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# Advancements in enzyme immobilization on magnetic nanomaterials: toward sustainable industrial applications

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Enzymes are widely used in biofuels, food, and pharmaceuticals. The immobilization of enzymes on solid supports, particularly magnetic nanomaterials, enhances their stability and catalytic activity. Magnetic nanomaterials are chosen for their versatility, large surface area, and superparamagnetic properties, which allow for easy separation and reuse in industrial processes. Researchers focus on the synthesis of appropriate nanomaterials tailored for specific purposes. Immobilization protocols are predefined and adapted to both enzymes and support requirements for optimal efficiency. This review provides a detailed exploration of the application of magnetic nanomaterials in enzyme immobilization protocols. It covers methods, challenges, advantages, and future perspectives, starting with general aspects of magnetic nanomaterials, their synthesis, and applications as matrices for solid enzyme stabilization. The discussion then delves into existing enzymatic immobilization methods on magnetic nanomaterials, highlighting advantages, challenges, and potential applications. Further sections explore the industrial use of various enzymes immobilized on these materials, the development of enzyme-based bioreactors, and prospects for these biocatalysts. In summary, this review provides a concise comparison of the use of magnetic nanomaterials for enzyme stabilization, highlighting potential industrial applications and contributing to manufacturing optimization.

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## 1. Introduction

Magnetic materials are widely used in modern technologies and devices with a broad classification of chemical and physical properties directly dependent on their structural composition.<sup>1–4</sup> Specifically, materials with constituents such as iron, nickel, aluminium, cobalt, and others that favour a response to a magnetic field are characterized as magnetic.<sup>5–11</sup> These materials can be classified as soft and complex, and magnets can attract both; however, soft materials are attracted only temporarily, while hard materials can be magnetized indefinitely.<sup>11–17</sup> Because of their versatility, they can be applied in various fields: sensing, smart devices, storage, biomedicine, immobilization and enzymatic stabilization, and adsorption of effluents and wastewaters.<sup>16–29</sup>

These materials can be used at different scales: macro metric, micrometric, and nanometric.<sup>11,16,30–34</sup>

Magnetic nanomaterials have attracted significant interest from various industries that synergistically apply nanoscience and nanotechnology to solve ongoing challenges.<sup>11,16,35–37</sup> The versatility of magnetic nanomaterial-based compounds is attributed to unique properties (*e.g.*, superparamagnetism), which result from the influence of thermal energy on a ferromagnetic nanoparticle.<sup>11,17,26</sup> When used as reinforcement materials, these compounds can enhance existing physical or chemical properties.<sup>24–28,36,38</sup> The synthesis of these nanomaterials is constantly evolving, and several routes have been proposed over the years.<sup>39–42</sup> The chemical or physical route used to produce this compound is defined based on its proposal of the final application. Several methods can be used, such as co-precipitation<sup>43–45</sup> aerosol route,<sup>46–48</sup> hydrothermal reaction<sup>49–51</sup> oxidative precipitation,<sup>52,53</sup> organic precursor method<sup>54,55</sup> sonochemical decomposition,<sup>56–59</sup> and sol-gel synthesis technique.<sup>60–62</sup>

Immobilization and enzymatic stabilization are among the most favourable application areas for these nanomaterials because several factors favour catalytic activity and stabilization in various reactive environments unsuitable for the use of soluble enzymes.<sup>63–66</sup> Advantages include high surface area, large surface-to-volume ratio and separation facilitated under

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external magnetic fields.<sup>67–70</sup> It should be noted that magnetite nanoparticles ( $\text{Fe}_3\text{O}_4$ ) are used more frequently compared to other types.<sup>71–73</sup> The low toxicity, good compatibility and high surface area justify the frequent use of this enzyme immobilization matrix.<sup>72,74</sup> One of the challenges to the efficient use of these materials in enzyme immobilization protocols is their high reactivity and easy degradation when exposed to specific environments, causing instability and better dispersion of the enzyme.<sup>66,72,75,76</sup>

However, several methods for modifying magnetic iron nanoparticles have been developed to improve the carriers for unrestricted use and with maximum efficiency.<sup>66,70,72,74,77–81</sup> Polymeric molecules such as polyethylene glycol (PEG)<sup>82,83</sup> polyvinylpyrrolidone (PVP),<sup>84–86</sup> poly(lactic-co-glycolic acid) (PLGA)<sup>87–89</sup> and polyvinyl alcohol (PVA)<sup>87–89</sup> have been used as coatings for these nanoparticles, mainly enhancing various chemical and physical properties.<sup>75,90–93</sup> In addition, the surface coating is made of natural and often abundant organic molecules such as chitosan, chitin, ethyl cellulose, gelatin, starch, (3-aminopropyl) triethoxysilane (APTES), carboxymethyl dextran among others<sup>94–103</sup> has been used. Therefore, chemical modifiers increase the versatility of these supports, allowing the immobilization of various biologically active and complex molecules.<sup>11,104–106</sup>

The enzyme immobilisation methods in magnetic nanoparticles are diverse, and their use depends on the final application of the biocatalyst.<sup>74,107,108</sup> Immobilization by physical adsorption is one of the most common methods and one of the first developed.<sup>66,69,72,74,109</sup> The interactions between matrix and enzyme are weak, such as electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic interactions.<sup>69,72</sup> The reaction conditions directly affect these interactions, which include pH, temperature, ionic strength, and biomolecule concentration.<sup>66,70,72,75,110,111</sup> The robustness of the support properties in these protocols is fundamental to the efficient use of these biocatalysts.<sup>66,75,112,113</sup> The protocol of immobilizing enzymes by covalent coupling is one of the most widely used due to increased enzymatic stability, which improves enzymatic activity.<sup>112–118</sup> In addition, other immobilization methods that use specific biologically mediated interactions are also used, such as ionic binding, trapping, and enzymatic encapsulation.<sup>119–127</sup>

For years, several reaction processes have experienced immobilisation and enzymatic stabilisation, improving protocols for synthesising and immobilising magnetic nanoparticle supports. Several enzymes have been immobilized in magnetic matrices belonging to the groups: xirreductases,<sup>128,129</sup> transferases,<sup>130,131</sup> hydrolases,<sup>132–134</sup> lyases,<sup>135–137</sup> isomerases,<sup>138</sup> lipases,<sup>139–143</sup> among others. The non-toxicity of magnetic nanoparticles and their surface area allow most of the above enzymes to interact efficiently, favouring immobilization parameters such as increased catalytic activity, better operational, thermal and pH stability, increased immobilization yield and more significant.<sup>144–150</sup>

Currently, enzymatic immobilization protocols are increasingly being analyzed to optimize processes, reduce costs, and improve immobilization parameters.<sup>151–154</sup> This experimental

design is a powerful tool to overcome the challenges of industrial scalability.<sup>155,156</sup> Variable analysis using experimental design is a promising alternative that has been increasingly used.<sup>157,158</sup> Numerous studies have been published discussing the main factors influencing enzymatic immobilization protocols.<sup>154,157,159,160</sup> Golmohammad Khoobakht *et al.* (2020) show that *Burkholderia cepacia* lipase was stabilized in magnetic nanoparticles of mesoporous silica shell-shell cores to synthesize biodiesel from residual soybean oil. Statistical optimization methods such as response surface methodology (RSM) with central composite design (CCD) were used. Notably, it predicted the biodiesel yield to be 92% under ideal conditions.<sup>161</sup> In this sense, there is a latent need for the use of statistical tools to optimize enzyme immobilization processes.<sup>154,159,162</sup>

In summary, immobilization protocols, the development of new supports, and process optimization are based on much of the current research on enzyme immobilization on solid media.<sup>163,164</sup> Deciding on the most appropriate immobilization protocol is fundamental to achieving maximum biocatalyst efficiency.<sup>165–167</sup> The development of hybrid supports, focusing on those of organic–inorganic composition with magnetic properties, taking advantage of organic residues, offers a promising option for the enzymatic immobilization process with the sustainable prerogative in its synthesis.<sup>168–172</sup> Experimental design, molecular simulation of biobehavioral, and analysis of variance are essential pillars for process optimization, focusing on the immobilization of active biomolecules, seeking maximum efficiency of support synthesis protocols, immobilization, and final application of the biocatalyst.<sup>154,159,162,166,167,173</sup> Therefore, the following study will address the most critical and current aspects of magnetic matrices for enzymatic immobilization. It will be based on the challenges and opportunities of this field, from the synthesis of magnetic nanomaterials to the immobilization protocols to the finalization of the essential aspects of process optimization.

## 2. Magnetic nanomaterials

### 2.1 Magnetic properties

The magnetic property of a material is determined by the magnetic moments per unit volume within the material.<sup>174</sup> Magnetic nanoparticles (MNPs) have inherent magnetic properties that make them versatile for various applications.<sup>175–178</sup> The magnetic properties of a material are classified based on its magnetic susceptibility ( $\chi_m$ ),<sup>179</sup> a fundamental response that indicates how a system interacts with an external magnetic field.

This parameter relates the magnetization of a material to the strength of an applied magnetic field.<sup>180</sup> The five basic types of magnetism are diamagnetism, paramagnetism, ferromagnetism, antiferromagnetism, and ferrimagnetism.<sup>179</sup>

MNPs exhibit unique properties, including high saturation magnetization/sizeable magnetic moment, response to moderate magnetic fields, and superparamagnetism.<sup>181</sup> These properties make them ideal for magnetic separation by application of a magnetic field.<sup>182,183</sup> Superparamagnetism, which results from the influence of thermal energy on ferromagnetic



nanoparticles, has attracted interest in recent decades.<sup>184</sup> High saturation magnetization ( $M_s$ ) and superparamagnetism are essential for applying an external magnetic field.<sup>185</sup>

MNPs address the challenges of handling and separating immobilized enzymes with low density and high dispersion, enhancing their reusability.<sup>176,186,187</sup> This allows for extended use in continuous mode while protecting against thermal and chemical changes during manufacturing and storage.<sup>187</sup> Magnetic recovery reduces production costs, and immobilized enzymes often exhibit higher activity and improved temperature and pH stability than unsupported enzymes.<sup>179</sup>

The excellent superparamagnetic property of MNPs allows easy separation from the reaction medium by simply applying an external magnetic field to the immobilized enzyme, followed by easy dispersion after the field is removed.<sup>183,188,189</sup> Unlike ferromagnetic nanoparticles, superparamagnetic nanoparticles do not retain their magnetic properties once the external magnetic field is removed, which is a significant advantage for reusing nanobiocatalysts.<sup>190</sup> Fig. 1 illustrates the importance of superparamagnetism in enzyme immobilization.

The magnetic properties of nanomaterials play a crucial role in the recovery process using an external magnetic field.<sup>178</sup> Higher  $M_s$  indicates superparamagnetic activity,<sup>191</sup> which is characterized by low coercivity ( $H_{ci}$ ) and retentivity ( $M_r$ ).<sup>192</sup> Superparamagnetism and high  $M_s$  allow reactor operation at relatively high flow rates and effective biocatalyst recovery.<sup>185</sup> However, magnetic properties can be reduced after modification and enzyme immobilization due to the presence of non-magnetic nanomaterials.<sup>193,194</sup> MNPs must be modified with functional groups to increase the enzyme binding tendency further and thus achieve efficient enzyme immobilization.<sup>195</sup> Enzyme immobilization on MNPs can reduce  $M_s$  if a biopolymer-based coating imparts a diamagnetic quality.<sup>188</sup> Coating MNPs improves multifunctionality and biocompatibility,<sup>196</sup> with both aggregation<sup>197,198</sup> and crystalline anisotropy<sup>197</sup> influencing  $M_s$ .

Among the supports, magnetite ( $Fe_3O_4$ ) stands out as the most widely used MNP for enzyme immobilization<sup>189</sup> because of its cost-effectiveness, biocompatibility, low toxicity, large surface area because of small particle size, high magnetic susceptibility, high saturation magnetization, and superparamagnetic properties at room temperature.<sup>189,194,199,200</sup> Consequently, immobilization of enzymes on MNPs significantly improves stability, catalytic performance, and reusability compared to pure enzymes.<sup>189,200,201</sup>

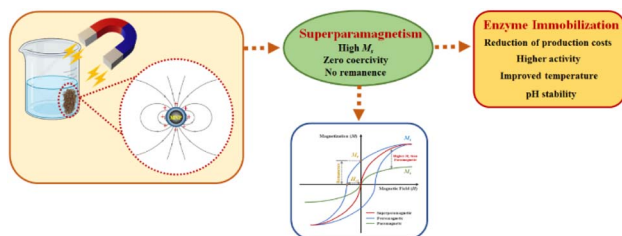


Fig. 1 The superparamagnetism of MNPs in enzyme immobilization.

## 2.2. Superparamagnetism

Among the properties of nanomaterials, superparamagnetism occurs when the magnetic material is reduced in size, such as between 10 nm and 150 nm in diameter<sup>202,203</sup> and presents a single-state domain in which the magnetic spins are aligned<sup>202</sup> (Fig. 2). In nanomagnetic particles with a single domain and smaller diameters, the superparamagnetic nanomaterial has less hysteresis, and demagnetization occurs more readily, with variations in hysteresis and magnetization becoming zero after a critical size radius is reached<sup>204–206</sup> (see Fig. 1). Overall, the practical advantages of superparamagnetic nanoparticles, such as biocompatibility, low toxicity, easy separation, and flexibility in modifying their surface, are relevant to their widespread use, especially in medicine.<sup>207,208</sup>

Several biomedical applications using superparamagnetic particles as drug-delivery systems can be found in the literature. In Neuberger *et al.* (2005), iron oxide-based superparamagnetic nanoparticles (SPIONs) are used as contrast agents in magnetic resonance imaging for the diagnosis of cartilage pathologies.<sup>203</sup> In addition, SPION can also be used as an oral contrast agent to diagnose gastrointestinal tumours or as an intravenous agent to detect other tumours in the body.<sup>207,209</sup> To use these particles as a drug-delivery system, the magnetic field is removed shortly after the particles are combined with an external magnetic field, which coordinates the delivery to the desired target area, making drug delivery more effective and less time-consuming.<sup>203,210</sup>

Using nanoparticles as a drug-delivery system is also advantageous for treating skin diseases. In Raviraj *et al.* (2021), SPIONs were developed to facilitate drug distribution in chemotherapy treatments of myeloma in rats in a non-invasive methodology, without needles and without controlling the local application of drugs.<sup>208,211</sup> The authors observed that the application of SPIONs with steric stabilization showed excellent penetration into the skin and promising results in treating skin tumours, with an increase in the immune response of the system associated with leukocyte infiltration in the studied tissue.<sup>208,212</sup> The results also suggest that using these nanomaterials as a drug-delivery system may contribute to the resumption of studies with drugs discontinued in chemotherapy regimens due to their high toxicity.<sup>208,212</sup>

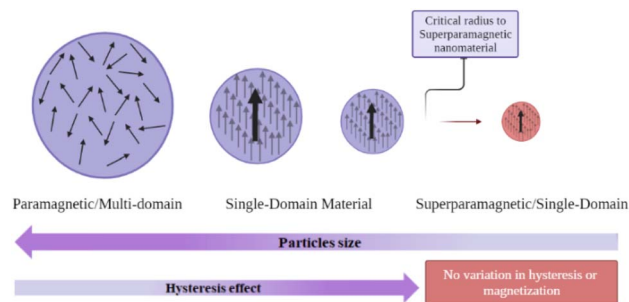


Fig. 2 Changes in the physicochemical properties of nanomaterials as a function of particle size. When particles are sized at the critical radius for superparamagnetic nanomaterials, the hysteresis and magnetization of the particle become zero.



### 2.3 Magnetic nanoparticle preparation

Magnetic nanoparticles (MNPs) consist of a magnetic material and a chemical component, and the functionality and application of MNPs are directly influenced by these two components.<sup>213</sup> The core of MNPs is primarily synthesized from  $\text{Fe}_3\text{O}_4$  and typically comprises iron oxides such as  $\text{Fe}_3\text{O}_4$ , hematite ( $\alpha\text{-Fe}_2\text{O}_3$ ), maghemite ( $\gamma\text{-Fe}_2\text{O}_3$ ), and  $\text{FeO}$  (iron(II) oxide).<sup>214</sup> The development and implementation of efficient techniques for the synthesis of high-quality nanomaterials is crucial.<sup>215,216</sup> The synthesis method and experimental conditions strongly influence the size and morphology of magnetic particles, which determine the material's magnetic properties and its applications.<sup>215–218</sup> Size and size distribution play a crucial role in determining the chemical and physical properties of MNPs, which further influence their functionality.<sup>215,216,218</sup> Because of their small particle size, MNPs often exhibit superparamagnetism,<sup>196,218,219</sup> characterized by dimensions approaching those of a single magnetic domain.<sup>196</sup> Tailoring of magnetic properties can be achieved by controlling the magnetic moment and crystallite size by incorporating high-entropy oxides.<sup>220</sup>

Precise control of the nanoparticle production parameters is essential, as the unique properties of MNPs are highly dependent on their size and morphology.<sup>221</sup> The synthesis method is selected based on the desired length, stability, morphology, and biocompatibility of the MNPs.<sup>218</sup> The primary synthesis methods for MNPs include physical, wet chemical, and a few biological approaches (e.g., green synthesis or biosynthesis).<sup>218,222,223</sup> Physical methods involve fractionating bulk material into smaller pieces through high-energy processes such as ball milling, considered a “top-down” approach.<sup>215,224</sup> In contrast, the “bottom-up” approach includes chemical and biological methods that involve particle formation through nucleation, growth, and precipitation.<sup>215,224</sup> Biological and chemical synthesis methods are the most widely used.<sup>218</sup> Physical methods, while providing better control over size and shape, have limitations, such as dispersed particle size distribution, time-consuming processes, and higher costs.<sup>215</sup> Standard methods for MNP synthesis include ball milling, coprecipitation, sol-gel, hydrothermal, thermal decomposition, microemulsion, and biological approaches,<sup>223</sup> as shown in Fig. 3.

Coprecipitation, a chemical synthesis method, uses a precursor salt and an essential precipitant,<sup>219,222</sup> often employing ferric ( $\text{FeCl}_3$ ) and ferrous ( $\text{FeCl}_2$ ) chlorides with ammonium hydroxide ( $\text{NH}_4\text{OH}$ ).<sup>222</sup> This method is preferred to achieve monodispersity of iron oxide MNPs and is considered the simplest for  $\text{Fe}_3\text{O}_4$  synthesis.<sup>218,219</sup> The oxidation states play a crucial role in controlling the dispersion behaviour of iron oxide MNPs, with the size and state of superparamagnetic iron oxide MNPs being modifiable by adjusting factors such as salt type, iron(II)/iron(III) molar ratio, ionic strength, temperature, and pH.<sup>218</sup> While coprecipitation is easy to perform, it tends to result in poorer crystalline quality and magnetic behaviour due to accumulation, although stability in aqueous media is maintained.<sup>216,222</sup> This synthesis process is suitable for applications

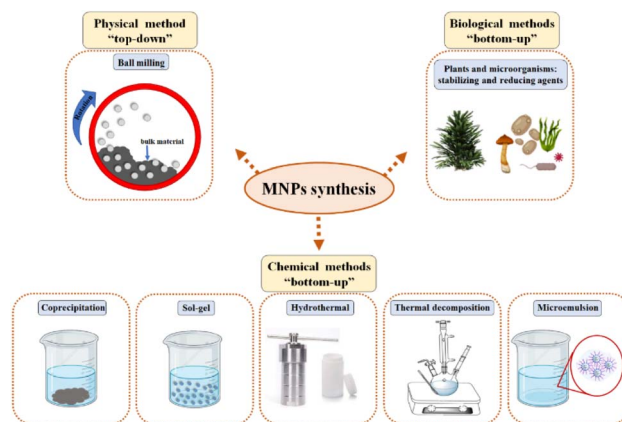


Fig. 3 Standard methods for MNP synthesis.

requiring large nanocrystals, where homogeneity in size and magnetic properties is not critical.<sup>223</sup> Compared to physical or vapour phase methods, coprecipitation provides better control over size and shape.<sup>222</sup>

Sol-gel synthesis involves gel formation at room temperature by polycondensation reactions of metal alkoxides and hydrolysis and does not require special equipment.<sup>223</sup> The sol or colloidal solution is prepared from metal salts dissolved in water, and the gel is obtained after drying the solvent by heating and roasting.<sup>223,225</sup> While size, shape, and composition can be controlled, the sol-gel method requires many toxic organic solvents and has some disadvantages, such as binding and high permeability.<sup>225</sup>

Hydrothermal synthesis involves using an autoclave with Teflon-lined stainless steel walls, typically containing water in a supercritical/sub-supercritical state.<sup>222,223,226</sup> This method operates under high temperature and pressure conditions, resulting in oxidation and hydrolysis reactions that produce MNPs of uniform size. Synthesis parameters affect crystallinity, crystallite size, particle size, purity, and magnetic properties.<sup>226</sup> Hydrothermal synthesis is preferred to produce highly crystalline MNPs with the desired size and shape.<sup>218</sup> It allows the development of different crystalline iron oxide nanoparticles, ensuring high crystallinity, size, shape and homogeneous composition.<sup>222</sup>

Thermal decomposition is an upscaled and extended synthesis process using organic solvents and nonmagnetic organometallic precursors.<sup>222</sup> It produces MNPs under extreme temperatures by decomposing organometallic precursors with organic surfactants.<sup>215,223</sup> The products' morphology, size, and monodispersity can be tuned by modifying the experimental conditions, and the annealing temperature affects the size and magnetic properties of the MNPs.<sup>219,227</sup> The obtained hydrophobic MNPs have limited applications (e.g., biomedical applications).<sup>225</sup> The hydrophobic MNPs obtained have limited applications, and thermal decomposition is energy-, material-, and time-consuming, using expensive and hazardous substances.<sup>215</sup>

In the microemulsion process, a mixture of oil, surfactant, and water is magnetically stirred at ambient temperature, with





surfactants and co-surfactants stabilizing the inter-phase.<sup>223,227,228</sup> The mixed system undergoes nucleation, crystal growth, aggregation and agglomeration, followed by precipitation of spherical microdroplets in a solid phase. The desired material is obtained after adding organic solvent and centrifugation.<sup>225</sup> While the method offers precise control over shape and particle size, high purity, good crystallinity, narrow size distribution, and the synthesis of various MNPs, it has drawbacks such as low-volume production, time consumption, and specialized equipment.<sup>229</sup>

In recent years, biosynthesis has gained popularity as an environmentally friendly and cost-effective approach compared to chemical or physical routes.<sup>230,231</sup> In this process, plants and microorganisms stabilize and reduce gents.<sup>223,230,232</sup> The produced MNPs are biocompatible and suitable for biomedical applications, and biosynthesis does not require expensive or hazardous chemicals, providing a simple and rapid processing route.<sup>223,231</sup> However, biosynthesis may result in poor dispersion of nanoparticles, and the related shortcomings, such as yield and MNP dispersion, still need to be investigated.<sup>223</sup>

#### 2.4 Structural characterizations

The techniques used to synthesize and characterize magnetic nanoparticles (MNPs) are vital in understanding their properties. Characterization is a critical preliminary step, especially given the diverse synthesis routes and applications of MNPs.<sup>175,183,184,223,233,234</sup> Structural characterizations are essential to verify the effects on MNPs after synthesis and modifications, and different techniques are used in MNPs research.<sup>175,181</sup> Fig. 4 summarizes the significant approaches to the structural characterization of MNPs.

Powder X-ray diffraction (XRD) is critical in determining nanomaterials' crystal structure and crystalline nature.<sup>233,234</sup> It provides insight into the crystallinity, diameter, and structural

changes introduced by coatings, functionalization, and immobilized materials on MNPs.<sup>196,233,235,236</sup> However, XRD alone may not distinguish iron oxide nanoparticles such as  $\gamma\text{-Fe}_2\text{O}_3$  and  $\text{Fe}_3\text{O}_4$  because of their similar patterns originating from identical cubic spinel structures. Mössbauer spectroscopy, a precise technique, is used to study the local structure of Fe and provides detailed information about the composition.<sup>192,237</sup> Mössbauer spectroscopy, a precise technique, is used to investigate the local structure of Fe, providing detailed information about the composition.<sup>238</sup> While giving a screening approach, Raman spectroscopy does not determine the exact amount of  $\text{Fe}_3\text{O}_4$ , a capability that Mössbauer spectroscopy has.<sup>239</sup>

Energy dispersive X-ray (EDX) analysis and energy dispersive spectroscopy (EDS) are used to identify the chemical composition of synthesized MNPs.<sup>178,230,240</sup> These techniques are critical to confirm the material's successful modification and evaluate the impact of surface modifications,<sup>178,181,230,234</sup> such as enzyme immobilization on MNPs.<sup>235,236,241</sup> X-ray photoelectron spectroscopy (XPS) confirms each magnetic nanoparticle surface modification step and successful enzyme binding.<sup>183,194</sup> XPS is a powerful method to verify the existence of the  $\text{Fe}_3\text{O}_4$  phase due to the coexistence of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  cations.<sup>237</sup> Fourier-transform infrared spectroscopy (FTIR) is used to evaluate functional groups and their possible interactions, providing insight into the formation of MNPs and surface modifications.<sup>189,194,214,235,241</sup>

Vibrating sample magnetometer (VSM) measurements assess nanostructured magnetic materials' magnetic behaviour and magnetic moment when subjected to vibrations perpendicular to a uniform magnetic field.<sup>233,242</sup> VSM reveals changes in magnetization that may indicate the presence of a non-magnetic layer at the core of the material.<sup>196</sup> The magnetization curve obtained from VSM can provide information about the behaviour of immobilized enzymes on MNPs and verify the superparamagnetic properties of the composite material.<sup>188,214</sup> Successful immobilization of enzymes on MNPs is often confirmed by studying their saturation magnetization  $M_s$ .<sup>241</sup>

Magnetization curves indicate superparamagnetism without hysteresis, visible coercivity and remanence, and a fully reversible magnetization process.<sup>194,196</sup> The analysis of superparamagnetism can be further refined by calculating the material's diamagnetic properties by comparing the sample's mass and the corresponding magnetic properties.<sup>233</sup>

### 3. Enzyme immobilization onto magnetic nanoparticles

#### 3.1 Enzymes and enzymes immobilization techniques

Enzymes are essential biocatalysts involved in biosynthesis and biodegradation that enable a wide range of human activities,<sup>243</sup> providing energy for most of the metabolic processes of the cell and assisting in a variety of biochemical reactions that generally occur under favourable conditions in the physiological environment.<sup>243–245</sup>

In addition, they have high chemo-, regio-, and stereo-selectivity, resulting in more pure and selective reactions that can even reduce the need for functional group protection,

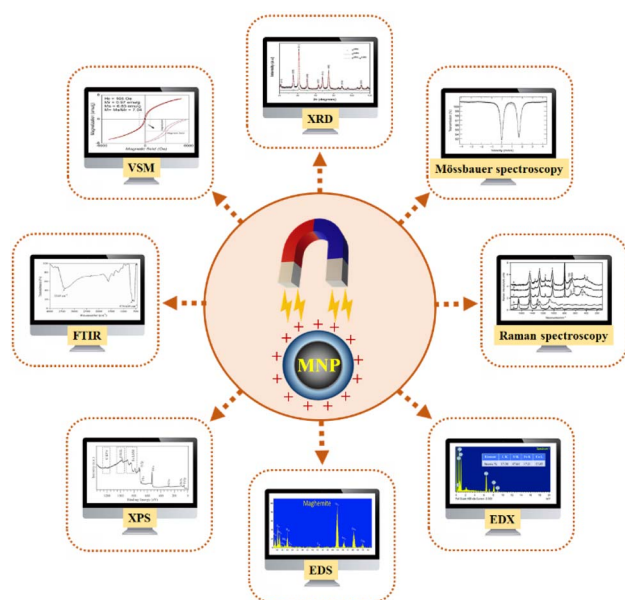


Fig. 4 The main structural characterization approaches of MNPs.



reduce purification steps, and increase the atom economy of the process, resulting in shorter synthetic routes.<sup>246,247</sup>

Become an essential pillar of the bio-economy, indispensable for the sustainable development of various scientific and technological sectors, industry, medicine, and society. Playing a notable role in several segments (such as energy production processes, biofuels, pharmacists, biosensors, the food industry, and textiles).<sup>248,249</sup>

However, enzymes' applications and desirable properties are constantly hampered by their instability at elevated temperatures or in aggressive solvents. Their inability to be recovered and reused makes their widespread use challenging. Immobilization can overcome these disadvantages, which enhances photocatalysts, making them more robust and resistant to thermal and solvent stress and preserving their catalytic activity under extreme conditions.<sup>248,249</sup>

Thus, immobilization improves the stability, selectivity, and kinetics of the enzyme, the main goal of which is to strengthen the biocatalyst's physical and enzymatic stability. Several methods are used to immobilize the enzymes. However, the industry always chooses the most accessible and economical ones based on physical or chemical immobilization, such as adsorption, covalent bonding, crosslinking, and encapsulation.<sup>250</sup> Fig. 5 shows the enzymatic immobilization techniques according to their classification and approaches.<sup>251</sup>

**3.1.1 Adsorption.** The adsorption process is simple, inexpensive, reagent-free, and generally does not cause chemical changes in the enzyme as it does not involve functionalization of the support.<sup>252,253</sup> It occurs through physical forces of attraction, and enzymes are immobilized on supports *via* van der Waals bonds, hydrophobic interactions, hydrogen bonds, and ionic bonds.<sup>254,255</sup>

**3.1.2 Covalent attachment.** Enzymes can be covalently immobilized on supports through chemical interactions, which provides high stability and enzymatic adherence to the support matrix, resulting in low leakage of the supported enzyme and attesting to the rigidity of its structure, which in turn can be naturally preserved against destructive agents such as heat, organic solvents, extreme pH, and others.<sup>256</sup>

The covalent immobilization method usually involves two steps. First, the support surface is activated by bifunctional

agents such as glutaraldehyde,<sup>257</sup> and then the enzyme is immobilized on the covalently activated surface. The cross-linkers, generally used in covalent binding, link the support material and the enzyme molecules.<sup>256,257</sup>

**3.1.3 Crosslinking.** In this method, enzymes are cross-linked to the support matrices by bifunctional reagents, of which glutaraldehyde is usually one of the most commonly used. Based on intermolecular reactions, the enzymes are thus immobilized with solidity through covalent bonds to improve reusability and stability. However, the catalytic activities of the enzymes may disappear during crosslinking.<sup>258,259</sup>

Essential cross-linking techniques are obtained by crystallizing, atomizing, and aggregating enzymes; at the end of cross-linking, the enzyme is immobilized, resulting in the production of cross-linked enzyme (CLE), cross-linked enzyme crystals (CLEC), bound enzyme aggregates (CLEA), and cross-linked spray drying enzyme (CSDE).<sup>258,259</sup>

**3.1.4 Encapsulation.** In immobilization by encapsulation, enzymes are held in polymeric structures with pores that allow substrates and products to pass through. Unlike adsorption, encapsulation protects the enzyme from direct contact with the reaction medium, minimizing inactivation effects because of the nature of the solvent in the medium. In addition, the method allows the enzymes to remain stable for a relatively long time and does not require extraction of the enzymes from the medium.<sup>258,259</sup>

## 3.2 Magnetic nanoparticles as supports

Good support material and its interaction with the enzyme are essential in immobilization, as the support properties can alter biocatalyst activity and enzyme loading.<sup>260</sup>

Thus, several nanostructured materials represent a relevant and new class of support matrices that have been investigated for the immobilization of various enzymes,<sup>261</sup> such as nanoparticles,<sup>262</sup> nanofibers,<sup>263</sup> nanotubes,<sup>264</sup> and nanosheets.<sup>265</sup>

Thus, they have promising applications in the biotechnology industry, as the catalytic activity of nanomaterials is similar to that of enzymes<sup>266</sup> because of their low cost, flexible catalytic activity, and high operational stability.<sup>267</sup>

In addition, the magnetization of substrates before use has shown great potential for recyclable applications.<sup>268</sup> Since nanomaterials can be easily collected and recycled by an appropriate external magnetic field through the incorporation of magnetic nanoparticles,<sup>268,269</sup> magnetite ( $\text{Fe}_3\text{O}_4$ ) is considered to be favourable as a nanocarrier for immobilization of enzymes due to its large surface area.<sup>268,269</sup>

Recently, several published works have developed such support for the presence of magnetic compounds to immobilize enzymes. The relevance of Fig. 6 can be seen in a Scopus search using the keywords nanoparticles and magnetic supports.

Yang *et al.* (2016) synthesized *in situ* rGO- $\text{Fe}_3\text{O}_4$  nanocomposites to support the immobilization of catalase enzyme (CAT) up to  $312.5 \pm 12.6 \text{ mg g}^{-1}$ , with almost no enzymatic leaching and the recovery of CAT activity can be increased to about 98% due to the high surface area of graphene and a magnetic field effect of  $\text{Fe}_3\text{O}_4$  nanoparticles. Studies direct

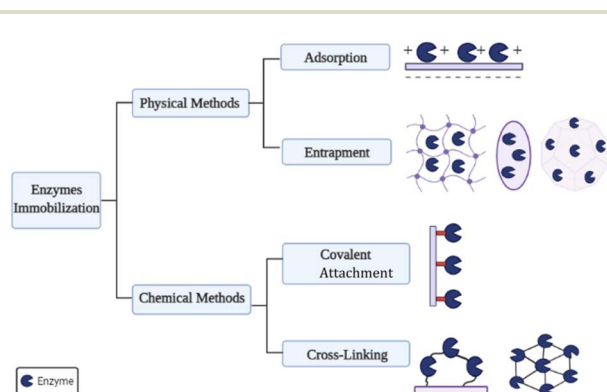


Fig. 5 Methods for enzymatic immobilization.



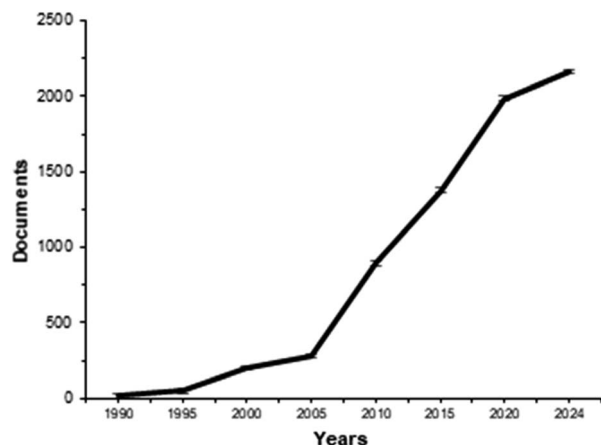


Fig. 6 Trends in the number of articles retrieved from Scopus using keywords such as "nanoparticles" and "magnetic supports".

nanocomposites as versatile nanosupports for biological or chemical reactions and separations.<sup>270</sup>

In this study, Lin *et al.* (2017) synthesized  $\text{Fe}_3\text{O}_4$  nanoparticles and coated them with chitosan, and glutaraldehyde was used as a cross-linking reagent for cellulase immobilization. The tests showed that the immobilized particles exhibited optimum cellulase loading efficiency (LE) of 99.6% and standard recovery rate (RR) of 68.5%, with a broader range of adaptability to pH and hydrolysis temperature compared with free cellulase, in addition to hydrolyzing efficiently for five experiments, maintaining an average of 80% of free cellulase activity. They were suggested to have promising potential in applying cellulose hydrolysis.<sup>271</sup>

Mehnti-Najafabadi *et al.* (2018) immobilized the xylanase in graphene oxide (GOMNP) superparamagnetic nanofilms functionalized with polyethylene glycol bisamine (PEGA). The results showed that the xylanase was bound to the functionalized nanocomposite, yielding 273 mg of enzyme per gram of PEGA-GOMNP. The immobilized enzyme retained approximately 40% of the initial activity after eight cycles and 35% of the initial catalytic activity after 90 days of storage at 4 °C. The study indicated that the support is biodegradable and suitable for bioengineering.<sup>272</sup>

Xue *et al.* (2019) immobilized lysozyme in a 1,2,3,4-butanotetracarboxylic acid-modified cellulose magnetic microsphere (BTCA), which exhibited better properties such as resistance to temperature, pH, and thermal and storage stability compared to free lysozyme. The apparent kinetics of immobilized lysozyme showed that its  $K_m$  value was 1.37 times higher than that of free lysozyme, and its  $V_{max}$  was slightly lower, with an acceptable reuse of  $51.9 \pm 2.2\%$  of activity after six cycles.<sup>273</sup>

Bezerra *et al.* (2020) immobilized *Thermomyces lanuginosus* (TLL) on a new hetero-functional divinyl sulfone (DVS) support in superparamagnetic nanoparticles functionalized with polyethyleneimine (SPMN@PEI-DVS), the remaining DVS groups were blocked with ethylenediamine (EDA), ethanolamine (ETA) and glycine (GLY) to prevent uncontrolled enzyme support reactions. As a result, 100% immobilization yield was achieved

in 1 hour at pH 10. However, at pH 5.0, they obtained the most excellent stability during thermal inactivation and good enantioselectivity for the hydrolysis of racemic methyl mandelate, the nanocatalysts blocked with EDA and ETA being 68% and 72%, respectively. They showed that the biocatalyst has excellent potential for industrial applications.<sup>274</sup>

Coutinho *et al.* (2020) used the co-precipitation method to synthesize hydroxyapatite (HA)/cobalt ferrite ( $\text{CoFe}_2\text{O}_4$ ) composites with different mass ratios to evaluate the viability support for the immobilization of  $\beta$ -glucosidase, phytase, and xylanase enzymes. The results showed that the composite with the highest cobalt ferrite content (2 : 1 ratio) was highly effective for immobilizing the three different enzymes, with immobilization yields (IYs) between 70 and 100% and recovered activities of 78 to 100%. Biocatalysts could be recovered rapidly, especially  $\beta$ -glucosidase, which could be reused 10 times while retaining about 70% of its initial activity.<sup>275</sup>

Carvalho *et al.* (2020) used magnetic nanoparticles ( $\text{Fe}_3\text{O}_4$ ) for the physical adsorption of *Yarrowia lipolytica* MUF RJ50682 lipase, achieving a high immobilization efficiency of 99%, and this biocatalyst was recycled 30 times with 70% lipase activity at the end. Moreover, they showed that immobilization on magnetic nanoparticles could achieve high pH tolerance and thermostability with a 40% improvement in thermodynamic parameters at 60 °C.<sup>276</sup>

Coşkun *et al.* (2021) aimed to increase the enzymatic activity and enantioselectivity of the lipase *Candida antarctica* B (Cal-B) by immobilization on graphene oxide (GO) nanoparticles, iron oxide ( $\text{Fe}_3\text{O}_4$ ) and graphene oxide/iron oxide nanocomposites (GO/ $\text{Fe}_3\text{O}_4$ ). The prepared samples were used as biocatalysts in the enantioselective transesterification reaction of (*R,S*)-1-phenylethanol reported for the first time in the literature.<sup>277</sup>

Perveen *et al.* (2021) fabricated a bioanode using nanocomposites containing magnetic particles of iron oxide ( $\text{Fe}_3\text{O}_4$ ), carbon nanotubes (CNT), gold nanoparticles (Au), and a conductive polypyrrole polymer (PPy), which was used as a support electrode for the immobilization of glucose oxidase (GOD) and investigated for its application in an enzymatic biofuel cell (EBFC) of glucose to improve the electron transfer kinetics and electrode stability. The bioanode was considered as a prospective material for the development of better electrochemical biosensors and biofuel anodes and showed promising results, such as the maximum current density of  $6.01 \text{ mA cm}^{-2}$  (0.22 V vs. Ag/AgCl) in 40 mM glucose concentration at 0.38 V open circuit potential (OCV).<sup>278</sup>

Paz-Cedeno *et al.* (2021) synthesized magnetic graphene oxide (GO-MNP). It was used as immobilization support for an industrial preparation containing cellulase and xylanase, which showed high activity for hydrolysis of pretreated sugarcane bagasse (PSB) and related activities of endoglucanase, xylanase,  $\beta$ -glucosidase and  $\beta$ -xilosidase of 70%, 66%, 88% and 70%, respectively, after 10 cycles, also maintained about 50% and 80% of their efficiency for cellulose and xylan hydrolysis. Thus, the study indicated the biocatalyst as a potential candidate for industrial applications such as second-generation ethanol production.<sup>279</sup>



Table 1 Studies described in the literature on the different supports of magnetic nanoparticles<sup>a</sup>

Enzyme	Magnetic carrier	Characteristics of NP	Coupling agent	Synthesis method	Recovered activity	Cycles	Application	References
<i>Aspergillus niger</i>	CMNP	Showed area amorphous and a thin chitosan layer	GLY and GLU	Covalent	—	15	Biotechnological processes involving magnetic separation	280
<i>C. rugosa</i>	Fe <sub>3</sub> O <sub>4</sub> -MCM-41	Spherical morphology	GLU	Covalent	—	4	Interesterification of soybean oil and lard	281
Trypsin (EC 3.4.21.4)	Fe <sub>3</sub> O <sub>4</sub> @GA	17 nm	—	Adsorption	—	8	Use in various protein degradation processes in the chemical and food industries	282
Lacase (EC 1.10.3.2)	Fe <sub>3</sub> O <sub>4</sub> @silica	—	GLU	Covalent	36.3 U L <sup>-1</sup>	6	—	283
Swine pancreas lipase (PPL)	Fe <sub>3</sub> O <sub>4</sub> @GO	Between 10 and 25 nm	APTES	Covalent	—	6	Hydrolysis of <i>p</i> -nitrophenyl dodecanoate	284
<i>Pseudomonas fluorescens</i>	Fe <sub>3</sub> O <sub>4</sub> @APTES	15 nm	GLU	Covalent	100%	5	Kinetic resolution of rac-secondary alcohols with potential application in heterogeneous catalysis	285
<i>C. rugosa</i>	Fe <sub>3</sub> O <sub>4</sub> @GO	—	EDC and NHS	Covalent	64.9%	5	Biodiesel production	286
<i>Lysozyme</i>	Fe <sub>3</sub> O <sub>4</sub> @cellulose	Mean size range was 400–500 nm	BTCA	Covalent	78.8%	6	Improve stability and bio-catalytic activities	287
<i>Thermomyces lanuginosus</i>	Fe <sub>3</sub> O <sub>4</sub> @PEI-DVS	—	DVS	Multipoint covalent attachment	—	7	Pharmaceutical, cosmetic, biotechnology and fine chemical industries	288
$\beta$ -Glucosidase, fitase e xylanase	HA-CoFe <sub>2</sub> O <sub>4</sub>	—	—	Adsorption	Range 75–100%	Up to 10	Biofuels, pharmaceutical drugs, paper and pulp, animal feed, food, beverage, among others	289
<i>Trichoderma asperellum</i> laccase	Fe <sub>3</sub> O <sub>4</sub> @SiO <sub>2</sub> -chitosan	From 350 to 700 nm	—	—	—	8	hydrolysis cycles to $\beta$ -glucosidase	290
<i>Candida antarctica</i> B	Fe <sub>3</sub> O <sub>4</sub> nanoparticles, GO nanosheets and GO/Fe <sub>3</sub> O <sub>4</sub> nanocomposite	Uneven surface after immobilization	GLU or EPH	Covalent	64.9%	5	Enhanced biohydrogen production from delignified lignocellulosic biomass	291



Table 1 (Contd.)

Enzyme	Magnetic carrier	Characteristics of NP	Coupling agent	Synthesis method	Recovered activity	Cycles	Application	References
<i>Inulinase</i>	Fe <sub>3</sub> O <sub>4</sub> @Ag	Spherical with the average particle diameter of around 100 nm	EDC and NHS	Covalent	—	—	Produce high-fructose syrup by hydrolysis of inulin	292
Cellulases and xylanases	GO-MNP-Enz	Morphological aspect of the graphite oxide surface has the shape of overlapping sheets	EDC and NHS	Covalent	$\beta$ -Glucosidase and $\beta$ -xylosidase presented the best results (110% and 78%, respectively) while xylanase (15%)	10	Producing second-generation ethanol (cellulosic ethanol) and other value-added bioproducts	293

<sup>a</sup> Note: (GLU) – glutaraldehyde; (AP) – 3-aminopropyl triethylenesilane; (EDC)– 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; (APTES) – 3-aminopropyltriethoxysilane; (GO) – graphene oxide; (NHS) – *N*-hydroxysulfosuccinimide; (BTCA) – 1,2,3,4-butanetetracarboxylic; (DVS) – divinyl sulfone; (EPIH) – epichlorohydrin; (GA) – gallic acid; (HA) – hydroxyapatite; glycidol – (GLY); chitosan-coated magnetic nanoparticles – (CMNP).

Thus, immobilising enzymes on magnetic supports has demonstrated efficacy and promise across chemical, biochemical, and industrial reactions. Studies have documented improvements in thermal stability and pH, facilitating fast and easy recovery for the reuse of biocatalysts at acceptable rates and over multiple cycles for diverse catalytic systems. In this sense, Table 1 presents the different enzyme carriers and their applications.

### 3.3 Immobilization *via* entrapment

Numerous methods are used for enzyme immobilization,<sup>294–297</sup> each designed to improve the physicochemical properties of enzymes for various applications.<sup>296</sup> The choice of immobilization technique and the properties of the enzymes significantly influence.

The degree of enzyme immobilization and the retained catalytic activity.<sup>295</sup> Enzyme immobilization can be carried out on a variety of organic and inorganic materials,<sup>294</sup> with factors such as the physicochemical properties of the enzyme and substrate, the need for robust attachment between the support and the enzyme, and the number of reuse cycles taken into account when determining the optimal immobilization method.<sup>297</sup>

The effectiveness of these methods is highly dependent on the support used. To optimize immobilization efficiency, the carrier should have a large surface area, good stability, substantial porous structures, and be readily adaptable to facilitate enzyme immobilization.<sup>296</sup> Improved enzyme activity and thermal stability are observed when the support's pore size matches the enzyme's hydrodynamic size. Although organic and inorganic materials can be used for enzyme immobilization, hydrophobic nanomaterials often exhibit higher immobilization.<sup>294</sup> An important consideration when selecting a matrix for immobilization *via* the entrapment approach is the efficient diffusion of substrate and product molecules.<sup>298</sup>

Among the various immobilisation methods, entrapment has proven to be among the most successful.<sup>294</sup> Entrapment is a physical process for immobilizing enzymes on carriers or transporters,<sup>299</sup> where enzymes are bound to a substrate by hydrogen bonding, ionic interactions, and van der Waals forces. This simple and inexpensive adsorption process can be purely physical or involve covalent bonding,<sup>294</sup> an irreversible immobilization.<sup>296</sup> Enzymes are confined with limited mobility in entrapment but remain in circulation as free entities.<sup>294</sup> The enzyme becomes entrapped within the material matrix as the carrier material grows, making it more stable and accessible to separate and recycle.<sup>300</sup> This entrapment prevents the unfolding of the enzyme, thus avoiding activity loss due to conformational changes.<sup>297</sup> Entrapment methods allow easy tuning and optimization of the support, creating an ideal environment to stabilize and enhance enzyme activity.<sup>300</sup> However, defects in the matrix<sup>300</sup> and minimal adsorption interactions<sup>298</sup> can lead to enzyme leaching, a challenge that can be overcome by combining entrapment with covalent immobilization methods.<sup>298</sup>

Enzyme entrapment can be achieved by four primary methods on gels: sol–gel methods (hard gel), cross-linking of



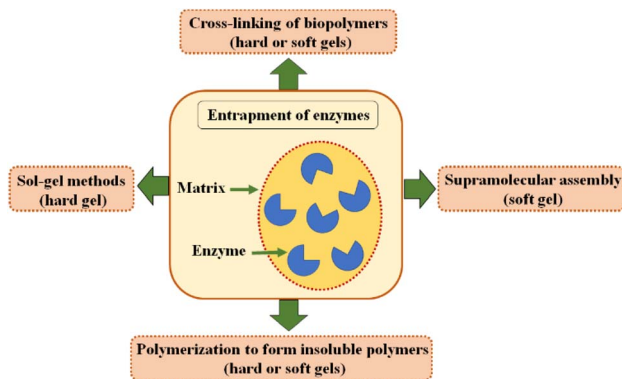


Fig. 7 The four main methods for the entrapment of enzymes out on gels.

biopolymers (hard or soft gels), polymerization to form insoluble polymers (hard or soft gels), and supramolecular assembly (soft gel),<sup>300</sup> as shown in Fig. 7. The sol-gel method is particularly suitable for immobilising or encapsulating labile enzymes in inorganic oxide matrices, offering the advantage of low temperature and pressure conditions. The extent to which the molecule retains its native properties depends on the interaction between the matrix network and the encapsulated enzyme. This process has successfully encapsulated various enzymes, including therapeutic enzymes, cellobiase, amylase, and lipase.<sup>301</sup> The sol-gel method, especially for hydrophobic enzymes such as lipase, forms a wide range of active biocatalytic materials, and the hybrid carriers produced can prevent enzyme leakage while providing increased mechanical stability.<sup>300,302</sup>

Recent advances in enzyme entrapment include 3D printing, metal-organic frameworks, smart gels (enzyme-responsive entrapment), ionic liquids, and hybrid materials.<sup>300</sup> The field of 3D printing for enzyme immobilization by entrapment is, with a few exceptions, primarily dominated by hydrogel-based 3D printing, with direct ink writing being the most commonly used method.<sup>303</sup> Enzymes such as  $\beta$ -galactosidase and laccase have been successfully used in 3D printable bioinks for enzyme entrapment. 3D printing is promising, particularly in industrial biocatalysis for flow reactions.<sup>300</sup>

### 3.4 Crosslinked enzymes

The aggregation of enzymes characterizes the method of enzyme immobilization *via* cross-linked enzyme aggregates (CLEAs) by cross-linking, where a collection of active molecules is linked by chemical interactions, forming cross-links that hold the enzymes together.<sup>304</sup> Moreover, cross-linked aggregates represent an irreversible technique of enzyme immobilization, providing autonomous and reusable stable biocatalysts with high enzyme activity retention, making them applicable in various industrial fields.<sup>305,306</sup>

Enzymatic aggregates are synthesized by crosslinking enzyme aggregates prepared by mixing an aqueous protein solution with organic solvents, polymers, or anionic salts and then crosslinking with a bifunctional chemical reagent.<sup>307</sup> The cross-linking reagent is a molecule with at least two reactive

ends that bind to specific regions of the enzyme that are not essential for catalytic activity, allowing the enzyme molecules to interact with macromolecular structures<sup>308,309</sup> physically. The resulting covalent cross-links are rigid and effectively prevent enzymatic denaturation, thereby preserving or enhancing catalytic activity and increasing enzyme stability.<sup>310</sup> In this method of enzyme immobilization, the enzyme is not attached to a solid support.<sup>311</sup>

This enzyme immobilization protocol highlights two cross-linking immobilization approaches: cross-linking enzyme aggregate (CLEA) and cross-linking enzyme crystal (CLEC).<sup>309,311</sup> The CLEA technique involves the addition of salts, organic solvents, or nonionic polymers to form enzyme aggregates with high catalytic activity.<sup>312</sup> The CLEC technique is more complex and involves controlled enzyme precipitation to produce microcrystals, followed by the formation of crystal aggregates through covalent bonding with a cross-linking agent to promote this chemical interaction.<sup>313</sup> Both protocols use cross-linking agents to form enzyme aggregates. CLECs offer several advantages, including high operational stability, catalytic activity, and ease of recycling. However, the protocol is complex and costly regarding time and resources.<sup>314-316</sup> CLEAs, on the other hand, are advantageous due to their simplicity, low cost of protein processing, and robustness of the biocatalyst. However, cross-linking agents can sometimes lead to structural changes or enzyme formation that may block active groups.<sup>317</sup>

In their innovative research, Akkas *et al.* (2020) presented a novel method for immobilizing ureases. This method involves a reticulated enzyme aggregation technique using lyophilization to enhance enzyme stabilization. In this study, lyophilization of bovine serum albumin (BSA), crosslinking with polyaldehyde dextran (DPA), and pH optimization of the crosslinker were used to immobilize jack bean urease (JBU). Notably, the relative catalytic activity of urea-CLEAs was approximately 1.47 times higher than free urease's. In addition, the biocatalyst exhibited enhanced thermal stability, allowing it to function in reactions at temperatures up to 85 °C while maintaining catalytic efficiency. According to the authors, the shelf life of the immobilized enzyme was extended to 4 weeks with unchanged catalytic activity. In addition, the recyclability of the enzyme was demonstrated, as its residual activity remained unchanged after 10 reaction cycles, and its thermal stability was nearly doubled. This approach opens new perspectives for enzyme engineering, providing access to new information and potential industrial applications.<sup>316</sup>

### 3.5 Covalent attachment

The covalent enzyme immobilization protocol is one of the most commonly used methods because it increases or maintains operational stability, thereby improving catalytic performance.<sup>318</sup> This method involves the formation of stable covalent complexes or bonds between the functional groups of the support and the enzyme, making it a chemical method of immobilization.<sup>319</sup> The enzyme-binding functional groups need not be essential for enzyme activity.<sup>311</sup> The primary functional groups involved in interactions with the support are typically



found in the side chains of the enzyme, such as cysteine (thiol group), aspartic acid, glutamic acid (carboxyl group), and lysine ( $\epsilon$ -amino group).<sup>320</sup> The major functional groups capable of interacting and forming a covalent bond with these side-chain enzyme groups include the imidazole, thiol, indole, hydroxyl, amino, and sulfhydryl groups.<sup>321–324</sup>

In most covalent coupling immobilization protocols, two essential steps are required to maintain the technique's efficiency.<sup>325</sup> The first step involves the activation of the surface of the solid support. In this step, one region of the ligand molecule covalently interacts with the surface of the support, activating the support while leaving the other region of the ligand molecule free for interaction with the enzyme.<sup>326</sup> Several activating reagents can be used for this purpose, including glutaraldehyde, carbodiimide, glycidol, epichlorohydrin, and formaldehyde.<sup>327</sup> The binding molecules bridge the support surface and the enzyme at this stage through covalent interactions. These binding reagents are multifunctional, thus allowing for this covalent coupling.<sup>328</sup> The next step involves the interaction between the activated solid support and the non-essential region of the enzyme. The pre-activated support forms a covalent bond with the enzyme, establishing a bond between the free portion of the binding reagent and the enzyme binding region.<sup>329,330</sup> The selection of the activating reagent and the immobilization protocol is determined based on the analysis of the support surface and the structural and conformational characteristics of the enzyme.<sup>331</sup>

Covalent fixation is characterized as an efficient technique for the immobilization of enzymes.<sup>332,333</sup> This protocol provides vital links between enzymes and solid support. Therefore, the leaching of the enzyme immobilized on the support is minimal, thus improving the stability of the immobilized enzymes and the immobilization yield.<sup>334,335</sup> Notably, the high uniformity of the bonds between enzyme and support allows reasonable control of immobilised enzyme amounts.<sup>328,335,336</sup>

Much of the work published in the last five years has shown that the parameters associated with enzyme immobilization (*e.g.*, immobilization yield, protein content, enzyme activity, thermal stability, and pH) are favourable.<sup>328</sup> In particular, the expressed activity of the biocatalyst is often maintained or increased after immobilization, as the conformations of the enzymes remain unchanged.<sup>335</sup> Therefore, the covalent fixation approach to immobilization mitigates the desorption phenomenon, reduces the spontaneous deactivation rate of the enzyme, and prolongs its useful life and operational stability.

The covalent fixation technique is often preferred as the primary immobilization protocol because of its proven efficacy in the literature. Helm *et al.* (2019) reported the covalent immobilization of the hydroxy-nitrile lyases HbHNL (from *Hevea brasiliensis* L.) and MeHNL (from *Manihot esculenta* C.) on porous silica substrates, achieving high immobilization performance. Because of the high enantioselectivity of these enzymes, biocatalysts have been used in kinetic resolution reactions to achieve superior chiral construction. As a result, a project was developed for a continuous flow micro-reactor with minimal HNL loads, resulting in a significant improvement in catalytic performance compared to the batch system.

The application of the constant flow system enabled the rapid production of chiral cyanohydrins with high conversion (97%) and high enantiomeric excess (98%) in only 3.2 minutes, using the lowest possible enzymatic load. The monolith immobilization protocol achieved high protein loads with immobilization yields of 89% (11.3 mg total protein; 1120 U per monolith) and 72% (17.4 mg total protein; 1310 U per monolith) for HbHNL and MeHNL, respectively, demonstrating the overall versatility of the covalent immobilization method. This protocol increased enzyme activity, improving substrate conversion rates and superior chiral construction.<sup>337</sup>

## 4. Enzymatic magnetic nanoparticle applications

### 4.1 Oxirreductases

Oxidoreductase (EC 1) enzymes include at least 26 subclasses of enzymes (<https://enzyme.expasy.org/enzyme-byclass.html>) that play a central role in metabolic pathways critical for cell function (<https://www.brenda-enzymes.org>). They catalyze oxidation–reduction reactions that involve the transfer of electrons, either as free entities or as hydrogen atoms, between a donor (reducing agent, which is oxidized) and an acceptor (oxidizing agent, which is reduced), or the transfer of oxygen atoms from O<sub>2</sub>, which is reduced, to an organic molecule, which is oxidized. These reactions account for at least one-third of all enzymatic reactions recorded in the BRENDA (Braunschweig ENzyme Database).<sup>338,339</sup> Oxidoreductases are a diverse group of enzymes that play a central role in various chemical oxidation–reduction reactions. These enzymes facilitate the transfer of electrons or the performance of oxidation and reduction reactions between different substrates.<sup>338,340</sup> They have a variety of properties that make them useful in many fields, including agriculture, environmental management, medicine, and analytical chemistry. These enzymes include oxidases, peroxidases, dehydrogenases, and oxygenases.<sup>338,341,342</sup>

Oxidoreductases can be functionally classified into several categories: (i) oxidases catalyze oxidation reactions using oxygen as the final electron acceptor. A well-known example is laccase, one of the first oxidases studied.<sup>338,343</sup> (ii) Peroxidases: these enzymes catalyze the oxidation of substrates using a peroxide, with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) being the most commonly used peroxide.<sup>344</sup> (iii) Dehydrogenases: these enzymes oxidize substrates by transferring electrons from hydrogen atoms to acceptor or donor cofactors. Some dehydrogenases can use molecules other than oxygen as electron acceptors.<sup>338,345</sup> (iv) Oxygenases: these enzymes catalyze oxidation reactions that directly incorporate oxygen into the substrate.<sup>346</sup>

Kouasse *et al.* (2020) used cholesterol oxidase (CHO) to be bound to magnetic nanoparticles *via* carbodiimide activation. FTIR spectroscopy analyses confirmed the binding between CHO and the nanoparticles, with efficiencies ranging from 98% to 100%. After nanoparticle association, comparative kinetic studies between free and immobilized CHO showed significant



stability and enzymatic activity improvements. In addition, the bound enzyme exhibited improved resistance to variations in pH, temperature, and substrate concentration.<sup>347</sup> Cholesterol oxidase has industrial and commercial importance, particularly in bioconversions, for clinically determining total or free serum cholesterol and agricultural applications.<sup>348,349</sup>

Huang *et al.* 2023 covalently bound lipase to magnetic iron nanoparticles using carbodiimide activation. The efficiency of lipase binding to magnetic nanoparticles was confirmed by FTIR analysis. Compared to the free enzyme, the nanoparticle-bound lipase showed a 1.41-fold increase in activity, a 31-fold improvement in stability, and better tolerance to temperature and pH variations.<sup>350</sup>

Ren *et al.* 2023 carried out the immobilization of yeast alcohol dehydrogenase (AmDH) on titanium nanoparticles. First, AmDH was coated with polyethyleneimine (PEI), which created a hydrophilic environment that stimulated the hydrolysis and condensation reaction of titanium, resulting in the formation of nanoparticles. This process created a rigid matrix that acted as a pocket, preventing the enzyme structure from unfolding. The immobilized enzyme, named AmDH-PEI-Ti, retained 80% of the activity observed in the free enzyme, with an entrapment efficiency of 90%, showing potential for industrial production.<sup>351,352</sup>

Oxidoreductases represent biocatalysts of great interest, with significant potential in producing polymeric building blocks, sustainable chemicals and materials derived from plant biomass in lignocellulose biorefineries. However, despite these promising applications, the chemical industry, especially in large-scale chemical manufacturing, has not yet widely adopted enzymatic oxidation reactions.<sup>353</sup>

This reluctance is mainly attributed to the lack of biocatalysts that possess the necessary selectivity, are commercially available, and are compatible with stringent process conditions. Such conditions include high substrate concentrations, use of solvents, and strongly oxidative environments. Overcoming these challenges is crucial for effectively integrating enzymatic oxidation reactions into industrial processes, presenting the potential to contribute significantly to the sustainable production of diverse chemical products and bio-based materials.<sup>353,354</sup>

#### 4.2. Transferases

Transferases are enzymes that are part of a group responsible for transferring various functional groups, such as the methyl group of a compound, to other groups that accept them, thereby creating a bond between the donor group and the acceptor group.<sup>355</sup> Transferase enzymes can be divided into subgroups based on the transferred functional groups. The first subgroup, known as glutathione *S*-transferases (GSTs), catalyzes the transfer of a methyl group from glutathione (GSH). The second subgroup, *N*-acetyltransferase (NATs), transfers an acetyl group. The third subgroup, sulfotransferases (SULTs), transfer one or more sulfate groups. The fourth subgroup, UDP-glucuronosyltransferases (UGTs), transfers a glycosyl group.<sup>356,357</sup> The scope and outcomes of transferases are still poorly understood and require further investigation. However,

it is known that transferase enzymes are mainly used in the health field, especially in pharmacology and biochemistry, as their primary function is to conjugate drug metabolites, making the studied drugs more hydrophilic, facilitating their absorption and allowing for their natural elimination.

Glutathione *S*-transferase enzymes (GSTs) are the most extensively studied of the transferases and are known as drug-metabolizing enzymes (DMEs). Based on the sequential catalysis analysis, these enzymes can be divided into phases I, II, and III, each with a different role.<sup>358</sup> Glutathione *S*-transferases (GSTs) are part of phase II and crucial enzymes in combating oxidative stress. They act as detoxification enzymes, conjugating products from phase I reactions. Phase I involves the oxidation of drugs, and the primary function of GSTs is to conjugate drug or xenobiotic metabolites to make them more hydrophilic. This occurs through several pathways, including methylation, glutathionylation, acetylation, and sulfation. Sulfotransferase enzymes (SULTs) are also part of phase II and are involved in the sulfation pathway.<sup>359</sup> In addition to their detoxification role, GSTs have isomerase and peroxidase functions and can bind to numerous endogenous substances and exogenous ligands.<sup>360,361</sup>

Transglutaminase (TGM) enzymes are a subgroup of transferase enzymes. Transglutaminases (TGMs) facilitate intramolecular and intermolecular cross-links between glutamine and lysine residues, with the former serving as acyl donors and the latter as acyl acceptors. These residues are commonly found in peptides and various proteins.<sup>362,363</sup> Transglutaminases (TGMs) play an essential role in the food industry, enhancing the properties of proteins and improving the texture and overall quality of food products. Viable technological methods contribute to more efficient use of raw materials, thereby improving the cost-benefit ratio of food production.<sup>364,365</sup> TGMs also have potential applications in other industries, including the leather and textile sectors.<sup>366</sup>

In a recent study, transglutaminase enzymes (TGMs) were subjected to enzymatic immobilization using magnetic nanoparticles (MNPs). Microbial TGMs were investigated to create cleaner and more environmentally friendly industrial applications. Immobilization of TGMs was achieved by covalent attachment, starting with the preparation of MNPs, which were then modified by a co-precipitation process with Fe<sup>2+</sup> and Fe<sup>3+</sup>. Subsequent modifications included carboxymethyl dextran (CMD) and CMD with oleic acid. The MNPs were activated with the crosslinking agent's pentamethylene hexamine (PEHA) and glutaraldehyde (GA), the latter being a common choice for enzyme immobilization techniques.<sup>367</sup> The immobilized TGMs on magnetic nanoparticles were thoroughly analyzed, including protein concentration, activity, thermal stability (in both free and immobilized forms), stirring speed, and reuse (cycles). The study concluded that the enzymes were quickly recovered from TGMs immobilized in CMD-oleic and CMD-MNPs, with CMD-MNPs showing the highest success rate in terms of immobilization. The enzymes were also hyperactivated, showing % residual activity of 110% and excellent thermal stability at 50 °C and 70 °C. This led to the conclusion that the immobilization studies with magnetic nanoparticles of TGMs were successful and could be used in industrial applications as an economical and





biodegradable biocatalyst, with potential applications mainly in the leather and wool industries.<sup>368–370</sup> In a recent study, transglutaminases (TGMs) were subjected to enzymatic immobilization, a widely used method for enzyme stability. Two different approaches were used, magnetic cross-linked enzyme aggregates (mCLEAs) and cross-linked enzyme aggregates (CLEAs), to obtain promising results regarding the enzymatic activity of the enzymes immobilized by these methods. After studying the techniques, the TGMs were subjected to a colourimetric hydroxamate procedure using CBZ-glutamine glycine (CBZ-Gln-Gly) as an amine acceptor substrate to identify the activity of the TGMs.<sup>371</sup> The TGMs showed the highest results among the enzymes included in the study. The ideal reagent for CLEAS TGMs was 2-propanol, resulting in a residual activity of 231%. In addition, both immobilization techniques, CLEAS and mCLEAS, showed more excellent storage stability when exposed to 4 °C for 44 days. mCLEAS TGMs showed very positive results compared to CLEAS TGMs, with residual activity of 53% under these conditions, providing more excellent stability of the immobilized TGMs. The study showed that mCLEAS had better operational stability and catalytic efficiency than CLEAS, demonstrating that magnetic nanoparticles significantly affect stabilization results. The two immobilization techniques have particular specificities and depend on the behaviour of the enzymes in specific environments and conditions to which they are subjected. Both immobilization techniques are promising, but the enzymes have no particular rules or parameters, whether CLEAS or mCLEAS. Both methods could be used in many future bioapplications.<sup>372</sup>

Scientists are increasingly studying and analysing transferases worldwide, with several studies presenting promising analyses for future applications. Although studies on

immobilization in magnetic nanoparticles (MNPs) and transferases are still in their infancy, guidelines for future scientific analysis already exist, especially in the food industry.<sup>373–375</sup> Although still in its infancy, scientific work on transferase enzymes aimed at enzymatic immobilization is already available, especially for TGMs. For example, an enzymatic membrane reactor (EMR) has been developed to recover whey protein.<sup>376</sup> In addition, a study describes the usefulness of TGM enzymes for immobilization in poly(*N*-isopropylacrylamide).<sup>377</sup> Glutathione *S*-transferases (GSTs) are used in enzymatic activity studies because of their high detoxifying properties.<sup>378</sup> They are also used to separate and purify proteins when labelled with GSTs. These enzymes protect the body from chemical carcinogenesis and conjugate glutathione (GSH) to various electrophilic substrates.<sup>379</sup> Transferases are expected to become the most studied group of enzymes for enzyme immobilization because they have desirable properties for all fields, especially for current studies focusing on cleaner, ecologically correct, and economically sustainable technologies. These enzymes have large-scale and industrial applications.

### 4.3. Hydrolases

Hydrolases (EC 3) catalyse hydrolysis reactions in living organisms.<sup>380,381</sup> They are divided into subclasses based on the particular bonds they target during chemical reactions. The diversity and adaptability of these enzymes in hydrolyzing a wide range of substrates, from small to large molecules, make them particularly attractive for industrial applications.<sup>382</sup>

Of the various subclasses of hydrolases, certain enzymes such as tannases,  $\alpha$ -amylases,  $\beta$ -galactosidases, proteases,

Table 2 Applications of immobilized hydrolases on magnetic nanoparticles

Enzyme	Support	Immobilization yield	Application	Reference
<i>Rhizomucor miehei</i> (RML) and <i>Thermomyces lanuginosa</i> (TLL)	Fe <sub>3</sub> O <sub>4</sub> @SiO <sub>2</sub> <sup>a</sup>	81–100%	Biodiesel production	390
<i>Bacillus subtilis</i>	ZnO nanoparticles	71.9–79.5%	Detergent formulation	391
<i>Rhizopus oryzae</i> (ROL)	Magnetite nanoparticles	74.7%	Synthesis of triacylglycerols	392
<i>Pseudomonas fluorescens</i> (PFL)	AGMNP-Co <sup>2+b</sup>	89%	Biodiesel production	393
<i>Thermomyces lanuginosus</i> (TLL)	Fe <sub>3</sub> O <sub>4</sub> @PEI <sup>c</sup>	69.6–74.4%	Synthesis of ethyl valerate	394
<i>Rhizomucor miehei</i> (RML)	Carbon nanotubes	95–98%	Hydrolysis of <i>p</i> -nitrophenyl butyrate	395
<i>Bacillus atrophaeus</i> (BaL)	Graphene oxide nanosheets	81.35%	Synthesis green apple flavor ester	396
Proteases produced by solid state fermentation	Magnetic iron oxide nanoparticles	93–96%	Hydrolysis of different protein sources	397
<i>Rhizopus oryzae</i> (ROL)	CoFe <sub>2</sub> O <sub>4</sub> <sup>d</sup>	77.43%	Biodiesel production	398
<i>Candida rugosa</i> (CRL)	Multiwalled carbon nanotubes with Co	88.5%	Synthesis of fruit flavors	399
Tannase from <i>Aspergillus ficuum</i>	mDE-PANI <sup>e</sup>	90%	Removing tannins from aromatic drinks	400
<i>Bacillus subtilis</i> A (BsLA)	Fe <sub>3</sub> O <sub>4</sub> @SiO <sub>2</sub>	89.94–93.72%	Hydrolysis of <i>p</i> -NPC	401
<i>Candida antarctica</i> B (CALB)	Fe <sub>3</sub> O <sub>4</sub> @CHI <sup>f</sup>	95%	Photo-curable functional esters	402
<i>Burkholderia cepacia</i> (BCL)	GTAMNPs <sup>g</sup>	98.8%	Standard esterification reaction between lauric acid and 1-dodecanol	403

<sup>a</sup> Note: magnetic nanoparticles coated with silica. <sup>b</sup> Magnetic nanoparticles with glycidoxypropyltrimethoxysilane (GOPTS), 5-AIPA and Co<sup>2+</sup>. <sup>c</sup> Superparamagnetic magnetite nanoparticles modified with polyethyleneimine (PEI). <sup>d</sup> Core-shell cobalt ferrite nanoparticles. <sup>e</sup> Magnetic nanoparticles composed of polyaniline-coated diatomaceous earth. <sup>f</sup> Magnetic nanoparticles cross-linked with chitosan. <sup>g</sup> Melamine-glutaraldehyde dendrimer-like polymers grafted on aminated magnetic nanoparticles.



phospholipases, and various lipases have been used in immobilization processes using magnetic nanoparticles as supports.<sup>383–389</sup> Table 2 provides some examples of these applications in industrial processes.

Studies indicate that proteases are increasingly used for enzymatic immobilization with magnetic nanoparticles.<sup>404–406</sup> Ibrahim *et al.* (2021) demonstrated that the nanobiocatalyst prepared by covalent immobilization of alkaline protease from *Salipaludibacillus agaradhaerens* in mesoporous double-core nanospheres (DMCSS) exhibited enhanced enzyme stability in high concentrations of NaCl, solvents, surfactants, and commercial detergents. Furthermore, the immobilized protease exhibited excellent operational stability, retaining 79.8% of its activity after ten cycles, thus proving to be a promising nanocatalyst for industrial applications.<sup>407</sup> Razzaghi *et al.* (2018) concluded that immobilization of the protease *Penaeus vannamei* in zinc sulfide (ZnS) nanoparticles improved the functionality of the enzyme at high temperatures, extreme pH conditions (pH 3 and 12) and during storage while also extending its optimal temperature range.<sup>408</sup>

In their study, Li *et al.* (2018) aimed to develop a novel biocatalyst for tea infusion clarification. To achieve this, they immobilized *Aspergillus niger* tannase on chitosan-coated magnetic nanoparticles (Fe<sub>3</sub>O<sub>4</sub>-CS). The immobilized tannase retained more than 50% of its initial activity even after eight reaction cycles. It exhibited improved pH and thermal stability and effectively enhanced the colour of both black and green tea infusions.<sup>385</sup>

In recent years, magnetite (Fe<sub>3</sub>O<sub>4</sub>)-based nanoparticles have emerged as a successful choice for immobilizing various lipases due to their numerous advantages, including high stability, low toxicity, and easy separation by an external magnetic field.<sup>392,401,409–411</sup> Sarno *et al.* (2017) used citric acid-functionalized Fe<sub>3</sub>O<sub>4</sub> nanoparticles to immobilize *Thermomyces lanuginosus* (TLL) lipase and applied the resulting biocatalyst in banana flavour synthesis. In particular, they achieved a remarkably high activity recovery compared with the free

lipase, with values reaching up to 144% at pH 7 and 323% at pH 7.5. Furthermore, the immobilized enzyme exhibited superior stability and improved reusability, retaining 75% of its initial activity after 60 days of storage during the third cycle of banana aroma production and 64% after 120 days. These results demonstrated the improved performance of the immobilized enzyme compared to its free counterpart.<sup>412</sup>

Lipases immobilized onto magnetic nanoparticles are also widely used in biodiesel production reactions.<sup>413–418</sup> In a recent study, Zulfiqar *et al.* (2021) developed a novel nanobio-catalyst by immobilizing lipase from *Aspergillus niger* onto titanium dioxide nanoparticle-modified polydopamine (PDA-TiO<sub>2</sub>). They used it to synthesise biodiesel *via* enzymatic transesterification using *Jatropha curcas* seed oil. The immobilized lipase exhibited greater resilience to changes in pH and temperature conditions. Moreover, the optimal biodiesel yield of 92% was achieved by conducting the transesterification process for 30 h at 37 °C with a 10% concentration of the nanobio-catalyst.<sup>419</sup>

Thus, research confirms hydrolases' considerable diversity and adaptability while demonstrating the significant benefits of using magnetic nanoparticles to aid immobilization. This has improved their properties and efficiency in various reactions, facilitating their application in numerous industrial processes.

#### 4.4 Lyases

Lyases (EC 4) are enzymes that catalyze addition and elimination reactions. They cleave chemical bonds but do not undergo this process by oxidation or hydrolysis.<sup>420–422</sup> Studies have demonstrated their use in vital areas such as agriculture and food<sup>423,424</sup> and medicine.<sup>425,426</sup> Some of the applications of these enzymes immobilized on magnetic nanoparticles are shown in Table 3.

Like other classes of enzymes, lyases have their subclasses. Among them are alginate lyases, which are synthesized by algae, bacteria, marine molluscs, fungi, and viruses and play essential roles in the degradation and assimilation of alginate.<sup>434–436</sup> In the study by Jiang *et al.* (2021), the alginate lyase AlgL17 was

Table 3 Applications of lyases immobilized onto magnetic nanoparticles

Enzyme	Support	Immobilization yield	Application	Reference
Alginate extracted from <i>Escherichia coli</i>	Fe <sub>3</sub> O <sub>4</sub>	97.8%	Antioxidant and antiapoptotic bioactivities in human umbilical vein endothelial cells	427
Pectate lyase from <i>Clostridium thermocellum</i>	Fe <sub>3</sub> O <sub>4</sub>	96.5%	Bioscouring of coarse cotton	428
Benzaldehyde lyase from <i>Pseudomonas fluorescens</i>	Epoxy-chelate magnetic support	87%	Synthesis of critical synthons for pharmaceutical products	429
Pectate lyase	Calcium hydroxyapatite nanoparticles and single-walled nanotube	>70%	Processes of both high and low temperatures	430
Phenylalanine ammonia lyase	Hybrid nanoflowers	90%	Biosensors	431
Cystathionine $\gamma$ -lyase	TiO <sub>2</sub>	95%	Biomining	432
Pectin lyase from <i>Acinetobacter calcoaceticus</i>	Magnetic carboxymethyl cellulose nanoparticles	80%	Purification of some fruit juices	433



immobilized onto magnetite (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles. In the end, they obtained a new biocatalyst that showed superior thermal and pH tolerance, excellent storage stability, and capacity for reuse. This biocatalyst was used to produce alginate oligosaccharides, which showed antioxidant activities and prevented cell self-destruction, being effective against hydrogen peroxide-induced oxidative stress in human umbilical vein endothelial cells.<sup>427</sup>

Another study by Shin *et al.* (2010) revealed a new biocatalyst prepared by immobilizing marine alginate lyase from *Streptomyces* sp. (ALG-5) in magnetic iron oxide and hybrid magnetic silica nanoparticles. It exhibited the most significant alginate degradation activity and could be reused more than ten times after magnetic separation.<sup>437</sup>

Another subclass of lyases is pectate lyases (PLs), which act on the degradation of pectin produced by pathogenic organisms and can potentially have industrial applications.<sup>438–441</sup> Chakraborty *et al.* (2017) immobilized the recombinant *Clostridium thermocellum* pectate lyase in magnetite nanoparticles and, from this process, produced a biocatalyst with more significant activity, improved thermal stability 32 times at 80 °C and 14 times at 90 °C, and with the ability to be reused for five cycles followed by 70% of the initial activity. Its application in the biofouling of cotton fabric showed an efficient removal of pectin from the fabric surface.<sup>428</sup>

An analysis of the existing research shows that lyases are enzymes that have not been widely studied in immobilization processes using magnetic nanoparticles as a support. However, the limited number of published studies highlights the potential applicability of these enzymes and the advantages of immobilizing them on these particles.

#### 4.5. Isomerases

Isomerases catalyze reactions that can induce intramolecular changes that convert the substrate into an isomer.

Immobilization magnetic nanoparticles increase isomerases' stability and offer advantages in the reaction medium because of their large surface area, ease of separation from the reaction medium by an external magnetic field, mobility, and mass transfer. Consequently, isomerases immobilized on magnetic particles find applications in various industrial sectors, as shown in Table 4.

The industrial production of phenylacetaldehyde is essential in the flavour and fragrance industry because it synthesises various products, including insecticides, disinfectants, and pharmaceuticals. This aromatic aldehyde is obtained from the isomerization of styrene oxide in the presence of alkali-treated silica or various zeolite compounds. In addition, oxidation of

2-phenylethanol can be performed using hexavalent chromium compounds or rhodium complexes.

The enzyme L-arabinose isomerase is of industrial importance because of its applicability. The process can occur *in vivo*, catalyzing the isomerization of L-arabinose to L-ribulose. *In vitro*, it can generate the reaction of D-galactose to D-tagatose in nutraceutical foods.<sup>446</sup> Therefore, the overall applicability of the process can be optimized when L-arabinose isomerase is immobilized on chitosan supports with magnetic nanoparticles.<sup>447,448</sup> Moreover, using chitosan with magnetic nanoparticles acts in applications, sewage, water treatment, and food preservatives in the food industry and presents a tremendous antioxidant specificity and antimicrobial practice.<sup>449</sup>

Lactose, a by-product of yoghurt and cheese production, is used in various food and pharmaceutical products.<sup>450</sup> However, industrial production of lactose is limited by its low solubility, sweetness, and bioavailability.<sup>451</sup> Concentrated lactose yields less sweet D-tagatose than sucrose.<sup>452</sup> D-Tagatose, an isomer of D-galactose, exists in  $\alpha$ -D-tagatose-2,6-pyranose,  $\beta$ -D-tagatose-2,6-pyranose,  $\alpha$ -D-tagatose-2,5-furanose, and  $\beta$ -D-tagatose-2,6-furanose structures catalyzed by the enzyme L-arabinose isomerase.<sup>453</sup> D-Tagatose synthesis involves catalytic isomerization at high pH using soluble bases that are neutralized with sulfuric acid after conversion. However, D-galactose isomerization with essential catalysts results in lower D-tagatose selectivity due to by-product formation.<sup>454</sup> Using magnesium-based catalysts (MgO) improves the isomerization of glucose, galactose, and arabinose with satisfactory yields. Supported by various materials such as carbon nanotubes, hydrotalcite, and aluminates, the stabilization of MgO during the reaction is improved.<sup>455–457</sup>

*Glucose isomerase*, an enzyme that catalyzes reversible isomerization reactions, is critical in the conversion of D-glucose and D-xylose to D-fructose and D-xylulose, which are widely used in industrial contexts.<sup>458</sup> In the food sector, its role is prominent in producing high fructose corn syrup (HFCS), a mixture of glucose and fructose suitable for people with diabetes.<sup>459,460</sup> The process can also involve the interconversion of xylose to xylulose by *glucose isomerase*. Therefore, magnetic particle immobilization provides an efficient method for easy recovery and reuse while reducing costs.<sup>461</sup> As a result, *glucose isomerase* and other enzymes present an opportunity for large-scale industrial mobilization and are widely used in various food industry sectors.<sup>462,463</sup>

The *disulfide isomerase* (PDI) enzyme is a redox chaperone with applications in thiol oxidoreductase and isomerase in nascent proteins in the endoplasmic reticulum.<sup>464</sup> The PDI enzyme has several functions at the cell surface, primarily

Table 4 Applications of industrial isomerases

Enzyme	Application	References
L-Arabinose isomerase	Food industry	442
Glucose isomerase	High fructose corn syrup (HFCS) production	443 and 444
Protein disulfide isomerase (PDI)	Thiol-disulfide of ADAM17 and $\alpha$ IIB $\beta$ 3	444
Triose phosphate isomerase	High definition proton magnetic resonance study	445
D- Psicose 3-epimerase	Food additives	444 and 445



maintaining redox homeostasis and the thiol-disulfide isomerization process of ADAM17 and  $\alpha$ IIB $\beta$ 3.<sup>465</sup> Therefore, the effects associated with thrombosis, platelet activation, and vascular thiol isomerases<sup>466</sup> can be reduced or inhibited by using the PDI process as an antithrombotic criterion.<sup>467</sup> Using chitosan-polyacrylic hybrid microspheres offers advantages regarding Hof stabilization, temperature, and operation during its application. Consequently, a technique is required for GI mobility with a high enzymatic reactivity process and stabilization at the junction of iron changes.<sup>468</sup>

*Triosephosphate isomerase* extracted from rabbits and chicken was analyzed by high-resolution proton magnetic resonance imaging. The analytical techniques detected five possible histidines in the chicken protein and one histidine in the rabbit enzyme over a pH 5.4 to 9 range in the amino acid sequences.<sup>469</sup> Specifically, the resonances of histidine 100 in chicken and rabbit and only histidine 195 in the chicken enzyme were considered.<sup>470</sup> Histidine 100 is destabilized by the addition of ligands such as D-glycerol-3-phosphate, which changes the conformation of the enzyme but remains stabilized in the presence of inhibitors such as phenyl phosphate. In this way, a peptide-NH proton exchange occurs in the histidine resonance regions, eliminating any deformation of the enzyme.<sup>471</sup>

D-Psicose production *via* the epimerization reaction of D-fructose using class 3 epimerases is under consideration.<sup>472</sup> D-Psicose, the carbon-3 epimer of the sugar D-fructose, is rare and has a lower sugar content than sucrose. It is a food additive with functions such as suppressing glucose in type 2 diabetes, producing a near-defensive effect, and inhibiting hepatic lipogenic proteins.<sup>473</sup> D-Tagatose 3-epimerase from *Pseudomonas cichorii* catalyzes the C-3 epimerization of D-fructose to produce D-psicose.<sup>474</sup> The D-psicose-3-epimerase from *Agrobacterium tumefaciens* was selected because of its substrate preference. This enzyme is also present in several other species, such as *Ruminococcus* sp. and *Clostridium bolted*.<sup>475</sup> However, certain factors limit the production of D-psicose-3-epimerase on an industrial scale. The immobilization process can optimize the reaction yield, and titanium dioxide (TiO<sub>2</sub>) is often used as a support material for nanoparticle immobilization due to its conductivity physical and chemical stability.<sup>476–478</sup>

#### 4.6. Other enzymes

*Trehalose* is an enzyme developed by a mechanism that maintains the processability and biological properties while preserving the activity of macromolecules.<sup>479</sup> Therefore, the reaction process through metal-organic frameworks (MOFs) is optimised using ZIF-8 to permeabilize two encapsulated enzymes from *Bacillus subtilis* coated with *glycemic isomerase*. The enzyme glucose isomerase converts trehalose synthase and the by-product glucose to fructose for industrial applications.<sup>480</sup> Because of its stability, the trehalose-protected enzyme is used in biological studies, agriculture, and the pharmaceutical and food industries. In addition, the food industry creates a stable, protective layer to delay nutrient loss and adjust food flavour.<sup>481,482</sup>

Lipases are enzymes widely used in biodiesel synthesis due to their mild reaction conditions, easy product recovery, and

environmental sustainability compared to chemical processes.<sup>483,484</sup> Therefore, immobilization of lipases allows reuse and improves stability and catalytic activity. The support choice provides the most suitable surface area and low cost.<sup>485</sup> Using magnetic silica/iron oxide nanoparticles promotes advantages in partitioning the material controlled by a magnetic field.<sup>486</sup> Covalent bonds have been used on various supports to produce the reaction between an active group on the support and the amino acid residue of the lipase.<sup>487</sup>

The enzyme *Burkholderia cepacialipase* (BCL) is considered a chiral high-resolution catalyst.<sup>488</sup> Immobilization in nanoparticles increases enzyme activity and stability. When immobilized in nanoparticles, they can be modified by dendrimer polymers to protect the structure of *Burkholderia cepacialipase* and thus increase the contact levels between substrates and the enzyme centre.<sup>489</sup> Applications in the pharmaceutical field through drugs are chiral drugs that present differences in dynamics and kinetics *in vivo*, requiring the separation of drug enantiomers. The surface modification controls the morphology (*i.e.*, improves the affinity of the compounds by the active groups), increasing the charge and favouring the recovery of the enzyme. Therefore, these chiral programs are widely used in the spice, textile, and pharmaceutical industries.<sup>490</sup>

The protein from *Thermomyces lanuginosus* (TLL) is a lipase with high catalytic efficiency due to its enantioselectivity and isoelectric point (pI).<sup>491</sup> Therefore, the use of the support with the reactive group divinyl sulfone (DVS) with polyethyleneimine (PEI) provides the hetero-functionality of the DVS-PEI support to generate intense multivalent covalent bonding.<sup>492</sup> Thus, the octyl DVS matrices in the immobilization process by superparamagnetic nanoparticles allow interfacial activation to occur, ensuring stabilization through the points of multivalent bonds.<sup>493,494</sup> The coating by nanoparticle immobilization protects the surface from oxidation and minimizes non-specific interactions. Thus, covalent nanoparticle immobilization optimized on DVS-containing supports provides a better pH ratio, incubation time, and different blocking intermediates. Therefore, its applications are targeted to the medical and fine chemical industries.<sup>495–497</sup>

*Glutathione* (GSH) enzyme is a tripeptide protein ( $\gamma$ -L-glutamyl-L-cysteinyl-glycine). When used with GSH-agarose, it allows the isolation and purification of recombinant protein with *glutathione S-transferase* (GST) activity by GSH-GST interaction chromatography.<sup>498</sup> The composition of GSH has one free amino group, two free carboxyl groups and several free thiol-reduced structures. Therefore, the GSH enzyme and agarose microbead are activated and cross-linked by the amino group medium.<sup>499</sup> Its application in closed GSH nanoparticles is used to conjugate folic acid in cancer cell detection.<sup>500,501</sup> Iron oxide nanoparticles are coated with GSH and are also used to promote the stability of immobilized GSH through covalent bonds such as tetraethoxysilane (TEOS).<sup>502</sup> Thus, magnetic nanoparticles attached to GSH can couple enzymes fused to GST through the GSH-GST interaction. In this way, the protease enzyme used with nanoparticles is bound to GSH in identifying the three-dimensional structure as they improve the stability and solubility of the protein.<sup>503,504</sup>





## 5. Bioreactor projects with enzymatic magnetic nanoparticles

The content presented above highlights a growing interest in nanotechnology applications in bioreactor projects, focusing on using enzymatic magnetic nanoparticles. Dutta S. *et al.* (2023)<sup>505</sup> highlight the use of nanoparticles in various stages of bio-ethanol production from lignocellulosic (LB) materials, helping to overcome challenges associated with complex compositions and inefficient degradation processes. The authors outline nanotechnological methods during pretreatment that offer significant advantages for different types of LB, improving both biofuel yield and quality. Recent experiments have shown that using magnetic nanoparticles offers remarkable advantages, facilitating the recovery and reuse of immobilized enzymes,<sup>506–508</sup> leading to an overall reduction in process costs.

The study by M. V. C. da Silva *et al.* (2023)<sup>509</sup> highlights the creation and verification of methods and processes for using 2-ethylhexyl oleate catalyzed by *Candida antarctica* lipase immobilized in poly(styrene-co-divinylbenzene) magnetic particles (STY-DVB-M). The detailed analysis focuses on the physical properties of the STY-DVB-M copolymer, such as the glass transition temperature of 85.68 °C and the onset of thermal degradation, which occurred at 406.66 °C, demonstrating the importance of support stability in bioreactors. Additionally, the work investigates the influence of magnetic field strength on reaction yield and productivity, emphasizing the versatility and control enabled by magnetization in the systems,<sup>510,511</sup> crucial aspects when exploring bioreactors with magnetic nanomaterials.

N. K. Abd-Elrahman *et al.* (2022)<sup>512</sup> deal with the production of biohydrogen by microbial electrolysis cells (MECs). By evaluating various parameters, including the configuration of MECs, electrode materials, substrates, pH, temperature, applied voltage, and nanomaterials, the MEC stands out for its efficiency in converting substrates to hydrogen, achieving between 80 and 100% efficiency, compared to dark fermentation with 33% and water electrolysis with 65%. The preferential choice of carbon materials due to their high porosity highlights the importance of careful material selection,<sup>513,514</sup> while the use of nanomaterials in MECs to increase the efficiency of anodic and cathodic reactions indicates the strategic potential of this application in bioreactors. Their approaches validate and suggest the feasibility of integrating both methods discussed in bioreactor projects with enzymes in magnetic nanoparticles.<sup>515</sup>

Although at an early stage of development, both studies emphasize the urgency of further research to ensure the improvement and effectiveness of bioreactors considering kinetic and engineering aspects, another emerging challenge is the aggregate use of AI and computer simulations.<sup>509,516,517</sup>

## 6. Study of countries, journals, and institutions

Bibliometric data analysis provides a quantitative and objective view of scientific publications, allowing the identification of the most influential countries in the production of knowledge in

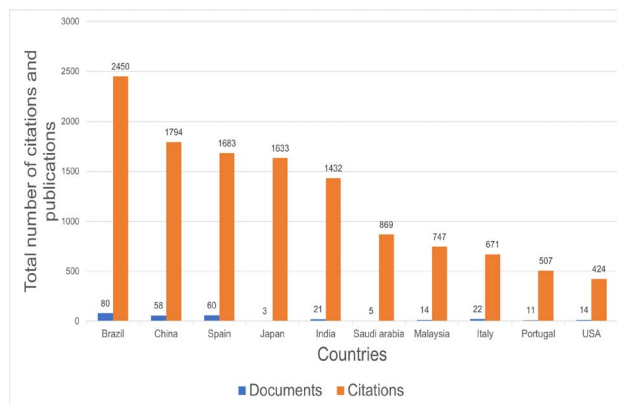


Fig. 8 Prominent countries have published the most and were most cited in magnetic nanomaterials for enzyme immobilization in the last five years.

a given research area. Brazil, China, and Spain emerged as the most important countries regarding citations and a number of publications on the research topic. As shown in Fig. 8, Brazil recorded 2450 citations associated with 80 publications, followed by China with 1794 citations and 58 publications, and Spain with 1683 citations and 60 publications during the study period. These data highlight the significant impact of research on the topic in the international academic and scientific sphere. The high production of articles and the substantial number of citations attributed to these countries indicate an active interest and a significant contribution to the advancement of knowledge in this specific field.

Fig. 9A illustrates the scientific collaboration between countries, with Brazil and China emerging as central players in this network. Brazil (58% of total citations) and China (42% of citations) are the most cited countries and have strong connections, as indicated by their size, the number of connections with other countries, and the thickness of the connecting lines. This suggests that both countries are major contributors to global research and have extensive international connections. Strengthening these collaborations could further promote the exchange of knowledge and resources worldwide. Fig. 9B visualises the temporal landscape of cross-country collaborations from 2016 to 2023. The map shows the strength of connections between countries and their clusters, with purple shades representing older years and light green shades representing more recent publications. This progression suggests a continued consolidation of research over time. In particular, clusters such as Brazil, China, Spain, Italy, and the United States maintain their relevance and significant contribution to knowledge in the field, laying a solid foundation for future scientific progress.

In Fig. 10A, we observe the importance of leading journals in different groupings (represented by clusters in yellow, red, and purple), with a focus on the journal with the highest impact, the Journal of Molecular Catalysis, which has 2441 citations across 63 publications, averaging 38.74 citations per article. This journal stands out as a central hub within its respective cluster



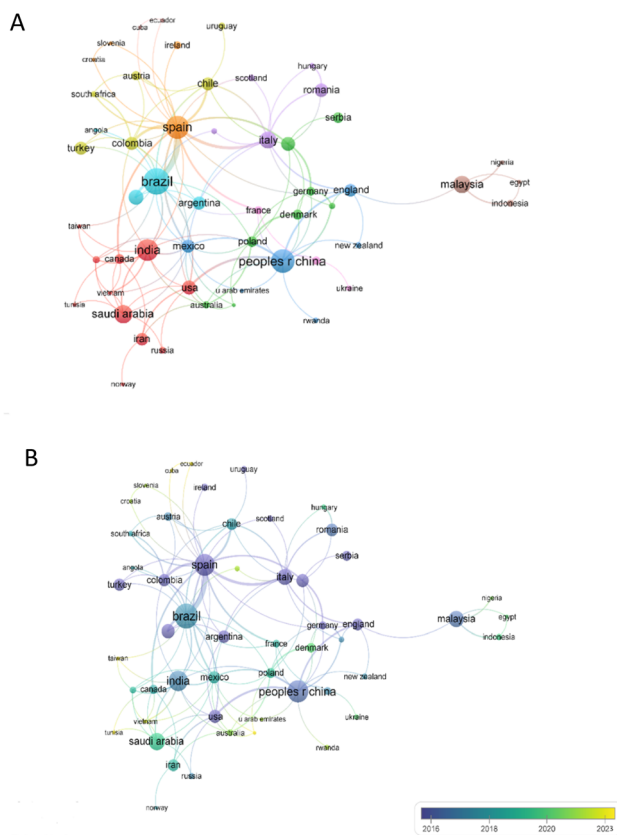


Fig. 9 Bibliometric maps of country co-authorship. (A) Network of clusters of most cited countries. (B) Overlay visualization of country link power.

(yellow) and explores the molecular and atomic aspects of catalytic activation and reaction mechanisms. We also highlight the Process Biochemistry cluster (purple), with 32 publications and 422 citations, focusing on processes related to bioactive molecules and living organisms. The Biochemical Engineering Journal (red), with 354 citations and 11 publications in the research topic clusters, is instrumental in developing biological processes, from preparing raw materials to recovering relevant products for industry.

The presence of these journals in the same thematic area underscores the concentration of efforts and interests in specific areas of science as well as the active networking and collaboration among researchers working in this field. This interconnectedness is fundamental to the exchange of knowledge. Furthermore, when analyzing Fig. 10B, which shows the number of publications over five years, the Journal of Molecular Catalysis accounts for 60% of the published documents, followed by Process Biochemistry with 30% of the publications and Biochemical Engineering Journal with 10%. This visual representation highlights the interactions between countries regarding citations and mutual collaborations. It also highlights the importance of the links between different research centres and academic institutions, underscoring the crucial role of international cooperation in driving scientific and technological progress on a global scale.

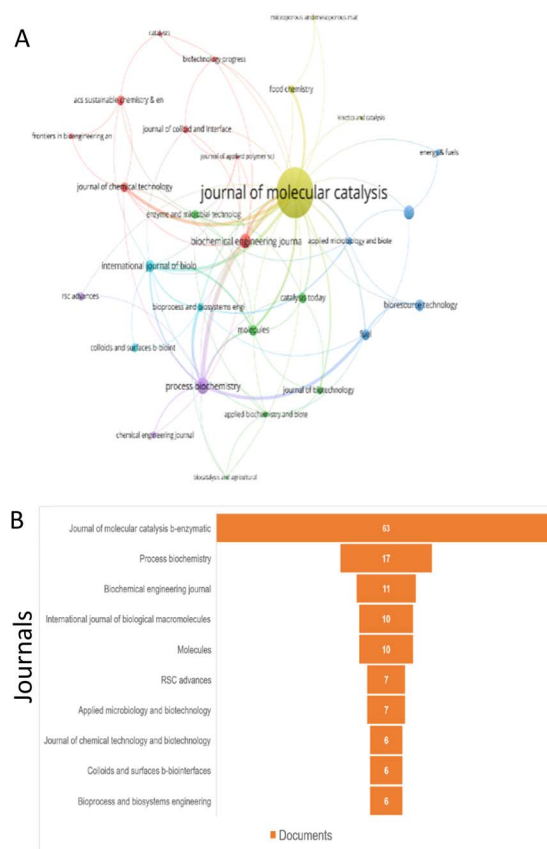


Fig. 10 Bibliometric analysis map of country networks. (A) The most influential journals. (B) Number of significant documents per journal.

In Fig. 11A, the top institutions are ranked by the number of published documents, most citations, and total link strength, highlighting those that have contributed the most to the research and their collaborative interactions. Aligarh Muslim University stands out, leading with 997 citations and 18 publications, reaffirming its dominance in research on magnetic nanomaterials for enzyme immobilization. The Superior Council for Scientific Investigations (CSIC) appears in second place with 841 citations and a higher number of 27 published documents, indicating increasing progress in research on this topic.

In third place is the Federal University of Ceará, in Brazil, with 722 citations and 14 published documents, highlighting Brazil's role in researching new technologies and advances. On the other hand, Fig. 11B provides a visualization map of the most important universities, identifying the clusters with the highest number of connections between universities. The Federal University of Ceará cluster (yellow) leads with 74 connections, followed by the CSIC cluster (orange) with 52 connections, and the University of São Paulo cluster (blue) with 36 connections. These results show that the total number of connections reinforces the strength of the overall connection of each institution. Of the 10 universities analyzed by the VOS-viewer software, six are of Brazilian origin, highlighting Brazil as an important research center in this area.



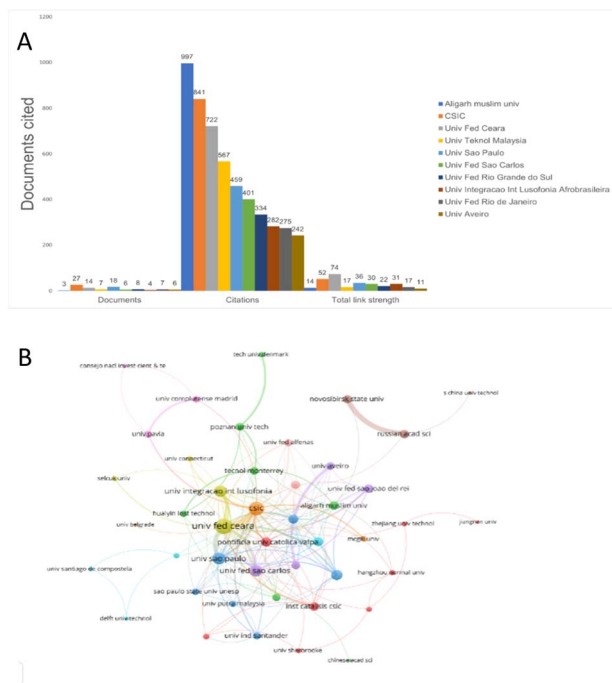


Fig. 11 Bibliometric analysis of institutions. (A) Main institutions that published and cited. (B) Map of collaboration networks between institutions.

## 7. Future trends (current challenges and prospects)

Magnetic nanoparticles are carriers with high potential for enzyme immobilization due to their easy separation and recovery from the reaction medium, large surface area, and high mass transfer capacity.<sup>518–521</sup> However, the specific interactions between the carrier and the enzyme require optimization protocols to enhance the enzymes' catalytic activity, stability, and recyclability.<sup>522–524</sup> Furthermore, the engineering and design of new magnetic nanomaterials with tailored structural properties and functionalities for specific applications while ensuring properties such as minimal toxicity, high biocompatibility, low environmental impact, and the selection of the appropriate immobilization method are critical considerations for industrial use.<sup>524–527</sup>

An example of a recently developed nanomaterial is cellulose nanocrystals, a versatile natural-based nanocarrier that has gained more attention in recent years due to its renewability, low cost, feasible synthesis and modification, high mechanical strength, and high stability against temperature and chemical compounds.<sup>528–534</sup> Incorporating magnetic nanoparticles into the support matrix can facilitate the recovery and reuse of biocatalysts in practical applications.<sup>528–535</sup> Another important aspect of using magnetic cellulose is that it allows for a single-step purification and immobilization of recombinant enzymes.

Enzymes fused with cellulose-binding domains.<sup>524–532</sup> On the other hand, the diversity of biocatalysts available and the difficulty in scaling up the production process of this biocatalyst indicate the need for new strategies to incorporate these

biotechnological units in the industrial sector.<sup>527,530,532</sup> Dopamine is a functionalization reagent that gives excellent results for this type of nanomaterials.<sup>525,533–535</sup> Dopamine is gaining attention for its versatility in anchoring various biologically active macromolecules such as antibodies, enzymes, DNA, and growth factors.<sup>525,534,536–539</sup> The presence of catechol and amine in dopamine provides efficient conjugation of enzymes to various nanocarriers and does not require additional coupling reagents/complex linkers or organic solvents.<sup>525,533–535</sup>

In addition to the aspects already discussed, for the use of magnetic nanoparticles on an industrial scale, it is also necessary to have a complete understanding of the functionalization effects of the support, the surface density of enzymes, the binding sites between nanoparticles and enzymes, how immobilization chemistry can affect the activity and stability of the biocatalyst, the involvement of conformational changes in the immobilization process, and the design and development of immobilized enzyme-based bioreactors.<sup>522,540,541</sup> From the topics discussed, it can be concluded that magnetic nanoparticles have a broad perspective in biocatalysis and several other fields.<sup>522,541–546</sup>

## 8. Conclusions

In summary, this review presents an overview of the development and construction of nanomaterial supports for possible applications in immobilising and stabilising enzymes. It is worth mentioning that enzymes immobilized on magnetic supports present advantages for commercial application due to their ease of separation and reuse, enabling greater scalability in the industrial sector. Furthermore, magnetic biocatalysts grouped by lipases have shown diverse applications and growing interest in the pharmaceutical and biofuel industries. It should also be noted that the bibliometric analysis explained that cooperation between countries and researchers is increasing on a large scale. Citations between journals and institutions generate high-impact articles and increase citations. The relevance of this topic and the high impact generated by the production of magnetic catalytic derivatives with biological content are increasingly apparent. However, magnetic biocatalysts still present some challenges that need to be overcome. Even though the advantages provided by enzyme immobilization add value, such as the possibility of recycling and improving their catalytic properties, the complexity of the synthesis and the different particularities of the lipase immobilization process still require more significant investments. Therefore, more research is needed to address these issues for long-term industrial applications of enzymes in addition to increased investment in this sector. To conclude, prospects are promising and have high industrial potential, allowing them to enhance existing processes further and produce new reaction routes that favour the sustainability and scalability of processes.

## Author contributions

Writing—original draft preparation, A. L. G. C., R. B. R. V., G. F. M., J. E. S. S., T. G. R., F. T. T. C., K. S. M., A. L. B. O., J. Y. N. H.





A., J. C. S. S.; writing—review and editing, A. L. G. C. and J. C. S. S. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

The authors declare no conflict of interest.

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