Biotechnology Advances 70 (2024) 108304

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Proteases immobilized on nanomaterials for biocatalytic, environmental and biomedical applications: Advantages and drawbacks

Muhammad Bilal [a,b,**], Sarmad Ahmad Qamar [c], Diego Carballares [d], Ángel Berenguer-Murcia [e], Roberto Fernandez-Lafuente [d,*]

[a] Department of Sanitary Engineering, Faculty of Civil and Environmental Engineering, Gdansk University of Technology, G. Narutowicza 11/12 Str., 80-233 Gdansk, Poland
[b] Advanced Materials Center, Gdansk University of Technology, 11/12 Narutowicza St., 80-233 Gdansk, Poland
[c] Department of Environmental, Biological & Pharmaceutical Sciences, and Technologies, University of Campania 'Luigi Vanvitelli', Via Vivaldi 43, 81100 Caserta, Italy
[d] Department of Biocatalysis, ICP-CSIC, C/ Marie Curie 2, Campus UAM-CSIC Cantoblanco, Madrid, Spain
[e] Departamento de Química Inorgánica e Instituto Universitario de Materiales, Universidad de Alicante, 03080 Alicante, Spain

ARTICLE INFO

Keywords:
Proteases
Immobilization
Nano-supports
Nanobiocatalysts
Green biotechnology

ABSTRACT

Proteases have gained significant scientific and industrial interest due to their unique biocatalytic characteristics and broad-spectrum applications in different industries. The development of robust nanobiocatalytic systems by attaching proteases onto various nanostructured materials as fascinating and novel nanocarriers has demonstrated exceptional biocatalytic performance, substantial stability, and ease of recyclability over multiple reaction cycles under different chemical and physical conditions. Proteases immobilized on nanocarriers may be much more resistant to denaturation caused by extreme temperatures or pH values, detergents, organic solvents, and other protein denaturants than free enzymes. Immobilized proteases may present a lower inhibition. The use of non-porous materials in the immobilization prevents diffusion and steric hindrances during the binding of the substrate to the active sites of enzymes compared to immobilization onto porous materials; when using very large or solid substrates, orientation of the enzyme must always be adequate. The advantages and problems of the immobilization of proteases on nanoparticles are discussed in this review. The continuous and batch reactor operations of nanocarrier-immobilized proteases have been successfully investigated for a variety of applications in the leather, detergent, biomedical, food, and pharmaceutical industries. Information about immobilized proteases on various nanocarriers and nanomaterials has been systematically compiled here. Furthermore, different industrial applications of immobilized proteases have also been highlighted in this review.

1. Introduction

1.1. Enzyme immobilization

Enzymes are biological catalysts that perform essential functions in different metabolic processes and are considered "green catalysts" due to their environmentally friendly nature, extreme selectivity and specificity, and extraordinary activity under mild conditions (Hauer, 2020; Schmid et al., 2001; Sheldon and Brady, 2019; Sheldon and Woodley, 2018). However, this biological origin of enzymess is the source of many problems for their industrial implementation. Enzymes are naturally designed to fulfill their physiological role under stress conditions. However, industry intends to use them under fixed and controlled conditions. Enzyme regulation is no longer necessary in this prospect, but it has become a problem. Enzymes are unstable and become inhibited by-products, substrates, and other reagents. Moreover, most enzymes with industrial interest are soluble, making their reuse and recovery complex (Schoemaker et al., 2003).

Enzyme stability and kinetic features may be tailored using many different tools. Metagenomics opens the possibility of utilizing all biodiversity (past and present enzymes may be utilized) (Fernández-Arrojo et al., 2010; Ferrer et al., 2008; Ferrer et al., 2016), directed

* Corresponding author.
** Corresponding author at: Department of Sanitary Engineering, Faculty of Civil and Environmental Engineering, Gdansk University of Technology, G. Narutowicza 11/12 Str., 80-233 Gdansk, Poland.
E-mail addresses: muhammad.bilal@pg.edu.pl (M. Bilal), rfl@icp.csic.es (R. Fernandez-Lafuente).

https://doi.org/10.1016/j.biotechadv.2023.108304
Received 25 August 2023; Received in revised form 30 November 2023; Accepted 15 December 2023
Available online 21 December 2023

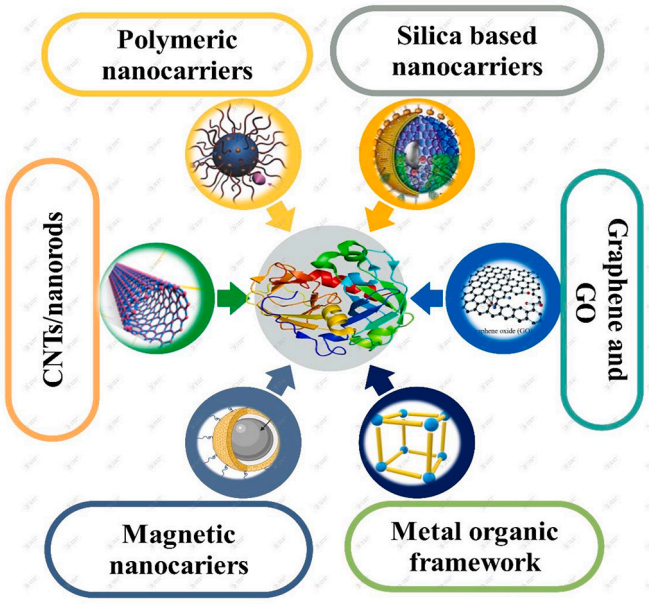

Fig. 1. Schematic representation of some of the most common nanomaterials used in enzyme immobilization.

evolution permits to improve a specific feature under the desired conditions mimicking natural evolution (Arnold and Volkov, 1999; Currin et al., 2015; Denard et al., 2015). Site-directed mutagenesis enables the improvement of the active center (Mateljak et al., 2019; Petrenko et al., 2020) or even the creation of new activities (Panis et al., 2020; Tong et al., 2021). Nowadays, enzymes bearing several active centers may be created (Carballares et al., 2022a), e.g., the case of pluryzymes (Alonso et al., 2020; Roda et al., 2022; Santiago et al., 2018). Enzyme chemical modification is more directed and efficient every day in altering enzyme features (Díaz-Rodríguez and Davis, 2011; Giri et al., 2021; Pagar et al., 2021).

Immobilization is a technique that was developed to permit enzyme recovery and, if the enzyme remains active, to reuse the enzymes in biocatalysts that were costly in their first moments (DiCosimo et al., 2013; Liese and Hilterhaus, 2013; Sheldon and van Pelt, 2013). Immobilization of enzymes may also simplify the down-stream processing and allow the use of a variety of reactor configurations and a stricter control of the reaction, avoiding product contamination by the enzyme (very important in food technology to prevent allergic reactions). Due to the considerable improvements in enzyme production, enzyme reuse is no longer required to make an enzymatic process viable (Bolivar et al., 2022). For example, Novozymes has launched a lipase to produce biodiesel (Eversa), designed to be used in free form and only represents 5% of the total process costs (Monteiro et al., 2021). Moreover, some strategies to recover and reuse free enzymes already exist (Woodley, 2017). However, enzyme immobilization has proved useful for far more than enabling enzyme recovery. Proper immobilization may improve enzyme stability for many different reasons, such as multipoint (Rodrigues et al., 2021) or multi-subunit immobilization (Fernandez-Lafuente, 2009), generation of micro-environments (Virgen-Ortiz et al., 2017), and fixation of more stable forms (Rodrigues et al., 2019), and this means widening the operational conditions range (drastic pH, temperatures, and presence of denaturing agents) (Rodrigues et al., 2021) and increase enzyme activity under drastic conditions (Dal Magro et al., 2019b; Dal Magro et al., 2020). If adequately designed, enzyme immobilization may be coupled to the purification of the enzyme (Barbosa et al., 2015). In

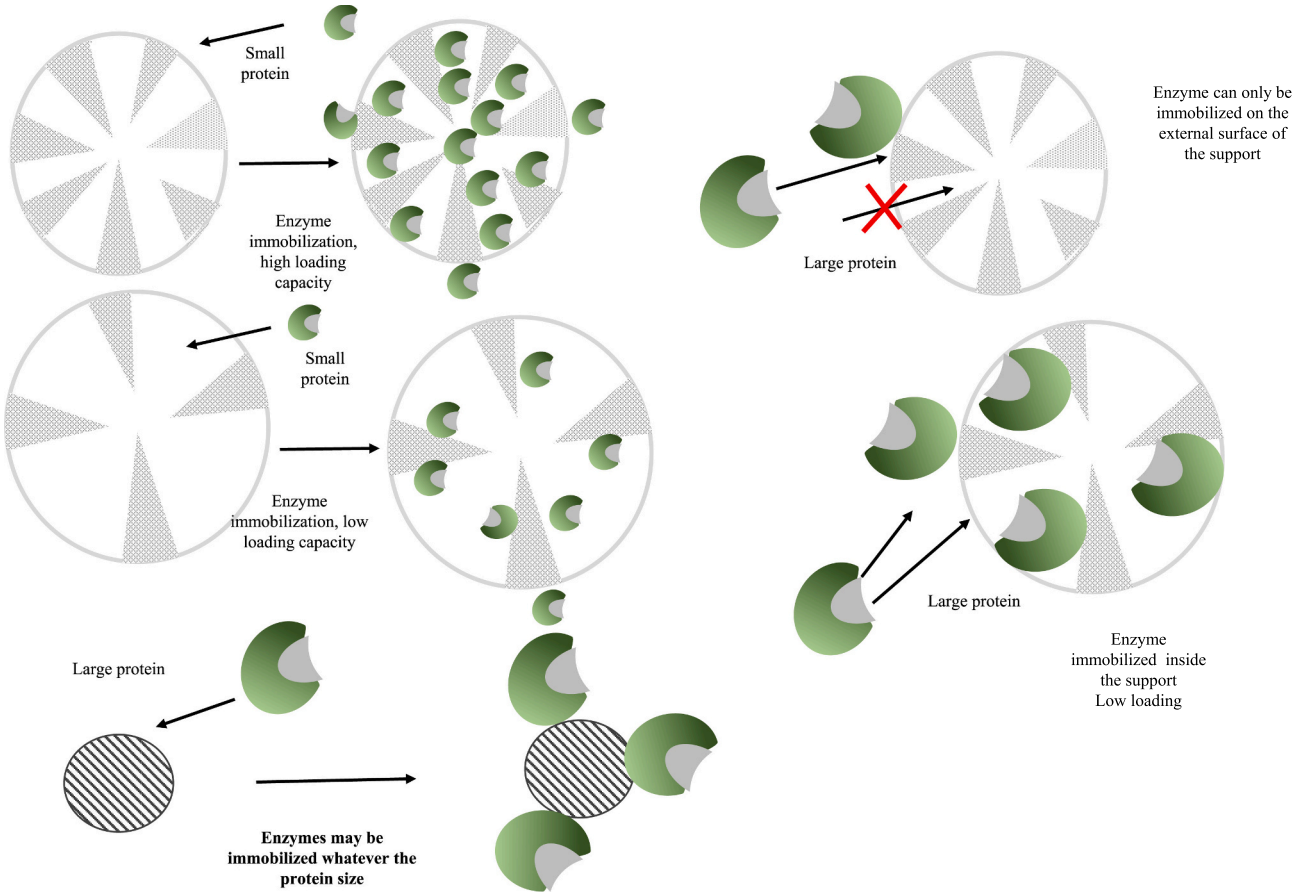

Fig. 2. Problems in the immobilization of large proteins in porous and non-porous matrices.

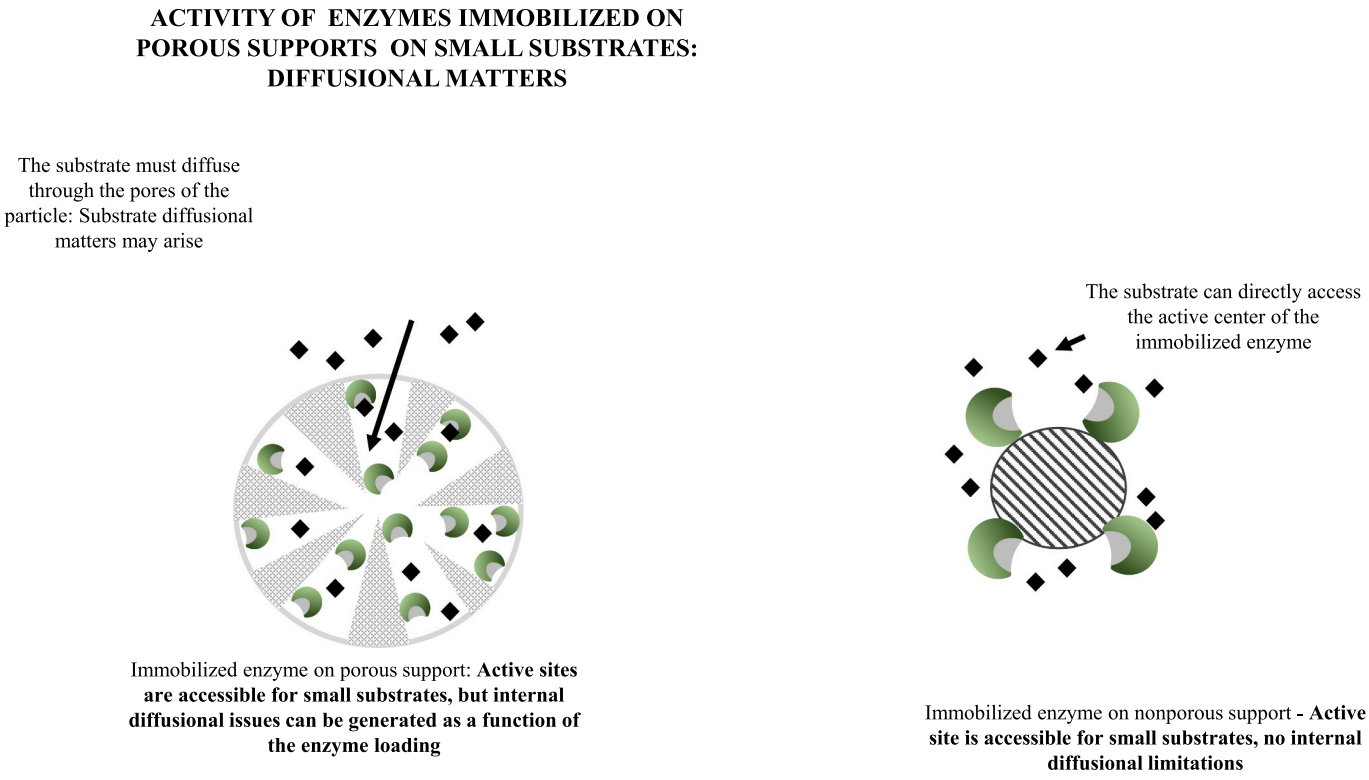

Fig. 3. Substrate diffusional limitations using porous and non-porous biocatalysts.

some instances, it is possible to immobilize hyperactivated forms of the enzyme, increasing enzyme activity (e.g., especially in the case of lipases immobilized through interfacial activation) (Manoel et al., 2015). Moreover, the enzyme interacts with the support surface, producing distortions (Souza et al., 2022; Souza et al., 2021). That way, new enzyme forms will be produced, in many instances with worse catalytic properties than the native enzyme, but if a high number of immobilization strategies are utilized, involving different immobilization events, immobilized enzymes with better selectivity, specificity or activity in a specific process may be achieved (Rodrigues et al., 2013). Enzyme inhibitions may also be decreased by diverse causes (Cowan et al., 1987; Mateo et al., 2004; Mateo et al., 2007). Very importantly, enzyme immobilization is compatible with all the previous techniques for improving enzyme features (Coscolín et al., 2018; Cowan and Fernandez-Lafuente, 2011; Madalozzo et al., 2015; Rodrigues et al., 2011; Rueda et al., 2016; Sánchez et al., 2021; Yao et al., 2014). Thus, enzyme immobilization becomes no longer just a means to recover the enzymes but may also become an essential step in preparing a biocatalyst with adequate features.

1.2. *Proteases in biocatalysis*

Protease enzymes can hydrolyze peptide bonds. They have impressive applications in a wide diversity of areas, ranging from food modifications (Mamo and Assefa, 2018; Tavano, 2013), cheese making (Alavi and Momen, 2020; Morellon-Sterling et al., 2020), and fine chemistry for the production of peptides, resolution of racemic mixtures (Tavano et al., 2018), textile (partial hydrolysis of fabrics) (Razzaq et al., 2019; Singh and Bajaj, 2017), medicine (Nitsche et al., 2014), production of antibody fragments (Morellon-Sterling et al., 2020) or biochemical studies (proteolytic maps), among others. One of the most important applications nowadays is the hydrolysis of proteins to achieve functional peptides (Castañeda-Valbuena et al., 2022; Görgüç et al., 2020; Karami and Akbari-Adergani, 2019; Nwachukwu and Aluko, 2019; Tacias-Pascacio et al., 2020).

As with any other enzyme, protease application may be improved if immobilized. They can be used in immobilized form even to produce milk clotting in the cheese-making industry, as long as some precautions are taken (e.g., performing the hydrolysis under coagulation temperature to prevent trapping of the immobilized enzyme) (Siar et al., 2020). However, they have some peculiarities that can make understanding the effects of their immobilization complex. When used to modify small substrates (e.g., in the synthesis of peptides, and resolution of racemic mixtures) (Tavano et al., 2018), they can behave as standard enzymes. However, when they are used as proteolytic agents, the substrate will be large and relatively rigid, perhaps larger than the enzyme. This will generate some problems using porous support.

The trouble with using immobilized enzymes is even bigger if applied in textiles to attack some solid fibers of textile fabrics. In this instance, it is required that the enzyme is correctly oriented (and at maximum loading; this may be more complex, as previously discussed) and, in the case of a solid, fully exposed to the medium. This reduces the utilization of proteases immobilized on porous supports. That way, the pores of the support where the protease is immobilized should be larger enough to permit the entry of the substrate, not only of the enzyme (Cipolatti et al., 2016; Garcia-Galan et al., 2011; Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021).

Using porous supports, protease immobilization can produce an enzyme with correct orientation, with the structure fully preserved but almost inactive in the target reaction because only the tiny fraction of enzyme molecules located on the support external surface will be active versus large substrates (Cipolatti et al., 2016; Garcia-Galan et al., 2011; Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021). As later discussed, this may be solved using nanomaterials to immobilize proteases. A second peculiarity of proteases is their capability to produce autolysis (Alexandre et al., 2001; Bickerstaff and Zhou, 1993; Kapust et al., 2001). This means that as they are also proteins, they can be attacked by other protease molecules and be proteolyzed. The proteolyzed enzymes may have lower stability or even change their selectivity or specificity. The immobilization on porous supports prevents this

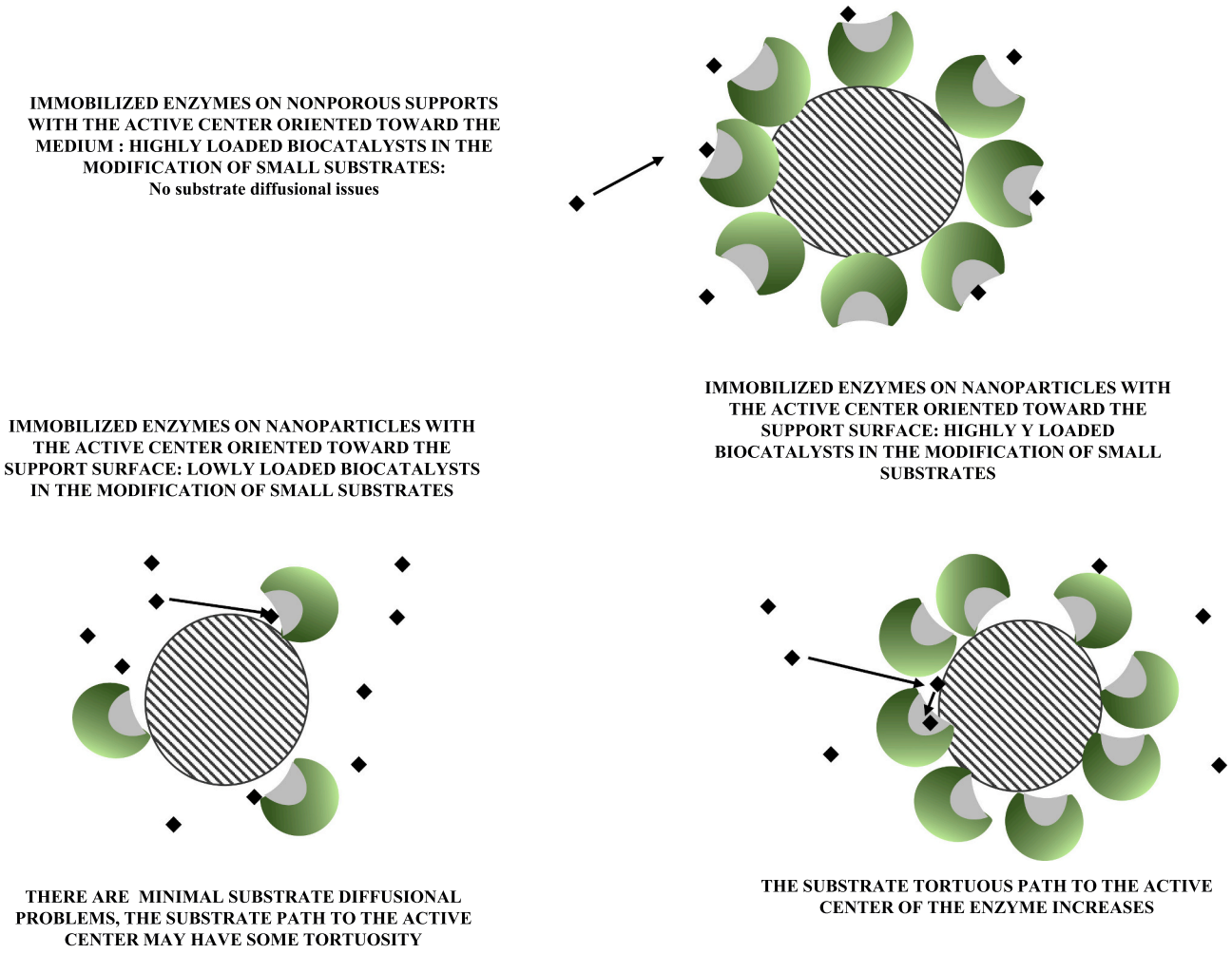

Fig. 4. Effect of enzyme orientation regarding the support surface and the enzyme loading on the substrate diffusional issues when immobilized on non-porous supports.

possibility entirely, as the enzyme will be dispersed and can no longer attack other enzyme molecules (except those on the external surface of the support, a very small fraction) (Garcia-Galan et al., 2011; Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021).

This protection is no longer offered when immobilizing the enzyme on a non-porous support, as all enzymes will be exposed to proteolysis (and to the interaction with interfaces and aggregation) (Cipolatti et al., 2016; Garcia-Galan et al., 2011; Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021). One academic problem in the prevention of protease autolysis is the increase in enzyme stability just by immobilization on porous supports (Bolivar et al., 2022). This can suggest that an increase in enzyme rigidity has been achieved when only enzyme proteolysis has been prevented, making the researcher reach the wrong conclusions (Rodrigues et al., 2021). Comparison of the target biocatalyst with a single point immobilized enzyme or in the presence of inhibitors may be a solution (Rodrigues et al., 2021). However, this is only of academic interest; the fact is that the operational stability of the immobilized proteases will be increased. In many instances, the use of proteases under drastic conditions is necessary. For example, to prevent contamination or because the enzyme is utilized to hydrolyze aggregated enzymes that are resolubilized in chaotropic agents. Only very stable biocatalysts will maintain the activity under concentrations of urea or guanidine able to resolubilize these aggregates, and the use of very stable enzymes further stabilized by multipoint covalent immobilization may be a requirement (Siar et al., 2017)

1.3. Bio-enzyme coordination with nanocarriers

Progress in nanobiocatalysis has devoted an impressive potential for refining enzyme functionality by immobilizing the enzymes on different nanocarriers (Ansari and Husain, 2012; Bilal et al., 2018; Ding et al., 2015; Jiang et al., 2009; Vaghari et al., 2016; Verma et al., 2013; Wang et al., 2009; Zhai et al., 2010). Nanobiocatalytic methodologies use several nano-structured constituents to immobilize enzymes, including nanoparticles (Bilal et al., 2018; Popat et al., 2011; Sharma et al., 2022; Vaghari et al., 2016; Zangi et al., 2023; Zhao et al., 2022), nanofibers (Khan et al., 2023; Rather et al., 2022; Ribeiro et al., 2021), nanotubes (Bilal et al., 2020; Feng and Ji, 2011), nanosheets (Monajati et al., 2018; Sáringer et al., 2021; Wang et al., 2016), nanocomposite scaffolds (Verma et al., 2013) (Fig. 1) for enzyme immobilization and the stabilization of their catalytic performance (Bilal et al., 2021; Reshmy et al., 2021).

Most of these are non-porous supports, bringing forth the pros and cons of these materials in enzyme immobilization (Cipolatti et al., 2016; Garcia-Galan et al., 2011). Taking as example nanoparticles, we can discuss some of these features. The main advantage is that by using non-porous nanoparticles, all enzyme molecules will be immobilized on the surface of the support (Cipolatti et al., 2016; Garcia-Galan et al., 2011). That way, any enzyme molecule can be immobilized on the support, independently of its size, while this did not happen using porous supports, where supports with larger pores must be utilized (Fig. 2) (Cipolatti et al., 2016; Garcia-Galan et al., 2011).

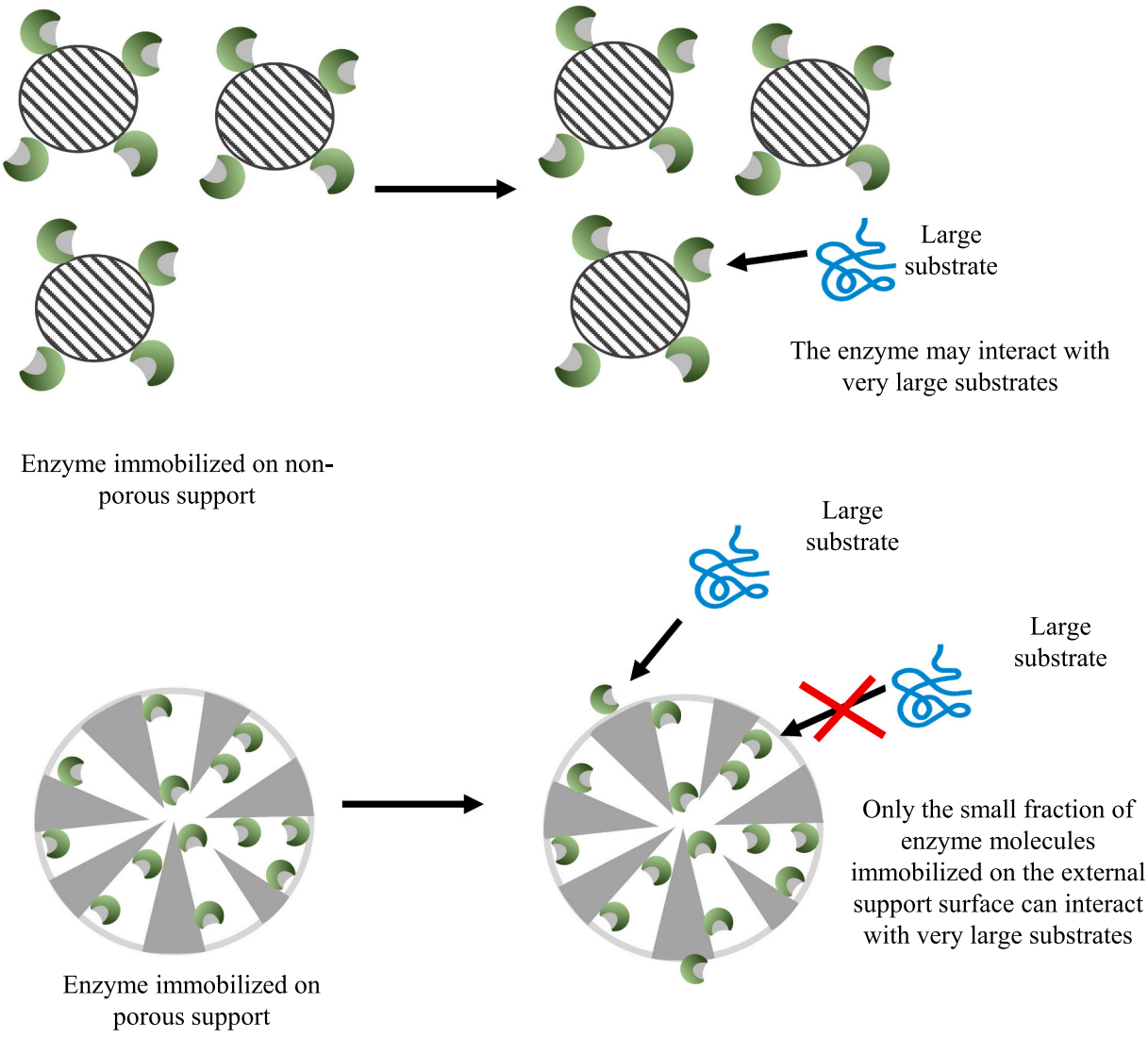

Fig. 5. Modification of very large substrates by enzymes immobilized on porous and non-porous supports.

Scarce substrate diffusional problems may be expected compared to the enzyme immobilized on porous support (Fig. 3). However, the use of fully loaded biocatalysts can present some small diffusional limitations even on small substrates when the enzyme does not present the correct orientation towards the reaction medium, as the substrate must diffuse between the two closely immobilized enzymes, increasing the tortuosity of substrate pathway to reach the enzyme active center (Fig. 4). This will not occur if the enzyme orientation concerning the active center is fully oriented towards the reaction medium.

Most importantly, as explained in the previous section, all of the immobilized enzyme molecules have access to any substrate, independently of their size, a clear advantage compared to porous supports (Cipolatti et al., 2016; Garcia-Galan et al., 2011) (Fig. 5). Using porous supports, only the tiny fraction of the enzyme that is immobilized on the outer surface of the bead can access very large substrates, meaning that <1% of the immobilized enzymes can attack the substrate. However, the fragments released from this first attack can penetrate inside the beads, and a progressive increase in enzyme activity may be detected (Fig. 6).

However, the activity cannot be taken for granted, even using non-porous nanomaterials. Only correctly oriented enzyme molecules will have the possibility of attacking large substrates (Cipolatti et al., 2016; Garcia-Galan et al., 2011; Hernandez and Fernandez-Lafuente, 2011) (Fig. 7). This proper orientation requirement is more important if the surface of the support becomes highly covered in an increasing amount of protease molecules (Garcia-Galan et al., 2011; Morellon-Sterling et al., 2020; Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021) (Fig. 8). Using low loadings, with protease molecules immobilized at large distances from one another, not fully correctly oriented enzyme molecules can access the large substrate; only when the active center is oriented towards the support surface, the activity will become null due to steric considerations (Fig. 8). (Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021). If we use a fully packed enzyme layer on the enzyme support, the enzyme molecules will have any activity versus larger substrates only as long as the enzyme active center has the correct orientation (Fig. 8). This is important as most immobilization methods give oriented enzyme molecules, even if in an uncontrolled way (Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021),

That means that we can have next to 100% activity using moderate enzyme loading, which drops to almost 0% using fully loaded biocatalysts (Garcia-Galan et al., 2011; Morellon-Sterling et al., 2020; Morellon-Sterling et al., 2022; Siar et al., 2017; Tacias-Pascacio et al., 2021). However, if the enzyme orientation is not entirely homogenous and some enzyme molecules are properly oriented, and others are not, the adequately oriented ones will attack the substrate. That means that the initial hydrolysis rate will be minimal. However, the small fragments released for the action of this fraction of enzymes may be attacked by some improperly oriented enzyme molecules, producing an increase in the reaction rate over time (Fig. 9). Properly oriented molecules at low

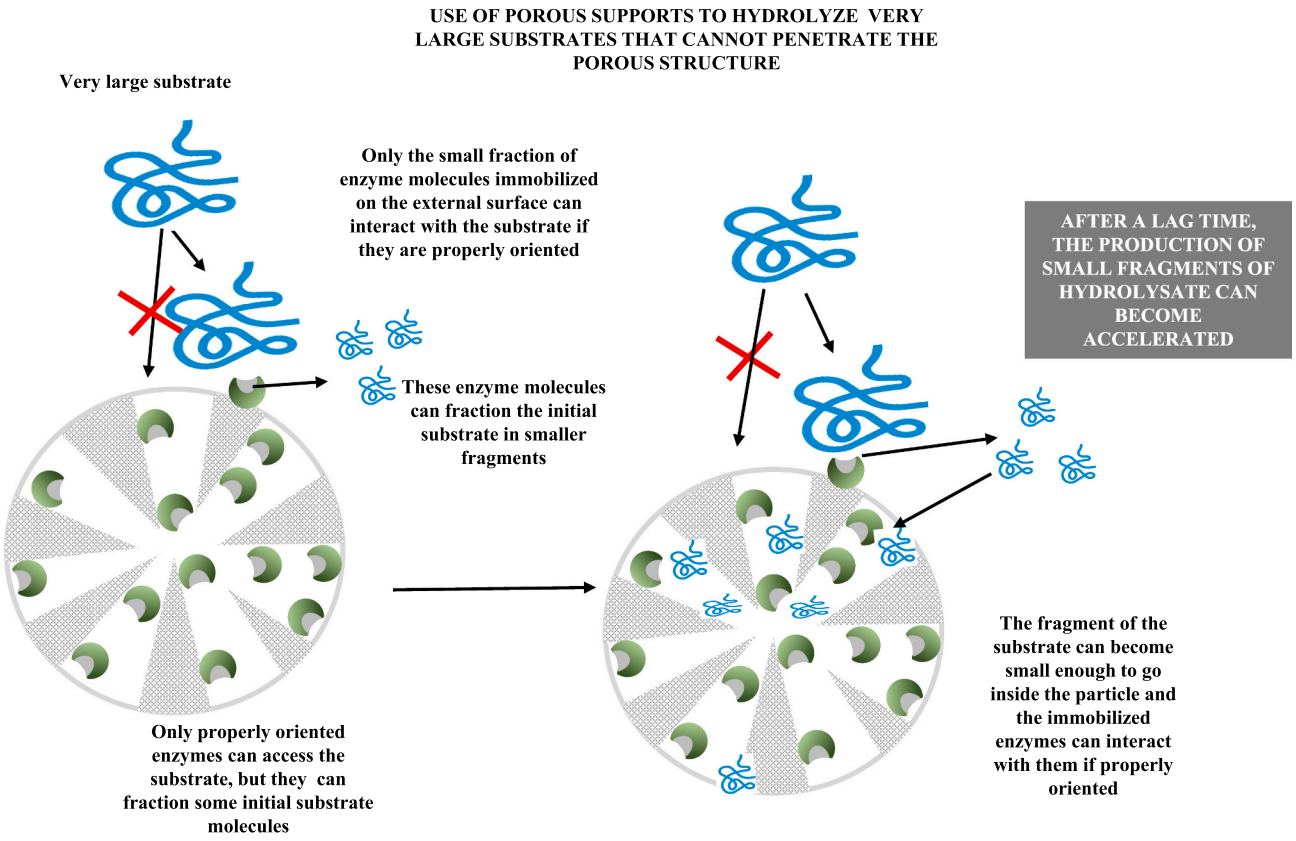

Fig. 6. Use of enzymes immobilized on porous supports to hydrolyze very large substrates: initially only the molecules located on the external surface of the bead can hydrolyze the substrate, but the fragments, of reduced size may go inside the porous structure of the support and become modified by the enzyme immobilized inside the porous structure.

loadings can attack from the first moment the large substrate and the smaller fragments (Fig. 10).

Activity recovery will not be too high using solids as substrates of the enzymes, such as fabrics, even under the best conditions (all enzymes are properly oriented). Only those enzyme molecules immobilized on one of the sides of the nanoparticle can attack the solid, as the other ones will not be in contact with it (Fig. 11). Using enzymes immobilized on suitable porous supports, only a small fraction on the surface may attack the substrate, and only those protease molecules on the support side may come in contact with the solid substrate. That can give initial activities lower than 50%, even if the enzyme molecules are perfectly active and well-oriented. Later, when some soluble fragments of the solid substrate are released, it becomes feasible for those enzyme molecules located in different areas to act (like the examples shown in Figs. 8 and 9). If the nanoparticle is magnetic, this has further advantages (Bilal et al., 2023; Bilal et al., 2018; Maroju et al., 2023; Vaghari et al., 2016). The immobilized enzyme may be used on a substrate composed of suspensions; there are no risks of pores obtrusion and, as the biocatalysts will be recovered by magnetism, the suspension of the substrate and the biocatalysts may be easily separated (Arco Arrieta et al., 2021) (Fig. 12). This situation may be widespread in food technology (juices clarification, for example) and constitute other of the "natural" niches of nanobiocatalysis (Dal Magro et al., 2019a; Dal Magro et al., 2018; Karataş et al., 2021; Kharazmi and Taheri-Kafrani, 2023).

Magnetic non-porous particles may permit a real one-step enzyme immobilization and purification of extracellular enzymes directly from the culture, as the presence of cells and debriding is not a problem in recovering the biocatalyst (Cao et al., 2017; Dold et al., 2016; Gennari et al., 2022; Jianping et al., 2003; Luo et al., 2021; Zhao et al., 2017) (the situation will be similar to the presence of solids in the reaction mixture, shown in Fig. 12). Using magnetic capture of the nanoparticles may generate a problem, as the breaking of the external surface of the nanoparticles can release non-magnetic fragments, bearing some enzyme molecules, or make the particle so small that it is not captured by the magnet, producing an apparently operational inactivation of the biocatalysts (Fig. 13).

Problems derived from the internal substrate/products/H^+ diffusion matters are unimportant using non-porous supports. The protease immobilized on non-porous supports can hydrolyze large and small substrates, and there are no discernible degrees in substrate concentration when going towards the core of the biocatalyst particles using high enzyme loadings as it occurs using porous supports (Bolivar et al., 2022) (Fig. 3). In this sense, as commented above, if the active center is oriented towards the support surface, even using a small substrate, there are some diffusional matters, mainly using fully loaded biocatalysts: the substrate must diffuse in the space between enzyme molecules (a short diameter pore, even being a pore of short length) and later into the pore formed by the enzyme and the support surface (Fig. 4). Using porous supports, this problem using highly loaded biocatalysts will be added to the problems of the entry of the substrate to the particle (Bolivar et al., 2022).

The lack of internal diffusional issues may not always be advantageous. For example, advantages of enzyme co-immobilization based on the promotion of concentration gradients of the product of the enzyme X that will be a substrate for enzyme Y are lost (however, synergy effects of co-immobilization will be maintained) (Arana-Peña et al., 2021; Monteiro et al., 2022). This turns enzyme co-immobilization on non-porous nanomaterials into a not-very-adequate biocatalyst design, considering the many problems derived from the design and utilization of co-immobilized enzymes (Arana-Peña et al., 2021). One option is to form enzyme successive layers ever the support surface, but there are no examples of this, and perhaps an enzyme layer is too short to promote a

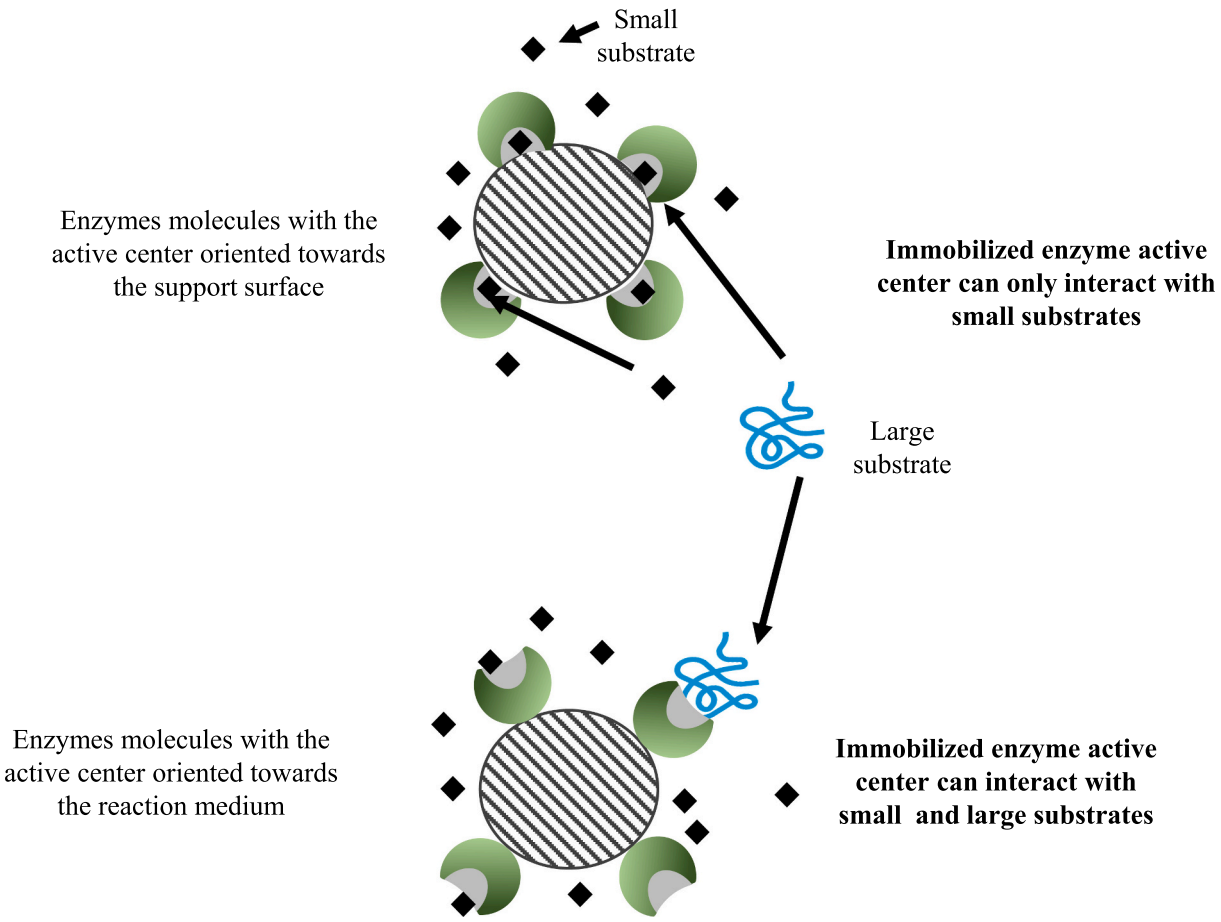

Fig. 7. Effect of the enzyme orientation on the modification of large and small substrates, when the enzyme is immobilized on a non-porous support.

real substrate gradient. It is impossible to fine tune over differences in internal and external pH values, although this possibility has been scarcely utilized even using porous supports. Still, it can have an undebatable interest. Using non-porous materials to immobilize enzymes and proteases presents some other specific problems, (Cipolatti et al., 2016; Garcia-Galan et al., 2011) as we will explain now. That means that nanomaterials should generally be selected as protease immobilization support if the advantages are evident (Cipolatti et al., 2016; Garcia-Galan et al., 2011).

First, as discussed in the previous section, non-porous supports expose the enzyme fully to the external medium. That means the enzymes could be rendered inactive by proteolysis, and interactions with interfaces (e.g., gas bubbles or drops of immiscible solvents); thus, one advantage of immobilizing enzymes is lost (Figs. 14–17). However, as previously discussed, this immobilization of the enzyme fully exposed to the medium is also the main reason for immobilizing enzymes on nanomaterials. Enzymes may be protected by coating with some polymer (Betancor et al., 2005) (Figs. 12–15), but this can also reduce the capability of the enzyme to attack large substrates in a similar way that cannot attack other protease molecules (but this may be interesting when using a suspension where a small substrate is present) (Fig. 18). If the enzyme orientation is not the "correct" one, it is possible to reduce or even eliminate the risks of autolysis. It is more challenging to hydrolyze an immobilized enzyme than a free protein; still, some activity versus large substrates may be found (Fig. 19). Also, these biocatalysts may be used to modify small substrates.

Secondly, it is frequently stated that the loading capacity of nanomaterials is higher than that of porous supports. However, many porous supports can immobilize 100 mg of enzyme per ml of packed support. In the case of 10% agarose beads, it is possible to immobilize 100 mg of enzyme per 100 mg of dried support (Zucca et al., 2016); few nanomaterials can provide these figures. The size is related to the loading capacity of non-porous nanomaterials; the particles must be very small to reach values like this. Tiny particles are difficult to handle in industry; recovery by standard filtration will not be possible, and the only way to recover may be by centrifugation (or by using membrane reactors) (Cipolatti et al., 2016; Garcia-Galan et al., 2011). Magnetic nanoparticles solved this problem, but they are quite expensive and incompatible with all media (too viscous a medium can make recovery difficult) and reactor size. Moreover, there is one additional problem usually overlooked in the literature: a nanoparticle with a small diameter will offer a lower geometric congruence with the enzyme; that way, the enzyme immobilization positive effects may be hindered or even lost (Pedroche et al., 2007; Rodrigues et al., 2021) (Fig. 20). If the support features are inadequate, this poor geometrical congruence may become positive, as the negative enzyme-support interactions will decrease (Santos et al., 2015). However, if that is the case, the proper solution is to change the support, when immobilizing an enzyme, we pursue to improve the enzyme features. In any case, if an intense multipoint covalent attachment is desired, large nanoparticle particles are necessary, which means a lower support loading capacity.

Thirdly, there is no possibility of ordering the enzyme molecules. Using porous supports, it becomes possible to co-immobilize enzymes forming crowns on the support pores as long as the immobilization rate is high enough (Diamanti et al., 2022; Rocha-Martín et al., 2012) (Fig. 21). Using nanoparticles, this is not possible (Fig. 22). Janus particles, bearing areas with some groups and other areas with other groups, may permit to co-immobilize enzymes following different strategies (Boujakhrout et al., 2015; Cao et al., 2015; Sánchez et al., 2013; Tang et al., 2020), but the real advantages of this are dubious, as

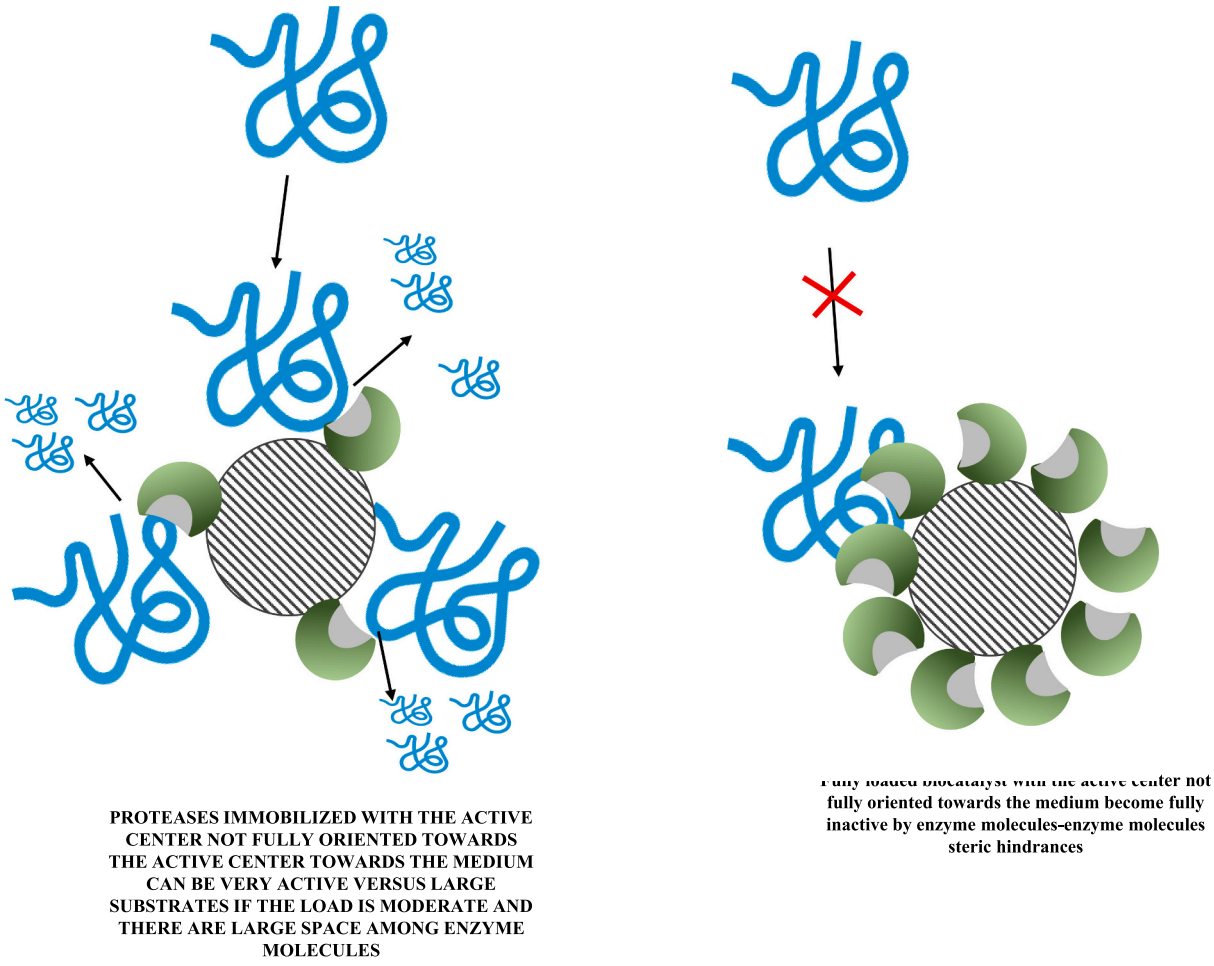

Fig. 8. Effect of the enzyme loading of non-porous supports when the biocatalyst is used in the modification (e.g., hydrolysis) of large substrates.

previously mentioned.

However, it is possible to have some s position ordering of one enzyme regarding the others, as successive enzyme layers can be formed (using adequate connection strategies, for example, using a polymer such as polyethyleneimine) (Arana-Peña et al., 2020a; Arana-Peña et al., 2020b; Carballares et al., 2022b; Hou et al., 1998; Kawakita et al., 2002; Kong et al., 1994; Rios et al., 2019; Willner et al., 1993). In multilayer construction, nonporous nanoparticles have advantages. In opposition to the use of porous supports, in nanoparticles, no apparent limits are described to the number of layers and the diameter, and that way, specific areas of the biocatalyst particle increase with each new enzyme layer. That way, the last enzyme layer will have a higher enzyme loading capacity than the first one, in contact with the support, in opposition to porous supports (Figs. 23–24). Only the last layer will be really exposed to the medium, so, only this layer can attack large or solid substrates (Fig. 25); the inner molecules may be useful to attack fragments or small substrates.

One problem with using nanomaterials is that, in many instances, the final biocatalyst is the product of nanoparticle aggregation (Cipolatti et al., 2016; Garcia-Galan et al., 2011; Morellon-Sterling et al., 2022; Tacias-Pascacio et al., 2021). Finally, in many instances, a "macroporous biocatalysts" is produced, losing the advantages of using non-porous nanoparticles (Fig. 26). This aggregation may be produced during particle activation (using bifunctional materials, it is likely that some nanoparticles can covalently form covalent aggregates) (Fig. 27) or during enzyme immobilization (Fig. 28). One way to prevent this aggregation is to use a large excess of "bifunctional agent", the activator or the enzyme (Figs. 27 and 29). This means a lower immobilization yield but it may be the only way to maintain the nanoparticles as individual and dispersed ones.

Nanobiocatalysts with high biochemical and industrial applicability, including enzymatic stability and working performance with processability and reusability, are to be trailed. The improvement of practical nanomaterials as enzyme transporters, maintenance, and restriction methods has been intensively studied in the previous few years (Murugappan et al., 2020; Qamar et al., 2020). The industrial-scale presentations of nanobiocatalysts and their assistance in bioprocesses have not been well-established. Bioprocess actions provide a distinct position for the construction of desired marketable products. With remarkable performance in recyclability and productivity, nanobiocatalysts have become attractive candidates for the pre-treatment of substrate, biotransformation, and biofuel production processes (Bilal et al., 2021). This review summarizes the current progress in using the protease based-nanobiocatalysts in engineering bioprocesses. Initially, protease immobilization on different innovative nanocarriers such as polymeric nanocarriers, silica nanocarriers, carbon nanotubes/rods, magnetic/non-magnetic nanocarriers, crosslinked enzyme aggregates (CLEAs), and metal-organic frameworks (MOFs) has been described in detail. Novel approaches for the functionalization and production of nanobiocatalysts are formerly addressed for diverse enzyme immobilization. The encapsulation and estimation of biocatalytic events and bioprocessing applications of subsequent nanobiocatalysts have been described with future research trends in this area.

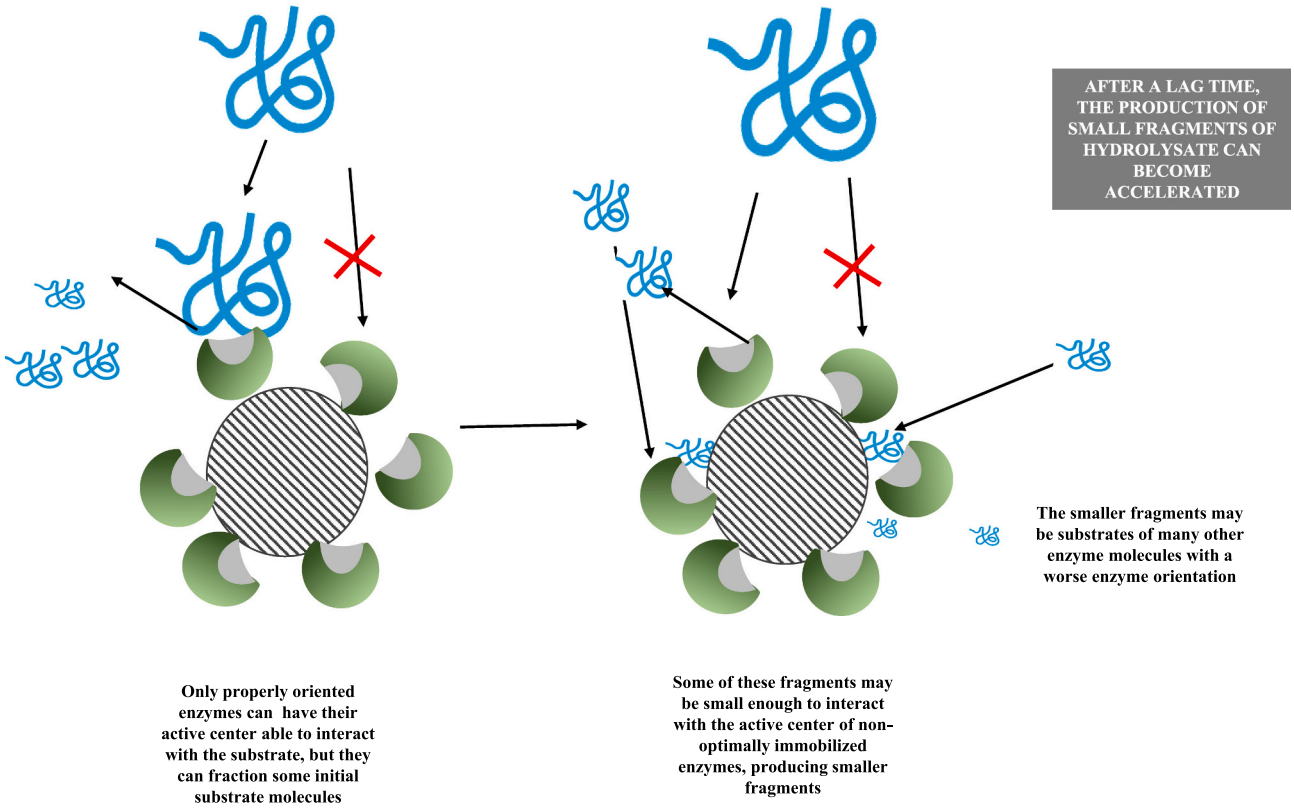

Fig. 9. Effects of a non-fully homogenous orientation of enzyme molecules on the surface of non-porous supports when using the biocatalyst for modifying large substrates.

2. Prospective nano-supports to develop protease-based nano-biocatalysts

2.1. Polymeric nanocarriers as immobilization supports

Nanomaterials can provide an excellent supportive environment for enzyme immobilization because they possess several desirable characteristics, such as mass transfer resistance, high surface area, and effective enzyme loading, which define the effectiveness of nanobiocatalysts. The inclusion of a homogeneous distribution of nanoparticles in polymeric nanocomposites results in a significantly increased interfacial area between the matrix and nanofiller, which alters the molecular mobility, relaxation behavior, and subsequent mechanical and thermal properties of the material (Bustamante-Torres et al., 2021). Natural biopolymeric materials, including alginate, cellulose, chitin, pectin, collagen, starch, carrageenan, sepharose, and chitosan, are commonly used for enzyme immobilization because of their biocompatibility, non-toxic nature, widespread availability, and ease of modification, which is facilitated by the presence of numerous functional groups on their surfaces. Chitosan-based nanostructured particles are natural materials with robust physicochemical and biological properties, which render them an environmentally friendly material for a wide range of bioproducts and applications, such as enzyme immobilization, tissue engineering, food packaging, diagnostics, biosensing, pharmaceutical, and wastewater treatment (Verma et al., 2020). For instance, Shojaei et al. (2017) conducted the immobilization of *Penaeus vannamei* protease onto chitosan nanoparticles using a non-covalent binding technique at varying pH and temperature ranges. The immobilized protease exhibited excellent reusability, demonstrating up to 10 cycles without any noticeable decline in activity. Recently, Ramalho and de Castro (2023) carried out the immobilization of alkaline protease from *Bacillus licheniformis* onto chitosan biopolymer, modified with ethylenediamine and glutaraldehyde. Results revealed that 5% chitosan modified with 5% glutaraldehyde furnished the highest protease immobilization yield of 69.9% yield. Additionally, it was observed that the immobilized biocatalyst exhibited high pH and thermal stabilities, with an activity retention of up to 47.08% after three consecutive cycles. In another study, glutaraldehyde-activated chitosan beads were used to immobilize protease from a UV-mutated *Bacillus* sp. The immobilized protease exhibited high immobilization efficiency and activity of 86.43% and 10.31 U/mL, respectively. Furthermore, it exhibited exceptional reusability (70.53%) following seven consecutive washings, along with enhanced thermal and pH stability (Kamal et al., 2021).

The hybrid polymeric nanoparticles are reported to be efficient support matrices for protease immobilization. In a recent study, Karakurt and Samsa (2023) reported protease immobilization onto silica-chitosan gel beads using glutaraldehyde as crosslinker. The hybrid polymer matrix, composed of silica and chitosan, achieved a high immobilization yield of 96% and retained over 50% of its activity after five consecutive cycles at 40 °C. Immobilized protease exhibited improved activity towards metal ions, and its activity was further improved (about 18%) in the presence of Mg^{2+} (5 mM) ions for 1 h. Furthermore, it presented higher stability in commercial laundry detergents than the free enzyme (Karakurt and Samsa, 2023). Similarly, Badoei-Dalfard et al. (2023) reported immobilization of *Serratia marcescens* protease using chitosan/cellulose acetate nanofibers functionalized with glutaraldehyde. High immobilization yield (85%) and activity recovery (121.3%) were reported at 4 h crosslinking in 5.0% glutaraldehyde using 210 mg protein/g support for 8 h at pH 9. About 52% increased protease activity was observed using immobilized protease rather than free enzyme in pH 10. High thermal stability (>75% activity retention) was reported at 60 °C for 180 min. In addition, immobilized protease exhibited 73% activity retention after three weeks of storage (Badoei-Dalfard et al., 2023).

Although plenty of reports describe protease immobilization using different (bio)polymeric nanocarriers, only a few studies have reported

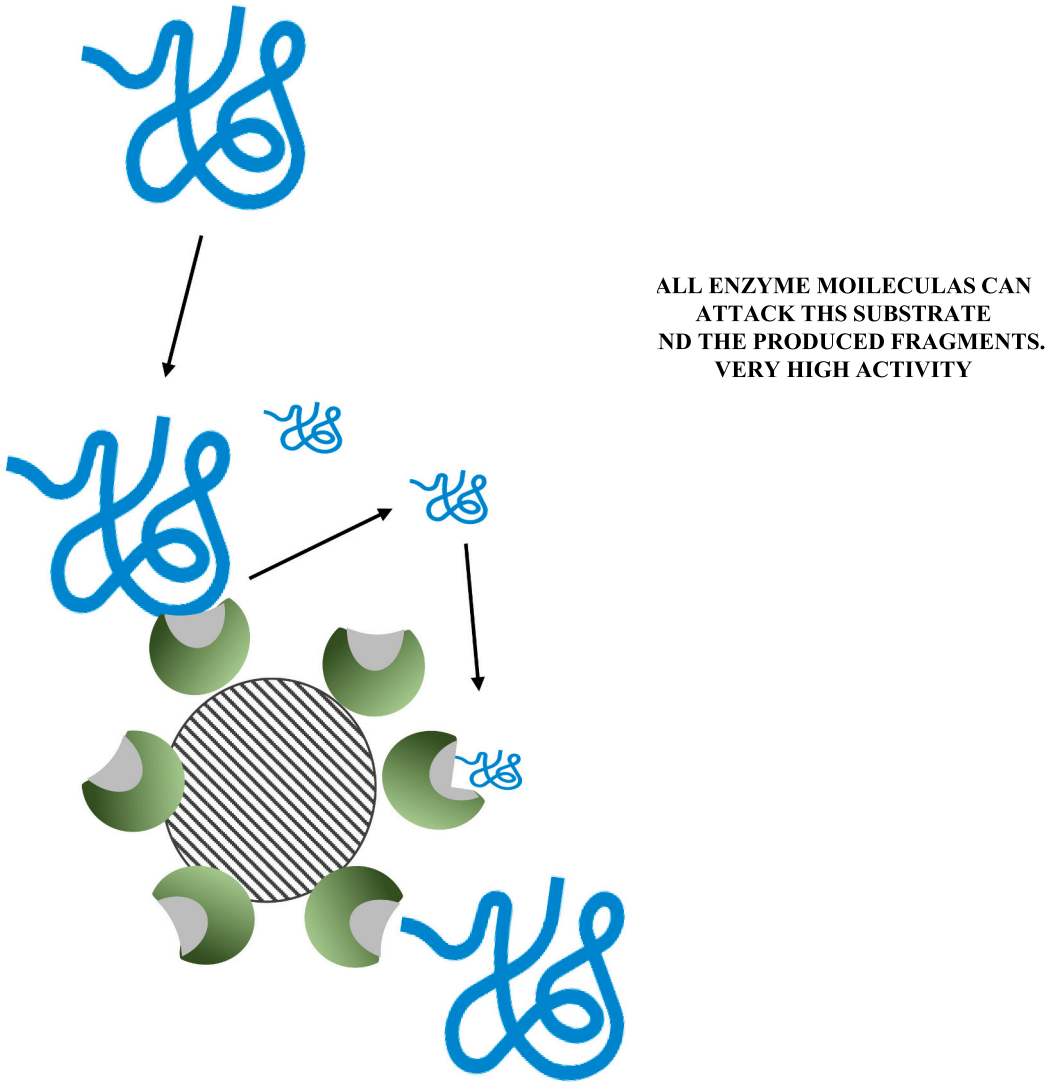

Fig. 10. Hydrolysis of large substrate catalyzed by enzymes immobilized on non-porous supports bearing the correct orientation regarding the support surface.

protease immobilization on a commercial scale. Some factors should be considered, such as the stability of protease, which should remain high during the intermediate steps, and the protocols must be robust, scalable, and reproducible. Additionally, bioprocesses must have minimal societal and environmental effects. Combining nanobiotechnology and green chemistry is necessary to develop renewable polymeric nanocarriers to improve the efficiency and industrial viability of processes, with a focus on sustainability. Table 1 provides a summary of current research that has reported the immobilization of protease onto polymeric (nano)carriers in terms of immobilization yield, key features, and recyclability potential.

2.2. *Silica-based nanocarriers as immobilization supports*

Silica-based nanoparticles (SNPs) and nanocarriers have shown significant research interest in biocatalysis and enzyme immobilization due to their structural stability, excellent biocompatibility, controllable particle size, larger surface area, and easy surface functionalization (Popat et al., 2011). In comparison to many other alternative materials, silica is environmentally friendly, relatively cheap, and exhibits notable chemical, mechanical, and thermal stability when employed as a support matrix. The pore dimensions of SNPs can be easily tailored to provide optimal diameters for the efficient loading of a large amount of enzyme molecules. Moreover, the biocompatibility and biosafety of SNPs make them highly promising carriers for the immobilization of enzymes, particularly in therapeutic contexts (Zhao et al., 2022). A serine alkaline protease from halotolerant alkaliphilic *Salipaludibacillus agaradhaerens* strain AK-R was covalently attached to activated double mesoporous core-shell silica (DMCSS) nanospheres. In comparison to the free enzyme, the DMCSS-incorporated AK-R protease derivative showed a displacement in the optimal pH and temperature from pH 10.0 to 10.5 and 60 to 65 °C, respectively. After the treatment for 1 h at 50 °C and 60 °C, the soluble enzyme maintained 47.2% and 9.1% of its original activity, whereas, the immobilized nanobiocatalyst preserved 87.7% and 48.3%, respectively. In addition, the immobilized AK-R construct demonstrated considerable improvement in enzyme stability at higher concentrations of surfactants, organic solvents, NaCl, and commercial detergents. It also presented excellent functional stability, retaining over 75% of its initial activity after 10 consecutive reuse cycles (Ibrahim et al., 2021).

Protease enzymes offer an efficient solution to the environmental degradation caused by chemical dehairing of animal hides in the tannery industry. Nevertheless, achieving cost-effective, reliable, and industrially acceptable manufacturing of alkaline protease poses a significant

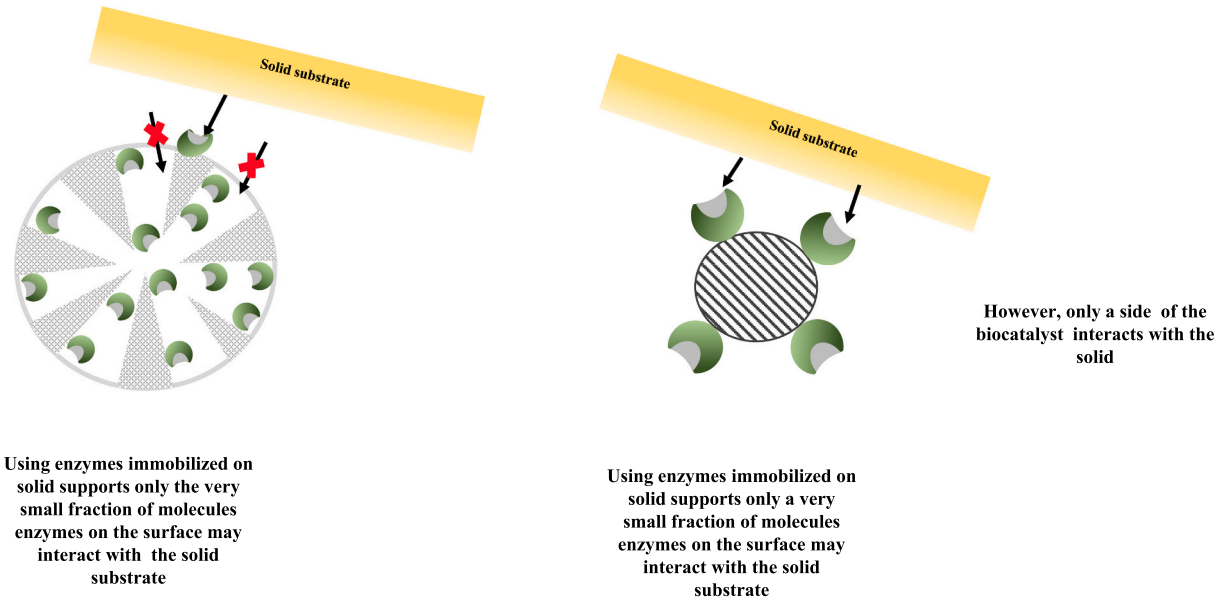

Fig. 11. Modification of solid substrates by enzymes immobilized on porous and non-porous supports.

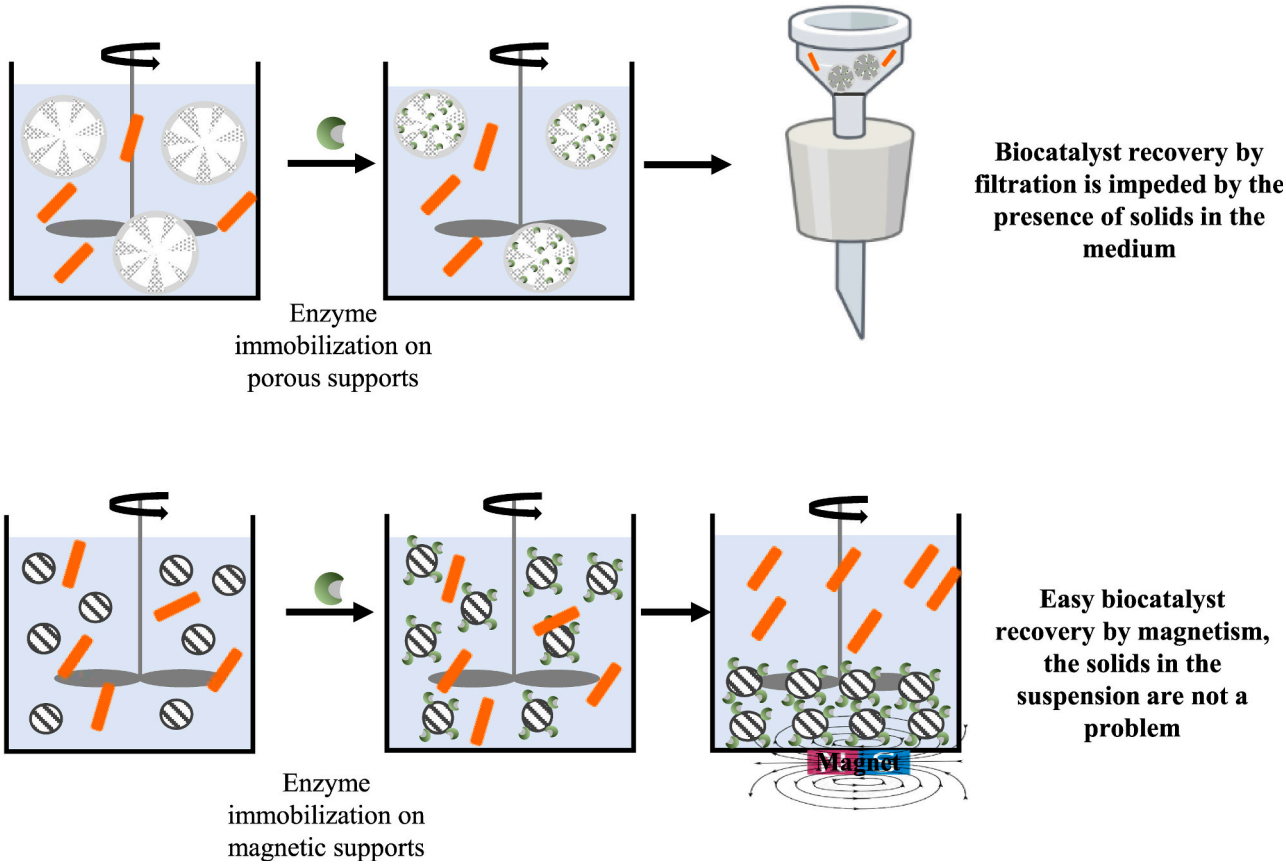

Fig. 12. Advantages of using magnetic materials when the reaction media is a suspension: easy biocatalyst recovery utilizing a magnet.

hurdle. Joshi et al. (2021) reported a one-pot green synthesis of nano-silica conjugates of alkaline protease from *Bacillus circulans* grown in a renewable waste-based medium. Acetone-precipitated alkaline protease was examined to be the best for the biogenic synthesis of nano-silica and the development of protease-based nano-conjugates. The developed protease nano-constructs (ranging from 100 to 200 nm), exhibited up to 1.7-folds of increased protease activity with excellent dehairing potential at 37 °C. In 2017, Özbek and Ünal conducted a study on the use of polymer-coated mesoporous nano-silica for immobilizing subtilisin by adsorption technique. In order to achieve this objective, chitosan and acrylic acid were combined to form a polymeric layer known as poly-acrylic acid and chitosan using in-situ polymerization. Both free and immobilized enzymes exhibited their maximum catalytic activity at pH 9.0. However, the immobilized biocatalyst showed greater thermal

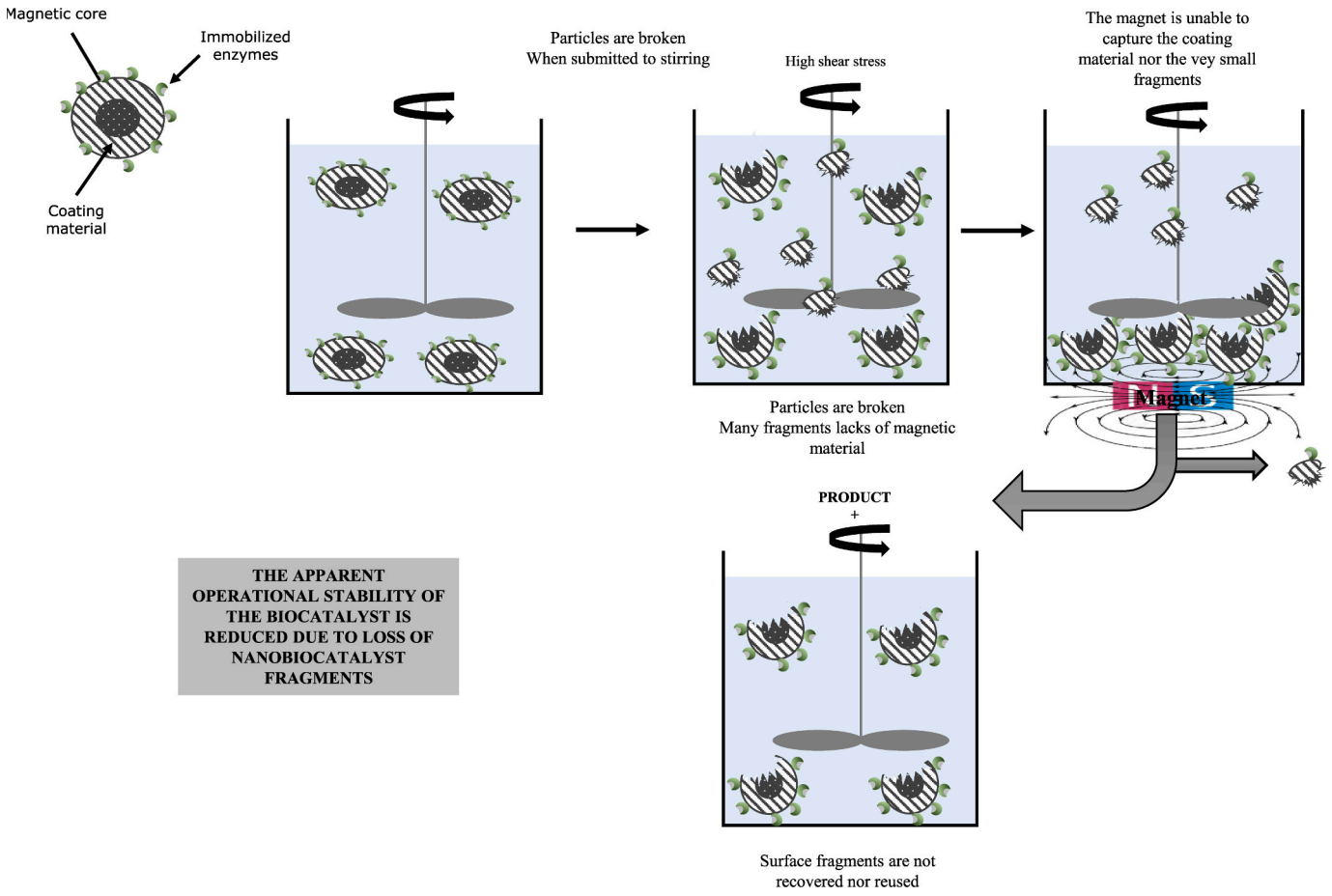

Fig. 13. Problems in the use of magnetic biocatalysts caused by the particle physical rupture: the breakage of the external surface leads to enzyme immobilized in non-magnetic materials, in the best-case scenario, the smaller magnetic nanoparticle cannot be retained by the used magnetic field.

stability compared to the free enzyme. After immobilization, the activity was reduced to 45.83% after 5 consecutive repeats. Further, the immobilized enzyme showed higher storage stability retaining 60% of its activity after 28 days of incubation (Özbek and Ünal, 2017).

In a recent study, Zhang et al. (2023) studied in-situ immobilization of a model enzyme (hemoglobin) using mesoporous silica nanoparticles. Zeolitic imidazolate framework-8 (ZIF-8) was used as a sacrificial template for the synthesis of Hb@ZIF-8. At a constant amount of Hb, the nano-construct was gradually changed from flake to granular. Further, the increased amount of Hb by fixing the molar ratio of 2-methylimidazole and Zn^{2+} resulted in the highest enzyme loading of 460 μg/mg possible. The nano-construct maintained over 74% of its initial activity in a hollow mesoporous silica nanoreactor under extreme conditions. Kim et al. (2022) reported protease immobilization onto halloysite nanotubes modified with 3-aminopropyltriethoxysilane and Fe_3O_4 nanoparticles. The protease nano-construct showed 59.56 mg/g enzyme loading capacity and excellent immobilization (87%) yield. As an immobilization matrix, silica nano-constructs provide good enzyme adsorption through physical and chemical interactions due to their large surface area. The pore size of approximately 3–10 nm is comparable with the hydrodynamic radius of the majority of enzymes in the biotechnology industry. Nevertheless, the application of microporous zeolites for enzyme immobilization is restricted by their small pore diameters, which typically range from 0.4 to 1.5 nm. In recent years, there has been a rise in the number of research reporting the development of zeolites with mesopores ranging from 2 to 10 nm for the immobilization of biomolecules and enzymes (Ibrahim et al., 2021; Kumari et al., 2015). Table 2 summarizes the results, important biocatalytic properties, and recyclability potential of protease immobilization onto silica-based (nano)carriers from recent investigations.

2.3. Carbon nanotubes as immobilization supports

Carbon nanotubes (CNTs) have garnered considerable attention due to their notable chemical stability, exceptional mechanical strength, and electrical conductivity (Mirsalari et al., 2021). Considering CNTs as potential materials for enzyme immobilization, Wang et al. (2008) studied the synthesis of polyaniline-coated Fe_3O_4/CNT nanocomposite to immobilize trypsin for efficient protein digestion. Polyaniline was coated onto Fe_3O_4/CNT by in-situ polymerization in the presence of an enzyme to create trypsin-based nanoconstructs. The performance and feasibility of the resultant biomaterial were demonstrated by the rapid digestion of lysozyme, myoglobin, and bovine serum albumin in 5 min. It showed promising applications in protein analysis due to its excellent dispersibility and high magnetic responsivity. The convenient separation from digests using an external magnetic field indicates its potential applications in proteome research (Wang et al., 2008). In another study, Homaei and Samari (2017) reported non-covalent immobilization of papain protease onto MWCNTs. Papain nano-conjugates showed improved pH and thermal stabilities and high recyclability as compared to free enzyme. A slight increase in K_m value of the immobilized enzyme suggests that the presence of MWCNTs did not substantially impede the interaction between the enzyme and substrate, nor the release of the product. Furthermore, the convenient separation from the product makes it a desired candidate to develop multi-enzyme systems (Homaei and Samari, 2017).

The immobilization and activity maintenance of protease absorbed onto monolayer organo-modified SWCNTs were studied by (Almarasy

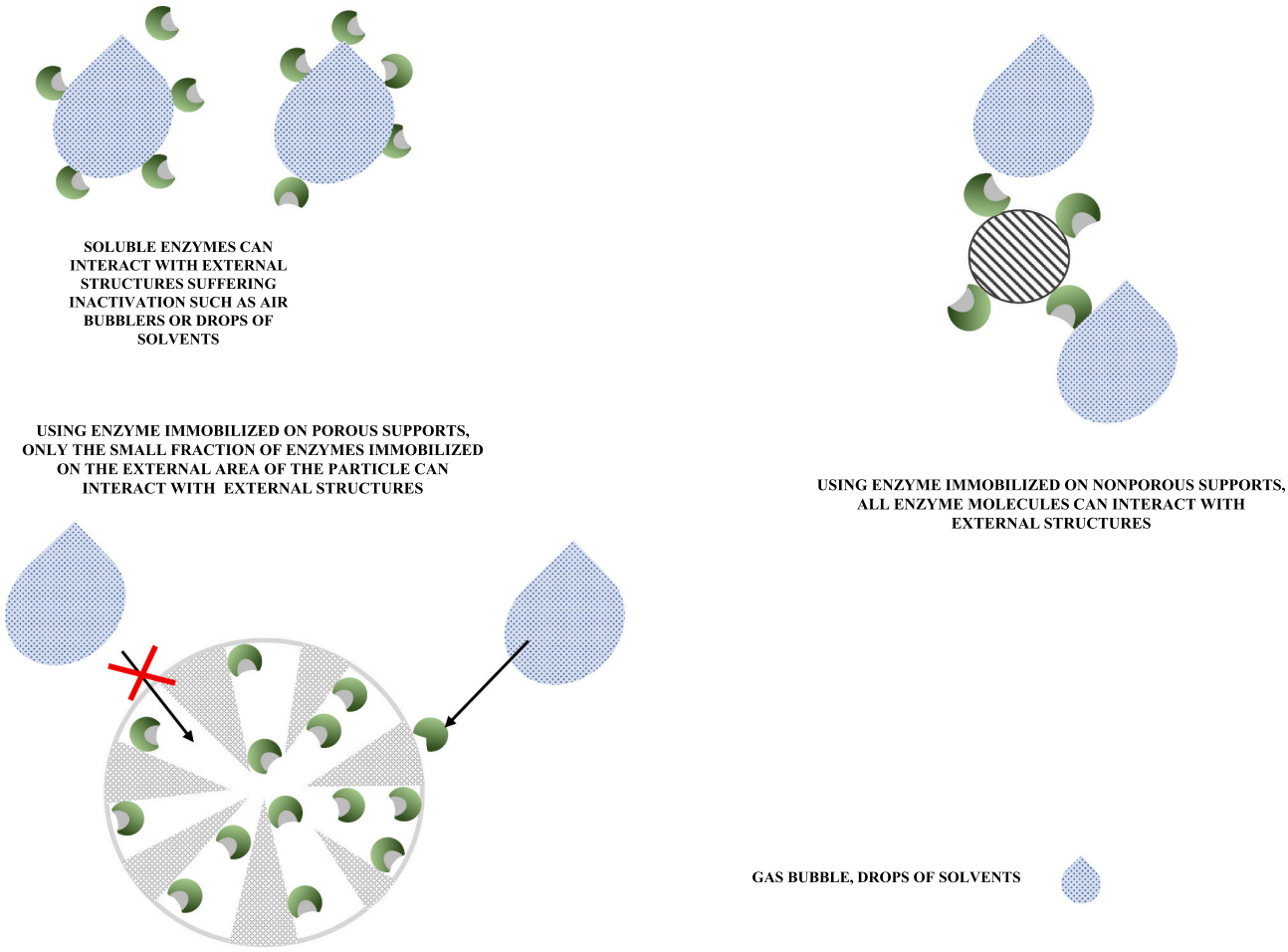

Fig. 14. Possibility of enzyme interaction with external interfaces when immobilizing the enzymes in porous and non-porous supports.

et al., 2021). Protease was immobilized onto the subphase after the formation of a monolayer on the waster surface of organo-modified SWCNTs. The enzyme nano-construct was evenly distributed in the solution containing luminous casein, resulting in the detection of fluorescence emission. The protease in a free state, when dissolved in water, demonstrated the capacity to break down molecular chains only at ambient temperature. However, the nano-construct exhibited functionality up to 160 °C, showing a high level of thermal stability. Additionally, it displayed a distinct and intense spectrum emission (Almarasy et al., 2021). The protease, purified from *Penaeus vanamei* shrimp, was immobilized onto MWCNTs after activating with nitric acid. To achieve this objective, a solution containing enzymes with a concentration of 7 mg/mL was introduced to the MWCNTs and maintained at a temperature of 10 °C for 4 h. The immobilized enzyme exhibited significant thermal stability within the temperature range of 60 to 80 °C. However, the pH stability was observed to be the same for both the free enzyme and the immobilized enzyme. Immobilization resulted in the modification of kinetic parameters (K_m *and* K_{cat}), suggesting their potential significance in many biotechnological applications (Deghan and Homaei, 2016).

Literature studies highlight that the immobilization of protease and other industrial enzymes onto CNTs and their derived nano-constructs provides a powerful tool for constituting the biocatalytic process. CNT-based materials are emerging as host matrices for protein/enzyme immobilization because of their favorable physicochemical characteristics, including high biomolecular loading, mechanical and thermal stability, and large surface area. However, the immobilization methods significantly influence the catalytic performance of CNTs-immobilized enzymes. Therefore, careful consideration of the immobilization method on CNT and target-specific enzymes is required for practical applications. There is an emerging concern about the precise functionalization of CNTs to construct robust and biocompatible nanocomposites that can serve as strong frameworks for immobilizing enzymes. CNTs-based nano-biocatalysts exhibit superior operational stability and catalytic performance compared to other types of nanomaterials. Despite the emergence of nano-engineering in enzyme biotechnology, additional studies are required to understand the interactions between CNTs and other counterparts with biocatalysts and their mutual influence on enzymatic structure and function.

2.4. *Magnetic/non-magnetic nanocarriers as immobilization support*

Magnetic nanocarriers for enzyme immobilization offer numerous advantages for commercial applications due to their convenient separation and high reusability potential. Magnetic nano-supports possess the advantage of lacking any external diffusional problems, which enhances their attractiveness for large-scale applications. For instance, Jin et al. (2010) studied covalent immobilization of alkaline protease onto (3-aminopropyl) trimethoxysilane-modified magnetic nanocarriers. A significant catalytic bioactivity of up to 47.8% was observed using casein as a substrate. Magnetic nano-constructs retained 98.63 ± 2.37% of catalytic activity after 60 days indicating a high level of storage stability. To carry out mix-and-separation experiments, rapeseed meal was used as a substrate, which showed 9.86% and 10.41% degree of hydrolysis in the presence of immobilized and free protease, respectively. The convenient recyclability of magnetic nano-constructs using a magnetic field could be a practical method for the continuous production of active peptides (Jin et al., 2010). Similarly, looking at the enzyme

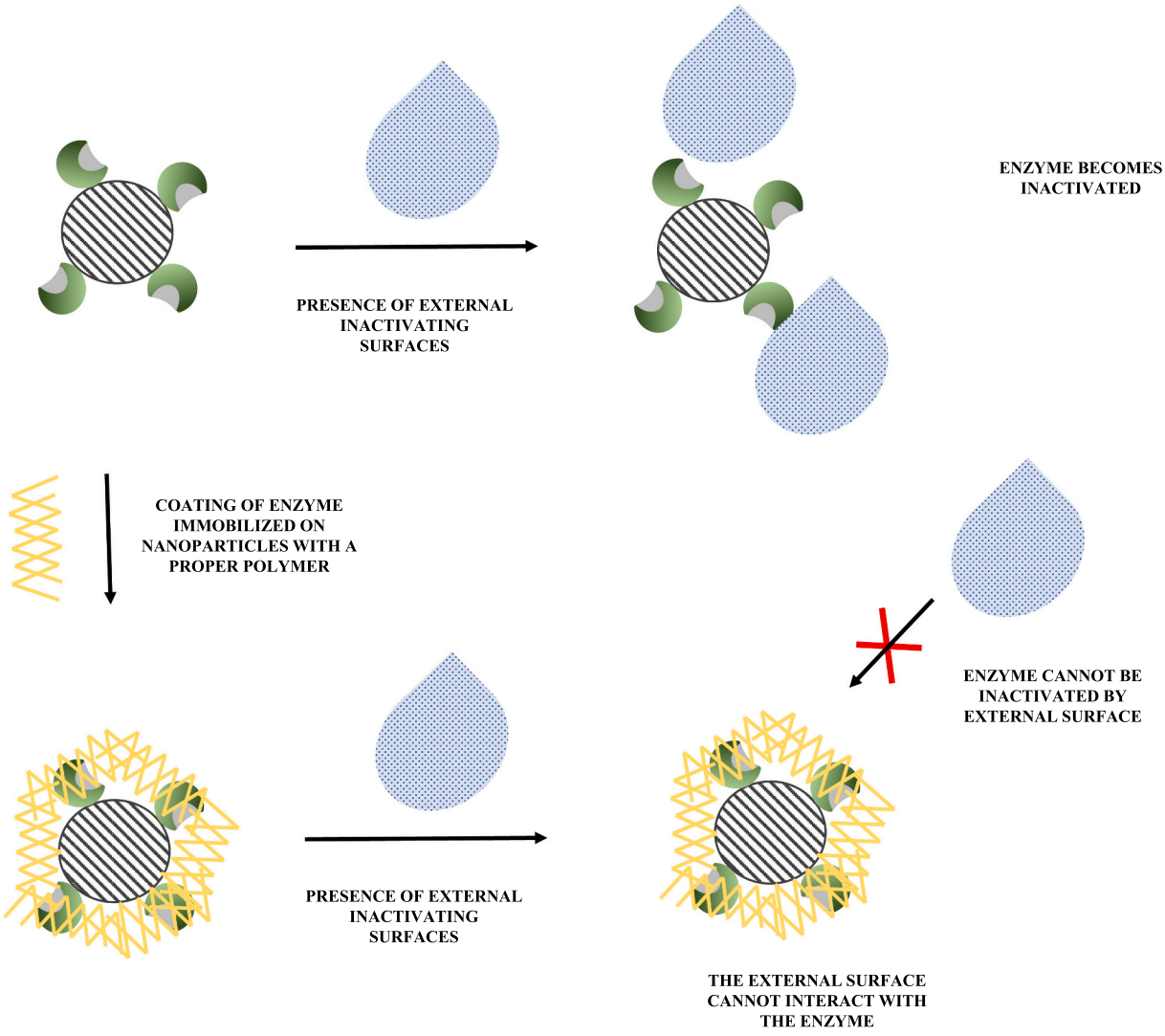

Fig. 15. Protection of enzymes immobilized on non-porous supports versus interaction with external interfaces by coating with some hydrophilic polymer.

reusability and cost perspectives, Glomm et al. (2021) conducted subtilisin A (a food-grade protease) immobilization onto magnetic nanoparticles. Glutaraldehyde-mediated immobilization was carried out using three different amine ligands: aminopropyl trimethoxy silane, Jeff amine, and chitosan. The nano-construct exhibited excellent storage stability, with a retention of ≤93% activity after 25 months, as well as remarkable reusability, with a retention of ≤85% after six repeated cycles. It was also able to catalytically degrade and extract protein from turkey tendons and chicken meat (Glomm et al., 2021).

Alkaline protease produced by soy residues and hair waste was immobilized onto functionalized magnetic nanoparticles using glutaraldehyde as a crosslinker (Yazid et al., 2016). High immobilization yield (93%–96%) was obtained using covalent binding from hair waste and soy residue-based proteases, respectively. However, simple adsorption resulted in 47%–54% yield after 8 h for both proteases. Both free and immobilized proteases showed identical pH stabilities; however, thermal stabilities were significantly improved (40 °C–60 °C) after immobilization. High storage stability (>85%) was reported after two months at 4 °C. Reusability and ease of separation were achieved using the immobilized protease as the activity retention was >60% after seven hydrolysis experiments (Yazid et al., 2016). Similarly, Masi et al. (2018) studied the protease production from *Enterococcus hirae* and *Pseudomonas aeruginosa* isolated from dairy industries. Immobilization was carried out using surface transformation techniques such as the development of silica-coated, amine, and cysteine-functionalized nanoconstructs. The highest catalytic activity of immobilized protease was found to be 290 μg/mL and 105 μg/mL for *Enterococcus* sp. and *Pseudomonas* sp., respectively. The immobilization capacity was found to be 6000 μM/g. Further, a high stability was also obtained as compared to the free protease (Masi et al., 2018).

da Silva et al. (2022) reported *Mucor subtilissimus* fibrinolytic protease immobilization onto Fe_3O_4 magnetic nanoparticles, activated with glutaraldehyde and coated with polyaniline. High immobilization yield (52.13%) was obtained using magnetic nanoparticles, which were capable of maintaining >60% of enzymatic activity at pH 7–10 and temperature of 40 to 60 °C as compared to free enzyme. Further, no cytotoxicity was found against J774A.1 and HEK-293 cells, which indicates its potential applications in the treatment of cardiovascular diseases with sustained release using an external magnetic field. Despite recent advancements in biocatalytic immobilization using magnetic nanocarriers, these systems still suffer from challenges, such as mass transfer limitation, changes in catalytic characteristics, and less efficiency against insoluble substrates. Therefore, further studies are needed to address such issues for industrial applications of proteases.

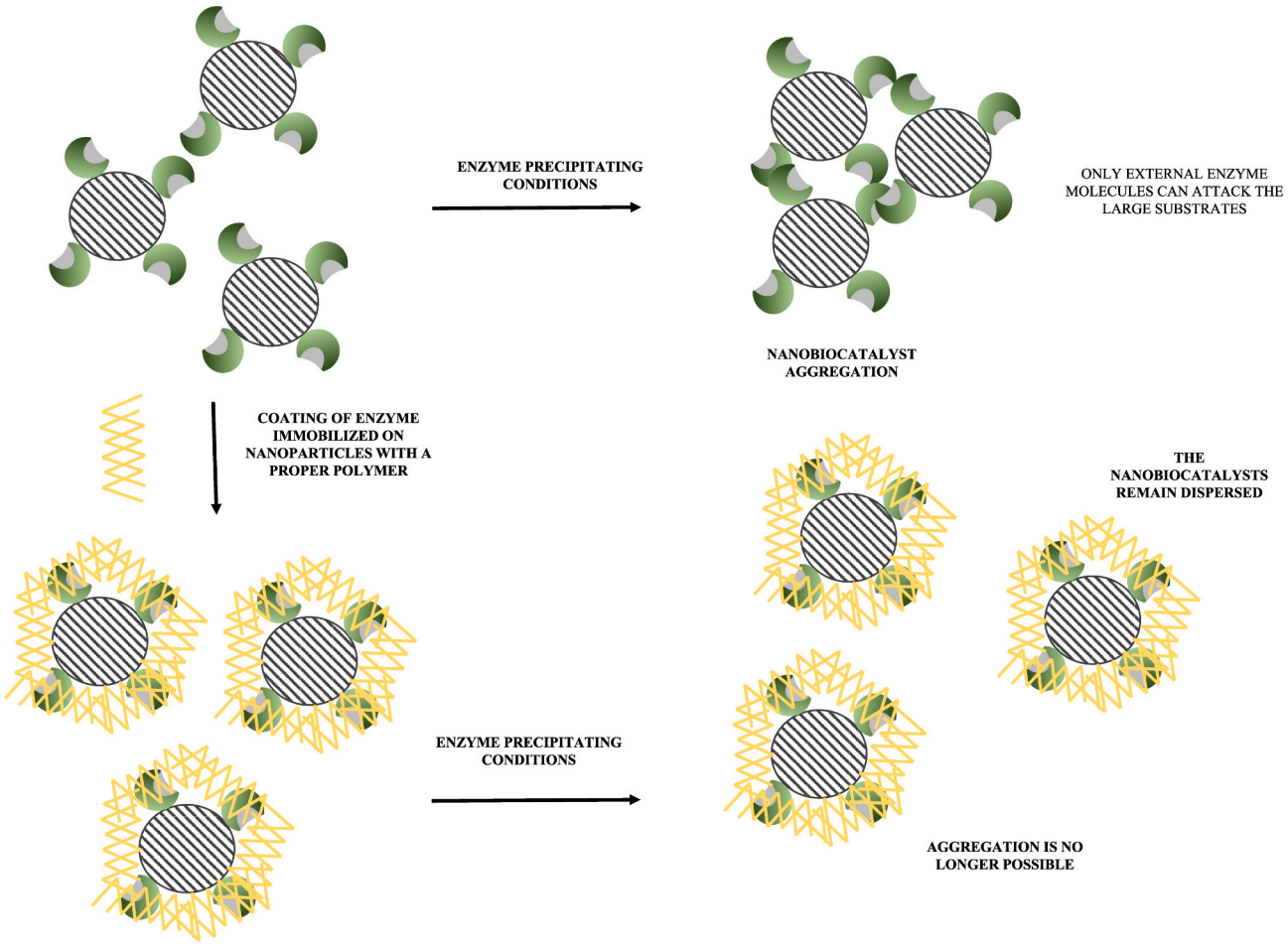

Fig. 16. Possibility of nanobiocatalyst aggregation during storage or operation. The coating with hydrophilic polymers can prevent this possibility.

2.5. Graphene/graphene oxide-based nanocarriers as immobilization support

The synthesis of graphite oxide first involves the oxidation of graphite to graphite oxide, followed by the exfoliation of this graphite oxide to obtain graphene oxide. This unique material has a two-dimensional structure, with carbon atoms that are sp^2 hybridized and organized in a hexagonal pattern (Raghavan et al., 2015). Its good optical transmission, thermal conductivity, high intrinsic mobility, and good electrical conductivity make it an excellent candidate for enzyme immobilization purposes. Moreover, it is environmentally friendly, biocompatible, and possesses a remarkable ratio of surface area to volume.

Graphene oxide nanosheets (GON) have intriguing properties for enzyme immobilization owing to their expansive specific surface area and numerous functional groups. In this sense, (Ranjbari et al., 2019) assessed the potential of GON as an immobilization matrix for *Penaeus vannamei* protease using glutaraldehyde as a coupling agent. The immobilization process had no impact on the optimal pH. However, the stability at higher pH levels and the thermal stability (activity at 90 °C) were enhanced after the enzyme immobilization on GON. The free enzyme exhibited a mere 10% of its original activity after being exposed to a temperature of 90 °C for 24 h. In contrast, the GON-conjugated protease maintained over 90% of its initial activity under the same conditions. The K_m and V_{max} values were found to be comparable for both the free and immobilized proteases. The hydrolysis studies, utilizing casein as a substrate, demonstrated the superior performance of the GON-immobilized enzyme compared to the free enzyme (Ranjbari et al., 2019). Similarly, (Mirzaei et al., 2022) studied papain immobilization using glutaraldehyde-functionalized, nitrogen-doped graphene quantum dots. After immobilization, the stability of papain was significantly improved (80% for 150 min) as compared to free enzyme (55% after 50 min). The stability of immobilized enzymes was improved especially in polar solvents. The K_m value decreased from 2.02 g/L to 1.20 g/L, while V_{max} decreased from 932.1 μmol/mg.min to 62.5 μmol/mg.min after immobilization (Mirzaei et al., 2022).

A graphene oxide silver nanoparticles hybrid was synthesized by Liu et al. (2021) to immobilize trypsin. The immobilization capacity of graphene oxide silver nanoparticles was higher as compared with bare graphene oxide. Further, it was confirmed that silver nanoparticles did not interfere with the adsorption of trypsin by GON. The amino acid microenvironment of trypsin was altered after immobilization. The enhanced activity of substrate hydrolysis was reported using bovine serum albumin as a substrate. Gu et al. (2018) reported the synthesis of GON-nanosheets that were functionalized with 3-aminopropyltriethoxysilane. The produced nano-constructs were utilized to immobilize papain. An immobilization efficiency and yield of over 80% were achieved at 35 °C and pH 8.0. The immobilized enzyme exhibited superior thermal stability in comparison to the free enzyme. In addition, the immobilized enzyme also exhibited significant storage stability, retaining around 63% of its activity after 30 days at 4 °C. The substrate binding affinity of the immobilized enzyme was enhanced due to the distinctive properties of graphene.

Shi et al. (2014) used polydopamine-coated magnetic graphene for the immobilization of trypsin via facile PDA chemistry rather than introducing other coupling agents. High immobilization efficiency (0.175 mg/mg) was obtained. High storage stability (about 100% activity retention at 4 °C after 30 days) was shown by the immobilized

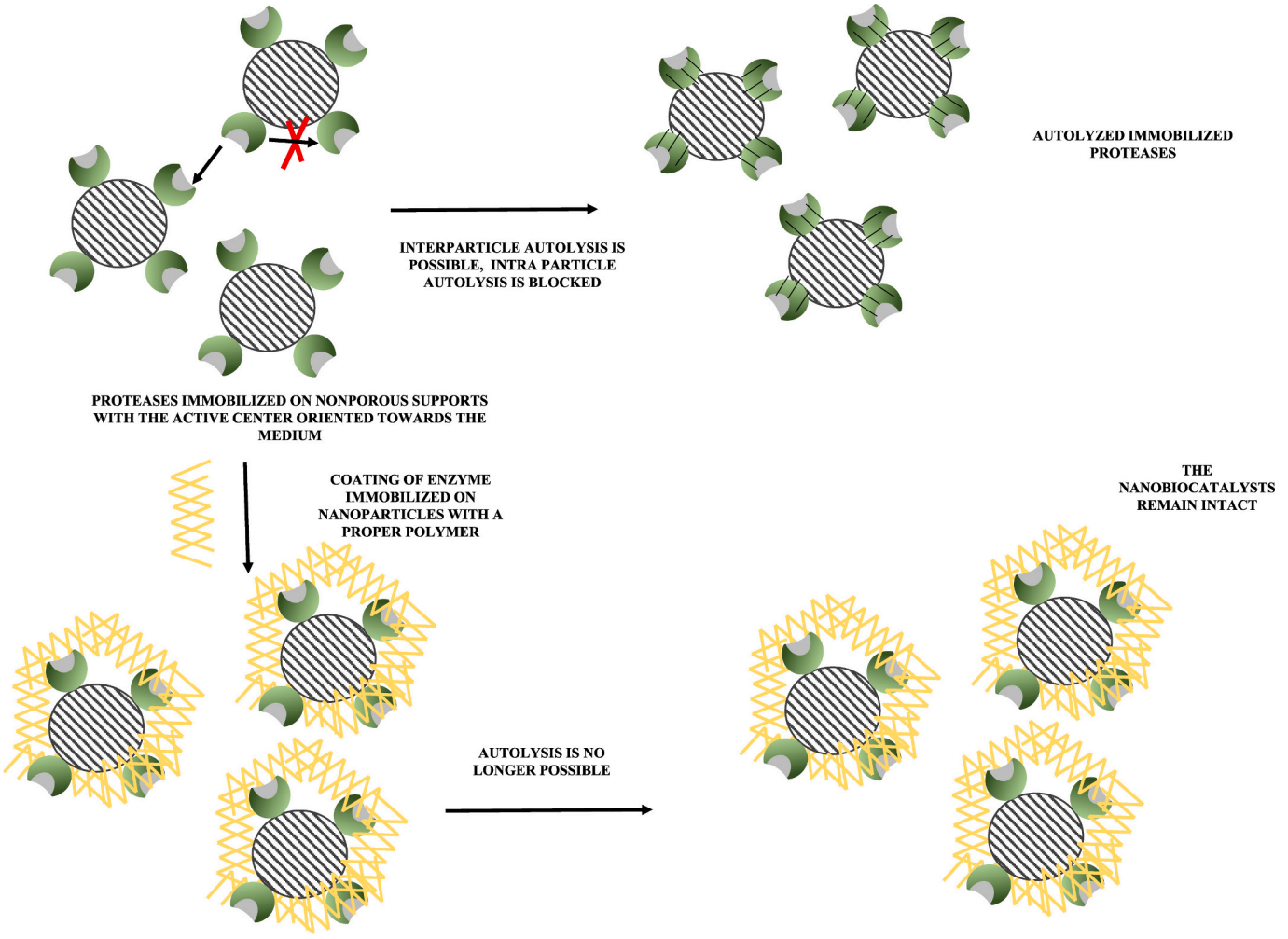

Fig. 17. Autolysis of proteases immobilized on non-porous supports may be prevented by coating with hydrophilic polymers.

enzyme. Further high recyclability (around 100% activity) after 5 repeated experiments indicates its potential for commercial-scale experiments. Because of their remarkable properties, graphene and graphene oxide-based support materials are promising candidates for immobilizing enzymes and other biomolecules. Furthermore, biocompatibility, high surface area, adequate conductivity, excellent processability, and abundance are the additional benefits of graphene derivatives over other counterparts such as fullerene, diamond, and nanotubes. The engineering of robust and cost-effective processes for protease immobilization onto graphene and its derivatives with a suitable immobilization strategy will result in the practical applications of nano-biocatalytic systems. Table 3 summarizes recent research that represents protease immobilization onto graphene oxide-based materials along with key characteristics, immobilization yield, and potential for recyclability.

2.6. Metal-organic frameworks as immobilization supports

Recently, numerous techniques have emerged to fabricate enzyme-incorporated nanomaterials, such as nanoflowers, capsules, and metal-organic frameworks (MOFs). These approaches have yielded notable advancements in elucidating the mechanisms involved and demonstrating their efficacy and potential for both industrial and academic applications of enzymes. Metal-organic frameworks are endowed with outstanding features like tunable frameworks, high porosity, structural diversity, ultrahigh surface area, a wide range of pore shapes, low toxicity, and easy chemical functionalization. Chulkaivalsucharit et al. (2015) achieved the one-pot synthesis of enzyme-encapsulated MOFs. In this regard, the enzyme was typically inserted over MOF crystal production, in which enzymes immobilized in MOFs showed excellent catalytic performance in contrast to the non-immobilized enzyme. Ultimately, MOFs offer an optimal framework for the immobilization of enzymes. Given their high porosity, superior crystallinity, and design versatility, there are an array of possibilities for protease immobilization. For example, proteases and other enzymes can be immobilized by studying factors such as pore size/structure, surface hydrophobicity, attachment of various functional groups, and BET surface area. Moreover, gaining a more comprehensive insight of the interplay between proteases and support matrices based on MOFs would contribute to advancing the commercialization of enzyme-based processes.

Gao et al. (2018) cloned the aspartic protease gene from *Geomyces pannorum* and expressed it in *Aspergillus oryzae* by cultivating at 20 °C. The resulting purified enzyme showed optimum activity at 60 °C and retained about 80% of its original activity at 50 to 70 °C. The protease with a specific activity of 585 U/mg, K_m 1.01 mg/mL, and V_{max} 4.4 × 10^{-2} mg/min.mL showed broad substrate specificity. Furthermore, its immobilization onto iron oxide nanoparticles increased the pH and thermal stability (Gao et al., 2018). Another study by Moslemi et al. (2018) described the introduction of amine groups through chemisorption of L-aspartic acid and the development of magnetic Fe $(OH)_3$@Fe_3O_4 nano-construct using a facile co-precipitation method. *Penaeus vannamei* protease immobilization. The immobilized enzyme showed higher thermal stability by 10 °C and pH stability (from pH 7 to pH 8), as compared to free enzyme. Furthermore, the pH, thermal, and

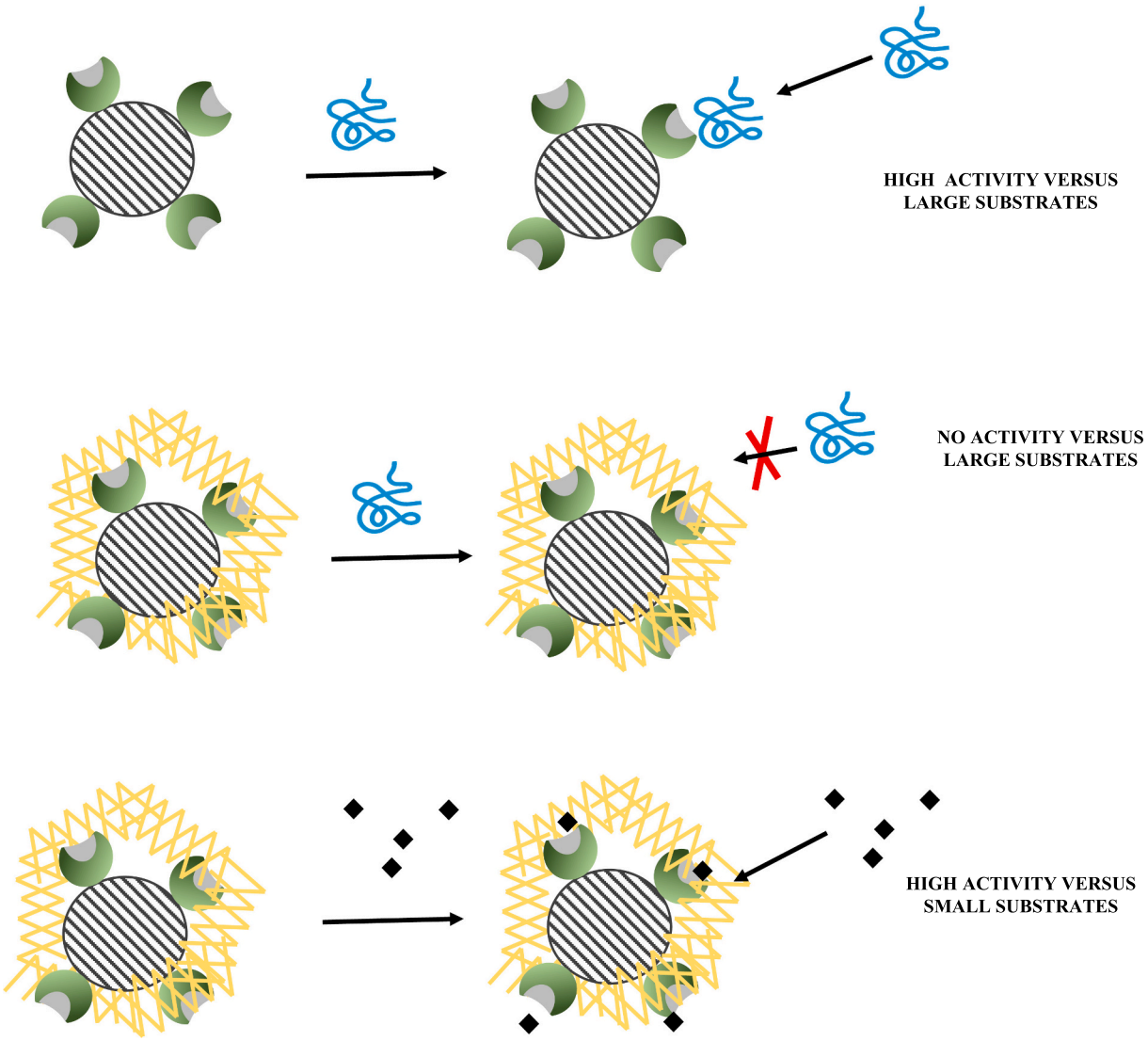

Fig. 18. Effect of nanobiocatalysts coating with polymers in the modification of large and small substrates.

storage stabilities were significantly improved after immobilization. High reusability (70% of its initial activity after 15 cycles).

Magnetic MOFs were used for the immobilization of *Bacillus* protease KHB3 by (Karami et al., 2022). The optimum temperature for free and mMOF-immobilized protease was 40 °C and 60 °C, respectively. Furthermore, an activity improvement (20%) was recorded for the immobilized protease at 80 °C as compared to the free enzyme. Higher V_{max} values (1.54 and 1.55-folds) after immobilization were recorded towards fibrin and gelatin, respectively. Two-folds higher activity retention of immobilized protease was recorded over the free enzyme after 21 days. Improved clot lysis was observed by the immobilized enzyme indicating its potential application in the protein hydrolysis experiments. Similarly, Badoei-Dalfard et al. (2020) studied MOF-immobilization of protease from *Bacillus* sp. CHA410. The optimum activity from both free and MOF-immobilized enzymes was obtained at pH 8 and pH 9, respectively. The relative activities after 160 min incubation showed 25% and 41% improvement after MOF-immobilization at 60 and 70 °C, respectively. The K_m value of immobilized protease was decreased from 0.685 to 0.033 mg/mL, while using fibrin as a substrate the K_m value was decreased from 0.292 to 0.145 mg/mL. Furthermore, the catalytic activity of protease was improved in the presence of divalent cations such as Zn^{2+}, Mg^{2+}, Mn^{2+}, etc. Improved thermal and storage stabilities were observed after MOF immobilization. Recent studies reporting protease immobilization onto metallic/metal-organic frameworks with immobilization yield, key features, and recyclability potential are summarized in Table 4, whereas Fig. 30 represents advanced nanocarriers to develop protease-based nano-biocatalysts.

3. Emerging applications of protease-based nanobiocatalysts

3.1. Protease-based nanobiocatalysts in the food sector

Although enzymes in their soluble form possess excellent functional properties, they have not been optimized to perform in large-scale industrial bioreactors operating under extreme pH conditions, rising temperatures, high substrate and product concentrations, mechanical agitation, or solvent use. Enzyme engineering is demonstrated as a prodigious approach to address these challenges, representing the most intriguing transdisciplinary and challenging objectives of industrial biotechnology (Guisan, 2006). In this context, advancements in enzyme immobilization technologies have been developed to enhance the characteristics of enzymes employed in the food industries. The utilization of immobilized biocatalysts has exhibited better performance in continuous operations at the industrial scale (Gómez et al., 2007). The protease from *Penaeus vannamei* was immobilized onto chitosan nanoparticles using a non-covalent method and employed for the clarification

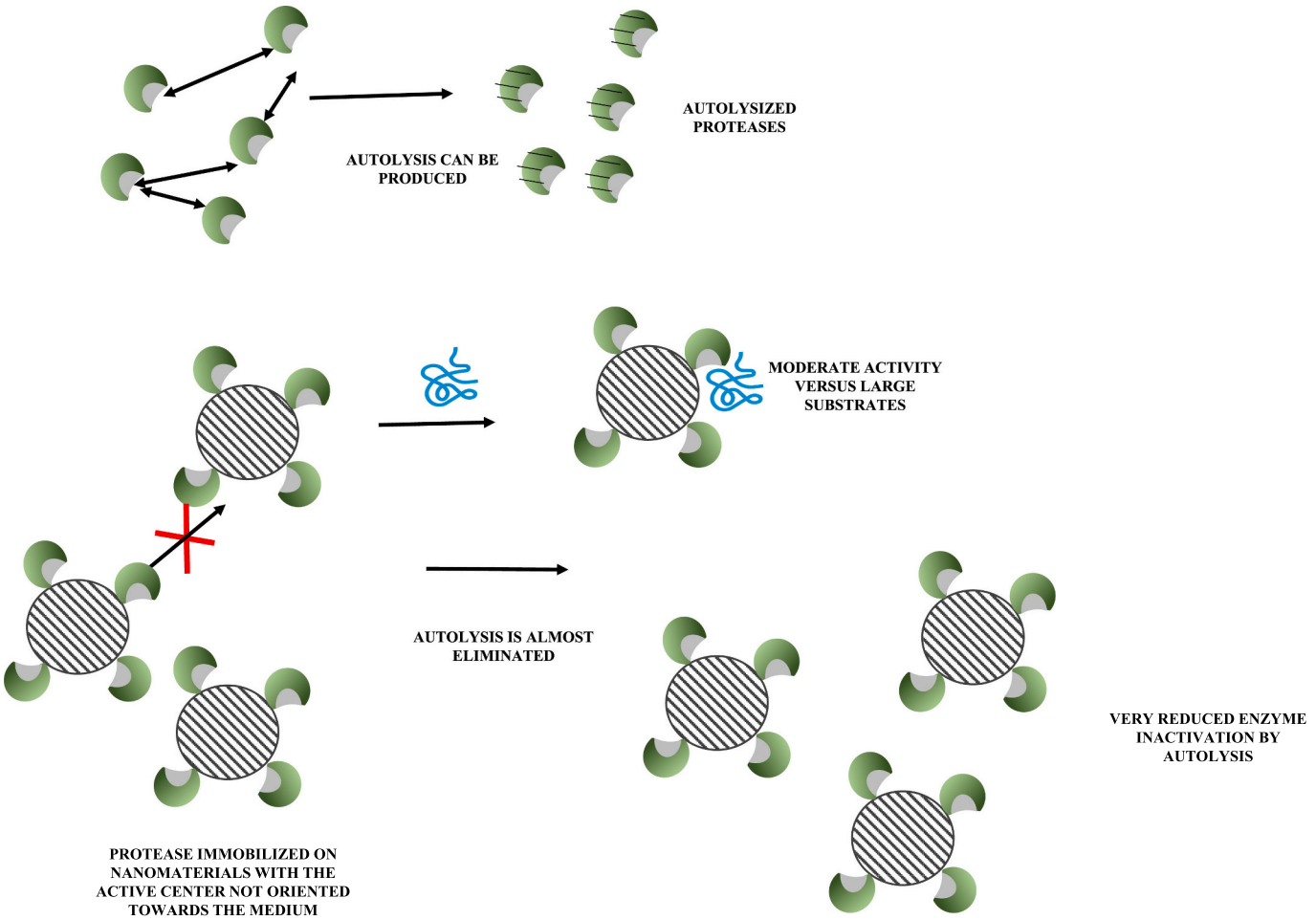

Fig. 19. Possibility of preventing autolysis via immobilization of proteases with a not full orientation towards the media: some activity versus large substrates may be maintained.

of orange juice (Shojaei et al., 2017). The protease enzyme mediates the efficient breakdown of proteins present in the juice, hence preventing the formation of complexes between positively charged proteins and negatively charged pectin. The occurrence of turbidity and insolubility is attributed to the interaction between proteins and cell components or phenolic compounds and can be mitigated through proteolytic activities (Pinelo et al., 2010). Using chitosan nanoparticles immobilized protease showed a significant reduction in turbidity formation over the cold storage period.

Recently, the fluidized-bed reactor has gained considerable popularity for implementing continuous processes, particularly in cases where substrates consist of dispersed particles or exhibit high viscosity (Benucci et al., 2020; Gómez et al., 2007). Therefore, the synthesis of food additives and food processing can be carried out with ease and in a more efficient way. A set of biocatalytic systems utilizing pectic enzymes was developed by covalent immobilization on glass beads, nylon granules, and polyacrylonitrile-based beads and employed for clarifying apple juice. Results revealed that the utilization of a fluidized bioreactor containing immobilized enzymes resulted in the complete hydrolysis of pectin within 41 mins, whereas packed reactors required 131 mins for the same process (Diano et al., 2008). The study highlights the potential of fluidized bed bioreactors in continuous juice clarifying for industrial implementation, emphasizing their ability to effectively reduce production costs.

The immobilization of a single enzyme has shown promise for several industrial applications. However, studies have indicated that employing a co-immobilization technique, which involves the collaboration of multiple enzymes, can significantly enhance their biocatalytic activity. The utilization of multi-enzyme systems in conjunction with a reasonable immobilization technique has been shown to improve productivity and reduce the cost associated with product recovery. Kharazmi and Taheri-Kafrani (2023) introduced an effective approach to fabricate a novel nanobiocatalytic system for fruit juice clarification by covalently co-immobilizing pectinase and xylanase onto functionalized magnetic nanoparticles. After 2 h of enzymatic treatment, the resulting co-immobilized system catalyzed over 50% turbidity reduction in pineapple juice. It retained over 60% of the original catalytic activity after nine successive cycles, suggesting that this bienzyme nanobiocatalyst has potential applications in industrial juice processing. In another study, Benucci et al. (2019) utilized a new multienzymatic system consisting of covalent immobilization of protease and pectinase on chitosan microspheres, for the clarification of pomegranate juice using a fluidized bed reactor.

The hydrolysis of proteins holds considerable significance within the food industry and in the synthesis of bioactive peptides. (Siddiqui and Husain, 2019) assessed the biocatalytic effectiveness of polydopamine-coated silver nanoparticles (Ag-PDA)-conjugated protease for the hydrolysis of various types of proteins, including casein, ovalbumin, and bovine serum albumin. Regarding the native enzyme, the enzymatic capability of Ag-PDA-immobilized trypsin was observed to be superior for protein hydrolysis. About 50% and 80% of the casein protein was hydrolyzed in 40 min by the catalytic action of free and immobilized trypsin, respectively. Similarly, the immobilized nanobiocatalyst demonstrated an improvement of 17% and 8% in the hydrolysis of ovalbumin and BSA in 2 h, respectively. The findings were comparable to protein digestion using immobilized trypsin onto tannin-capped

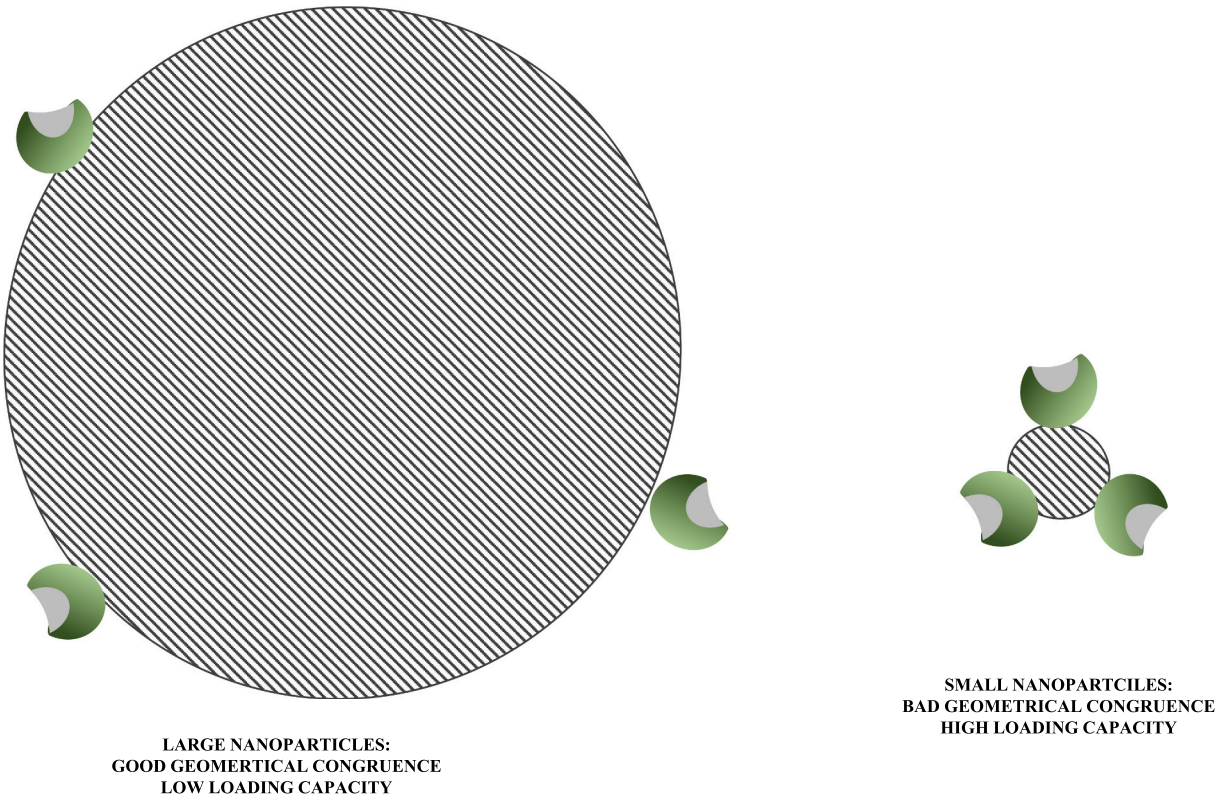

Fig. 20. Enzyme-nanoparticles geometrical congruence dependence on the diameter of the nanoparticle: the smaller the nanoparticle, the smaller the percentage of enzyme surface that can interact with the support.

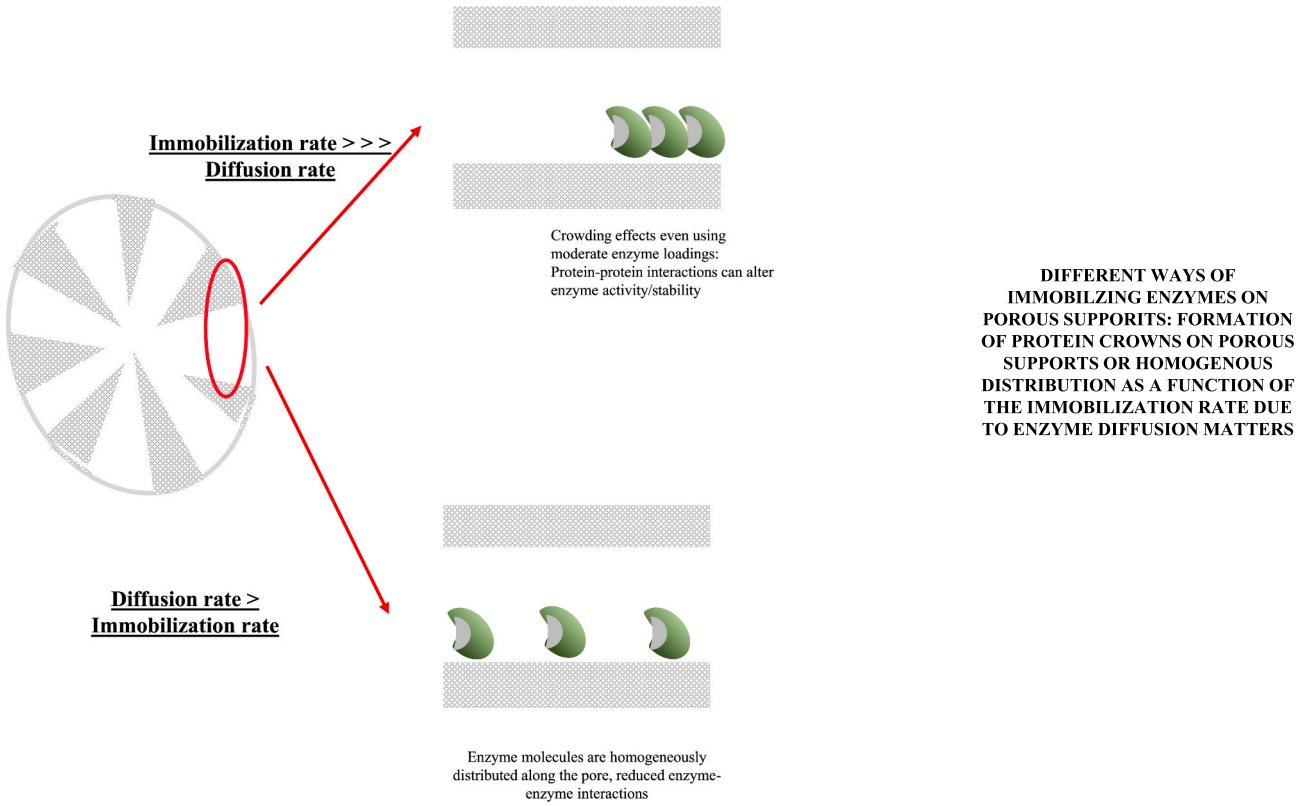

Fig. 21. Enzyme distribution on a porous support depending on the ratios immobilization rate/enzyme diffusion rate.

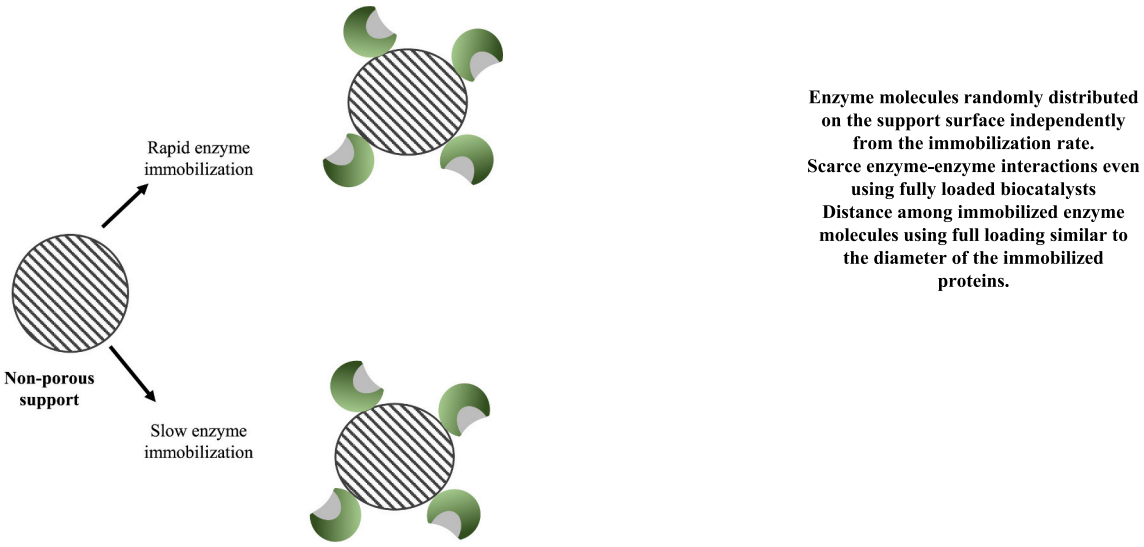

Fig. 22. Enzyme distribution on nanoparticles is not possible, independently of the immobilization rate. Moreover, it should not have a real impact on enzyme features.

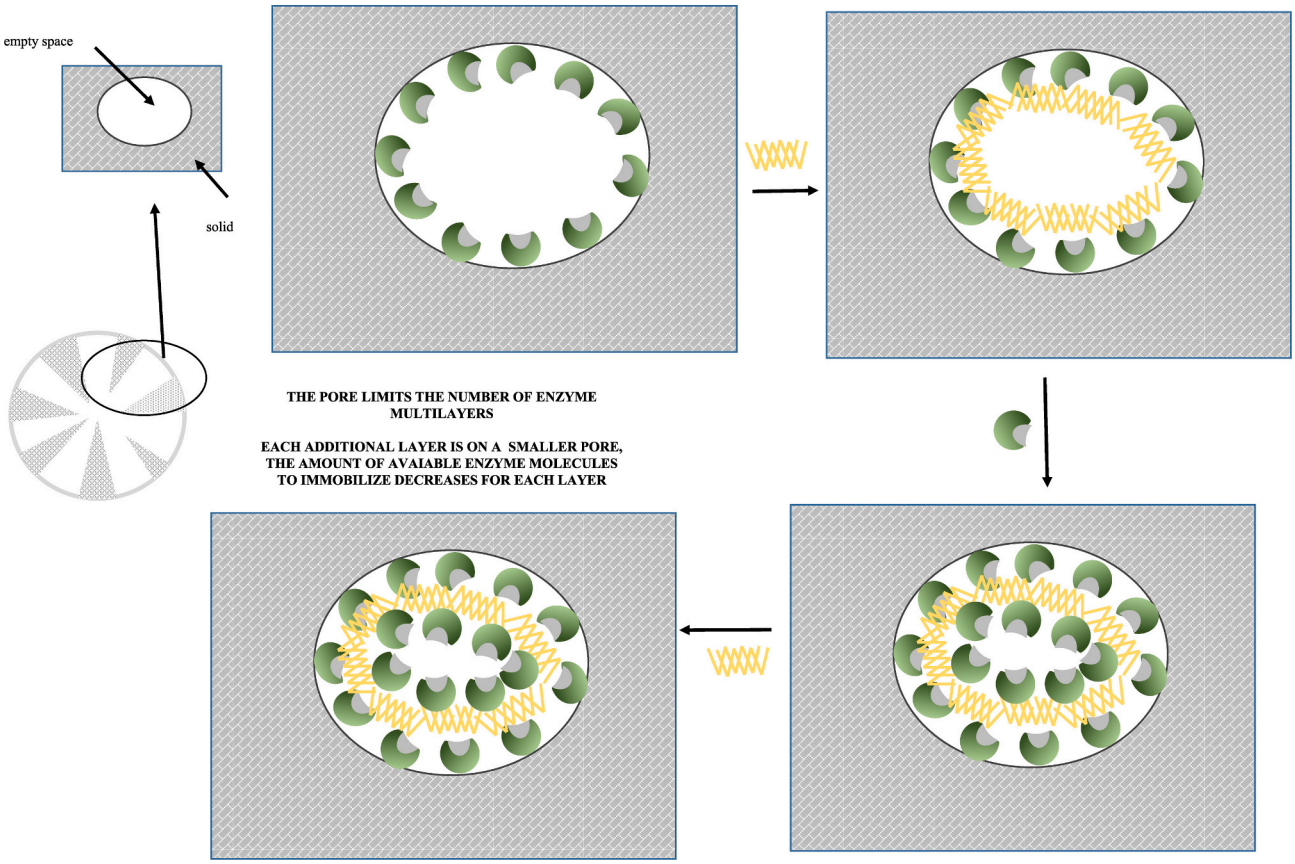

Fig. 23. Building of multilayer-enzyme biocatalysts using a porous support: each enzyme layer is smaller and there is a physical limit to the number of enzyme layers.

magnetic nanoparticles (Atacan et al., 2017). The low hydrolytic ability of native trypsin compared to Ag-PDA-linked derivative may be attributed to the inadequate proteolytic process resulting from steric obstruction or autolysis. On the other hand, the enhanced digesting effectiveness of trypsin attached to Ag-PDA can be ascribed to many factors. Firstly, the minimum steric hindrance allows optimal interaction between the enzyme and the protein substrate, facilitating efficient digestion. Secondly, the limited autolysis of the trypsin molecule ensures its stability and prolonged activity during digestion. Lastly, the presence of AgNPs in the PDA matrix provides a greater surface area, allowing for a higher enzyme-to-protein substrate ratio and accelerating digestion ((Siddiqui and Husain, 2019).

3.2. *Protease-based nanobiocatalysts in detergent formulations*

Proteases are of paramount importance in detergent formulations

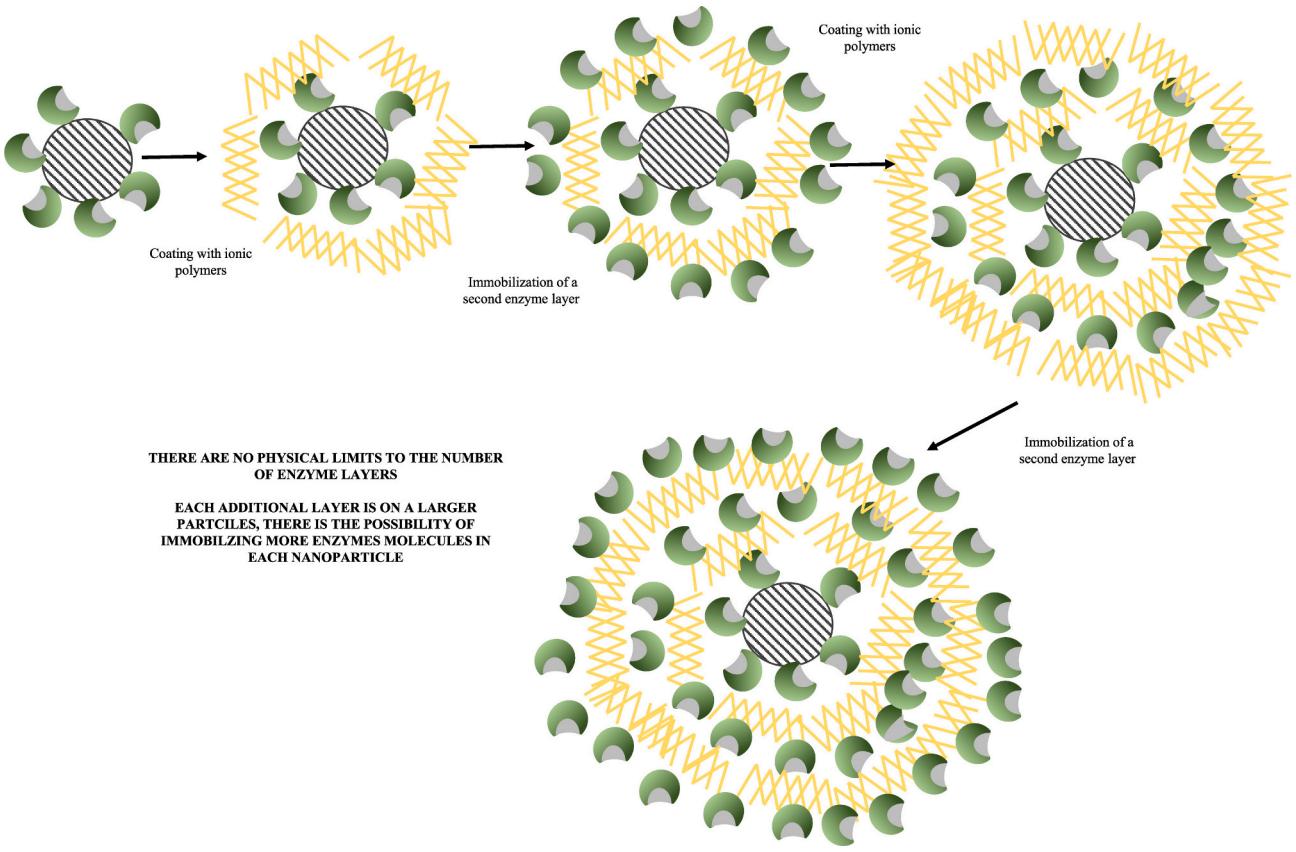

Fig. 24. Building of multilayer-enzyme biocatalysts using a non-porous support: each layer is larger and there is not a physical limit to the number of enzyme layers, just the size of the nanobiocatalyst that loses this condition.

since they contribute to removing protein-based stains and enhance the overall efficacy of detergents. Incorporating protease enzymes in detergents has evolved from minor additives to integral constituents. Nevertheless, the stability of enzymes is compromised by an alkaline environment, elevated washing temperatures, and the presence of different chemical agents (Maurer, 2004). Proteases compatible with detergents and particularly resistant to alkaline conditions and washing temperatures are direly needed in the industrial sector. Immobilization is one of the intriguing ways to enhance the adaptability and resilience of enzymes against harsh reaction conditions. The stability of protease immobilized on glutaraldehyde-cross-linked chitosan-silica gel beads was observed to be higher when compared to the free enzyme in the presence of various commercial laundry detergents such as Bingo, Omo, and Perwoll. The free and immobilized protease exhibited 84%, 131%, and 85%, and 87% of activity in *Omo* and *Ariel* detergents, respectively. According to Karakurt and Samsa (2023), the immobilized biocatalytic system demonstrated approximately 10% higher biocatalytic efficiency compared to the free enzyme in the presence of Perwoll and Bingo.

In their study, Gulmez et al. (2022) investigated the stability characteristics of an organic-inorganic hybrid nanoflower by a thermal treatment process. The nanoflower-incorporated enzyme was subjected to heating at temperatures of 30, 40, and 50 °C for 2 h in liquid and solid laundry detergents including *Omo, ART, ABC, Ariel, Alo*, and *Perwoll*. The activity of the immobilized nanobiocatalyst was shown to be superior to that of the free counterpart across all evaluated temperatures. The recombinant subtilisin protease derived from *Bacillus subtilis* PTTC 1023 exhibited full retention of its original enzymatic activity when exposed to Ariel, Tursil, Cosla, and Persil detergents at temperatures of 30, 40, and 50 °C (Gulmez et al., 2018). The utilization of protease immobilized on silica nanoparticles resulted in enhanced efficacy in removing protein-based stains in laundry detergents. Conversely, the unmodified enzyme exhibited little impact on enzymatic activity when exposed to identical soil substrates (Soleimani et al., 2013). The aforementioned findings indicate that using immobilized protease in detergent formulations may be highly effective under high temperature and alkalinity conditions. This suggests that there is potential for practical application in detergent formulations.

3.3. *Protease-based nanobiocatalysts in leather processing*

In recent years, enzyme-mediated bioprocesses have largely replaced chemical-based procedures in the leather industry for dehairing and opening skin fibers before their conversion into leather. This involves the exploitation of protease for hair removal and amylase for fiber opening, as alternatives to sodium sulfide and calcium hydroxide, respectively. Protease-assisted dehairing techniques on raw skin/hides have proven to be a significant development in the leather industry. The enzymes participating in this process facilitate the hydrolysis of non-collagenous substances, such as albumin, casein, elastin, and proteoglycans, while ensuring the integrity of keratin and collagen within the skin (Anzani et al., 2017). Proteases have demonstrated efficacy in cleaving the peptide bonds of proteins and proteoglycans inside the hair bulb region, leading to hair detachment from the skin matrix (Saravanan et al., 2014).

In contrast to the traditional sodium sulfide treatment, the utilization of protease immobilized on zinc oxide nanoparticles (ZnNPs) has emerged as a more sustainable and environmentally friendly alternative for the dehairing of skin. The utilization of the ZnNP-based nano-biocatalytic system resulted in a notable decrease in the duration necessary to attain equivalent dehairing efficiencies (Murugappan et al., 2020). During the dehairing procedure, it was noticed that the pristine protease-treated skins had signs of putrefaction, as evidenced by a pronounced odor. However, skins treated with nano-biocatalyst were less vulnerable to microbial attack and did not exhibit any adverse

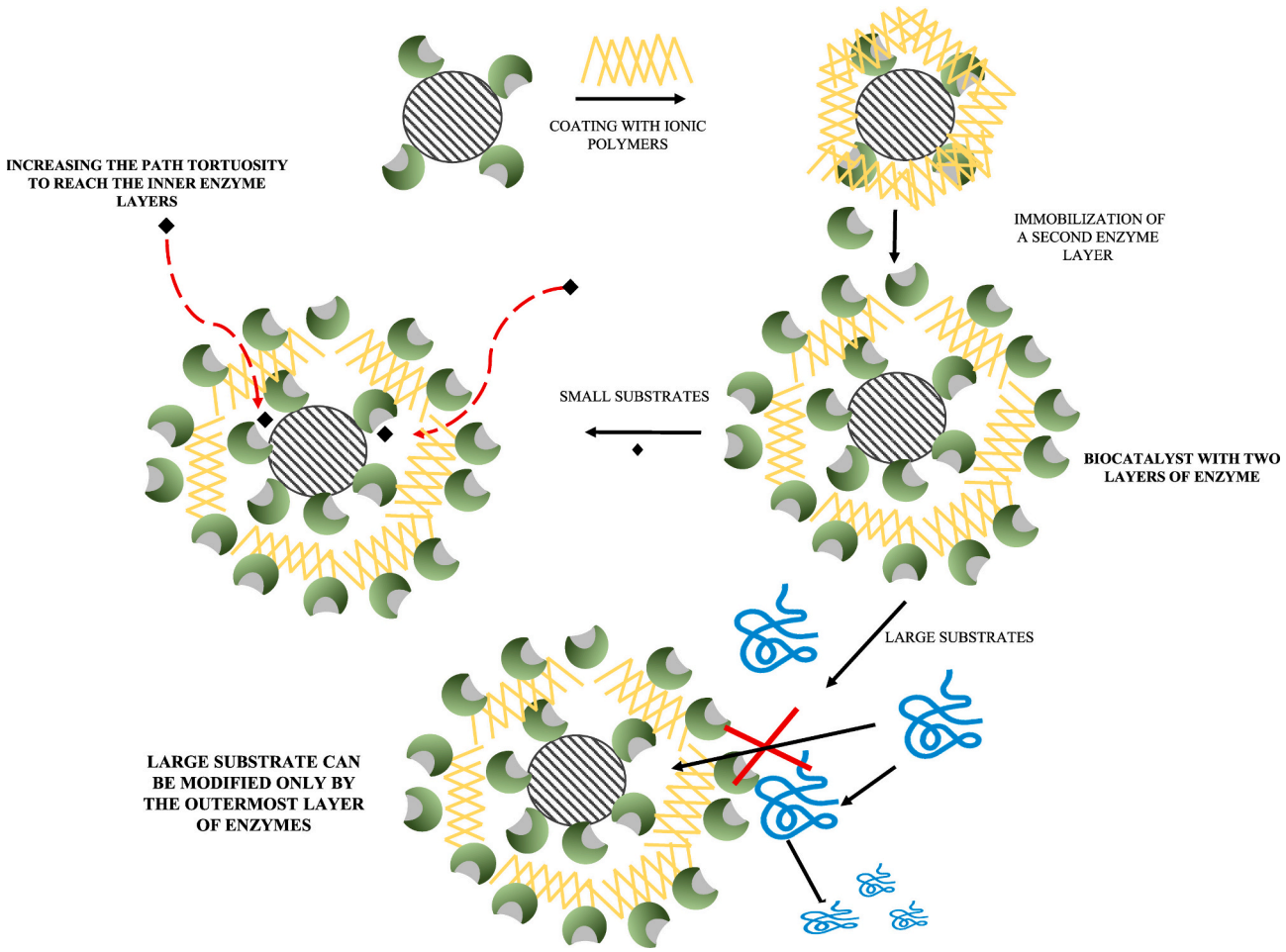

Fig. 25. The formation of a multilayer-enzyme biocatalyst produces substrate diffusional limitations and steric hindrances for the activity of the enzymes in the inner layer, raising some problems similar to porous supports.

alterations, which might be ascribed to the antimicrobial activities of zinc oxide nanoparticles. Additionally, compared to the control, the nano-biocatalyst demonstrated a higher level of effectiveness in removing intra-fibrillary material from the skin, indicating increased protease activity upon nanoparticle immobilization.

Alkaline proteases derived from protein-rich waste materials, i.e., soya residues and hair waste, were immobilized onto glutaraldehyde-functionalized iron oxide nanoparticles (MNPs), and the resulting MNPs-immobilized proteases were then used for the hydrolysis of different types of proteins, including casein, oat bran protein isolate, and egg white albumin. The immobilized Phw (protease derived from hair waste) exhibited a 24% greater degree of hydrolysis than the free Phw enzyme within the initial 30-min treatment process. Furthermore, the immobilized Phw enzyme achieved a peak hydrolysis rate of 80% after a 24-h duration. In a similar vein, the efficacy of MNPs-immobilized Phw and Psr (protease produced from soya residues) was confirmed through the hydrolysis of oat bran protein isolates. Notably, both MNPs-immobilized Phw and Psr exhibited superior hydrolysis efficiency compared to their free counterparts. The findings were consistent with earlier studies, which utilized immobilized proteases on highly active agarose supports and magnetic chitosan nanoparticles for the hydrolysis of whey and soya protein isolates (Lamas et al., 2001; Wang et al., 2014). When utilizing a vegetable protein source, both the free and immobilized forms of Psr exhibited a greater degree of hydrolysis compared to Phw. Regarding the hydrolysis of egg white albumin, it was observed that the immobilized Psr enzyme achieved a maximum hydrolysis rate of 60% within a 24-h period. On the other hand, the immobilized Phw enzyme exhibited a maximum degree of hydrolysis of 70%. Hence, it can be shown that proteases originating from various sources exhibit distinct catalytic characteristics and effectiveness, which depend on the specific substrate employed.

The alkaline protease derived from *Bacillus circulans* MTCC 7906 was immobilized onto silica nanoparticles to create a bioconjugate formulation of nano-silica alkaline protease. The nanobiocatalytic system that was developed demonstrated potential utility in the dehairing of goatskin while also avoiding any histopathological degradation (Joshi et al., 2021). After 12 h of treatment at 37 °C, the same group demonstrated an admirable dehairing performance with nano-silver enzyme bioconjugate complex containing alkaline protease. The histological examination revealed a complete removal of hairs with minimal rarefication, demonstrating the superior performance of the nano-silver enzyme bio-conjugate compared to both the crude enzyme and commercial preparation (Joshi et al., 2020). Rai and Mukherjee (2011) further supported the suitability of alkaline protease for dehairing goat skin. Overall, the research indicates that enzymatic processes have emerged as the preferable way for dehairing, surpassing chemical methods due to their ability to mitigate hazardous waste and enhance leather quality.

3.4. *Protease-based nanobiocatalysts for silver recovery.*

Microbial proteases are of significant importance in the metal recovery process, particularly in the context of silver recovery from X-ray films. The conventional techniques for silver recovery encompass the

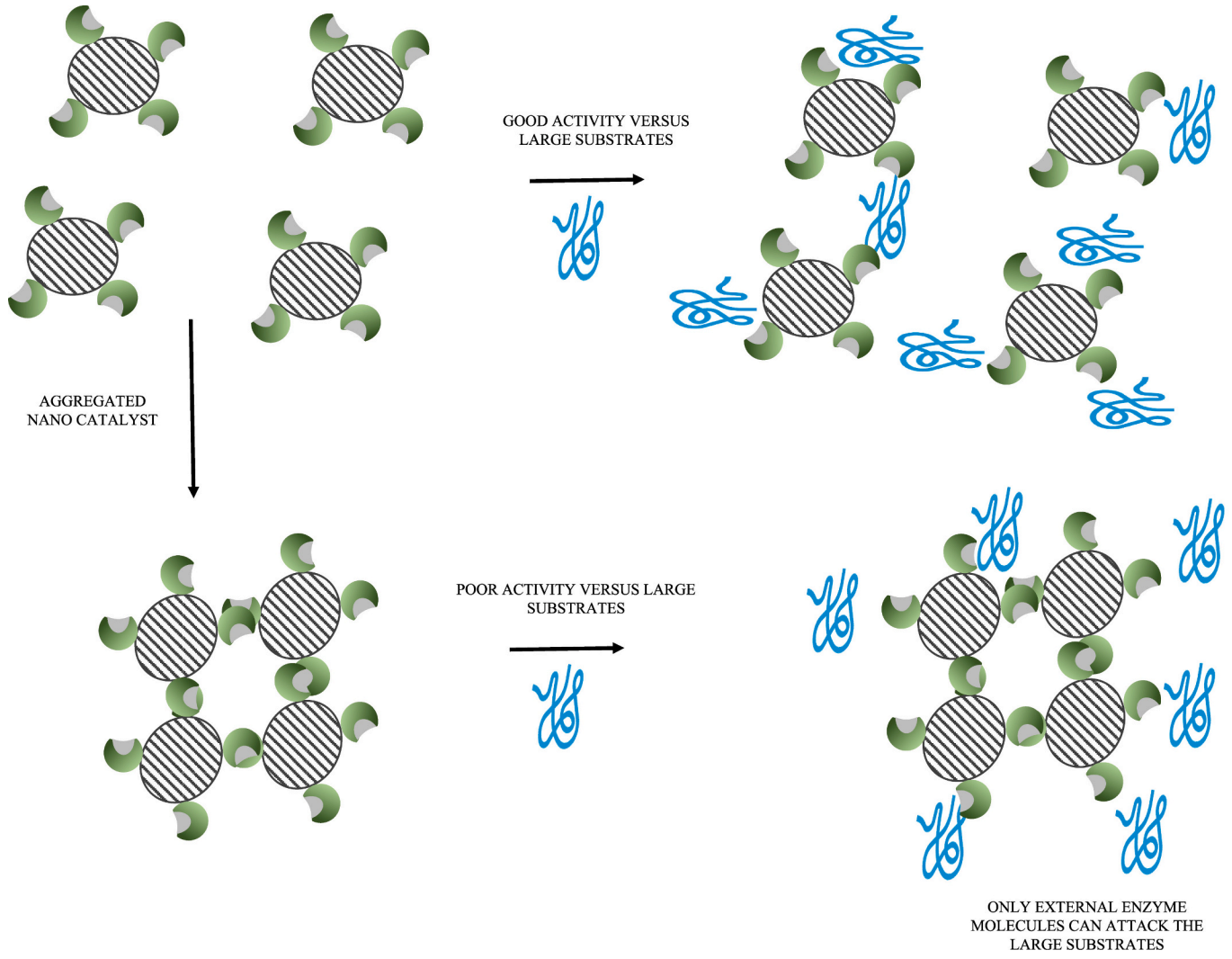

Fig. 26. Nanobiocatalyst aggregation leads to the loss of the advantages/problems of nanobiocatalyst, forming a porous macrosupport.

incineration of X-ray films, the extraction of silver through smelting from the resulting ashes, and the chemical exfoliation of silver from the film. However, it is important to note that these methods have adverse effects on the environment and may lead to a low-grade silver recovery. The implementation of environmentally sustainable techniques for the recovery of silver utilizing hydrolyzing enzymes, such as proteases and keratinases, has the potential to enhance the quality of the recovered silver while concurrently diminishing the labor and chemical consumption associated with conventional processes (Liya et al., 2023). Nevertheless, the cost associated with the recovery of silver from X-ray films can be further reduced with the immobilization of protease, enabling their repeated utilization.

Using free and Ca-alginate immobilized proteases, Qamar et al. (2020) successfully hydrolyzed the gelatin layer from discarded X-ray sheets. While the free enzyme required 40 min of immersion time at 8 pH and 40 °C to dissolve the gelatinous layer, the Ca-alginate entrapped protease only took 20 min to completely dissolve the layer and release silver particles. Gelatin attached to silver was removed from X-ray films with the use of a magnetic CLEA-protease nanocomposite treatment. The formation of a clear zone following a 2-h treatment signifies the breakdown of gelatin and the release of silver from it. Additionally, it was discovered that protease in its immobilized form hydrolyzed gelatin more than the enzyme in its free form (Hashemabadi and Badoei-Dalfard, 2019). In addition to its cost effectiveness and environmental friendliness, protease-assisted hydrolysis offers the notable advantage of enabling the reuse of films after silver extraction. These films can be employed in various applications, such as the development of X-ray film and the production of recording tapes, fabric, packaging materials, and soft-drink bottles (Radha and Arun, 2010).

3.5. *Protease-based nanobiocatalysts in bioremediation.*

Numerous industrial operations and processes, including those within the food sector, such as the manufacturing of leather from animal hide, the extraction of gelatin from animal bones, and the processing of fish and meat, resulted in the generation of wastewater with elevated levels of total dissolved solids (TDS). This is primarily attributed to the utilization of sodium chloride and calcium chloride salts. The discharge of untreated saline wastewater that contains high levels of TDS and organic constituents has a detrimental impact on the environment (Sivaprakasam et al., 2011). Physicochemical methods are commonly employed for the treatment of these effluents; however, these approaches are associated with high costs. Furthermore, the presence of organic impurities and pollutants in the wastewater contributes to the issue of air pollution (Boopathy et al., 2013).

Recently, reports have established the potential of employing enzyme-mediated biocatalytic methods to treat high TDS-containing wastewater and the degradation of organic matter in highly saline environments. For example, Pounsamy et al. (2019) developed a nanoporous carbon catalyst utilizing a halotolerant protease for protein

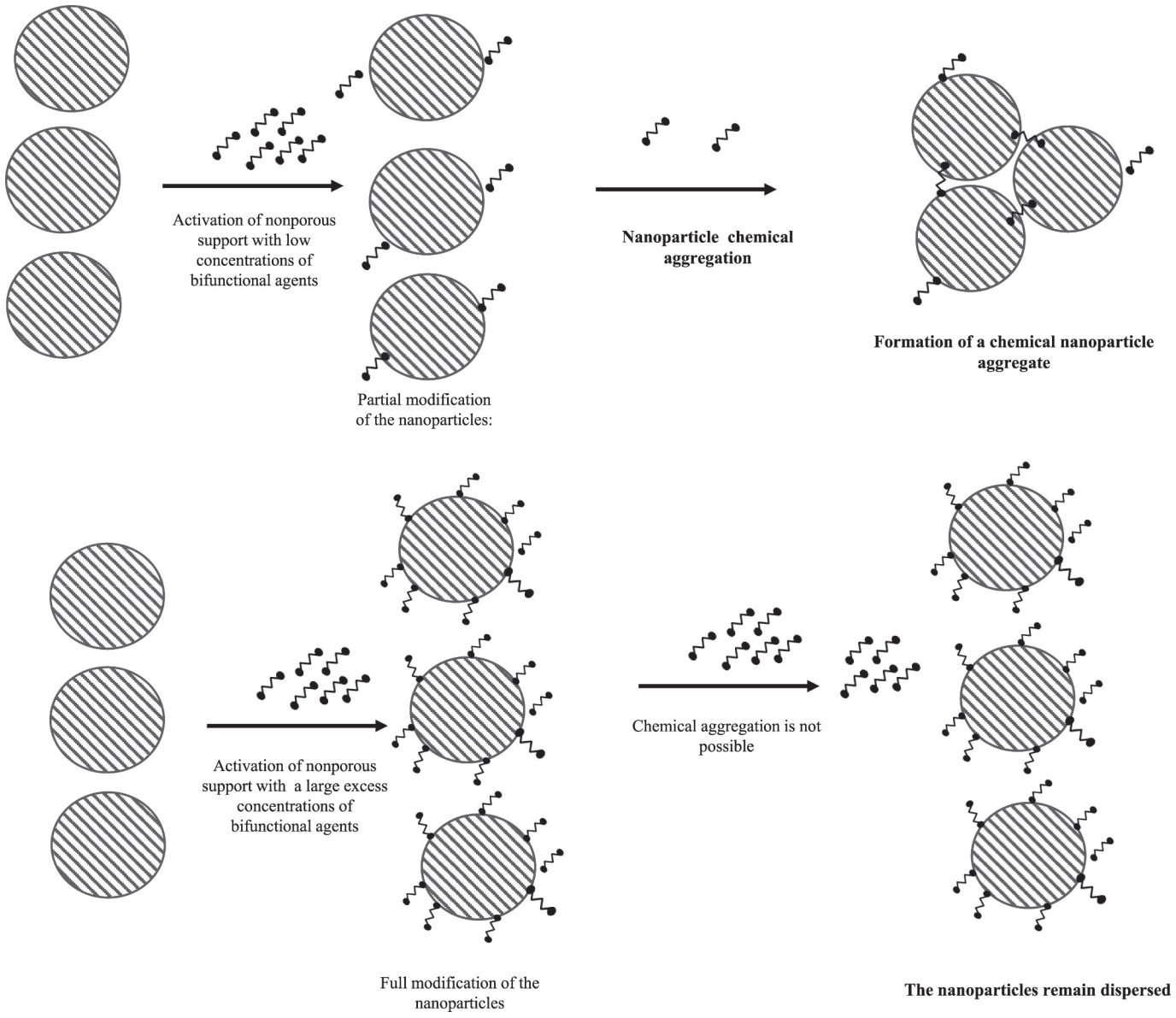

Fig. 27. Chemical nanoparticles aggregation using bifunctional reagents in the activation step (e.g., glutaraldehyde). The use of a large excess of this reagent prevents aggregation.

breakdown in tannery-TDS wastewater produced during the soaking procedure. Following a treatment duration of 1.5 h, the catalytic activity of nanoporous-activated carbon-immobilized protease resulted in over 95% protein hydrolysis at pH 6.0 and 7.0. However, the catalytic activity was gradually decreased in alkaline environments. A protein fragmentation rate of 96% was observed at a temperature of 30 °C, and the nanobiocatalyst maintained its efficacy, with protein fragmentation levels ranging from 70% to 75%, even at elevated temperatures of 50 °C and 60 °C.

In another study, Maharaja et al. (2017) investigated the remediation capabilities of protease immobilized functionalized nano porous activated carbon (Pr-NPAC) in the breakdown of protein found in the soaking liquor discharged from the leather industry. The findings of the study indicate that the protein content of the soak liquor decreased by 91.5% when the temperature was raised to 40 °C. Additionally, it was observed that further temperature increases resulted in a decrease in protein degradation and amino acid formation. This phenomenon may be attributed to the increased kinetic motion, which hinders the adsorption of protein molecules onto the immobilized matrix. The immobilized nanobiocatalyst retained its activity over 25 consecutive cycles when exposed to the protein content of the soak liquor. The catalytic efficacy of PersiProtease1, a thermo-halo-alkali-stable protease derived from tannery wastewater, was demonstrated in the biodegradation of various substances, such as real sample tannery wastewater protein, whey protein, chicken feathers, dehairing sheepskin, and waste X-ray films. The results revealed that PersiProtease1 has shown considerable potential in efficiently decomposing both liquid and solid industrial waste materials containing high protein levels (Ariaeenejad et al., 2022).

The rapid expansion of the poultry industry has led to significant waste generation within poultry farms and slaughterhouses. This issue can potentially present a dilemma for both human and ecological systems (Brandelli et al., 2015). Owing to the escalating environmental contamination associated with the poultry industry, particularly concerning keratin waste, developing effective biocatalysts for hydrolysis keratin has become a compelling objective for researchers. In this respect, a Bacillus keratinase was immobilized onto the functionalized magnetic nanoparticles using the cross-linked enzyme aggregates

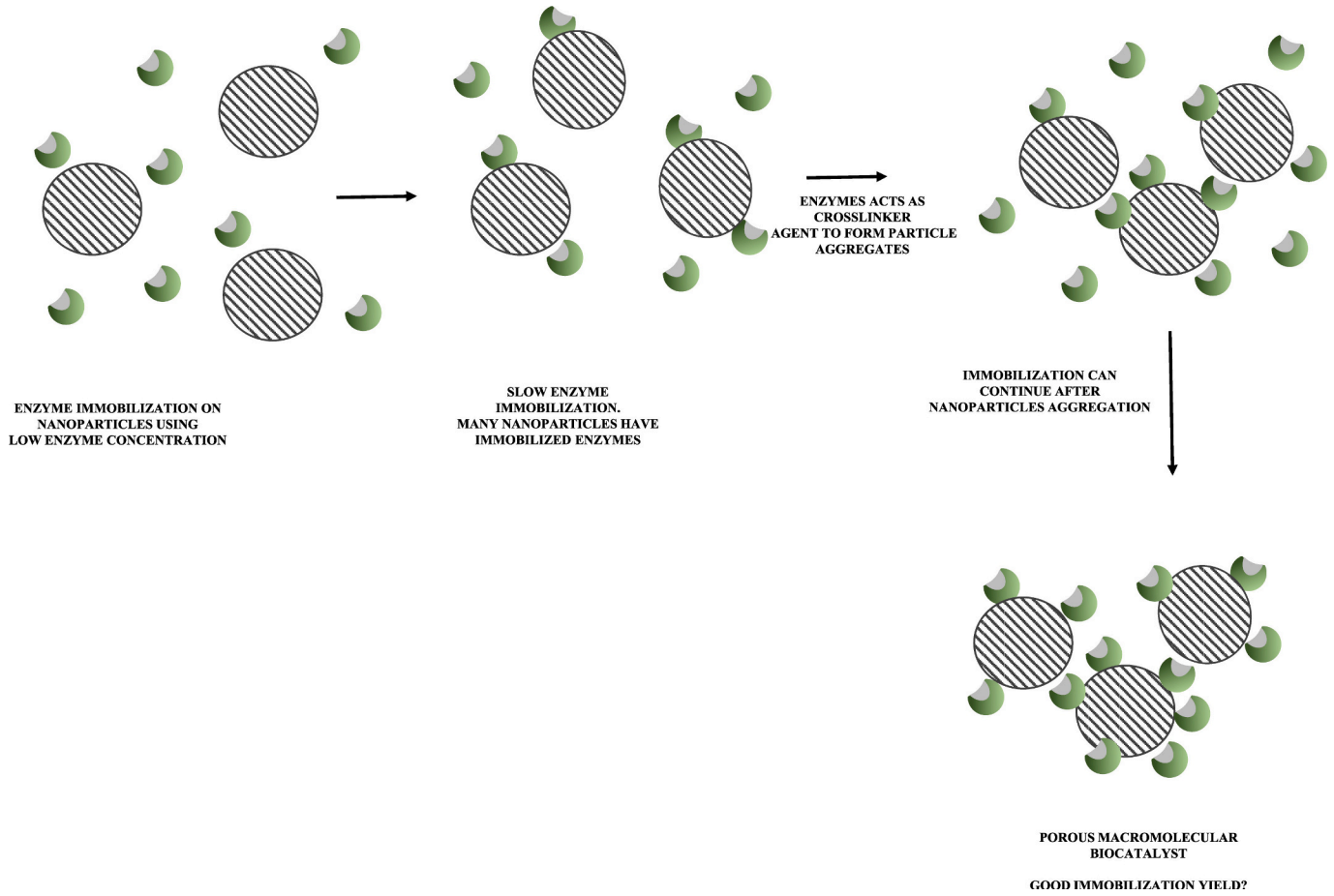

Fig. 28. Chemical nanobiocatalyst aggregation during enzyme immobilization step, where the enzyme acts as crosslinking reagent.

(CLEAs) technique. Following a 2-h incubation period at 42 °C, it was observed that the immobilized magnetic nanobiocatalyst exhibited a threefold increase in the hydrolysis degree of keratin waste compared to the free enzyme (Lotfi et al., 2021). In their study, Sarathi et al. (2019) examined the viability of utilizing electrospun polyvinyl alcohol chitosan nanofibers electrospun as a support material for immobilizing keratinases for the degradation of chicken feathers. The researchers found that the immobilized keratinase achieved a hydrolysis efficiency of approximately 88% in degrading chicken feathers after 72 h. Abdel-Fattah et al. (2018) found that the crude keratinolytic protease from *Bacillus licheniformis* ALW1 demonstrated a hydrolysis degree of up to 63% for natural feathers after a 24-h treatment. In conclusion, the application of immobilized proteases in the treatment of wastewater effluents offers many significant benefits compared to the utilization of free enzymes, such as enhanced stability, simplified handling, reusability, and a resulting reduction in operational costs.

3.6. *Protease-based nanobiocatalysts in the biomedical sector*

So far, numerous studies have demonstrated the potential benefits of proteolytic enzymes as wound-healing agents. The enhancement of antibacterial therapeutic efficacy by proteases can be achieved by accelerating the breakdown of the protein constituents inside the biofilm matrix. In their study, Baidamshina et al. (2020) documented the capabilities of chitosan-immobilized Ficin, a nonspecific sulfhydryl protease, in inhibiting biofilm formation and promoting wound healing. The chitosan-immobilized Ficin could disrupt biofilms induced by staphylococcal bacteria and enhance the effectiveness of antimicrobial drugs against bacteria embedded inside the biofilms. In an in vivo setting, the application of Ficin, whether in soluble or immobilized form, resulted in a 50% reduction in the size of skin wound regions infected with *Staphylococcus aureus* in rats following a treatment period of 4 days. In addition, the implementation of an immobilized enzyme led to a three-logarithmic reduction in the number of *S. aureus* cells on the surfaces of the wounds over six days. This contrasts with the control group, which required more than ten days to reach the same outcome. Other benefits include a smoother reepithelialization process and the development of new tissue that closely resembles the collagen structure found in the native tissue. The findings indicate that the utilization of immobilized Ficin exhibits potential advantages in addressing biofilm-associated infections while also facilitating wound healing and microbial decontamination at an accelerated rate. In another study, the same group also showed that immobilized endolytic cysteine protease papain on chitosan matrices of medium (200 kDa) and high molecular weight (350 kDa) increases the efficiency of antimicrobials against bacteria embedded in biofilms (Baidamshina et al., 2021). Both the soluble and immobilized forms of papain shown high efficacy in eradicating biofilms formed by *S. aureus* and *S. epidermidis*. As a result, papain can enhance the efficacy of antimicrobial agents against Staphylococci embedded within biofilms, making it potentially useful for the treatment of external wounds.

Fabricating novel nano-supports with multifunctional properties is immensely important for protease immobilization. Kim et al. (2022) designed a 3-aminopropyltriethoxysilane and Fe_3O_4 NPs functionalized halloysite nanotubes-based multifunctional nano-support to immobilize protease type I enzyme. The as-synthesized nanomaterial construct deciphered robust anti-biofilm activities against *Escherichia coli*, *Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus*, and antibacterial potential. Prabhawathi et al. (2019) fabricated gold core and silver shell nanoparticles (AuAgNPs) and employed them to conjugate

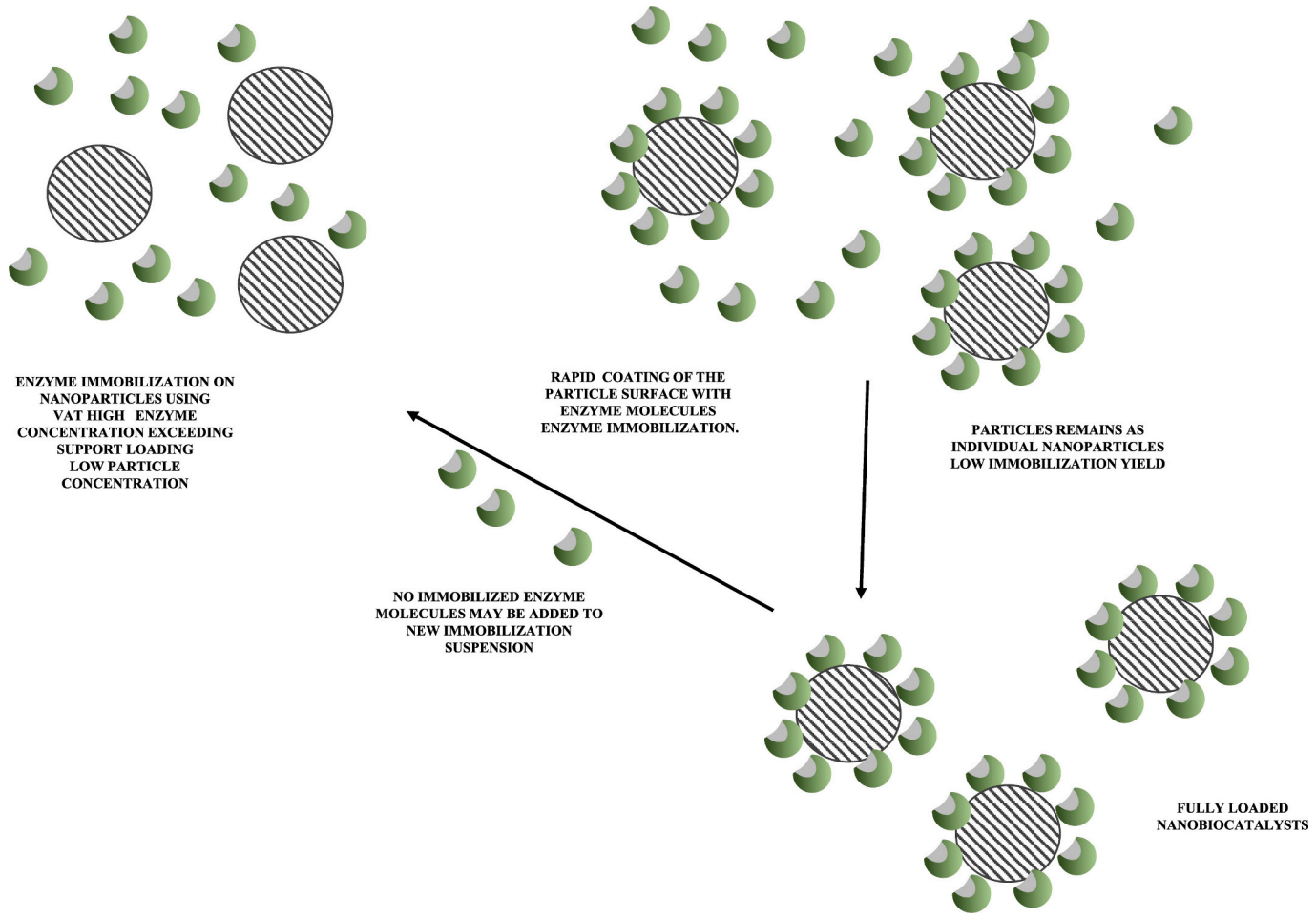

Fig. 29. The use of a large excess of enzyme during immobilization on non-porous nanomaterials can prevent the biocatalyst crosslinking via the enzyme, although this can lead to very poor immobilization yields.

Table 1
Protease immobilization onto polymeric (nano)carriers with immobilization yield, key features, and recyclability potential.

Immobilization matrix	Immobilization yield (%)	Key features	Recyclability	References
Alginate/dextrose	77.6	Lower activation energy and deactivation constant, improved thermostability	92.7 and 52.4% after 8 and 12 hydrolysis cycles, respectively.	(Abdella et al., 2023)
Calcium alginate	>70	Lower activation energy, and improved thermal and pH stability profiles	>80% activity retention during initial 8 cycles that was decreased to 73% and 68% in next experiments	(Qamar et al., 2020)
Calcium alginate	68.76	Improved thermal and pH stability with 80.88% storage stability at 4 °C for 30 days	32.97% activity retention after 7 consecutive cycles	(Adetunji and Olaniran, 2023)
Porous chitosan microspheres	76.7 ± 1.7	Improved catalytic activity and stability after immobilization	70.3% activity retention after 7 repeated hydrolysis cycles	(Tang et al., 2023)
Chitosan	69.9	Increased optimum temperature range (50–70 °C)	47.08% activity retention after three cycles	(Ramalho and de Castro, 2023)
Chitosan-silica gel beads	96	High stability on metal ions and catalytic performance	50% activity retention after 5 repetitive cycles	(Karakurt and Samsa, 2023)
Chitosan/cellulose acetate nanofiber	85	52% increased activity than free protease at pH 10. High storage stability about 75% at 60 °C and 180 min at pH 9	About 81% activity rendition after 10 cycles of reuse	(Badoei-Dalfard et al., 2020)
Chitosan beads	86.43 ± 1.15	High pH and thermal stability than free protease	70.53% activity retention after 5 repeated cycles	(Kamal et al., 2021)
Chitosan nanoparticles	262.32	High thermostability	73.53% activity retention after 15 cycles	(de Oliveira et al., 2021)
κ-carrageenan/ hyperbranched poly (amidoamine)	84.38 ± 0.83% at pH 9.3	High thermostability and increased half-life	89% activity retention after 8-weeks storage at 4 °C	(Awad et al., 2020)
Magnetic chitosan	–	52% activity enhancement by the immobilized enzyme	70% activity retention after 10 cycles	(Khankari et al., 2021)

Table 2
Protease immobilization onto silica-based (nano)carriers with immobilization yield, key features, and recyclability potential.

Immobilization matrix	Immobilization yield (%)	Key features	Recyclability	References
Double mesoporous core-shell silica nanospheres	75.3	High stability in commercial detergents, salt, organic solvents, and surfactants	79.8% activity retention after 10 repeated cycles	(Ibrahim et al., 2021)
Nano-silica	–	1.7-folds increased in enzyme activity. High catalytic stability	–	(Joshi et al., 2021)
Polymer-coated mesoporous silica NPs	–	60% storage stability after 28 days of incubation.	45.83% activity retention after 5 repeated cycles	(Özbek and Ünal, 2017)
Fe_3O_4@SiO_2 –NH_2	–	Improved thermostability of trypsin	85% activity retention after 6 repeated cycles	(Aslani et al., 2018)
Hollow mesoporous silica nanoreactors	460 μg/mg	Over 74% enzyme activity maintenance under extreme conditions	80% activity retention after 5 repeated cycles	(Zhang et al., 2023)
Magnetic silica nanoparticles	–	High storage stability (up to 93%) after 25 months.	About 85% activity retention after six consecutive cycles	(Glomm et al., 2021)
Cellulose monoacetate/ polycaprolactone	82	No significant change in activity and stability of the enzyme	59% activity retention after 7 repeated cycles	(Aykut et al., 2017)
Magnetic core/ mesoporous shell silica NPs	91.2	Improved activity and stability of enzyme	75% activity retention after 10 repeated cycles	(Ibrahim et al., 2016)
Fumed silica nanoparticles	–	High exopeptidase activity	–	(Katić et al., 2021)
Supermagnetic halloysite nanotubes	–	Antibacterial effect and antibiofilm effect on *E. coli* and *S. aureus*	43% activity retention after 5 repeated cycles	(Kim et al., 2022)
Magnetic halloysite nanotube	185.5 mg/g	Improved kinetics, thermodynamics, and storage stability	Up to 56% activity retention after 10 repeated cycles	(Sillu et al., 2022)

Table 3
Recent studies reporting protease immobilization onto graphene oxide with immobilization yield, key features, and recyclability potential.

Immobilization matrix	Immobilization yield (%)	Key features	Recyclability	References
Graphene oxide nanosheets activated with glutaraldehyde	57.35	Improved thermal and pH stability. 92% storage stability at 4 °C for 30 days	–	(Ranjbari et al., 2019)
Nitrogen-doped graphene quantum dots	78	Stability enhancement in polar solvents. Improved thermal stability	65% of initial activity retention after 5 repeated cycles	(Mirzaei et al., 2022)
Graphene oxide silver NPs	–	Higher immobilization capacity and improved catalytic activity	–	(Liu et al., 2021)
Graphene oxide nanosheets	80	35% activity retention at 25 °C for 30 days. Improved thermal stability after GO binding	54% activity retention after 10 repeated cycles	(Movahedi et al., 2019)
Graphene oxide nanosheets	80	Improved thermal stability. 63% activity retention at 4 °C after 30 days	–	(Gu et al., 2018)
Graphene oxide	–	High thermostability and storage stability (89.9% after 5 days)	Up to 40% of initial activity after 3 repeated cycles	(Su et al., 2012)
Graphene oxide	–	Improved storage stability of *nano*-immobilized protease	–	(Su et al., 2013)
Polydopamine-coated magnetic graphene	0.175 mg/mg	High storage stability (almost 100% activity at 4 °C after 30 days)	Almost 100% (like first cycle) activity retention after 5 repeated experiments	(Shi et al., 2014)
Graphene oxide	–	Improve and maintain the activity of protease enzyme	–	(Xu et al., 2012)
Graphene oxide nanosheets	–	Improved thermal and chemical stability (up to 80% at 50 °C for 6 h, whereas free enzyme showed only 4%)	–	(Sakata et al., 2012)

subtilisin, aiming to explore their potential in biomedical applications. The obtained biomaterial AuAgSNPs demonstrates antibiofilm characteristics against *E. coli* and *S. aureus*, though with a lower effectiveness level than the biomaterial made with bare AuAgNPs. It was observed that subtilisin has the ability to disrupt the adhesive surface proteins of bacteria, hence impeding the process of biofilm development. In contrast to AuAgNP, the AuAgSNPs did not exhibit any cytotoxic effects on 3 T3 cells. Furthermore, the presence of subtilisin was found to enhance the proliferation of fibroblast cells. The biocompatibility, antibacterial, and antibiofilm activities of AuAgSNPs suggest its utilization in medical devices and implants.

Urokinase is an enzyme that is involved in the breakdown of blood clots. As a thrombolytic agent, it can dissolve blood clots by converting proenzyme plasminogen into active enzyme plasmin, which plays its role in fibrinolysis. Immobilizing urokinase onto agar-coated magnetic nanoparticles has been studied for its potential utility in thrombus clearance. Thrombolytic analysis revealed a noteworthy improvement in the thrombus removal efficiency using magnetically controlled urokinase-coated MNPs compared to a pure urokinase preparation. There was an average 50% increase in the thrombolysis rate when urokinase-coated MNPs were introduced. The in vitro thrombolysis test conducted in a microfluidic channel demonstrates an almost complete eradication of thrombus, which can be attributed to the clot-dissolving properties of urokinase. This effect is particularly notable since it prevents the formation of blood clots during the magnetically-activated microablation process. In addition, the experiment shows that a 10.32 mg thrombus mass may be removed entirely in the microchannel within 180 s (Chang et al., 2017).

Utilizing a non-toxic natural hydrogel to encapsulate cross-linked enzyme aggregates (CLEAs) is highly advantageous for orally delivering bioactive protein drugs, peptides, and enzymes. Sangeetha and Abraham (2008) studied the controlled protein release efficacy of subtilisin encapsulated in alginate: guar gum hydrogel composite beads. The beads encapsulated with CLEAs exhibited a protein loading of 66.65%. Protein release was observed to be extremely slow at pH 1.2. Nevertheless, the entrapped protein was released quickly due to the hydrogel swelling at pH 7.4. The hydrogel exhibited a lower protein release at pH 1.2 compared to pH 7.4, which was mainly attributed to

Table 4
Recent studies reporting protease immobilization onto metallic/metal-organic frameworks with immobilization yield, key features, and recyclability potential.

Protease enzyme type	Nanomaterials for immobilization	Key features	References
Alkaline protease	Gold–TiO_2 Core–Shell Nanowire	Improved catalytic stability	(Farhadyar and Sadjadi, 2011)
Protease	γ-Fe_2O_3/Fe_3O_4 Magnetic NPs	Improved stability	(Xin et al., 2010)
Protease	Zinc oxide nanoparticls	Improved catalytic and thermal stability	(Diyanat et al., 2018)
Aspartic protease	Iron oxide nanoparticles	Improved catalytic activity	(Gao et al., 2018)
Aspartic protease	$Fe(OH)_3$@Fe_3O_4	Improved stability	(Moslemi et al., 2018)
Trypsin and chymotrypsin	Vinyl sulfone agarose beads	Improved stability and catalytic activity	(Morellon-Sterling et al., 2021)
Protease co-immobilized with amylase	Copper oxide	Improved catalytic stability	(Murugappan and Sreeram, 2021)
Keratinase	Fe_3O_4 nanoparticles	Improved catalytic activity	(Lotfi et al., 2021)
Protease	Magnetic metal–organic frameworks	Improved catalytic activity and thermal stability	(Karami et al., 2022)
Protease	Metal–organic frameworks	Improved thermal and storage stability with lower activation energy	(Badoei-Dalfard et al., 2020)
Trypsin	Nanoporous metal-organic frameworks	High catalytic stability	(Liu et al., 2013)
Proteases	Nanoflower-like metal organic frameworks	High catalytic activity. Improved thermla and organic solvent stability	(Li et al., 2023)

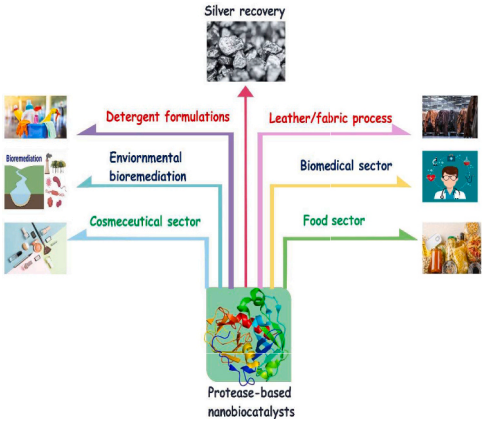

Fig. 30. Different uses of nanobiocatalysts of proteases.

the pH-sensitive properties of the alginate component rather than the guar gum. The incorporation of guar gum, a nonionic polysaccharide, has enabled the controlled release of protein under pH 7.4 conditions. The findings indicate that the subtilisin enzyme, when encapsulated within hydrogel beads, exhibits potential for facilitating the oral administration of peptides. This is attributed to the enzyme-controlled release occurrence at pH 7.4, which corresponds to the physiological pH of the jejunum region. Fig. 31 demonstrates the emerging biotechnological applications of protease-based nanobiocatalysts.

4. Concluding views and outlook

Progress in nano-structured immobilized enzymes has devoted a great potential for refining enzyme functionality. Nanobiocatalytic methods use several nano-structured constituents, including nanoparticles, nanofibers, nanotubes, and nanoporous materials, for enzyme immobilization and maintaining their catalytic performance. Nanostructured materials have been shown to possess various superior characteristics over conventional solid supports for enzyme immobilization. Nanocarriers immobilized proteases were reported as more resistant to denaturation caused by the change in temperature and pH, detergents, organic solvents, and various other denaturants. Nanocarrier-immobilized proteases have been successfully studied for their continuous and batch reactor operations for different purposes in leather, detergents, biomedical, food, and pharmaceutical industries. Furthermore, the high catalytic efficiency of protease in organic solvents has been reported after immobilization onto nano-support matrices. Immobilization technology in the near future will provide cost-effective alternative methods for bio-industry.

The emerging applications of immobilized enzymes in biocatalytic, environmental, and biomedical sectors are often confronted by non-biodegradable support materials, the elevated cost of support fabrication, and the incapability of using the biocatalytic system on a larger scale, mass transfer efficiency, and translation from lab-scale processes to industrial level. The most significant obstacles in transforming this technology towards their deployment are the elevated costs of constructing novel support materials with unique features, adequate enzyme production, and satisfactory enzyme immobilization. Additional research is necessary to overcome the issues of biodegradability, bioavailability, and high cost of enzyme-conjugated carriers for making this technology industrially and commercially feasible.

The development of an immobilization bio-system should be meticulously considered in terms of using an adequately designed support carrier, an appropriate functional group on the surface of the support, and the right immobilization procedure (i.e., support activation, conditions for enzyme immobilization, multi-interaction conditions between support and enzyme). If any of these factors are not considered while immobilizing the enzyme, the optimal results might not be achieved, leading to an enzyme with even poorer biocatalytic properties compared to the free form of the enzyme in some instances. On the other hand, a properly adopted immobilized protocol can yield enzyme systems with high stability (even thousands of folds) and improved properties than soluble counterparts.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

The NOBELIUM JOINING GDANSK TECH RESEARCH COMMUNITY program is graciously acknowledged for supporting this work under the international collaboration project awarded to Muhammad Bilal (Grant number 44/2023/IDUB/I.1). We gratefully recognize the financial support from Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (PID2022-136535OB-I00). A.B.M. thanks the Generalitat Valenciana (CIPROM/2021/70) for financial support and PID2021-123079OB-I00 project funded by MCIN/AEI/10.13039/501100011033 and "ERDF A way of making Europe".

References

Abdel-Fattah, A.M., El-Gamal, M.S., Ismail, S.A., Emran, M.A., Hashem, A.M., 2018. Biodegradation of feather waste by keratinase produced from newly isolated Bacillus licheniformis ALW1. J. Genet. Eng. Biotechnol. 16 (2), 311–318.

Abdella, M.A., Ahmed, S.A., Hassan, M.E., 2023. Protease immobilization on a novel activated carrier alginate/dextrose beads: Improved stability and catalytic activity via covalent binding. International Journal of Biological Macromolecules 230, 123139.

Adetunji, A.I., Olaniran, A.O., 2023. Biocatalytic profiling of free and immobilized partially purified alkaline protease from an autochthonous Bacillus aryabhattai Ab15-ES. Reactions 4 (2), 231–245.

Alavi, F., Momen, S., 2020. Aspartic proteases from thistle flowers: traditional coagulants used in the modern cheese industry. Int. Dairy J. 107, 104709.

Alexandre, H., Heintz, D., Chassagne, D., Guilloux-Benatier, M., Charpentier, C., Feuillat, M., 2001. Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. J. Ind. Microbiol. Biotechnol. 26, 235–240.

Almarasy, A.A., Yamada, Y., Mashiyama, Y., Maruyama, H., Kimura, Y., Fujimori, A., 2021. Activity maintenance characteristics and protease adsorption on Langmuir monolayer of Organo-modified single-walled carbon nanotubes. ChemistrySelect 6 (21), 5329–5337.

Alonso, S., Santiago, G., Cea-Rama, I., Fernandez-Lopez, L., Coscolín, C., Modregger, J., Bargiela, R., 2020. Genetically engineered proteins with two active sites for enhanced biocatalysis and synergistic chemo-and biocatalysis. Nat. Catal. 3 (3), 319–328.

Ansari, S.A., Husain, Q., 2012. Potential applications of enzymes immobilized on/in nano materials: A review. Biotechnol. Adv. 30 (3), 512–523.

Anzani, C., Prandi, B., Buhler, S., Tedeschi, T., Baldinelli, C., Sorlini, G., Sforza, S., 2017. Towards environmentally friendly skin unhairing process: a comparison between enzymatic and oxidative methods and analysis of the protein fraction of the related wastewaters. J. Clean. Prod. 164, 1446–1454.

Arana-Peña, S., Rios, N.S., Mendez-Sanchez, C., Lokha, Y., Carballares, D., Gonçalves, L. R., Fernandez-Lafuente, R., 2020a. Coimmobilization of different lipases: simple layer by layer enzyme spatial ordering. Int. J. Biol. Macromol. 145, 856–864.

Arana-Peña, S., Rios, N.S., Mendez-Sanchez, C., Lokha, Y., Gonçalves, L.R., Fernández-Lafuente, R., 2020b. Use of polyethylenimine to produce immobilized lipase multilayers biocatalysts with very high volumetric activity using octyl-agarose beads: avoiding enzyme release during multilayer production. Enzym. Microb. Technol. 137, 109535.

Arana-Peña, S., Carballares, D., Morellon-Sterlling, R., Berenguer-Murcia, A., Alcantara, A.R., Rodrigues, R.C., Fernandez-Lafuente, R., 2021. Enzyme co-immobilization: always the biocatalyst designers' choice… or not? Biotechnol. Adv. 51, 107584.

Arco Arrieta, J.D., Alcántara, A.R., Fernández Lafuente, R., Fernández Lucas, J., 2021. Magnetic micro-macro biocatalysts applied to industrial bioprocesses.

Ariaeenejad, S., Kavousi, K., Mamaghani, A.S.A., Ghasemitabesh, R., Salekdeh, G.H., 2022. Simultaneous hydrolysis of various protein-rich industrial wastes by a naturally evolved protease from tannery wastewater microbiota. Sci. Total Environ. 815, 152796.

Arnold, F.H., Volkov, A.A., 1999. Directed evolution of biocatalysts. Curr. Opin. Chem. Biol. 3 (1), 54–59.

Aslani, E., Abri, A., Pazhang, M., 2018. Immobilization of trypsin onto Fe_3O_4@ SiO_2–NH_2 and study of its activity and stability. Colloids and Surfaces B: Biointerfaces 170, 553–562.

Atacan, K., Çakıroğlu, B., Özacar, M., 2017. Efficient protein digestion using immobilized trypsin onto tannin modified Fe3O4 magnetic nanoparticles. Colloids Surf. B: Biointerfaces 156, 9–18.

Awad, G.E., Ghanem, A.F., Wahab, W.A.A., Wahba, M.I., 2020. Functionalized κ-carrageenan/hyperbranched poly (amidoamine) for protease immobilization: thermodynamics and stability studies. International Journal of Biological Macromolecules 148, 1140–1155.

Aykut, Y., Sevgi, T., Demirkan, E., 2017. Cellulose monoacetate/polycaprolactone and cellulose monoacetate/polycaprolactam blended nanofibers for protease immobilization. Journal of Applied Polymer Science 134 (44), 45479.

Badoei-Dalfard, A., Khankari, S., Karami, Z., 2020. One-pot synthesis and biochemical characterization of protease metal organic framework (protease@ MOF) and its application on the hydrolysis of fish protein-waste. Colloids Surf. B: Biointerfaces 196, 111318.

Badoei-Dalfard, A., Saeed, M., Karami, Z., 2023. Protease immobilization on activated chitosan/cellulose acetate electrospun nanofibrous polymers: biochemical characterization and efficient protein waste digestion. Biocatal. Biotransform. 41 (4), 279–298.

Baidamshina, D.R., Koroleva, V.A., Trizna, E.Y., Pankova, S.M., Agafonova, M.N., Chirkova, M.N., Porfiryev, A.G., 2020. Anti-biofilm and wound-healing activity of chitosan-immobilized Ficin. Int. J. Biol. Macromol. 164, 4205–4217.

Baidamshina, D.R., Koroleva, V.A., Olshannikova, S.S., Trizna, E.Y., Bogachev, M.I., Artyukhov, V.G., Kayumov, A.R., 2021. Biochemical properties and anti-biofilm activity of chitosan-immobilized papain. Mar. Drugs 19 (4), 197.

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., 2015. Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. Biotechnol. Adv. 33 (5), 435–456.

Benucci, I., Mazzocchi, C., Lombardelli, C., Cacciotti, I., Esti, M., 2019. Multi-enzymatic systems immobilized on chitosan beads for pomegranate juice treatment in fluidized bed reactor: effect on haze-active molecules and chromatic properties. Food Bioprocess Technol. 12, 1559–1572.

Benucci, I., Caso, M.C., Bavaro, T., Masci, S., Keršienė, M., Esti, M., 2020. Prolyl endopeptidase from Aspergillus niger immobilized on a food-grade carrier for the production of gluten-reduced beer. Food Control 110, 106987.

Betancor, L., Fuentes, M., Dellamora-Ortiz, G., López-Gallego, F., Hidalgo, A., Alonso-Morales, N., Fernández-Lafuente, R., 2005. Dextran aldehyde coating of glucose oxidase immobilized on magnetic nanoparticles prevents its inactivation by gas bubbles. J. Mol. Catal. B Enzym. 32 (3), 97–101.

Bickerstaff, G.F., Zhou, H., 1993. Protease activity and autodigestion (autolysis) assays using Coomassie blue dye binding. Anal. Biochem. 210 (1), 155–158.

Bilal, M., Zhao, Y., Rasheed, T., Iqbal, H.M., 2018. Magnetic nanoparticles as versatile carriers for enzymes immobilization: a review. Int. J. Biol. Macromol. 120, 2530–2544.

Bilal, M., Nguyen, T.A., Iqbal, H.M., 2020. Multifunctional carbon nanotubes and their derived nano-constructs for enzyme immobilization–a paradigm shift in biocatalyst design. Coord. Chem. Rev. 422, 213475.

Bilal, M., Qamar, S.A., Ashraf, S.S., Rodríguez-Couto, S., Iqbal, H.M., 2021. Robust nanocarriers to engineer nanobiocatalysts for bioprocessing applications. Adv. Colloid Interf. Sci. 293, 102438.

Bilal, M., Rashid, E.U., Munawar, J., Iqbal, H.M., Cui, J., Zdarta, J., Jesionowski, T., 2023. Magnetic metal-organic frameworks immobilized enzyme-based nano-biocatalytic systems for sustainable biotechnology—a review. Int. J. Biol. Macromol. 123968.

Bolivar, J.M., Woodley, J.M., Fernandez-Lafuente, R., 2022. Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. 51, 6251–6290.

Boopathy, R., Karthikeyan, S., Mandal, A., Sekaran, G., 2013. Characterisation and recovery of sodium chloride from salt-laden solid waste generated from leather industry. Clean Techn. Environ. Policy 15 (1), 117–124.

Boujakhrout, A., Sánchez, E., Díez, P., Sánchez, A., Martínez-Ruiz, P., Parrado, C., Villalonga, R., 2015. Single-walled carbon nanotubes/au–mesoporous silica Janus nanoparticles as building blocks for the preparation of a bienzyme biosensor. ChemElectroChem 2 (11), 1735–1741.

Brandelli, A., Sala, L., Kalil, S.J., 2015. Microbial enzymes for bioconversion of poultry waste into added-value products. Food Res. Int. 73, 3–12.

Bustamante-Torres, M., Romero-Fierro, D., Arcentales-Vera, B., Pardo, S., Bucio, E., 2021. Interaction between filler and polymeric matrix in nanocomposites: magnetic approach and applications. Polymers 13 (17), 2998.

Cao, W., Huang, R., Qi, W., Su, R., He, Z., 2015. Self-assembly of amphiphilic Janus particles into monolayer capsules for enhanced enzyme catalysis in organic media. ACS Appl. Mater. Interfaces 7 (1), 465–473.

Cao, G., Gao, J., Zhou, L., Huang, Z., He, Y., Zhu, M., Jiang, Y., 2017. Fabrication of Ni2+−nitrilotriacetic acid functionalized magnetic mesoporous silica nanoflowers for one pot purification and immobilization of His-tagged ω-transaminase. Biochem. Eng. J. 128, 116–125.

Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R., 2022a. Design of artificial enzymes bearing several active centers: new trends, opportunities and problems. Int. J. Mol. Sci. 23 (10), 5304.

Carballares, D., Rocha-Martin, J., Fernandez-Lafuente, R., 2022b. Preparation of a six-enzyme multilayer combi-biocatalyst: reuse of the most stable enzymes after inactivation of the least stable one. ACS Sustain. Chem. Eng. 10 (12), 3920–3934.

Castañeda-Valbuena, D., Berenguer-Murcia, Á., Fernandez-Lafuente, R., Morellon-Sterling, R., Tacias-Pascacio, V.G., 2022. Biological activities of peptides obtained by pepsin hydrolysis of fishery products. Process Biochem. 120, 53–63.

Chang, M., Lin, Y.-H., Gabayno, J.L., Li, Q., Liu, X., 2017. Thrombolysis based on magnetically-controlled surface-functionalized Fe3O4 nanoparticle. Bioengineered 8 (1), 29–35.

Chulkaivalsucharit, P., Wu, X., Ge, J., 2015. Synthesis of enzyme-embedded metal–organic framework nanocrystals in reverse micelles. RSC Adv. 5 (123), 101293–101296.

Cipolatti, E.P., Valério, A., Henriques, R.O., Moritz, D.E., Ninow, J.L., Freire, D.M., de Oliveira, D., 2016. Nanomaterials for biocatalyst immobilization–state of the art and future trends. RSC Adv. 6 (106), 104675–104692.

Coscolín, C., Beloqui, A., Martínez-Martínez, M., Bargiela, R., Santiago, G., Blanco, R.M., Ferrer, M., 2018. Controlled manipulation of enzyme specificity through immobilization-induced flexibility constraints. Appl. Catal. A Gen. 565, 59–67.

Cowan, D.A., Fernandez-Lafuente, R., 2011. Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. Enzym. Microb. Technol. 49 (4), 326–346.

Cowan, D.A., Daniel, R.M., Morgan, H.W., 1987. Some observations on the inhibition and activation of a thermophilic protease. Int. J. BioChemiPhysics 19 (5), 483–486.

Currin, A., Swainston, N., Day, P.J., Kell, D.B., 2015. Synthetic biology for the directed evolution of protein biocatalysts: navigating sequence space intelligently. Chem. Soc. Rev. 44 (5), 1172–1239.

da Silva, M.M., Neto, J.M.W.D., Regueira, B.V.B., do Couto, M.T.T., da Silva Sobral, R.V., Conniff, A.E.S., Pastrana, L., 2022. Immobilization of fibrinolytic protease from Mucor subtilissimus UCP 1262 in magnetic nanoparticles. Protein Expr. Purif. 192, 106044.

Dal Magro, L., Silveira, V.C., de Menezes, E.W., Benvenutti, E.V., Nicolodi, S., Hertz, P.F., Rodrigues, R.C., 2018. Magnetic biocatalysts of pectinase and cellulase: synthesis and characterization of two preparations for application in grape juice clarification. Int. J. Biol. Macromol. 115, 35–44.

Dal Magro, L., de Moura, K.S., Backes, B.E., de Menezes, E.W., Benvenutti, E.V., Nicolodi, S., Rodrigues, R.C., 2019a. Immobilization of pectinase on chitosan-magnetic particles: influence of particle preparation protocol on enzyme properties for fruit juice clarification. Biotechnol. Rep. 24, e00373.

Dal Magro, L., Kornecki, J.F., Klein, M.P., Rodrigues, R.C., Fernandez-Lafuente, R., 2019b. Optimized immobilization of polygalacturonase from Aspergillus niger following different protocols: improved stability and activity under drastic conditions. Int. J. Biol. Macromol. 138, 234–243.
Dal Magro, L., Kornecki, J.F., Klein, M.P., Rodrigues, R.C., Fernandez-Lafuente, R., 2020. Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range. Enzym. Microb. Technol. 132, 109397.
de Oliveira, J.M., Fernandes, P., Benevides, R.G., de Assis, S.A., 2021. Production, characterization, and immobilization of protease from the yeast Rhodotorula oryzicola. Biotechnology and Applied Biochemistry 68 (5), 1033–1043.
Deghan, P., Homaei, A., 2016. Immobilization of Penaeus vanamei protease on carbon nanotubes and investigation of its activity and stability. J. Aquat. Physiol. Biotechnol. 4 (2), 35–56.
Denard, C.A., Ren, H., Zhao, H., 2015. Improving and repurposing biocatalysts via directed evolution. Curr. Opin. Chem. Biol. 25, 55–64.
Diamanti, E., Arana-Peña, S., Ramos-Cabrer, P., Comino, N., Carballares, D., Fernandez-Lafuente, R., López-Gallego, F., 2022. Intraparticle macromolecular migration alters the structure and function of proteins reversibly immobilized on porous microbeads. Adv. Mater. Interfaces 9 (18), 2200263.
Diano, N., Grimaldi, T., Bianco, M., Rossi, S., Gabrovska, K., Yordanova, G., Mita, L., 2008. Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. J. Agric. Food Chem. 56 (23), 11471–11477.
Díaz-Rodríguez, A., Davis, B.G., 2011. Chemical modification in the creation of novel biocatalysts. Curr. Opin. Chem. Biol. 15 (2), 211–219.
DiCosimo, R., McAuliffe, J., Poulose, A.J., Bohlmann, G., 2013. Industrial use of immobilized enzymes. Chem. Soc. Rev. 42 (15), 6437–6474.
Ding, S., Cargill, A.A., Medintz, I.L., Claussen, J.C., 2015. Increasing the activity of immobilized enzymes with nanoparticle conjugation. Curr. Opin. Biotechnol. 34, 242–250.
Diyanat, S., Homaei, A., Mosaddegh, E., 2018. Immobilization of Penaeus vannamei protease on ZnO nanoparticles for long-term use. International Journal of Biological Macromolecules 118, 92–98.
Dold, S.M., Cai, L., Rudat, J., 2016. One-step purification and immobilization of a β-amino acid aminotransferase using magnetic (M-PVA) beads. Eng. Life Sci. 16 (6), 568–576.
Farhadyar, N., Sadjadi, M., 2011. Fabrication and Characterization of Gold–TiO_2 Core–Shell Nanowire for Immobilization of Alkaline Protease. Journal of Biomedical Nanotechnology 7 (3), 466–470.
Feng, W., Ji, P., 2011. Enzymes immobilized on carbon nanotubes. Biotechnol. Adv. 29 (6), 889–895.
Fernández-Arrojo, L., Guazzaroni, M.-E., López-Cortés, N., Beloqui, A., Ferrer, M., 2010. Metagenomic era for biocatalyst identification. Curr. Opin. Biotechnol. 21 (6), 725–733.
Fernandez-Lafuente, R., 2009. Stabilization of multimeric enzymes: strategies to prevent subunit dissociation. Enzym. Microb. Technol. 45 (6–7), 405–418.
Ferrer, M., Beloqui, A., Timmis, K.N., Golyshin, P.N., 2008. Metagenomics for mining new genetic resources of microbial communities. Microbial Physiol. 16 (1–2), 109–123.
Ferrer, M., Martínez-Martínez, M., Bargiela, R., Streit, W.R., Golyshina, O.V., Golyshin, P.N., 2016. Estimating the success of enzyme bioprospecting through metagenomics: current status and future trends. Microb. Biotechnol. 9 (1), 22–34.
Gao, B., He, L., Wei, D., Zhang, L., 2018. Identification and magnetic immobilization of a pyrophilous aspartic protease from Antarctic psychrophilic fungus. J. Food Biochem. 42 (6), e12691.
Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., Rodrigues, R.C., 2011. Potential of different enzyme immobilization strategies to improve enzyme performance. Adv. Synth. Catal. 353 (16), 2885–2904.
Gennari, A., Simon, R., de Moura Sperotto, N.D., Bizarro, C.V., Basso, L.A., Machado, P., Renard, G., 2022. One-step purification of a recombinant beta-galactosidase using magnetic cellulose as a support: rapid immobilization and high thermal stability. Bioresour. Technol. 345, 126497.
Giri, P., Pagar, A.D., Patil, M.D., Yun, H., 2021. Chemical modification of enzymes to improve biocatalytic performance. Biotechnol. Adv. 53, 107868.
Glomm, W.R., Wubshet, S.G., Lindberg, D., Dankel, K.R., Afseth, N.K., Stenstad, P.M., Johnsen, H., 2021. Immobilized protease on magnetic particles for enzymatic protein hydrolysis of poultry by-products. LWT 152, 112327.
Gómez, J.L., Bódalo, A., Gómez, E., Hidalgo, A.M., Gómez, M., Murcia, M.D., 2007. Experimental behaviour and design model of a fluidized bed reactor with immobilized peroxidase for phenol removal. Chem. Eng. J. 127 (1–3), 47–57.
Görgüç, A., Gençdağ, E., Yılmaz, F.M., 2020. Bioactive peptides derived from plant origin by-products: biological activities and techno-functional utilizations in food developments–a review. Food Res. Int. 136, 109504.
Gu, X., Gao, J., Li, X., Wang, Y., 2018. Immobilization of Papain onto graphene oxide nanosheets. J. Nanosci. Nanotechnol. 18 (5), 3543–3547.
Guisan, J.M., 2006. Immobilization of enzymes as the 21st century begins: an already solved problem or still an exciting challenge?. In: Immobilization of Enzymes and Cells, pp. 1–13.
Gulmez, C., Altinkaynak, C., Özdemir, N., Atakisi, O., 2018. Proteinase K hybrid nanoflowers (P-hNFs) as a novel nanobiocatalytic detergent additive. Int. J. Biol. Macromol. 119, 803–810.
Gulmez, C., Altinkaynak, C., Turk, M., Ozdemir, N., Atakisi, O., 2022. Hemoglobin-inorganic hybrid nanoflowers with different metal ions as potential oxygen carrying systems. Chem. Biodivers. 19 (1), e202100683.
Hashemabadi, M., Badoei-Dalfard, A., 2019. Fabrication of magnetic CLEA-protease nanocomposite: high progression in biotechnology and protein waste management. Catal. Lett. 149, 1753–1764.
Hauer, B., 2020. Embracing nature's catalysts: a viewpoint on the future of biocatalysis. ACS Catal. 10 (15), 8418–8427.
Hernandez, K., Fernandez-Lafuente, R., 2011. Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. Enzym. Microb. Technol. 48 (2), 107–122.
Homaei, A., Samari, F., 2017. Investigation of activity and stability of papain by adsorption on multi-wall carbon nanotubes. Int. J. Biol. Macromol. 105, 1630–1635.
Hou, S.-F., Yang, K.-S., Fang, H.-Q., Chen, H.-Y., 1998. Amperometric glucose enzyme electrode by immobilizing glucose oxidase in multilayers on self-assembled monolayers surface. Talanta 47 (3), 561–567.
Ibrahim, A.S.S., Al-Salamah, A.A., El-Toni, A.M., Almaary, K.S., El-Tayeb, M.A., Elbadawi, Y.B., Antranikian, G., 2016. Enhancement of alkaline protease activity and stability via covalent immobilization onto hollow core-mesoporous shell silica nanospheres. International journal of molecular sciences 17 (2), 184.
Ibrahim, A.S., Elbadawi, Y.B., El-Toni, A.M., Almaary, K.S., El-Tayeb, M.A., Elagib, A.A., Maany, D.A.F., 2021. Stabilization and improved properties of Salipaludibacillus agaradhaerens alkaline protease by immobilization onto double mesoporous core-shell nanospheres. Int. J. Biol. Macromol. 166, 557–566.
Jiang, Y., Guo, C., Xia, H., Mahmood, I., Liu, C., Liu, H., 2009. Magnetic nanoparticles supported ionic liquids for lipase immobilization: enzyme activity in catalyzing esterification. J. Mol. Catal. B Enzym. 58 (1–4), 103–109.
Jianping, W., Lei, P., Xiaojing, X., Zhiyong, Z., 2003. Nitrifying treatment of wastewater from fertilizer production in a three-phase flow airlift loop bioreactor. Eng. Life Sci. 3 (6), 271–275.
Jin, X., Li, J.-F., Huang, P.-Y., Dong, X.-Y., Guo, L.-L., Yang, L., Chen, H., 2010. Immobilized protease on the magnetic nanoparticles used for the hydrolysis of rapeseed meals. J. Magn. Magn. Mater. 322 (14), 2031–2037.
Joshi, N., Kocher, G.S., Kalia, A., Banga, H.S., 2020. Development of nano-silver alkaline protease bio-conjugate depilating eco-benign formulation by utilizing potato peel based medium. Int. J. Biol. Macromol. 152, 261–271.
Joshi, N., Kocher, G.S., Kalia, A., Banga, H.S., 2021. Bacillus circulans MTCC 7906 aided facile development of bioconjugate nano-silica alkaline protease formulation with superlative dehairing potential. Environ. Pollut. 285, 117181.
Kamal, S., Hussain, F., Bibi, I., Azeem, M., Ahmad, T., Iqbal, H.M., 2021. Mutagenesis and immobilization of ChitB-protease for induced de-staining and goat skin dehairing potentialities. Catal. Lett. 1–16.
Kapust, R.B., Tözsér, J., Fox, J.D., Anderson, D.E., Cherry, S., Copeland, T.D., Waugh, D. S., 2001. Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. Protein Eng. 14 (12), 993–1000.
Karakurt, V., Samsa, C.G., 2023. Immobilization of protease on chitosan–silica gel beads for high detergent and surfactant stability and high tolerance against metallic ions and organic solvents. Chem. Pap. 77 (6), 3361–3372.
Karami, Z., Akbari-Adergani, B., 2019. Bioactive food derived peptides: A review on correlation between structure of bioactive peptides and their functional properties. J. Food Sci. Technol. 56, 535–547.
Karami, Z., Tamri, H., Badoei-dalfard, A., 2022. Immobilization of protease KHB3 onto magnetic metal–organic frameworks and investigation of its biotechnological applications. Catal. Lett. 1–14.
Karataş, E., Tülek, A., Yildirim, D., Tamtürk, F., Binay, B., 2021. Immobilization of Sporothrix schenckii 1099-18 exo-polygalacturonase in magnetic mesoporous silica yolk-shell spheres: highly reusable biocatalysts for apple juice clarification. Food Biosci. 43, 101324.
Katić, K., Banjanac, K., Simović, M., Ćorović, M., Milivojević, A., Marinković, A., Bezbradica, D., 2021. Development of protease nanobiocatalysts and their application in hydrolysis of sunflower meal protein isolate. International Journal of Food Science & Technology 56 (9), 4287–4297.
Kawakita, H., Sugita, K., Saito, K., Tamada, M., Sugo, T., Kawamoto, H., 2002. Production of cycloisomaltooligosaccharides from dextran using enzyme immobilized in multilayers onto porous membranes. Biotechnol. Prog. 18 (3), 465–469.
Khan, R.S., Rather, A.H., Wani, T.U., Rather, S.U., Amna, T., Hassan, M.S., Sheikh, F.A., 2023. Recent trends using natural polymeric nanofibers as supports for enzyme immobilization and catalysis. Biotechnol. Bioeng. 120 (1), 22–40.
Khankari, S., Badoei-Dalfard, A., Karami, Z., 2021. Cross-linked enzyme aggregates of fibrinolytic protease BC1 immobilized on magnetic chitosan nanoparticles (CLEAs-Fib-mChi): synthesis, purification, and characterization. Applied Biochemistry and Biotechnology 193, 2004–2027.
Kharazmi, S., Taheri-Kafrani, A., 2023. Bi-enzymatic nanobiocatalyst based on immobilization of xylanase and pectinase onto functionalized magnetic nanoparticles for efficient fruit juice clarification. LWT 114914.
Kim, M., Shin, M.K., Sung, J.-S., Kadam, A.A., 2022. Supermagnetic halloysite nanotubes surface-tuned with aminosilane for protease immobilization and applied for eradication of bacterial biofilm. Appl. Surf. Sci. 593, 153469.
Kong, W., Zhang, X., Gao, M.L., Zhou, H., Li, W., Shen, J.C., 1994. A new kind of immobilized enzyme multilayer based on cationic and anionic interaction. Macromol. Rapid Commun. 15 (5), 405–409.
Kumari, A., Kaur, B., Srivastava, R., Sangwan, R.S., 2015. Isolation and immobilization of alkaline protease on mesoporous silica and mesoporous ZSM-5 zeolite materials for improved catalytic properties. Biochem. Biophysics Rep. 2, 108–114.
Lamas, E.M., Barros, R.M., Balcão, V.M., Malcata, F.X., 2001. Hydrolysis of whey proteins by proteases extracted from Cynara cardunculus and immobilized onto highly activated supports. Enzym. Microb. Technol. 28 (7–8), 642–652.
Li, H., Tu, Y., Tu, T., Zhang, Q., 2023. Immobilization of Proteases on Nanoflower-Like Metal Organic Framework. Chinese Journal of Chemistry 41 (12), 1504–1508.
Liese, A., Hilterhaus, L., 2013. Evaluation of immobilized enzymes for industrial applications. Chem. Soc. Rev. 42 (15), 6236–6249.

Liu, H., Hao, C., Zhang, Y., Yang, H., Sun, R., 2021. The interaction of graphene oxide-silver nanoparticles with trypsin: Insights from adsorption behaviors, conformational structure and enzymatic activity investigations. Colloids and Surfaces B: Biointerfaces 202, 111688.
Liu, W.-L., Lo, S.-H., Singco, B., Yang, C.-C., Huang, H.-Y., Lin, C.-H., 2013. Novel trypsin–FITC@ MOF bioreactor efficiently catalyzes protein digestion. Journal of Materials Chemistry B 1 (7), 928–932.
Liya, S.M., Umesh, M., Nag, A., Chinnathambi, A., Alharbi, S.A., Jhanani, G., Brindhadevi, K., 2023. Optimized production of keratinolytic proteases from Bacillus tropicus LS27 and its application as a sustainable alternative for dehairing, destaining and metal recovery. Environ. Res. 221, 115283.
Lotfi, F., Badoei-dalfard, A., Hassanshahian, M., 2021. Immobilization and biochemical characterization of keratinase 2S1 onto magnetic cross-linked enzyme aggregates and its application on the hydrolysis of keratin waste. Catal. Lett. 1–17.
Luo, M., Zhang, M., Chen, G., Zhao, J., Guo, H., 2021. A potential method for one-step purification and direct immobilization of target protein in cell lysate with magnetic microbeads. Biochem. Eng. J. 176, 108182.
Madalozzo, A.D., Martini, V.P., Kuniyoshi, K.K., de Souza, E.M., Pedrosa, F.O., Glogauer, A., Krieger, N., 2015. Immobilization of LipC12, a new lipase obtained by metagenomics, and its application in the synthesis of biodiesel esters. J. Mol. Catal. B Enzym. 116, 45–51.
Maharaja, P., Mahesh, M., Sunkapur, L.K., Swarnalatha, S., Sekaran, G., 2017. Treatment of tannery saline wastewater by using effective immobilized protease catalyst produced from salt tolerant Enterococcus feacalis. J. Environ. Chem. Eng. 5 (2), 2042–2055.
Mamo, J., Assefa, F., 2018. The role of microbial aspartic protease enzyme in food and beverage industries. J. Food Qual. 2018.
Manoel, E.A., Dos Santos, J.C., Freire, D.M., Rueda, N., Fernandez-Lafuente, R., 2015. Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. Enzym. Microb. Technol. 71, 53–57.
Maroju, P.A., Ganesan, R., Dutta, J.R., 2023. Biofuel generation from food waste through immobilized enzymes on magnetic nanoparticles. Mater. Today: Proc. 72, 62–66.
Masi, C., Chandramohan, C., Ahmed, M.F., 2018. Immobilization of the magnetic nanoparticles with alkaline protease enzyme produced by Enterococcus hirae and Pseudomonas aeruginosa isolated from dairy effluents. Braz. Arch. Biol. Technol. 60.
Mateljak, I., Monza, E., Lucas, M.F., Guallar, V., Aleksejeva, O., Ludwig, R., Alcalde, M., 2019. Increasing redox potential, redox mediator activity, and stability in a fungal laccase by computer-guided mutagenesis and directed evolution. ACS Catal. 9 (5), 4561–4572.
Mateo, C., Monti, R., Pessela, B.C., Fuentes, M., Torres, R., Manuel Guisán, J., Fernández-Lafuente, R., 2004. Immobilization of lactase from Kluyveromyces lactis greatly reduces the inhibition promoted by glucose. Full hydrolysis of lactose in milk. Biotechnol. Prog. 20 (4), 1259–1262.
Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzym. Microb. Technol. 40 (6), 1451–1463.
Maurer, K.-H., 2004. Detergent proteases. Curr. Opin. Biotechnol. 15 (4), 330–334.
Mirsalari, H., Maleki, A., Raissi, H., Soltanabadi, A., 2021. Investigation of the pristine and functionalized carbon nanotubes as a delivery system for the anticancer drug dacarbazine: drug encapsulation. J. Pharm. Sci. 110 (5), 2005–2016.
Mirzaei, F., Valizadeh, H., Pazhang, M., 2022. Immobilization of papain on nitrogen-doped graphene quantum dots improves the enzymatic properties and makes it a biosensor for cystatin C. Process Biochem. 118, 307–316.
Monajati, M., Borandeh, S., Hesami, A., Mansouri, D., Tamaddon, A.M., 2018. Immobilization of L-asparaginase on aspartic acid functionalized graphene oxide nanosheet: enzyme kinetics and stability studies. Chem. Eng. J. 354, 1153–1163.
Monteiro, R.R., Arana-Peña, S., da Rocha, T.N., Miranda, L.P., Berenguer-Murcia, Á., Tardioli, P.W., Fernandez-Lafuente, R., 2021. Liquid lipase preparations designed for industrial production of biodiesel. Is it really an optimal solution? Renew. Energy 164, 1566–1587.
Monteiro, R.R., da Silva, S.S., Cavalcante Jr., C.L., de Luna, F.M.T., Bolivar, J.M., Vieira, R.S., Fernandez-Lafuente, R., 2022. Biosynthesis of alkanes/alkenes from fatty acids or derivatives (triacylglycerols or fatty aldehydes). Biotechnol. Adv. 108045.
Morellon-Sterling, R., Carballares, D., Arana-Peña, S., Siar, E.-H., Braham, S.A., Fernandez-Lafuente, R., 2021. Advantages of supports activated with divinyl sulfone in enzyme coimmobilization: Possibility of multipoint covalent immobilization of the most stable enzyme and immobilization via ion exchange of the least stable enzyme. ACS Sustainable Chemistry & Engineering 9 (22), 7508–7518.
Morellon-Sterling, R., El-Siar, H., Tavano, O.L., Berenguer-Murcia, Á., Fernández-Lafuente, R., 2020. Ficin: a protease extract with relevance in biotechnology and biocatalysis. Int. J. Biol. Macromol. 162, 394–404.
Morellon-Sterling, R., Tavano, O., Bolivar, J.M., Berenguer-Murcia, Á., Vela-Gutiérrez, G., Sabir, J.S., Fernandez-Lafuente, R., 2022. A review on the immobilization of pepsin: a Lys-poor enzyme that is unstable at alkaline pH values. Int. J. Biol. Macromol. 210, 682–702.
Moslemi, M., Homaei, A., Toiserkani, H., 2018. Aspartic acid introduce the functional amine groups on the surface of superparamagnetic Fe(OH)3@Fe_3O_4 nanoparticles for efficient immobilization of penaeus vannamei protease. Bioprocess and Biosystems Engineering 41, 749–756.
Movahedi, M., Samsam Shariat, S.Z.A., Nazem, H., 2019. Immobilization of lactoperoxidase on graphene oxide nanosheets and copper oxide nanoparticles and evaluation of their stability. Catalysis Letters 149, 562–573.
Murugappan, G., Khambhaty, Y., Sreeram, K.J., 2020. Protease immobilized nanoparticles: a cleaner and sustainable approach to dehairing of skin. Appl. Nanosci. 10, 213–221.
Murugappan, G., Sreeram, K.J., 2021. Nano-biocatalyst: Bi-functionalization of protease and amylase on copper oxide nanoparticles. Colloids and Surfaces B: Biointerfaces 197, 111386.
Nitsche, C., Holloway, S., Schirmeister, T., Klein, C.D., 2014. Biochemistry and medicinal chemistry of the dengue virus protease. Chem. Rev. 114 (22), 11348–11381.
Nwachukwu, I.D., Aluko, R.E., 2019. Structural and functional properties of food protein-derived antioxidant peptides. J. Food Biochem. 43 (1), e12761.
Özbek, B., Ünal, Ş., 2017. Preparation and characterization of polymer-coated mesoporous silica nanoparticles and their application in Subtilisin immobilization. Korean J. Chem. Eng. 34, 1992–2001.
Pagar, A.D., Patil, M.D., Flood, D.T., Yoo, T.H., Dawson, P.E., Yun, H., 2021. Recent advances in biocatalysis with chemical modification and expanded amino acid alphabet. Chem. Rev. 121 (10), 6173–6245.
Panis, F., Kampatsikas, I., Bijelic, A., Rompel, A., 2020. Conversion of walnut tyrosinase into a catechol oxidase by site directed mutagenesis. Sci. Rep. 10 (1), 1659.
Pedroche, J., del Mar Yust, M., Mateo, C., Fernández-Lafuente, R., Girón-Calle, J., Alaiz, M., Millán, F., 2007. Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: correlation between enzyme–support linkages and thermal stability. Enzym. Microb. Technol. 40 (5), 1160–1166.
Petrenko, D.E., Mikhailova, A.G., Timofeev, V.I., Agapova, Y.K., Karlinsky, D.M., Komolov, A.S., Rakitina, T.V., 2020. Molecular dynamics complemented by site-directed mutagenesis reveals significant difference between the interdomain salt bridge networks stabilizing oligopeptidases B from bacteria and protozoa in their active conformations. J. Biomol. Struct. Dyn. 38 (16), 4868–4882.
Pinelo, M., Zeuner, B., Meyer, A.S., 2010. Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. Food Bioprod. Process. 88 (2–3), 259–265.
Popat, A., Hartono, S.B., Stahr, F., Liu, J., Qiao, S.Z., Lu, G.Q.M., 2011. Mesoporous silica nanoparticles for bioadsorption, enzyme immobilisation, and delivery carriers. Nanoscale 3 (7), 2801–2818.
Pounsamy, M., Somasundaram, S., Palanivel, S., Balasubramani, R., Chang, S.W., Nguyen, D.D., Ganesan, S., 2019. A novel protease-immobilized carbon catalyst for the effective fragmentation of proteins in high-TDS wastewater generated in tanneries: spectral and electrochemical studies. Environ. Res. 172, 408–419.
Prabhawathi, V., Sivakumar, P.M., Boobalan, T., Manohar, C.M., Doble, M., 2019. Design of antimicrobial polycaprolactam nanocomposite by immobilizing subtilisin conjugated Au/Ag core-shell nanoparticles for biomedical applications. Mater. Sci. Eng. C 94, 656–665.
Qamar, S.A., Asgher, M., Bilal, M., 2020. Immobilization of alkaline protease from Bacillus brevis using Ca-alginate entrapment strategy for improved catalytic stability, silver recovery, and dehairing potentialities. Catal. Lett. 150, 3572–3583.
Radha, K., Arun, C., 2010. Recycling of exposed photographic X-ray films and recovery of silver using Bromelain. Sustain. World 142, 421–430.
Raghavan, N., Thangavel, S., Venugopal, G., 2015. Enhanced photocatalytic degradation of methylene blue by reduced graphene-oxide/titanium dioxide/zinc oxide ternary nanocomposites. Mater. Sci. Semicond. Process. 30, 321–329.
Rai, S.K., Mukherjee, A.K., 2011. Optimization of production of an oxidant and detergent-stable alkaline β-keratinase from Brevibacillus sp. strain AS-S10-II: application of enzyme in laundry detergent formulations and in leather industry. Biochem. Eng. J. 54 (1), 47–56.
Ramalho, E.X., de Castro, R.J.S., 2023. Covalent bonding immobilization of a Bacillus licheniformis protease on chitosan and its application in protein hydrolysis. Biocatal. Agric. Biotechnol. 50, 102713.
Ranjbari, N., Razzaghi, M., Fernandez-Lafuente, R., Shojaei, F., Satari, M., Homaei, A., 2019. Improved features of a highly stable protease from Penaeus vannamei by immobilization on glutaraldehyde activated graphene oxide nanosheets. Int. J. Biol. Macromol. 130, 564–572.
Rather, A.H., Khan, R.S., Wani, T.U., Beigh, M.A., Sheikh, F.A., 2022. Overview on immobilization of enzymes on synthetic polymeric nanofibers fabricated by electrospinning. Biotechnol. Bioeng. 119 (1), 9–33.
Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A., Ashraf, M., 2019. Microbial proteases applications. Front. Bioeng. Biotechnol. 7, 110.
Reshmy, R., Philip, E., Sirohi, R., Tarafdar, A., Arun, K., Madhavan, A., Szakacs, G., 2021. Nanobiocatalysts: advancements and applications in enzyme technology. Bioresour. Technol. 337, 125491.
Ribeiro, E.S., de Farias, B.S., Junior, T.R.S.A.C., de Almeida Pinto, L.A., Diaz, P.S., 2021. Chitosan–based nanofibers for enzyme immobilization. Int. J. Biol. Macromol. 183, 1959–1970.
Rios, N.S., Arana-Peña, S., Mendez-Sanchez, C., Lokha, Y., Cortes-Corberan, V., Gonçalves, L.R., Fernandez-Lafuente, R., 2019. Increasing the enzyme loading capacity of porous supports by a layer-by-layer immobilization strategy using PEI as glue. Catalysts 9 (7), 576.
Rocha-Martín, J., Rivas, B.D.L., Muñoz, R., Guisán, J.M., López-Gallego, F., 2012. Rational co-immobilization of bi-enzyme cascades on porous supports and their applications in bio-redox reactions with in situ recycling of soluble cofactors. ChemCatChem 4 (9), 1279–1288.
Roda, S., Fernandez-Lopez, L., Benedens, M., Bollinger, A., Thies, S., Schumacher, J., Gertzen, C.G., 2022. A plurizyme with transaminase and hydrolase activity catalyzes cascade reactions. Angew. Chem. 134 (37), e202207344.
Rodrigues, R.C., Berenguer-Murcia, Á., Fernandez-Lafuente, R., 2011. Coupling chemical modification and immobilization to improve the catalytic performance of enzymes. Adv. Synth. Catal. 353 (13), 2216–2238.
Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Fernández-Lafuente, R., 2013. Modifying enzyme activity and selectivity by immobilization. Chem. Soc. Rev. 42 (15), 6290–6307.

Rodrigues, R.C., Virgen-Ortíz, J.J., Dos Santos, J.C., Berenguer-Murcia, Á., Alcantara, A. R., Barbosa, O., Fernandez-Lafuente, R., 2019. Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. Biotechnol. Adv. 37 (5), 746–770.
Rodrigues, R.C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R., 2021. Stabilization of enzymes via immobilization: multipoint covalent attachment and other stabilization strategies. Biotechnol. Adv. 52, 107821.
Rueda, N., Dos Santos, J.C., Ortiz, C., Torres, R., Barbosa, O., Rodrigues, R.C., Fernandez-Lafuente, R., 2016. Chemical modification in the design of immobilized enzyme biocatalysts: drawbacks and opportunities. Chem. Rec. 16 (3), 1436–1455.
Sánchez, A., Díez, P., Martínez-Ruíz, P., Villalonga, R., Pingarrón, J.M., 2013. Janus Au-mesoporous silica nanoparticles as electrochemical biorecognition-signaling system. Electrochem. Commun. 30, 51–54.
Sakata, M., Funatsu, A., Sonoda, S., Ogata, T., Taniguchi, T., Matsumoto, Y., 2012. Immobilization of trypsin on graphene oxide nanosheets for increased proteolytic Stability. Chemistry Letters 41 (12), 1625–1627.
Sánchez, D.A., Alnoch, R.C., Tonetto, G.M., Krieger, N., Ferreira, M.L., 2021. Immobilization and bioimprinting strategies to enhance the performance in organic medium of the metagenomic lipase LipC12. J. Biotechnol. 342, 13–27.
Sangeetha, K., Abraham, T.E., 2008. Preparation and characterization of cross-linked enzyme aggregates (CLEA) of subtilisin for controlled release applications. Int. J. Biol. Macromol. 43 (3), 314–319.
Santiago, G., Martínez-Martínez, M., Alonso, S., Bargiela, R., Coscolín, C., Golyshin, P.N., Ferrer, M., 2018. Rational engineering of multiple active sites in an ester hydrolase. Biochemistry 57 (15), 2245–2255.
Santos, J.C.S.D., Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C., Fernandez-Lafuente, R., 2015. Importance of the support properties for immobilization or purification of enzymes. ChemCatChem 7 (16), 2413–2432.
Sarathi, M., Doraiswamy, N., Pennathur, G., 2019. Enhanced stability of immobilized keratinolytic protease on electrospun nanofibers. Prep. Biochem. Biotechnol. 49 (7), 695–703.
Saravanan, P., Renitha, T.S., Gowthaman, M., Kamini, N., 2014. Understanding the chemical free enzyme based cleaner unhairing process in leather manufacturing. J. Clean. Prod. 79, 258–264.
Sáringer, S., Rouster, P., Szilagyi, I., 2021. Co-immobilization of antioxidant enzymes on titania nanosheets for reduction of oxidative stress in colloid systems. J. Colloid Interface Sci. 590, 28–37.
Schmid, A., Dordick, J., Hauer, B., Kiener, A., Wubbolts, M., Witholt, B., 2001. Industrial biocatalysis today and tomorrow. Nature 409 (6817), 258–268.
Schoemaker, H.E., Mink, D., Wubbolts, M.G., 2003. Dispelling the myths–biocatalysis in industrial synthesis. Science 299 (5613), 1694–1697.
Sharma, S., Gupta, S., Arya, S.K., Kaur, A., 2022. Enzyme immobilization: implementation of nanoparticles and an insight into polystyrene as the contemporary immobilization matrix. Process Biochem. 120, 22–34.
Sheldon, R.A., Brady, D., 2019. Broadening the scope of biocatalysis in sustainable organic synthesis. ChemSusChem 12 (13), 2859–2881.
Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: why, what and how. Chem. Soc. Rev. 42 (15), 6223–6235.
Sheldon, R.A., Woodley, J.M., 2018. Role of biocatalysis in sustainable chemistry. Chem. Rev. 118 (2), 801–838.
Shi, C., Deng, C., Li, Y., Zhang, X., Yang, P., 2014. Hydrophilic polydopamine-coated magnetic graphene nanocomposites for highly efficient tryptic immobilization. Proteomics 14 (12), 1457–1463.
Shojaei, F., Homaei, A., Taherizadeh, M.R., Kamrani, E., 2017. Characterization of biosynthesized chitosan nanoparticles from Penaeus vannamei for the immobilization of P. Vannamei protease: an eco-friendly nanobiocatalyst. Int. J. Food Prop. 20 (sup2), 1413–1423.
Siar, E.-H., Zaak, H., Kornecki, J.F., Zidoune, M.N., Barbosa, O., Fernandez-Lafuente, R., 2017. Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins. Process Biochem. 58, 98–104.
Siar, E.-H., Morellon-Sterling, R., Zidoune, M.N., Fernandez-Lafuente, R., 2020. Use of glyoxyl-agarose immobilized ficin extract in milk coagulation: unexpected importance of the ficin loading on the biocatalysts. Int. J. Biol. Macromol. 144, 419–426.
Siddiqui, I., Husain, Q., 2019. Stabilization of polydopamine modified silver nanoparticles bound trypsin: insights on protein hydrolysis. Colloids Surf. B: Biointerfaces 173, 733–741.
Sillu, D., Agnihotri, S., Reddy, M.S., 2022. Magnetic Halloysite Nanotube/α-Amylase Based Nanobiocatalytic Transformation of Food Processing Waste into an Active Fermentation Ingredient. ACS Sustainable Chemistry & Engineering 10 (31), 10169–10180.
Singh, S., Bajaj, B.K., 2017. Potential application spectrum of microbial proteases for clean and green industrial production. Energy Ecol. Environ. 2, 370–386.
Sivaprakasam, S., Dhandapani, B., Mahadevan, S., 2011. Optimization studies on production of a salt-tolerant protease from Pseudomonas aeruginosa strain BC1 and its application on tannery saline wastewater treatment. Braz. J. Microbiol. 42, 1506–1515.
Soleimani, M., Khani, A., Dalali, N., Behbehani, G.R., 2013. Improvement in the cleaning performance towards protein soils in laundry detergents by protease immobilization on the silica nanoparticles. J. Surfactant Deterg. 16 (3), 421–426.
Souza, P.M.P., Carballares, D., Lopez-Carrobles, N., Gonçalves, L.R., Lopez-Gallego, F., Rodrigues, S., Fernandez-Lafuente, R., 2021. Enzyme-support interactions and inactivation conditions determine Thermomyces lanuginosus lipase inactivation pathways: functional and florescence studies. Int. J. Biol. Macromol. 191, 79–91.
Souza, P.M., Carballares, D., Gonçalves, L.R., Fernandez-Lafuente, R., Rodrigues, S., 2022. Immobilization of lipase B from Candida antarctica in octyl-vinyl sulfone agarose: effect of the enzyme-support interactions on enzyme activity, specificity, structure and inactivation pathway. Int. J. Mol. Sci. 23 (22), 14268.
Tacias-Pascacio, V.G., Morellon-Sterling, R., Siar, E.-H., Tavano, O., Berenguer-Murcia, A., Fernandez-Lafuente, R., 2020. Use of Alcalase in the production of bioactive peptides: a review. Int. J. Biol. Macromol. 165, 2143–2196.
Su, R., Shi, P., Zhu, M., Hong, F., Li, D., 2012. Studies on the properties of graphene oxide–alkaline protease bio-composites. Bioresource Technology 115, 136–140.
Su, R., Zhang, W., Zhu, M., Xu, S., Yang, M., Li, D., 2013. Alkaline Protease Immobilized on Graphene Oxide: Highly Efficient Catalysts for the Proteolysis of Waste-Activated Sludge. Polish Journal of Environmental Studies 22 (3).
Tacias-Pascacio, V.G., Morellon-Sterling, R., Castaneda-Valbuena, D., Berenguer-Murcia, Á., Kamli, M.R., Tavano, O., Fernandez-Lafuente, R., 2021. Immobilization of papain: a review. Int. J. Biol. Macromol. 188, 94–113.
Tang, Y., Wang, P., Zeng, H., Rui, Z., 2023. Construction of porous chitosan macrospheres via dual pore-forming strategy as host for alkaline protease immobilization with high activity and stability. Carbohydrate Polymers 305, 120476.
Tang, S., Zhang, F., Gong, H., Wei, F., Zhuang, J., Karshalev, E., Li, Z., 2020. Enzyme-powered Janus platelet cell robots for active and targeted drug delivery. Sci. Robot. 5 (43), eaba6137.
Tavano, O.L., 2013. Protein hydrolysis using proteases: an important tool for food biotechnology. J. Mol. Catal. B Enzym. 90, 1–11.
Tavano, O.L., Berenguer-Murcia, A., Secundo, F., Fernandez-Lafuente, R., 2018. Biotechnological applications of proteases in food technology. Compr. Rev. Food Sci. Food Saf. 17 (2), 412–436.
Tong, L., Zheng, J., Wang, X., Wang, X., Huang, H., Yang, H., Yao, B., 2021. Improvement of thermostability and catalytic efficiency of glucoamylase from Talaromyces leycettanus JCM12802 via site-directed mutagenesis to enhance industrial saccharification applications. Biotechnol. Biofuels 14, 1–9.
Vaghari, H., Jafarizadeh-Malmiri, H., Mohammadlou, M., Berenjian, A., Anarjan, N., Jafari, N., Nasiri, S., 2016. Application of magnetic nanoparticles in smart enzyme immobilization. Biotechnol. Lett. 38, 223–233.
Verma, M.L., Barrow, C.J., Puri, M., 2013. Nanobiotechnology as a novel paradigm for enzyme immobilisation and stabilisation with potential applications in biodiesel production. Appl. Microbiol. Biotechnol. 97, 23–39.
Verma, M.L., Kumar, S., Das, A., Randhawa, J.S., Chamundeeswari, M., 2020. Chitin and chitosan-based support materials for enzyme immobilization and biotechnological applications. Environ. Chem. Lett. 18, 315–323.
Virgen-Ortiz, J.J., Dos Santos, J.C., Berenguer-Murcia, Á., Barbosa, O., Rodrigues, R.C., Fernandez-Lafuente, R., 2017. Polyethylenimine: a very useful ionic polymer in the design of immobilized enzyme biocatalysts. J. Mater. Chem. B 5 (36), 7461–7490.
Wang, S., Bao, H., Yang, P., Chen, G., 2008. Immobilization of trypsin in polyaniline-coated nano-Fe3O4/carbon nanotube composite for protein digestion. Anal. Chim. Acta 612 (2), 182–189.
Wang, Z.-G., Wan, L.-S., Liu, Z.-M., Huang, X.-J., Xu, Z.-K., 2009. Enzyme immobilization on electrospun polymer nanofibers: an overview. J. Mol. Catal. B Enzym. 56 (4), 189–195.
Wang, S.-N., Zhang, C.-R., Qi, B.-K., Sui, X.-N., Jiang, L.-Z., Li, Y., Zhang, Q.-Z., 2014. Immobilized alcalase alkaline protease on the magnetic chitosan nanoparticles used for soy protein isolate hydrolysis. Eur. Food Res. Technol. 239, 1051–1059.
Wang, Y., Hou, C., Zhang, Y., He, F., Liu, M., Li, X., 2016. Preparation of graphene nano-sheet bonded PDA/MOF microcapsules with immobilized glucose oxidase as a mimetic multi-enzyme system for electrochemical sensing of glucose. J. Mater. Chem. B 4 (21), 3695–3702.
Willner, I., Riklin, A., Shoham, B., Rivenzon, D., Katz, E., 1993. Development of novel biosensor enzyme electrodes: glucose oxidase multilayer arrays immobilized onto self-assembled monolayers on electrodes. Adv. Mater. 5 (12), 912–915.
Woodley, J.M., 2017. Bioprocess intensification for the effective production of chemical products. Comput. Chem. Eng. 105, 297–307.
Xin, B.j., Si, S.f., Xing, G.w., 2010. Protease immobilization on γ-Fe_2O_3/Fe_3O_4 magnetic nanoparticles for the synthesis of oligopeptides in organic solvents. Chemistry–An Asian Journal 5 (6), 1389–1394.
Xu, G., Chen, X., Hu, J., Yang, P., Yang, D., Wei, L., 2012. Immobilization of trypsin on graphene oxide for microwave-assisted on-plate proteolysis combined with MALDI-MS analysis. Analyst 137 (12), 2757–2761.
Yao, J., Chen, Q., Zhong, G., Cao, W., Yu, A., Liu, Y., 2014. Immobilization and characterization of tannase from a metagenomic library and its use for removal of tannins from green tea infusion. J. Microbiol. Biotechnol. 24 (1), 80–86.
Yazid, N.A., Barrena, R., Sánchez, A., 2016. The immobilisation of proteases produced by SSF onto functionalized magnetic nanoparticles: application in the hydrolysis of different protein sources. J. Mol. Catal. B Enzym. 133, S230–S242.
Zangi, A.R., Amiri, A., Borzouee, F., Bagherifar, R., Pazooki, P., Hamishehkar, H., Javadzadeh, Y., 2023. Immobilized nanoparticles-mediated enzyme therapy; promising way into clinical development. Discover Nano 18 (1), 55.
Zhai, R., Zhang, B., Liu, L., Xie, Y., Zhang, H., Liu, J., 2010. Immobilization of enzyme biocatalyst on natural halloysite nanotubes. Catal. Commun. 12 (4), 259–263.
Zhang, Y., Qi, C., Tu, J., 2023. Fabrication of hollow mesoporous silica-based nanoreactors for enzyme immobilization: high loading capacity, effective protection, and recyclability. Mater. Today Chem. 27, 101298.

Zhao, X., Hong, H., Cheng, X., Liu, S., Deng, T., Guo, Z., Wu, Z., 2017. One-step purification and immobilization of extracellularly expressed sortase A by magnetic particles to develop a robust and recyclable biocatalyst. Sci. Rep. 7 (1), 6561.
Zhao, L., Zhang, Y., Yang, Y., Yu, C., 2022. Silica-based nanoparticles for enzyme immobilization and delivery. Chem. Asian J. 17 (17), e202200573.
Zucca, P., Fernandez-Lafuente, R., Sanjust, E., 2016. Agarose and its derivatives as supports for enzyme immobilization. Molecules 21 (11), 1577.