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Vibrational Spectroscopy as a Promising Tool to Study Enzyme-Carrier Interactions: A Review.

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Abstract: The high selectivity and specificity together with the catalytic activity ofenzymes lead them to be a key tool in biotechnology industries. The enzyme immobilization on a carrier material facilitates the reuse of the enzyme and improve sits stability. This article is a comprehensive review that reports papers in which different enzymes have been immobilized on distinct carrier materials and enzyme-carrier interactions were evaluated by infrared and/or Raman spectroscopy. Vibrational spectroscopy was used to inspect the spectral bands of different classes of enzymes before and after their immobilization on carriers. In fact, the characteristic spectral bands that prove the interaction enzyme-carrier are related with the amide functional groups of enzymes. In addition, those interactions are characterized by the shift, broadening, or increment on intensity of specific bands in reference to the spectrum of their carrier material. In this review, 20 of these studies used infrared spectroscopy and only three used Raman spectroscopy. The use of both vibrational spectroscopy techniques is increasingly due to their proved capacity to characterize the enzyme-carrier interactions without damaging them.

Keywords: Enzyme immobilization; carrier materials; enzyme-carrier interactions; infrared spectroscopy; Raman spectroscopy.

1.Introduction

Enzymes are biocatalysts in the nanometer scale with extreme importance in the chemistry processes regulation of organisms and cells (1–2). They are proteins highly specialized that have the ability to catalyze chemistry reactions in an efficient and selective manner (3–4). Without catalysis, chemical reactions, which are required to sustain biological life systems, do not occur in a useful time scale (3). Enzymes exhibit a high degree of specificity to their substrates, accelerate chemical reactions in a formidable way, and work in aqueous solutions under mild conditions of temperature and pH (3, 5). However, extreme conditions of temperature, pH, and ionic strength induce the inactivation of the enzymes due to denaturation caused by changes in the conformational structure (6). Although the high specificity of enzymes offers great challenges for several applications—such as chemical conversions, biosensing, and bioreactors—the short catalytic lifetimes of enzymes represent a severe limitation in their use (2, 4, 7–8). Enzymatic processes are more environmentally friendly, cost-effective, and even more sustainable than the chemical processes. Because of that, in the last two decades biocatalysis has emerged to develop green and sustainable chemical manufacture in the field of pharmaceuticals, the food industry, detergents, and textile, among others (4–5, 7).

It is important to mention that enzymes, when used for industrial purposes, are affected by a low operational stability, difficult recovery, and reuse of the enzyme (7–8). Therefore, the immobilization of enzymes has attracted general scientific and industrial interest. Compared with free enzymes, enzymes immobilization brings the improvement of thermal and pH stability. Furthermore, it confers easy product separation, which avoids the unwanted presence of the enzyme in the final product (8–12). When the enzyme is coupled to an adequate solid carrier, the resulting biocatalyst can be reused several times, reducing the costs of the process (4, 7, 12–13). The techniques used to immobilize enzymes are key factors for a successful process (14). Figure 1 illustrates schematically the immobilization methods used to date. Enzymes can be immobilized on solid carriers using different methods, such as physical adsorption, entrapment, or covalent binding. The technical applications, as well as the enzyme stability, are affected by the method used. Physical adsorption and entrapment are considered the simplest methods (11, 13). In these two methods, the weak binding between the enzyme and the carrier can maintain the native structure of the enzyme (8, 11). However, the loss of the enzyme, due to low adsorption, could occur. This can lead to low stability and low catalytic activity of the enzymes (13). By contrast, covalent binding is characterized by a strong binding

between the enzyme and the solid carrier, which could lead to the distortion of the enzyme's native structure (13). That is a disadvantage, as it decreases the enzyme activity (9, 13). This method is suitable if the enzyme is not required in the final product.

The material carrier is also an important factor, if the matrices should be biocompatible and inert, not affecting the native structure of the enzyme (10). The used carriers include a wide variety of materials, which can be divided into different groups depending on the origin: organic or inorganic (11). The organic group includes natural (agarose, alginate, chitosan, cellulose, etc.) and synthetic (acrylic resins, polyacrylamide, polystyrene, etc.) materials (8, 12). The low stability and nonresistance to organic solvents become a disadvantage when using these materials in the immobilization process (7–8). However, with these organic matrices it is easier to chemically modify the conditions fora given enzyme (8). Inorganic matrices (e.g., silica, alumina, and hydroxyapatite) exhibit thermal and mechanical stability and better resistance to solvents (7–8, 12). This group of materials has been explored, as it is possible to control the diameter of the pore and the surface area (7).

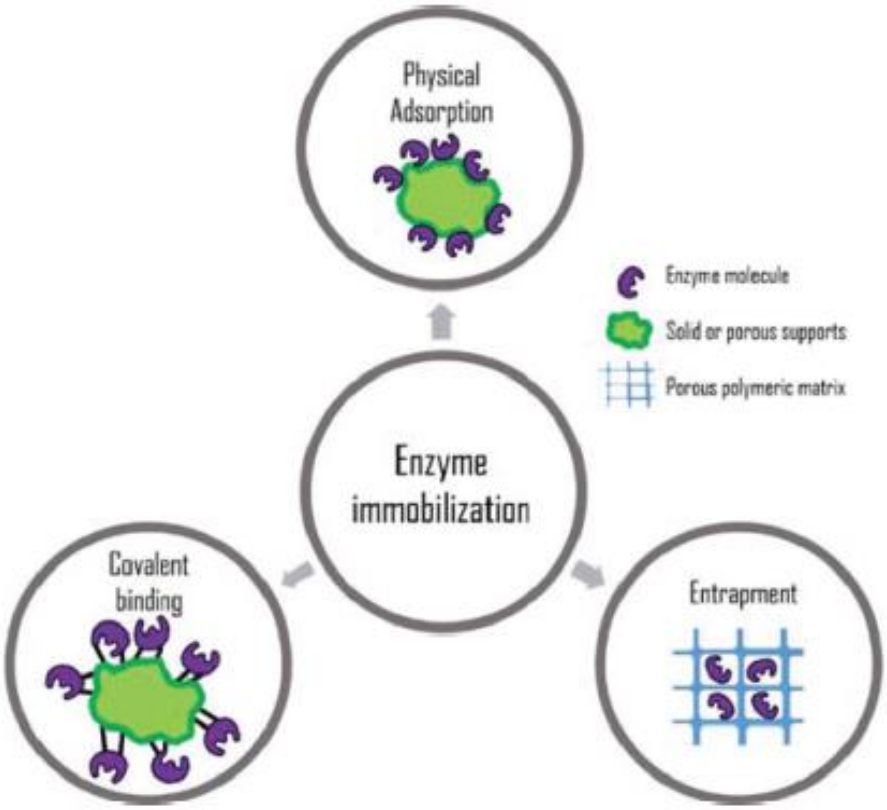

Figure 1. Methods of enzyme immobilization.

In order to evaluate the enzyme immobilization process, its physical-chemistry characterization is essential. Several studies deal with the process of enzyme immobilization and focus on the characterization of the materials carrier by using different techniques. Many of them verify the carrier's morphology using transmission electron microscopy (TEM) or scanning electron microscopy (SEM; 12, 15–17). Nitrogen adsorption/desorption is usually required to evaluate the specific surface area, the mesopore volume, and the cavity diameter (7, 12, 15). With Fourier transform infrared spectroscopy (FTIR), some researchers have confirmed the presence of certain chemical compounds in the carrier's structure, mainly when they attached different materials to activate the surface area (7, 14, 17). The amount of immobilized enzyme is typically quantified by thermogravimetric analysis (TGA) based on the weight loss when temperature increases (7, 17). Sometimes X-ray techniques are necessary to observe the crystal structure of the material's carrier (15). Once examined the carrier's structure is essential to evaluate the immobilized enzyme activity to be compared with the free enzyme activity. Thereby, the benefits of the carrier's immobilization are disclosed. Commonly, this is carried out by the reaction of the enzyme with a specific substrate. When the reaction is finished, the solution absorbance is measured with UV-vis spectroscopy (7, 12, 15, 17–21). Other times, the immobilized enzyme activity is measure by the bio-rad protein assay (13, 16). Typically, the enzyme's activity is examined by evaluating the pH and temperature effect before and after the immobilization process.

Vibrational spectroscopy provides powerful features to identify different compounds by analyzing their vibrational modes. Some studies have used infrared (IR) and Raman spectroscopy to characterize the chemical compounds of the free enzyme and the free carrier, but also the functional groups that are relevant in the linkage interaction between them. Therefore, the aim of this work is to critically review the literature applying different vibrational spectroscopic techniques to study the interactions between the immobilized enzyme and its carrier.

Vibrational Spectroscopy Applied to Study Enzyme-Carrier Interactions

Vibrational spectroscopy is included in the group of spectroscopic techniques, and the two most important are IR and Raman spectroscopy (22–23). These analytical techniques are nondestructive and noninvasive (23–24). Today, the number of researchers using these techniques is increasing due to their capacity to characterize

the structure of different molecular species without damaging them. These techniques measure vibrational energy levels, which are associated with chemical bonds in the specie (23). For a given sample, spectroscopic analysis generates a characteristic spectrum. This “chemical finger-print” is based on its composition and is used for identification, characterization, and qualitative and quantitative analysis (22–23, 25). The spectrum acquired contains information about the chemical composition of species represented by characteristic bands. Then, the data processing and the chemical identification can be favored by chemometric analysis (23, 25). IR and Raman spectroscopy are complementary techniques (22). Although in both techniques the fundaments are based on molecular vibrations, each one presents different processes and selection rules providing different chemical information. In general, infrared spectroscopy tends to emphasize asymmetric vibrations of polar groups (O-H, C-H, N-H, C=O), whereas Raman spectroscopy tends to emphasize asymmetric vibrations of nonpolar groups (C=C and C-C stretches; 22).

Because enzymes are composed mostly of polypeptides, vibrational spectroscopy can be a useful technique to give important information about the characteristic functional groups of these molecules. Thus, the material carrier, the native enzyme, and the interactions between them can be analyzed by vibrational spectroscopy. Because the molecular vibrations are different for each sample, these techniques are capable of evaluating if the immobilization process affects the enzyme. Furthermore, the intensity of the spectra signals or the shifting of typical bands could represent modifications in the conformation of the enzyme. As a consequence, the next sections will focus on the potential of IR and Raman spectroscopy in the study of enzyme-carrier interactions.

Infrared Spectroscopy

The atoms that make up the molecules vibrate in order to achieve their equilibrium positions. These molecular vibrations are stimulated by radiation. In IR spectroscopy, if the radiation frequency matches the natural vibration bond frequency, the molecular specie will vibrate. The vibration is IR active when there is a change in the dipole moment.

Today, IR continues to be the most used technique to identify functional groups for a wide group of organic and inorganic materials. Regarding the subject of this review, this technique was mostly applied to characterize enzyme-carrier interactions.

Table 1 shows the immobilized enzymes and carriers used for the immobilization (with and without modifier agents), methods for immobilization in different carriers' materials, and the typical IR bands, reported to date in the literature, that can characterize the molecular vibrations of enzyme-carrier interactions. It is important to note that the spectra of the raw carrier and the free enzyme were compared with the spectra of the immobilized enzyme on the carrier. Each band modification, which characterizes the molecular vibrations during the interaction, has been classified as new, shifted, broadening, or increased intensity referred to the raw carrier spectra.

Following, a revision of the IR spectroscopic studies on different enzyme-carrier interactions is outlined according to the enzyme type, which has also been established in accordance to the enzyme commission number (EC number).

Table 1. Enzyme-carrier interactions studied by infrared spectroscopy.

				Characteristic bands[a]				
Enzyme (source)	Carrier (Carrier modifier)	Immobilization method	Cross-linking agent	Wavenumber (cm^{-1})	Vibrational mode	Chemical group	Change	Ref.
Lipase (*Candida rugosa*)	MSU-H silica (APTES)	Physical adsorption Covalent binding	Glutaraldehyde	1540 1650 3500	δ (N—H) ν (C=O) ν (N—H)	Amide II Amide I Amide A	New Increased intensity Broadening	(26)
[b] Lipase (*Pseudomonas cepacia*)	Polyurethane containing β-cyclodextrin	Physical adsorption	CDI MDI	1643	ν (C=O)	Amide I	New	(27)
Lipase (*Porcine pancreatic*)	SBA-15 silica (carboxyl-ionic liquid)	Physical adsorption Covalent binding	—	1550 1650	δ (N—H) ν (C=O)	Amide II Amide I	New and increased intensity New and increased intensity	(28)
Lipase (*Candida rugosa*)	Vesicular silica	Physical adsorption	—	1540 1647 3700–3200	δ (N—H) ν (C=O) ν (N—H)	Amide II Amide I Amide A	New New New	(29)
Lipase (*Burkholderia sp.*)	Magnetic silica nanoparticles (alkyl group)	Physical adsorption	—	2420 2500–3000	ν (C—H)	Alkyl-lipase Alkyl	New Soft broadening	(30)
Lipase (*Candida rugosa*)	Magnetic chitosan microspheres	Cross-linking	Glutaraldehyde	1544 1662	δ (N—H) ν (C=N)	Amide II Carbonyl + amine	New New	(31)
Lipase (*Candida rugosa*)	Exfoliated graphene oxide nanocomposite (APTES)	Physical adsorption	—	1078 1633	ν (C—N) ν (C=O)	Aliphatic amines Amide I	New Increased intensity	(32)
[b] β-galactosidase (*Kluyveromyces lactis*)	Silicon dioxide nanoparticles (glutaraldehyde)	Covalent binding	—	1410 1642 2886, 2942 3000–3500	δ (C—H) ν (C=O) ν (N—CH_3) ν(O—H) and ν(N—H)	Amide II Amide I	Shifted Increased intensity Shifted Shifted and increased intensity	(16)

Table 1. Enzyme-carrier interactions studied by infrared spectroscopy (continued).

Enzyme (source)	Carrier (Carrier modifier)	Immobilization method	Cross-linking agent	Characteristic bands[a] Wavenumber (cm^{-1})	Vibrational mode	Chemical group	Change	Ref.
[b] Lactase (*Aspergillus oryzae*)	Magnetic nanoparticles (acid carboxylic)	Covalent binding	—	1232	ν (C—N)	Amide III	New	(34)
				1535	δ (N—H)	Amide II	Increased intensity	
				1643	ν (C=O)	Amide I	New	
				3273	ν (N—H)	Amide A	Increased intensity	
α-Amylase (*Bacillus subtilis*)	Alumina	Physical adsorption	—	1553	δ (N—H)	Amide II	New	(35)
				1401	ν (C—H)	CH_2	New	
				3400	ν (N—H)	Amide A	Increased intensity	
α-Amylase (*Bacillus subtilis*)	Zirconia	Physical adsorption	—	1400	δ (C—H)	Scissoring	Broadening	(36)
				1500	δ (N—H)	Amide II	New	
Cellulase	Magnetic-chitosan nanoparticles (glutaraldehyde)	Covalent binding	—	1541	δ (N—H)	Amide II	New	(20)
				1651	ν (C=O)	Amide I	New and increased intensity	
Cellulase (*Trichoderma viride*)	Silica (Polyamidoamine dendrimer)	Physical adsorption Cross-linking	Glutaraldehyde	1552	δ (N—H)	Amide II	Shifted	(10)
				1656	ν (C=O)	Amide I	Shifted and broadening	
β-glucosidase	Magnetic nanoparticles (sodium citrate)	Cross-linking	Glutaraldehyde	1553	δ (N—H)	Amide II	New and increased intensity	(38)
				1642	ν (C=O)	Amide I	New and increased intensity	
Pectinase	Silica- magnetite nanoparticles ((3-Chloropropyl) trimethoxysilane)	Covalent binding	—	1543	δ (N—H)	Amide II	New	(42)
				1656	ν (C=O)	Amide I	New	
Papain	Silica- magnetic nanoparticles ((3-Chloropropyl) trimethoxysilane)	Covalent binding	—	1542	δ (N—H)	Amide II	New	(51)
				1650	ν (C=O)	Amide I	Increased intensity	
α-chymotrypsin	Magnetic-chitosan nanoparticles	Covalent binding	—	3400-3500	ν (NH_2) ν (O—H)	Amine	Broadening	(48)
Carbonic anhydrase (*Bovine*)	SBA-15 silica (APTES)	Covalent binding Physical adsorption Cross-linked	Glutaraldehyde	1453	δ (C—H)	Aromatic ring	New	(53)
				1538	δ (N—H)	Amide II	New	
				1644	ν (C=N)	Amide I	Increased intensity	
				3072-3745	N—H, NH_2, O—H,C—H		Broadening and new	
[b]Peroxidase	Porous silicon (APDES)	Covalent binding	Glutaraldehyde	1691	ν (C=O, C—N)	Amide I	New	(58)
				3350	ν (NH_2)	Amide I	New	
Chloroperoxidase (*Caldariomyces fumago*)	SBA-15 silica (APTEOSB)	Physical adsorption Covalent binding	—	1531	δ (N—H)	Amide II	New and increased intensity	(59)
				1640	ν (C=O)	Amide I		
				3410	ν (N—H)	Amide A	New	
Superoxide dismutase (*Bovine erythrocytes*)	Mesoporous silica (Aminopropyl KIT-6)	Physical adsorption	—	1528	δ (N—H)	Amide II	New	(63)
				1656	ν (C=O)	Amide I	New	

[a]Summary of the bands that undergoes a change due to the enzyme-support interaction comparing with the raw material.
[b]Studied by Attenuated total reflectance (ATR)-FTIR.
δ: bending; ν: stretching.

Hydrolases

Hydrolases (EC 3) are enzymes with high interest for industrial applications as they catalyze the hydrolysis of a chemical bond present in various natural substances (5). Examples of hydrolases are lipases, glycosylases, and proteases. As described above, enzymes, in their free form, present some drawbacks for industrial purposes. For that, different studies have adopted the strategy of enzyme immobilization. Following, these studies are discussed, emphasizing their IR spectral characterization.

Lipases. Lipases (EC 3.1) belong to the group of hydrolases with action on ester bonds. There are many lipases that hydrolyze esters and carboxylic acids, with particular interest to the industry (5). According to Jesionowski et al. (8), lipases have been the most studied enzymes in immobilized form. With the increasing use of such enzymes in practical applications, the use of immobilization techniques has grown. Until now, lipases have been immobilized on distinct materials: organic (chitosan beads, poly

(methylolacrylamide)) and inorganic (mesoporous silica, magnetic nanoparticles). Studies have shown an activity for immobilized lipases higher than for free enzymes (8).

In their free form, lipases are mainly characterized by the amide A group and the asymmetrical NH_3C bending vibration. Once this enzyme has been attached on different surfaces, the IR spectra of the interaction lipase-carrier depends on each carrier's material. Yu et al. (26) immobilized lipases on mesoporous silica (MSU-H type) using two different methods: covalent binding and physical adsorption followed by cross-linking. The second method followed by a cross-linking process was carried out by adding glutaraldehyde. The free lipase spectrum showed the presence of the amide A group around 3500 $cm^{-1}$and the NH_3C vibration at 1540 cm^{-1}. When they attached lipases on mesoporous silica by a cross-linking process, the IR spectrum showed a band at around 600 cm^{-1}, which is characteristic of the glutaraldehyde linkage. The marked band at 1100 cm^{-1} was attributed to Si-O-Si vibrations characteristic of silica materials. In this same spectrum, the appearance of a new band at 1540 cm^{-1} was assigned to N-H bending vibration, and the broadening of the band around 3500 cm^{-1} to amide functional groups, which proved the immobilization of lipases on the silica material by cross-linking process (indicated in Table 1). Regarding the covalent binding process, the silica carrier was previously functionalized with (3-aminopropyl) triethoxysilane (APTES), and the glutaralde-hyde was used to bind lipases. The spectra showed the typical band vibrations of the carrier components (silica and glutaraldehyde), but the band at 1650 cm^{-1} (C=O stretching vibration), representative of amide I, also appeared strong and suggested the successful attachment of the enzyme on the carrier by covalent binding. When researchers added a surfactant (Pluronic 123) on the surface of MSU-H silica carrier, the presence of this chemical reagent was evident in the spectra. The bands that indicate the presence of the lipase continued to be at the same positions (1540, 1650, and 3500 cm^{-1}). After the addition of the surfactant, the immobilized lipases on silica MSU-H exhibited higher activity. The Pluronic 123 worked as an activator of lipases leading to some conformation all changes that improved the enzyme activity.

To immobilize lipases, Dhake et al. (27) decided to use a new approach on physical adsorption. They used polyurethane copolymers containing b-cyclodextrin and two types of cross-linking agents: 4, 40-dicyclohexylmethane diisocyanate (CDI) and 4, 40-diphenylme-thane diisocyanate (MDI). The IR spectra of the immobilized lipase on copolymer surfaces howed the appearance of the amide I band at 1643 cm^{-1}, which clearly represented the successful adsorption of the enzyme on the carrier (see Table 1). Therefore, after immobilization by physical adsorption the enzyme maintained its native conformation. However, it was difficult to identify other typical bands of lipases in

the spectra of the enzyme-carrier system. Probably, the amide vibrations were masked by the urethane vibrations.

Yang et al. (28) modified mesoporous silica (SBA-15 type) with carboxylic-functionalized ionic liquid to immobilize lipases. The immobilization process was carried out by physical adsorption and covalent binding. All IR spectra of the carrier's material showed the characteristic bands of Si-O-Si vibrations at 1080, 780, and 470 cm^{-1}. In the presence of the ionic liquid (imidazole), the modified carrier exhibited two new bands at 1550 and 1470 cm^{-1}, representing the imidazolium ring symmetrical stretching vibration. After immobilization, the IR spectra revealed the appearance of the bands corresponding to the amide I and the amide II vibrations modes at 1650 and 1550 cm^{-1}, respectively, which indicates the presence of lipases on the carrier (as indicated in Table 1). It should be noted that these characteristic bands from lipases had a higher intensity when immobilized on the modified carriers. This evidence was also confirmed with the results from enzyme loading capacity, in which the modified carriers exhibited higher values than parentSBA-15 type. When the enzyme activity was analyzed, the immobilized enzymes also displayed higher values. However, lipases immobilized by physical adsorption showed higher activity than lipases immobilized by covalent binding. This was justified by the chemical bonding forming between the enzyme and the carrier, which can affect the conformation of the native enzyme and decrease the enzyme activity. On the other hand, this chemical bonding conferred more stability and protected more efficiently the enzyme from the external conditions (like pH and temperature).

Wu et al. (29) prepared silica vesicles as carriers, with two different pore sizes, to immobilize lipases by physical adsorption. The pore size was controlled by the molar ratio of 1,3,5-triisopropylbenzene (TIPB) and sodium dodecyl sulfate (SDS). The spectra of silica vesicles showed the characteristic bands of Si-O-Si (1100, 806, and 470 cm^{-1}) and Si-OH (3700–3200 and 966 cm^{-1}) vibrations. The interaction between lipases and silica vesicles was revealed by the binding established between them (–CONH–), which was characterized by the presence of two new bands at 1647 cm^{-1}(C=O stretching vibration) and 1540 cm^{-1} (N-H bending vibration). It was also possible to identify another band between 3700 and 3200 cm^{-1}, representing the amide A functional group, which was not present in the carrier material (as shown in Table 1). It is important to note that in this same spectrum, the typical bands of silica materials were clearly recognized without displacement. Relating to enzyme activity, the immobilized enzyme showed higher activity than the native enzyme in a broad range of pH and temperatures. Moreover, the silica vesicles with higher pore size showed great stability and retained enzyme

activity, due to the larger pore area, which facilitates the immobilization and consequently improves the enzyme reactions.

The application of magnetic (Fe_3O_4) nanoparticles as carriers to immobilize enzymes has received special attention due to its easy magnetic separation and appropriated physical properties. However, its reactivity and the non porosity of magnetic materials could lead to high degradability. To overcome this drawback, Tran et al. (30) prepared magnetic nanoparticles coated with silica and then functionalized with an alkyl group. Then, lipases were immobilized on these matrices by physical adsorption. Figure 2 shows these different materials (parent magnetic nanoparticles, silica-magnetic nanoparticles, and silica-magnetic nanoparticles functionalized before and after lipase immobilization). All of them exhibited the band at 582 cm^{-1} corresponding to the Fe-O vibration characteristic of magnetic materials. When silica was in the carrier material, it was also possible to identify the Si-O-Si vibrations by the band at around 1100 cm^{-1}, indicating the successful attachment between silica and magnetic nanoparticles. The presence of the alkyl group was characterized by the band at 1850 cm^{-1}, which was attributed to the alkyl-SiO_2 vibrations. Finally, the appearance of the band at around 2400 cm^{-1} corresponded to the alkyl-lipase binding, and the soft broadening of the band at around 2500–3000 cm^{-1} confirmed the presence of lipases on the magnetic-silica functionalized carrier (as indicated in Table 1). This alkyl functionalized carrier exhibited the highest adsorption capacity due to its abundant binding sites and good affinity for lipases.

Xie and Wang (31) prepared magnetic chitosan microspheres to immobilize lipases. They used the glutaraldehyde as a cross-linking agent to the covalent attachment of lipases. The IR spectra of the magnetic chitosan microspheres exhibited the typical bands of the parent magnetic material and chitosan. The band at 580 cm^{-1} was found to be related to Fe-O stretching vibrations, and the slight shifting of the bands in the range of 1300–1600 cm^{-1} was due to the C-O and N-H bending vibrations, which indicated the presence of alcoholic groups in chitosan. This proved a successful use of chitosan on magnetic microspheres surface. The spectra of the immobilized lipase on the chitosan magnetic microspheres showed the appearance of a notable band at 1662 cm^{-1} relating to the C=N stretching vibration, which represented the interaction between carbonyl group of glutaraldhyde and amine groups of lipases. Another band at 1544 cm^{-1}, typical of amide groups, was interpreted as the presence of immobilized lipases on the carrier material (as indicated in Table 1). Once more, this study proved the improvement of lipases' operational stability after the immobilization process.

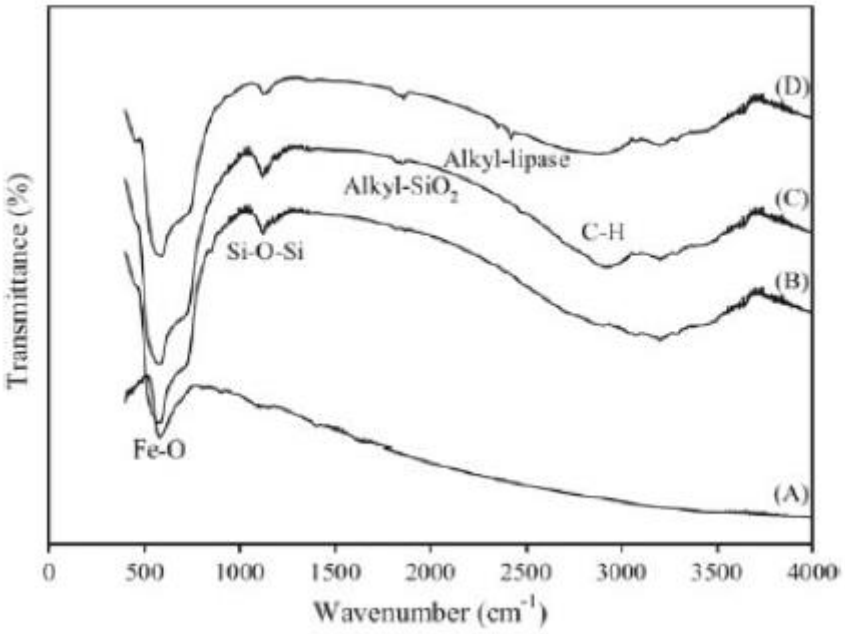

Figure 2. FTIR spectra of (A) magnetic nanoparticles, (B) silica-magnetic nanoparticles, (C) alkyl-silica-magnetic nanoparticles, (D) lipases immobilized on alkyl-silica-magnetic nanoparticles. Reprinted from Journal of Biotechnology, 158, Dang-Thuan Tran, Ching-Lung Chen, and Jo-Shu Chang, Immobilization of Burkholderia sp. Lipase on ferric silica nanocomposite for biodiesel production, 112–119, Copyright (2012), with permission from Elsevier.

Patel et al. (32) explored the functionalization of exfoliated graphene oxide as a carrier to immobilize lipases. First, they functionalized the exfoliated graphene oxide surface with APTES; then, the lipases were immobilized by physical adsorption. The IR spectra of the carrier material before and after enzyme immobilization showed a band at 3400 cm^{-1}, representative of O-H stretching vibrations. This band con-firmed the presence of the carboxyl functional groups characteristics of the graphene oxide. The presence of the band at 2900 cm^{-1} was related to the C-H stretching vibrations, characteristic of methylene groups, and the band at around 550 cm^{-1} was attributed to the epoxy groups. These three regions of the spectra were related to the carrier material. In the spectrum of the functionalized exfoliated graphene oxide, a band at 1100 cm^{-1} appeared, characteristic of Si-O vibrations, and it was considered an indication of the successful attachment of APTES on the carrier's surface. At last, the lipase adsorption on the functionalized surface was characterized by the broadening of the band at 1078 cm^{-1}, which represented the C-N stretching vibration, and the increased intensity of the band at 1633 cm^{-1}, corresponding to the C=O stretching vibration (as shown in Table1). These two bands are characteristic of amide functional groups present in lipases.

As a brief summary, the IR spectra of the immobilized lipases, either on mesoporous silica or on magnetic nanoparticles, clearly showed lipase carrier interactions. Additionally, graphene oxide was also considered an excellent candidate to be used as a suitable carrier, due to its biocompatibility and mechanical, thermal, and electrical properties. In the IR spectra the presence of lipases on the graphene oxide carrier is quite noticeable. Another advantage of this material is its hydrophilic character, which allows its easy dispersibility in water and other organic solvents containing oxygen. There are many ways to functionalize graphene oxide in order to change its properties and get a chemically modified graphenes oxide more adjustable for many applications (33). However, a drawback of this process is that it can be complex and time consuming.

Glycosylases. Glycosylases (EC 3.2) are another group of hydrolases with relevant interest for industry (5, 9). More specifically, carbohydrases obtained from microbial sources are used often in the food industry (9). In general, the group of carbohydrases includes enzymes involved in hydrolysis and synthesis of carbohydrates, such as b-galactosidase, cellulases, pectinases, and b-glucosidases. To be cost-effective and to improve the enzymes' stability conditions, researchers have used immobilization techniques to immobilize carbohydrases into different carriers, including gels, synthetic polymers, and magnetic nanoparticles (9). Next, these studies will be discussed in more detail for the corresponding enzyme.

B-galactosidase: Belonging to the group of glycosylases, b-galactosidase (EC3.2.1.23), most commonly known as lactase, catalyzes the breakdown of lactose into glucose and galactose. Its action on the hydrolysis of lactose, the predominant sugar in milk, plays an important role in human nutrition (15, 18). This enzyme has already been immobilized on several materials—such as chitosan, agarose, and cellulose—by physical and chemical interactions (12, 34). In order to find a sturdier carrier-system, Verma et al. (16) immobilized b-galactosidase on silicon dioxide nanoparticles, an inorganic material with high thermal and mechanical stability and ideal for maintaining the enzyme's conditions. They used the glutaraldehyde to activate the carrier's surface and thereafter covalently attach the enzyme. The IR spectrum of the native b-galactosidase showed bands in the range between 3000 and 3500 cm^{-1}, due to O-H and N-H stretching vibrations present in the enzyme. The bands at 2886 and 2942 cm^{-1} represented the N-CH_3 stretching vibrations. Finally, the band at 1642 cm^{-1} arose from the C=O stretching vibration, which represented amide I. Concerning to the IR spectrum of the interaction b-galactosidase-nanoparticles, in Figure 3, it can be seen how the amide characteristic bands from the enzyme appeared slightly displaced, confirming the binding of the enzyme on the carrier (as shown in Table 1). The carrier material showed

the silicon characteristic bands at1059 cm^{-1}, from Si-O-Si stretching vibration. This band suffered a shift to 1038 cm^{-1} after immobilization of the enzyme. The band at 436 cm^{-1} arose from the presence of the glutaraldehyde. The activity of the immobilized enzyme showed a slow decrease with temperature when compared with the native enzyme. In turn, the immobilized enzyme was revealed to be more stable at higher pH values.

In another study developed by Talbert et al. (35), b-galactosidase was immobilized on magnetic nanoparticles functionalized with carboxylic-acid, through covalent binding. Once again, the amide linkages confirmed the presence of the enzyme on the carrier. The bands at 3273 and 1535 cm^{-1}, corresponding to N-H stretching vibrations, suffered an increment in the intensity (as indicated in Table 1). The appearance of the band at 1643, owed to C=O stretching vibrations, proved the presence of amide vibrations features from b-galactosidases. However, the activity of the immobilized enzyme was affected by the interaction with carboxylic acid present on the surface of the nanoparticles. With the increase of temperature, the energy of the system rose. For that, the interactions enzyme- carrier increased abruptly, leading to a decrease in enzyme's activity. Varying the pH, the enzyme stability in native or immobilized form did not change.

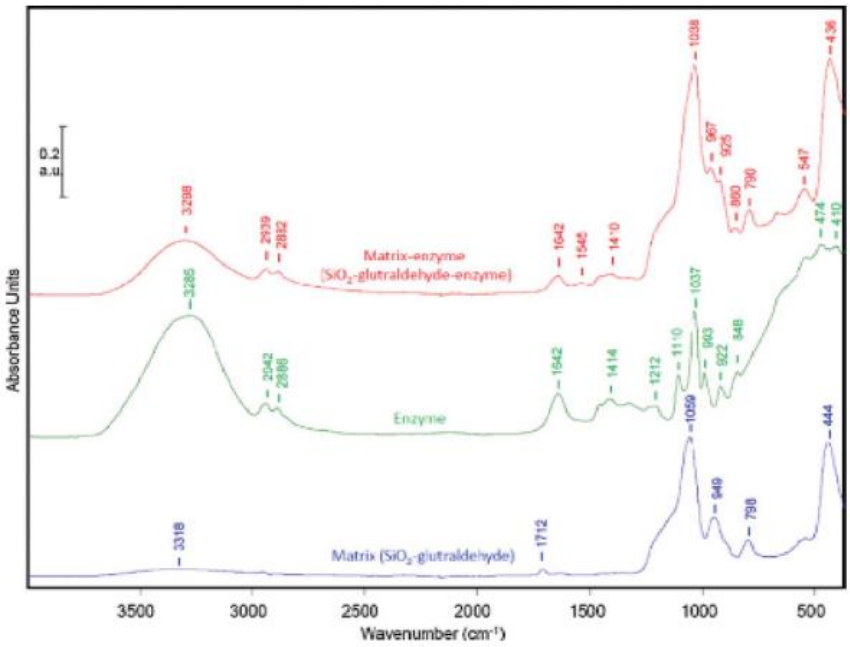

Figure 3. FTIR spectra of silicon dioxide carrier material, free b-galactosidase and b-galactosidase immobilized on silicon dioxide. Reprinted from International Journal of Biological Macromolecules, 50, Madan Lal Verma, Colin James Barrow, J. F. Kennedy, and Munish Puri, Immobilization of b-galactosidase from Kluyveromyces lactis on functionalized silicon dioxide nanoparticles: Characterization and lactose hydrolysis, 432–437, Copyright (2012), with permission from Elsevier.

As described above, the covalent binding has the drawback of changing an enzyme's conformation, which affects its activity. However, the success of the previous studies on immobilization of b-galactosidase may be attributed to the covalent binding. This process avoided the leaching of protein by the strong binding established between the enzyme and the carrier. In the presence of carboxylic acid on the surface of magnetic nanoparticles, the enzyme's activity was affected. On the other hand, the immobilized b-galactosidase on the silicon dioxide nanoparticles, activated with glutaraldhyde, exhibited higher activities when compared to the native enzyme. If the carboxylic acid was changed by another chemical reactive, the process probably could be improved. Comparing these two inorganic supports forb-galactosidase immobilization (silicon dioxide nanoparticles and magnetic nanoparticles) in terms of reusability and cost, the second one seems to be more advantageous, as it is easy to separate from the reaction mixture by applying only a magnetic field.

Amylase: Amylases (EC 3.2.1.1) present many interesting properties for food industry applications. They catalyze the hydrolysis ofa-1,4-glycosidic linkages present in polysaccharides like starch and glycogen. In particular, a-amylases break down long-chain carbohydrates such as maltose and glucose (36, 37). This enzyme has already been immobilized on distinct carriers, among them soluble polymers (38). Reshmi et al. (36) proposed the immobilization of a-amylases on alumina by physical adsorption. The IR spectra of the samples before immobilization presented broad bands at 3440, 1630, and1381 cm^{-1}, a feature of the bending and stretching vibrations of the O-H bond of water. The binding of Al-O-OH was assigned by another two bands at 579 and 672 $cm^{-1.}$ After immobilization, the band at 1381 cm^{-1} disappeared, which might prove the adsorption of amylases through O-H bindings (as indicated in Table 1). Beyond that, a little increase in intensity of the band at 3400 cm^{-1}also confirmed the immobilization of the enzyme on the alumina surface. In addition, it was possible to identify the typical amide groups from the enzyme represented by N-H bending vibrations at 1553 cm^{-1} and the attachment of CH_2 to the amide groups at 1401 cm^{-1}. Regarding enzyme stability, it was improved after immobilization at higher pH conditions.

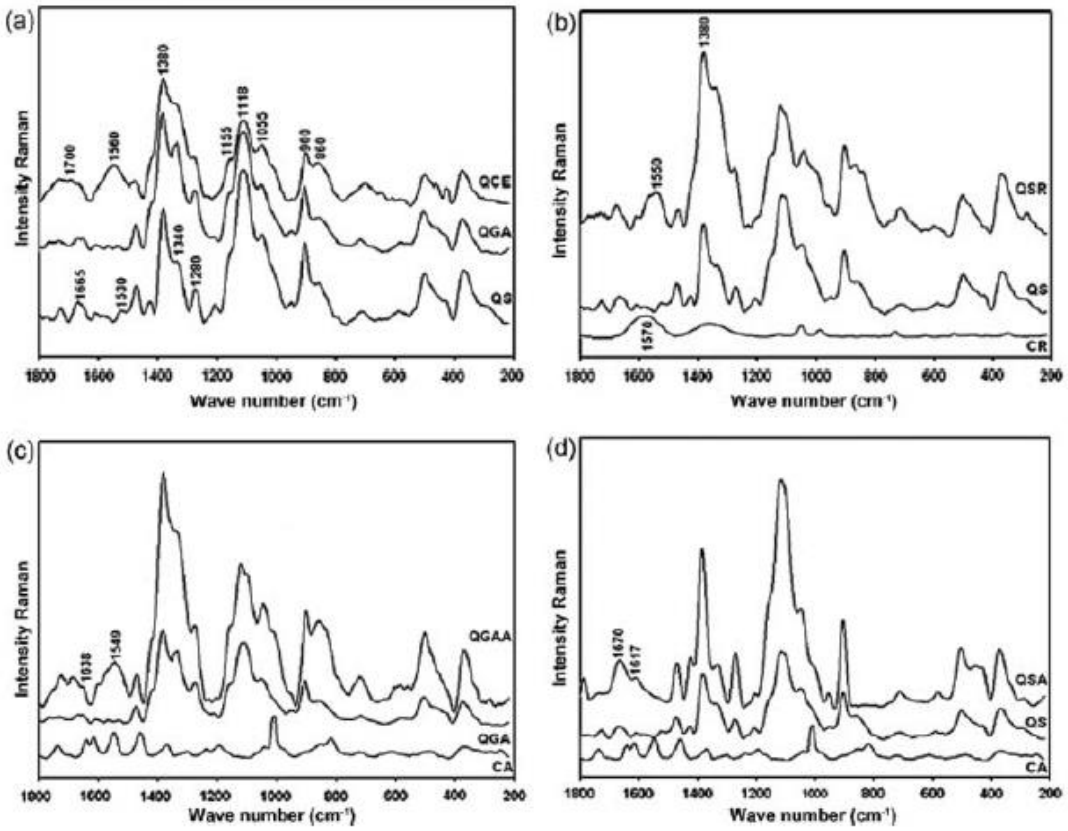

Figure 4. Raman spectra of lipases immobilized on: (a) chitosan (QS), chitosan treated with glutar-aldehyde (QGA), chitosan treated with 18-crown-6 (QCE); (b)Candida rugosa lipase (CR), QS and CR immobilized on QS (QSR); (c)Candida antarctica lipase (CA), QGA and CA immobilized on QGA (QGAA); (d) CA, QS and CA immobilized on QS (QSA). Reprinted from Carbohydrate Polymers, 79, C.E. Orrego, N. Salgado, J. S. Valencia, G. I. Giraldo, O. H. Giraldo, and C. A. Cardon, Novel chitosan membranes as support for lipases immobilization: Characterization aspects, 9–16, Copyright (2010), with permission from Elsevier.

The same researchers (37) also immobilized a-amylase on zirconia by physical adsorption. Before immobilization, the IR spectra showed the bands at 3440 and 1625 cm^{-1}, a feature of the bending and stretching vibrations of OH of water. The bands at 580 and 730 cm^{-1} were attributed to the Zr-O binding. After immobilization, the adsorption of amylases on the carrier was explained by the broadening of the band at around 1500 cm^{-1}, characteristic of the N-H bending vibrations, and the appearance of the band at 1400 cm^{-1}, due to CH_2 scissoring mode (as shown in Table 1). When compared with the native enzyme, the immobilized enzyme showed a higher stability and high activity in a wide range of pH levels.

Looking at the IR spectra of both studies, the immobilization of a-amylases on these metal oxides showed similar spectral results. However, the spectra of zirconia presented more instability, because the zirconia is a transition metal and its crystal structure varies with temperature and also probably with the laser intensity.

Cellulase: Another example of glycosylases are cellulases (EC 3.2.1.4). These enzymes hydrolyze cellulose into glucose (10, 20). Because its hydrophilic nature leads to susceptible denaturation, some researchers have immobilized cellulase on different matrices. Due to the main advantages of magnetic supports to immobilize biological materials, Zang et al. (20) synthetized Fe_3O_4-chitosan nanoparticles to immobilize cellulase. They made use of glutaraldehyde's abilities to improve the mechanical properties of chitosan and to establish the covalent binding between the enzyme and the carrier. The IR spectrum of the Fe_3O_4-chitosan nanoparticles exhibited bands of both components. Fe_3O_4 was characterized by Fe-O stretching vibration at 600 cm^{-1}, and chitosan was characterized by: C-O stretching vibration at 1381 cm^{-1} and N-H bending vibration at 1595 cm^{-1}. The spectrum of the conjugation carrier (Fe_3O_4-chitosan-glutaraldehyde) showed the Fe-O stretching vibration of Fe_3O_4, C-O stretching and N-H bending vibrations of chitosan, and a new band at 1630 cm^{-1} from C=N vibration, corresponding to the successful interaction between the chitosan and glutaraldehyde. The spectra of the immobilized enzyme on the carrier showed the predominant bands of the carrier, and the amide bands from cellulase appeared at 1541 and 1651 cm^{-1} (as indicated in Table 1). Different analytical experiments revealed the increase of the enzyme's stability and activity when immobilized, concerning pH and temperature, in comparison to the free enzyme.

In another study, Wang et al. (10) immobilized cellulase on a silica surface functionalized with polyamidoamine dendrimer through adsorption and cross-linking methods. The glutaraldehyde was used as a cross-linking agent. The IR spectra of the bare silica presented the typical bands of Si-O-Si vibrations at 1097 cm^{-1} vibrations and Si-OH vibrations at around 3400 cm^{-1}. When the silica surface was functionalized with polyamidoamine dendrimer, the IR spectrum presented an increment in the intensity of the bands at 1637 and 1556 cm^{-1}, relating to C=O stretching and N-H bending vibrations, suggesting the success of the grafting process. Comparing these spectra with the spectrum of the immobilized enzyme, it was possible to recognize the broadening of the band between 3000 and 3500 cm^{-1}, characteristic of the amide II functional group. Addition-ally, the spectra exhibited a shift and a slight broadening of the band 1637 to 1656 cm^{-1}(as indicated in Table 1), which was attributed to the absorbance of the cellulase on the carrier. Compared to the free enzyme, the immobilized cellulase showed

high activity in a wider range of temperatures and pH levels. Furthermore, the adsorbed cellulose retained more activity than the cross-linked cellulase. However, the cross-linking method presented higher stability due to the strong binding established between the enzyme and the carrier. In light of the literature results, the adsorption method is a simple method, and enzyme structure is not affected, although sometimes the enzyme connection is not strong enough to keep it immobilized on the carrier. On the other hand, the cross-linking method is able to protect the enzyme from the external conditions and improve stability. In spite of this, the chemical binding can affect the enzyme activity.

According to these works, the immobilization of cellulase on the silica carrier was made clear by IR. Probably, the presence of the polyamidoamine dendrimer made easier the linkage process. Furthermore, the use of magnetic-chitosan nanoparticles also revealed good results. However, the use of glutaraldehyde to bind the enzyme on the magnetic surface is not advised, because it is a toxic molecule that can lead to an easy polymerization with the surrounding molecules.

B-glucosidase: b-glucosidase (EC 3.2.1.21) hydrolyzes several glycosides, as the terminal nonreducing residues in b-D-glucosides, by realizing glucose (39). It is used, for instance, in the decomposition of cellulose and other related polysaccharides. In its native form, this enzyme presents low storage stability and reusability, fundamental features for industrial applications (39). b-glucosidase has already been immobilized on sol-gel beads(40), Eupergit C (41), and sodium alginate supports (42). Zhou et al. (39) used super para-magnetic nanoparticles for the immobilization of b-glucosidase to improve the yield activity of this enzyme. In this study, the glutaraldehyde was used as a cross-linking agent to link the enzyme on the material. The IR spectrum of b-glucosidase immobilized on magnetic nanoparticles revealed a band at 581 cm^{-1}, which was attributed to Fe-O stretching vibration characteristic of magnetic materials. In the same spectrum, the band sat 1543 cm^{-1}, from N-H bending vibration, and at 1656 cm^{-1}, from C=O stretching vibration, were used to prove the interaction b-glucosidase-carrier (see Table 1). The rigidity of the system increased with the cross-linking method. For that, compared to the native enzyme, the immobilized enzyme showed more stability and the range of pH and temperature reaction were broadened. Although the literature reports that this enzyme has mostly been immobilized on natural carriers, it presents a lack of mechanical properties and durability. Thus, the use of an inorganic carrier like magnetic nanoparticles can be a good option to immobilize b-glucosidase.

Pectinase: Pectinase (EC 3.2.1.15) degrades pectin, which is a polysaccharide found in plant cell walls. This is another important glycosylase used to decrease the

turbidity of fruit and vegetable juices and wines (43). Pectinase has been immobilized on different organic synthetic supports, including nylon (44), ion-exchange resins (45), and polyacrylamide (46). Mosafa et al. (43) studied the covalent immobilization of pectinase on silica magnetic nanoparticles functionalized with (3-chloropropyl) trimethoxysilane. In its native form, pectinase's IR spectrum presented characteristic bands of amide functional groups. Therefore, the IR spectrum of the interaction pectinase-nanoparticles showed a band at 1642 cm^{-1}, relating to the C=O stretching vibration, and another band at1553 cm^{-1}, corresponding to N-H bending vibration (see Table 1). This confirmed the covalent binding (-CONH-) between the components. An improvement on the stability of the immobilized enzyme was verified. The covalent binding established between the pectinase and the nanoparticles was strong enough to resist conformational changes by varying temperature or pH level, or in the presence of other substrate. For that, the immobilization of pectinase on this inorganic carrier can be more advantageous than in other organic carriers in which this enzyme has been immobilized. In addition, the synthetic carriers present a slow degradability and high toxicity, limiting their use in food grade manufacturing.

Proteases. Proteases (EC 3.4) are considered to be the dominant enzyme type because of their extensive applications in the detergent industry, aminoacids analysis and developing of protein hydrolysates used, for instance, as nutritional support for certain patients (47). This group of enzymes is responsible for the hydrolysis of peptide bonds and for that related with biological pathways. Over the years, researchers have studied several methods to improve the proteases' performance, by managing proteases' efficiency (controlling pH, temperature, and ionic interactions), chemical modifications (coupling reactive functional groups), and proceeding to enzyme immobilization (47). Different studies have shown that the choice of immobilization process improves the stability of proteases in certain environmental conditions. Functionalized silica microparticles, magnetic-chitosan nanoparticles, and cellulose membranes are examples of materials that have already been used as carriers in proteases immobilization (48–50). In the next sections, studies that prove the success of the immobilization process are presented.

Papain: Papain (EC 3.4.22.2), an enzyme present in papaya, hydrolyzes proteins with high specificity for peptide bonds (51). It is mostly used in the food industry, on the process of meat tenderization to increase flavor (52). Papain has been immobilized on silica spheres and alginate beads (53), among others. As described previously, Tran et al. (30) synthetized magnetic nanoparticles coated with silica to immobilize lipases. To immobilize papain, Mosafa et al. (52) prepared the same carrier material, and the

resulting nanoparticles were functionalized with (3-chloropropyl) trimethoxysilane. The spectrum of the conjugate nanoparticles after immobilization of papain showed the characteristic bands of the nanoparticles components, and also the typical bands of the enzyme functional groups. The band at 588 cm^{-1} was related to Fe-O stretching vibrations, and the band at 1082 cm^{-1} was assigned to Si-O-Si stretching vibrations. The band at 1650 cm^{-1} was attributed to the C=O stretching vibrations, and the band at 1542 cm^{-1} to N-H bending vibrations, as shown in Table 1. The last ones come from functional groups of papain immobilized on the nanoparticles. Once again, the immobilized enzymes exhibited an improvement in stability and optimum activity in a broad range of temperature and pH levels.

a-chymotrypsin: Another example of proteases are a-chymotrypsins (EC 3.4.21.1). These are digestive enzymes present in the pancreactic juice (51). They act in the duode-num, catalyzing the breakdown of proteins and polypeptides. This is another important enzyme in the food industry, used, for instance, to prepare high-energy supplements (49). Ju et al. (49) focused their work on the immobilization of a-chymotrypsin on magnetic-chitosan nanoparticles by covalent binding. The IR spectrum of the free enzyme exhibited three distinct bands at 1646, 1532. and 1425 cm^{-1}, a feature of the amide functional groups of a-chymotrypsin. After immobilization on the magnetic-chitosan nanoparticles, a band appeared at 579 cm^{-1}, from Fe-O bond, and another band at 1082 cm^{-1}, attributed to the C-O stretching vibration present in the chitosan. Additionally, this spectrum revealed the broadening of the band located between 3400 and 3500 cm^{-1}, assigned to the NH_2 stretching vibration, and the presence of OH vibrations (as shown in Table 1). All this evidence indicated the success of the attachment of the a-chymotrypsin on magnetic-chitosan nanoparticles. Regarding the enzyme activity, first the immobilized enzyme activity increased and, after three hours, it remained constant. Studies also proved that pH and temperature significantly influence the enzyme activity.

Lyases

Lyases (EC 4) catalyze the breakdown of molecules into two parts by the formation of a double bond or rings. Because lyases may produce amino acids, they are currently applied in industrial processes, whereas they are not widely used in laboratory applications. Following, a study focused on the immobilization of a lyase named carbonic anhydrase will be discussed.

Carbonic Anhydrase. Carbonic anhydrase (EC 4.2.1.1) is a zinc-containing enzyme that catalyzes the fast interconversion of carbon dioxide and water into carbonic acid, free protons, and bicarbonate ions (51). This enzyme is used to catalyze the hydration of CO_2 molecules (54). Carbonic anhydrase has been immobilized on different carriers, such as polyurethane (55) and gold nanoparticles (56), among others. Vinoba et al. (54) immobilized carbonic anhydrase on spherical SBA-15 using three distinct methods: covalent binding, physical adsorption, and cross-linking. For the immobilization of the enzyme by covalent binding, the spherical SBA-15 surface was functionalized with APTES, and then glutaraldehyde was added. For the adsorption process, the researchers used the spherical SBA-15 without functionalization and without glutaraldehyde. Finally, the cross-linking process was carried out by SBA-15 without functionalization and with glutaraldehyde as cross-linking agent. Regardless of the immobilization process, the IR spectrum confirmed the attachment of carbonic anhydrases. Compared with the raw carrier material, the broadening of the band in the range between 3745 and 3072 cm^{-1} con-firmed the presence of the amide groups represented by N-H, NH_2, O-H, and C-H vibrations. The increase in the intensity of the band at 1644 cm^{-1} was attributed to the C=N stretching vibration. The two new bands at 1534 and 1453 cm^{-1} were assigned to the N-H bending vibration and the presence of an aromatic ring in carbonic anhydrase (as shown in Table 1). Finally, the presence of the Si-O-Si vibration at 1050 cm^{-1} was the most important region that proved the interaction enzyme-carrier. However, the system stability and the enzyme activity varied with the immobilization process. The cross-linking method led to the enzyme-carrier system with the highest stability. This probably occurred because of the cross-linking within the silica pores that protects the enzyme from the external conditions, like pH level and temperature. The covalent binding is usually characterized by a high stability process, although the use of APTES to functionalize the spherical silica and glutaraldehyde to bind it to the enzyme may have affected the enzyme conformation. Therefore, the cross-linking method fixed the enzyme in a simple way and showed best results, but the toxicity of the glutaraldehyde can be unsafe for use in the food industry.

Oxidoreductases

Oxidoreductases (EC 1) catalyzes the oxidation and reduction processes, in which electrons are added or removed from molecules during chemical reactions (57). Although the use of oxidoreductases is important in many industrial processes, it is still minimal in comparison with hydrolases (57). However, the use of oxidoreductases is promising to

carry out synthetic transformations in many interesting areas, such as polymer synthesis and pollution control, among others (58). Following, the different approaches reported to immobilize different oxidoreductases will be discussed, emphasizing the IR spectroscopic characteristics when they were immobilized.

Peroxidase. Peroxidases (EC 1.11.1.7) belongs to the group of oxidoreductases that oxidize organic substrates of living organisms (59). Due to its instability under operational conditions, Sahare et al. (59) immobilized peroxidases on porous silicon activated with 3-aminopropyldiethoxysilane (APDES). They used glutaraldehyde to attach the enzyme by covalent binding. First, the IR spectrum of the carrier material presented a shift of the band attributed to silicon vibrations from 1050 to 1034 cm^{-1}, which proved the functionalization of the surface with APDES. Beyond that, the ethoxy groups present in APDES led to the appearance of the band at 1440 cm^{-1}, assigned to the asymmetric and symmetric deformation of CH_3. Once more, the IR spectrum allowed researchers to confirm the success of the immobilization process due to the appearance of the amide groups vibrations at 1691 cm^{-1} in the spectrum of the carrier's material (as shown Table 1). The C=O and C-N stretching vibrations were attributed to amide I groups. The appearance of the band at 3350 cm^{-1} was assigned to NH_2 stretching vibrations. The typical groups of the porous silicon were also represented by Si-O and Si-OH vibrations at 1034 and 825 cm^{-1}, respectively. Contrary to the previous studies reported in this review, the immobilized peroxidase showed lower thermal stability. In particular, the thermal stability of the immobilized peroxidase was studied at 50C and compared with the free enzyme. The thermal conductance of silicon affected the system and, because of that, the thermal stability of the immobilized peroxidase decreased when compared with the free enzyme.

Chloroperoxidase. Chloroperoxidase (EC 1.11.1.10) is another example of oxidoreductase participating in a variety of reactions, such as sulfoxidation, epoxidation, oxidation of alcohols to aldehydes, hydrogen peroxide dismutation, and halogenation. For that, chloroperoxidases can be applied to generate new clean fuels, such as gas oil, diesel, and gasoline (60). This enzyme has already been immobilized on celite (61), polyurethane (62), and aminopropyl-glass (63). Montiel et al. (60) immobilized chloroperoxidase on amorphous silica gel and mesoporous silica SBA-15 through physical and covalent adsorption. The covalent binding was carried out by functionalizing SBA-15 surface with APTES. The IR spectrum of the immobilized chloroperoxidase on SBA-15, by physical adsorption and covalent binding, showed the appearance of the characteristic bands of amide A, I and II at 3410 cm^{-1}, 1640 cm^{-1}, and 1531 cm^{-1}, respectively (as shown in Table 1). In the same spectra, it was also possible to identify

a notable band at1083 cm^{-1}, which was assigned to the Si-O-Si stretching vibration characteristic of the SBA-15 mesoporous silica. These proved the interaction chloroperoxidase-carrier. The immobilized enzyme by covalent binding exhibited the highest activity and stability due to the strong binding enzyme-carrier. However, the IR spectrum did not show the immobilization of chloroperoxidase on amorphous silica gel, and other techniques were used to evaluate this carrier system. Those studies revealed low enzymatic load and, consequently, low activity due to the lack of pores on the surface material. The presence of pores on the silica surface materials is an advantage for immobilizing enzymes. Mesoporous silica exhibited high surface area and large pore volume, which facilitates the interaction's chloroperoxidase-carrier.

Superoxidase Dismutase. Superoxide dismutases (EC 1.15.1.1) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (51). They work in the protection of cells against toxic products released during aerobic respiration (51, 64). To optimize the stability and the activity of superoxide dismutase, Falahati et al. (64) immobilized this enzyme on mesoporous silica nanoparticles KIT-6 functionalized with aminopropyl by physical adsorption. The IR spectra of the enzyme before and after the immobilization process showed the bands at 1656 and 1528 cm^{-1}, attributed to the amide I and II, respectively (see Table 1). Regardless of immobilization, the bands did not exhibit a notable shift, but showed a different ratio in their amide bands' intensity. There was a change from 1.6 to 1.8 for free and immobilized enzyme, respectively. This was interpreted as no changes in the enzyme structure when it was adsorbed on the aminopropyl-functionalized mesoporous silica carriers. Moreover, the activity of the immobilized enzyme was higher than the free enzyme and did not change with increasing temperature. After immobilization, the enzyme was more stable and kept its functional structure due to the pore protection.

Raman Spectroscopy

In Raman spectroscopy, the spectrum is originated from the scattering of radiation, due toa change in polarizability of the bound. The sample is excited with a laser beam of a single wavelength and it is scattered by the molecules. Most of the scattered radiation will have the same energy as the original light (Rayleigh scattering), but a small part of the laser light undergoes a slight energy change, which means that the original frequency of the light is shifted, in what is usually called Raman shift. Because this represents only a small number of photons, intense excitation sources must be used (i.e., lasers).

Today, this technique has emerged as a powerful tool to characterize typical functional groups in different areas of knowledge (e.g., medical, forensic, and food science). There is a review that reports a detailed study of Raman spectroscopy as a useful tool for the measurement and analysis of individual proteins (51). However, this technique has scarcely been applied to characterize immobilized enzymes on carriers, in particular for lipases. In Table 2, the main information reported on different enzyme-carrier studies by Raman spectroscopy is outlined, following the same criteria and nomenclature used in Table 1.

As previously mentioned, Dhake et al. immobilized lipases on polyurethane copolymers containing b-cyclodextrin (27). The IR analysis was not clear, probably due to the polyurethane signal, which obscured the lipases signal. Regardless of the cross-linking agent (CDI or MDI), in potassium phosphate buffer at pH 7, the Raman spectra did not present any significant difference before and after the immobilization process. However, different strategies can be used to enhance the Raman signal. In this study researchers replaced H2O by D2O at different pH values. Once lipases presented highest stability at neutral pH, the Raman spectra showed signals in this range. For lipases immobilized with CDI, it was possible to identify the amide IV from lipases at around 550 cm^{-1}, and the C-C, C-N, C-O vibrations at 1100 cm^{-1}, notable in the free lipase spectrum as well (see Table 2). Another important thing was the appearance of a substantial broad band at 2500 cm^{-1}, attributed to the spectral signal of native lipases. After the interaction with the carrier, this broadening disappeared. This was evidence of the change in the molecular environment of lipases after the immobilization process. Compared to CDI, lipases immobilized with MDI only exhibited a notable difference in the aromatic C-H vibration present in this cross-linking agent, at 3100 cm^{-1}, and the intense band at 2500 cm^{-1} derived from lipases was present again. However, in this spectrum it was not possible to identify another characteristic lipase band, which might not prove the success of the immobilization process.

Orrego et al. (65) prepared three different chitosan membranes to immobilize lipases. The chitosan membranes were modified with glutaraldehyde and 18-crown-6 ether. The immobilization process of lipases was carried out by physical adsorption. The Raman spectra of chitosan and modified chitosan carriers showed three Raman bands. The first band observed between 800 and 940 cm^{-1} was attributed to NH_2 vibrations. The second band in the range of 1000–1200 cm^{-1} was related to C-O, C-O-C, C-C, and C-N vibrations, from saccharide structures. The last band located between 1300 and 1500 cm^{-1} exhibited different peaks due to C-H, CH_2, and CH_3 vibrations, from the amide groups of chitosan. These bands are typical of chitosan and they are present in the three

different carriers. Even the treated carriers preserve the original structure of chitosan. Figure 4 shows the Raman spectra of lipases immobilized on those carriers. The bands at 1638 and 1549 cm^{-1} are assigned to amide I and amide II groups, respectively. However, in the spectrum of the candida rugosa lipases, it is only possible to verify a band centered at 1570 cm^{-1}. When lipases were immobilized on chitosan membranes, the spectrum showed a new characteristic band of amide groups at around 1550 cm^{-1}, derived from the enzyme, and an increase in intensity of the band at around 1380 cm^{-1}, corresponding to CH_3 vibrations (as shown in Table 2). This fact was attributed to the success of the binding between the enzyme and the carrier material. Lipases immobilized on chitosan membranes treated with glutaraldehyde revealed the highest catalytic activity. This system was highly organized due to its high crystallinity, which is directly linked to the ability to bind amide groups. Thus, high crystallinity leads to a greater ability to load enzymes.

In order to get additional information about the study of molecular vibrations performed by IR analysis, Patel et al. (32) also used Raman spectroscopy to evaluate lipases immobilization on functionalized graphene oxide. In the spectra of the systems before and after lipases' immobilization, the two intense bands at 1300 and 1600 cm^{-1} correspond to C-C vibrations and carboxylic groups characteristic of the carrier material (as shown in Table 2). The only difference in the functionalized carrier was a small shift of these two peaks. The spectra of the immobilized lipase exhibited the appearance of a new band at around 950 cm^{-1}, which was attributed to the success of the immobilization process. Furthermore, the system of the immobilized enzyme revealed itself to be more efficient and sturdier against external conditions.

Table 2. Enzyme-carrier interactions studied by Raman spectroscopy.

				Characteristic bands[a]				
Enzyme (source)	Carrier (Carrier modifier)	Immobilization method	Cross-linking agent	Wavenumber (cm^{-1})	Vibrational mode	Chemical group	Change	Ref.
Lipase (*Pseudomonas cepacia*)	Polyurethane containing β-cyclodextrin	Physical adsorption	CDI MDI	550 1100 2500	ν(C—C, C—N, C—O)	Amide IV	New New Disappearance for CDI	(27)
Lipases (*Candida antarctica and Candida rugosa*)	Membranes of chitosan with glutaraldehyde and 18-crown-6-ether	Physical adsorption	—	1380 1550	CH_3 δ(N—H)	Amide groups of chitosan Amide II	Increased intensity New	(64)
Lipase (*Candida rugosa*)	Exfoliated graphene oxide nanocomposite (APTES)	Physical adsorption	—	950 1300 1600	C—C Carboxylic groups		New Shifted Shifted	(32)

[a]Summary of the bands that undergoes a change due to the enzyme-support interaction comparing with the raw material.
δ: bending; ν: stretching

Conclusions and Future Trends

The immobilization of enzymes can be considered an excellent alternative to improve enzyme catalytic properties. The choice of the immobilization method, as well as the material's carrier, is fundamental to ensure the success of the immobilization process. Inorganic materials are preferable than organic materials, due to mechanical and chemical stability properties, which improve the enzyme activity. Today, silica materials and magnetic nanoparticles are the most common materials used as carriers of enzymes.

Different studies showed relevant information, proving the success of IR and Raman spectroscopy to identify the interaction enzyme-carrier. These interactions are confirmed with the displacement and increment on the intensity of some spectral bands or the appearance of new ones. Mainly, the functional groups of amides (amide I, amide II, or amide A) represent the most sensible bands that prove the interaction enzyme-carrier. The stretching vibration of C=O at 1650 cm^{-1}, the bending vibration of N-H at 1540 cm^{-1}, and the stretching vibration of N-H at 3500 cm^{-1} are usually the bands that identify the presence of enzymes immobilized on the carrier.

Regarding vibrational spectroscopy techniques to evaluate the enzyme-carrier inter-action, the large majority of studies have used IR spectroscopy instead of Raman spectroscopy. However, the few results of Raman spectra seem to be highly promising for a non invasive and easy-to-interpret study of enzyme-carrier interactions. As a consequence, further applications of Raman spectroscopy to study enzyme-carrier interactions are expected in the near future.

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