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REVIEW ARTICLE

A systematic review about affinity tags for one-step purification and immobilization of recombinant proteins: integrated bioprocesses aiming both economic and environmental sustainability

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Abstract

The present study reviewed and discussed the promising affinity tags for one-step purification and immobilization of recombinant proteins. The approach used to structure this systematic review was The Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) methodology. The Scopus and Web of Science databases were used to perform the bibliographic survey by which 267 articles were selected. After the inclusion/exclusion criteria and the screening process, from 25 chosen documents, we identified 7 types of tags used in the last 10 years, carbohydrate-binding module tag (CBM), polyhistidine (His-tag), elastin-like polypeptides (ELPs), silaffin-3-derived pentalysine cluster (Sil3k tag), N-acetylmuramidase (AcmA tag), modified haloalkane dehalogenase (HaloTag®), and aldehyde from a lipase polypeptide (Aldehyde tag). The most used bacterial host for expressing the targeted protein was *Escherichia coli* and the most used expression vector was pET-28a. The results demonstrated two main immobilization and purification methods: the use of supports and the use of self-aggregating tags without the need of support, depending on the tag used. Besides, the chosen terminal for cloning the tag proved to be very important once it could alter enzyme activity. In conclusion, the best tag for protein one-step purification and immobilization was CBM tag, due to the eco-friendly supports that can be provided from industry wastes, the fast immobilization with high specificity, and the reduced cost of the process.

Keywords Cloning vectors · Enzyme immobilization · Expression host · Fusion tag · Plasmid label · Single-step purification

Introduction

Biocatalysis performed by enzymes has been widely used in industrial bioprocesses, since the use of enzymes presents process advantages, which enable its application. The main reasons for applying enzyme biocatalysis are the low energy expenditure and the environmental appeal, causing an increase in process sustainability when compared to other methods (Saldarriaga-Hernández et al. 2020). Although enzymes present various advantages in several biotechnological applications, they also exhibit a high-cost production that impairs its industrial use (Jemli et al. 2014). Aiming to reduce process costs, the heterologous production of proteins using recombinant DNA technology has been employed as a promising way to improve enzyme productivity in large-scale production (Liu et al. 2020; Vo-Nguyen et al. 2022).

This technology allows the cloning of genes of interest into plasmid vectors to perform the heterologous expression of its respective protein in a host (Bernal et al. 2018). Several systems as prokaryotic, *Escherichia coli* (*E. coli*) (Bernardes et al. 2019; Zhang et al. 2020a), and eukaryotic cells, *Aspergillus* spp. (Ichikawa et al. 2022), *Saccharomyces cerevisiae* (Qi et al. 2020), and *Pichia pastoris* (Wang et al.

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2019; Garrigós-Martínez et al. 2021) are used for protein expression using recombinant technology. The recombinant enzyme expression by fast-growing hosts, such as *E. coli*, may enhance process efficiency (Asemoloye et al. 2021).

The industrial application of enzymes, mainly used in food and pharmaceutical processes, requires previous purification steps to separate the protein of interest from other existing metabolites in the crude cell lysate or from culture medium components (Trabelsi et al. 2019). Protein purification was performed by different classic methods for many years, such as chromatography (Peschke et al. 2019) and ultrafiltration (Lin et al. 2019). Even though recombinant protein technology enhances process feasibility, the use of purified enzymes in free form is also related to high-cost bioprocess and presents some challenges like efficient storage, reusability—due to the difficult separation from the substrate—and stability (Olajuyigbe et al. 2019; Huang et al. 2020; de Andrade et al. 2021).

Immobilization corresponds to the binding of an enzyme onto the surface of an insoluble matrix (Liang et al. 2017; Kumar et al. 2019; Srinivasan et al. 2020) or may be related to the formation of self-assembled complexes without the use of supports (Peschke et al. 2017; Lin et al. 2020). In this respect, the immobilization technique is widely performed with the recombinant DNA technique to promote industrial enzyme applications (Bloess et al. 2019). The immobilization may be performed in two ways, using affinity markers to direct the binding of the support onto a specific site of the enzyme (Han et al. 2021), or without tags, randomly binding or subtly directing the bind of the support to the enzyme (Zhang et al. 2021a).

Affinity tags are peptides, amino acid sequences or protein domains that are used for oriented immobilization, such as polyhistidine (His-tag), carbohydrate-binding modules (CBM), and elastin-like polypeptides (ELPs). The tags are cloned into the plasmid during the cloning process, at the N or C-terminal of the enzyme, or pre-exist in its structure (Sun et al. 2017; Chang et al. 2018; Flores et al. 2022). The main reason to apply protein oriented immobilization is the facilitated purification and possibility of reuse, due to the fact that tags bind the enzyme to a support which has affinity to the marker (Xue et al. 2019).

The use of recombinant DNA techniques combined with oriented immobilization, in some cases, can provide the one-step purification and immobilization of the enzyme with the use of affinity tags, making the process a more efficient, time-saving, and cost-effective method, compared to traditional procedures that are usually very expensive and complex (Qin et al. 2019; Tian et al. 2021). Freitas et al. (2022a) conducted a review about the one-step purification and immobilization of enzymes using affinity tags. However, the authors did not focus only on simultaneous purification and immobilization bioprocesses, they presented tags for either only purification or only immobilization, and mainly described protein-engineered advanced materials. So, this review article aims to present the studies that performed tag-mediated single-step purification and immobilization, providing an update on the most used affinity tags, their supports, and methods used for simultaneous purification and immobilization, and the respective advantages or disadvantages. Furthermore, this review provides new trends about simultaneous immobilization and purification of recombinant enzymes, considering its cost and time advantages, since these bioprocesses are still usually performed in multiple steps.

Methodology

The approach used to organize this review article was The Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) methodology (Salameh et al. 2020). The bibliographic survey was performed in two databases, Scopus and Web of Science, using the following keywords, (protein* OR enzyme*) AND ("tag*" OR "affinity tag*" OR "affinity binder*" OR "affinity marker*") AND ("protein immobilization" OR "enzyme immobilization" OR "oriented immobilization") AND ("protein purification" OR "enzyme purification") AND ("recombinant protein*" OR "recombinant enzyme*") AND ("expression" OR "recombinant expression") NOT (antibod*).

The eligibility criteria used were (a) year: articles between 2011 and 2022 were selected; (b) document type: articles and reviews were included; (c) language: only articles written in English were included; and c) source type: only journal articles were selected. The article screening process was initially performed by reading the article abstracts; those which did not present results related to the aim of this review were excluded. The selected articles were read in full and screened again, those that did not present methodologies and results with the same criteria were excluded. The results were arranged by type of tag used for the one-step purification and immobilization of recombinant enzymes and the data were tabulated.

Results and discussion

The search in Scopus using the keywords resulted in 265 documents, while the search in Web of Science resulted in only 2. Using the inclusion/exclusion criteria, the number of Scopus documents was reduced to 171 articles, and the documents of Web of Science were reduced to 1. There were no duplicated documents, resulting in a total of 172 articles. These articles were subjected to a screening trial. The first screening resulted in 38 selected articles, and the

main reason for exclusions was for those articles that did not present the protein purification process in only one step and those that did not purify and immobilize the enzyme simultaneously. Papers that did not explain whether the purification and immobilization process complied with the above criteria in the abstract were subjected to the second trial, which was based on reading the full documents. This final process selected 25 articles out of these 38. Figure 1 describes the document's selection steps through the PRISMA method, including the identification and the screening process.

The selected studies performed single-step purification and oriented immobilization mediated by affinity tags. The oriented immobilization method, demonstrated in these articles, has been used in many cases aiming at its application in large-scale production in the industry due to its advantages, such as increased enzyme activity and stability (Zhou et al. 2021b), and allowing the reuse of immobilized enzymes in catalysis cycles for successive batches (Pagolu et al. 2021). Moreover, the purification of enzymes is substantial for its application in some industry bioprocesses (Tasgin et al. 2020). In these papers selected through PRISMA, the authors were able to purify and immobilize the enzyme in just one step, uniting two important approaches, reducing the cost and time of the processes.

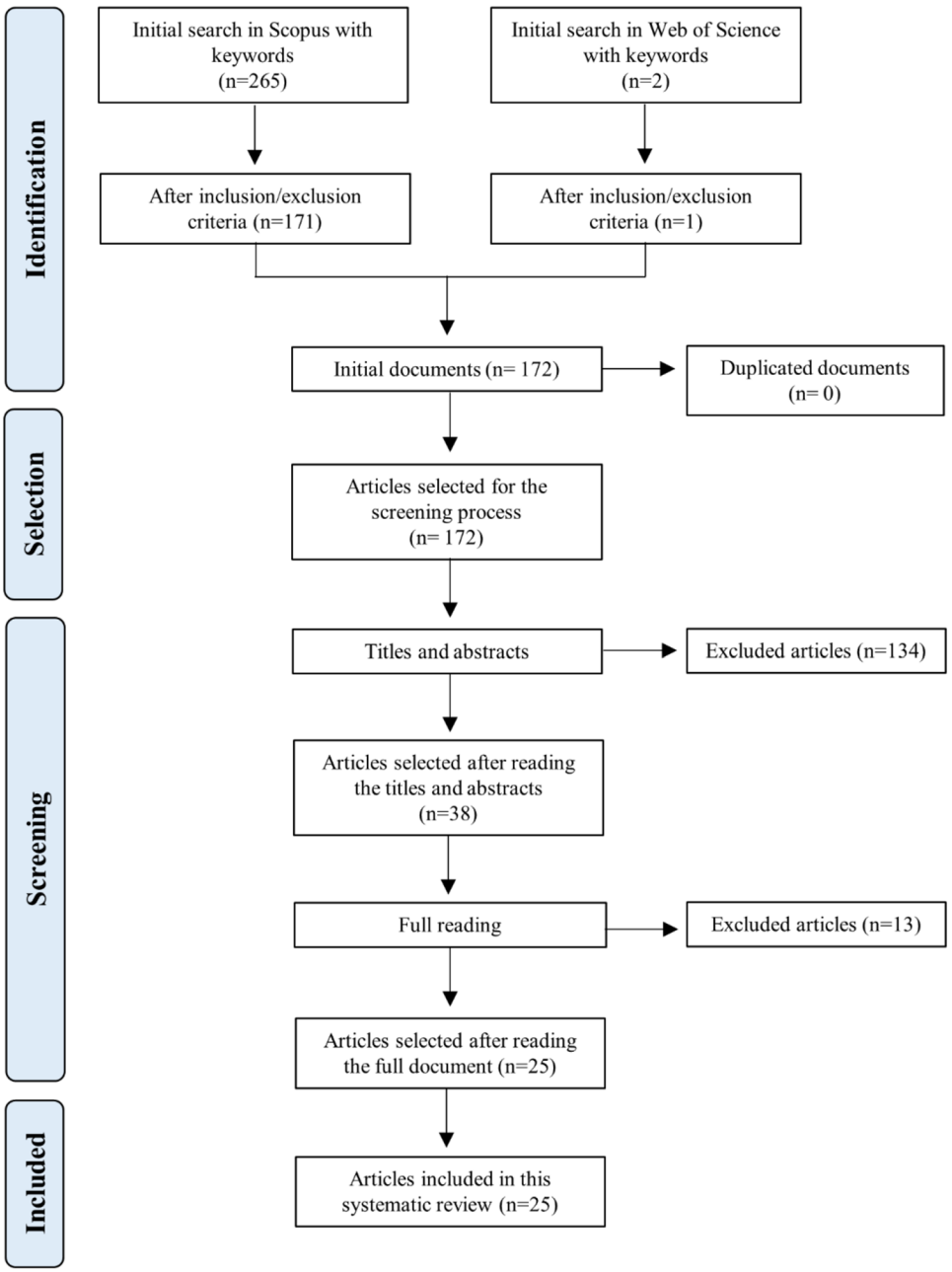

Fig. 1 Research flowchart describes the steps employed for article selection through the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) methodology

After analyzing the articles selected for this review, the use of expression vectors to express the recombinant proteins with the chosen tags was analyzed. Figure 2 shows the quantification of plasmids used in the studies, among the research articles that reported this information. It was observed that seven studies used the plasmid pET-28 as the expression vector, two used pET-35, other two used pETDuet-1, and a couple of authors chose both pCG and pCIG vectors. Each of the remaining studies used different plasmids.

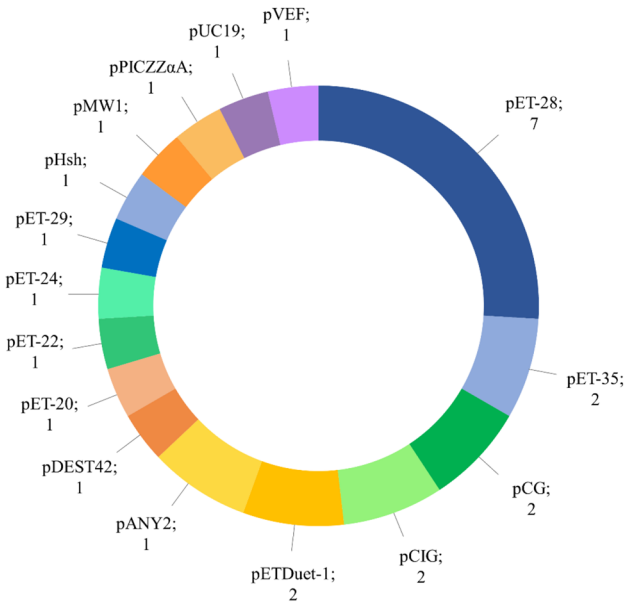

Fig. 2 Distribution of the number of expression vectors used in studies selected through the bibliographic search (Preferred Reporting Items for Systematic Review and Meta-analysis methodology—PRISMA) that performed tag-mediated one-step purification and immobilization of recombinant enzymes

Furthermore, the selected documents provided relevant information about the tags used in single-step purification and immobilization per year, between 2011 and 2022, as shown in Fig. 3. The most used tag was CBM, which was mentioned in nine studies in 7 different years, mostly in 2022. The second most employed tag was His-tag, used eight times, and ELPs tag used in a couple of studies.

The main results of one-step purification and immobilization of targeted enzymes—protein, plasmid, expression strains, affinity tags, and supports—from research articles published between 2011 and 2022 are shown in Table 1. Table 1 shows that approximately 91.67% of the selected articles used *E. coli* cells for enzyme expression and, among them, 77.27% used *E. coli* BL21 strains. *E. coli* is the most widely used system to express recombinant proteins in bacterial cells because of its rapid growth and ability to incorporate foreign genes for expressing the recombinant protein (Kaur et al. 2018; Asemoloye et al. 2021).

One of the disadvantages of using *E. coli* strains for expressing proteins is the formation of insoluble aggregates, inclusion bodies. These aggregates are expressed due to the accumulation of folding intermediates during recombinant protein expression, mainly caused by the high speed of growth. The main disadvantage of expressing inclusion bodies corresponds to the formation of a large proportion of denatured, non-active, and unfolded protein (Kaur et al. 2021; Hu et al. 2021). *Pichia pastoris* was also used for expressing a recombinant protein. This eukaryotic is one of the promising systems for recombinant enzymes production, due to its commercial availability, high capacity for

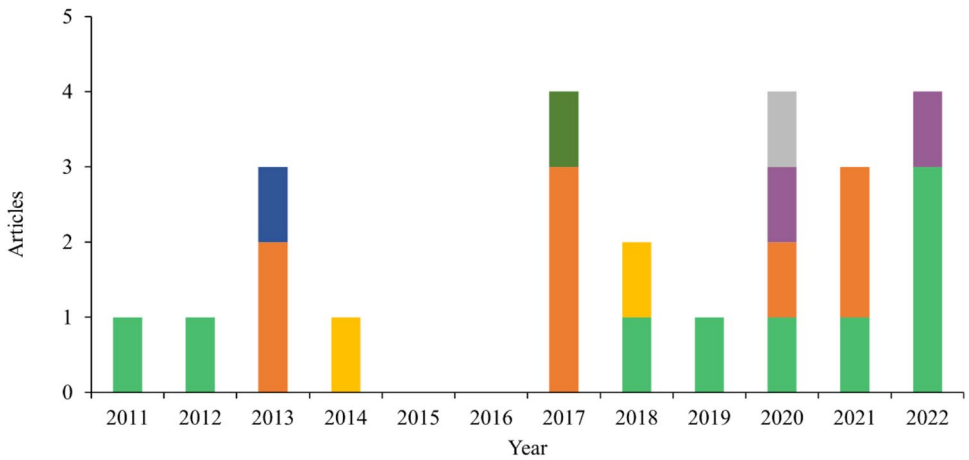

Fig. 3 Affinity markers used in articles that reported one-step purification and immobilization. The articles were selected through the bibliographic survey (Preferred Reporting Items for Systematic Review and Meta-analysis methodology—PRISMA) per year between 2011 and 2022. Legend chart: light green square: carbohydrate-binding module; purple square: aldehyde tag; dark green square: Halotag®; grey square: *N*-acetylmuramidase; orange square: polyhistidine; pink square: silaffin-3-derived pentalysine cluster; yellow square: elastin-like polypeptides

Table 1 Recombinant enzyme, expression vector, and microorganism chosen for recombinant DNA technology, affinity binder selected for enzyme fusion, and support designated for protein immobilization from studies selected through Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) methodology

Enzyme or complex	Affinity tag	Expression plasmid	Expression microorganism	Support	References
Alcohol dehydrogenase	Carbohydrate-binding modules	pVEF[b]	*E. coli* M15Δ*gly*A	Regenerated amorphous celulose	Benito et al. (2022)
Lipase A	Silaffin-3-derived pentalysine cluster	pETDuet-1[b]	*E. coli* Rosetta-2(DE3)	Diatom biosilica	Abdelhamid et al. (2022)
β-Galactosidase	Cellulose-binding domain	pET-35b(+)[a]	*E. coli* BL21(DE3), C41(DE3), and Rosetta(DE3)	Microcrystalline cellulose, nanocellulose obtained by alkaline hydrolysis, nanocellulose obtained by acid route and solvent-treated cellulose	Gennari et al. (2022a)
β-Galactosidase	Cellulose-binding domain	pET-35b(+)[a]	*E. coli* BL21(DE3), C41(DE3), and Rosetta(DE3)	Microcrystalline magnetic cellulose produced by different methods	Gennari et al. (2022b)
Self-assembly multienzyme complex (α-glucan phosphorylase, phosphoglucomutase, inositol 1-phosphate synthase, and inositol monophosphatase)	Carbohydrate-binding module 3	pET-20b[b]	Uninformed	Surface of *E. coli* cells	Liu et al. (2021)
β-Xylanase XynII	Polyhistidine	pANY2[a]	*E. coli* BL21(DE3)	Curdlan-based and Ca^{2+}-chelated magnetic microspheres	Zhang et al. (2021b)
Glucose dehydrogenase	Polyhistidine	Uninformed	*E. coli* BL21(DE3)	$NiFe_2O_4$ magnetic nanoparticles	Zhou et al. (2021a)
Catalase	Silaffin-3-derived pentalysine cluster	pETDuet-1[b]	*E. coli* Rosetta-2(DE3)	Bare silica particles	Abdelhamid et al. (2020)
L-Glutamate oxidase	Chitin-binding domains	pET-29a[b]	*E. coli* BL21(DE3)	Ultrasonic wave chitin powder	Zhou et al. (2020)
Peptide-N-glycosidase F	Polyhistidine	pET-28a[b]	*E. coli* BL21(DE3)	Magnetic particles modified with IDA-Ni^{2+}	Zhang et al. (2020b)
α-Amylase	N-acetylmuramidase	pET-28a(+)[b]	*E. coli* BL21(DE3)	Gram-positive enhancer matrix particles	Zhao et al. (2020)
Fructosyl peptide oxidase	Cellulose-binding domain	pET-24a[b]	*E. coli* BL21(DE3)	Bacterial cellulose nanofibrils	Chen et al. (2019)
Xylanase–lichenase chimera (multienzyme chimera)	Elastin-like polypeptides	pET-22b(+)[b]	*E. coli* BL21(DE3)	No support	Lin et al. (2018)
β-Glucosidase	Cellulose-binding module 3	PPICZαA[b]	*Pichia pastoris* KM71H	Regenerated amorphous celulose	Hu et al. (2018)
Hydantoinase and carbamoylase	Polyhistidine	pDEST42, pET-28a and pMW1[b]	*E. coli* BL21(DE3)	Cobalt functionalized magnetic beads, Dynabeads®	Slomka et al. (2017)
Pullulanase	Polyhistidine	PHsh[b]	*E. coli* JM109	Nickel (II)-modified magnetic nanoparticles	Wang et al. (2017)
Benzaldehyde lyase	Halotag®	pET-28[b]	*E. coli* BL21(DE3)	HaloLink™ resins (Sepharose® beads)	Döbber and Pohl (2017)
ω-Transaminase	Polyhistidine	pET-28a[a]	*E. coli* Rosetta(DE3)	Ni^{2+}-nitrilotriacetic acid functionalized magnetic mesoporous silica nanoflowers	Cao et al. (2017)

Table 1 (continued)

Enzyme or complex	Affinity tag	Expression plasmid	Expression microorganism	Support	References
Xylanase	Elastin-like polypeptides	PET-22b[b]	*E. coli* BL21, and *E. coli* BLR(DE3)	No support	Li and Zhang (2014)
Fructosyl peptide oxidase	Polyhistidine	pET-28b(+)[a]	*E. coli* BL21	Uniform magnetic core/shell microspheres functionalized with Ni^{2+}	Zhang et al. (2013)
Lipase	Aldehyde tag	pET-28b(+)[b]	*E. coli* BL21(DE3)	Amino-functionalized mesocellular siliceous foam support	Wang et al. (2013)
Benzaldehyde lyase	Polyhistidine	pUC19[b]	*E. coli* BL21(DE3)	Heterofunctional chelate-epoxy modified magnetic nanoparticles	Tural et al. (2013)
Thermophilic polyphosphate glucokinase	Cellulose-binding module 3	pCG and PCIG[b]	*E. coli* BL21Star(DE3)	Regenerated amorphous cellulose	Liao et al. (2012)
Putative phosphoglucose isomerase	Cellulose-binding module 3	pCG and PCIG[b]	*E. coli* BL21Star(DE3)	Regenerated amorphous cellulose	Myung et al. (2011)

E. coli Escherichia coli, *Halotag*® modified haloalkane dehalogenase

[a]Tag pre-exists in plasmid structure

[b]Tag cloned into plasmid.

large-scale production (Lima-Pérez et al. 2018; Taghizadeh et al. 2021), and production of secretory proteins, directly into the culture medium (Türkanoğlu Özçelik et al. 2019).

Carbohydrate binding module tags

CBM tags correspond to the most used affinity binders for protein single-step purification and immobilization in the last 10 years. These tags are a general group that includes carbohydrate-binding module tags (Danaeifar et al. 2022), cellulose-binding domain tags (also known as cellulose-binding module tags) (Yu et al. 2017), and chitin-binding domain tags (Godigamuwa et al. 2021).

Different supports and enzymes were used to perform each study reported in this review. Benito et al. (2022) reported the application of a CBM tag from family 3 (CMB3) and from family 9 (CBM9) for targeting a *Saccharomyces cerevisiae* alcohol dehydrogenase. The resulting fusion enzyme was used to compare this system to a His-tagged alcohol dehydrogenase. The immobilization/purification step occurred using regenerated amorphous cellulose (RAC) as support in bioreactor fed-batch experiments. The cloning process used the pVEF plasmid and the targeted enzyme was expressed in *E. coli* M15Δ*gly*A. The retained activity corresponded to 86.1% for CBM3 and 97.7% for CBM9, presenting an immobilization yield of 98.4% (CBM3) and 99.7% (CBM9). The specific activity using His was higher than CBM alcohol dehydrogenase. The CBM enzymes proved to be suitable for single-step purification and immobilization, while the CBM9 was successful for the purification process.

Employing the same support used above, Hu et al. (2018) purified and immobilized a *Thermoascus aurantiacus* IFO9748 β-glucosidase fused to a CBM3 tag. The process employed pPICZαA plasmid and *Pichia pastoris* KM71H. After 15 min, 96.0% enzyme was immobilized onto support. Moreover, after 30 cycles of reuse, 96.9% and 98.9% of the substrates (daidzin and genistin, respectively) could still pass through the hydrolyzing process. In 2011, another study performed by Myung et al. (2011) employed the same RAC support using a putative *Clostridium thermocellum* phosphoglucose isomerase also target to a CBM3 tag. The plasmids pCIG and pCG, and the *E. coli* BL21Star (DE3) strain were used. The obtained immobilized enzyme was extremely stable at the temperature of 60 ºC.

Additionally, the CBM3 tag was applied for the immobilization of a thermophilic polyphosphate glucokinase (*Thermobifida fusca* YX) onto RAC. The *E. coli* BL21Star (DE3) strain and the vectors pCG and pCIG were used. This CBM-enzyme system is the first thermostable polyphosphate glucokinase described, with improved stability as well. This better stability was observed at 55 °C, pH 9.0, in the presence of magnesium ions (Liao et al. 2012). Moreover, Chen

et al. (2019) purified/immobilized a fructosyl peptide oxidase fused to a cellulose-binding domain on bacterial cellulose nanofibrils. The expression system was based on the pET-24a plasmid and *E. coli* BL21(DE3) cells. The enzyme/support interaction occurred fast, binding 0.24 mg targeted enzyme to 1 mg cellulosic support.

In addition, another study used the respective tag to immobilize a β-galactosidase (*Kluyveromyces* sp.) in four different supports: nanocellulose obtained by alkaline hydrolysis, microcrystalline cellulose, solvent-treated cellulose, and nanocellulose obtained by acid route. The authors chose pET-35b(+) and *E. coli* BL21(DE3), C41(DE3), and Rosetta(DE3) for expressing the fusion proteins. In this study, the load of 150 U/$g_{support}$ demonstrated 55–106 U/$g_{support}$ activity. The enzymatic activity was between 53.0 and 64.0% after 40 cycles of reuse (Gennari et al. 2022a). Gennari et al. (2022b) performed another investigation about the fusion of the same enzyme, tag, and expression system, using microcrystalline magnetic cellulose produced by different methods. The chosen load for immobilization was 30 U/$g_{support}$, based on the highest yields. Both microcrystalline celluloses showed 90.0% efficiency in immobilizing the protein. The enzyme demonstrated 50.0% of activity after 15 cycles of reuse.

Aiming to perform a biofilm-mediated immobilization, Liu et al. (2021) reported a self-assembly multienzyme complex composed of α-glucan phosphorylase, phosphoglucomutase, inositol 1-phosphate synthase, and inositol monophosphatase using a carbohydrate-binding module 3 affinity marker fused to pET-20b plasmid. These enzymes self-assembled into a complex through the specific interaction between dockerins and cohesins. The complex was immobilized on the surface of *E. coli* cells, due to the interaction between SpyTag and SpyCatcher. The biofilm-immobilized enzyme presented high recycling ability and showed a significant increase (4.28×) in initial reaction rate for producing myo-inositol from maltodextrin.

Employing a different type of CBM, a chitin-binding domain (ChBD), Zhou et al. (2020) fused a L-glutamate oxidase and immobilized it on chitin powder. The vector and strain used to express the recombinant protein were pET-29a and *E. coli* BL21(DE3), respectively. The immobilized enzyme showed better thermal and pH stability than the free oxidase.

The related cellulosic and chitin supports demonstrate many application advantages. These materials contribute to the low cost of the process compared to traditional purification methods and, due to their natural matrix, they are considered eco-friendly (Chávez-Guerrero et al. 2018; Yang et al. 2020; Maaloul et al. 2020). Besides being natural materials, cellulose can also be obtained from industry residues as rice husk (Rashid and Dutta 2020), carrot (Berglund et al. 2016), walnut shell (Hemmati et al. 2018), sugarcane bagasse (Ejaz et al. 2020), pomegranate peel, corncob, grape stalk, corn husk, marc of strawberry-tree fruit and fava pod (Vallejo et al. 2021). This wide range of residues demonstrate the environmental appeal of cellulosic supports, once they can be obtained from industrial wastes. Furthermore, CBM tags, in general, present a high binding affinity and specificity (Jia et al. 2021), the main features for performing tag-mediated single-step purification with simultaneous immobilization.

Polyhistidine tags

His-tags are amino acid sequences that usually contain six histidine residues and are widely used as affinity markers for recombinant enzyme technology (Freitas et al. 2022b). Histidine binders are commonly used with magnetic supports (Coscolín et al. 2018); therefore, magnetic nanoparticles have been significantly used (Shirzadi et al. 2020). Zhou et al. (2021a) demonstrated the functionalities of the fusion between a His-tag and a glucose dehydrogenase expressed in *E. coli* BL21(DE3). The immobilization was performed using $NiFe_2O_4$ magnetic nanoparticles. As a result, the highest activity recovery was 71.4%. The immobilized enzyme showed better pH and thermal stability and could be reused for ten cycles retaining more than 65.0% of its initial activity.

Wang et al. (2017) reported that using His fused to a pullulanase (*Anoxybacillus* sp. WB42), it was possible to perform a single-step immobilization/purification. The study showed the differences between a N-terminal and a C-terminal His-tag. The His-pullulanase system was expressed in *E. coli* JM109 using the pHsh vector. Different core shell structured nickel (II)-modified magnetic nanoparticles were responsible for adsorbing the enzyme, immobilizing it. The N-terminal His-tag showed a higher reusability than the C-terminal His. After six successive batches, immobilized N-terminal-His-pullulanase retained 100.0% from its initial activity, while the C-terminal one retained 75.0%. This information proves that the position of the tag plays a critical role in the enzyme properties, such as reusability or pH and thermal stability. Moreover, chelate-epoxy-modified magnetic nanoparticles were developed to immobilize a *Pseudomonas fluorescens* Biovar I benzaldehyde lyase fused to a His-tag in pUC19 plasmid and expressed in *E. coli* BL21(DE3). This system enhanced enzyme stability and activity (Tural et al. 2013).

According to Zhang et al. (2021b), Ca^{2+}-chelated and curdlan-based magnetic microspheres can be used to perform the immobilization of a *Trichoderma reesei* QM6a β-xylanase XynII labeled with a histidine marker. The protein expression was performed using pANY2 vector, containing the His-tag in its structure, and *E. coli* BL21(DE3). The immobilized enzyme presented a better performance in buffers containing solvents and metal ions than the free

enzyme, and also showed an enhancement in storage and thermal stability. After 35 days of storage, the immobilized xylanase retained 82.0% of its initial activity. The enzyme also showed 60.0% of activity after ten cycles of reuse.

Zhang et al. (2013) performed the immobilization of a His-tagged fructosyl peptide oxidase onto magnetic core/shell microspheres functionalized with Ni^{2+}. *E. coli* BL21 strains were used to express the target protein cloned into the pET-28b(+) vector. The microspheres were capable of separating the histidine enzymes with a high performance, presenting a binding capacity of 103 $mg_{enzyme}/g_{support}$.

Aiming to perform one-step purification of a peptide-N-glycosidase F fused to a histidine tag, Zhang et al. (2020b) immobilized the enzyme on magnetic particles modified with iminodiacetic acid-Ni^{2+}. *E. coli* BL21(DE3) and pET-28a were used as the expression system and the enzyme was expressed in the form of inclusion bodies aiming to increase its yield. The immobilization was responsible for improving the thermal stability of the enzyme. The enzyme also could be reused for five cycles exhibiting an excellent remaining activity. The targeted protein showed a binding capacity as high as 120 mg/g. The authors also demonstrated that, combined with microwave irradiation, the glycoprotein deglycosylation could be done faster with oriented immobilization of the enzyme.

Moreover, a *Arthrobacter crystallopoietes* DSM20117 hydantoinase and a carbamoylase were immobilized on cobalt functionalized magnetic beads, Dynabeads®, using a C-terminal His-tag (Slomka et al. 2017). A codon-optimized hydantoin was also expressed by adding an N-terminal SBP-tag. The expression system used *E. coli* BL21(DE3) strain, and pDEST42, pET-28a, and pMW1 plasmids. The activity assay showed the highest value for the codon-optimized immobilized hydantoin with the value of 105.47 mU/$mg_{substrate}$ about the hydrolysis of hydroxymethylhydantoin, demonstrating an increase in specific activity by 200.0% compared to the free enzyme. The carbamoylase showed its highest specific activity of 12.91 U/$mg_{substrate}$ using *N*-carbamoylphenylalanine as substrate. Cao et al. (2017) produced Ni^{2+}-nitrilotriacetic acid functionalized magnetic mesoporous silica nanoflowers for single-step purification and immobilization of a His-tagged ω-transaminase. The enzyme was cloned into pET-28a and expressed in *E. coli* Rosetta (DE3). The immobilized protein demonstrated a better thermal and pH stability, and also presented a desirable storage stability compared to the free one. The immobilized enzyme retained 67.38% of initial activity after being reused for 12 cycles with a protein loading of 35.54 $mg/g_{support}$.

Therefore, His-tags correspond to one of the most used markers for one-step purification and immobilization. His-tags are usually employed in studies with nickel supports due to their high affinity (Rashid et al. 2017). In addition, other metals can be used to immobilize His-proteins, such as cobalt (Auer et al. 2017), copper, and zinc (Ge et al. 2021). In general, histidine binders provide better stability for the targeted enzyme, in terms of pH, temperature, and storage, and present a low-cost process, which may be positive aspects for industrial applications.

Elastin-like polypeptides tags

ELPs are a type of peptide tags, specifically polymers composed of pentapeptide (Val-Pro-Gly-X-Gly) repeats, where X corresponds to a variable amino acid, except for proline (Roberts et al. 2015). ELPs are used for enzyme immobilization, usually without support due to the capacity of ELPs to self-assemble into insoluble aggregates (Wu et al. 2016). Li and Zhang (2014) immobilized a xylanase using ELP at the C-terminal. The tag was self-assembled into an insoluble particle at a specific temperature by inverse transition cycling (ITC) method, meaning that the tag is responsible for carrying the immobilization without requiring any support. The enzyme was expressed in *E. coli* BL21(DE3) and *E. coli* BLR(DE3) using pET-22b vector. The immobilized xylanase was responsible for 92.0% of the total enzyme activity, and 81.0% of the native enzyme (without ELP) activity could be harvested in the active insoluble form.

In addition, another study performed one-step purification and immobilization of two enzymes, a xylanase-–ichenase chimera, via SpyTag/SpyCatcher reaction (Lin et al. 2018). The multicomplex was formed by xylanase and lichenase attached to a SpyTag and another chimera connecting to ELPs and a SpyCatcher. The chimera self-assembled into an insoluble active particle during the purification process by ITC protocol. The marker responsible for the purification and self-assembled immobilization was the ELPs tag. The chosen enzymes were cloned into pET-22b(+) and expressed in *E. coli* BL21(DE3). After ten cycles of reuse, the xylanase retained 44.0% of its initial activity and lichenase, 56.0%. In addition, the multicomplex showed higher thermal stability than the free enzymes. The industrial importance of this study is related to the enzymatic breakdown of the proteins, producing xylan and lichenan that may be converted into bioethanol.

Due to the fact that ELPs did not need any support carrier for the simultaneous immobilization and one-pot purification, they represent, through an economic point of view, a time and cost-efficient process, easy to scale-up.

Other types of tags

The silaffin-3-derived pentalysine cluster (Sil3K) from *Thalassiosira pseudonana*, a peptide with the sequence KAAKIFKGKSGK, and its mutant penta-arginine peptide, Sil3R, RAARIFRGRSGR, were used to perform the immobilization of a *Bacillus subtilis* catalase. These

sequences were cloned into pETDuet-1 and expressed in *E. coli* Rosetta-2 (DE3), using bare silica particles. The Sil3K-catalase showed a catalytic activity of 2724 ± 75 U/$mg_{protein}$ while the catalase, without the tag, showed 2696 ± 80 U/$mg_{protein}$. These results suggest that the fused Sil3K tag did not interfere in enzyme features. The Sil3K-catalase showed an enhancement in enzyme thermal stability and reusability, compared to the free and the Sil3R enzyme, and also demonstrated a better enzyme activity recovered after immobilization. The Sil3K enzyme retained 72.0% of its initial activity after five cycles of reuse (Abdelhamid et al. 2020).

The same authors performed another study using the Sil3K and Sil3R tags and another one named Sil3H, a mutant variant from Sil3K, composed of a penta-histidine peptide. They used *Bacillus subtilis* lipase A as a model for performing the immobilization, and the same expression system. The immobilization occurred using economic diatom biosilica matrix. The binding of enzymes onto support and the expressed activity were, respectively, 90% and 279 U/$g_{biossilica}$ for Sil3K-LipaseA, 86% and 251 U/$g_{biosilica}$ for Sil3R-LipaseA and, finally, 10% and 37 U/$g_{biosilica}$ for Sil3H-LipaseA. The study also evaluated the fusion of tags at both termini of the enzyme. The multivalent Sil3K-LipaseA-Sil3K proved to have a better activity recovery and loading capacity, than the single tagged enzyme (Abdelhamid et al. 2022).

Zhao et al. (2020) fused a *N*-acetylmuramidase tag (AcmA tag) to the C-terminal of a α-amylase. They immobilized the enzyme on Gram-positive enhancer matrix (GEM) particles, composed mainly by peptidoglycan. The pET-28a (+) and *E. coli* BL21(DE3) were used for the expression system. The binding of the enzyme onto GEM occurred fast due to the tag's high specificity. The immobilized enzyme showed a high storage stability. After eight cycles of reuse, the enzyme retained 37% of its initial activity.

Döbber and Pohl (2017) performed the immobilization of a *Pseudomonas fluorescens* benzaldehyde lyase targeted with a modified haloalkane dehalogenase (HaloTag®) onto HaloLink™ resins (Sepharose® beads). The authors used pET-28a and *E. coli* BL21(DE3) for expressing the protein. When immobilized, the enzyme presented storage stability being stable for months at 4 ºC in buffer. The immobilized enzyme (120 µg) attached to 1 mg support was responsible for catalyzing 12 µmol of the desirable product. The reusability showed a decrease in enzyme initial activity around 30–40% at each cycle.

An aldehyde from a lipase polypeptide (Aldehyde tag), also known as a sulfatase motif, was fused to a lipase. The recombinant technology was performed using pET-28a(+) and *E. coli* BL21(DE3). The enzyme was immobilized on amino-functionalized mesocellular siliceous foam support. This method demonstrates environmental importance due to the fact that it does not need to use glutaraldehyde for the immobilizing process, a hazardous chemical crosslinking reagent (Wang et al. 2013), widely used in traditional methods (Asmat et al. 2019). In addition, the novel immobilized lipase showed higher thermal stability than the traditional immobilized one, using glutaraldehyde (Wang et al. 2013).

Methods for simultaneous immobilization and purification of tagged recombinant proteins

The articles selected through PRISMA presented very specific purification and immobilization methods, despite that they could be divided in two main groups: immobilization and purification using supports—specific to each tag—and using self-aggregating tags without the need of support. Figure 4 presents the tag-mediated one-step purification and immobilization methods for recombinant enzymes: immobilization and purification using support and, in a distinct way, using self-aggregating tags.

The first was reported in articles that used CBM, His, Sil3K, AcmA, Halotag®, and Aldehyde tags. In summary, the crude cell lysate containing cell debris and metabolites including the enzyme of interest is incubated with specific support for a variable time with different protein loads, immobilizing the targeted enzymes onto the support due to the affinity between support and the marker. The purification occurs when the immobilized enzymes are harvested by centrifugation, by magnetic separation—using a magnetic device—or by filtration, eliminating the cell debris.

Among these studies, 54.55% used centrifugation to separate the cell debris from the targeted immobilized enzyme (Myung et al. 2011; Liao et al. 2012; Wang et al. 2013; Döbber and Pohl 2017; Hu et al. 2018; Chen et al. 2019; Zhao et al. 2020; Zhou et al. 2020; Abdelhamid et al. 2020, 2022; Liu et al. 2021; Gennari et al. 2022a), while 42.86% performed a magnetic separation (Zhang et al. 2013, 2020b, 2021b; Tural et al. 2013; Wang et al. 2017; Slomka et al. 2017; Cao et al. 2017; Zhou et al. 2021a; Gennari et al. 2022b), and 4.54% filtered the immobilized enzyme (Benito et al. 2022).

Among the nine articles where the authors performed a magnetic separation for purifying the enzyme, in eight, the enzyme was fused to a His-tag. This number could be explained by the affinity of His-tag for metal supports composed of cobalt or nickel which can be separated from a solution with a magnetic device. Due to this characteristic, His-tags are also used to purify recombinant enzymes through immobilized metal ion affinity chromatography (IMAC) which is based on the affinity interaction between the polyhistidine and chelated transition metal ions (Salimi et al. 2017). The other article used a CBM tag for immobilizing the enzyme onto magnetic cellulose. Centrifugation was the main method used for harvesting the targeted proteins,

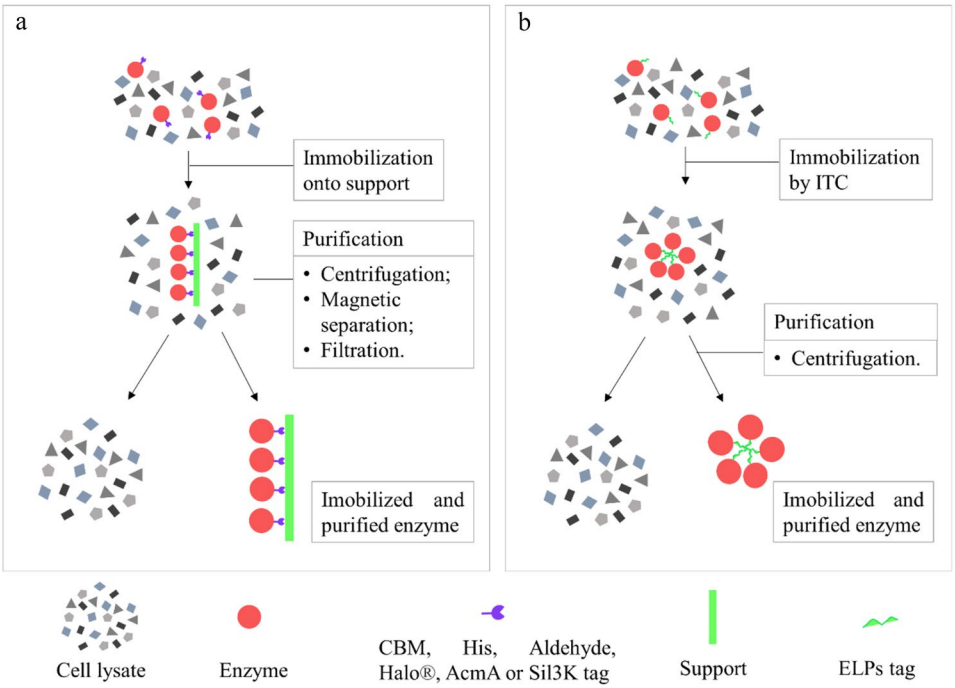

Fig. 4 Scheme of tag-mediated one-step purification and immobilization of recombinant enzymes. **a** Immobilization and purification using support, specific to each tag; **b** immobilization and purification with self-aggregating tags, without the use of support. *AcmA N*-acetylmuramidase, *Aldehyde* aldehyde from a lipase polypeptide, *CBM* carbohydrate-binding module, *ELPs* elastin-like polypeptides, *Halotag*® modified haloalkane dehalogenase, *His* polyhistidine; *ITC* inverse transition cycling, *Sil3K* silaffin-3-derived pentalysine cluster

with CBM, AcmA, Halotag®; Aldehyde, and Sil3K tag with diverse supports.

The immobilization without the use of supports was performed by Li and Zhang (2014) and Lin et al. (2018). Essentially, this type of immobilization is performed with ELPs as fusion tags and occurs by ITC. This protocol uses the phase transition behavior of the tag to separate the cell debris from the targeted enzyme, due to the transformation of the soluble enzymes in insoluble particles, considered immobilized enzymes. This transformation occurs through the application of temperatures above the transition temperature of ELPs. At the end, the cell debris and metabolites are removed by centrifugation.

Moreover, the ELPs have been widely studied for their use as tags for protein purification by ITC protocol through affinity precipitation (Mullerpatan et al. 2020). ITC corresponds to a simple and low-cost procedure and, mostly, shows a high purification efficiency. These positive aspects are turning the use of ELPs, a promising methodology for industry large-scale production, (Han et al. 2022) and, consequently, contributing for its application in double objective processes that simultaneously immobilize and purify enzymes through only one step.

Affinity tag fused to amino (N) or carboxy (C) terminals

During the cloning process, the affinity marker may be fused to amino (N) or carboxy (C) terminals of the enzyme of interest (Zhao and Huang 2016; Esen et al. 2019). The determination of the enzyme terminal to apply a tag aiming to perform, subsequently, the one-step purification and immobilization of the cloned protein depends on many factors to be feasible and to succeed. Figure 5 shows the predicted structure of different tags at the N- or C-terminal of a model protein (*Porphyromonas gingivalis* peptidase).

The folding of the protein may interfere in the choice to which terminal the tag will be fused, due to the locality of N- and C-terminal in the enzyme tertiary structure: the overall folding forming the 3D structure of each enzyme places the terminal and the catalytic site in different spatial positions, that means that the terminal may be in a strategic place for tag fusion or, negatively, may difficult tag interaction between linker and support—if the tag is located in the middle of the folding, making the fusion unfeasible, as shown in Fig. 5. In this case, the N-terminal tags may compromise the interaction between binder and support (Booth et al. 2018).

Moreover, the introduction of a tag in enzyme structure may alter the protein behavior, once it could modify enzyme conformation and folding, reducing biocatalyst functionality (Effer et al. 2019). In that regard, with different enzymes, the affinity marker may behave in a distinct way, negatively or positively (Booth et al. 2018).

Among the nine documents selected through PRISMA that performed single-step purification and oriented immobilization using CBM tags, six used the CBM fused to the enzyme N-terminal (Liao et al. 2012; Chen et al. 2019; Zhou et al. 2020; Gennari et al. 2022b, a; Benito et al. 2022), while only one fused the tag to C-terminal (Hu et al. 2018) and the other couple of articles did not report this information.

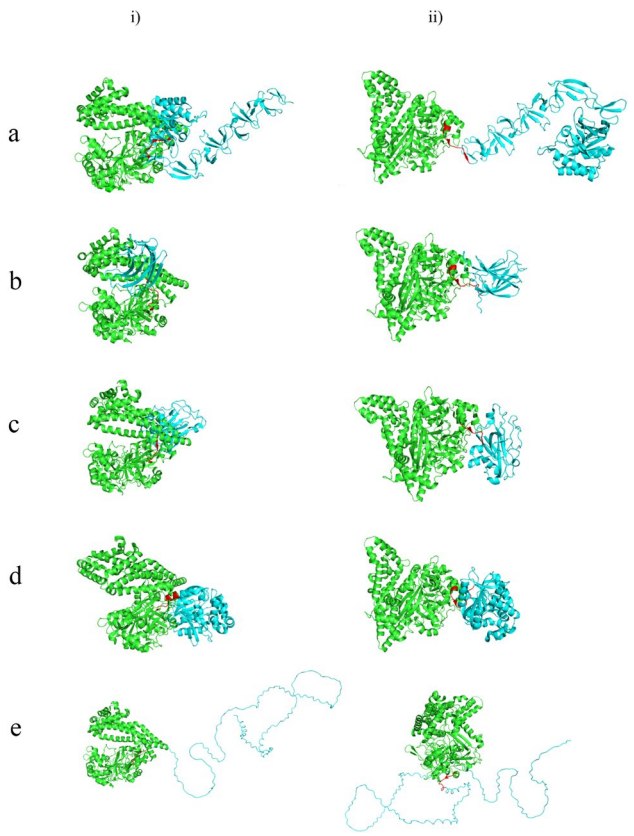

Fig. 5 Predicted structures of different tags located at the (i) N- or (ii) C-terminal of a model protein (*Porphyromonas gingivalis* peptidase). **a** *N*-acetylmuramidase; **b** cellulose-binding domain; **c** chitin-binding domain; **d** modified haloalkane dehalogenase; **e** silaffin-3-derived pentalysine cluster. Legend chart: cyan—affinity tag; green—model protein; red—position of the terminal where the tag is cloned. The protein structures were constructed using the software PyMOL (The PyMOL Molecular Graphics System, Version 25.4 Schrödinger, LLC; https://pymol.org)

In addition, most articles that conducted His-tag-mediated one-step purification and immobilization fused the binder to the enzyme N-terminal (Zhang et al. 2013, 2020b; Cao et al. 2017), while a couple of studies fused to the C-terminal (Tural et al. 2013; Slomka et al. 2017) and Wang et al. (2017) fused to both terminals aiming to compare the enzyme behavior in each fusion position. The remaining studies did not report this information. Aldehyde tag (Wang et al. 2013), AcmA tag (Zhao et al. 2020), and Sil3K tag (Abdelhamid et al. 2020, 2022) were all fused to the C-terminal, and on the other hand, HaloTag® (Döbber and Pohl 2017) was fused to N.

The different tag location in the searched documents, at the C- or N-terminal of the encoding enzyme, could affect the protein expression levels (Parshin et al. 2020), the catalytic activity (Meng et al. 2020), and protein stability (Aslantas and Surmeli 2019). The expression system chosen could also interfere in protein folding and conformation (Parshin et al. 2020). Thus, changing the enzyme profile or the fusion tag may alter the system's parameters, as catalytic activity, turning the system feasible or not for application, or may maintain the same results.

Applications of tagged recombinant proteins

The immobilized targeted enzymes must present some positive features for implementation in industry large-scale production, as low-cost supports (Facin et al. 2020), fast and efficient purification and immobilization processes (Zhao et al. 2019), and eco-friendly procedures and matrixes (de Souza et al. 2020; Carvalho et al. 2021).

The documents selected for this systematic review presented different tags that showed distinct characteristics. CBM was the most used tag for immobilization and purification of enzymes in a single step. The best features about using this type of tag are its high specificity, the use of low-cost and eco-friendly supports for immobilizing the targeted enzyme and fast purification procedures. His-tag, as well as CBM, are one of the most used affinity binders, due to the low-cost process to purify the protein. And ELPs, used to immobilize without support, correspond to an economic and time-efficient procedure.

Another important feature to apply the tag in the cloning of an enzyme to perform its purification and immobilization is related to the size and the molecular weight of the binder, once small tags have lower possibility to affect enzyme functionality (Zhao et al. 2013). In that regard, the His-tags, composed of six histidine residues, correspond to a molecular weight of 0.8 kDa (Ye et al. 2017). The molecular weight of this amino acid sequence is very low compared to peptides as ELPs, whose composition is easy to modify and may correspond to molecular weights ranging from 9 to 110 kDa (Bataille et al. 2016; Kuna et al. 2018) and protein domains as CBM tags, with different molecular weights as 11.6 kDa (Abouhmad et al. 2016), 17.7 kDa (Lin et al. 2017), and 25.4 kDa (Li et al. 2016). That means that His-tags have a lower chance to compromise enzyme structure and function after expression.

Despite that, His-tags may not purify the protein from the cell lysate in some situations. Many proteins contain histidine in their structure (Moro et al. 2020), and if this kind of protein exists in the crude lysate, they could, possibly, bind to the support as the cloned His-enzyme. That probably means that, although the cloned enzyme could be immobilized onto the support, other proteins could also bind, and so there would be no purification.

Thus, the best affinity tag for applying in a single-pot purification and immobilization depends on the main interest and also on which protein will be cloned. Despite that, in general, CBM corresponds to the best option of fusion tag according to the reviewed information, mainly by the wide

range of materials for developing carbohydrate supports that bind CBM—mostly eco-friendly and sustainable materials which can be obtained from industry residues—by the fast immobilization that occurs with high specificity and affinity and by the low-cost process, one of the most important factors for industry.

Future perspectives

The future perspectives for applying the one-step purification and oriented immobilization in industry in the next few years may be related to the development of new technologies associated with large-scale production, cost reduction, and enzyme stability. A technique which has already been used is the encapsulation of immobilized enzymes, mainly by core–shell systems. The core–shell capsules may enhance operational stability and enzyme reusability, aiming for industrial purposes (Wang et al. 2021; İspirli Doğaç and Teke 2021; Feng et al. 2022). Immobilizing enzymes in core–shell particles and performing simultaneous one-step purification have already been demonstrated and proved to be a potential alternative for the application of enzymes in industry, due to the reusability properties of the core–shell system (Zhang et al. 2013; Shin et al. 2016), aiming for industrial catalytic batches.

In addition, it can be noted that the one-step purification and immobilization process of recombinant enzymes is limited to the use of the enzymes shown in Table 1. In this context, the application of different enzymes of industrial interest in these processes under study is promising, as well as the development of new plasmid constructions and different combinations of the enzyme-tag system to further increase the economic viability of the process.

Furthermore, immobilized enzyme reactors are flow-through devices used for enzyme catalytic reactions due to its capacity of repeating cycles of catalysis with a continuous flow, while the enzyme is retained in the reactor. This application of immobilized enzymes may enhance the productivity and reusability of the enzyme (Böhmer et al. 2020; Goldhahn et al. 2020) by facilitating continuous batch biocatalysis in industry.

Besides, the future trends in enzyme immobilization are strongly directed toward the development of materials obtained from industry wastes or formulated with these residues. In that sense, the agroindustrial wastes may be used to produce enzyme support since they generate a low-cost product, in addition to giving a different purpose to a residue that would be discarded. In that regard, the field of material science is highly rated for the development of new supports using these residues aiming resistance, enzyme protection from adverse conditions, and high affinity to the tag.

Another perspective, for further research, may be related to the fact that most studies selected through PRISMA presented *E. coli* as the expression host, and the recombinant proteins, in these cases, were expressed intracellularly, in need of a disruption process to release these proteins. Therefore, the addition of signal peptides compatible with *E. coli* secretion system in the plasmid structure may be a positive alternative to reduce processes operating time and cost, once it would theoretically direct the production of the recombinant protein to the extracellular environment (Seo et al. 2019), and, consequently, would skip the disruption process.

Thus, studies about core–shell encapsulation, continuous flow reactors, supports made from industrial wastes, and inclusion of signal peptides in the cloning process may be proposed for improving tag-mediated single-step purification and immobilization of recombinant enzymes and increase their applications in industrial large-scale production.

Concluding remark

Tag-mediated one-step purification and immobilization of recombinant enzymes is a process performed using the recombinant DNA and enzyme oriented immobilization technique. The aim of this process is related to cost reduction of enzyme purification methods and improvement of enzyme performance; promoting protein reuse, and improving pH, temperature, and storage stability, factors that may be responsible for the decrease in the entire process cost. In this review article, we described the affinity tags used for recombinant protein single-step purification and immobilization in the last 10 years by a systematic review, resulting in 25 documents reporting the use of 7 different types of tag. CBM was the most used tag due to low-cost process and environmental appeal. His-tags are the second most used, mostly due to the reduced cost for protein purification and higher enzyme stability. Moreover, the research demonstrated that ELPs are used without support, due to their capacity to self-assemble into insoluble aggregates by inverse transition cycling. AcmA tag, Aldehyde tag, Sil3K, and HaloTag® were also used to perform simultaneous purification and immobilization, and improved enzyme stability.

The aforementioned tags present various positive aspects, such as stability and reusability, and also show different possibilities for industrial application, even though they were less reported than CBM, His, and ELPs tag. Beyond, the tag location, at C- or N-terminal, demonstrated to be important once it could make the interaction between binder and support difficult, besides the use of a tag also could alter protein behavior. Finally, the CBM tag was considered the best tag for single-step purification and immobilization of enzymes aiming both economic and environmental sustainability of bioprocesses, due to the wide range of eco-friendly supports which can be supplied by agroindustry wastes, by the fast

immobilization with high specificity, and the reduced cost of the process.

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Declarations

Conflict of interest The authors declare they have no conflicts of interest.

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