Biocatalizzatori

Tecniche di immobilizzazione e applicazioni biotecnologiche di cellule ed enzimi

BIOCATALIZZATORI

Catalizzatori biologici per ottenere un prodotto di interesse mediante biotrasformazioni

Cellule in coltura

- l'organismo viene cresciuto nel terreno A
- le cellule vengono raccolte e risospese nel terreno B che contiene il substrato da trasformare
- condizioni di crescita e di biotrasformazione da ottimizzare separatamente

Enzimi purificati

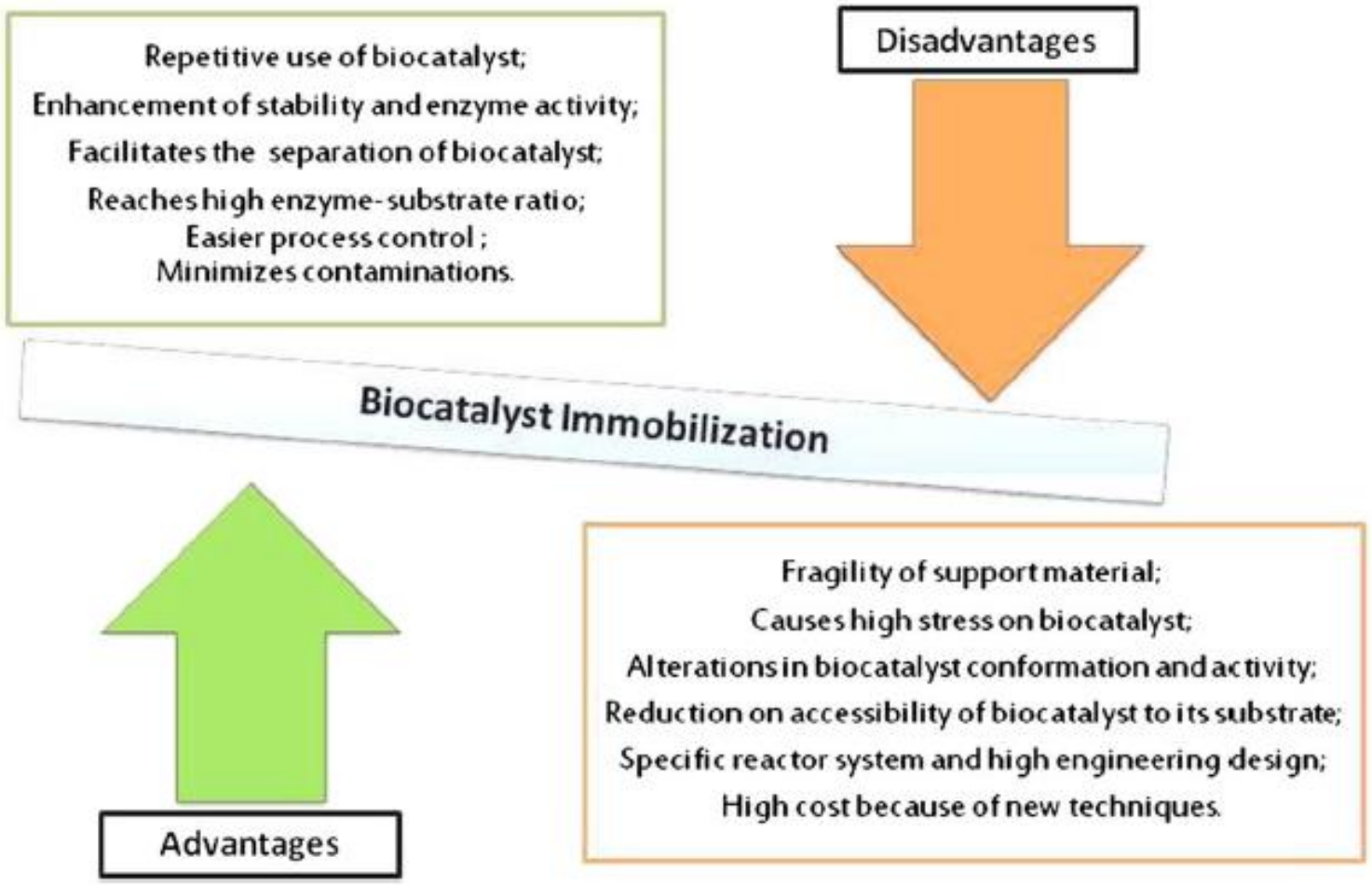
- si risolvono eventuali problemi legati alla permeabilità della membrana
- si evita la formazione di prodotti collaterali
- possibili problemi di stabilità
- è necessario purificare l'enzima!

BIOCATALIZZATORI

I biocatalizzatori si possono utilizzare in forma libera o immobilizzata

E' necessario mantenere l'attività catalitica nell'immobilizzazione

BIOCATALIZZATORI IMMOBILIZZATI VS SOLUBILI



BIOCATALIZZATORI IMMOBILIZZATI

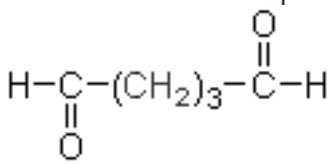
- Immobilizzazione senza supporto (carrier-free)
- Immobilizzazione su supporto (carrier)

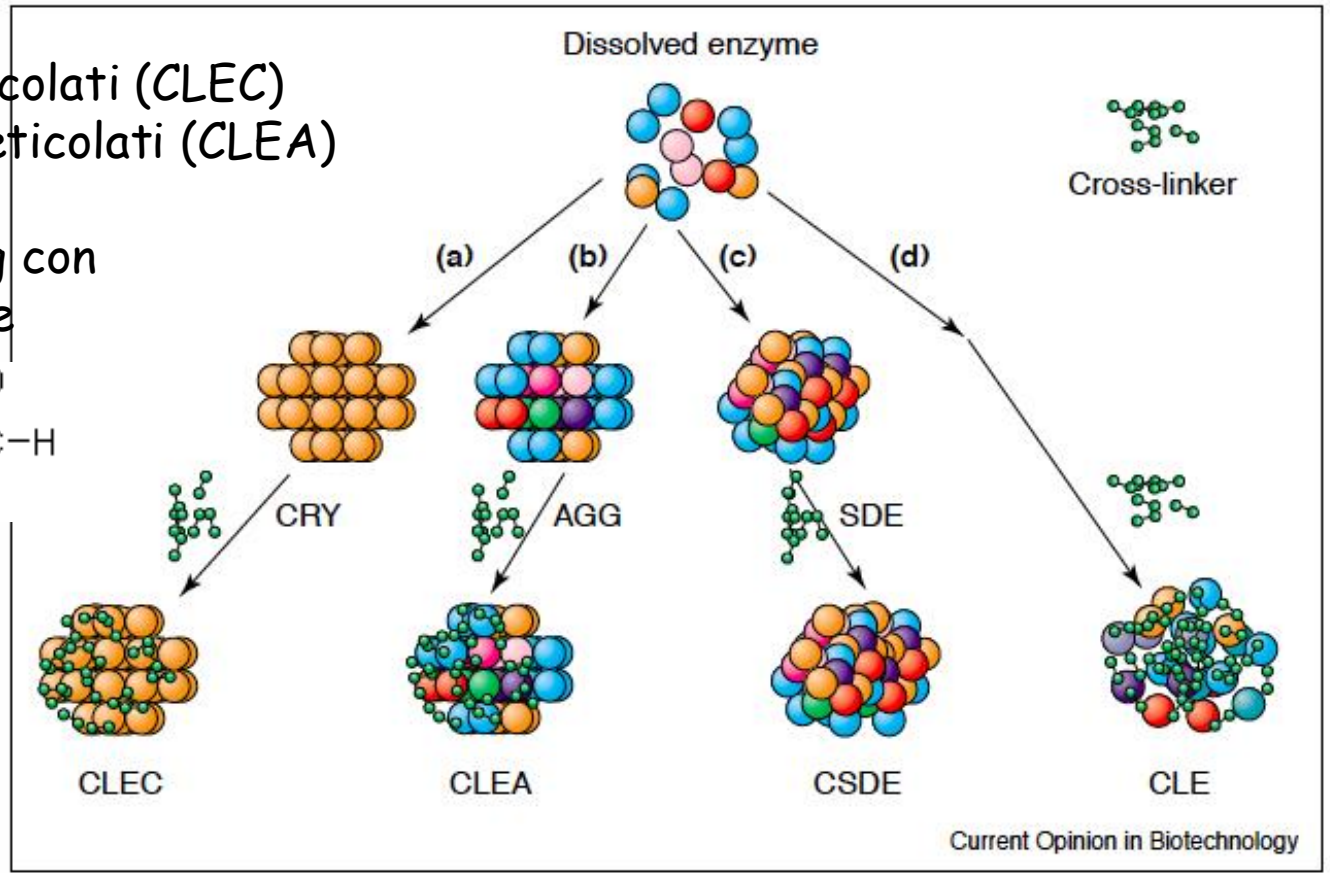
- Principali gruppi reattivi sul biocatalizzatore che possono essere usati per l'immobilizzazione con formazione di legami covalenti:
  - NH_2 (N-terminale e catena laterale di Lys)
  - COOH (C-terminale e catena laterale di Glu e Asp)
  - SH (catena laterale di Cys)

Metodi di immobilizzazione carrier-free (self-immobilizzazione)

Cristalli reticolati (CLEC)
Aggregati reticolati (CLEA)

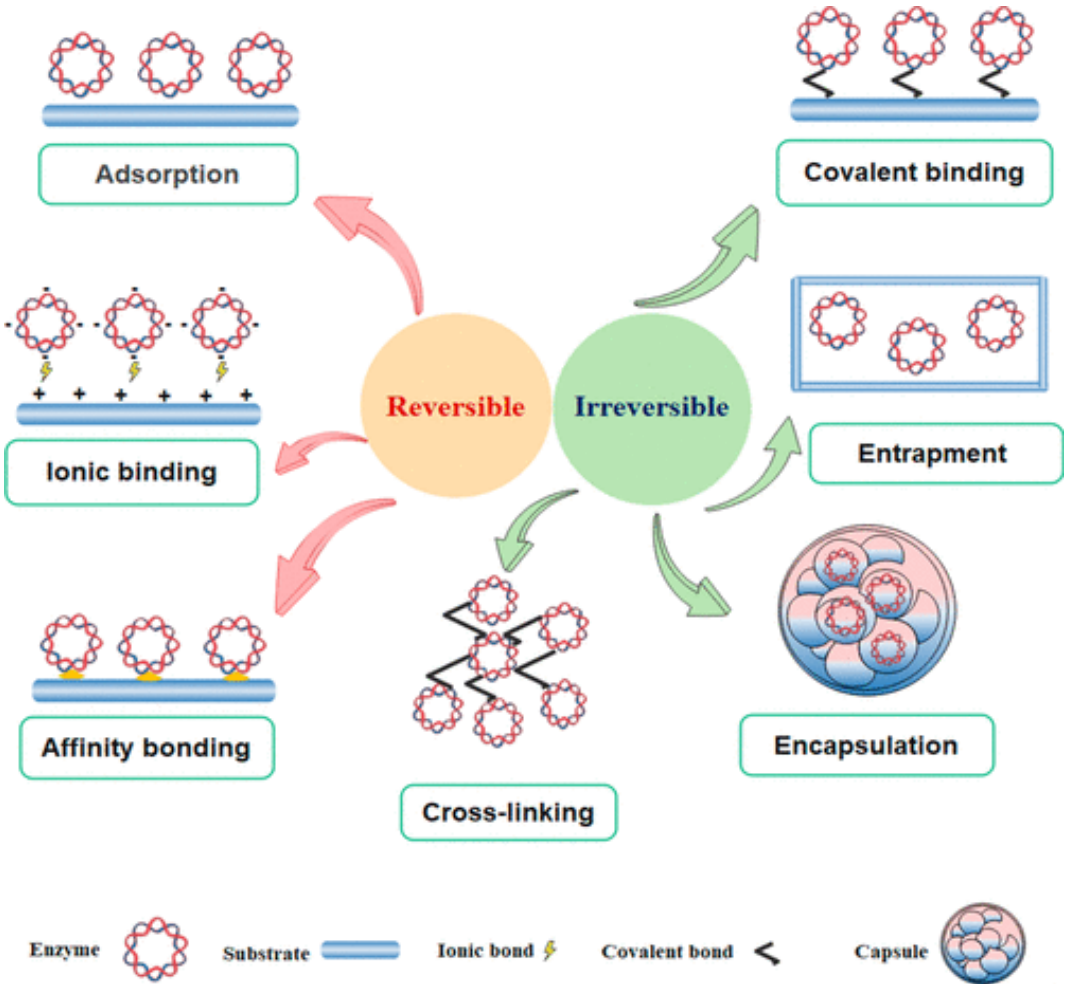
Cross-linking con glutaraldeide



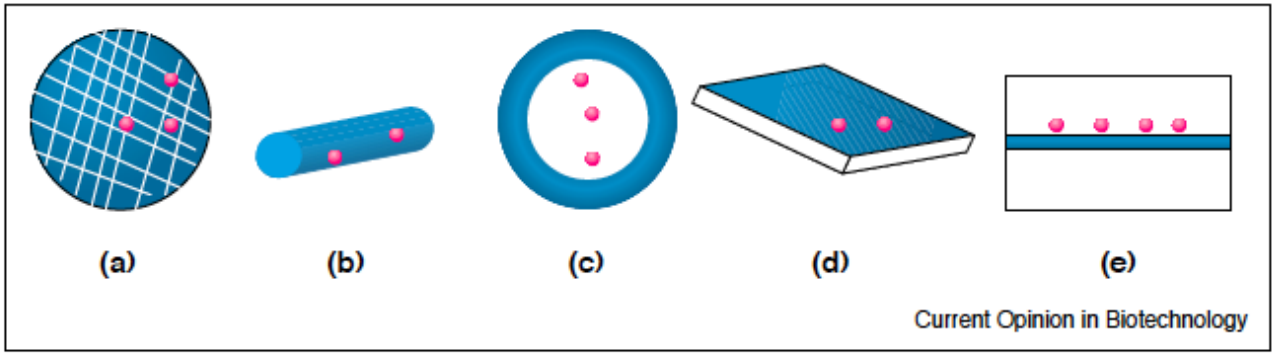


The different approaches to the production of carrier-free immobilised enzymes: **(a)** crystallization; **(b)** aggregation; **(c)** spray-drying; **(d)** direct cross-linking. AGG, aggregates; CRY, crystals; SDE, spray-dried enzyme.

Metodi di immobilizzazione di biocatalizzatori



Formati dei supporti (carrier)



Carrier-bound immobilised enzymes of defined size and shape. Insoluble carriers vary in their geometric parameters, different shapes and types of enzyme carrier are illustrated: **(a)** bead, **(b)** fibre, **(c)** capsule, **(d)** film and **(e)** membrane.

Tecniche di immobilizzazione: pregi e difetti

Adsorbimento
Intrappolamento
Incapsulamento
Legame covalente

Cross-linking

Table 1
Comparative evaluation of merits and demerits of various immobilization types.

Immobilization type	Merits	Demerits
Adsorption	✓ Easy to carry out ✓ No reagents are required ✓ No pore diffusion limitation ✓ Minimum activation steps involved ✓ Comparatively cheap method of immobilization ✓ Less disruptive to enzyme than chemical methods	× Lower efficacy level × Desorption of enzymes from the carrier
Covalent bonding	✓ Wider applicability ✓ Comparatively simple method ✓ No leakage or desorption problem ✓ A variety of support/carrier available ✓ Strong linkage of enzyme to the support ✓ Multifunctional groups availability from the support/carrier	× Competitive inhibition issues × Chemical modification of enzyme × Loss of functional conformation of enzyme
Entrapment	✓ Mild conditions are required ✓ Easy to practice at small scale ✓ Fast method of immobilization ✓ Can be used for sensing application ✓ Cheap (low cost matrixes available) ✓ Less chance of conformational changes	× Leakage of enzyme × Pore diffusion limitation × Chance of microbial contamination × Lower level of industrial implementation
Cross-linking	No matrix or support involved Comparatively simple method Widely used in industrial applications	Poly-functional reagents are required e.g. glutaraldehyde Denaturation or structural modification by cross-linker
Encapsulation	✓ Cost effective method ✓ Enzymes are stable for long time ✓ No extraction/purification steps are required ✓ "One-pot" immobilization of multiple enzymes ✓ Native conformation of enzyme is best maintained ✓ Cell organelles e.g. mitochondria can be immobilized	× Less concentration of enzymes × Generation of unwanted products × Modification of end products by other enzymes

Supporti per immobilizzazione

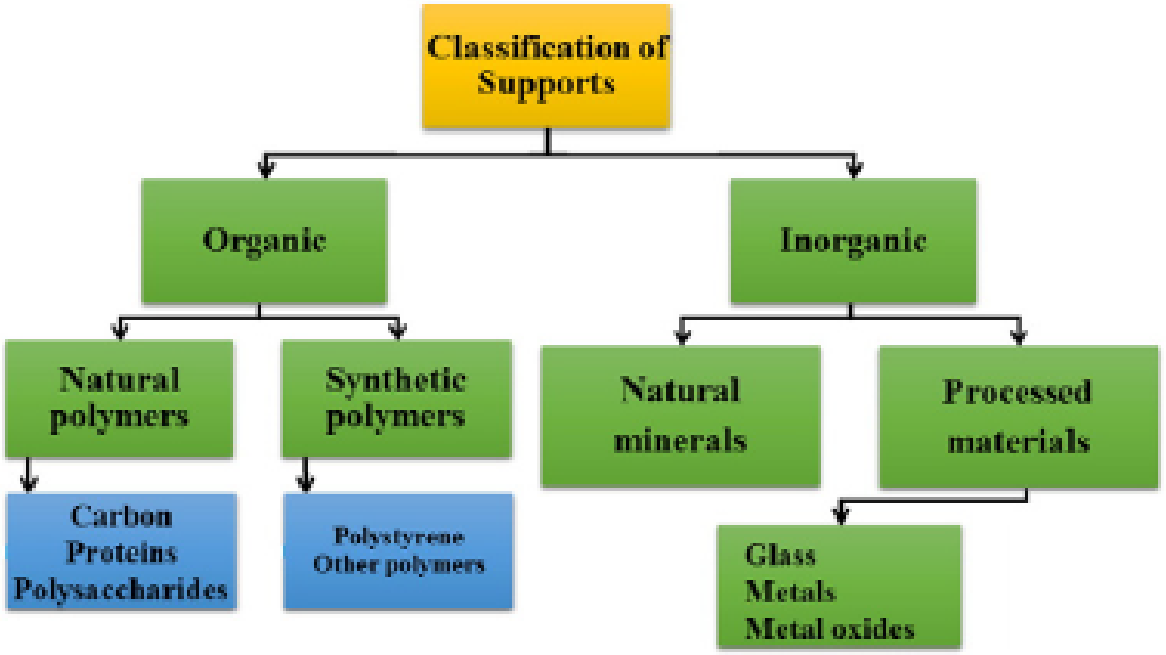


Fig. 1. A schematic illustration of the classification of support materials used for immobilization purposes.

Supporti per immobilizzazione

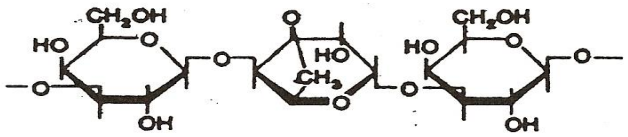
Le matrici organiche naturali o sintetiche usate per l'immobilizzazione di cellule o enzimi devono essere stabili e non reattive.
Le matrici devono essere attivate.

Table 1. Organic Supports for Cell Immobilization

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

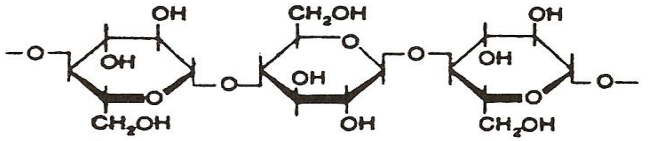
Polysaccharides and polyamides frequently serve as matrices

Agarose

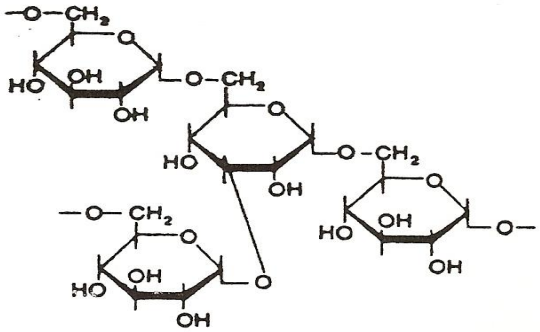


—o-galactose-3, 6-anhydro-L-galactose-o-galactose—

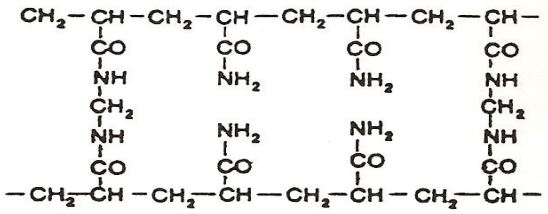
Cellulose



Crosslinked dextran (Sephadex)

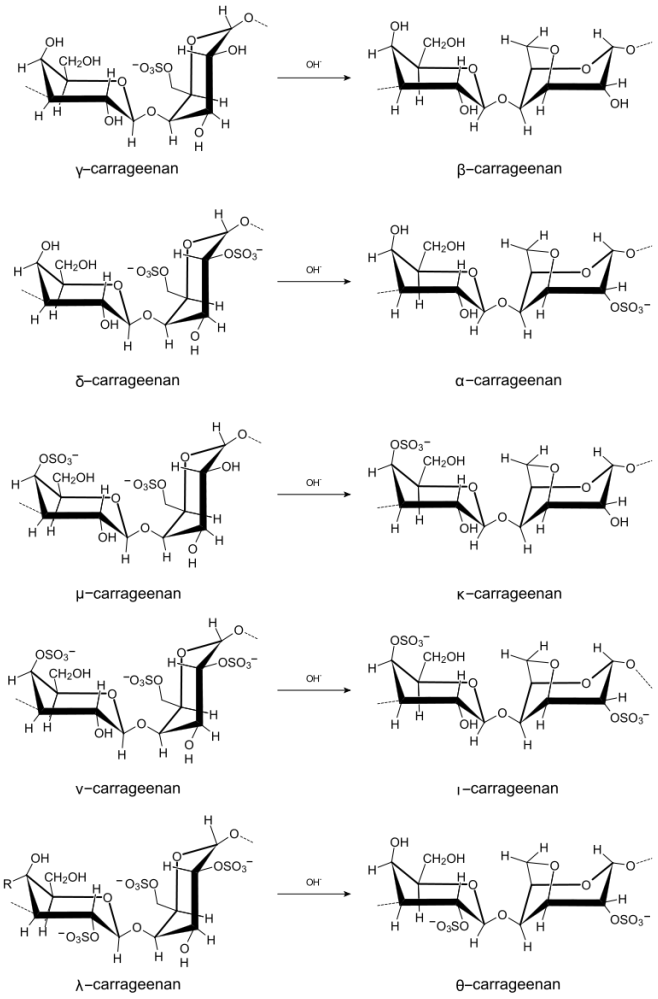


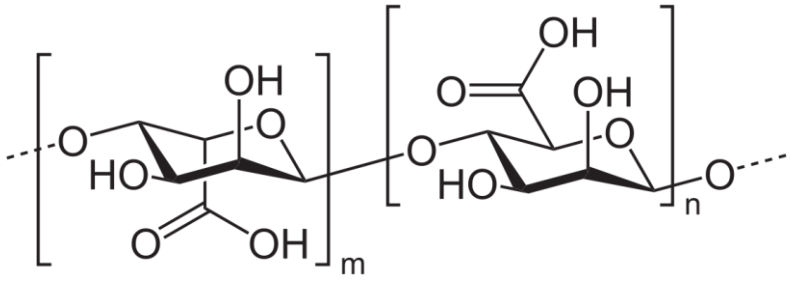
Crosslinked polyacrylamide



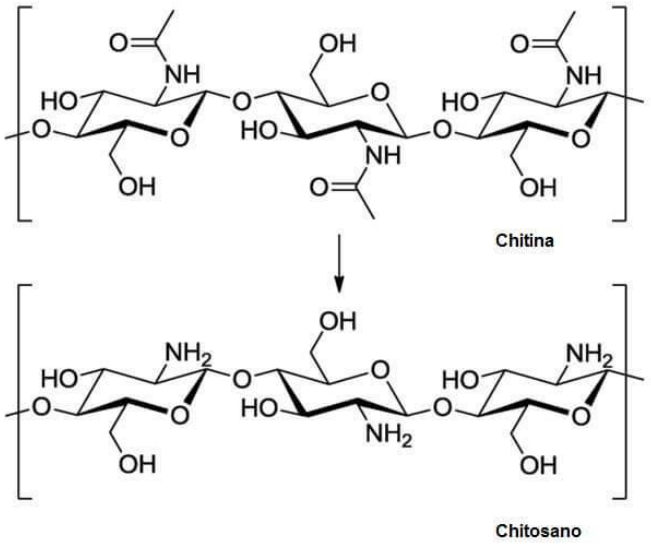
Matrici polisaccaridiche biocompatibili per intrappolamento/incapsulamento

Alginato e carrageenano dalle alghe: gelificazione mediata dal calcio

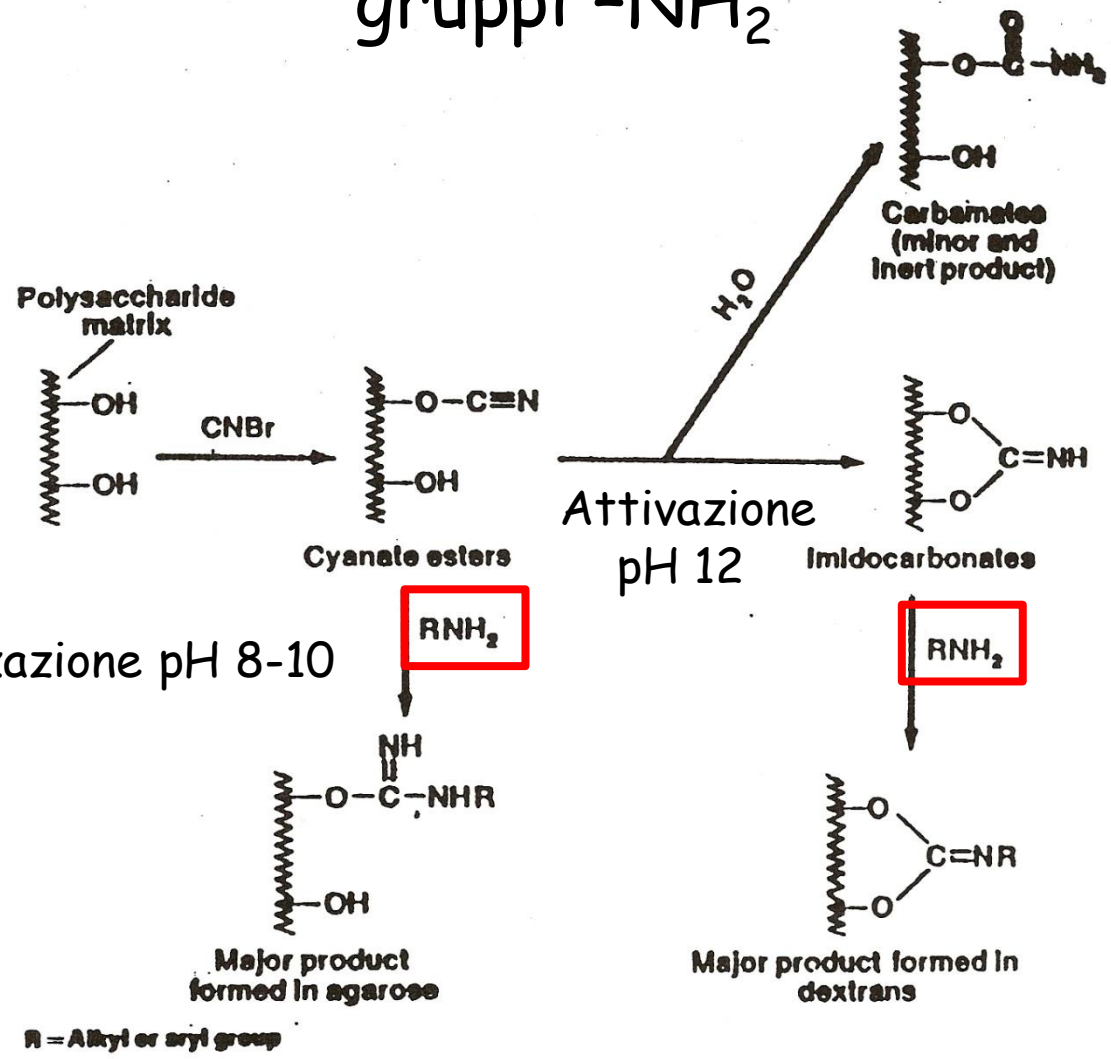




Chitosano da crostacei e funghi



Attivazione di una matrice polisaccaridica con bromuro di cianogeno (CNBr) per immobilizzare gruppi $-NH_2$



Matrici derivatizzate e attivate per reagire con diversi gruppi sulle proteine (-NH_2, -COOH, -SH, aminoacidi aromatici ecc.)

La presenza di un braccio spaziatore sulla matrice riduce problemi legati all'ingombro sterico e all'accessibilità del sito attivo dell'enzima immobilizzato

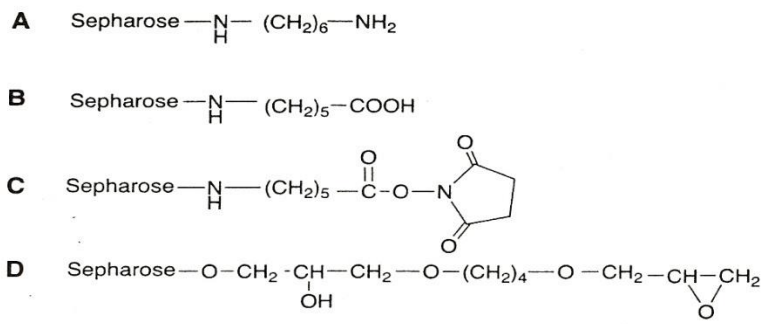


Figura 4.9 Strutture parziali di (A) AH-Sepharose, (B) CH-Sepharose 4B, (C) CH-Sepharose 4B attivato e (D) Sepharose 6B epossi-attivato. Gentile concessione di Pharmacia.

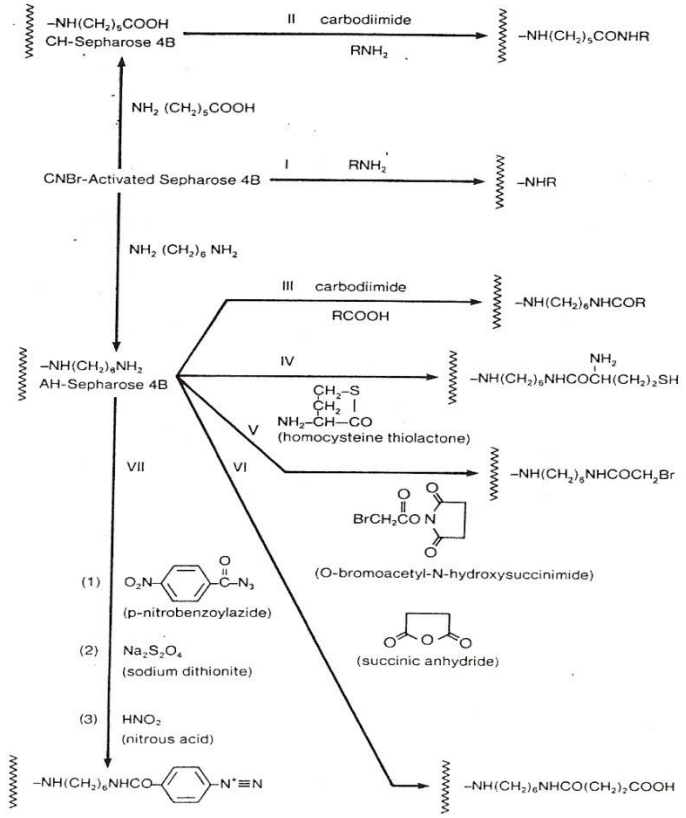
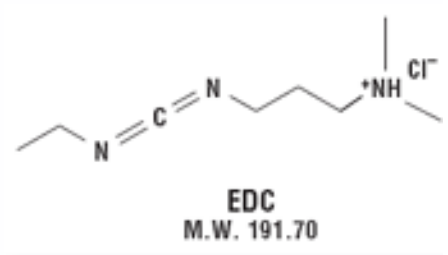


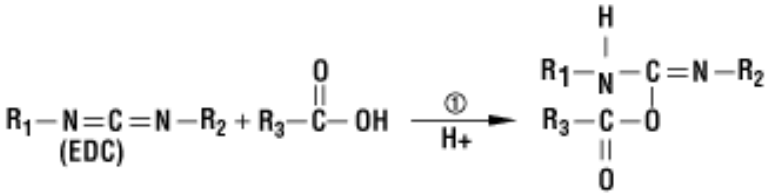
Fig. 3.6. Reactions used to couple ligands to Sepharose.

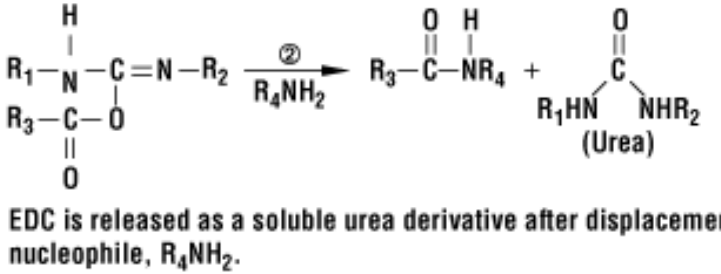
Le carbodiimidi: cross-linker specifici per gruppi carbossilici/amminici

EDC

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride



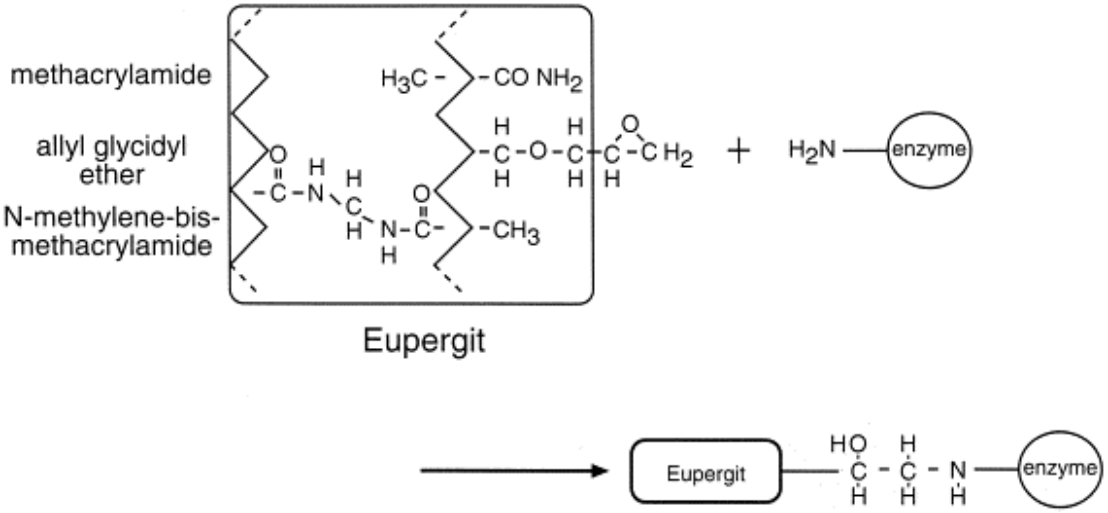




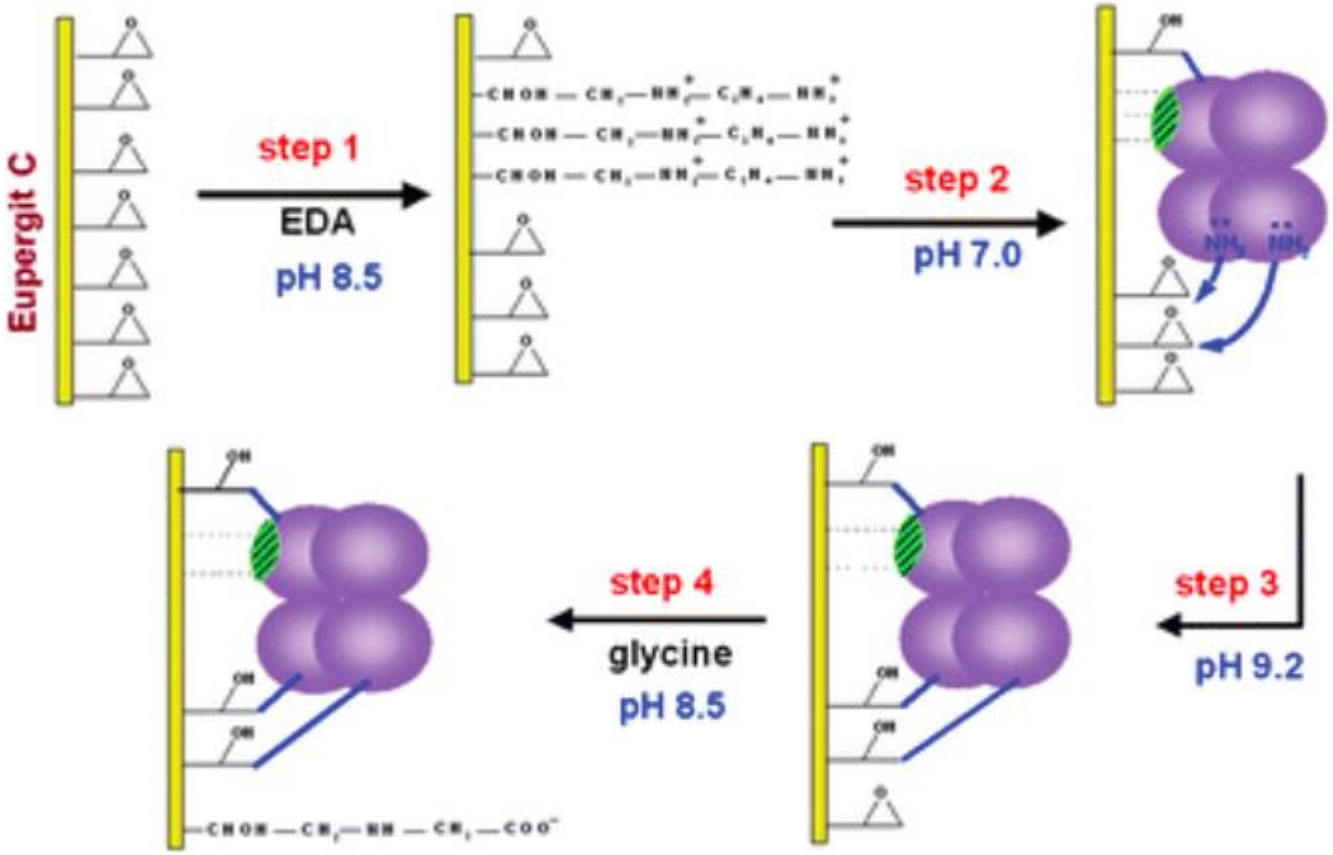
EDC reagisce con un gruppo carbossilico sulla molecola 1 formando un intermedio *O*-acylisourea. Questo intermedio può reagire con un gruppo amminico sulla molecola 2, formando un legame covalente tra le due molecole.

Eupergit C

Sfere macroporose di co-polimeri derivati dall'acrylamide, attivata con epossidi per l'immobilizzazione covalente mediante reazione preferenziale con gruppi amminici
Pori r = 100 nm
Oxirane density 300 μmol/g dry beads



Multi-point attachment su Eupergit C



Bioreattori

- Supporti polimerici: particelle, membrane e nanofibre
- Packed-bed reactors (biocatalizzatore 'impaccato' su colonna)
- Fluidized-bed reactors (biocatalizzatore mantenuto 'in movimento' da un flusso continuo di substrato)
- Continuous flow stirred reactors (biocatalizzatore mescolato con il substrato a flusso continuo)
- Membrane reactors (biocatalizzatore 'separato' da una membrana)

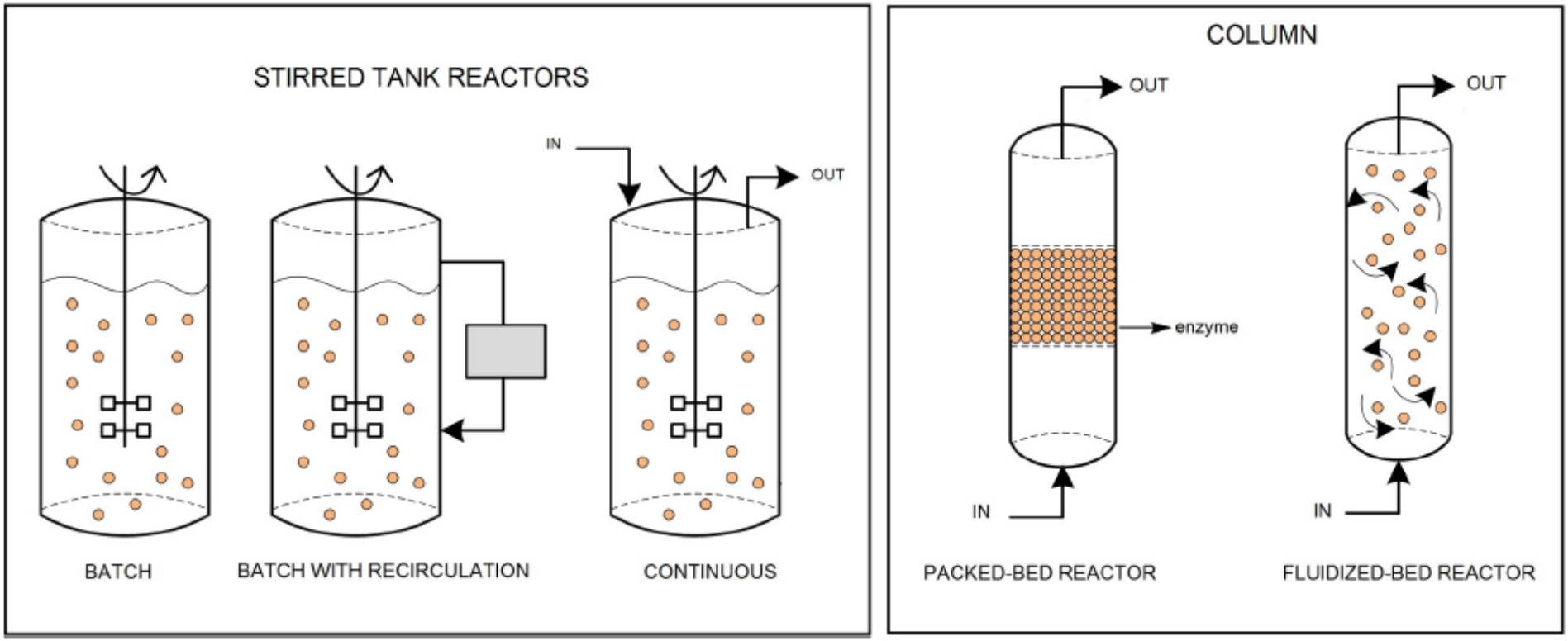


Fig. 3. Schematic representation of the main types of reactors.

Comprehensive REVIEWS in Food Science and Food Safety WILEY

Biocatalysis in the winemaking industry: Challenges and opportunities for immobilized enzymes

Carminna Ottone | Oscar Romero | Carla Aburto | Andrés Illanes | Lorena Wilson

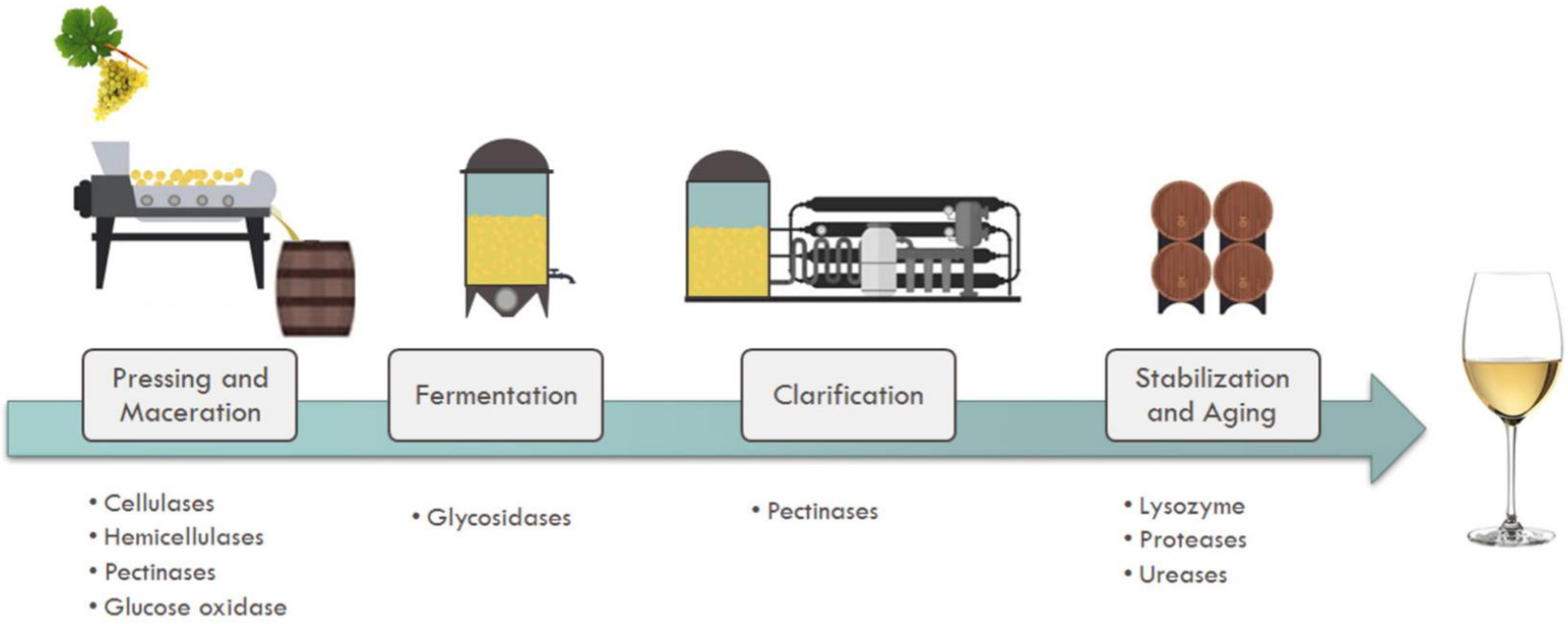


FIGURE 1 Diagram of the main steps in the winemaking process

Applicazioni biotecnologiche di enzimi immobilizzati per la produzione di aromi nel vino

Molti componenti degli aromi del vino sono costituiti da un terpene volatile legato a un residuo di glucosio a sua volta legato ad un altro zucchero (arabinosio, ramnosio o apiosio). Il terpene viene liberato dall'azione sequenziale di glicosidasi specifiche.

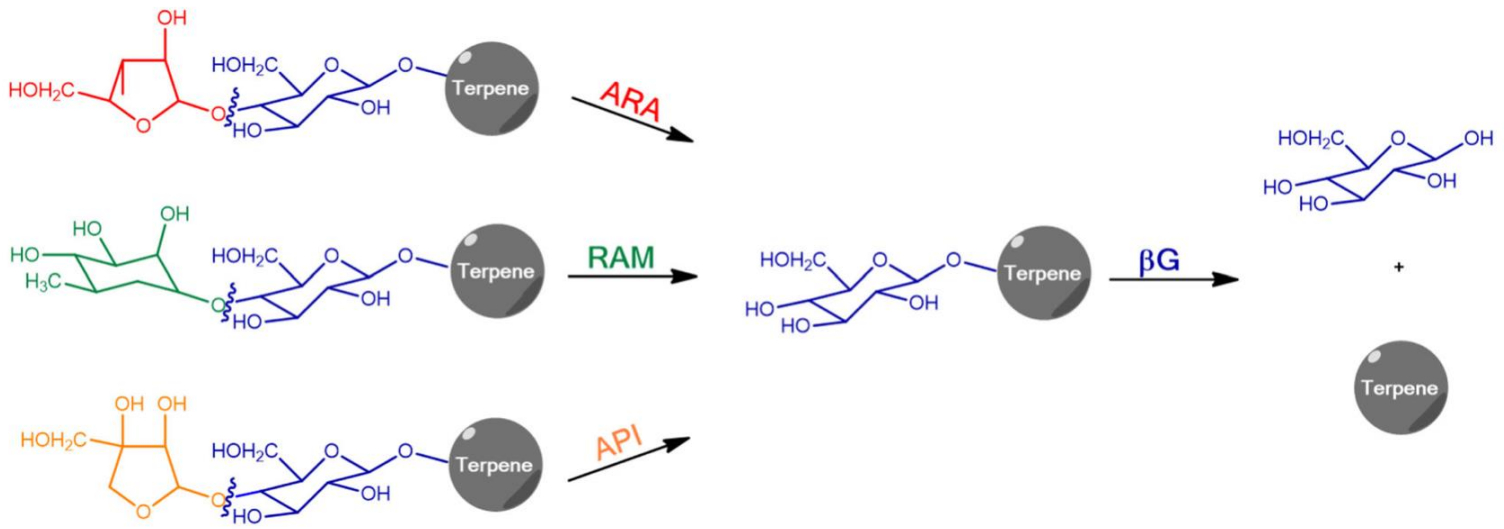
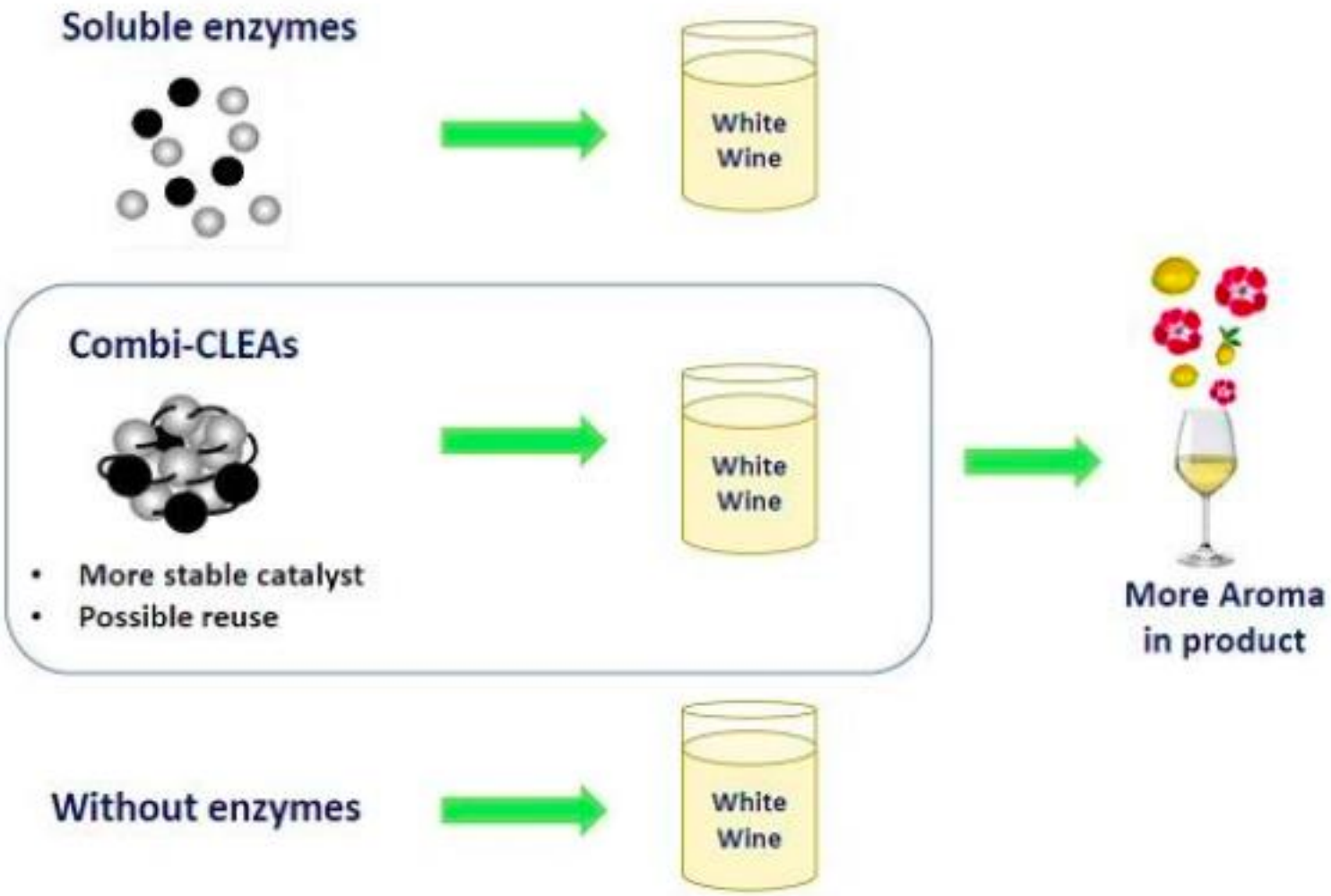


FIGURE 2 Scheme of the cascade reaction mechanism for the release of the glycosylated precursor molecules catalyzed by four different glycosidases: α-L-arabinofuranosidase (ARA), α-L-rhamnopyranosidase (RAM) and β-D-apiofuranosidase (API), and β-D-glucopyranosidase (βG). Modified from (Ahumada et al., 2016)

β-glycosidases for aroma liberation



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Aroma enhancement in wines using co-immobilized *Aspergillus niger* glycosidases

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ABSTRACT

A major fraction of monoterpenes and norisoprenoids in young wines is conjugated to sugars representing a significant reservoir of aromatic precursors. To promote their release, β-glucosidase, α-arabinosidase, and α-rhamnosidase from a commercial *Aspergillus niger* preparation, were immobilized onto acrylic beads. The aim of this work was the development and application of an immobilized biocatalyst, due to the well-known advantages over soluble enzyme preparations: control of the reaction progress and preparation of enzyme-free products. In addition, the obtained derivative showed increased stability in simile wine conditions. After the treatment of Muscat wine with the biocatalyst for 20 days, free monoterpenes increased significantly (from 1119 to 2132 μg/L, $p < 0.01$) with respect to the control wine. Geraniol was increased 3,4-fold over its flavor thresholds, and accordingly its impact on sensorial properties was very relevant: nine of ten judges considered treated wine more intense in fruit and floral notes.

Table 1
Effect of protein load on the immobilization efficiency.

mg Applied protein/g support	Bound protein		Immobilization yield (%)			Immobilization efficiency (%)		
	mg/g	%	BG	Ara	Rha	BG	Ara	Rha
35	22 ± 2.1	63 ± 6.0	68 ± 7.0	85 ± 8.5	77 ± 7.5	83 ± 8.4	91 ± 9.0	100 ± 9.0
70	42 ± 4.6	60 ± 6.2	70 ± 7.1	65 ± 6.9	75 ± 7.5	89 ± 8.5	92 ± 9.0	88 ± 9.0
145	48 ± 5.1	33 ± 4.1	33 ± 3.8	43 ± 5.5	49 ± 5.2	76 ± 7.8	55 ± 6.3	80 ± 8.5

Mean ± standard deviation (S.D.).

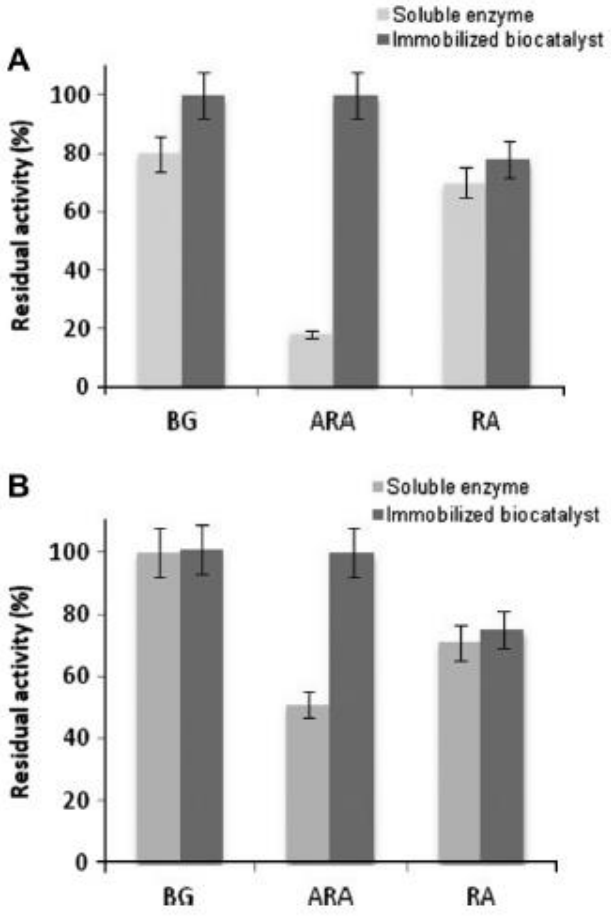


Fig. 4. Stability of glycosidases in model wine at pH 3.5 (A) and pH 4.0 (B), after 70 days of incubation at 23 °C. Model wine consisted of ethanol 12% v/v, containing 3.5 g/L tartaric acid, 2.5 g/L malic acid and 60 mg/L sodium metabisulfite.

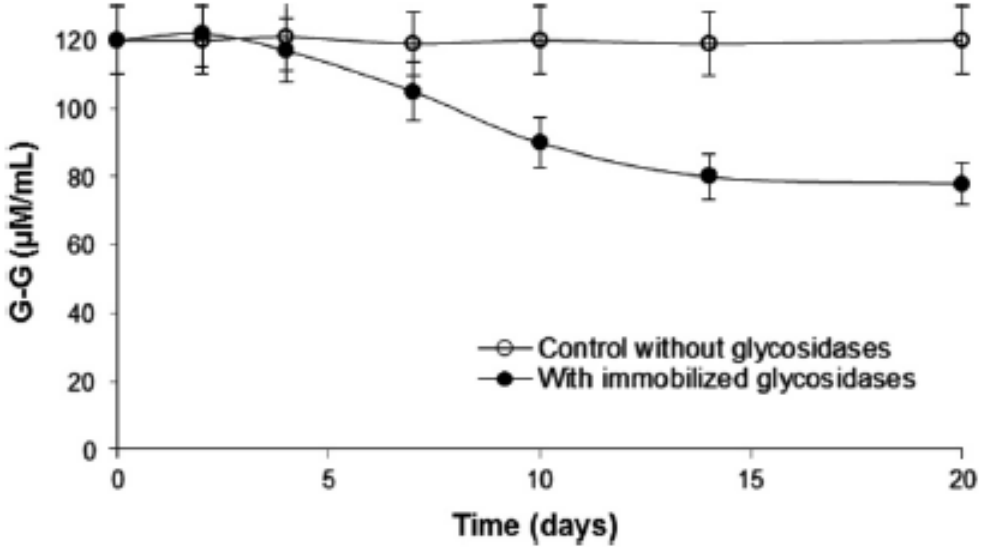


Fig. 5. Variation of glycoside content (G–G values) of Muscat wine at pH 4.0 and 23 °C, incubated with immobilized glycosidases or without glycosidases (control).

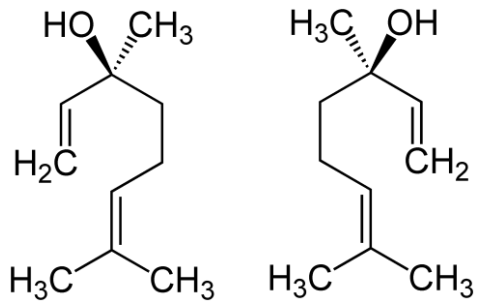
Table 2
Effect of immobilized glycosidases on the concentration of monoterpens and norisoprenoids in Muscat wine. Mean concentrations of compounds (μg/L) and relative standard deviations ($n = 3$).

Aromatic compounds	Descriptor	Odor threshold (μg/L)	Control wine without glycosidases (μg/L)	Wine treated with immobilized glycosidases (μg/L)	Significance (p value)
Linalool	Rose	50[a]	555 ± 86	615 ± 25	n.s.
α-Terpineol	Floral, pine	400[a]	182 ± 20	246 ± 17	<0.05
Geraniol	Fruit, floral	130[a]	98 ± 11	438 ± 26	<0.001
Oxide A (trans-furanic of linalool)	Leafy, sweet, floral, creamy, earthy	>6000[b]	47 ± 15	213 ± 79	<0.05
Oxide B (cis-furanic of linalool)	Leafy, sweet, floral, creamy, earthy	>6000[b]	28 ± 9	100 ± 3	<0.001
Oxide C (trans-piranic of linalool)	Leafy, sweet, floral, creamy, earthy	3000-5000[b]	151 ± 53	386 ± 28	<0.01
Oxide D (cis-piranic of linalool)	Leafy, sweet, floral, creamy, earthy	3000-5000[b]	59 ± 12	135 ± 56	n.s.
Total terpenes			1119 ± 182	2132 ± 211	<0.01
Vomifoliol	Dried fruit, raisins	_	nd	20 ± 3	<0.001
3-Oxo-α-ionol	Honey, apricots	_	nd	7 ± 1	<0.001
Total norisoprenoids			nd	27 ± 3	<0.001

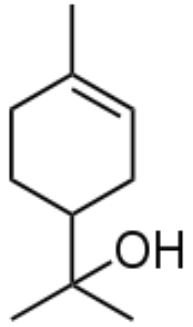
nd: Below the limit of detection, ns: not significant.

[a] Riberau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, & D. (1998). Handbook of Enology, vol. 2, The chemistry of wine. Stabilization and treatments (2nd ed.), Wiley.

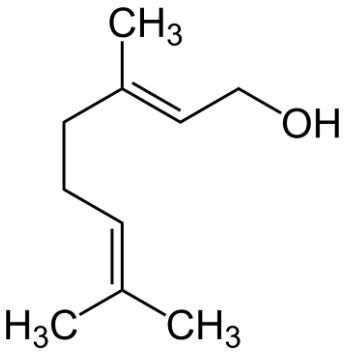
[b] Ribérau-Gayon. P., Boidron, J. N., Terrier, A. (1975) Aroma of muscat grape varieties. J Agricultural Food Chem 23, 1042–1047.



Linalool



Terpineol



Geraniol

Applicazione di enzimi immobilizzati su Eupergit C per la biotrasformazione del lattosio

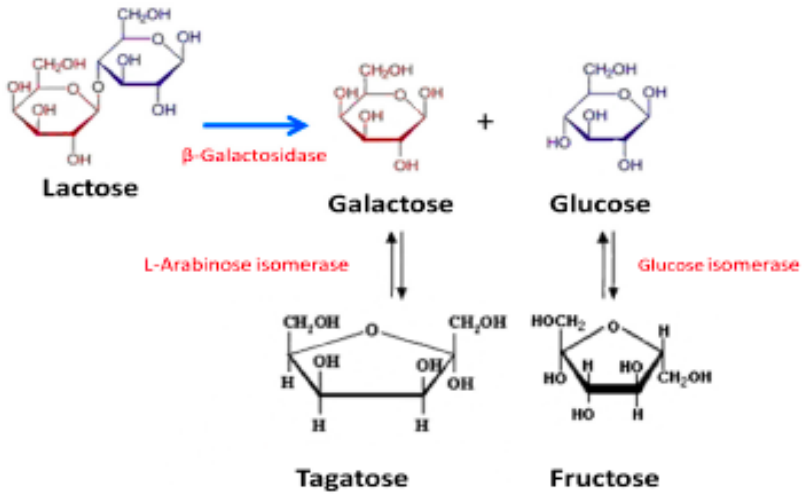


Figure 1. Diagram of reactions for enzymatic production of ketohexoses from lactose. Structures of main products of interest are depicted with enlarged sizes.

Table 5. Lactolysis and isomerization in Mozzarella cheese whey at 50 °C by tri-enzymatic systems [1].

System	Lactolysis (%)	Tagatose (%) [2]	Fructose (%) [2]
Soluble enzymes	76 ± 1	22 ± 3	21 ± 1
Immobilized derivatives (sequential use)	86 ± 1	31 ± 2	24 ± 2
Immobilized derivatives (simultaneous use)	93 ± 3	40 ± 1	29 ± 3

[1] Results are means of triplicate determinations ± SD; [2] Conversion percentages (6 h operation) according to HPLC analysis (see supporting information in supplementary materials).

Applicazione di enzimi nella degradazione dell'amido. Proprietà ed alcune applicazioni dei prodotti di idrolisi dell'amido

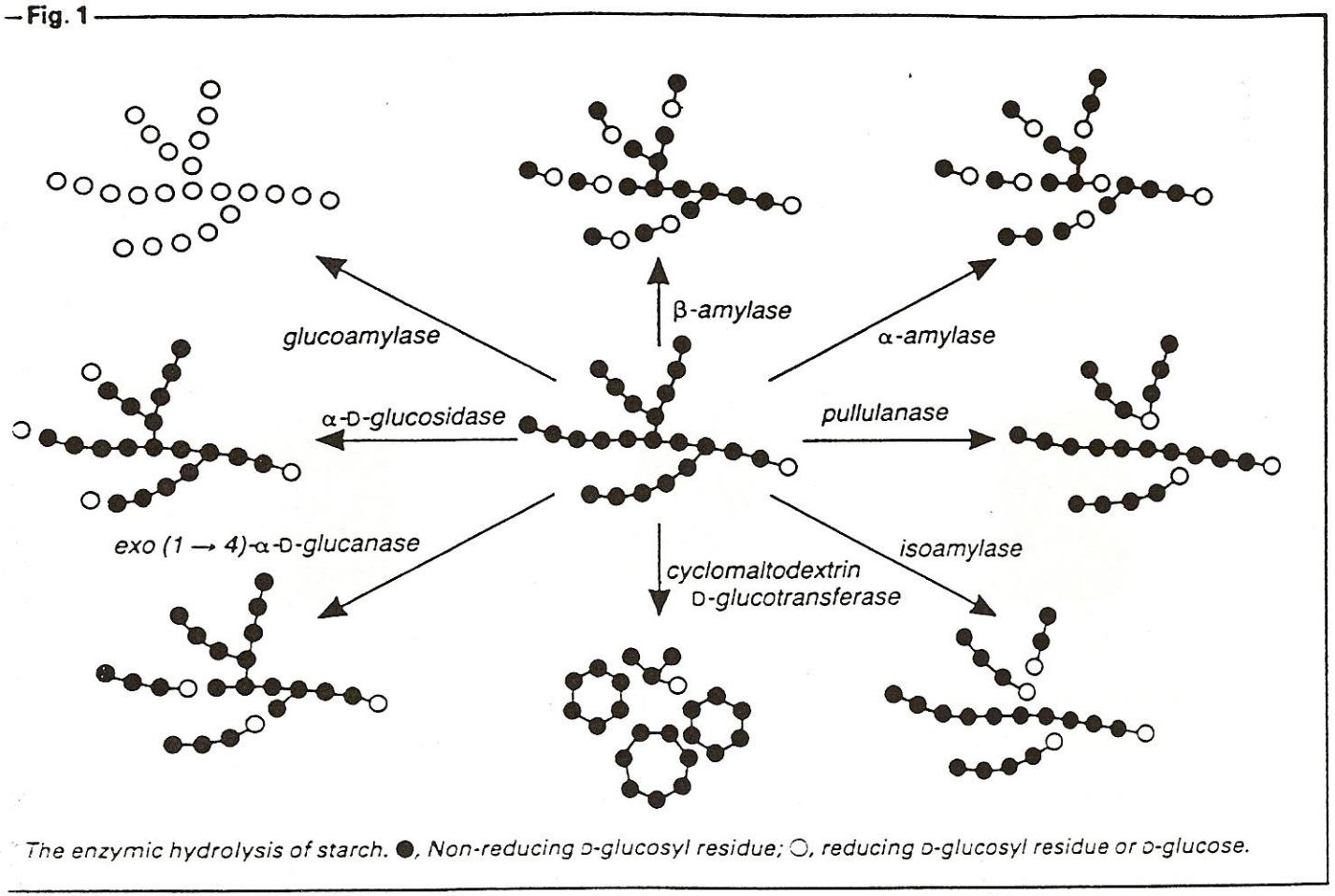
Type of syrup	DE[a]	Composition (%)
Low DE maltodextrins	15–30	1–20 D-glucose 4–13 maltose 6–22 maltotriose 50–80 higher oligomers
Maltose syrups	40–45	16–20 D-glucose 41–44 maltose 36–43 higher oligomers
High maltose syrups	48–55	2–9 D-glucose 48–55 maltose 15–16 maltotriose
High DE syrups	56–68	25–35 D-glucose 40–48 maltose
Glucose syrups	96–98	95–98 D-glucose 1–2 maltose 0.5–2 isomaltose
Fructose syrups	98	48 D-glucose 52 D-fructose

Product type	Properties	Application
Maltodextrins	Low osmolaritygelability	Carbohydrate component for baby food, thickeners, fillers, stabilizers
Maltose syrup	Increased sweetness	Hard pastry and bakery products
High sugar syrup	Low hygroscopicity and viscosity; increased water retention, sweetness and fermentation	Confectionery, soft drinks, fermentation products, jams, canned foods, sauces
Molasses for baby food	Moderate sweetness, reduced crystallisability	Sweeteners for baby food, confectionery, soft drinks, jams, jelly, canned food, ice cream
Glucose Syrups	Increased sweetness	Non-alcoholic beverages, fermentation products, raw materials
Glucose-fructose syrups	High sweetness	Soft drinks, canned food, sauces, canned fruit
Cereal syrups	Moderate sweetness, non-crystallizability, high nutritional value	Flour confectionery and bakery products

Dextrose equivalent (DE): indica il grado di idrolisi dell'amido

$$DE = \frac{M_{glucose}}{M_n} \times 100$$

Enzimi coinvolti nella degradazione dell'amido



Applicazione di enzimi nella degradazione dell'amido

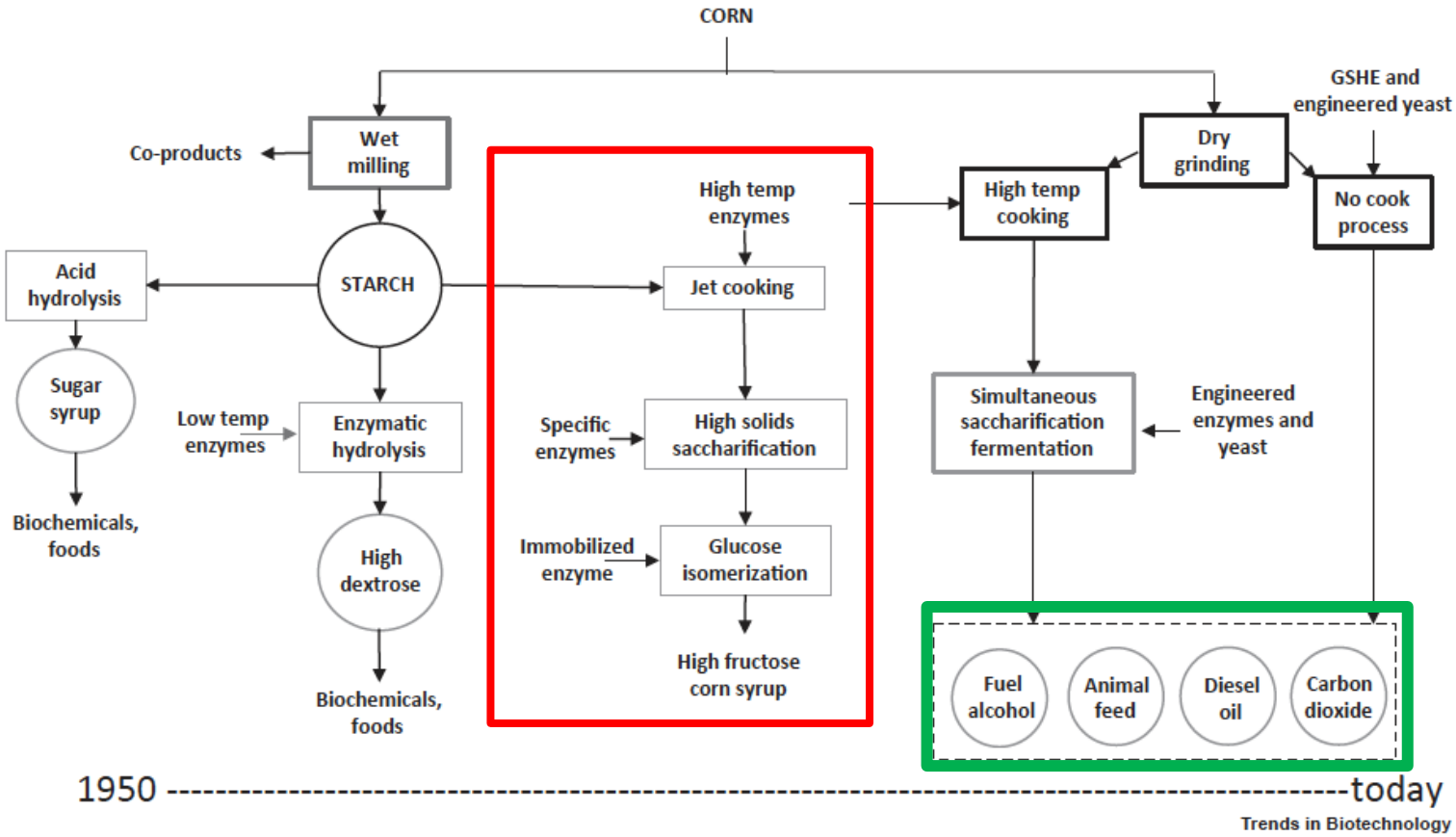


Figure 1. Enzyme Technology impact on Corn Processing from 1950 to today. Abbreviation: GSHE, granular starch hydrolyzing enzyme.

Applicazione di enzimi nella degradazione dell'amido per la produzione di sciroppo ad alto contenuto di fruttosio

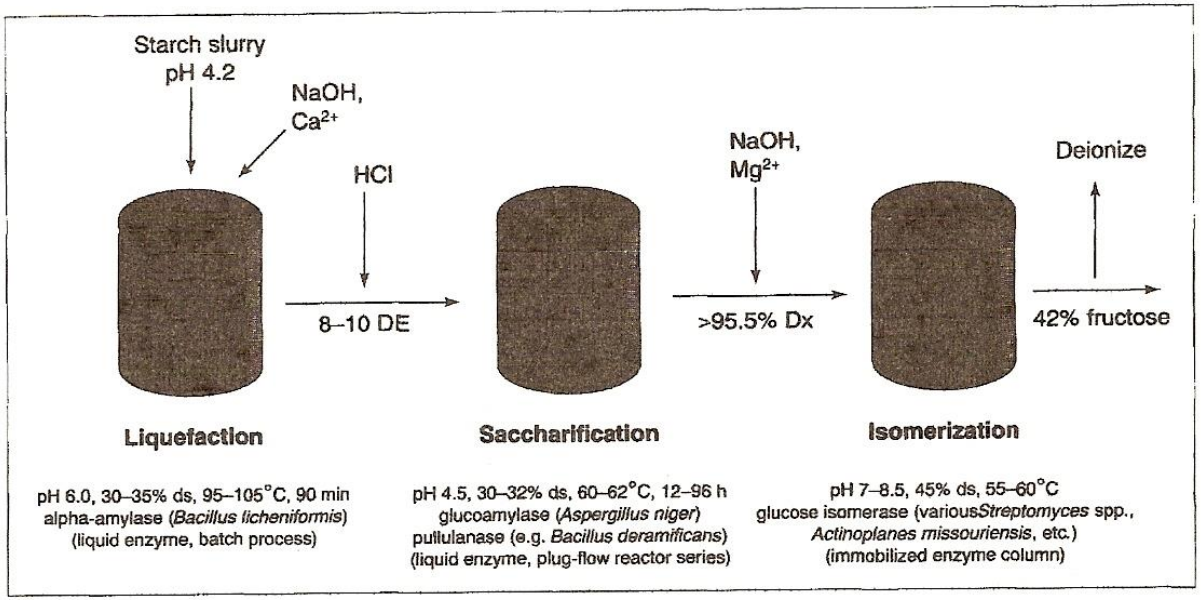


Figure 1

The starch process for high-fructose corn syrup. Schematic outline of the enzymatic steps in the processing of slurried corn starch to fructose, showing individual enzyme-usage conditions and typical processing parameters. Arrows indicate adjustment points within the process for pH and/or ion components. The process parameters may be different when producing ethanol from corn. The term 'ds' refers to the percentage of starch or glucose dry solids suspended in the slurry. DE is 'dextrose equivalent', a measure of the number of reducing ends present in a starch hydrolysate; each reducing end of an oligosaccharide is equivalent to a single dextrose residue. The greater the degree of starch liquefaction or hydrolysis, the higher the DE. Undegraded starch has a DE approaching zero; a fully hydrolysed starch would have a DE of 100. DE is related to average chain length of the oligosaccharide by the following formula: $DE = 180/(162n + 18) \times 100$, where n is the average oligosaccharide chain length. For example, a starch slurry with a DE of eight has an average chain length of ten glucose residues. The term '%Dx' is the percent of dextrose in the solution. In the example shown, after saccharification, the process stream would have 32% dry solids with greater than 95.5% dextrose (DX).

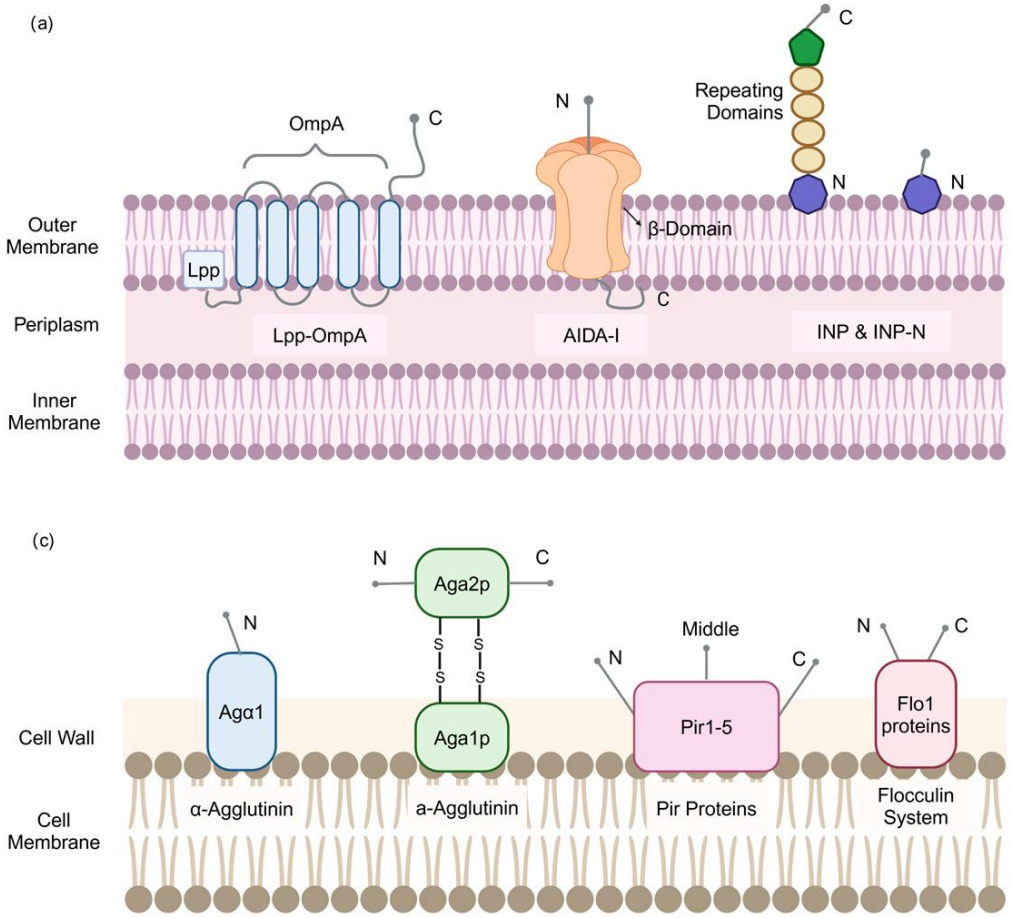
Ingegnerizzazione di enzimi per la degradazione dell'amido

Application	Enzymes altered	Value creation
Starch liquefaction	Thermostable amylase	More starch solubilized
	Low pH, thermostable amylase	Decreased chemicals needed
	Amylase without calcium needed	Less ion exchange cost
	Rapid viscosity reduction amylase	Less water, more capacity
Saccharification	Debranching specific pullulanase	Higher glucose yield
Glucose isomerization	Immobilized enzyme	Minimum fructose loss
No-cook starch process	Granular Starch Hydrolyzing Enzymes (GSHEs)	Less energy, higher yield
Starch to fermentation	Enzymes expressed in yeast	Lower cost process
	Pathway enzymes engineered	Increased ethanol, decreased glycerol, more sustainable process
	Enzyme cocktail (amylase, protease, and lipase)	Higher revenue and margin

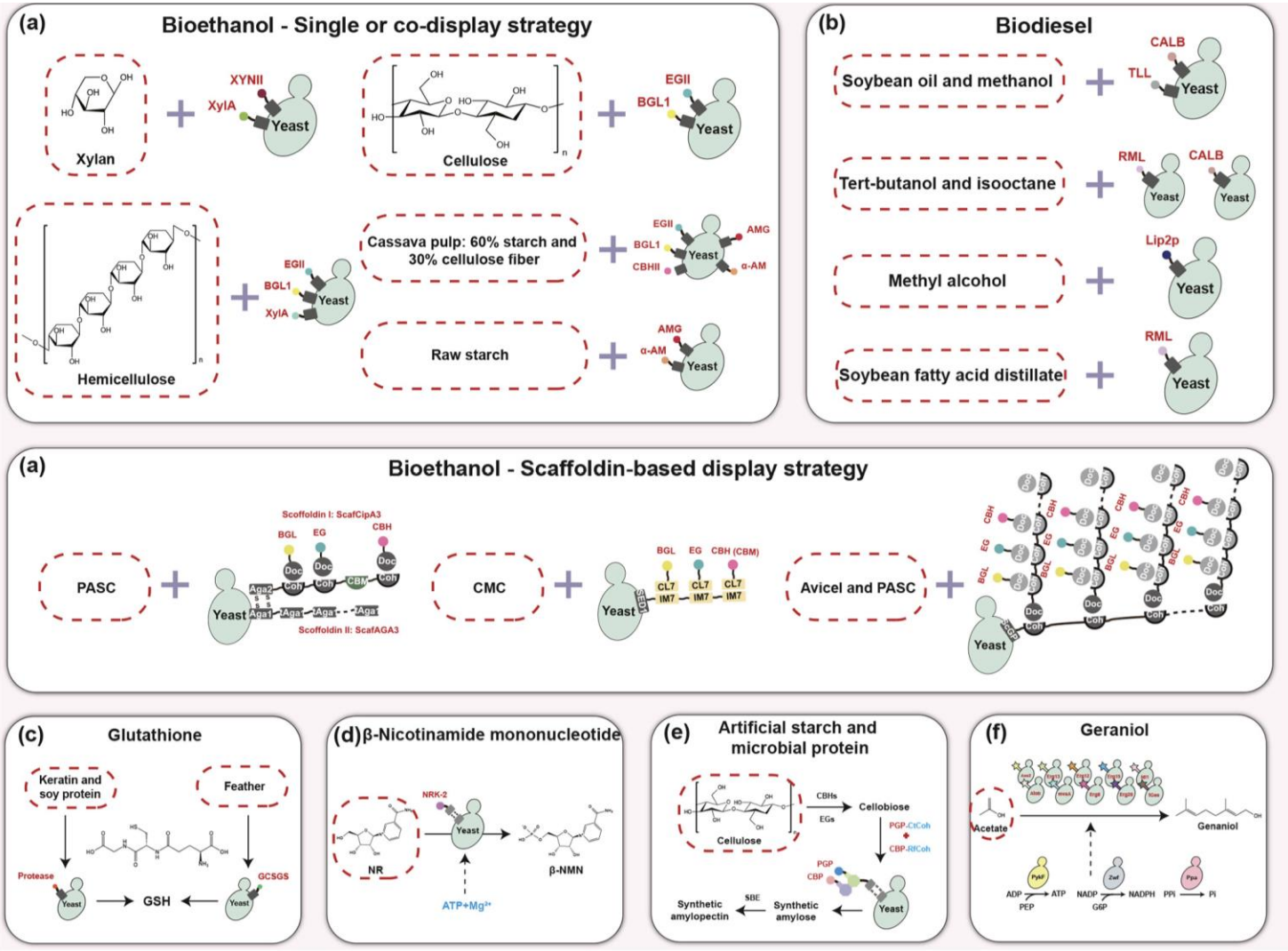
Cell surface display come strategia di immobilizzazione alternativa?

- Cosa si intende con 'cell surface display'?
  - Esposizione di una proteina ricombinante sulla superficie di una cellula
- Che cosa è richiesto per eseguire 'cell surface display'?
  - Una cellula microbica (batterio o lievito)
  - Una proteina (endogena?) per ancorare la proteina di interesse sulla superficie della cellula
  - Ingegneria proteica per progettare la proteina di fusione

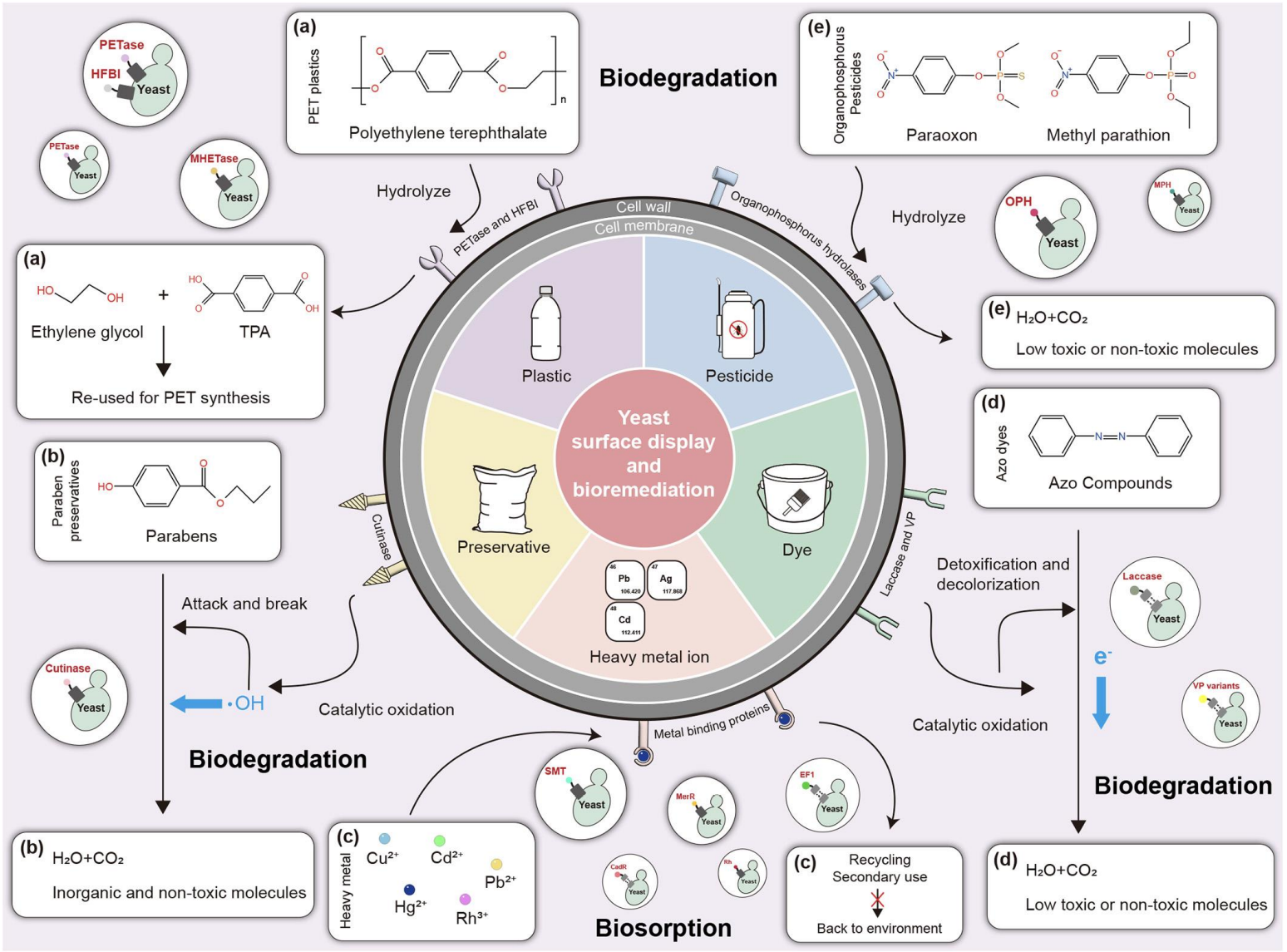
Proteine di ancoraggio per cell surface display in batteri e lieviti



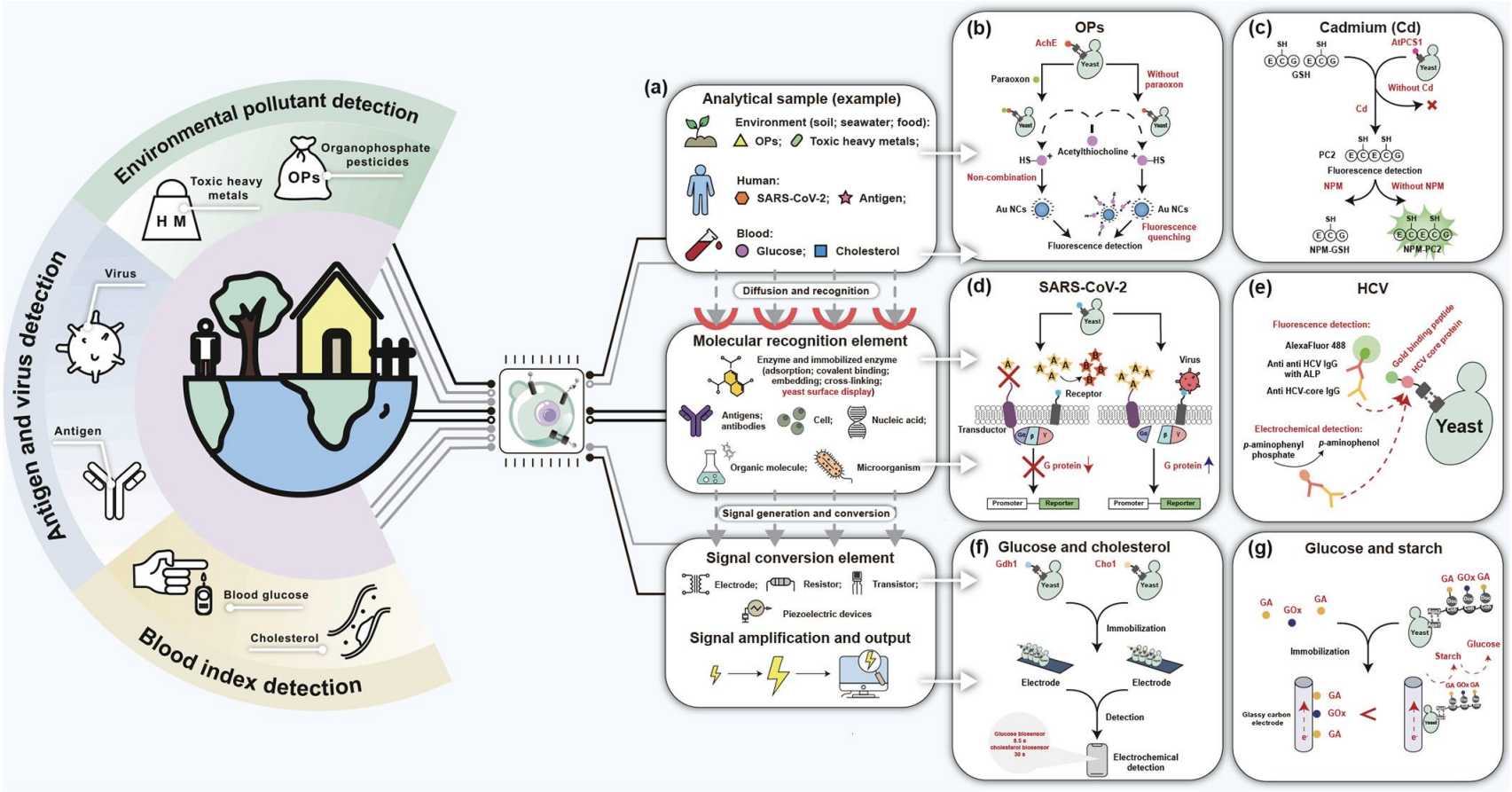
Applicazioni di cell surface display: biocatalisi



Applicazioni di cell surface display: biorimedio



Applicazioni di cell surface display: biosensori



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RESEARCH ARTICLE Open Access

Functional expression of a novel α-amylase from Antarctic psychrotolerant fungus for baking industry and its magnetic immobilization

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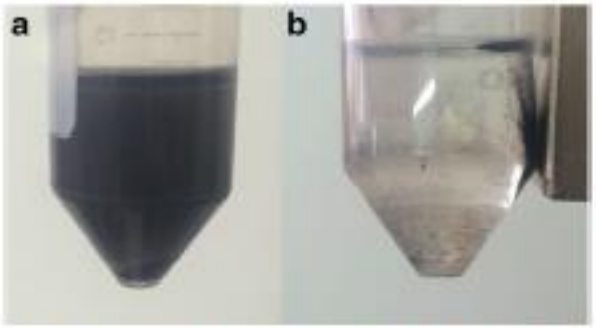

Fig. 5 **a** AmyA1 immobilized on pretreated IONPs; **b** AmyA1-IONPs under magnetic field

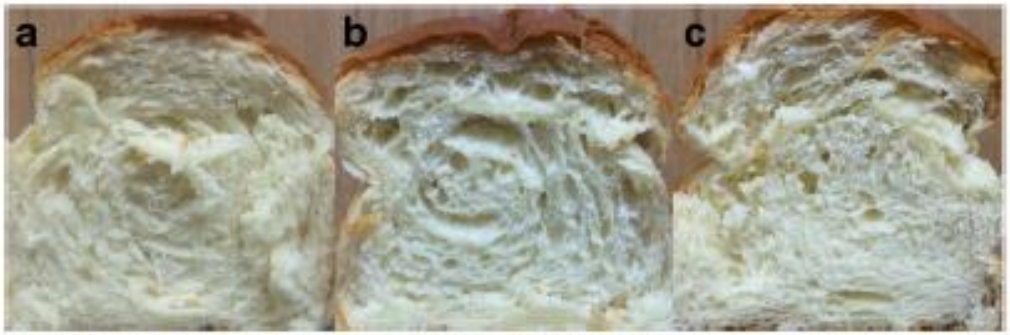

Fig. 4 Crumb structure of the loaf supplemented with: control (without enzyme) (**a**); AmyA1 (**b**); α-amylase TAA from *A. oryzae* (**c**)