industries [1-3]. However, to be profitable at the industrial level, industrial enzymes must allow easy handling and operation procedures, stability and reuse. For this purpose, enzyme immobilization has emerged as a tool to produce robust industrial biocatalysts; and it has been employed over the last forty years for the successful utilization of enzymes in industrial processes [4].

Immobilization is defined as the confinement of enzymes in a defined space, retaining their catalytic activity and allowing their repeated and continuous use [5]. Furthermore, immobilization of enzymes must meet other criteria to obtain robust biocatalysts. Any immobilized enzyme should comprise non-catalytic requirements (shape, size, thickness, length, etc.) designed to aid separation and reuse; as well as, catalytic requirements (activity, selectivity, stability, pH and temperature optimum, etc.) designed to convert the target compounds [6].

The immobilization of enzymes affects their conformation, rigidity and aggregation state, modifying their reactivity [7]. Thus, enzyme immobilization has been also utilized to modulate enzyme selectivity [8-10].

The immobilization of enzymes offers the following advantages: 1) The possibility of reuse, 2) The increase in stability, such as resistance to high temperatures, extreme pHs, high substrate concentration, polar solvents, mechanical shear, among others, 3) Increased volumetric activity, and 4) Increase and modulation of selectivity, such as regio-, acyl-, chemo- and enantioselectivity [11-14].

Methods with physical or chemical interactions are used for enzyme immobilization. When physical methods are used, the enzyme does not undergo structural changes as it is physically attached or trapped by the support structure. The main advantage being that the immobilized enzyme, trapped or encapsulated, nearly maintains its native state; allowing its reuse and, in most cases, an improvement in the enzyme stability [15-17]. However, the main disadvantage is that the union between the support and the enzyme is very weak; causing enzyme leakage while the biocatalyst is operating in aqueous medium. Adsorption, entrapment, and encapsulation are the...
more representative types of physical immobilization. In contrast, chemical immobilization may alter the protein structure because enzymes are attached on the surface and/or inside the support by chemical bonds which may modify its shape, rigidity and aggregation state [18]. Covalent bonding to substrates and self-immobilization are within these types of immobilization.

Immobilization of enzymes by covalent attachment to a support is a method in which proteins are irreversibly linked. To achieve immobilization, the reactive amino groups of amino acids such as lysine, typically present in the surface of proteins, are mainly used. With these amino acids, the covalent attachment of enzymes to a reactive group of the support leads to a highly stable covalent bond. Epoxides are commonly used as reactive groups on supports to react with the amino groups of lysine within the enzyme [19]. Covalent attachment usually provides the strongest bond between the enzyme and the support compared to other types of immobilization, being able to minimize the leakage of enzyme from the support [18]. Besides allowing its reuse, the majority of supports provide the enzyme with a protective barrier [14]. Nonetheless, in many cases, the supports used have high prices raising the cost of production of the thereby immobilized biocatalysts [20]; on the other hand, carrier-free immobilization or self-immobilization is possible since it achieves linking of two enzyme molecules through bifunctional cross-linking agents or simply cross-linkers without the use of a support. The bifunctional compound most commonly used for this purpose is glutaraldehyde (GA) [21], which is a small dialdehyde molecule. Some advantages of self-immobilization are greater volumetric activity per biocatalyst mass, increased specific activity, easier production, reduced production costs, higher purity, less interferences and/or contaminations by the support and lower mass transfer limitations [6,22]. Carrier-free immobilized enzymes are advantageous catalysts in processes requiring high yields and productivities, where enzymes cannot be stabilized through the conventional solid supports [23].

Although there are many immobilization strategies, none of them overcome all enzymes’ limitations in all kinds of processes. The selection of specific strategies of immobilization will depend on many factors such as limitations inherent to the enzyme and the process in which it will be used [26]. Thus, the selection of the best enzyme immobilization technique will depend on its application demands as well as the balance between cost-production and the potential uses of the immobilized biocatalyst on its direct application. In this context, cross-linked enzyme aggregates (CLEA) technology has been successfully used through the last fifteen years over a wide range of enzymes, and has proved to be an easy, fast, efficient and suitable approach in enzyme optimization; therefore, in this mini-review we summarize a battery of examples where enzymes properties has been improved by this technology.

2 Carrier-free immobilization types, advantages and disadvantages.

The carrier-free immobilized enzymes do not need an extra inactive mass, the support. They are usually produced by direct cross-linking of dissolved enzymes (CLE), crystallized enzymes (CLEC), and aggregated enzymes (CLEA).

The first report of such structures was described by Quiocho and Richards [25], who found that insoluble proteins linkage could be obtained by using bifunctional cross-linking chemical groups, such as glutaraldehyde. These linked proteins known as cross-linked enzymes (CLE) retained their catalytic activity. When the link is based on purified enzymes, linked proteins are known as cross-linked enzyme crystals (CLEC) [26]. However, at the time the work of Quiocho and Richards was published, the efforts in immobilization research were focused towards immobilization by adsorption, entrapment and chemical bonding to supports, due to the low activity and stability retention that CLE exhibited. These structures began to be studied more intensively in the 1990’s [11,27].

The CLE are formed with proteins in solution that are linked together by a cross-linking agent having sizes from 1 to 100 μm. Compared to the native enzymes, this type of biocatalyst improves their thermal stability, but requires the care of various factors such as pH, the amount of cross-linking agent, ionic strength and temperature. However, CLE present disadvantages such as low native activity retention (usually less than 50%), poor reproducibility, and low mechanical stability [6]. These inconveniences have been partially overcome by entrapping CLE in a gel matrix or membranes [28-30].

Along with CLE, studies were directed to the production of CLEC, that are suitable for biotransformations in non-aqueous media, due to its high stability under harsh conditions [25,31]. CLEC are crystalline proteins, bonded together with a cross-linking agent. They achieved improved mechanical strength and high stability against temperature, pH, organic solvents and proteolysis [22,32,33]. For instance, lipase-CLEC have been developed in order to improve drug delivery under natural low-pH gastric conditions [34]. The catalytic activity obtained
with CLEC can be improved by controlling the size of the enzyme crystals. It was found that minimizing the crystal size increases the catalytic activity retained. However, it requires laborious steps of crystallization of proteins, which increases the production’s cost [35]. Compared to crystallization, a less expensive method to aggregate the enzyme molecules and further cross-linking is precipitating the proteins by adding salts, organic solvents or non-ionic polymers [20,36]. This idea was first introduced with penicillin G amidase in the Sheldon’s laboratories [37] and subsequently marketed by CLEA Technologies (Holland). Cross-linked enzyme aggregates (CLEA) are obtained from non-purified precipitated enzymes forming solid particles that remain together through non-covalently binding. These CLEA remain permanently insoluble after covalent binding with a cross-linking agent such as glutaraldehyde (Fig. 1) [27,37]. CLEC and CLEA exhibit 10 to 1000 times greater activity (U g⁻¹ of biocatalyst) than their corresponding biocatalysts attached to a support [22,38-41]. Consequently, the use of carrier-free immobilized enzymes could be beneficial for processes requiring high productivity and yield, long stability and for labile enzymes that cannot be efficiently stabilized by traditional methods of immobilization on supports. Besides, the use of carrier-free immobilized enzymes is clearly advantageous since it is possible to feed reactors with a higher amount of enzyme, which compensates the loss of activity during reuse, without prolonging the reaction time [6,42]. In contrast, the enzymes immobilized on supports occupy more than a 10–20% volume of the reactor, which causes the prolongation of reaction time in order to achieve the same conversion levels [43]. The selectivity of carrier-free immobilized enzymes as CLEC and CLEA strongly depends on the conformation of the crystals or the enzyme aggregates [37]. Crystallization or aggregation conditions to obtain CLEC or CLEA can alter the selectivity and/or activity of biocatalysts [21]. However, although CLEA eliminates the need for crystallization of the enzyme of interest, there remains an inherent disadvantage; since a precise protocol of aggregation and cross-linking must be established for each enzyme, implying that significant efforts must be made to optimize preparation conditions [44]. CLEA stability and activity are similar to those of CLEC [6,37]. The catalytic properties of CLEA depend on the precipitating agent. Different precipitating agents induce distinct conformations of enzyme aggregates, in such a way that the selectivity of CLEA strongly depends on the conformation of the enzyme aggregates [37].

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Simple process for the preparation of CLEA in an organic solvent. Reprinted from reference [70] with the permission of Springer (license number 3687170268753).
Similar to carrier-immobilized enzymes, CLEA present a better stability than the free enzyme under severe conditions of reaction such as pH, organic solvents, temperature, and higher resistance to substrates and inhibitory products or denaturants [20,45,46]; attributes that turn them into promising insoluble biocatalysis for industrial applications.

An important property of CLEA for their large scale application is the particle size, which has a direct effect on mass transfer limitations and filterability [47]. Typically, the particle size of a CLEA varies between 0.1−200 μm. However, during its application in industrial processes, where recovery operations such as filtration and centrifugation are required, increase in size particle will promote mass transfer limitations with the consequent reduction in its overall activity [48]. Among the factors determining the CLEA size, the amount of enzyme and the concentration of the cross-linker play a major role [21]. Both parameters can modify the final result of the biocatalyst, as reported for CLEA of lipase from Candida rugosa, whose optimum activity in the hydrolysis of lauric acid was obtained with 40−50 µm particles [49].

According to their morphology, CLEA can be classified as: Type I. These CLEA measure around 1 µm in diameter and are formed from low glycosylated enzymes with large hydrophobic surfaces such as CAL-B, which contains $8 \times 10^8$ enzyme molecules per CLEA and forms aggregates of a spherical shape (Fig. 2A); and Type II. Which measure less than 0.1 µm in diameter and are formed from enzymes with large hydrophilic surfaces and high glycosylation levels, such as lipases from Candida rugosa and oxynitrilase from Prunus amygdalus. These type of CLEA present around $1 \times 10^4$ enzyme molecules per CLEA, and are aggregates of slightly irregular shapes and are smaller than those in type 1 (Fig. 2B) [50].

CLEA might form aggregates of larger size (> 1 µm), called “clusters”, presenting mass transfer limitation. Future studies are aimed at the formation of CLEA with fewer tendencies to form “clusters”. It has recently been reported that increasing the stirring speed and time avoids the formation of “clusters” as they decrease the size of CLEA without inactivating them, achieving an increase in mass transfer and allowing 100% recovery of the original activity of the free enzyme. With this purpose, the reciprocating cell disruptor (FastPrep®) has been used. In comparison with the use of a vortex, use of FastPrep® allows decreasing the size of CLEA in just 30−60 s of treatment, thus recovering 100% of activity. In contrast, the use of a vortex requires stirring for 90 minutes to recover 80% of the initial activity [48,51,52]. Another effort to increase CLEA’s mass transfer proved that porous CLEA (p-CLEA) can be obtained by adding starch as a pore-forming agent, which at the end of the process was removed with α-amylase [53]. This will allow the use of macromolecular substrates.

Despite the advantages of carrier-free immobilized enzymes, there are certain inconveniences to solve since no one knows exactly how to control the size of enzyme aggregates, how to increase their flexibility, as well as the exact methodologies to modulate the stability, selectivity and activity [6]. Unfortunately, the design of immobilized enzymes for its optimal performance is still largely empirical [27] and deep characterization studies are missing.

3 Cross-linking chemical nature and most employed cross-linkers.

Glutaraldehyde (GA) is the cross-linking agent most frequently used for the preparation of CLEA, since it
achieves high conversion efficiency, besides being low cost and having high market availability [47]. However, with some enzymes, such as nitrilases, retention of enzymatic activity is very low or almost null when using this dialdehyde as cross-linking agent [27]. Mateo et al. tested a large polyaldehyde molecule (dextran-polyaldehyde) [54]. CLEA formed with this cross-linker presented higher activity (10−90 times) in comparison to the one commonly formed with GA. Furthermore, CLEA of nitrilases cross-linked with GA totally lose their activity; such effect is attributed to the small size of the agent (0.1 kDa) that manages to go inside the protein and destroy its tertiary structure, resulting in the complete loss of activity. The latter effect was not observed with the dextran polyaldehyde, which is a long chain polymer (100−200 kDa). However, despite being less denaturing, the dextran polyaldehyde (DP) is not always superior to GA. Valdés et al., [55] compared the activity, size, stability and reusability of CLEA of lipase PS from Burkholderia cepacia cross-linked with GA and DP, finding that the small dialdehyde allows obtaining smaller, properly cross-linked, active, stable and reusable biocatalysts than those obtained with the larger dextran polyaldehyde.

Few studies have explored other cross-linking agents different from GA. Among them, the work of Mateo et al., [54] used for the first time the dextran polyaldehyde. Latter, Miletic et al., [56] prepared CLEA of the lipase from CAL-B using diepoxides of different chain length finding a strong dependence of the thermostability of the enzyme with the degree of cross-linking and chain size of the diepoxide used. Recently, Wang et al., [57] reported the use of p-benzoquinone as a cross-linking agent of the recombinant lipase of Geobacillus sp., which is five times more thermostable at 50°C for 90 minutes than CLEA prepared with GA. Similar results were obtained with L-lysine as the cross-linker for the preparation of higher biocompatible peroxydase and urease-CLEA [58]. However, all these cross-linker agents are based on the formation of covalent bindings between protein-amino groups and the reactive group of the cross-linking agents. More recently, Talekar et al., [59] showed that pectin is a more suitable cross-linker of glucoamilase than GA, attaining more active and stable pectin-CLEA than GA-CLEA.

Another available alternative is the cross-linking of carboxyl-activated protein amino groups with chitosan as cross-linker for lacasse-CLEA [60] and polyethylenamines for lipase-CLEA [61]. The carboxyl-activated cross-linking of proteins has the advantage that carboxyl groups are more abundant than amino groups. Thereby, a higher chemical modification degree is attained with the consequent increase in protein rigidity and thermal stability [61,62].

On the other hand, the concentration of cross-linking agent regulates the degree of cross-linking and therefore modifies the final structure of the CLEA, a result impacting their activity and stability. Wilson et al. [63] found that excess in the degree of cross-linking sites reduces productivity, stability and performance of an immobilized enzyme of CLEA of penicillin acylase for the production of cephalixin. Majumder et al., [64] demonstrated that activity, stability and enantioselectivity of CLEA of lipase from Pseudomonas cepacia, can be significantly modified by using different GA concentrations. Their findings show that there is an increase in the thermostability of CLEA at a higher degree of cross-linking; however, activity and enantioselectivity are compromised, because of the higher degree of enzyme rigidity. They also proved that the morphology of the CLEA depends on the cross-linking degree, which increases by incrementing the concentration thereof, in turn increasing the degree of rigidity of the molecule resulting in lower catalytic activity. In addition, cross-linking time and temperature are also implicated in the final activity of CLEA, since both higher times [65,66] and temperatures [60,67] drive higher cross-linking degrees thus altering this property. pH also plays an important role in the step of cross-linking since GA can be in monomeric or polymeric forms depending upon the conditions of pH of the medium. The final products of reaction obtained in the step of cross-linking under alkaline and acidic conditions are different [68] since GA tends to polymerize at high pH values, which does not occur at pH 7 [55,63]. Also, Yu et al., [49] found that the size of the particle of CLEA of CRL is influenced by pH, having a larger size in alkaline medium.

4 Improvement of enzymatic catalytic features by CLEA's technology.

Several factors are responsible for the final catalytic behavior of CLEA. Some works have dedicated their efforts to describe and understand such factors and their effects on CLEA's technology preparation [21,47,69]. Herein, we summarize different strategies to improve of catalytic properties of enzymes via their immobilization as CLEA.

4.1 Enhancement of CLEA's activity

Activity of CLEA depends on several factors such as precipitant agent, additive, cross-linker, cross-linking time, enzyme concentration, temperature, pH and agitation,
among others. Precipitation agents and precipitation conditions play a crucial role in the preparation of CLEA. They can induce a more active conformation of the enzyme [70]. However, one cannot generalize the use of one precipitating agent for all enzymes since this agent can drive opposite results with different enzymes. For that, a variety of precipitating agents with each enzyme should be evaluated, taking into account that the optimal precipitant may not be the one that will ultimately produce the optimal CLEA [47].

A recent study demonstrated that during CLEA preparation, the period of time between the precipitation of enzymes and the cross-linking step influences a lot the structural organization of the resulting enzyme preparation. As an example of these different properties, CLEA of penicillin acylase (PA), fresh (which were cross-linked immediately after precipitation) and mature (which were cross-linked after spending seven days at 4°C), precipitating both with polyethyleneglycol (PEG6000) and cross-linking with GA were evaluated [71]. It was observed that mature CLEA presented larger sizes than the fresh CLEA; besides, kinetic studies showed that the mature CLEA were more effective in both synthesis and hydrolysis reactions. Therefore, they demonstrated that the size of the aggregates could regulate the extent of covalent modifications of the PA and thus influence the catalytic properties of their respective CLEA. Likewise, it has been found that the size of CLEA is also affected by the type and concentration of precipitating salts employed [49,51]. Organic solvents used as precipitant agents may be denaturing for enzymes. The damage caused by such agents is primarily because solvents remove water bound to the protein diminishing its flexibility. To solve this problem, Wang et al. [72] studied the preparation of CLEA in assisted precipitation with sugars such as glucose, trehalose and sucrose. Resulted CLEA were stabilized towards several denaturant solvents.

On the other hand, addition of additives during the precipitation step has been evaluated. Use of Triton X-100, sodium dodecyl sulphate and crown ethers has demonstrated their influence on the activity of CLEA [50,70,73]. Addition of Triton X-100 increases the activity of those lipases which tend to form dimers such as that of Thermomyces lanuginosus (TLL), whereas the Candida antarctica B (CAL-B) is hardly influenced by the presence of Triton X-100 [76].

The activation of lipases due to additives such as surfactants and crown ethers is generally attributed to the freezing and more active conformation of the enzyme during the preparation step [75]. Since additives are not covalently bound to the enzyme, they can be easily rinsed from CLEA using an appropriate organic solvent leaving the immobilized enzyme in a fixed favorable catalytic conformation [27].

CLEA preparation from proteins with low content of lysine becomes difficult because lysine residues play a main role in the cross-linking step when GA is used. This problem can be solved by precipitating proteins with polymers containing primary amino groups, such as polylysine [76] and polyethyleneimine [74,77]; the latter forms a positively charged microenvironment that prevents the contact between the organic solvents with the protein providing it with greater stability in the presence thereof [78,79]. Another alternative is the use of bovine serum albumin (BSA) as a protein feeder that serves as source of protein and amino groups to carry out the cross-linking of the enzyme of interest [64,80]. It has been reported that CLEA prepared in the presence of BSA retain higher catalytic native activity than those prepared without BSA. Addition of BSA to obtain CLEA of lipase from Pseudomonas cepacia results in obtaining 100% of its native activity, while the same immobilized enzyme with GA in the absence of BSA retained only 0.4% [80]. The addition of BSA in the preparation of CLEA improves the performance of cross-linking, the size and flexibility [52], and stability of the CLEA [61,65,80]. Moreover, the addition of BSA to the CLEA preparation prevents proteolysis, increasing the biocatalysts stability [81].

Bioimpression with specific substrates during CLEA preparation allows maintaining native enzyme activities favoring the cross-linking of more active configuration. Imprinting substrate treatments have been applied for phenylalanine ammonia lyase (PAL) with trans-cinnamic acids, which were more active and stable in comparison with the free enzyme during the synthesis of L-phenylalanine [82]. Moreover, substrate imprinting might be an alternative for the protection of active residues during the cross-linking step as it was observed for lipase CLEA imprinted with trioctanoin, which were 2-fold more active in the presence than in the absence of the employed triglyceride [61].

4.2 Enhancement of CLEA’s stability

Frequently, the low native enzyme stability under large-scale reaction conditions is a main drawback hampering their fast incorporation to industrial bioprocesses. Hence, stabilization of native enzymes at several harsh production conditions must be implemented. Temperature and pH are operational parameters with detrimental effects on enzyme activity at the industrial level. Enzyme stability
towards denaturing temperatures is related to the degree of rigidity of the molecule [83]. Namely, the greater number of inter and intra molecular covalent bonds, the higher the thermal stability. Therefore, the covalent immobilization of enzymes is highly correlated with the stabilization of enzymes towards temperature. Several examples of thermostabilization are described by immobilization of different enzymes as CLEA [46, 60, 62, 84]. During CLEA preparation, the effect of cross-linking time, cross-linker concentration and cross-linker type are directly implicated in the thermostabilization effect of the final CLEA. Thermostability might also depend on the type of precipitant agent used for CLEA obtention. Thermostability of penicillin G acylase CLEA precipitated by tert-buthanol was higher than that obtained with DME and PEG [85]. On the other hand, the preparation of CLEA in the presence of PEI (as an additive or cross-linker) has demonstrated beneficial effects increasing thermal stability of lipase CLEA [61, 86].

Another weakness of native enzymes is the negative effect of high substrate or product concentrations. In regards, CLEA's approach has been used as an effective tool to reduce substrate or product inhibition. Cu et al., [82] demonstrated that imprinted CLEA of phenylalanine ammonia lyase imprinted with trans-cinnamic acids significantly reduces substrate inhibition.

Besides temperature and substrate/product inhibition, acid or alkaline conditions are important issues in enzyme stabilization. Enzymes are more active at certain pH intervals, showing maximum activity at a specific pH value. However, several hydrolytic and synthetic reactions entail acidic or alkaline conditions that frequently denature enzymes, thus hampering their direct application. CLEA immobilization has also been successfully used to enhance the stability of enzymes at a broader pH range, for instance, the CLEA of peroxidase from Brassica rapa present higher activity at acidic conditions as compared to the free enzyme [87]. On the other hand, enhancement of stability of glutamate decarboxylase at alkaline values allows the production of γ-amino butyric acid which is characterized by the pH increase during the reaction [88]. A similar effect was observed for the CLEA of phenylalanine lyase during the synthesis of L-phenylalanine [82].

Since Klibanov demonstrated that hydrolytic enzymes catalyze a large number of synthetic reactions in organic solvents, scientists began the intensive research of stable biocatalysts in the presence of denaturing solvents. In this field, CLEA technology has also confirmed its competence in providing solvent-resistant enzymatic biocatalysts [42, 89]. The addition of charged polymers as PEI during CLEA preparation has protective effects against solvent denaturation since it hampers the contact of the solvent and the enzyme [74, 79, 90].

As well, industrial biocatalysts must entail high storage stability. To this aim, CLEA have demonstrated high storage-stabilization of enzymes. For instance, the amyloglucosidase-CLEA maintained 98% of its initial activity after 60 days at 4°C, whereas the free enzyme lost more than 50% of its initial activity after 5 days at the same storage conditions [91]. Moreover, protease-CLEA, which are applied in sea paints, have been well stabilized under artificial seawater exhibiting a hyperactivation of 900% after 28 days of storage at such oxidizing conditions [92]. Other good examples of high storage stability of CLEA are also addressed in literature [93, 94].

### 4.3 Enhancement of CLEA's selectivity

Enzyme selectivity is one of the attractive catalytic features that make enzymes into powerful tools in the preparation of fine valuable compounds. Hence, the modification and modulation of a desired selectivity is the main goal of the scientific community. Recently, Sheldon has summarized several enantioselective asymmetric synthetic reactions catalyzed by CLEA of lipases, proteases, amidases, esterases, nitritases, peroxidases and nitrile hydratase [89]. The improvement in enzyme selectivity is related to several factors. Particularly, Majumder [64] demonstrated that GA concentration affects differently the activity, stability and enantioselectivity of CLEA of lipase from Pseudomonas cepacia.

The selectivity of the biocatalyst is also affected by the use of additives during the preparation of CLEA. Wilson and coworkers [74] evaluated the enantioselectivity of CLEA of lipase from Alcaligenes sp. in presence of polyethyleneimine and dextran sulphate as precipitating agents; addition of Triton X-100 modify the activity and enantioselectivity in the hydrolysis of glycidyl butyrate. Furthermore, the addition of BSA during lipase-CLEA preparation also showed a marked effect on the enzyme enantioselectivity during the hydrolytic resolution of S-mandelic acid [61]. Moreover, it has been recently reported that enantioselectivity and activity of magnetic-CLEA of Yarrowia lipolitica lipase were highly influenced by an alternating magnetic field during the resolution of S-2-octanol [95]. This latter effect was mainly attributed to the behavior of magnetic-CLEA as microscopic stirrers.
5 Combi-CLEA in cascade-reactions.

Nowadays, cascade reactions represent an advantageous approach for the preparation of multistep reaction processes which may be conducted in a single step instead of separate operations [96]. Immobilized multi-enzymatic systems offer several advantages including avoidance of the accumulation of unstable intermediates, reduction of secondary undesirable reactions, achievement of mult cascade reaction processes and the regeneration in situ of cofactors [24]. Similar strategies are used to produce combi-CLEA, which contain more than one type of enzyme. Combi-CLEA have been successfully employed for cascade reactions as the resolution of S-mandelic acid [97,98] and in processes with multipurpose reactions. Examples of that are combi-CLEA of pectinase, xylanase, and cellulase which perform the three activities at the same time [99] and combi-CLEA of several feruloyl esterases for the production of alkyl ferulates [100]. Besides the combi-CLEA approach has also been applied for the biodegradation of acetaminophen in wastewaters by the combined action of laccase and tyrosinase based CLEA, which exhibited high stability under harsh conditions of temperature and chemical denaturation [101].

Some multistep reactions frequently imply the formation of labile intermediates that must be protected in order to prevent their degradation. Combi-CLEA composed of (S)-hydroxynitrile lyase and a nitrilase were excellent examples of such application. This combi-CLEA were applied in the two step cascade synthesis of (S)-2-hydroxycarboxylic acid amides, that needs the prevention of the racemization of the (S)-2-hydroxynitrile intermediate by the protection of the nitrile-converting enzyme [102]. The latter bioconversion has been also achieved with whole cells as catalysts; however the combi-CLEA strategy presents higher stability in the required solvent (diisopropylether) and is an easier recycling biocatalyst than the entire cells [103].

6 Coupling CLEA with solid matrixes and nano-particles.

Carrier-free immobilized enzymes have the disadvantage that they are not yet fully scalable to industrial levels since they exhibit low mechanical stability against shear stress and harsh conditions of stirring [21,69,104].

In order to improve the mechanical stability of CLEA, several research groups have worked on the encapsulation, coating and entrapment of these in polymers and matrices that allow them to increase their stability. Moon et al. [105] prepared chymotrypsin and Mucor javanicus lipase CLEA that were trapped inside the pores of a mesoporous mesocellular silica matrix with pores hierarchically ordered (HMMS). This material has cavities of 37 nm where the CLEA are retained after having been cross-linked with GA and cannot get out because each of these small holes is joined by channels of 13 nm in diameter through which the CLEA cannot pass through and are retained in the pores. With this type of entrapment, it was possible to increase the stability of CLEA up to 2 weeks stirring at 200 rpm, which prevented lixiviation of CLEA of the support and the inhibition of chymotrypsin autoysis.

Likewise, penicillin acylase CLEA were encapsulated inside a hydrogel of rigid polymer matrix of polyvinylalcohol (LentiKats®) which turned out quite stable in organic solvents, while maintaining 95% of its activity during 50 days in mechanical stirring at 20°C [104].

A different alternative is the stabilization through the cross-linking of CLEA with amino-functionalized magnetic nanoparticles (magnetic-CLEA) [106] or by direct cross-linking of enzymes on magnetic particles (CLEMPA) [107]. Such structures have driven biocatalysts with higher operational stability, easier separation and reutilization, besides CLEA clumping is avoided [69]. Further, CLEA have also been cross-linked to magnetic beads as magnetic-coated mesoporous silica maintaining their initial activity after shaking during 35 days and the biocatalyst could be also recycled 35 times [108].

Immobilization technology in the form of CLEA has proven to be successfully applicable to many and varied enzymes, becoming a new option for the tools catalogue of industrial biocatalysis. In this area, some types of reactor configurations have been proposed such as those of membranes in which the immobilized aggregates remain retained and substrates and products are pumped through the reactor, [109,110] the ones of microchannels in which a CLEA membrane is formed around the surface of a microchannel [111] and those of a perfusion basket [112]. Also, CLEA have been employed in continuous reactors [113].

7 Conclusions, future challenges and trends.

Within the vast range of immobilization techniques, cross-linked enzyme aggregates (CLEA) offer attractive advantages such as simplicity of preparation, low production cost and fast optimization. CLEA technology has proven to be applicable to a wide range of enzymes turning it into a useful tool for the catalogue of biocatalysis.
processes. Nevertheless, there will be numerous studies aimed to improve CLEA’s stability, performance in industrial processes and finding new cross-linking agents. Likewise, for rational modulation of the selectivity, activity and stability of enzymes, future efforts will focus the understanding of the phenomena governing the behavior and performance of enzymes in the immobilized form.

References


Galvis M., Barbosa O., Ruiz M., Cruz J., Ortiz C., Torres R., Fernandez-Lafuente R. Chemical amination of lipase B from Candida antarctica is an efficient solution for the preparation of crosslinked enzyme aggregates. Process Biochem., 2012,47,2373-2378.


Cross-linked enzyme aggregates (CLEA) in enzyme improvement – a review


