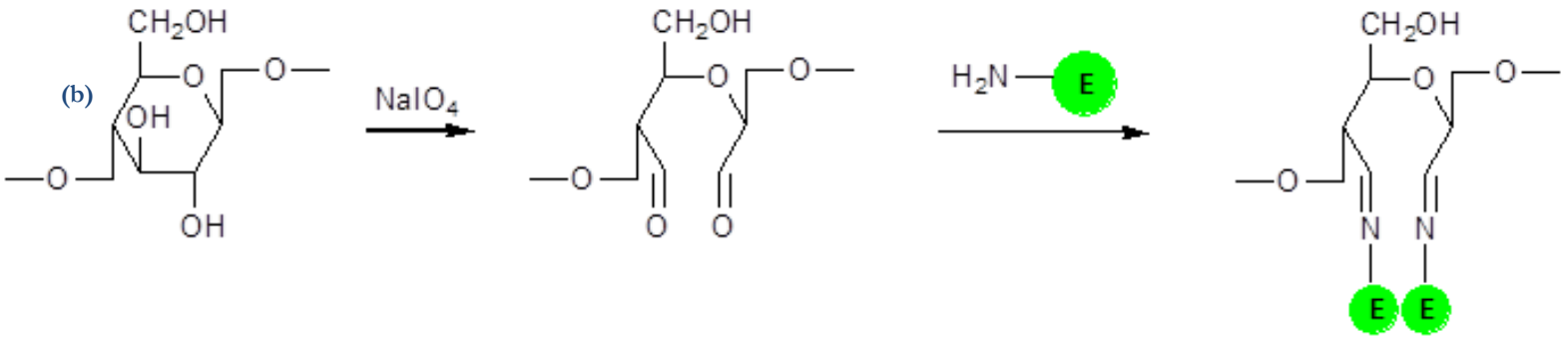




glutaraldehyde

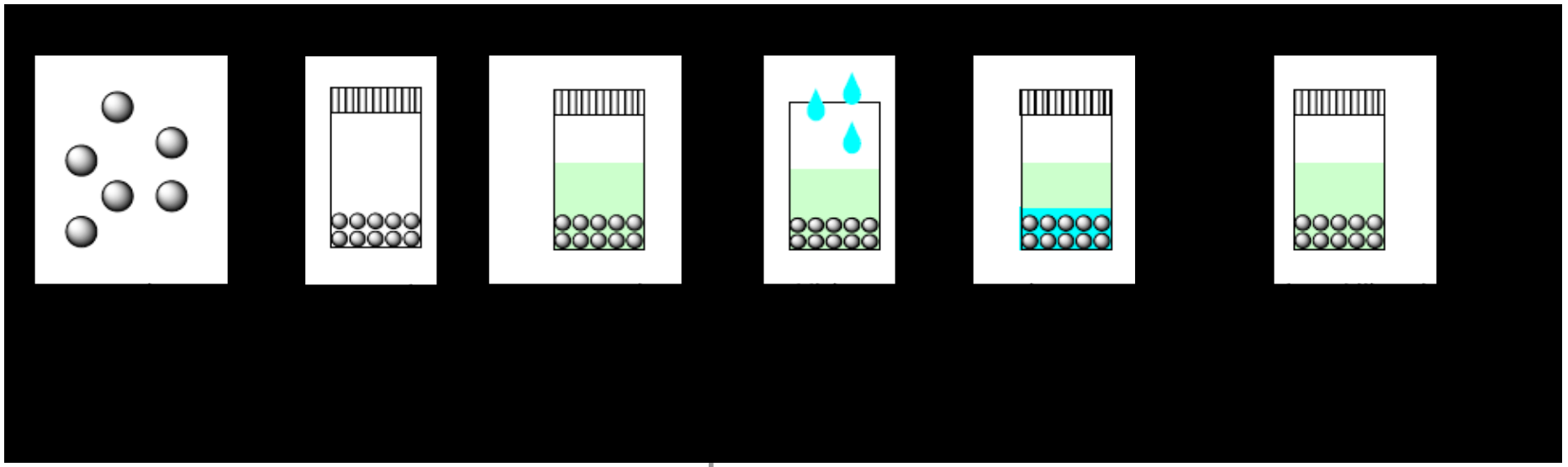
Immobilization by covalent coupling of enzyme on oxidized CELLULOSE support.

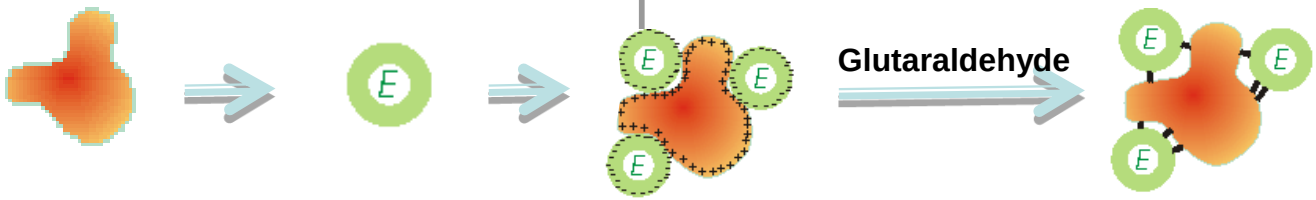
The carrier is activated by a process involving oxidation of cellulose to provide aldehyde groups, and covalent binding of enzyme molecules on aldehyde activated support.



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.

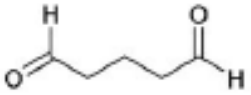
Covalent Enzyme immobilization via adsorption + crossInking





Adsorption

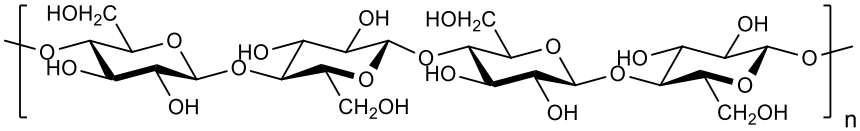
Adsorption + cross-linking



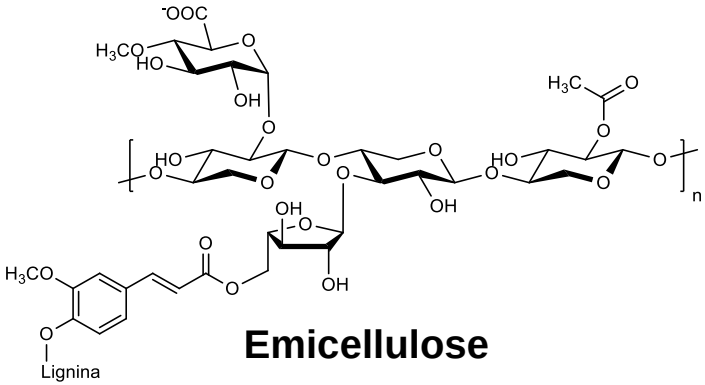
Use of a difunctional chemica reagent: imino group formation

Lignocellulosic natural biomass as carriers

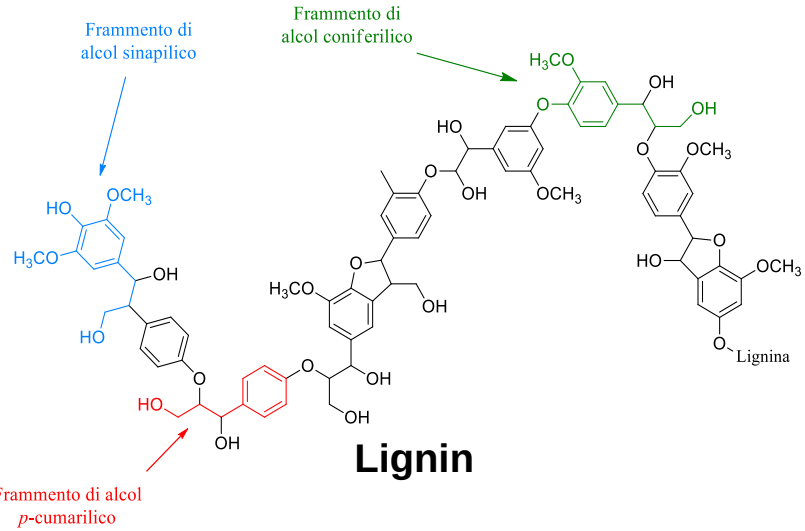
lignocellulosic



Cellulose



Emicellulose



Lignin

Cost and environmental impact of solid carriers

Organic Process Research & Development **2011**, *15*, 266–274

Guidelines and Cost Analysis for Catalyst Production in Biocatalytic Processes

Pär Tufvesson,* Joana Lima-Ramos, Mathias Nordblad, and John M. Woodley

Center for Process Engineering and Technology, Department of Chemical and Biochemical Engineering, Technical University of Denmark, 2800 Lyngby, Denmark

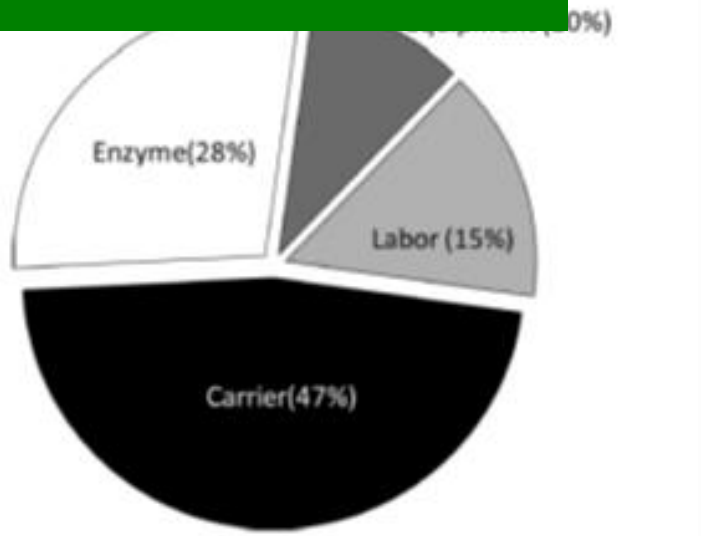
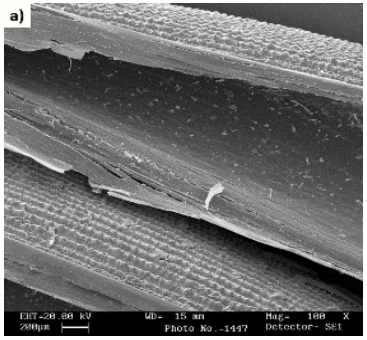


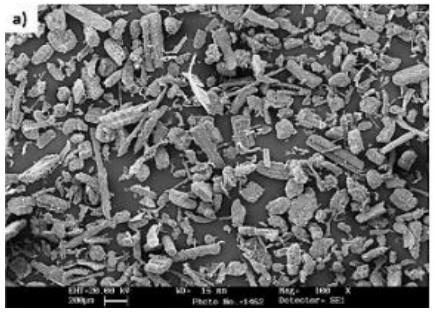
Figure shows the distribution of costs in a case study for adsorption immobilization; e.g. raw material accounts for 75% of the costs of the catalyst. With these conditions, the immobilization increases the specific enzyme cost by a factor of 4, from 500 €/kg to 2000 €/kg, although the per kilogram cost of the catalyst is of course lower, 100 €/kg of immobilized enzyme.

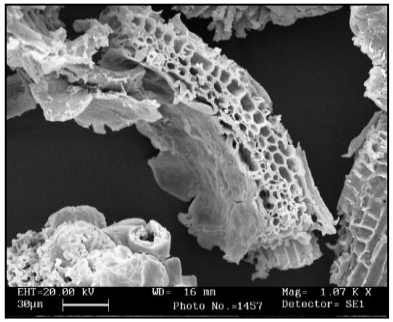
Rice husk as renewable carrier for enzyme immobilization

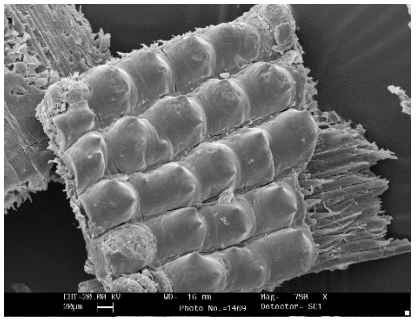
120 Mt of rice husk / y
- **Only 20 Mt used**
- **Continuous supply**

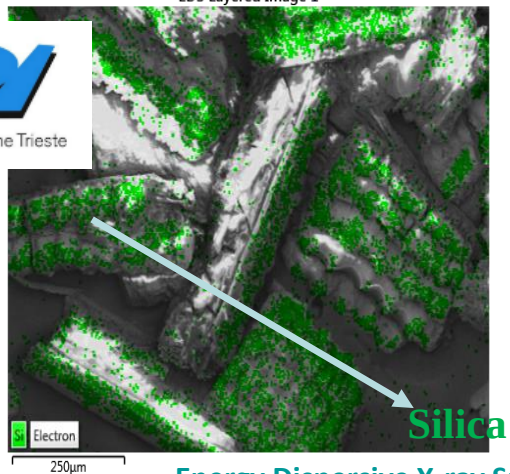
Composition	% weight
Silica	20-25
Organic	80-75
Cellulose	46.5
Lignin	31.9
Hemicellulose	22.1











Energy Dispersive X-ray Spectrometry (EDS)

L. Corici, et al *RSC Advances* 6, 63256–70,2016

Rice husk as enzyme carrier

120 M tons year , inexpensive

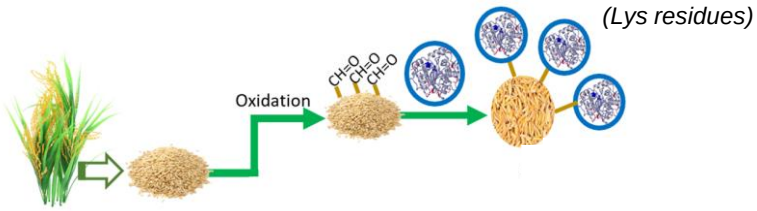
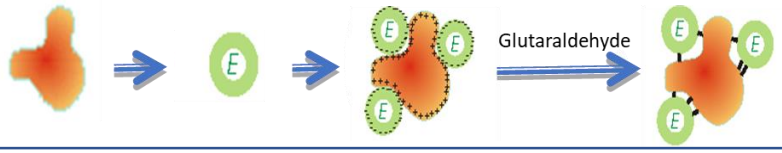
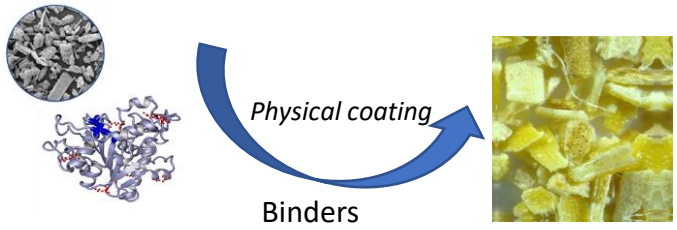
Composition

- **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**

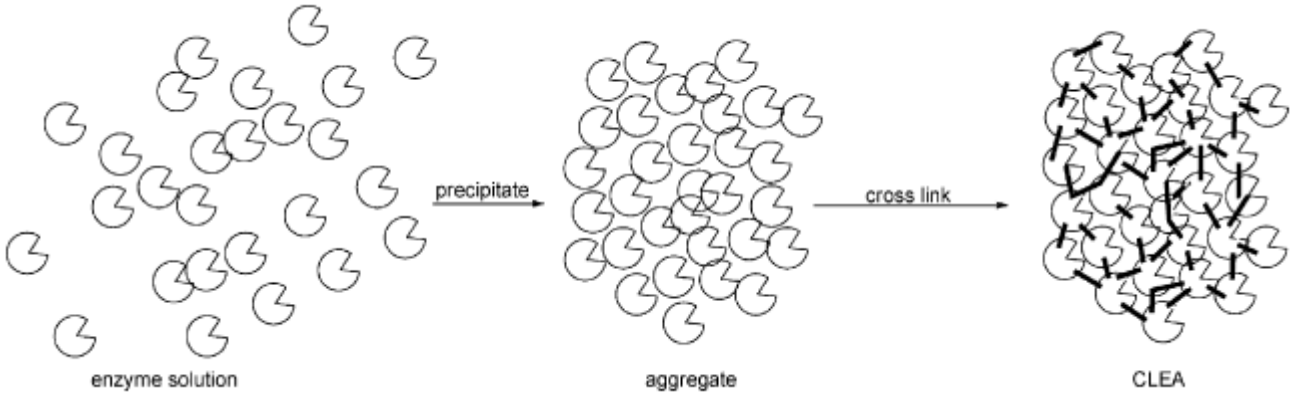
Corici, L.; Ferrario, V.; Pellis, A.; Ebert, C.; Lotteria, S.; Cantone, S.; Voinovich, D.; Gardossi, L. *RSC Advances* 2016, 6, 63256-63270.

Immobilization of enzymes on rice husk

1.Covalent bonds with functional groups on rice husk surface		Higher stability. Proteins do not detach from the carrier. Suitable for applications in aqueous and also non-aqueous solvents, as well as for viscous systems.	Functionalization increases costs and complexity in the protocols. Partial loss of enzyme activity due to covalent modifications and conformational stress.
2. Adsorption + crosslinking with a di-aldehyde		Proteins covalently bond on the carrier. Suitable for applications in aqueous and also non-aqueous solvents.	Glutaraldehyde toxicity. Covalent modifications of the protein cause some loss of activity.
3. Spray drying of aqueous solutions of enzyme + binders		Inexpensive, minimal stress applied to the enzyme.	Proteins detach from the support when exposed to aqueous media.Suitable for applications in hydrophobic solvents/media, not viscous to avoid mechanical stress.

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.



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A different immobilization technique for each system

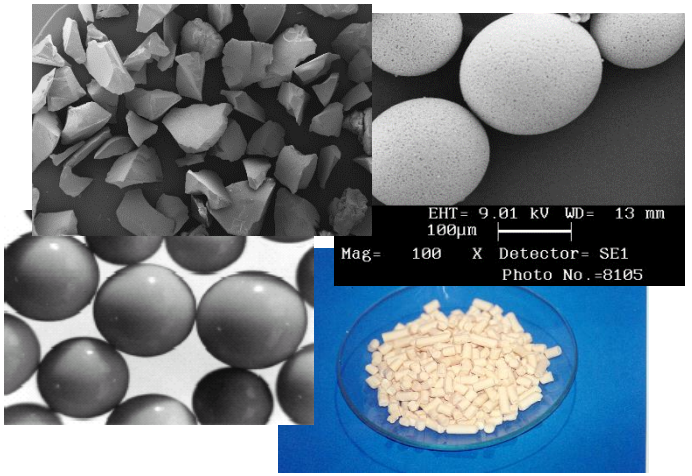
	Aqueous	Aqueous/solvent	Highly viscous	Hydrophobic solvent
Covalent	X	X	X	X
Adsorption				X
Adsorption + cross-linking	X	X	X	X

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.

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Industrial use of immobilized enzymes

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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

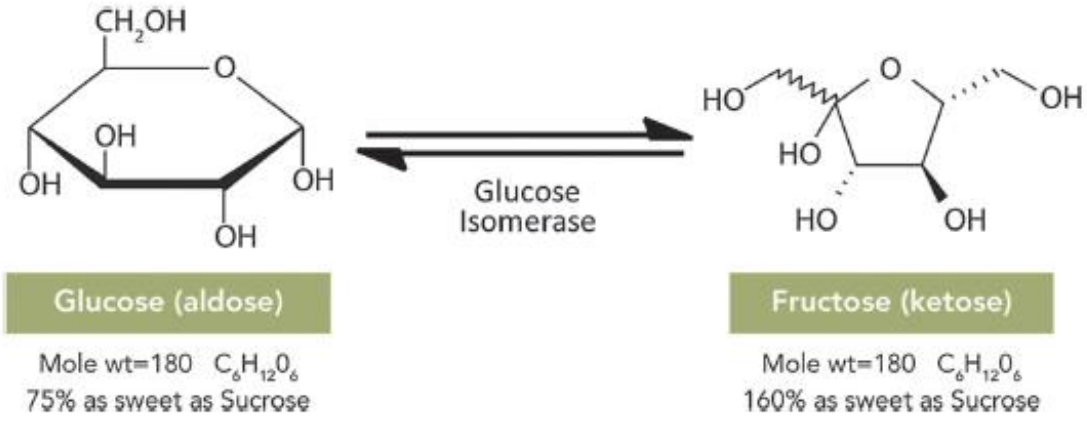
Table 2 Large scale industrial processes utilizing immobilized biocatalysts

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.

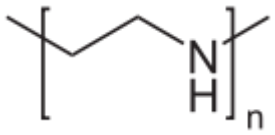
Although D-xylose is the native substrate, the enzyme has broad substrate specificity and efficiently converts D-glucose to D-fructose (Scheme 1).



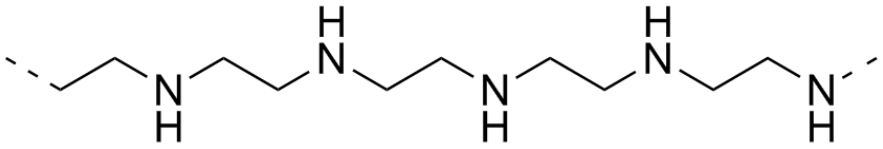
Scheme 1 Isomerization of D-glucose to D-fructose.

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

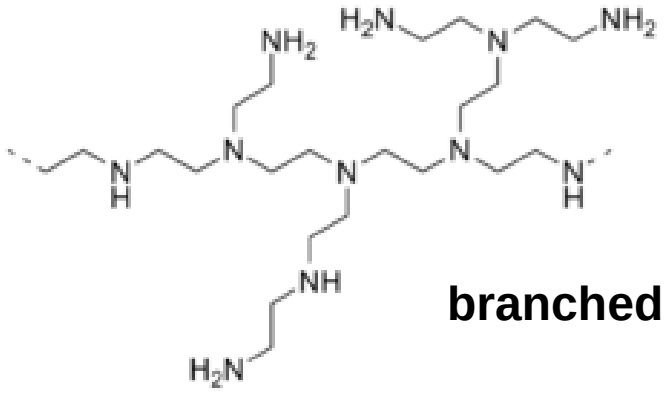
Product	Producer	GI source	Description	Currently sold?
Optisweet® 22	Miles-Kali/Solvay	*S. rubiginosus*	Adsorption of GI on to SiO_2 followed by crosslinking with glutaraldehyde	N
TakaSweet®	Miles Labs/Solvay	*Flavobacterium arborescens*	Polyamine/glutaraldehyde crosslinked cells extruded and spheronized	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



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How to describe a biocatalysed process and report a biocatalysed experiment

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

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

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- Rigorous experimental planning
- Detailed reporting of experimental conditions