



Cite this: *React. Chem. Eng.*, 2023, 8, 2689

## A review of lipase immobilization on hydrophobic supports incorporating systematic mapping principles†

José Renato Guimarães,<sup>ab</sup> Kaíque Souza Gonçalves Cordeiro Oliveira,<sup>b</sup> Maria Carolina Pereira Gonçalves, <sup>b</sup> João Paulo Romanelli,<sup>c</sup> Laiane Antunes Lopes,<sup>b</sup> Ángel Berenguer-Murcia, <sup>d</sup> Roberto Fernandez-Lafuente \*<sup>e</sup> and Paulo Waldir Tardioli \*<sup>b</sup>

A review of the literature covering research on the immobilization of lipases on hydrophobic supports was performed using systematic mapping (SM) concepts. This approach consists of a rigorous review of the methodology used to catalog evidence, to identify gaps at the frontier of knowledge, to identify unknown trends, and to list research groups. Our results show a wide variety of available lipases, including commercial, wild-type and recombinant strains. However, the most commonly used lipases are lipases from *Thermomyces lanuginosus* (TLL), *Candida rugosa* (CRL) or *Rhizomucor miehei* (RML) and lipase B from *Candida antarctica* (CALB). A wide variety of supports with different degrees of hydrophobicity were identified and the supports activated with a layer of octyl or octadecyl groups were the most commonly used. The advantages of lipase immobilization on these supports are discussed. Among them, the immobilization, purification, stabilization and hyperactivation of lipases in a single step stand out. Moreover, problems related to lipase immobilization by interfacial activation are highlighted (mainly enzyme release). Strategies to overcome these problems include immobilization on heterofunctional supports or intermolecular crosslinking of enzymes immobilized by physical and/or chemical agents. The possibility of increasing the capacity of supports by lipase multilayer immobilization is also discussed. Finally, the structure, distribution of the network and the frequency of co-occurrence between lipases and supports are elucidated to determine the possible hotspots and hitherto unexplored advances in knowledge.

Received 4th August 2023,  
Accepted 2nd September 2023

DOI: 10.1039/d3re00420a

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## Introduction

Biocatalytic processes have been expanding due to their promising role in the development and optimization of

industrial technologies in different areas. Among the most commonly used biocatalysts, lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are gaining a privileged position due to their numerous advantages over the use of traditional chemical catalysts, especially those concerning the high selectivity and specificity of these enzymes.<sup>1–5</sup> However, the use of such enzymes in their soluble form for large-scale industrial processes is not very attractive, because of their high production cost, moderate operational stability and difficulties in the downstream product raised by lipases.<sup>6</sup> To overcome these drawbacks, the immobilization of these biocatalysts on a solid support has been widely exploited. If properly designed, this approach can provide several advantages from an industrial point of view, such as an increase in the operational stability of the biocatalyst, a widening of the operational window, easy recovery from the product stream, and the possibility of lipase reuse.<sup>6–11</sup>

Lipases have been extensively immobilized by adsorption on supports bearing hydrophobic surfaces<sup>12–16</sup> due to their peculiar catalytic mechanism, which is called interfacial activation (Fig. 1). This mechanism relies on the existence of

<sup>a</sup> Institute of Natural Resources, Graduate in Bioprocess Engineering, Laboratory of Fermentative and Enzymatic Processes (LPFEnz), Federal University of Itajubá, Av. Benedito Pereira dos Santos, 1303, 37500-903, Itajubá, MG, Brazil.

E-mail: [jrenatoguimaraes@unifei.edu.br](mailto:jrenatoguimaraes@unifei.edu.br)

<sup>b</sup> Graduate Program in Chemical Engineering (PPGEQ), Laboratory of Enzyme Technologies (LabEnz), Department of Chemical Engineering, Federal University of São Carlos (DEQ/UFSCar), Rod. Washington Luís, km 235, 13565-905 São Carlos, SP, Brazil. E-mail: [kaiquesg.eq@gmail.com](mailto:kaiquesg.eq@gmail.com), [mariacarolinapgoncalves@gmail.com](mailto:mariacarolinapgoncalves@gmail.com), [laiane\\_antunes@hotmail.com](mailto:laiane_antunes@hotmail.com), [pwtardioli@ufscar.br](mailto:pwtardioli@ufscar.br); Tel: +55 16 3351 9362

<sup>c</sup> Laboratory of Ecology and Forest Restoration, Department of Forest Sciences, "Luiz de Queiroz" College of Agriculture, University of São Paulo, 11 Av. Pádua Dias, 13418-900 Piracicaba, SP, Brazil. E-mail: [joaopromanelli@usp.br](mailto:joaopromanelli@usp.br)

<sup>d</sup> Departamento de Química Inorgánica e Instituto Universitario de Materiales, Universidad de Alicante, 03080 Alicante, Spain. E-mail: [a.berenguer@ua.es](mailto:a.berenguer@ua.es)

<sup>e</sup> Departamento de Biocatálisis, ICP-CSIC, Campus UAM-CSIC, 28049 Madrid, Spain. E-mail: [rfl@icp.csic.es](mailto:rfl@icp.csic.es); Tel: +34 91594804

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3re00420a>



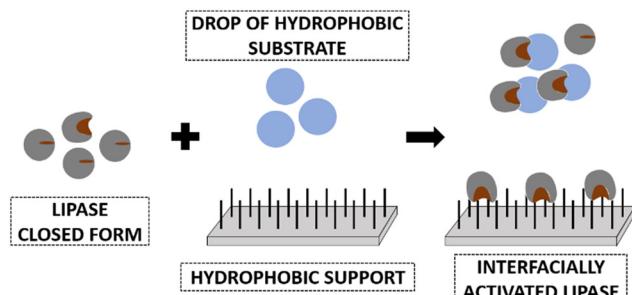


Fig. 1 Interfacial activation of lipases versus drops of insoluble substrates or the surfaces of hydrophobic supports.

two forms of the lipase in equilibrium: 1) a closed form (hydrophobic regions of the lid interact with hydrophobic regions around the active center, isolating it from the reaction medium); 2) an open form (the lid moves and allows lipase adsorption on a hydrophobic surfaces (e.g., drops of oils or air bubbles),<sup>17–20</sup> exposing the active center to the medium).<sup>20</sup>

This immobilization protocol is a simple technique and presents several advantages: 1) pH is not a limiting factor as long as the enzyme is soluble and stable at the utilized pH;<sup>21</sup> 2) the use of low ionic strength can lead to the simultaneous immobilization and purification of lipases;<sup>21–25</sup> 3) the immobilization method is reversible; 4) the open and adsorbed form of this enzyme is very stable,<sup>26–28</sup> even regarding covalently immobilized multipoint lipases;<sup>29,30</sup> 5) lipases immobilized on hydrophobic supports have the active center more exposed to the medium and the lid does not move in the presence of a medium of high ionic strength;<sup>31</sup> 6) reduction in the accumulation of hydrophilic compounds on the porous biocatalyst surface;<sup>32–34</sup> 7) higher enzymatic activities can be achieved regarding the free enzyme, due to stabilization of open-form lipase.<sup>22</sup>

Moreover, the relevance of the research addressing the immobilization of lipases on hydrophobic supports is indisputable, since this theme has led to an increased number of published scientific research papers over the years (Fig. 2). Accordingly, in an effort to advance rigorous methods for conducting evidence reviews in this topic, we incorporated some principles of systematic mapping (SM) to evaluate research involving the immobilization of lipases on hydrophobic supports. SM methodologies investigate and reveal trends, knowledge gaps, and existing heterogeneity in studies conducted on benches. This is because SM retrieves comprehensive research from various bibliographic sources, transparently screens articles, and critically assesses the quality of these studies.<sup>35–39</sup>

Guidelines to standardize SM methods are formulated by formal coordinating review bodies from various disciplines, such as Cochrane in healthcare, the Campbell Collaboration in social welfare, and the Collaboration for Environmental Evidence (CEE) in conservation and environmental management.<sup>40</sup> Nonetheless, there is no specialized organization devoted to guiding the conduct of evidence

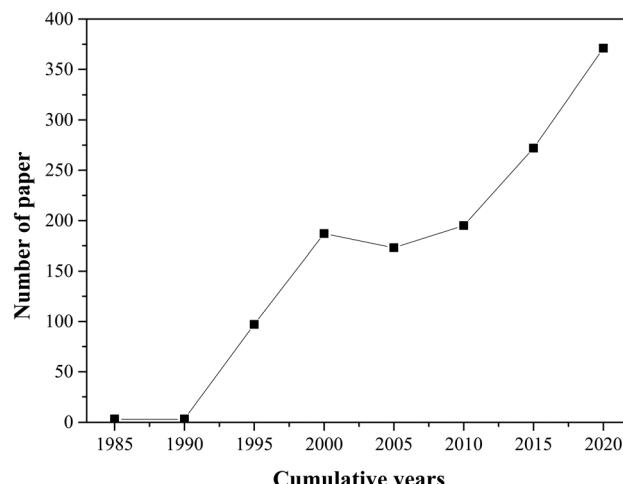


Fig. 2 Annual distribution of all records retrieved through a bibliographic search for the immobilization of lipases on hydrophobic supports in the Web of Science. The data shown every five years represent the cumulative number of papers published.

synthesis in the chemical engineering sector, which suggests that the majority of reviews in this area are classified as nonsystematic. Consequently, using SM methods to conduct a literature review in this field may allow the extraction of relevant information from our research subject.

In this context, the purpose of this review is to present trends and gaps across the literature on the immobilization of lipases on hydrophobic supports in order to support future studies on this topic. Our evaluation is restricted to a specific subset of scientific articles retrieved from four bibliographic sources: Web of Science (Core Collection: SCI-E and ESCI), Scopus, PubMed (Central; PMC), and SciELO.

## Methodology

### Literature search and database building

This review was designed by following the Collaboration for Environmental Evidence guidelines,<sup>41</sup> in close collaboration with environmentalists and specialists on the subject. There is no dedicated body for retrieving evidence in the area of chemical engineering. The following search string was used to retrieve titles, abstracts, and keywords of related publications: “interfacial activation\*” OR “activation immobilization\*” OR “interfacial immobilization\*” OR “interfacial activation immobilization\*” OR “interfacial adsorption\*” OR “adsorption immobilization\*” OR “hydrophobic adsorption\*” OR “hydrophobic immobilization\*” OR “hydrophobic adsorption immobilization\*” OR “hydrophobic support\*” OR “hydrophobic interaction\*” AND “lipase\*”. Searches were restricted to articles published between 2011 and 2020 in order to obtain a sample of recent publications. The database was updated on April 26, 2021 (see the complete search procedures in Fig. S1 – ESI†).



Bibliographic searches were performed in four different databases to minimize possible bias in the review process, returning a total of 1855 articles. We retrieved 368 publications from Scopus, 644 from Web of Science, 843 from PubMed, and none from SciELO (Table S1 – ESI†). The SCI-E database present within the Web of Science platform was used because it covers most significant scientific results and other online databases that also contain citation information, such as Science Direct and Google Scholar. The ESCI was also used because it contains complete records of articles indexed by journals not yet covered by the SCI-E which are nevertheless under evaluation for indexing in the SCI-E. Thus, relevant scientific results that can be found in this database may have an influence on bibliometric metrics. After an analysis of coverage and overlap to remove replicates, the database was reduced to 1433 publications. Eligibility criteria were used to eliminate unrelated publications inadvertently included in the reference list after this search. Initially, the screening process was conducted by analyzing titles and abstracts of primary research publications (*i.e.*, original data for a specific research study), considering the following inclusion criteria: (i) lipase immobilization; (ii) lipase immobilization on solid supports; (iii) lipase immobilization on hydrophobic supports. A total of 370 records were then submitted to a full-text analysis and 264 primary studies were selected to be included in this study. A summary of the methodology employed is presented in Fig. 3 and details of the papers eliminated after title/abstract screening and full-text analysis are shown in Table S2 and Fig. S2 (ESI†), respectively.

A sample of 30 random articles was double screened by two different assessors (J. R. G. and L. A. L.) to account for subjective decisions in the inclusion/exclusion of eligible studies.<sup>41</sup> Decisions were compared using the kappa test of agreement to ensure the repeatability of the process. Kappa values range from 0 to 1: high values indicate greater agreement and values lower than 0.6 indicate inconsistency between raters when the inclusion criteria should be redefined.<sup>42</sup> In this analysis, a kappa score of 0.91 (0.95 lower/upper confidence limit) was obtained, which indicates almost perfect agreement between the reviewers, as well as decisions being sufficiently repeatable (Table S3 – ESI†).

### Data synthesis

Articles meeting our inclusion criteria ( $n = 264$ ) were subjected to data coding and synthesis of results. Two categories of keyword (types of lipase and hydrophobic support) were created and assigned to each paper to build network maps. Despite the extensive literature that exists on immobilization of lipases on hydrophobic supports, important aspects of this immobilization protocol remain to be clarified. Considering this, here we elucidate some of the relevant aspects, including the main enzymes and supports used, besides problems and solutions that are related to this research topic.

### Data handling

For data processing, Origin® (version 9.0) and MS Excel (v. 2016) were used to perform the calculations and prepare the

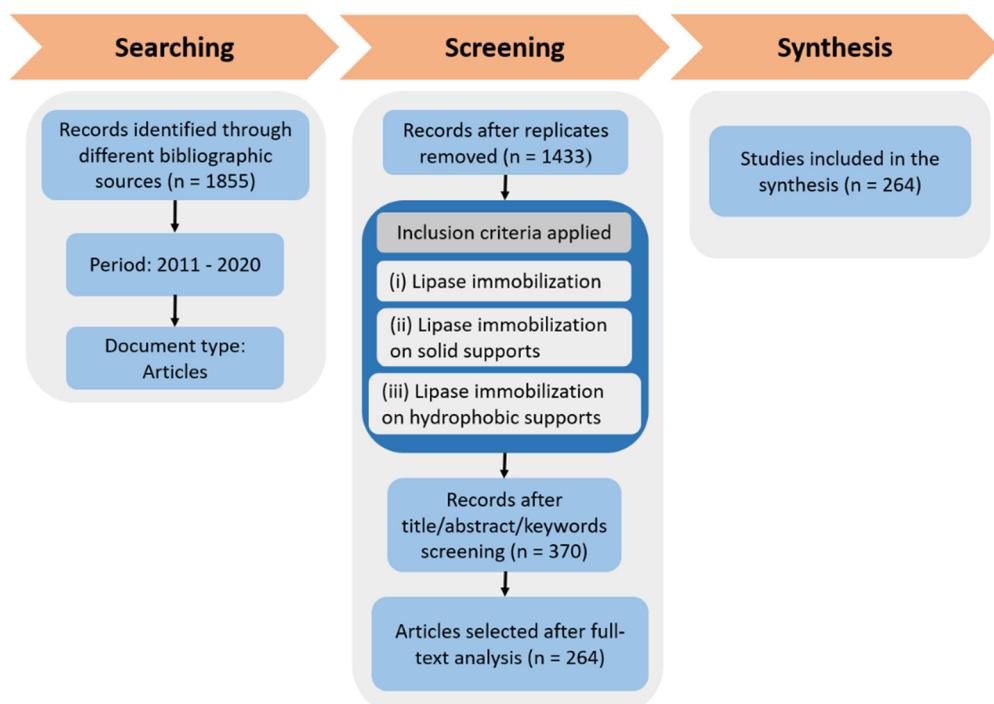


Fig. 3 Flow diagram for the selection of studies (adapted from ROSES Flow Diagram for Systematic Maps. Version 1.0).



graphs. R software was used to perform the screening process – ‘revtools package’.<sup>43</sup> This analysis allows the removal of duplicate publications that were retrieved from different academic bibliographic sources (a necessary step in systematic mapping).

Furthermore, the set of keywords created was applied to VOSviewer software (version 1.16.15) through the criterion of co-occurrence of terms to build network maps containing information related to the important topics of immobilization of lipases on hydrophobic supports. The purpose of co-occurrence analysis is to examine the degree of association between the elements of a “collection unit”. If pairing is performed multiple times, then the association is strengthened with every additional match. However, if only one match is made as part of the support, the association is tenuous or spurious.

## Results and discussion

The different lipases and solid supports present in our database are available in Table S4 (ESI†).

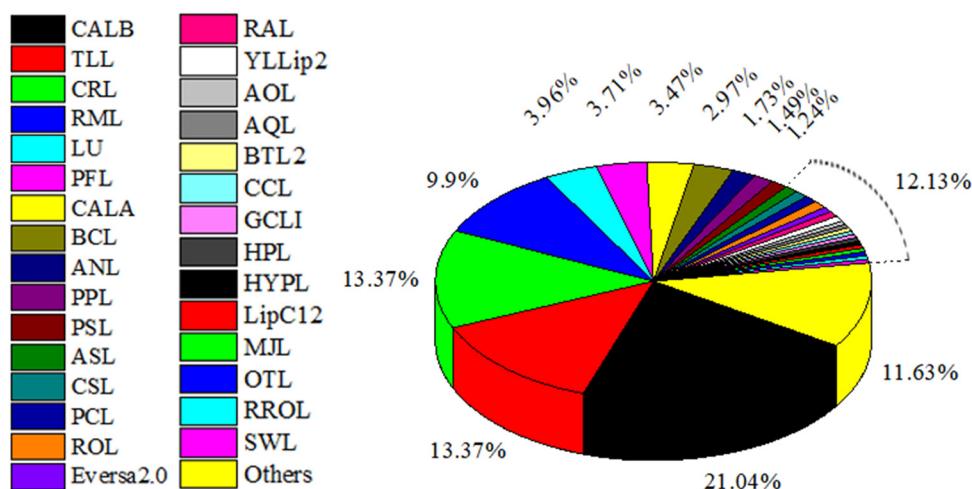
### Enzymes and supports used in hydrophobic immobilization

The immobilization of a lipase on a hydrophobic support can allow the synthesis of a library of biocatalysts with modulated catalytic properties for application in industrial biotransformation processes. This is possible due to the structure and properties of the different existing supports<sup>44–47</sup> and the affinity of the lipases with these carriers,<sup>48,49</sup> as well as the immobilization conditions.<sup>50–54</sup> These factors, alone or collectively, can alter the selectivity,

specificity and final activity of lipases and in some cases lead to an immobilized biocatalyst design that is much more suitable for the production process than the free-enzyme formulation.<sup>51,55–58</sup> Thus, there is no way to say whether a specific lipase is adequate or inadequate for a specific process as its properties may be strongly tailored upon immobilization.<sup>57</sup>

As shown in Fig. 4, the most frequently immobilized enzymes on hydrophobic supports are lipases from *Candida antarctica* (form B) (CALB), *Thermomyces lanuginosus* (TLL) and *Candida rugosa* (CRL). These lipases have probably been used due to their stability and activity after immobilization. CALB and TLL are enzymes that have high stability and can be used in various industrial conditions, and are already commercialized as immobilized formulations in resins.<sup>59,60</sup> On the other hand, CRL presents moderate stability<sup>61</sup> and an immobilized formulation is not available on the market. The use of stability is a delicate factor to infer the sovereignty of these lipases in this bibliographic survey, considering that lipase A from *Candida antarctica* (CALA) has high stability and is in the seventh position of the most used lipases in the survey. In addition, Eversa® Transform 2.0 (EV2.0) is a commercial evolution of TLL and has high stability, but is not in the top 10 due to its recent appearance in the market.<sup>62</sup>

The cost of acquiring enzymes is a factor that reduces the technical and economic viability of biocatalytic processes, but it is probably not the main variable that determined the ranking of lipases in our study. Lipases such as CRL and porcine pancreas lipase (PPL) are formulations that have a low cost compared to other



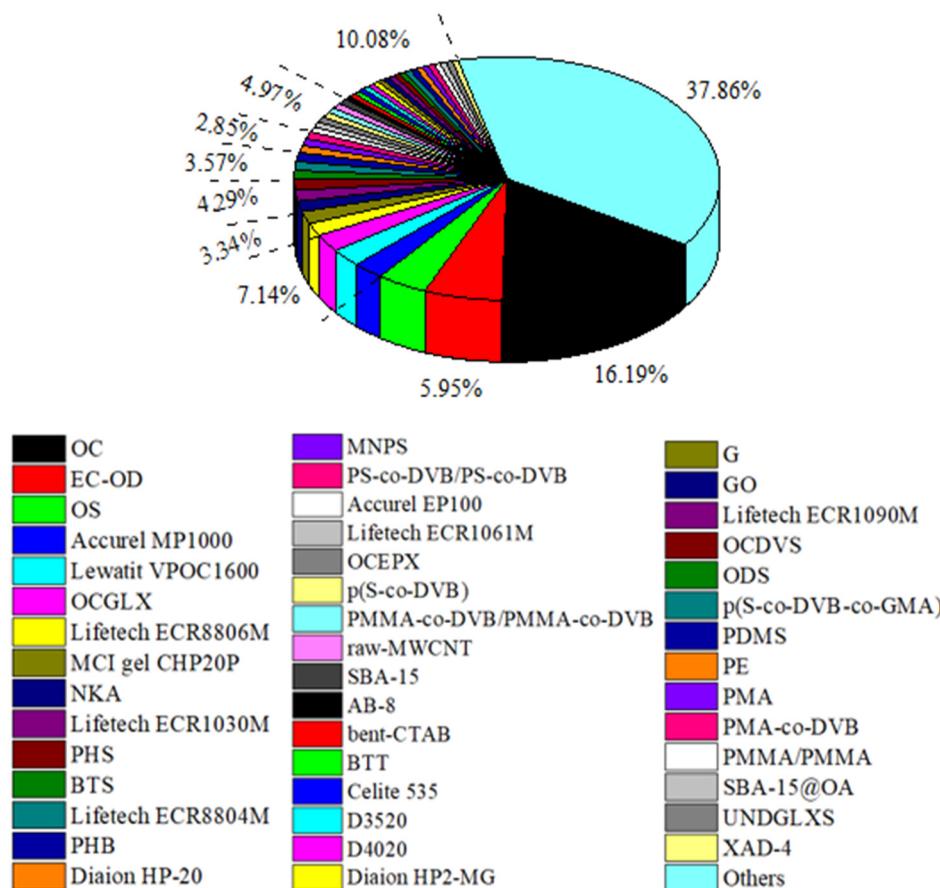
**Fig. 4** Lipases used for immobilization by interfacial adsorption. The category “others” represents lipases or hydrophobic supports with less than 1% occurrence in our dataset. CALB: lipase B from *Candida antarctica*; TLL: lipase from *Thermomyces lanuginosus*; CRL: lipase from *Candida rugosa*; RML: lipase *Rhizomucor miehei*; LU: phospholipase Lecitase Ultra; PFL: lipase from *Pseudomonas fluorescens*; CALA: lipase A from *Candida antarctica*; BCL: lipase from *Burkholderia cepacia*; ANL: lipase from *Aspergillus niger*; PPL: lipase from porcine pancreatic; PSL: lipase *Pseudomonas stutzeri*; ASL: lipase from *Alcaligenes* sp.; CSL: lipase from *Candida* sp. 99–125; PCL: lipase from *Pseudomonas cepacia*; ROL: lipase from *Rhizopus oryzae*; Eversa2.0: Eversa® Transform 2.0; RAL: lipase from *Rhizopus arrhizus*; YLLip2: lipase *Yarrowia lipolytica* LIP2; AOL: lipase from *Aspergillus oryzae*; BTL2: lipase from *Bacillus thermocatenulatus* 2; CCL: lipase from *Candida cylindracea*; GCLI: lipase from *Geotrichum candidum*; HPL: lipase from hog pancreas; HYPL: lipase from *Hypocrea pseudokonigii*; LipC12: metagenomic lipase; MJL: lipase from *Mucor javanicus*; OTL: lipase from *Chinook salmon*; RROL: recombinant lipase from *Rhizopus oryzae*; SWL: lipase of *Staphylococcus warneri* EX17.



lipases,<sup>61,63</sup> but PPL is the tenth most used lipase in the bibliographic survey. Probably, the specificity of these lipases is the main factor that led to their classification in this survey. Non-specific enzymes can be applied in numerous industrial processes, but enzymes that present strict specificity are limited to restricted reactions, but are no less important than non-specific lipases. Some lipases, such as TLL and Eversa® Transform, are 1,3-specific enzymes whose specificities are affected when immobilized mainly on hydrophobic supports. Thus, this increases the range of application of these enzymes.<sup>15,51</sup>

Fig. 5 shows a compilation of the most commonly used hydrophobic supports from 2011 to 2020. It is possible to observe a wide variety of matrices with different degrees of hydrophobicity used for lipase immobilization. Among them, there are commercial supports, such as octyl-agarose, (and

some derivatives such as octyl-glyoxyl agarose), EC-octadecyl Sepabeads™, Accurel MP 1000, octyl and phenyl silica and Lewatit VP OC 1600. This last support is used in the industrial production of Novozym® 435.<sup>59</sup> Moreover, the use of supports prepared from agro-industrial residues is also observed, such as rice husk silica (RHS) with triethoxy(phenyl)silane, chitin-polyhedral oligomeric silsesquioxanes support, and cellulose/Fe<sub>2</sub>O<sub>3</sub> hydrogel microspheres. Given the variety of available supports, a library of lipase biocatalysts with different properties for industrial application can be obtained. This is only possible because the structure and properties of the supports condition the catalytic properties of the lipases. This effect can produce changes in the specificity and selectivity of the enzymes, modulating the biocatalyst to the desired biotechnological process.<sup>52–54,64–66</sup>



**Fig. 5** Hydrophobic supports used for immobilization by interfacial adsorption. The category others represent lipases or hydrophobic supports with less than 1% occurrence in our dataset. OC: Octyl-Sepharose; EC-OD: EC-octadecyl Sepabeads; OS: octyl silica; OCGLX: octyl-glyoxyl agarose; PHS: silica modified with phenyl; BTS: Butyl-Sepharose; poly-hydroxybutyrate particles; MNPS: hydrophobic magnetic nanoparticles from magnetite; PS-co-DVB/PS-co-DVB: core-shell particles of poly(styrene-co-divinylbenzene); OCEPX: Octyl-epoxide Sepharose; p(S-co-DVB): poly(styrene-co-divinylbenzene); PMMA-co-DVB/PMMA-co-DVB: poly(methyl methacrylate-co-divinylbenzene)/poly(methyl methacrylate-co-divinylbenzene); raw-MWCNT: multi walled carbon nanotubes; SBA-15: Santa Barbara Amorphous-15; bent-CTAB: Na-bentonite-modified with cetyltrimethyl ammonium bromide; BTT: Butyl-Toyopearl; D3520: polystyrene-based hydrophobic resin; D4020: Relizyme OD4030; G: graphene; GO: oxidized graphene; OCDVS: divinyl sulfone-activated octyl agarose; ODS: silica modified with octadecyl; p(S-co-DVB-co-GMA): poly(styrene-co-divinylbenzene-co-glycidyl methacrylate); PDMS: silk fibers functionalized with amino-functional polydimethylsiloxane; PE: poly(ethylene glycol) decorated polystyrene nanoparticles modified by the adsorption of Congo red; PMA: poly-methacrylate particles; PMA-co-DVB: poly(methacrylate-co-divinyl benzene); PMMA/PMMA: poly(methyl methacrylate)/poly(methyl methacrylate); SBA-15@OA: oleic acid-modified SBA-15; UNDGLXS: undecanol-glyoxyl silica. The other supports are presented with the commercial names.



The main acyl groups present on supports are the octyl and octadecyl groups. In general, the use of these acyl groups can lead to the formation of a lipase biocatalyst with different properties, even using the same conditions during hydrophobic immobilization. TLL was immobilized on octyl-agarose (OC) and Purolite® C18 (Lifetech ECR8806M) and the results demonstrated that the biocatalyst immobilized on resin with octadecyl groups led to better catalytic properties in certain processes and higher stabilization compared to the biocatalyst immobilized on OC.<sup>67</sup> Furthermore, the greater degree of hydrophobicity of octadecyl groups can improve the lipase adsorption strength and in that way reduce enzyme leakage.<sup>24,68</sup>

### Problems of lipase immobilization on hydrophobic supports

The main problem with lipase immobilization on a hydrophobic support is the possibility of enzyme desorption when the biocatalyst is subjected to drastic conditions, such as high temperature or the presence of an organic solvent.<sup>69</sup> Furthermore, when applied in biosurfactant synthesis, reactions where a biosurfactant is an intermediate product of the process or in a heterogeneous medium containing substances with detergent properties can also facilitate enzyme release.<sup>70</sup> A strategy used to minimize this problem

is lipase immobilization on a heterofunctional support. This type of support has a unique surface exhibiting various physicochemical capabilities, *e.g.*, a layer of acyl groups to obtain lipase interfacial activation or a layer of groups able to give other physical interactions (*e.g.*, ionic exchange<sup>71</sup>) or a covalent bond that will make immobilization irreversible.<sup>72–76</sup> These new biocatalysts are generally even more stable than standard biocatalysts immobilized only by hydrophobic interaction.<sup>77</sup>

The use of heterofunctional supports can provide different modulating effects on the catalytic properties of lipases due to changes in the interactions between enzyme and support,<sup>78</sup> and it can also provide new systems for the co-immobilization of lipases by different mechanisms,<sup>94</sup> increasing the possibilities for application of the biocatalyst in industry.<sup>77,79,92</sup> On heterofunctional supports, the enzyme is usually initially immobilized by interfacial activation under conditions of low ionic strength and pH in the range of 5.0–7.0, and later, the conditions are modified to favor covalent bonds, such as incubation in alkaline solution. The limitation of the use of these supports is related to the sensitivity of some lipases to alkaline pH when incubated to produce covalent immobilization or the lack of nucleophiles near the active center.<sup>72</sup> Some heterofunctional supports and their

**Table 1** Functional groups present in heterofunctional supports used in the immobilization of lipases by interfacial activation and covalent bonding

Lipase source	Support	Functional groups	Reference
CRL; RML; TLL	OCDVS	Octyl and vinyl sulfone	79
CALB	Lifetech™ ECR 8285F	Butyl and epoxy	80
CALB	SMMP-octyl-glu	Octyl and aldehyde	81
CRL; LipC12	Aga-C8-GLU	Octyl and aldehyde	82
BaL	GO-NH <sub>2</sub> -PMAO	Hydrophobic groups present on the support and aldehyde	83
PLL	PBA-PAD	Inner hydrophobic PBA domain and aldehyde	84
CRL	Lifetech™ ECR1030M; Lifetech™ ECR8285	Butyl and epoxy	85
CRL; CALB; RAL; ASL; HPL; MML; PCL; RNL	P(SAN-DVB)-GMA	Hydrophobic groups of the matrix poly(styrene- <i>co</i> -acrylonitrile- <i>co</i> -divinylbenzene) and epoxide	86
LipC12	OCA; ODA	Octyl and aldehyde; octadecyl and aldehyde	87
SHL	PMA- <i>co</i> -DVB	Inner hydrophobic PBA domain and aldehyde	88
CALB; CCL; PCL; PFL; HPL	ChiS-G	Hydrophobic portion of chitosan and aldehyde	89
RML	OCEPX	Octyl and epoxide	90
CALB	UndGLX, OCEPX; UndGLXS; OCEPXS	Undecanol and glyoxyl; octyl and epoxide	75
BTL2	UndGLXS; OCEPX	Undecanol and glyoxyl; octyl and epoxide	91
PsL; ASL	OGS	Octyl and glyoxyl	74
CALA; CALB; CRL; TLL; RML; LU	OCDVS; OCGLX	Octyl and vinyl sulfone; octyl and glyoxyl	77
CALB	OCGLX	Octyl and glyoxyl	92
CALB; TLL	OCGLX	Octyl and glyoxyl	93
LU; RML; PFL	OCGLX	Octyl and glyoxyl	94
CALB; TLL; RML	OCGLX	Octyl and glyoxyl	95
PFL	OCGLX	Octyl and glyoxyl	96
CRL; CALA	OCGLX	Octyl and glyoxyl	73
RML; CRL	OCGLX	Octyl and glyoxyl	97
CALB; TLL; RML	OCGLX	Octyl and glyoxyl	72
CALB; RML; LEU	OCGLX; OCGLXR	Octyl and glyoxyl	98
PsL; ASL	OCGLXS	Octyl and glyoxyl	99
CALA; CALB; TLL; RML; CRL; LU	OCEDA; OCHDA	Octyl and glyoxyl	71



functional groups are presented in this review, as shown in Table 1.

Table 1 demonstrates the predominance of heterofunctional supports activated with octyl and glyoxyl groups for the immobilization of different lipases,<sup>71,73–75,77,91,92,94,95,97–99</sup> followed by the use of supports activated with octyl and epoxy groups.<sup>75,90,91</sup> The other heterofunctional supports present distinct groups for immobilization by interfacial and covalent activation. A support activated with octyl and vinyl sulfone groups has not been used frequently, very likely because it has only recently been proposed. However, it should be highlighted as a support that can lead to the construction of a library of biocatalysts.<sup>77,79</sup> This is possible because at the end of the covalent immobilization, there is a need for a final step that requires the blocking of the other reactive groups of the support with a nucleophile.<sup>77,79</sup> This step allows the adaptation of enzyme–support interactions using reagents with very different physical properties.<sup>77,79</sup> Thus, the use of different blocking reagents can lead to the formation of biocatalysts with different structures and functional properties, even starting from a collection of immobilized enzymes with exactly the same distribution of enzymes present on the support, orientation in relation to the support surface and number of bindings of enzymes to the support,<sup>30,100,101</sup> as shown in Fig. 6.

The most commonly used acyl group in the supports is octyl, but the octadecyl group can lead to higher lipase stabilization.<sup>67</sup> No paper showing a combination of octadecyl and vinyl sulfone groups in a heterofunctional support was retrieved in the systemic analysis, although

one was found in 2023.<sup>102</sup> Also, no record was found when performing a brief analysis of data from the last years extracted from the Web of Science using “octadecyl” AND “vinyl sulfone” as a search criterion. This is a gap in the advancement of knowledge to be explored for the study of the synthesis of heterofunctional supports with these characteristics. A possible problem is the size mismatch of the spacer spleens of vinyl sulfone and octadecyl groups. In this case, strategies must be sought to minimize this problem.<sup>102</sup>

Another strategy that has been used to minimize the desorption problems of hydrophobic supports is intermolecular crosslinking of immobilized enzymes with physical and/or chemical agents. The association of these agents and the immobilization of the lipase on the support provides the formation of a biocatalyst with large enzyme planar aggregates simultaneously interacting with the support at multiple sites.<sup>103–106</sup> Therefore, to achieve desorption of the lipase, it will be necessary to release all the enzyme molecules forming each planar physical or chemical aggregate, which is much more difficult than the release of a single lipase protein. Another advantage of crosslinking with physical or chemical agents is the modulation of catalytic properties and, in some cases, improvements in the stability of the biocatalyst.<sup>66,107–109</sup> However, recovery of the support after enzyme inactivation becomes complicated if intermolecular chemical crosslinking of all enzyme molecules is achieved, physical crosslinking being better in this respect. Table 2 shows the main physical and/or chemical agents used in the papers obtained in the bibliographic survey.

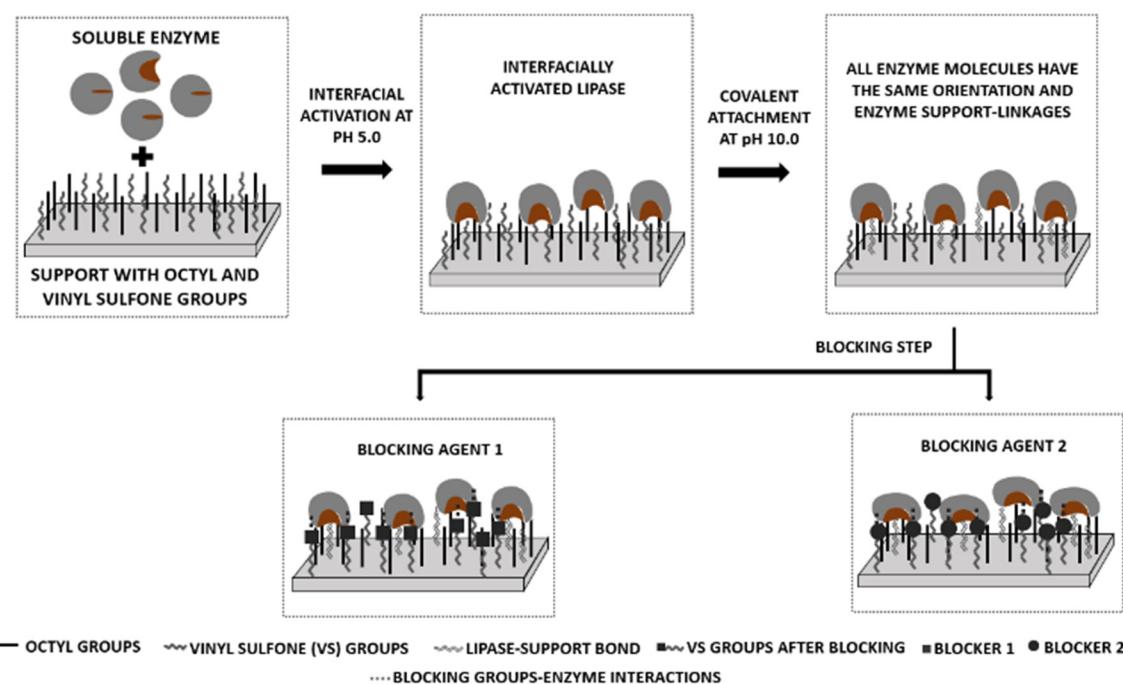


Fig. 6 Schematic representation of enzyme immobilization on octyl and vinyl sulfone heterofunctionalized support.



**Table 2** Building-block chemicals used for the modification of the enzymatic surface after immobilization by interfacial adsorption

Lipase source	Support	Chemical or physical agent	Reference
TLL	Lifetech™ ECR8806M; EC-OD; Lewatit VP OC 1600	PEG	15
CALB; TLL; RML	OCGLX	EDA	95
TLL	MNPs	EDA, GA and DA	120
CALA; CALB; RML; TLL; LU	OC	PEI and GA	121
RML	OC	DA	69
CALB; RML	OC	PEI and DS	103
CALB	OC	PEI	122
CALB	OC	DA	123
AFE	EC-OD	GA	111
RML	OC	Poly-allylamine (PAA) and DA	104
CALB	OC	EDA and TNBS	64
CALB; TLL; RML	OC	EDA, amino groups with succinic anhydride and PEI	66
CALA; CALB; TLL; RML; LU	OC; OVS	PEI	124
LipC12	OCA; ODA	PEG	87
CALB	Lewatit VP OC1600	TNBS, EDA, PEI	125
CALB	OC	PEI and DS	119
CALB	NKA	PEG	16
RML	OC	PEI and DS	118
CALB; TLL; RML	EC-OD	PEI and DS	65
CALB	OC	PEI	105
TLL; RML; CALB	OC	EDA and DA	106
CALB; TLL; RML	Lewatit VP OC1600	PEI	126
TLL	EC-OD	PEG	127
LU	OC	EDA and TNBS	109
CALB	OS	PEG	128
CALA; CALB; RML; TLL; LU	OC	PEI and GA	129
CALB	PTMOS	GA	76
CRL	PHB	GA	112
CRL	MSU-H	GA	113
Eversa2.0; CALB	OC	PEI	130
CALA; CALB	OC	PEI	131

Physical or chemical crosslinking agents have different characteristics. Glutaraldehyde (GA) is a small bifunctional crosslinking molecule<sup>110</sup> that has been used in situations where the enzymes are immobilized at high load on the support<sup>76,111–113</sup> or when the surface of the immobilized enzyme is aminated with ethylenediamine (EDA),<sup>109</sup> since close proximity between molecules is required so that the amine groups located in different enzyme molecules are clustered together for intermolecular crosslinking to be efficient.<sup>114</sup> On the other hand, dextran aldehyde (DA) is a large multifunctional molecule<sup>115</sup> that does not demonstrate the limitations involved with crosslinking with glutaraldehyde. Due to its being a multifunctional reagent, it provides very intense crosslinking that makes immobilization of the enzyme on the support irreversible. This makes it impossible to reuse the support after enzyme inactivation.<sup>116</sup> Poly-ionic polymers, such as polyethyleneimine (PEI)<sup>117</sup> or dextran sulfate (DS), are alternatives to dextran aldehydes, as they provide highly efficient crosslinking, but do not preclude the reuse of the support after enzyme inactivation.<sup>103,118,119</sup>

The crosslinking of the enzyme immobilized on a hydrophobic support has been carried out with physical and chemical agents of carbonic origin; however, a possibility that should be studied is the crosslinking of immobilized

lipases with metal phosphate. This strategy arises from the frontier of knowledge related to the use of metal phosphates for free-enzyme mineralization by an immobilization technique called nanoflower self-assembly.<sup>132</sup> In this case, the metal phosphate interacts with a protein nucleation center and starts the self-assembly of an organic-inorganic flower, leading to the formation of an insoluble biocatalyst. Expanding this approach to an immobilized enzyme, an immobilized biocatalyst with intermolecular crosslinking using an inorganic agent can be obtained. In this case, the metal phosphate binds to the enzyme nucleation point and begins the growth of the inorganic structure with the formation of an inorganic film on the immobilized enzyme. The nanoflower self-assembly step is probably not reached because the enzyme is immobilized in a plane.<sup>132</sup> The combination of immobilization and inorganic crosslinking can combine the benefits of both protocols and contribute to the formation of a biocatalyst with catalytic properties and greater stability than the biocatalyst without crosslinking.<sup>67,102</sup>

Another problem that is related to all preexisting supports, not restricted to hydrophobic supports, is their limited load capacity: that is, the amount of enzyme that can be immobilized per gram of support did not permit the filling of the particle volume with enzyme when using preexisting



supports. If the loading capacity of the support is increased, the impact of the cost of the support on the total expenses of biocatalyst production can be reduced.<sup>133</sup> An alternative is the construction of biocatalysts with a three-dimensional design composed of multilayers of enzymes (an enzyme layer immobilized over the previous one to multiply the final load capacity of the support). This allows the use of a smaller amount of support by increasing the volumetric or mass activity of the biocatalyst.<sup>124,129</sup> In addition, this strategy allows the synthesis of combilipases that can be used in a cascade reaction.<sup>121,124</sup>

PEI is a crosslinking agent that can be used to build a multilayer biocatalyst starting from a first layer of immobilized enzyme.<sup>117</sup> This polymer is able to adsorb onto immobilized enzyme molecules through strong ionic exchange. In addition, the PEI-coated enzyme is able to immobilize other enzyme molecules also *via* ion exchange<sup>121,122,124</sup> involving the enzyme in a three-dimensional structure instead of a flat surface.<sup>134</sup> After each overlapping enzyme layer, crosslinking with glutaraldehyde of the structural network formed by PEI and enzyme can be carried out; this strategy of immobilization by covalent bonding is adopted to prevent the release of the enzyme during the PEI incubation<sup>121</sup> (Fig. 7). Dextran sulfate is an alternative polymer to PEI in the construction of multilayer biocatalysts.<sup>135</sup>

## Co-occurrence analysis of keywords

We performed a co-occurrence analysis between the main lipases and supports in our database. Based on the retrieved publications, it is possible to elucidate the structure, distribution of the network and the frequency of co-occurrence of these keywords to determine the possible hotspots and frontiers of knowledge not yet explored.

Bibliometric maps revealed that 75 lipases and 45 hydrophobic supports were cited by the studies we investigated (Fig. 8). Fig. 8a shows the use of more than one lipase in ~23% of the studies, which may indicate that these studies are looking for an enzyme with catalytic properties suitable for the productive process being evaluated, since there is no universal biocatalyst for application in all processes. CALB showed the highest number of co-occurrences with different lipases ( $N = 34$ ), the main ones being TLL, CRL, RML, PFL, CALA and Lecitase Ultra (LU). Next, RML ( $N = 32$ ), TLL ( $N = 28$ ) and CRL ( $N = 17$ ) were the lipases that showed the highest co-occurrence. The use of different lipases in the same publication may be related to the development of new methods of immobilization and/or stabilization of the final biocatalyst, making it necessary to validate these strategies with different lipases that have different characteristics. In addition, this may demonstrate the need to develop a library of biocatalysts with suitable

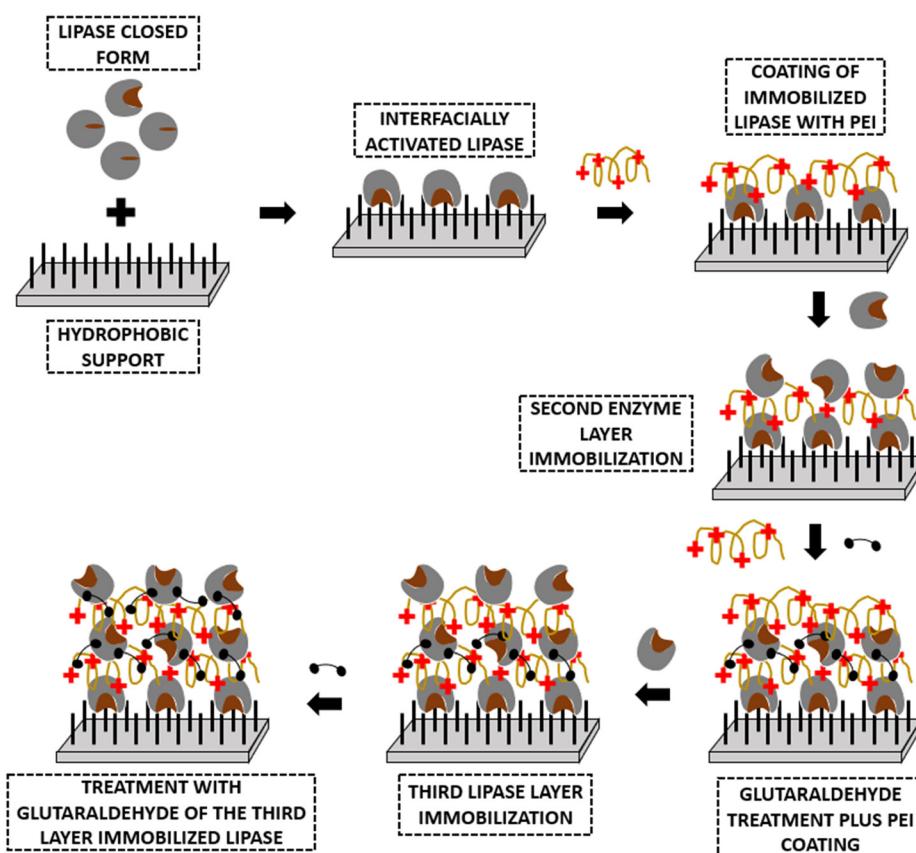
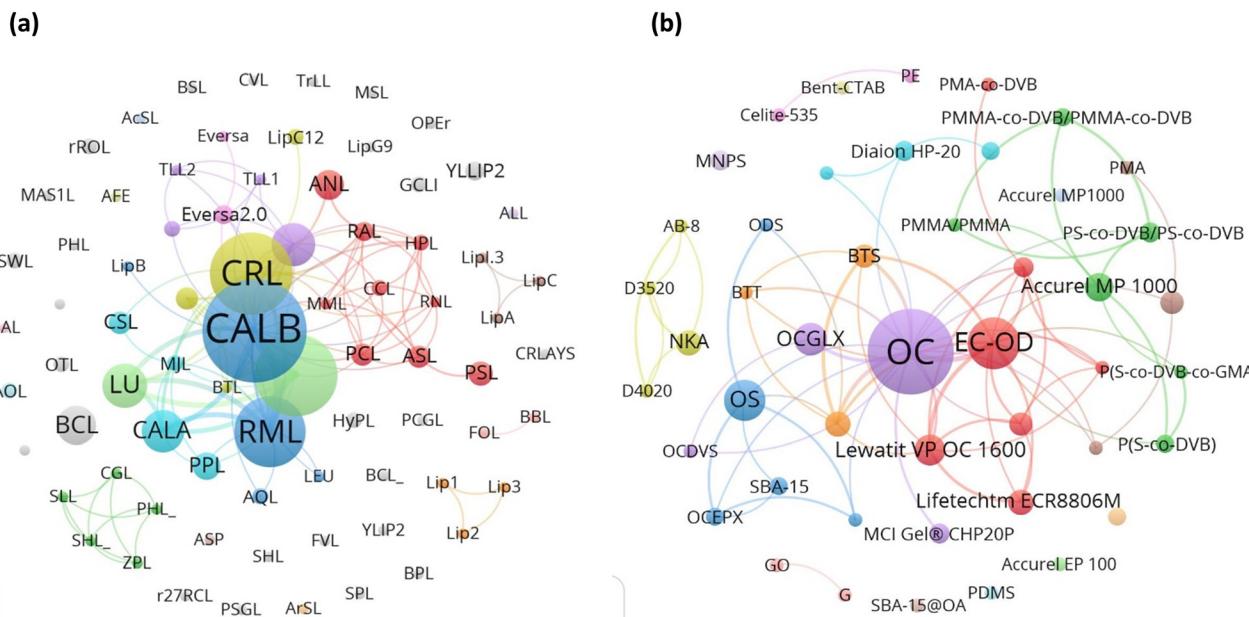


Fig. 7 Use of PEI as an adhesive agent for the construction of a lipase multilayer biocatalyst.



**Fig. 8** Network analysis of lipases (a) and hydrophobic supports (b) addressed in our dataset for enzymatic immobilization by interfacial adsorption. The size of the node and the thickness of the edges are proportional to the number of occurrences.

catalytic properties for the industrial process to be studied.<sup>120,122</sup>

As can be seen in this systemic analysis, several studies are focused on the immobilization of CALB, TLL, CRL, RML, PFL and CALA for application in industrial processes or studies of new stabilization strategies for these enzymes. However, Eversa Transform is a new lipase on the market that has been gaining pace in several applications in aqueous and organic media but only presented 3 co-occurrences. This is an enzyme that emerged as an improved version of TLL and has low cost compared to the mentioned enzymes, high thermal stability and greater resistance to the presence of methanol.<sup>62</sup> Another enzyme worth mentioning is PPL, which presented only one co-occurrence. This enzyme has a very low cost, so this factor associated with the immobilization technique by interfacial adsorption can minimize the costs of the process and the simultaneous purification and immobilization of this enzyme may be carried out. The main limitation is its specificity, since it is a 1,3-specific lipase.<sup>63</sup>

In Fig. 8b, a co-occurrence network between hydrophobic supports occurs in ~30% of publications. This result suggests that the use of supports with different degrees of hydrophobicity served as priorities in studies to obtain biocatalysts with different catalytic properties. We found a high number of co-occurrences between commercial and non-commercial supports ( $N = 22$ ). This demonstrates two possible issues: the first would be the use of commercial supports to validate the advantages of non-commercial supports; the second would be the use of commercially pre-existing supports, aiming at easing their acquisition and, possibly, reducing process costs. There is also the co-occurrence of heterofunctional supports ( $N = 5$ ) and the occurrence of many supports prepared using materials that

are agro-industrial residues, although there is no co-occurrence between them. Even though supports prepared from agro-industrial residues are evident, there is a gap and there is a need to explore other materials, such as glycerol, lignin, and soybean husk, among others. These residues can be used for the synthesis of activated carbon, are rich in oxygen and nitrogen surface groups, and can be used *in natura* or activated with desired reactive groups.<sup>136,137</sup>

## Limitations

Our strategy was mapped using two Web of Science databases (SCI-E and ESCI), Scopus, PubMed and SciELO. We chose not to use scientific evidence, such as theses and dissertations, committee or government reports, documents from congresses, conferences and seminars, or ongoing research. This limits it to a set of primary data published in scientific journals; that is, the information extracted from publications obtained in the bibliographic survey may have been underestimated. Thus, our conclusions must be evaluated under these circumstances. However, the contribution of this review is very important to show the direction of research related to the immobilization of lipases on hydrophobic supports.

## Conclusions

The results presented in this research demonstrate a systematic review of the immobilization of lipases on hydrophobic supports. Here, a wide variety of lipases with different applicability are presented. It is not possible to obtain an optimal universal biocatalyst for use in different applications. However, the choice of support and enzyme

immobilization and stabilization strategies are factors that can influence the catalytic properties and final stability of the immobilized biocatalyst. Various supports with different degrees of hydrophobicity are presented. Furthermore, problems related to immobilization by interfacial adsorption and the strategies used to overcome them are described. Immobilization on a heterofunctional support and crosslinking with physical or chemical reagents are the main solutions to this problem. A co-occurrence analysis between the main lipases and supports in our database was performed. Furthermore, the structure, network distribution and frequency of co-occurrence between lipases and supports to determine possible hotspots and as yet unexplored frontiers of knowledge were elucidated. Therefore, the contribution of this review is very important for showing the direction of research related to the immobilization of lipases on hydrophobic supports, their bottlenecks and solutions to problems.

## Author contributions

J. R. G.: conceptualization, methodology, investigation, visualization, formal analysis, writing – original draft, review and editing. K. S. G. C. O.: visualization, formal analysis, review and editing. M. C. P. G.: visualization, formal analysis. J. P. R.: methodology, visualization, formal analysis; L. A. L.: visualization, formal analysis. A. B.-M.: visualization, formal analysis, review and editing. R. F.-L.: resources, conceptualization, methodology, writing – review and editing, supervision. P. W. T.: resources, conceptualization, methodology, writing – review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

The authors declare no conflict of interest.

## Acknowledgements

This work was funded by São Paulo Research Foundation (FAPESP, grant number 2016/10636-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Finance Code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, project numbers 405889/2016-0 and 308212/2017-7), Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (project PID2022-136535OB-I00 and MCIN/AEI/10.13039/501100011033) and by FEDER A Way of Making Europe funds (project PID2021-123079OB-I00), and Generalitat Valenciana (CIPROM/2021/70).

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