Immobilization techniques applied to the development of biocatalysts for the synthesis of nucleoside analogue derivatives

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Abstract: ***Background*:** Nucleoside analogue (NAs) derivatives comprise a large family of pharmaceuticals clinically used as antitumoral and antiviral compounds. Originally, the production of NAs involved chemical synthesis, but a greener bioproduction alternative exists and involves the use of enzymes that catalyze transglycosylation reactions between modified purinic or pyrimidinic bases and sugars. To be considered as an option for industrial application, it is vital to immobilize these biocatalysts. ***Methods*:** This article describes current methodologies for whole cell and protein immobilization mostly applied to the synthesis of important NAs. Immobilization describes ways of cell or enzyme confinement to diverse of surfaces or matrixes. It is important to be familiar with the variety of matrixes and supports available prior to biocatalyst immobilization so the most adequate can be selected for the purpose sought. ***Results*:** From the different articles compiled it can be acknowledged that the main methods for protein or cell stabilization are immobilization by adsorption, covalent, cross-linking and entrapment. The most widely used matrixes and supports are agar, alginate, polyacrylamide, sepharose derivatives, and acrylic resins, among others. Protein or cell stabilization has the advantage of stabilizing immobilization, favoring their facile separation from the reaction medium for further reuse and also making the purification of the final product easier. Moreover, biocatalyst stabilization allows a facile estimation of the economic cost of the bioprocess and of an eventual scale-up, being a basic requirement for industrial application. ***Conclusion*:** In order to achieve successful biocatalyst immobilization, parameters such as biocatalyst stability, mechanical resistance, and reusability should be considered. This review describes and summarizes the methods used for the immobilization of biocatalysts for the synthesis of NAs in the last years.

Keywords: Entrapment, adsorption, matrix, support, hydrogel, covalent bonding, nanoclays, reusability

1. INTRODUCTION

Nucleosides represent the basic unit of one of the most important biomolecules in living systems, the nucleic acids (DNA and RNA). As components of these macromolecules, nucleosides are involved in all cellular processes related with the synthesis of RNA and DNA, cell signaling, enzymatic regulation and metabolism. Natural nucleosides are formed by the association of a nucleobase (purinic or pirimidinic) with a five carbon sugar or pentose. The nucleobases can be purines (adenine and guanine) or pyrimidines (thymine, cytosine and uracil) and the pentose residues can be either β-D-ribofuranose or β-D-deoxyribofuranose for RNA or DNA, respectively (Fig. **1**). Analogues of these nucleosides are synthetic nucleosides specially produced to imitate the physiological functions of natural counterparts in order to interfere with the cellular metabolism [1, 2].

Nucleoside analogues (NAs) enter the nascent DNA and RNA chains, preventing cellular division and replication of the viruses. Once included in DNA, suppression of chain elongation, mutations accumulate in the viral progeny or apoptosis induction occur [3]. The potential therapeutic effects of these actions are inhibition of cancer cell growth and viral replication [3].

Nucleoside analogues are synthesized mostly chemically in a process that involve numerous stages of protection and deprotection of functional groups, involving the use of many toxic organic solvents, harsh reaction conditions, and the appearance of species very difficult to purificate (racemic mixtures)[4]. Bearing this in mind, biocatalytic synthesis of nucleosides (employing enzymes) appeared as a different choice for the bioproduction of NAs due to the nature of enzymatic reactivity: performing reactions with high selectivity, efficiency and easy recovery and reuse [5]. The advantages of using these biocatalysts are high stereo- and regioselectivity, reactions that take place in a mild environment, offering a greener bioprocess [6]. NAs have been synthesized via transglycosylation reactions using two

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types of enzymes: nucleoside phosphorylases (NPs) and N-deoxyribosyltransferases (NDTs), which transfer glycosyl residues from nucleosides to acceptor bases [5]. NPs catalyze the reversible phosphorolysis of both ribo- and deoxyribonucleosides by the cleavage of N-glycosidic bonds of nucleosides without addition of ATP, to form a free base and its respective activated pentose moiety (pentose-1-P), which is then coupled to the desired modified base either by the same or a different NP to give a NA [7]. They can be classified according to their substrate specificity as purine NPs (PNPs; EC 2.4.2.1) or pyrimidine NPs (PyNPs; EC 2.4.2.2)[7, 8]. Alternatively, NDTs (EC 2.4.2.6) catalyze the exchange between the purine or pyrimidine base of 2′-deoxyribonucleosides and free purine or pyrimidine bases. In the reaction, an intermediate of a covalently bound 2-deoxy-α-D-ribofuranosyl moiety is formed, where the glycosidic hydroxyl of 2′-deoxyribose is esterified by a glutamic acid of the active site of NDTs [5, 9].

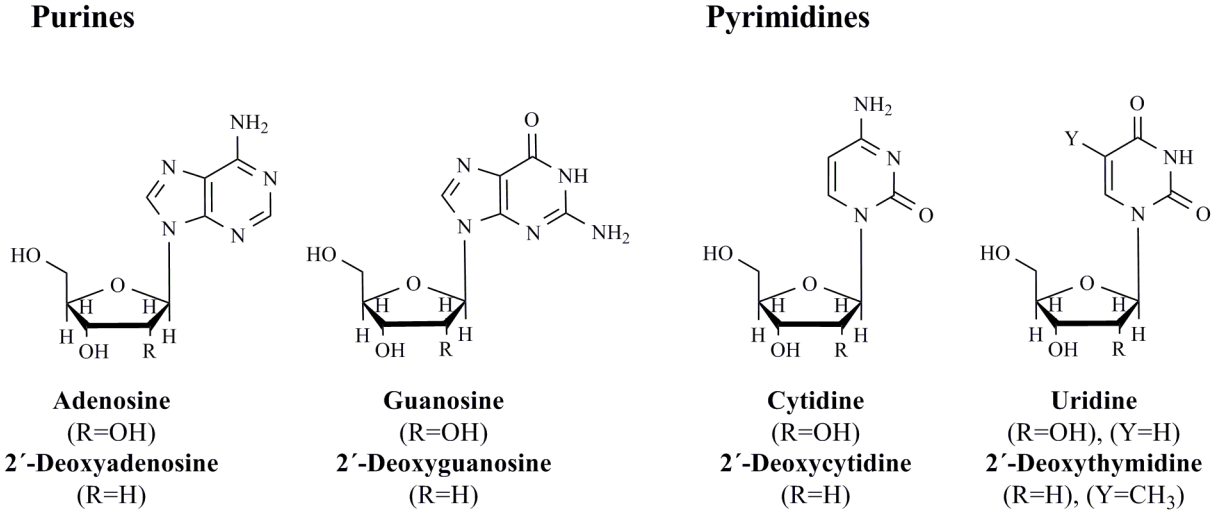
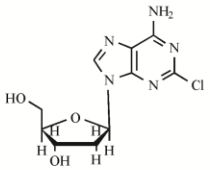
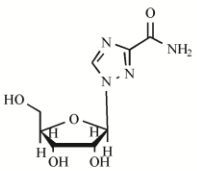
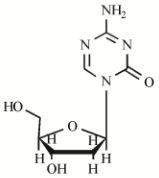
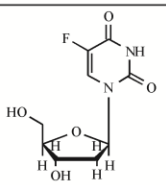
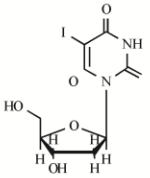
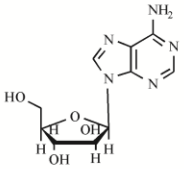
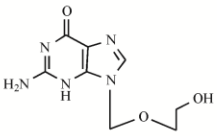
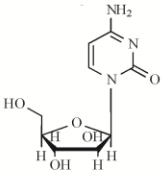
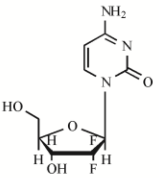
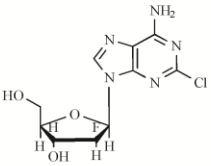
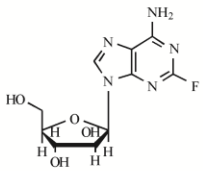
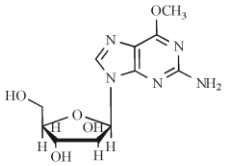
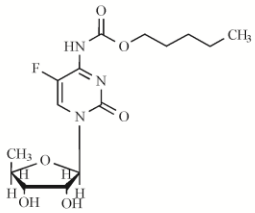
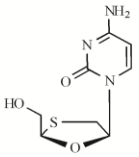
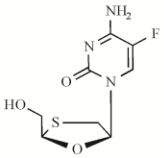


Fig. (1). Chemical structure of natural nucleosides present in RNA and DNA. The modification of these nucleosides represents the starting point for nucleoside analogues development.

A number of analogues of pyrimidine and purine nucleosides have been employed as anticancer and antiviral pharmacological drugs. In cancer, the purines NAs (PNAs) cladribine, fludarabine, clofarabine and nelarabine are employed in the treatment of malignant disorders of the blood (Table **1**). As regards pyrimidine analogues, cytarabine is extensively used in the treatment of acute leukemia; gemcitabine is indicated for the treatment of several solid tumors; and decitabine is used for the treatment of myelodysplastic syndromes (MDS). The fluoropyrimidine floxuridine has activity against various types of cancer [2]. Also, antiviral NAs constitute the first-line therapy against specific viral infections, and the discovery of NAs effective in HIV therapy marked a breakthrough in AIDS treatment. The cytidine analogues lamivudine and emtricitabine are used in combination with other drugs in HIV therapy [5]. As they can be used for long periods of time in chronic viral infections, NAs with improved bioavailability and reduced side effects have been extensively explored. NAs used in antiviral therapies include ribavirin, iduviran, vidarabine, acyclovir, among others [2].

Concerning their mechanism of action, they enter the cell through specialized nucleoside transporter proteins whose tissue distribution determine its cellular specificity and susceptibility to nucleoside analogues [10]. The clinically administered compounds are generally prodrugs, meaning that they must be modified after administration in order to exert their action. The most common example of this is phosphorylation that occurs after entering the cell, performed by enzymes involved in deoxynucleoside salvage pathways. The resultant triphosphorylated nucleoside (nucleotide) is responsible for producing their activity by being incorporated into DNA during replication or repair [11], acting as chain terminators and stopping viral DNA polymerase [5]. However, each NA has a unique drug target [12]. These compounds are considered polyvalent because they contain multiple hydroxyl and amino groups which confer them a highly hydrophilic and polar nature. They have a rather short plasma half-life due to they suffer hydrolysis by nucleoside phosphorylases or deamination by nucleoside deaminases present in the body (agregar cita). So, in order to maintain the plasma concentration required for effective treatment, large doses and frequent administration are necessary, which in many cases augment the undesirable side effects. Therefore, new strategies for NA modification are in continuous search. Chemical modification of parent drugs is one of the most successful strategies to overcome the drawbacks mentioned above. However, selective modification of a single functional group when there is repetitive functional groups with the same chemical reactivity in a molecule represents a problematic task using classic organic chemistry. An example of this difficulty is the regioselective acylation of polyhydroxyl compounds such as nucleosides [13]

Table 1. Classification of NAs in current clinical use

	Adenosine analogues	Guanosine analogues	Cytidine analogues	Uridine/Thymidine analogues
Modification in the base	 Cladribine	 Ribavirin	 Decitabine	 Floxuridine  Idoxuridine
Modification in the sugar residue	 Vidarabine	 Acyclovir	 Cytarabine  Gemcitabine	
Modification in the base and in the sugar residue	 Clofarabine  Fludarabine	 Nelarabine	 Capecitabine  Lamivudine  Emtricitabine	

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Moreover, to avoid some adverse side effects, existing NAs have been modified by different techniques such as glycosylation, halogenation, deamination, and acylation, this last strategy being one of the most studied to create derivatives with improved activity. Different enzymes other than NPs and PNPs have been used for this purpose, for instance, lipases, glycosidases, deaminases. These biocatalysts can be used as enzymes (isolated by purification or produced by genetic engineering) or as wild type or recombinant whole cell microorganisms [14-17].As a general term, immobilization describes many different forms of cell or enzyme confinement to different types of surfaces or matrixes [18]. The bond established between the surface and the biocatalyst can be reversible or not, and the interactions can be grouped as adsorption, covalent, entrapment and cross-linking immobilization (Table **2**) [19, 20]. Adsorption immobilization employ labile interactions such as Van der Waals, ionic or hydrophobic bonding; the nature of the bond is nonspecific, and as a disadvantage, desorption may occur. In covalent bonding, enzyme or cells are permanently linked to the functional groups of the support, which is attractive as there is no biocatalyst leakage, although the matrix cannot be reused. A kind of non-permanent covalent immobilization involves the formation of disulfide bonds or bridges (S-S), which are generally reserved for enzyme-support interaction. Entrapment is a versatile method for enzyme or whole cell stabilization, this last biocatalyst type being the preferred one for this immobilization method. Also, these methods can be combined, such as an enzyme first immobilized by adsorption and then entrapped in a porous polymer [19]. Biocatalyst immobilization allows for its stabilization, favors separation from the reaction medium for further reuse, and also makes the purification of the final product easier [5].

Table 2. Immobilization techniques. Whole cell and enzyme immobilization methods can be broadly classified as irreversible and reversible, *this bonding type is mostly applied to enzyme stabilization

Immobilization	Irreversible	Entrapment	Alginate, polyacrylamide, agar, agarose, cellulose
		Covalent bonding	EC-EP, IDA-Agarose, EDA-Agarose, Glyoxyl- Agarose, and CNBr-Sepharose
		Cross-linking	Using bifunctional reagents: glutaraldehyde, tannic acid, etc
	Reversible	Adsorption	DEAE-Sepharose, Q-Agarose, Lewatit-Agarose, Octyl-agarose
		Covalent bonding* via disulfide bonds (S-S)	Thiol-Agarose

As mentioned above, biocatalysts can be used in two forms, as isolated enzymes or as whole cell. On the one hand, microbial whole cells represent an excellent alternative since they carry their cofactors with them, are effective, ecological and low-cost catalysts. Nowadays, cell stabilization is the mostly used procedure for bacterial stabilization ; basically, the microorganisms are incorporated inside a rigid network that prevents cell release but is sufficiently permeable to permit the circulation of substrates and products [18]. Different kinds of matrixes have been used for this purpose and can be divided by their physicochemical properties into hydrogels, thermogels and synthetic polymers [18, 21, 22]. On the other hand, enzyme immobilization represents an excellent alternative for the biotechnology industry. However, to be used as biocatalysts, enzymes are needed in high amounts, and since the methods to obtain them are expensive, it is necessary to recover and reuse them to make an economically feasible process. Enzymes can be stabilized by adsorption, covalent conjugation, entrapment, and affinity binding [5, 23]. A great variety of supports can be used for enzyme stabilization, and the immobilization methods and matrixes used influence the activity of immobilized enzymes. A versatile matrix must ensure different features. Preferably, it must not react with nonspecific reagents, must be inert, must be strong enough to resist mechanical stress, be stable in a range of conditions, and should avoid product inhibition, and bacterial contamination [23]. The advantages and disadvantages of immobilization are depicted in Table **3**. Examples of the use of these matrixes for immobilizing biocatalysts for NAs production are described later in this text.

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Table 3. Summarized advantages and disadvantages of biocatalyst stabilization

Advantages	Disadvantages
Biocatalyst reusability and recyclability	Immobilization costs
Broad working conditions (pH, T°)	Immobilization time
Different choice of reactors	Diffusion problems
Easier product purification and downstream processing	Increased reaction volume due to increased biocatalyst surface/volume
Increased activity and stability	Reduced activity

2. IMMOBILIZATION

2.1. Cell Immobilization

There are a great variety of methods for immobilizing whole bacterial cells, but generally whole cell immobilization can be performed by entrapment in porous polymers or microcapsules or by interacting with inorganic or organic supports [24, 25]. The interaction of cells is caused by Van der Waals forces and ionic or covalent bonding, specially when microbial exopolymers participate in the procedure [25]. As mentioned earlier, cell entrapment is the preferred technique for whole cell immobilization. Basically, cells are included within a rigid network porous enough to allow the diffusion of substrates and products and protect the selected microorganism from the reaction medium (cita trelles springer 2013). Cells are entrapped within a gel in the free spaces of a polymeric net composed by natural or synthetic polymer. Gellation is then reached by the supplementation of one or more cross-linking agents. The mixture of the soluble polymer and the cells is passed through a needle or micropipette tip into a gelling solution to create the beads[26]. There is gelation induced by temperature and by ions (ionotropic).

Apart from being one of the most widely used, this method is considered to be an specially easy, simple and safe technique for stabilization. The main advantages of this methodology lie in their high operational stability, easy upstream separation and bioprocess scale-up feasibility [5]. The matrixes employed in this methodology can be classified in hydrogels, thermogels and synthetic polymers [18]. Of these matrixes, alginate, agarose, and polyacrylamide have been widely used for biocatalyst immobilization for NAs synthesis.

It is important to mention that there are a number of cell immobilization methods that are different from entrapment and are less used for biotransformations of NAs. These include:

*Adsorption or adhesion on polymeric surfaces. [25, 27].
*Adhesion onto covalent supports [28].
*Cell encapsulation. [28], [29], [30].

At the moment of choosing a support to immobilize a biocatalyst, different parameters will define the most appropriate selection, such as ability to retain the biocatalyst in the matrix and maintaining or increasing its stability. To determine which support acts as better stabilizer, different factors summarized in Table **4** should be considered.

Table 4. Parameters that permit characterizing the immobilization

Support characterization
Biomass loading capacity
Biomass release
Diffusion between substrates and products (mass transfer)
Maintenance of the enzymatic activity after stabilization
Operational stability
Storage stability

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2.2. Enzyme immobilization

Enzymes are broadly exploited in by biotechnological, food and pharmaceutical companies [31]. These biocatalysts have a better performance in aqueous enviroments, pH arround 7, and temperatures under 50°C; these features are not the desired ones to optimize a reaction where substrates and/or products are not easily soluble in water [31]. Also, they are in need in high amounts and due to their elevated costs great productivity is demanded [32]. One of the best ways to prevent these limitations to ocurr is to stabilize the enzymes. Immobilization confers enzymes improved activity, stability in a larger pH and temperature range in comparison to native enzymes. Also, protein stabilization expands applicability in organic solvents, causes increased recyclability and allows process scale-up and easier downstream and purification processes [23].

Different techniques are employed for protein immobilization and depend on the chemistry of the interaction. Enzymes can be stabilized by adsorption, covalent bonding and also by entrapment. A large variety of supports can be used for enzyme stabilization. Generally, it is better to immobilize soluble enzymes in non soluble solid supports [33]. This allows the biocatalyst to be subjected to continuous uses without losing its activity, making possible the economical recovery of the protein. Enzyme biotransformations can take place in very diverse reaction solutions: aqueous, anhydrous organic solvents, supercritical fluids, ionic liquids, etc. The choice of supports and the immobilization procedure will be determined almost entirely on the final reaction conditions (medium, temperature, pH) where the stabililized will be employed [23, 26, 33]. Matrixes can be classified according to their chemical properties as natural or synthetic polymers, and also inorganic materials, such as alumina, silica, zeolites and mesoporous silicas, are usually used as carrier matrixes [34, 35]. A great advantage of supports like agarose beads, zeolites, porous glass, epoxy resins like Sepabeads, is that they confer a big large surface for enzyme–support binding. [32, 36, 37].

Protein engineering helps to defeat the previously mentioned drawbacks of natural enzymes when used as biocatalysts [33]. The development of process-specific engineered biocatalysts comprise enhancing different features of the chemical nature of them such as regioselectivity chemoselectivity, and stereoselectivity. Advances in stabilization methodologies, mainly in relation to oriented immobilization, have resulted in higher activity in comparison to random immobilization, because enzyme accessibility is improved and its active site remains unaltered [33]. Regarding immobilization, with the aim of guaranteeing a highly cost-effective process development, several parameters defined in Table **5** should be considered.

Table 5. Considerations for industrial enzyme immobilization

Support choice
Retain or enhance enzyme native activity
Structural stability
High enzyme loading
Low reaction volumes
High storage and operational stability
Low cost of immobilization and carrier

3. TYPES OF MATRIXES/SUPPORTS

Generally, enzyme carriers are classified as organic and inorganic depending on their source. Organic carriers include natural polymers such as alginate, chitin, chitosan and cellulose, and synthetic polymers like polyacrylamide, polyurethane, polyvinyl alcohol, among others [27]. Other macromolecular sugars, apart from alginate, chitosan and agarose, such as starch and cellulose, have been used for enzyme immobilization, which is very economical [38]. However, starch is not often used now because of its poor mechanical properties and its susceptibility to other microbial contamination. Also, the cellulose immobilization procedure employs harsh reaction conditions due to its particular structure [39].

Derivatized agarose constitutes an excellent alternative due to its hydrophilic nature, compatibility with lots of activation strategies, ready availability with a great range of pore sizes and resistance to mechanical stress [40]. Other options, like dextran derivatives (i.e., Sephadex® and Sephacryl®), are rather costly and have reduced mechanical resistance [41].

Regarding inorganic carriers, there are many compounds of this group including silica- and metal-oxide-based matrixes such as zeolites, mesoporous silicas, alumina, ceramics, mesoporous glasses, magnetic nanoparticles. Among these silicas, titania and hydroxyapatita are the most common [23]. Immobilization by adsorption is the favourite choice, since it is the simplest stabilization strategy. With respect to development and industrial process, the most important aspects concerning the support are cost and amount needed, mechanical properties, which will depend of the reaction conditions (agitation) and reactor; physical surface and pore size, hydrophilicity, chemical stability, biocatalyst retention, among others.

3.1. Organic Carriers

3.1.1. Natural Polymers

Hydrogels (alginate, κ-carrageenan, chitosan)

Alginate is a general name employed to group the salts and derivatives of alginic acid. Alginates have been used in food and pharmaceutical companies for over 85 years as thickening, emulsifying, film forming, and gelling agents [42]. Alginates are polysaccharide that are composed by different proportions of uronic acid: D-mannuronic acid (M) and L-guluronic acid (G) (Fig. **2**), and the source of seaweed species (*Laminaria yperborea* and *Macrocystis pyrifera)* is responsible for the variation in the amount of M and G [43]. Alginates properties and functionality, such as gelling capacity and gel strength are given by the difference in the ratio of M and G and also by its block arrangement. A remarkable feature of sodium alginate is the ability to gelate when exposed to multivalent cations [44]. Also, alginate can be used as a polymer for immobilization by cell encapsulation by using different technologies such as emulsion techniques or extrusion methods [29].

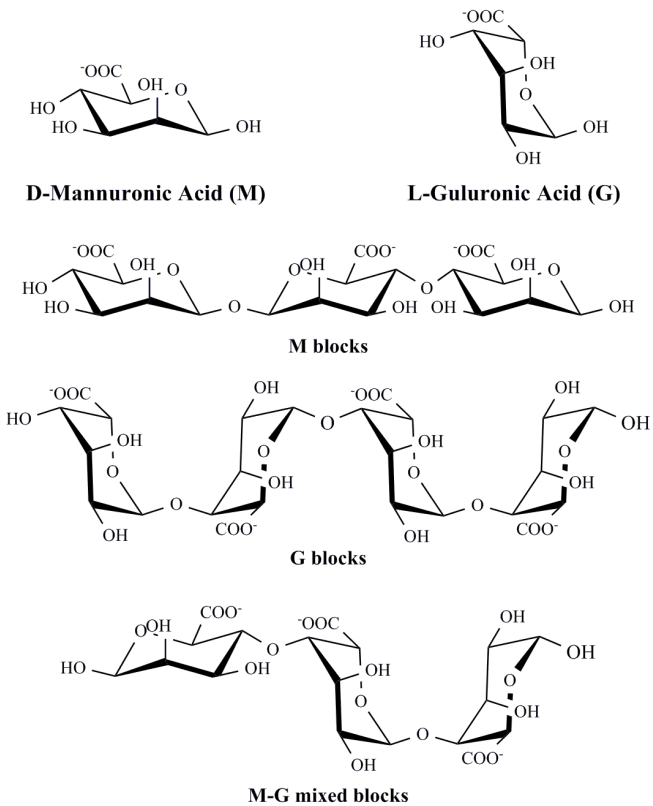

Fig. (2). Alginate structure and formation of M and G blocks arrangements.

Chitin is one of the most common natural polysaccharides isolated from crustaceans, insects and fungi; it is a linear polymer composed of b-(1-4)-linked D-glucosamine (deacetylated unit) an N-acetyl-D-glucosamine (acetylated unit)(Fig. **3**) [45]. **Chitosan** is the deacetylated product of chitin (Fig. **3**). As chitosan has less than 40% of N-acetyl groups, it is more soluble in pH below 6.5. This polymer is nontoxic, it can be obtained in several presentations (powder, gel, fibers, and membranes), and easily derivatizable. Therefore, a chemical treatment of chitosan at low pH conditions is required to maintain its insolubility, a key feature of the support for the success of enzyme immobilization [23].

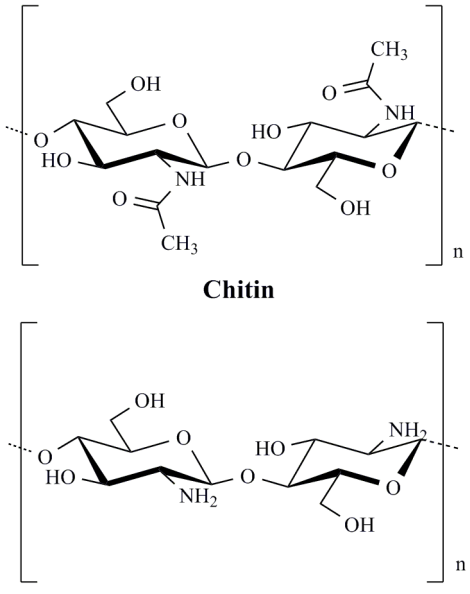

Fig (3). Chitin and chitosan structure

Thermogels (agar, agarose, cellulose)

These polymers are formed upon temperature-induced gelation. They have the advantage of being nontoxic and biocompatible.

Agar is a linear polysaccharide obtained from the cell walls of some red seaweeds that belong to the *Rhodophyceae* class. The commercially available agar belongs mostly to the genera *Gelidium* and *Gracilaria* [46]. Agar has been widely used in different areas, in genetic engineering it is used as raw material for the preparation of electrophoresis gels and in microbiology it is the preferred solid culture media [47]. Agar is composed by two distinctive components: agarose and agaropectin [48]. There are two monosaccharides present: β--galactose and 3,6-anhydro-α-L-galactose, linked by glycosidic bonds β(1–4) (between β-D-galactose and 3,6-anhydro-α-L-galactose, originating the disaccharide basic unit called neoagarobiose) and α(1–3) (between 3,6-anhydro-α-L-galactose and β-D-galactose, giving the disaccharide basic unit called agarobiose) (Fig. **4**).

Agarose, as aforementioned, represents one of the subunits of agar, and it is isolated from agar by separation from the

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other constituent, agaropectin. Agarose is composed by repetitive units of agarobiose (Fig. **4**). Similarly to the alginate extrusion technique, to immobilize a biocatalyst in agarose, a mixed solution containing agarose. and whole cells is dropped into a stirred oil solution were spherical shaped beads are produced [18].

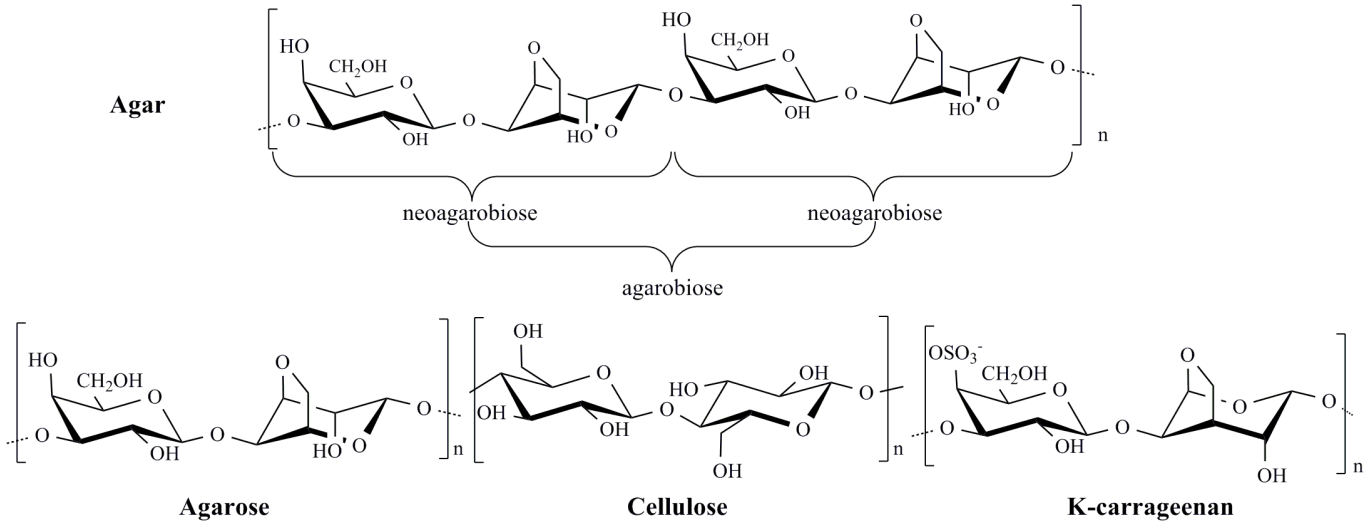

Fig. (4). Chemical structure of natural polymers agar, agarose, cellulose and k-carrageenan

Cellulose is the most abundant natural polymer and also the cheapest support available as an enzyme carrier. The hydroxyl group of the monomer (glucose) can bind with the aminoacids present in the enzymes (Fig. **4**). Cellulose has a peculiar 3D structure that requires costly treatments to render a support suitable for immobilization techniques [49]. In fact, cellulose chains form quite compact strands, linked to each other by a regular set of interchain hydrogen bonds. be classified into four different methods: the gel, the droplet, the emulsion and the dehydration method [50].

K-carrageenan. Carrageenans belong to a family of gel-forming and viscosifying linear sulfated polysaccharides that are extracted from several species of the red seaweeds (*Rhodophyta fillum*)[50]. Carrageenans, in the same manner that alginates, represent essential supplies in diverse industries, for example, in the manufacture of food products, and also as additives in pharmaceutical and cosmetic formulations[50]. The most widespread types of carrageenan are identified according to their number and position of sulfate groups by a Greek prefix: ι, κ, and λ-carrageenan. The κ-carrageenan basic units are composed by alternating 3-linked β-galactopyranose and 4-linked 3,6-anhydro-α-D-galactopyranose disaccharides [51] (Fig. **4**). K-carrageenans have only one sulfate group per disaccharide attached to the O3 galactopyranose ring. The ability of forming gels is the main attribute of these polymers and a requisite for their employment as immobilization martrixes [52]. The solidification of κ-carrageenan is directed by certain monovalent cations (K^+, Cs^+, Rb^+, and NH_4^+). Immobilization techniques carried out with carrageenans can

3.1.2. Synthetic Polymers (polyacrylamide, polyvinyl alcohol, polyurethane)

In many cases, these polymers have better mechanical performance than the natural carrier but are not easily biodegradable.

Polyacrylamide gel entrapment is a generally employed technique for synthetic immobilization. A mixture composed of whole cells, the acrylamide monomer and a cross-linker reagent (bisacrylamide) is mixed and polymerized by starting the reaction with an initiator (ammonium persulfate) in the presence of a catalytic enhancer (N,N,N′,N′-tetramethylethylenediamine, TEMED) (Fig. **5**)[18]. Then, the mixture is placed horizontally in a container where it polymerizes and gels, and then the gel is cut into cubes. The disadvantage of the use of this matrix is that it may be toxic to some microorganisms[18].

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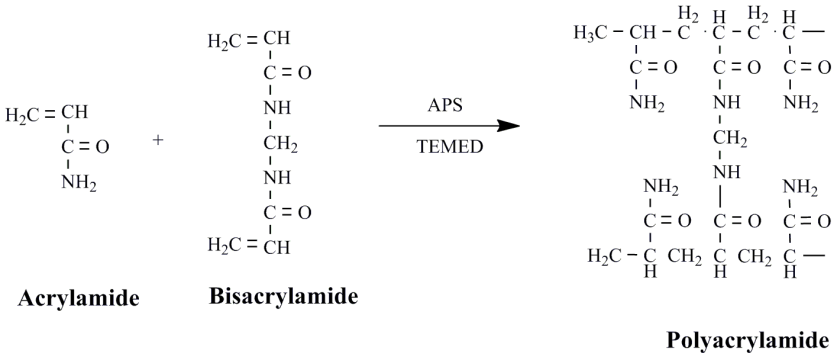

Fig. (5). Acrylamide gelling process

Polyvinyl alcohol

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer used in diverse applications from papermaking, to textiles as well as in various biomedical applications including ophthalmic materials, tendon repair, and drug delivery [53] (Fig. **6**). It is used in various cross-linked forms, giving hydrogels or membranes to be employed in protein purification and as immobilization supports [54, 55]. It is very suitable for a large number of purposes due to its hydrophilic nature, which confer the capacity to be modified through its hydroxylic groups by acetalization and/or acylation [56].

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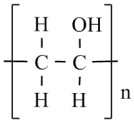
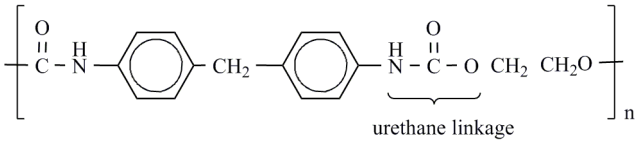


Polyvinyl alcohol (PVA)

Fig. (6). PVA structure

Polyurethane

Polyurethanes (PU) are one of the most versatile materials in industrial use ranging from foam and adhesives making to automobile parts or surface coatings (Fig. **7**). Also, it has been shown to be useful for biochemical and biotechnological applications, and flexible polyurethane foams have gained relevance as microbial carriers for their good mechanical properties, high porosity, large adsorption surface, resistance to organic solvents and microbial attack, easy handling, regenerability, and convenient price [57, 58]. One advantage of PU is that it can be used to scale up bioprocesses due to easy preparation of large volumes of this carrier. An important aspect, is that the high rates of absorption of positive charge and hydrophobic character of polyurethane permit the interaction with most microbial cell surfaces. Pus are inexpensive and easily regenerated by extraction or washing with solvents [28, 58].



Polyurethane

Fig. (7). Chemical structure of a polyurethane

Agarose derivatives

Agarose is also used as a solid support for bioligands or enzymes; it is mostly used in two different categories: separation and catalysis at both laboratory and industrial scale. The trade name for a cross-linked, beaded-form of agarose is Sepharose (from Separation-Pharmacia-Agarose). The most common application for this material is in chromatographic separations of biomolecules and also in immobilization of biocatalysts. This support can be chemically activated using different functional groups, allowing it to bind enzymes, antibodies and other proteins through covalent attachment to the resin [40]. Several bifunctional agents are well known as cross-linkers for agarose beads: among them, epichlorohydrin, 2,3-dibromopropanol, divinylsulfone, bis-oxiranes [40]. Common activation chemistries include cyanogen bromide (CNBr) activation. In this method, cyanogen bromide reacts with hydroxyl groups of a carrier to yield the reactive imidocarbonate derivative (the activated carrier). The subsequent reaction is between this activated carrier and amine groups of the enzyme. Also, other functional groups, such as iodoacetyl, can be added to selectively bind cysteine side chains from the proteins to be stabilized. The coupling of enzyme occurs entirely or at least predominantly through the free amino groups of the ligand protein. Also, the electrophilic groups can react with the thiol groups of the enzyme, and also may be attached by weaker nucleophiles, such as phenolic hydroxyls of tyrosine residues.

Various grades and chemistries of activated sepharose are commercially available. The ready-made activated carriers

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are available commercially in the market, for example: CNBr-activated Sepharose 4B (Fig. **8**). An advantage of activated agarose is that it can be preserved in a dry stabilized state by cold storage for long periods of time. It is important to mention that the supports used for adsorption immobilization can be derivatized with other functional groups, such as aldehyde groups, and can be used as covalent supports [59]. Such supports are named heterofunctional and may be defined as supports that have several distinct functionalities on their surface being able to interact with a protein. In this respect, glutaraldehyde-activated supports are the oldest multifunctional supports [60, 61].

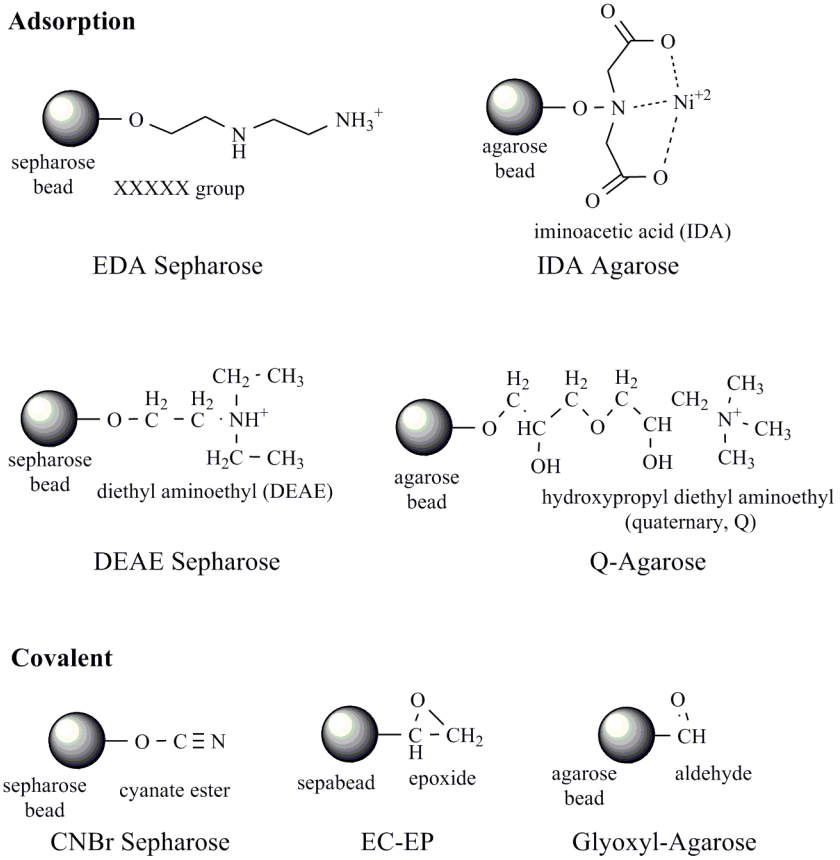

Fig. (8). Different agarose-derived supports employed for adsorption and covalent immobilization

Another widely used support, not agarose-derived, is commercial Eupergit® C (Fig. **9**). This support consists of macroporous beads with a diameter of 100–250 μm, made by copolymerization of N,N′-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether, and methacrylamide. Eupergit® C interacts with enzymes trough its oxirane groups with the amino groups of the proteins, at neutral and alkaline pH [62].

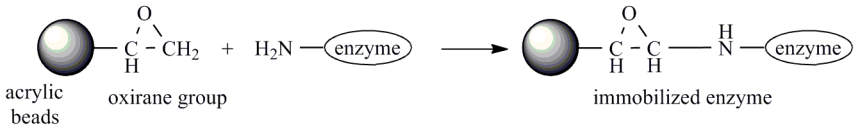

Fig. (9). Eupergit C coupling mechanism

3.2. Inorganic Carriers

There is a large variety of inorganic solids that can be used as carrier matrixes. Examples of these materials used for the immobilization of enzymes are: zeolites, clays, anthracites, porous glass, activated charcoal, ceramics, alumina and mesoporous silicas [23]. As a result of the immobilization, enzymes gain enhanced activity, stability in a wider pH and temperature range in comparison to free enzymes and also gives them possibility to be reused [33]. Regading the various inorganic carriers employed for stabilizing enzymes by adsorption, silicas may be those carriers that aquire more

notability [27]. Another mineral employed as a carrier for absorption stabilization is hydroxyapatite, a mineral composed by calcium phosphate and naturally present in nature. Besides, bentonite has been described as an enzyme carrier with high protein adsorption capacity. Interestingly, one of the features of bentonite is that it is not soluble in water, but instead it swells, which is why enzymes adsorbed in bentonite can be employed in aqueous solutions [27].

Nanocomposites have been used over the past decades for enzyme immobilization, and their use has recently been reviewed [63]. These compounds are composed of a variety of clay minerals. Clay minerals are a naturally occurring group of hydrous aluminum or magnesium phyllosilicates with a two-dimensional layered structure on a nanometer scale [63]. That particular structure gave the clay minerals an distinctive physicochemical features including a large surface area, swelling and ion exchangeability [63]. Also, this particular structure permits this clay minerals to be altered by organic, polymeric or biological molecules. In this regard, modified clay minerals bear other functional groups, which confer them a bigger adhesion area and enhanced hydrophobicity along with less steric hindrance. Clay minerals include: bentonite, montmorillonite, smectites, halloysite, sepiolite, laponite, palygorskite, among others. Enzymes are the preferred substrate for immobilization on to these nanoclay surfaces, which can be either by adsorption or by covalent bonding.

4. CHEMISTRY IN THE IMMOBILIZATION PROCESS

A broader way of classifying the approaches to immobilize a biocatalyst is by categorizing them as reversible and irreversible immobilization methods [19] (Table **2**). Another way to classify them is according to the chemical nature of the bonding as: adsorption, covalent, entrapment and cross-linking immobilization (Fig. **10**).

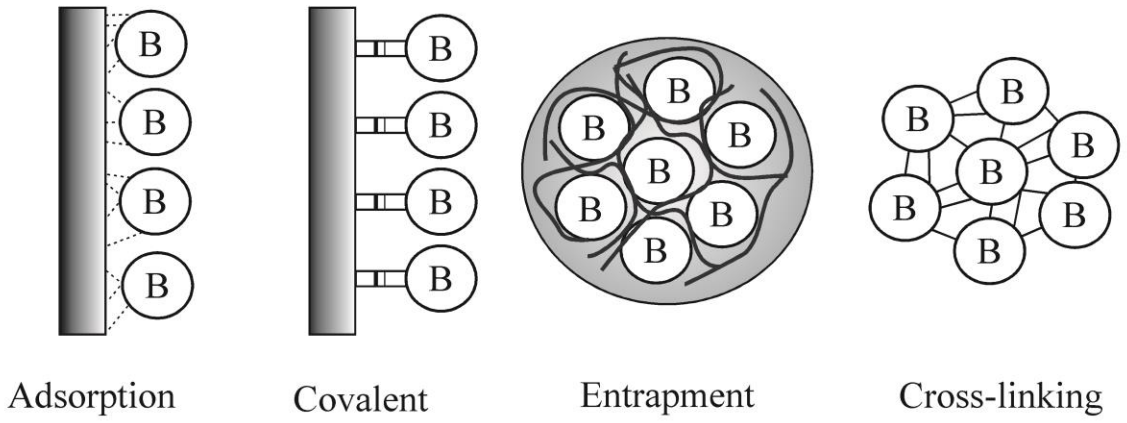

Fig. (10). Immobilization methods employed for biocatalyst (B) stabilization.

4.1. Adsorption

One of the most relevant and practical stabilization methods is immobilization by adsorption or carrier binding. Adsorption immobilization involves weak and nonspecific bonds between the carrier and the biocatalyst such as Van der Waals, ionic or hydrophobic interactions, as well as hydrogen bonding [19]. Therefore, the adsorbed biocatalysts can be easily removed by minor changes in pH, ionic strength or temperature, which represents a disadvantage for the industrial use of enzymes. It is also worth noting that due to the weak nature of the bonds the native structure of the enzymes is not altered, which allows the enzyme to retain its activity. In addition, almost every carrier could be used for enzyme adsorption, but not viceversa [27].

So, for the successful adsorption of the enzyme to occur it is necessary to have enzyme-carrier affinity, which is guaranteed by the existence of specific functional groups on the carrier surface, which allows the formation of the biocatalyst–carrier interactions. However, if these functional groups are not present, intermediate agents can be employed (carrier modifiers) to modify biocatalyst–carrier interactions [27]. Enzyme stabilization on to the solid carriers can be performed via a great range of chemical and physical methods [64, 65]. It is important to point out that the adsorption stabilization method is not exclusively restricted to enzymes, and carrier groups can also interact with the groups present on the enzyme surface [25]. For example, *Lactobacillus animalis* has been successfully immobilized in DEAE-Sepharose, obtaining a biocatalyst able to bioproduce a variety of halogenated nucleosides including the antitumoral floxuridine by transglycosylation. DEAE-Sepharose was the support that had the greatest bacterial binding capacity, with a productivity of 53 mg/L h and 248 h of operational stability [66]. In a later report, a biocatalyst with 2'-N-deoxyribosyltransferase activity (NDT) was

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developed from *Lactobacillus animalis* cell free extracts, resulting in a derivative with an activity of 2.6 U/g for the biosynthesis of floxuridine and other halogenated nucleosides. The obtained activity was better than that previously reported using immobilized enzymes. Furthermore, this biocatalyst was successfully employed to bioproduce other halogenated pyrimidine and purine 2´-deoxynucleosides [67].

As previously mentioned, the immobilization of enzymes may broaden the protein capacities in nonaqueous environments. The typical case is lipases, which in aqueous solution catalyze the hydrolysis of esters to alcohols, whereas in organic environments they perform transesterifications of the previously mentioned substrates [27]. The hyperactivation of lipases can be accomplished by adsorption of these enzymes on hydrophobic supports, which allows enhanced activity due to the correct immobilization of the open conformation of the lipases [26, 68]. Therefore, several commercially available lipases immobilized on inert supports have been used extensively to esterify a number of NAs. Among these are polymerizable acyclovir derivatives transesterified enzymatically with divinyl dicarboxylates using *Candida antarctica* lipase (CAL-B immobilized in acrylic resin from Sigma) as catalyst in anhydrous acetone or DMSO at 50°C and 250 rpm [69]. Also, this lipase was employed for the Ara-C (cytarabine) acylation with vinyl stearate (VS) in different binary organic solvents, showing the highest 5′-OH regioselectivity (>99.9%) and conversion (90.8%) among other enzymes tested [70]. In a later study, the synthesis of polymer-drug conjugates was attempted using different commercial lipases, organic solvents and substrate molar ratios for gemcitabine acylation with divinyl dicarboxylates of different chain length. After choosing CAL-B (Sigma) as catalyst, four polymerizable vinyl gemcitabine esters were obtained by very selective transesterification in acetone. Subsequently, radical homopolymerization of the obtained 5´-O-vinyladipyl-gemcitabine monomers and their copolymerization with different saccharides such as galactose, glucose and lactose were performed. Three saccharide polymer derivatives of gemcitabine were synthesized. Of these, the derivatives containing galactose or lactose could have hepatoma-targeting function [71]. In a different research, also using a commercial CAL-B (Novozym 435®, from Novo Nordisk), the use of various solvents (organic solvents and ionic liquids) and immobilization materials (acrylic resin and carbon nanotubes) allowed defining the best environment for the regioselective in one step and one-pot production of gemcitabine-5´-O-lipoate. The derivative obtained, proved to be more effective in comparison to the parent drug against non-small cell lung cancer [72]. Also using Novozym 435 (*C. antarctica* lipase B or Chirazyme L-2) the process for the regioselective acylation of ribavirin was designed, bearing in mind that the alanine showed superior results (better bioavailability and less side effects) a series of preclinical evaluations. So, regioselective acylation of ribavirin with the oxime ester of l-carbobenzyloxy(Cbz)-alanine was carried out using Novozym 435. After 24 h at 60 °C, the acylated product was purified with 85% yield [73]. In the same manner, Kryger *et al*. proposed the synthesis of a macromolecular prodrug of ribavirin. The synthesis of ribavirin acrylate was carried out chemoenzymaticaly using acetone oxime acrylate, ribavirin, and Novozym 435 lipase. After optimizing reaction conditions the final product yielded more than 85%. The effect of this ribavirin MP was tested in erythrocytes, hepatocytes and macrophages by an internalization assay, which showed reduced uptake by erythrocytes without altering the drug interaction with the other relevant hepatic cell lines, which suggests an effective elimination of the main side effect of ribavirin [74].

Another lipase, from *Pseudomonas cepacia* (PSC-L), has also been widely used to acylate NAs. Li *et al*. reported the use of various lipases for the enzymatic acylation of nucleoside analogues in different ionic liquid-containing systems. The selected PSC-L (commercial, immobilized on ceramic from Amano Enzyme Inc. Japan) mediated floxuridine benzoylation, obtaining excellent conversion (>99%) and great 3´-regioselectivity (92%) in anhydrous THF with the addition of 1-butyl-2,3-dimethylimidazolium hexafluorophosphate ($[C_4MIm]PF^6$) [75]. In a later report, the specificity of this lipase in the acylation of nucleosides was investigated by the use of substrate engineering. *P. cepacia* lipase showed great 3´-regioselectivities (96-99%) in the lauroylation of 2´-deoxynucleosides and low to moderate 3´-regioselectivities (59–89%) in the lauroylation of ribonucleosides. This could be explained to the the unfavorable hydrogen bond interaction between the 2-hydroxyl groups of ribonucleosides [76].

Also, *Penicillium expansum* lipase (PEL) immobilized in macroporous adsorbent resin D4020 was evaluated for the acylation of vidarabine and other purine analogues, using 2-methyltetrahydrofuran (MeTHF), a biomass-derived compound that represents an attractive medium for biocatalysis. Crude PEL powder (Leveking Bioengineering Co., Ltd., Shenzhen, China) and macroporous adsorbent resin D4020 (composed of cross-linked polystyrene with a specific surface area of 540–580 m^2/g, Chemical Co. of Nankai University, Tianjin, China) were immobilized. The protein binding capacity of this support was 35.5 mg protein/g resin, determined by Bradford. The regioselective acylation of ara-A was performed using immobilized *Penicillium expansum* lipase in 2-methyltetrahydrofuran (MeTHF), a biosolvent derived from biomass, obtaining good yields (89%) and excellent 5´-OH regioselectivity (>99%) after 42 h using undecylenic acid vinyl ester as acyl donor [77]. Lastly, crude *Penicillium expansum* lipase (PEL) powder was stabilized in the macroporous adsorbent resin D4020. Then a great variety of pyrimidine nucleosides, including floxuridine, idoxuridine, stavudine (d4T) and zidovudine, were modified with this lipase in MeTHF. [78].

4.2. Covalent Bonding

This method has been extensively investigated for enzyme-stabilization and is one of the mostly employed. The benefits of the use of this method rely on the permanent nature of the bonding enzymes, they are not desorbed in the media, so a highly stable biocatalyst is developed. This technique is not the preferred one for the immobilization of cells because the reagents used are often toxic to the cells

and finding the conditions in wich cells can be successfully stabilized with no harm is an almost imposible task [79]. The enzymes bind covalently to the support through their functional groups that are not vital for its catalytic activity. Therefore, in order to protect the enzyme active site, it is a good approach to perform the stabilization protocol including a competitive inhibitor or the natural substrate [80]. The functional groups involved in the interactions are side chains of the amino acids, such as the amino group from lysine, carboxyl from aspartic and glutamic acid, the thiol group from cysteine and the phenolic group of tyrosine [19]. As aforementioned, due to the covalent nature of the bond once the biocatalyst has lost all its activity after successive reaction cycles it must be discarded, and the support cannot be reused like the adsorption ones.

An attractive approach of this methodology is that via multipoint covalent attachment a great stabilization of the 3-D structure of the immobilized enzyme can be achieved [26]. This is performed by stabilizing the enzyme onto highly activated preexisting supports with short spacer arms. In this way, a number of residues in the enzyme surface can interact with the functional groups present in the support, achieving higher stability and activity [26]. Besides, multipoint and multi-subunit covalent attachments of enzymes to supports modified with linkers help stabilizing the enzyme by provide rigidity to the immobilized enzyme structure [81]. Multi-subunit covalent attachments allow stabilizing, for example, both subunits of a dimeric protein on very highly activated supports. A more difficult task is to immobilize multimeric proteins, but this can be achieved by the covalent attachment of several subunits (two or three) to the support and the subsequent cross-linking of the other subunits to the immobilized subunits by using functional or polyfunctional cross-linking agents (e.g., glutaraldehyde and aldehyde-dextran). In this way, complex multimeric structures can be stabilized [26, 81]. An example of this immobilization procedure is cited in this report. A phosphopentomutase (PPM) from *Escherichia coli* ATCC 4157 was overexpressed, purified, and immobilized on agarose activated with cyanogen bromide (CNBr-Ag), monoaminoethyl-N-aminoethyl agarose (MANAE-Ag), MANAE-glutaraldehyde (MANAE-G) and glyoxyl agarose (Gx-Ag). A molecular modeling allowed predicting that the orientation of the PPM immobilized on various agarose supports affects the thermal stability of the heterogeneous biocatalyst. Several orientations provided different intensity in the covalent attachment between the protein and solid surface, and moreover the junctions occur in regions with different rigidity. Glyoxyl-agarose was the support with better results. The immobilization of PPM on glyoxyl (aldehyde) activated supports required the incubation of the enzyme at pH 10. The soluble enzyme incubated at pH 7 maintained around 90% activity for more than 24 h and 30% of its initial activity after 18 h of incubation at pH 10. This shows that the stability of the enzyme is extremely low at alkaline pH, a condition necessary to perform the multipoint covalent immobilization on a glyoxyl support. As the protective effects of additives such as PEG, trehalose and glycerol on the enzymatic activity under drastic conditions are well known, these additives were studied obtaining a highly stable derivative using glycerol 10%. The developed biocatalyst was able to bioproduce ribavirin from 1,2,4-triazole-3-carboxamide (TCA) and D-ribose-5-phosphate (R5P), in the presence of a purine nucleoside phosphorylase [82].

Another interesting methodology is enzyme immobilization using thiol-disulfide exchange reactions, which offer the possibility of forming a stable and reversible covalent bond, the disulfide bridges (-S-S-). Enzymes that with exposed thiol (SH) groups that are not vital for their functionality can be immobilized onto thiol-reactive supports using friendly reaction conditions, such as neutral pH, ambient temperature, etc. Also, enzymes that do not have exposed thiol groups can be altered either chemically or by means of a genetic engineering approach to present these groups and be used as substrates of these supports [83].

Regarding other disadvantages of covalent immobilization, in some cases when stabilizing an enzyme on to a porous material, diffusion limitation might occur as a result of the unequal distribution of the enzyme within a porous carrier, leading to a reduction of pore volume available to the substrate and product diffusion, this situation is particularly often when overloading the carrier with high protein amounts [33].

It is important to mention that the selection of the immobilization support will depend on the characteristics of the protein, and variations occur according to the one selected and the immobilization procedure. For example, these authors employed three different covalent stabilization strategies to immobilize *Candida rugosa* lipase on Eupergit® C supports. Different parameters such as the amount of binding enzyme, activities and coupling yields were compared. The strategy that yields the highest activity retention (43.3%) is based on coupling lipase via its carbohydrate moiety previously modified by periodate oxidation. Also, the study of thermal deactivation kinetics at three different temperatures (37, 50 and 75°C) discovered that the lipase stabilization onto the support altered the thermal deactivation profile of the biocatalyst, resulting therefore in an enzyme with improved thermoresistence (>2-fold in comparison with the original immobilization procedure and 18-fold more than free lipase)[84]. Also using Eupergit-C support, a cytidine deaminase from *E. coli* was immobilized in order to perform the enzymatic resolution of a racemic mixture of (+/-) Lamivudine, 3TC. The (+) isomer was selectively deaminated from the mixture, and after a two-column process, optically pure 3TC was obtained with 76% crystalline yield [85]. In a similar manner, Osborne *et al*. used an immobilized esterase from a microbial source for the preparation of the (−)-FTC isomer (Emtricitabine). Liophilized cholesterol esterase (Boehringer) was immobilized on Accurel microporous polypropylene (Accurel PP). After many immobilization steps, glutaraldehyde (0.25% v/v) was added and incubated to allow crosslinking between the esterase and the matrix. The resultant stabilized biocatalyst was filtrated and dried by rotary evaporation. The biocatalyst was mixed into a solution of racemic FTC butyrate ester (±) and notably the cleavage of the required isomer to the corresponding alcohol, which when isolated as the hydrochloride, gave 31% yield and 98%

enantiomer selectivity. The immobilized biocatalyst was recycled 14 times, and the process was successfully scaled up allowing the obtainment of 200 g/L of racemic FTC butyrate using 1-pentanol/potassium dihydrogen phosphate buffer as solvents to give (−)-FTC HCl (98% enantiomer selectivity, 2.17 kg, 31% molar yield based on racemic FTC butyrate) [86].

Pure recombinant *Lactobacillus reuteri* 2′-deoxyribosyltransferase (*Lr*NDT) was stabilized on epoxy activated Sepabeads EC-EP303 whose principal chemical features including concentration of activated oxirane groups have been described [87]. Fernández-Lucas *et al.* described the biosynthesis of various naturally occurring nucleosides as well as other antiviral and antitumoral nucleosides in therapeutic use, among which decitabine, floxuridine, idoxuridine were produced using S*Lr*NDT4 as biocatalyst [15]. Also, the therapeutical nucleosides 5-ethyl-2′-deoxyuridine and 5-trifluorothymidine are reported to be produced enzymatically for the first time.The most relevant features of the developed biocatalyst S*Lr*NDT4 for its employment at an industrial scale are that showed a recyclability of 26 times in the synthesis of 2,6-diaminopurine-2′-deoxyriboside with activity loss.

Again, enzyme immobilization onto different supports yields different results. In this study, a screening with different hydrolases (esterases, lipases and proteases) was performed, and the commercial neutral protease from *Bacillus subtilis* showed the better activity and regioselectivity [88]. Therefore, it covalent inmmobilization on to various supports was assayed. First, it was immobilized on to the hydrophobic epoxy carriers Eupergit® C and Sepabeads EC-EP. Both this immobilization procedures were laborious and time consuming (24 h for first hydrophobic adsorption at pH 8), then, it is attached covalently to the support by interaction between lysine amino groups and the epoxy groups of the carrier. Both this biocatalyst were not stable and lost more than 50% of its initial activity after 24 h and suffer about 50% protease N release.

Then, different hydrophilic carriers (agarose) were assayed using various activation chemistries: glyoxyl-agarose, glutaraldehyde-agarose and cyanogen bromide agarose. Interestingly, comparable results were obtained independently of the activation chemistry used for the carrier, as no desorption of protease N was observed. Besides, all these agarose stabilized derivatives resulted to be much more stable in comparison with both the free enzyme and the epoxy-carriers derivatives. In order to elucidate the reason or the behavior of the protease N immobilized in the different supports, a 3D structure model of *B. subtilis* protease N built *in silico* using the structure of *Staphylococcus aureus* metalloproteinase as the template to predict the hypothetical localization of lysines on the enzyme surface. The stable biocatalyst obtained was successfully used in the development of efficient preparative bioprocesses for the regioselective hydrolysis of acetylated nucleosides, giving new intermediates for the synthesis of capecitabine in high yield [88]. Capecitabine is an antitumor agent employed in the therapeutics of various cancers, such as colon cancer (advanced stage), as well as breast and ovary cancers [89].

The enzymes Puo-phosphorylase and uridine phosphorylases, obtained by genetic engineering from overproducing *E. coli* BL21(DE3)/pERPUPHO1, were immobilized in aminopropylated macroporous glass AP-CPG-170 (Sigma) by the glutaric dialdehyde method. A covalent bond between the aldehyde groups and the amino groups of the sorbent and proteins is formed in the presence of sodium borohydride. The use of these immobilized enzymatic preparations significantly simplifies the technology of preparation of the modified nucleosides and allows a repeated use without loss of the catalytic activity of the enzymatic preparations (up to 15 manufacturing cycles). The obtained biocatalyst was employed for vidarabine and ribavirin production, obtaining 56% yields after 24 h and 36.8 mM of ribavirin with a molar yield of 92% after 36 h [90]. More recently, the multienzymatic synthesis of this drug, also using arabinosyluracil and adenine, was reported; uridine phosphorylase from *Clostridium perfringens* (*Cp*UP) and a PNP from *Aeromonas hydrophila* (*Ah*PNP) were used as covalently immobilized biocatalysts. Immobilization was performed on aldehyde agarose. The synthesis of ara-A was then developed in a 2 L scale, and the product was isolated with 53% yield (3.5 g/ L) and 98.7% purity [91].

4.3. Entrapment

In this method, biocatalysts are physically entrapped within a rigid network that is porous enough to allow penetration of substrate but still rigid to prevent biocatalyst diffusion into the surrounding medium. The bonds involved in its stabilization can be covalent or not. This methodology has been mostly used for cell entrapment [18] and less used for enzyme stabilization since due to their small molecular size in comparison with cells may cause slow leakage during continuous use; this is why pore size must be adjusted with the concentration of the polymer used [33]. Biocatalysts have been entrapped in natural polymers like agar, agarose and gelatine through thermoreversal polymerization, but in alginate and carrageenan by ionotropic gelation. The polymer matrixes used in this method have a porous structure, which allows substrate and products to diffuse easily through and into the matrix. Among the many advantages of this technique is that it allows high mechanical strength, is a fast and simple method, but shows limitations like biocatalyst leakage, diffusional problems and loss of matrix integrity after successive uses [38].

Different parameters must be set for each immobilization. Regarding ionotropic gelation of alginate, a different behavior of the same biocatalyst immobilized in this matrix and using different divalent ions (Ca^{2+}, $Ba^{2+}$and Sr^{2+}) as cross-linking solution has been reported. Various parameters such as exposure time and concentration of the solution in the gelation process were optimized, Sr-alginate being the condition with better performance, obtaining biocatalyst with better mechanical properties (compression strength, swelling ratio and fracture frequency), which is favorable for a future scale-up. Also, the obtained biocatalyst had higher operational stability in comparison with Ca-alginate [92]. In a later report, the addition of the nanoclay bentonite resulted in an improvement of more than 90% of storage stability,

and the reusability of the biocatalysts was greater compared with control conditions, allowing bioprocess scale-up to obtain floxuridine with high productivity per gram of biocatalyst [93].

Regarding agarose matrix, as aforementioned, it has been extensively used for the biotransformation of several clinically relevant NAs. The guanosine analogue ribavirin, commonly used as an antiviral for the treatment of Hepatitis C virus (HCV) infection, was bioproduced using *Escherichia coli* ATCC 12407 immobilized in agarose, obtaining good product yields and enhancing the biocatalyst stability with the immobilization. The developed immobilized biocatalyst was operationally active for more than 270 h and could be stored for 4 months without activity loss. This microorganism was also stabilized in polyacrylamide; but this matrix resulted more labile than the agarose matrix, considering that integrity loss was apparent after 80 h of continuous reaction. Regarding storage stability, the agarose immobilized biocatalyst remained stable for over 120 days while the polyacrylamide stabilized remained active for 80 days [94]. Another report describes the use of Geobacillus kaustophilus ATCC 8005, an extremophile microorganism, as biocatalyst stabilized into an agarose matrix complemented with the nanoclay bentonite (Ag-Bent), which was defined as a bionanocomposite. This reinforced biocatalyst proved to be stable for over 580 h without loss of activity, significantly improving operational stability and mechanical properties in comparison with the non supplemented agarose matrix (Ag-Ctrl, 150 h)[95].Besides, process was successfully scaled up to a packed-bed bioreactor, achieving 370 mg L^{-1} of ribavirin. Also, when the mechanical properties were studied, even though Ag-Ctrl had an increased mass after incubation; no appreciable swelling was observed for Ag-Bent, reinforcing the role of bentonite addition on matrix stabilization [95]. In another study, *E. coli* immobilized cells on agarose with the same substrates yielded 72 mM ribavirin after 4 h of reaction time at pH 7.0, 60 ºC, the same conditions as those of the previously mentioned assays [16].

4.4. Cross-linking

In cross-linking immobilization enzymes molecules are attached to each other via covalent bonds [61]. This method, also called copolymerization, employs bi- or multifunctional reagents to bind with the functional groups of the enzyme to form an insoluble cross-linked complex. The most commonly used cross-linking reagent is glutaraldehyde, which binds with the amino group of the enzyme via its own carbonyl group. Several molecules of glutaraldehyde can bind together to form oligoglutaraldehyde. Other cross-linking reagents such as diazobenzidine, tannic acid, dimethyl adipimidate, and hexamethylene diisocyanate are also used [64]. Among the benefits of this technique is that is simple and economical, but the disadvantage is that polyfunctional reagents may alter the enzyme structure causing a significant loss in catalytic properties.

Covalent attachment and cross-linking are effective and durable to enzymes, but they are not often used for immobilization of cells[38]. This is mainly due to the fact that the agents used for covalent bond formation are usually cytotoxic and it is difficult to find conditions under which cells can be immobilized without any damage. Also, bifunctional reagents may react with the enzymes present in the cells reducing their biocatalytic activities. There are only some reported cases of successful cell covalent binding and generally involve yeast [58, 96].

Robust catalysts were produced via the cross-linking of enzyme crystals (CLECs) [97, 98] and also, promising results were obtained with the so-called cross-linked enzyme aggregates (CLEAs) [99]. Although CLECs are chemically and mechanically quite robust catalysts, the development of suitable protocols for crystallization and cross-linking is lengthy and laborious task, representing the main drawback in comparison with CLEAs [99]. Cross-linking of protein precipitates affords a higher degree of control over the properties of the final product.

Different CLECs have been patented for the resolution of enantiomerical mixtures. One is the process for preparing a substantially pure β-D or β-L-1,3-dioxolane nucleosides, compounds with antiviral HIV activity [100]. Similarly, the enzyme-mediated enantioselective synthesis of anti-viral compounds, such as 2-hydroxymethyl-5-(5-flurocytosin-1-yl)-1,3-oxathiolane (FTC) and its analogues, in a non-homogenous reaction system using CLECs from different hydrolases is described [101].

McClean *et al*. assayed the hydrolysis of the l-valine ester prodrug of acyclovir (valacyclovir) to acyclovir using 62 immobilized and free commercial enzyme preparations. From this screening 19 of them proved to be catalysts capable to hydrolyze valacyclovir to acyclovir. The subsequent studies focused on subtilisin A (subtilisin protease from *Bacillus licheniformis*, also known as Subtilisin Carlsberg) and Chiro CLEC-BL, a cross-linked form of the same enzyme, which displayed the highest activity. However, all attempts to esterify l-valine with acyclovir failed to yield the desired product with the enzymes tested. By testing l-valine methyl ester instead, the synthesis of enantiopure valacyclovir could be achieved by using ChiroCLEC-BL, and the best results were obtained in the presence of neat methyl l-valinate or with tert-butanol cosolvent [102]. Table **6**. summarizes different methodologies implied for the synthesis of NAs.

Table 6. Different supports used for the synthesis of NAs

A. Adsorption

Matrix/ Support	Biocatalyst	NAs	Operational stability (h)	Preparation Time (h)	Reaction conditions	Advantage	Refs.
DEAE-Sepharose	*L. animalis* (whole cell)	Floxuridine	248	18	20 mM Tris–HCl buffer pH 7, 30°C	Desorption and matrix reusability	[66]
DEAE-Sepharose	*L. animalis NDT from cell extracts (LaNDT)*	Floxuridine and other halogenated nucleosides	64	8			[67]
Q- Agarose			24	8			[67]
Hydrophobic acrylic resin (Sigma)	*Candida antarctica* lipase (CAL-B)	Acyclovir derivatives (divinyl dicarboxylates)	ND	-	anhydrous acetone or DMSO at 50°C and 250 rpm		[69]
		Gemcitabine derivatives (divinyl dicarboxylates of various chain lengths)	ND	-	anhydrous acetone at 50°C and 250 rpm		[71]
Novozym 435 (Novo Nordisk)		Gemcitabine-5´-O-lipoate	72 h	-	acetonitrile, at 60°C		[72]
Macroporous acrylic support Novozym 435 (Novozymes)		Cytarabine (Ara-C) derivatives (vinyl stearate)	ND	-	25% hexane, 0.07 initial water activity,50°C		[70]
Novozym 435		Ribavirin oxime ester of l-carbobenzyloxy (Cbz)-alanine	ND	-	24 h at 60 ◦C		[73]
Novozym 435		Ribavirin acrylate	ND	-			[74]
	Pseudomonas cepacia (PSC-L)	Floxuridine derivatives (vinyl benzoate)	ND	-	THF-[C4MIm]PF6 (10%, v/v), 50 ◦C, 200 rpm		[75]
Macroporous adsorbent resin D4020	*Penicillium expansum Lipase (PEL)*	Pyrimidine nucleosides floxuridine, idoxuridine, stavudine (d4T) and zidovudine	ND	**30**			[78]
		ara-A			MeTHF 42 h undecylenic acid vinyl ester		[77]

B. Covalent

Matrix/ Support	Biocatalyst	NAs	Operational stability (h)	Preparation Time (h)	Reaction conditions	Advantage	Refs.
epoxy-activated Sepabeads EC-EP303 (multipoint attachment)	*L. reuteri NDT* (LrNDT)	Decitabine, floxuridine, etc.	ND	48 h	pH 6.5, 40°C	100% Immobilization yield,	[15]
Eupergit-C	*E. coli* cytidine deaminase	Lamivudine (racemic resolution)			pH 7.0, 32°C		[85]
Accurel microporous polypropylene Accurel PP	Lyophilized cholesterol esterase (Boehringer)	Emtricitabine (racemic resolution)	15 successive cycles	24	Potassium dihydrogen orthophosphate solution ,pH 7.0 and 30°C		[86]
Agarose CNBr Agarose glutaraldehyde	B. subtilis protease	Capecitabine	-	ND	10% CH3CN in 25 mM phosphate buffer pH 7	Increased activity and stability compared to epoxy supports	[103]
Aminopropylated macroporous glass AP-CPG-170 (Sigma)	*Puo-phosphorylase and uridine phosphorylase*	Ribavirin, Vidarabine	15 cycles	31 h	Phosphate buffer 50 mM for 36 h at 60°C.		[90]
Aldehyde agarose	UP from *Clostridium perfringens* (CpUP) and PNP from *Aeromonas hydrophila* (AhPNP)	Vidarabine	240 h	-	Phosphate buffer 25 mM, pH 7.5, 25°C, DMF (30%)		[91]
Glyoxyl-Agarose	PPM from *E.coli*	Ribavirin	ND	9 h	Phosphate buffer 25 mM, pH 7, 25°C Manganese 0,1 mM, 1 μM Glucose 1,6 biphosphate	Multipoint attachment	[82]

C. Entrapment

Matrix/ Support	Biocatalyst	NAs	Operational stability (h)	Preparation Time (h)	Reaction conditions	Advantage	Refs.
Ca- Alginate	*L. animalis* In Ca-alginate	Floxuridine	16 h	2 h	20 mM Tris–HCl buffer pH 7, 30°C		[92]
Sr- Alginate	*L. animalis* In Sr-alginate		40 h	2 h			
Alginate-Bentonite	*L.animalis* in alginate supplemented with bentonite		80 h	2 h		Enhanced mechanical properties compared to control	[93]
Agarose	*Escherichia coli* ATCC 12407	Ribavirin	270 h	2 h	Phosphate buffer 30 mM pH 7.0, 30 ◦C	120-day storage stability 4°C	[94].
Polyacrylamide			80 h	1 h		80-day storage stability 4°C	
Agarose	*Geobacillus kaustophilus* ATCC 8005	Ribavirin	150 h	2 h	Phosphate buffer 30 mM pH 7.0, 60 ◦C		[95].
Agarose bentonite			580 h	2 h		Enhanced mechanical properties compared to control	
Agarose	*E. coli*	Ribavirin	480 h	2 h	Phosphate buffer 50 mM pH 7.0, 60 ◦C		[16].

ND: not determined

Combined methods

As previously mentioned, two or three different immobilization methods can be combined to create a new one, which in many cases synergizes the advantages of the separate techniques. The classic example is immobilizing an enzyme first by adsorption, and then by entrapment in a porous polymer (Fig. **11**).

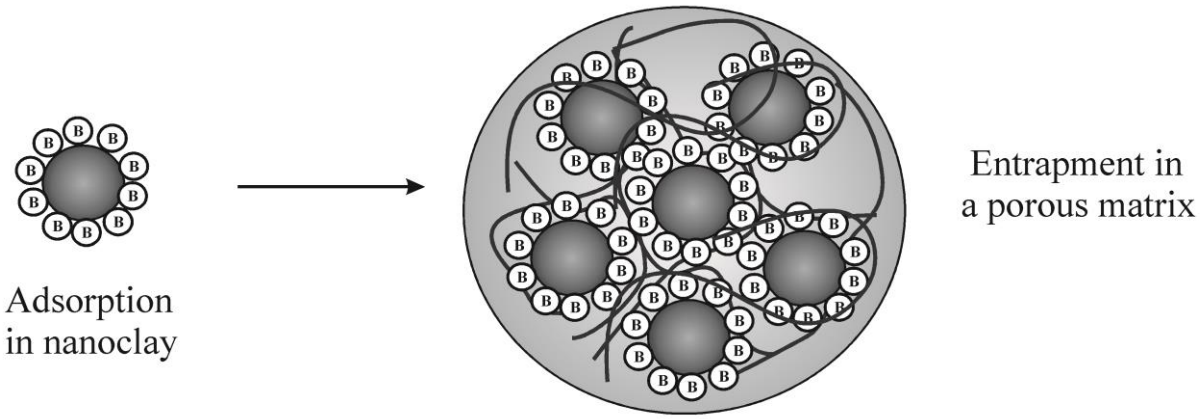

Fig. (11). Combined immobilization. The biocatalyst (B) is first immobilized by absorption onto a nanoclay and then is entrapped in a porous matrix.

Different approaches have been used to merge matrixes, and one of them is chitosan combined with alginate. As a result, chitosan-coated enzymes was better than alginate alone due to the enhanced physical and ionic interactions between the enzyme and the matrix showing less protein leakage[104]. In the same way, a wet composite of chitosan and clay resulted more effective in trapping enzymes mainly due to the presence of hydroxyl and amino groups as well as with high porosity and hydrophilicity. Chitosan in the form of beads can entrap twice as much of the enzymes [23, 105].

Another example is the mixed matrix developed using polyacrylamide supplemented with natural chitosan nanofibers (CNFs) via *in situ* free-radical polymerization. Different properties of this matrix were studied, and it was found that CNFs interacted strongly with polyacrylamide via hydrogen and covalent bonds In this hydrogel system, chitosan nanofibers work as a multifunctional cross-linker and a reinforcing agent. Different mechanical parameters of this mixed matrix were improved in comparison with polyacrylamide, such as compressive strength, storage modulus, and swelling ratio (SR). It was stablished that the best CNF contents was 1.5 wt%, which was the one that yielded the best combined swelling and mechanical properties for the hydrogels [106].

Lately, polymer matrix based nanocomposites have gained lots of attention in the nanotechnology area [107]. Using inorganic nanoparticles in combination with polymeric matrixes can provide high-performance novel materials useful in many industrial fields. The integration of inorganic nanoparticles into a polymer matrix allows both properties from inorganic nanoparticles and polymer to be combined/enhanced and thus advanced new functions[108]. This come from the recent acknowledgement that exfoliated clays could improve significantly existing polymers by conferring enhanced mechanical properties. While the reinforcement aspects of nanocomposites are the primary area of interest, a number of other properties and potential applications are present. Understanding the property changes as the particle (or fiber) dimensions decrease to the nanoscale level is important to optimize the resultant nanocomposite [107]. The combination of polymers and nanoclays yields nanocomposites with different "molecular morphology" determined by wide-angle X-ray scattering (WAXS) and transmission electron microscopy (TEM) analyses. Away from a precise or graphic classification, these are addressed to three types of morphologies: immiscible (conventional or microcomposite), intercalated, and miscible or exfoliated, which are schematically represented in Fig. **12**. For most purposes, complete exfoliation of the clay platelets, meaning almost complete dispersion of the nanoclays plates within the polymer matrix, represent the best scenario of the mixed matrix formation [107].

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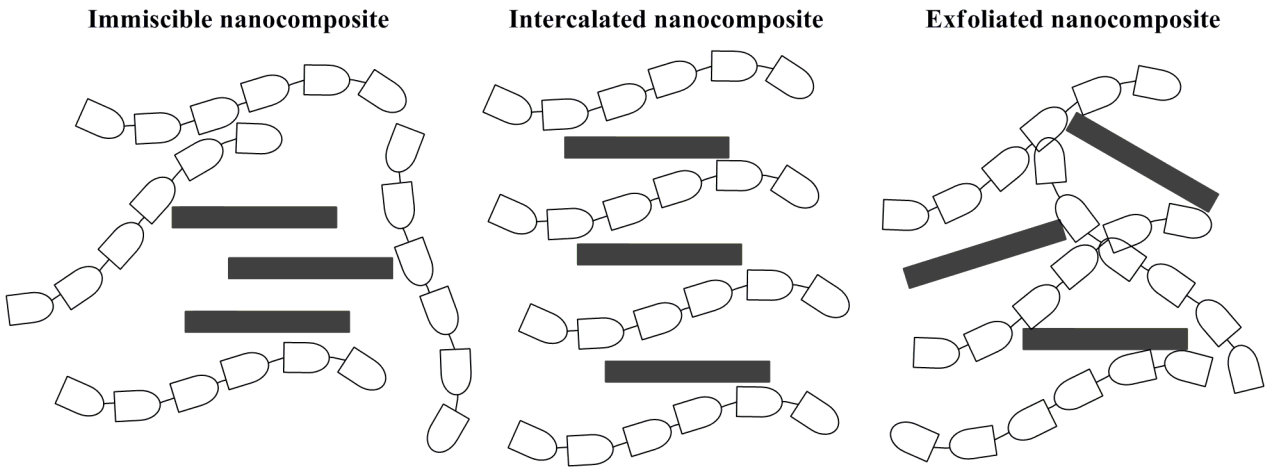

Fig. (12). Different nanocomposite arrangements. Adapted from [107]

As mentioned, the addition of nanocomposites to the immobilization process, among other features, improves the mechanical properties of the biocatalysts obtained [109] facilitating the design of bioreactors for further scale bioprocess [93]. Moreover, the biopolymer chitosan/vermiculite (VMT) nanocomposites were prepared by mixing process of the cationic biopolymer chitosan with three different modified VMT (HVMT, NVMT, and OVMT), that were previously incubated with hydrochloride, sodium, and cetyl trimethyl ammonium bromide (CTAB), correspondingly. Different studies, such as wide-angle X-ray diffraction, transmission electron microscopy and thermogravimetric analysis, were performed to characterize chitosan/ nanocomposite matrixes. The results showed that the silicate layers were disorderedly dispersed into the chitosan matrix at nanoscale, and bared ehnhanced thermal stability compared to the others evaluated, providing promising high-performance and low-cost chitosan nanocomposites.

Apart from that, interesting results were obtained by combining nanocomposites (alginate-clay) with essential oils, resulting in the development of an antimicrobial nanocomposite with excellent activity against three of the most often foodborne pathogens: *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. Interestingly, after incorporation of essential oils to the nanocomposite film the antibacterial activity of them was not altered and corresponded to the nature and amount of the essential oils added [110].

In the past years, many immobilization methods and supports were developed to obtain chromatographic stationary phases with immobilized enzymes. These are used in batch experiments or packed into columns, and in flow systems such as immobilized enzyme reactors (IMERs). In an IMER the enzyme can be immobilized either on a suitable chromatographic support or on the inner surface of silica capillaries, where the catalytic reaction occurs upon substrate injection during its chromatographic elution. IMERs are generally combined with detection technologies such as capillary electrophoresis (CE) or HPLC, allowing in this cases a real time monitoring [66]. A very promising approach has been described in the work by Calleri *et al*., where they report the development of a novel IMER, consisting of a purine NP from *Aeromonas hydrophila* (*Ah*PNP) immobilized covalently on a fused silica open tubular capillary (OTC) via Schiff base chemistry. This biochromatographic system was used for the evaluation of the substrate specificity on nucleoside libraries. Interestingly, about 60 reactions were performed with negligible lost of activity, therefore supporting the importance and confirming the reliability of this IMER as a novel instrument for nucleoside analogues screening. Apart from that, the *Ah*PNP-IMER proved to be very stable after long-term storage retaining its full activity after 1 month storage [67].

Nanoclays

The organic modification of clay minerals and addition of linking molecules are made to improve the immobilization so as to increase the loading, activity and stability of enzymes [63]. Different natural and modified nanoclays have been used to stabilize *Candida rugosa* lipase [111]. Satisfactory results were obtained in the immobilization of this enzyme onto modified and unmodified bentonites [112], using a 3-aminopropyltriethoxysilane-modified montmorillonite K-10 (Mt–S) support via glutaraldehyde spacer (Mt–G) [113] and using two organobentonites with different hydrophobicities as supports, expanding the range of pH and thermal application [114]. Also, the use of three

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smectite nanoclays (Laponite, SWy-2 and Kunipia) organic modified with octadecyltrimethyl-ammonium surfactant, as suitable host matrices has been reported. The immobilized enzyme retains a significant part of its activity after repeated use under drastic reaction conditions originating from the use of oxidants [115]. Even though these enzyme preparations have not been yet studied for the synthesis of NAs, they have a lot of potential for acylation of clinically relevant NAs.

Magnetized supports

Biologically active compounds and cells immobilized on magnetic carriers can be removed from the system simply by using an external magnetic field [116]. In a situation in which two enzymes are simultaneously used in a process and one enzyme deactivates first, one can be immobilized on a magnetic support and the other on a nonmagnetic one, so it can be separated from the medium when it has lost its activity, allowing the other enzyme to be reused. Many magnetic carriers bearing adequate functional groups (e.g., hydroxyl, amino, oxirane, sulfhydryl groups), streptavidin, or in activated form are commercially available [26]. Other types, such as silanized magnetite or fine magnetite particles obtained from magnetotactic bacteria, have also been used for immobilization of antibodies, DNA, RNA, phospholipids, polysaccharides, enzymes, and cells [117] [118].

These supports consist of magnetic microspheres prepared by the suspension polymerization of glycidyl methacrylate, ethylene glycol dimethacrylate, and vinyl acetate in the presence of oleic acid-coated magnetite and then, alcoholyzed to improve their hydrophilicity [119] (Fig. **13**). The immobilization of enzymes onto magnetic beads offers many advantages in comparison to other immobilization methods, such as easy manipulation and separation from the medium by the use of a magnet, and the use of a variety of commercially available supports [120]. Noteworthy, bacterial cells have also been immobilized with this technology, obtaining promising results for wastewater treatment [121].

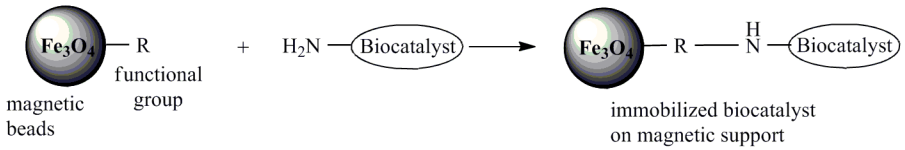

Fig. (13). Immobilization of magnetite supports

Magnetic immobilization of *Lr*NDT onto magnetic chitosan was performed by Fernández-Lucas *et al.* The obtained biocatalyst proved to be effective for performing the enzymatic synthesis of 2′-deoxyribonucleoside analogues as well as arabinosylnucleosides such as vidarabine (ara-A) and cytarabine (ara-C). The enzyme attached to magnetic chitosan beads was reused up to 30 times with negligible loss of catalytic activity in the synthesis of 2,6-diaminopurine-2′-deoxyriboside and 5-trifluorothymidine. Besides, immobilized *Lr*NDT retained 50% of its maximal activity after 56.3 h at 60°C, whereas no activity alteration was observed after storage at 40°C for 144 h. Cross-linked magnetic chitosan beads were prepared in the presence of epichlorohydrin under alkaline conditions, and subsequently incubated with glutaraldehyde in order to obtain an activated support for covalent attachment of nucleoside 2′-deoxyribosyltransferase from *Lactobacillus reuteri* (*Lr*NDT) [122]. Different amount of magnetite (Fe_3O_4) and epichlorohydrin (EPI) led to different macroscopic beads to be used as supports for enzyme immobilization, whose morphology and properties were characterized by scanning electron microscopy, spin electron resonance (ESR), and vibrating sample magnetometry (VSM). [122].

CONCLUSION

Biocatalysis has been widely accepted in diverse sectors owing to its ease of production, substrate specificity, and green chemistry. The enzymes used as biocatalysts represent a remarkable discovery in the field of bioprocess technology. In order to obtain them, laborious and costly processes must be carried on. Whole cells, on the other hand, represent an excellent alternative due to their low cost and great and fast availability. They offer many advantages such as fast reaction conditions, and the enzymes are protected within the cell in their natural environment.

The choice of support is of vital importance for a successful biocatalyst development; a number of matters must be taken in consideration, the most relevant being cost, nature and intended use of the biocatalyst, reaction conditions (temperature, pH, media), scale of production, among others.

As thoroughly described, immobilization offers a great number of advantages for the development of biocatalytic processes, such as easy downstream separation, recyclability and reusability of the biocatalyst, being a prerequisite of industrial application. Also, it offers the possibility of broadening the enzyme pH and temperature ranges, as well

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as allowing better performances in nonaqueous solvents. Entrapment stabilization represents the preferred method for the immobilization of cells, but the bigger pores may cause enzyme leakage. Covalent stabilization is widely used for enzyme stabilization; permanent bonds guarantee high biocatalyst stability, but the process may be long and laborious. Absorption stabilization, as reversible bond formation occurs, gives the possibility of reutilizing the supports after the biocatalyst has lost its activity, but slight changes in reaction conditions (pH, ionic strength) may cause desorption of the biocatalyst. Finally, cross-linking stabilization offers a fast and excellent immobilization alternative without the use of a support. The biocatalyst nature, reaction conditions and scale of the bioprocess will define the best immobilization procedure and the most suitable support.

CONFLICT OF INTEREST

The authors declare no conflicts of interest. This research was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-2658), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2014-KA5-00805) and Universidad Nacional de Quilmes (PUNQ 1409/15).

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Received: March 20, 2014 Revised: April 16, 2014 Accepted: April 20, 2014