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Research review paper

Design and biocatalytic applications of genetically fused multifunctional enzymes

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ABSTRACT

Fusion proteins, understood as those created by joining two or more genes that originally encoded independent proteins, have numerous applications in biotechnology, from analytical methods to metabolic engineering. The use of fusion enzymes in biocatalysis may be even more interesting due to the physical connection of enzymes catalyzing successive reactions into covalently linked complexes. The proximity of the active sites of two enzymes in multi-enzyme complexes can make a significant contribution to the catalytic efficiency of the reaction. However, the physical proximity of the active sites does not guarantee this result. Other aspects, such as the nature and length of the linker used for the fusion or the order in which the enzymes are fused, must be considered and optimized to achieve the expected increase in catalytic efficiency. In this review, we will relate the new advances in the design, creation, and use of fused enzymes with those achieved in biocatalysis over the past 20 years. Thus, we will discuss some examples of genetically fused enzymes and their application in carbon-carbon bond formation and oxidative reactions, generation of chiral amines, synthesis of carbohydrates, biodegradation of plant biomass and plastics, and in the preparation of other high-value products.

1. Introduction

Nature has always been a constant source of inspiration for chemists, not only because of the enormous variety of compounds that living beings can produce, but also because of the extraordinary ways (synthesis strategies) used to produce them. The natural synthetic strategy is based on three key aspects: i) the use of enzymes as catalysts; ii) the compartmentalization of enzymes or even entire biosynthetic pathways, e.g. in cellular organelles; and iii) the sequential use of enzymes in complex metabolic pathways that allow living beings to build complex structures from simple elements (García-Junceda et al., 2015).

Biosynthetic pathways can turn a reversible process into an irreversible one, eliminate inhibition problems due to excess product, or prevent the lack of substrate, leading to extraordinary synthetic efficiency (Burek et al., 2022; López-Gallego and Schmidt-Dannert, 2010; Oroz-Guinea and García-Junceda, 2013; Sánchez-Moreno et al., 2012; Schmidt et al., 2021; Siedentop et al., 2021). The shift of thermodynamic equilibria by product elimination could be another advantage of the sequential work of several enzymes. During evolution, some of these processes catalysed by multiple enzymes were optimized for more efficient metabolism by combining two or more activities in only one polypeptide chain, resulting in multifunctional enzymes. This evolution appears to occur through the combination of genes encoding the monofunctional precursors of these enzymes. Natural multifunctional enzymes have been discovered in both primary and secondary metabolism and are found in a wide variety of organisms (Bernhardsgrütter et al., 2018; Conrado et al., 2008). A notable example of natural multifunctional enzymes is tryptophan biosynthesis mediated by the

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polyaromatic pathway in fungi. This comprises the AROM complex, which consists of five different enzymatic activities in a single polypeptide (Charles et al., 1986). Interestingly, the same overall reaction in prokaryotes is carried out by five independent enzymes (Hawkins, 1987).

Multiple strategies have been developed to mimic nature's organization of sequentially assembled enzymes such as enzyme encapsulation, covalent co-immobilization of enzymes, scaffold-mediated co-localization of enzymes, post-translational selective enzyme conjugation or gene fusion (Fig. 1). Encapsulation (Fig. 1A) is the confinement of the enzyme in a porous membrane that forms a bilayer (Jegannathan et al., 2008; Mazurek et al., 2020). Encapsulation prevents direct contact between the enzyme and the bulk medium and prevents the enzyme from leaking. This type of immobilization is technologically straightforward. Perhaps the biggest disadvantage is that diffusion can be significantly limited due to the high load of the encapsulated enzyme. Therefore, to overcome the mass transfer problems, encapsulation in microcapsules has been developed. The microcapsules, such as liposomes and polymersomes, are usually spherical with diameters in the micro to millimeter range and have core-shell structures of varying complexity (Rother and Nidetzky, 2014). Cross-linking of enzyme aggregates (CLEAs; Fig. 1B) with a bifunctional reagent is used to prepare carrier-free macroparticles, avoiding the inevitable dilution of activity that leads to the incorporation of a large non-catalytic mass, which can range from 90 to >99% of the mass of the biocatalyst (Sheldon, 2011; Sheldon and van Pelt, 2013). The main advantages of CLEAs are to provide highly concentrated enzymatic activity in a highly stabilized biocatalyst and, most importantly, the low cost due to the elimination of any support, which in some cases can be even more expensive than the enzyme itself (Zdarta et al., 2018). Binding to a carrier (Fig. 1E) can be physical (such as hydrophobic and van der Waals interactions), ionic, or covalent. Physical binding is generally too weak to fix the enzyme to the support under conditions of high reactant and product concentrations and high ionic strength. Ionic binding is generally stronger than physical union but in both cases the enzymes are not covalently attached to the support and enzyme leaching remains a problem (Sheldon and van Pelt, 2013). In contrast, covalent binding of the enzyme to the support would generally prevent leaching of the enzyme from the surface. Furthermore, if multiple bonds are formed between the enzyme and the support, a highly rigidized region can be formed that stabilizes the enzyme against interfering factors (Guisan et al., 2022). The disadvantage of this approach is that this type of very intense multipoint immobilization is not easy to achieve and if the enzyme is irreversibly deactivated, both the enzyme and the support must be discarded. Synthetic fusion enzymes are formed mainly by genetic fusions of two or more genes that originally encoded independent proteins (Fig. 1F). Most fusion proteins are obtained by gene splicing through overlap extension (Horton et al., 1989). To date, fusion proteins have been constructed mainly by the end-to-end fusion approach, but some examples of insertional gene fusions have also been reported, in which the gene of the first protein is inserted in-frame into the gene of the second protein (Aÿ et al., 1998; Doi and Yanagawa, 1999; Furtado et al., 2013). While the protein fusion approach works well when two enzymes are to be recruited in close proximity to each other, this strategy is less feasible for three or more enzymes. In these cases, other strategies can be used, such as scaffold-mediated co-localization of enzymes (Fig. 1D) or post-translational conjugation (Fig. 1C).

The best documented example of scaffold-mediated co-localization of enzymes is based on natural cellulosomes (Fierobe et al., 2001). The engineered complexes consist of two main components derived from natural cellulosomes: (1) the scaffold, which contains a cellulose-binding domain (CBD) and multiple cohesin-binding domains in a single polypeptide, and (2) the enzyme dockerin, which binds post-translationally to the scaffold via a high-affinity dockerin-cohesin interaction. Protein scaffolds based on different combinations of protein-protein interaction domains, such as the PDZ domain, the SH3 domain, or the GBD domain have been developed (Dueber et al., 2009). With this approach, the stoichiometry of the enzymes can be optimized to balance and maximize the flux of the pathway. Another interesting approach for post-traslational generation of fusion enzymes is the use of Cu-free click

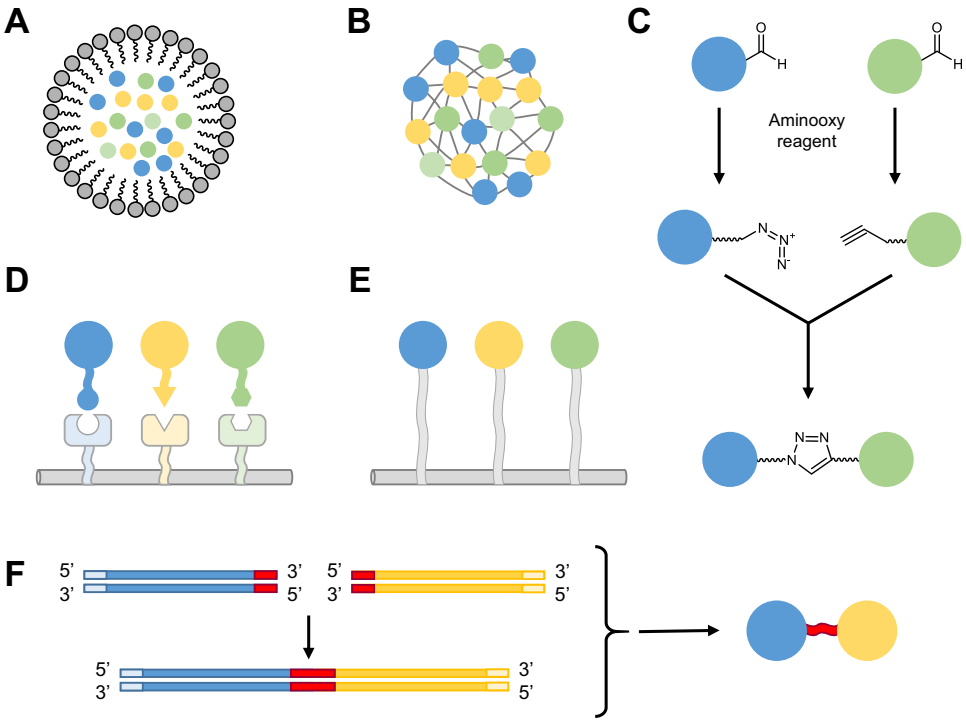

Fig. 1. Different strategies to mimic nature's organization of assembled enzymes. (A) Enzyme encapsulation. (B) Cross-linked enzyme aggregates (CLEAs). (C) Protein assembly using Cu-free click chemistry in combination with the aldehyde tag protein modification strategy. (D) Scaffold-mediated co-localization of enzymes. (E) Covalent immobilization in a support matrix. (F) Genetically fused enzymes.

chemistry in combination with the aldehyde tag protein modification strategy (Fig. 1C; Hudak et al., 2012). The aldehyde tag is a sequence with five residues (CxPxR) that is recognized by the formylglycine (fGly)-generating enzyme (FGE) (Appel and Bertozzi, 2015). This enzyme catalyzes the post-translational oxidation of the cysteine residue in the aldehyde tag, allowing site-specific protein modification. The aldehyde in the fGly can then be modified with an aminooxy reagent to form a stable oxime. In this way, it is possible to introduce azides or cyclooctins into recombinant proteins in a site-specific manner via small linkers. Finally, Cu-free click chemistry can be performed for the covalent assembly of protein-protein conjugates. SpyTag and SpyCatcher protein ligase coupling is based in the use of proteins which are capable of spontaneous isopeptide bond formation, to develop peptide tag/binding partner pairs which covalently bind to each other (Howarth, 2011; Keeble and Howarth, 2020). Finally, the dock and lock (DNL) method (Chang et al., 2007; Rossi et al., 2006) is based on the use of protein domains involved in the natural association between protein kinase A (PKA; cyclic AMP-dependent protein kinase) and A-kinase anchor proteins (Baillie et al., 2005; Wong and Scott, 2004). These domains are the dimerization docking domain (RIDD, 44 residues) and the anchor domain (RIAD, 18 residues), respectively. Each domain can be fused with the N- or C-terminus of the target enzymes and, when the resulting derivatives are combined, form a stably tethered complex through various non-covalent protein-protein interactions. The resulting complex has a defined composition that, in principle, retains the functions of the individual components.

Among all the aforementioned strategies to bring enzymes in close proximity, this review will focus on the design and use of fusion enzymes for biocatalysis. These synthetic enzymes may entail interesting features when applied for the synthesis of organic compounds in terms of enzymatic activity and product conversion increase. In this sense, substrate channeling is one of the most commonly pointed causes to explain the catalytic improvement of the fused enzymes when compared to the mix of individual parental proteins. Substrate channeling is defined as a process in which two or more successive enzymes in a metabolic pathway interact to transfer a metabolite (or intermediate) from the active site of the earlier enzyme to the active site of the later enzyme without allowing diffusion of the metabolite into the bulk phase (Ovádi, 1991). Therefore, an increase in catalytic efficiency can be expected because reaction rates are independent of bulk metabolite concentrations; a decrease in the transit time required for an intermediate to reach the active site of the subsequent enzyme and for the system to reach the new steady state; protection of chemically labile intermediates; and separation of intermediates from competing reactions (James and Viola, 2002; Spivey and Merz, 1989). There are a large number of examples of substrate channeling in natural enzyme complexes (Zhang, 2011). However, the existence of this effect must not be assumed lightly, since the sole fusion of two enzymes does not directly imply the appearance of this phenomenon and proper experiments to confirm it should be performed. Two general channeling mechanisms have been proposed: perfect channeling and leaky channeling (Wheeldon et al., 2016; Zhang, 2011). Perfect channeling entails a direct substrate transfer mechanism, while leaky channeling would rely exclusively on a proximity mechanism, thus solely requiring the spatial closeness of the two enzymes. Nonetheless, there is some controversy about the accuracy of the proximity mechanism, resulting on a stimulating debate on the topic (Kuzmak et al., 2019; Poshyvailo et al., 2017; Sweetlove and Fernie, 2018), which is out of the scope of this review.

In addition to substrate channeling, other causes may positively influence the reaction outcome when genetically joining two enzymes (Yang et al., 2016). For instance, the fusion between the enzyme sequences can improve the stability of one or both enzymes (e.g., thermostability and pH stability), thus improving product synthesis. This is due to the fact that said fusion can enhance the interior hydrophobic interactions and anchor the N- and/or C-terminal regions, which are generally very flexible parts of the protein (Xu et al., 2020) (see examples in Sections 2.2, 2.4, 3.3.2. and by Huang et al., 2013; Aalbers and Fraaije, 2017; and Adlakha et al., 2012 respectively). Additionally, fusion of highly charged proteins can improve enzyme kinetics. Abdallah et al. (2019), showed that GFP with high negative surface charge fused to an alcohol dehydrogenase (AdhD) increases the local ionic strength and improves the second-order rate constant. This effect was further studied by Hess's group (Zhang et al., 2016), using the cascade reaction of glucose oxidase (GOx) and horseradish peroxidase (HRP) located on DNA scaffolds as a model system. In this case, no neighborhood effect was found and suggested that the reason for the increase in enzyme activity is that the pH near the surface of the negatively charged DNA nanostructures is lower than in the bulk solution, creating a more optimal pH environment for the scaffold-bound enzymes. Indeed, when enzymes colocalize with materials that have fixed charges, this can lead to the accumulation or depletion of protons and ions that can cause a significant change in local pH of up to 3 units (Abdallah et al., 2022). Another beneficial trait could be, the potential increase of overexpression and solubility of one of the parental enzymes when fused, can be the reason behind the performance improvement in in vivo reactions. Usually, if one of the proteins should perform as a solubility enhancer partner, it is placed at the N-terminus providing a reliable open reading frame for an efficient translation (Yang et al., 2016). For instance, although production of β-carotens was achieved by expressing the bifunctional lycopene cyclase/phytoene synthase (CrtYB) and the phytoene desaturase enzyme (CrtI) from Xanthophyllomyces dendrorhous in *Saccharomyces cerevisiae*, a main bottleneck is the overexpression of CrtI (Verwaal et al., 2007). To alleviate this issue, Rabeharindranto et al. (2019) designed and created numerous fusion enzymes including different combinations of the domains CrtY, CrtB and CrtI, showing that the N- or C-terminal positioning of CrtI or CrtB catalytic domains strongly affected the protein production level. Additionally, positive (and negative) variations in activity can be also due to changes in protein conformation derived from the steric hindrance, as well as changes in flexibility and folding of the joined proteins (Elleuche, 2015).

2. General considerations, challenges and limitations in the development of fusion enzymes

When developing fusion enzymes, some essential aspects must be considered to avoid potential pitfalls. With this in mind, we will describe the compatibility of fusion partners, followed by the fusion design strategy, the order in which genes should be incorporated into the construct, and the evaluation of linker properties.

2.1. Quaternary structure of the parent proteins

When designing fusion proteins, it is important to consider the possible subunit interactions that may occur in the context of the fused enzyme. When two monomeric proteins are fused, the resulting protein is expected to retain the monomeric character of the parent enzymes. However, when the parent proteins have a dimeric or oligomeric character, it is difficult to predict the quaternary structure of the fusion protein, and macromolecular complexes of varying size and activity may result (Bülow and Mosbach, 1991; Fig. 2). For example, in the fusion of an *R*-ω-transaminase (RTA) with a D-amino acid oxidase (DAAO), the fusion enzyme showed no activity because both enzymes were dimeric, possibly because the high molecular size of the multimeric enzymes RTA and DAAO prevented proper folding of the fusion enzyme (Du et al., 2019). In another example, the union of the tetrameric β-galactosidase from *E. coli* with a dimeric galactose dehydrogenase from *Pseudomonas fluorescens* resulted in a fusion protein that was a mixture of tetramers and hexamers. Both quaternary structures dissociated to dimers by heating at 60 °C for 5 min, resulting in complete loss of β-galactosidase activity (Ljungcrantz et al., 1989).

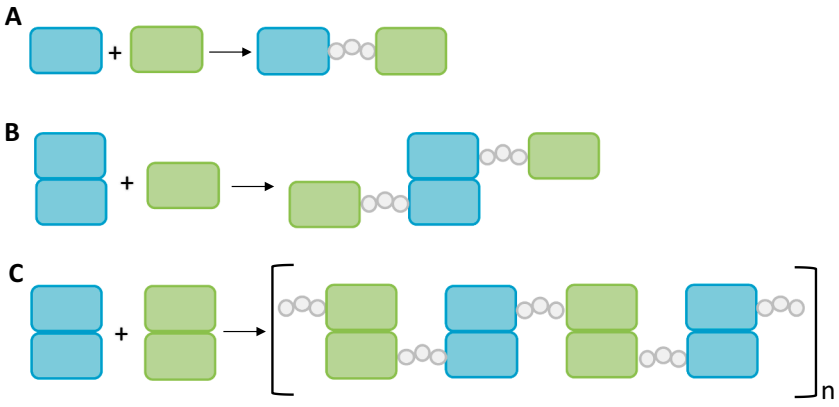

Fig. 2. Schematic representation of the interactions that can occur between subunits in a fusion protein. (A) Fusion of two monomeric proteins retain the monomeric structure. (B) A monomeric protein fused with a dimeric or multimeric one, retain the dimeric or oligomeric structure. (C) Two dimeric or oligomeric proteins may form macromolecular complexes of different size (Bülow and Mosbach, 1991).

2.2. Gene fusion order

The order in which genes are placed is also an important step in fusion design. Although in some cases the order of fusion does not have a significant impact on activity (Torres Pazmiño et al., 2008), in other cases it has been shown to be critical for the correct function of the system (Aalbers and Fraaije, 2017). Indeed, one order might be superior to the other because of the presence of important structural elements near the ends that may contribute to proper enzyme folding, oligomerization, and cofactor coordination. Fusion of enzymes could lead to structural changes in the individual enzymes in the fusion constructs, resulting in the observed changes in catalytic properties (Elleuche, 2015; Yu et al., 2015), thus it is important to test as many possible alignments of genes in the fusion protein as possible. For example, as part of a study aimed at obtaining heparinase I (HepA) in a cost-effective manner and making it industrially useful by improving its productivity and thermostability, Huang et al. (2013) developed a strategy in which three proteins-HepA, maltose-binding protein (MBP), and green fluorescent protein (GFP)-were fused to enable the purification of HepA in one step and real-time detection of its activity by fluorescence. After a systematic review, they found that the order of fusion was important for MBP binding affinity and HepA activity, while linker sequences had significant effects on protein expression, HepA activity and thermostability, and GFP fluorescence. In a flavin-containing monooxygenase (FMO-E)/horse liver alcohol dehydrogenase (HLDH) fusion enzyme, the sequence of proteins in the fusion enzyme was critical for the success of the strategy (Huang et al., 2019). When HLADH was positioned at the C-terminus of the fused enzyme, it retained more activity than the reverse enzyme, likely due to disruption of oligomers formed by alcohol dehydrogenase, as previously described (Aalbers and Fraaije, 2017).

If one of the proteins is to act as a partner for the solubility enhancer, it is usually more efficient to place it at the N-terminus because it (i) provides a reliable open reading frame in which the solubility enhancer can take advantage of efficient translation, and (ii) can be easily cleaved to yield the native protein (Costa et al., 2014). However, N-terminal fusion would still not be suitable if steric limitations or other drawbacks were encountered.

2.3. Design and selection of the linker sequence

As mentioned earlier, end-to-end gene fusion is the most common approach for fusion enzymes and, if this is the chosen technique, the choice of an appropriate linker is of utmost importance to obtain stable and active systems. Linkers not only join two proteins, they can also improve folding, stability, and biological activity, increase expression yield, and help obtain targeted pharmacokinetic profiles (Patel et al., 2022). Nature is a good repository of naturally occurring linkers in multidomain proteins, and they can serve as inspiration for the development of rational empirical linkers (Papaleo et al., 2016). George and Heringa (2002), analyzed a total of 638 multidomain protein chains and concluded that the average length of linkers in nature is 10.0 ± 5.8 residues. In addition, linkers are slightly hydrophobic (with increased hydrophilicity for longer linkers) and mainly have an α-helix or disordered secondary structure (Chen et al., 2013; George and Heringa, 2002). These are also the most important properties to consider in the rational design of linkers, summarized in composition (rigidity and hydrophobicity) and length. Flexibility, hydrophilicity, and extended conformation of the linker have been described as important factors to avoid disrupting the functionality of the domains (Guo et al., 2017).

When some degree of movement/interaction between parts is required, flexible linkers can be made with small polar or nonpolar amino acids (Chen et al., 2013), and it was shown that flexibility can be fine-tuned by varying glycine content (van Rosmalen et al., 2017). Flexible linkers were the first options explored in protein fusion design. Gly-rich linkers can be considered as independent entities that do not interfere with the function of the individual proteins they bind. In this way, the fused proteins behave independently, so that the single-chain enzymes have the combined activity of the parental proteins. However, early examples failed due to domain contacts showing that rigid linkers were also necessary (Amet et al., 2009; Maeda et al., 1997). Helical rigid linkers with the general structure $A(EAAAK)_nA$ (n = 2–5) were subsequently evaluated to effectively separate domains (Arai et al., 2001), and their use and applicability in fusion design has recently been studied in detail (Arai, 2021). The seminal work by Arai et al. (2001) is also one of the typical examples of how linker length can affect the performance of fusion proteins. The authors tested different repeats of the α-helix motif and measured how fluorescence between the fused proteins was affected, showing that rigid linkers separated the parts more efficiently. Similarly, another rigorous example using fusion enzymes evaluated linkers from 11 to 32 amino acids in P450 fusions and found an overall improvement in stability, activity, and coupling efficiency only by optimizing linker length (Hoffmann et al., 2016).

Despite a rational linker design, it is difficult to predict whether the chosen sequence is the best choice or whether its amino acid sequence will have any effect on the fusion design (Torres Pazmiño et al., 2009). For this reason, some studies are cautious when designing a fusion protein and perform a combinatorial design for linker optimization that includes different rigid and/or flexible linkers with different sequence composition and length (Li et al., 2016). Using this approach, both activities of a bifunctional β-glucanase/xylanase were greatly improved after testing 13 linkers, both flexible and rigid. Interestingly, the authors found that both a glycine-rich flexible linker and a rigid helical linker

were excellent at fusing these two enzymes and enhancing both activities (Lu and Feng, 2008).

An interesting study on the effect of linker length on the behavior of the fusion enzyme was described by Nakashima et al. (2014) for the degradation of crystalline cellulose. To produce the fusion protein, the authors use an enzyme originally discovered in plants as responsible for cell wall enlargement (McQueen-Mason et al., 1992) and later described in fungi (Chen et al., 2010) and bacteria (Kerff et al., 2008), expansin, which is capable of degrading crystalline cellulose. Expasin from *Bacillus subtilis* (EXLX1) was assembled with an endoglucanase from *Clostridium thermocellum* (CelD) using a flexible glycine-serine-peptide linker (GS linker = GGGGS). The authors produced three different types of fused enzymes that combined the parental enzymes without linker (EXLX1-CelD) and with three (EXLX1-GS3-CelD) or six copies (EXLX1-GS6-CelD) of the GS linker. After optimization, the fused and parental enzymes were successfully expressed in *E. coli*. The activity of the fused enzymes was tested using cellulose with different crystallinity index. In the first 24 h of the reaction, EXLX1-GS3-CelD and EXLX1-GS6-CelD provided higher digestibility compared to CelD. The increase in digestibility by EXLX1-GS3-CelD and EXLX1-GS6-CelD was due to the corresponding affinity of the protein for the cellulose substrate due to the presence of EXLX1. The results showed a strong influence of the linker on the activity and fused enzymes. Thus, direct fusion of the two proteins caused a decrease in endoglucanase activity. On the other hand, the enzyme with the shorter link proved to be more effective for all cellulose substrates tested.

Another successful example of this approach is the optimization of peroxygenase-alcohol oxidase (UPO-AAO) fusions for self-sufficient peroxygenase reactions (Gomez De Santos et al., 2020). In this case, flexible linkers (mainly glycine-rich linkers) and rigid linkers (α-helical structures) with different lengths were tested. The authors also examined the effects of the relative position of the enzymes. Finally, an optimal long linker combining a rigid region with a small flexible part allowing some degree of freedom between the biocatalytic units ensured the maximum activity of the two partner enzymes.

2.4. Biochemical compatibility of the fused enzymes

Once the fusion enzyme is created, there are perhaps two major issues that fusion enzymes can face. The first is the need for both fusion partners to operate under similar conditions (pH, temperature, etc.), and the second is the one-to-one ratio of fused enzymatic activities imposed by the genetic fusion method.

Regarding the biochemical compatibility of the fusion partners, Ribeiro et al. (2011) were challenged by the differences between the optimal pH values of the laccase and xylanase activities of the parent enzymes when developing xylanase-laccase chimeras. At the optimal pH of each parent enzyme (4.5 for laccase and 7.5 for xylanase), <20% activity was observed in the other enzyme. However, after fusion, the overlap in pH dependence of the two activities improved significantly, and at a pH of 5.0, the fusion enzyme had 96% of the maximum laccase activity and about 50% of the maximum xylanase activity. These differences may be due to changes in the microenvironment of the active sites, which may lead to changes in the pK_a values of the catalytic residues, or to conformational changes resulting from the fusion of the enzymes (Ribeiro et al., 2011). Investigation of the biochemical compatibility of the fusion partners is more necessary when a thermophilic enzyme is fused to a mesophilic one, since the former usually have relatively modest catalytic rates at low temperatures, which is not the case for mesophilic enzymes that can be inactivated at high temperatures. It should also be noted that cases have been described in which stability transfer occurs from the most thermophilic enzyme to the mesophilic one. This is the case described by Aalbers and Fraaije (2017) in the development of a fusion enzyme that could efficiently convert cyclohexanol to ε-caprolactone in vitro. For this purpose, the authors chose as BVMO partner the cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO). This enzyme is more thermostable and has a higher tolerance to solvents than the more commonly used BVMO from *Acinetobacter calcoaceticus* NCIMB 9871, and therefore has greater potential for use at the industrial level. As ADH partners, the authors tested three enzymes based on their preference for $NADP^+$ and their thermostability and found that the most stables ADH transfers some stability to TmCHMO and increases its T_m by up to 3 °C.

Concerning the quaternary structure of the proteins involved, the fixed ratio of fused enzyme activities imposed by the gene fusion approach, is even more critical. One of the key features to ensure that a multi-enzyme system works optimally is that the product of the first enzyme must be produced at the same rate that the second consumes it, in such a way as to avoid its accumulation and minimize its degradation or loss in the reaction medium. However, this adjustment is not possible when using a fusion enzyme in which the relationship between the activities is structurally fixed. Sometimes a similar effect could be achieved by modulating the ratio of the substrates (Iturrate et al., 2010) when it is imperative to modulating the activities ratio, one can resort to post-translational conjugation methods as those described in the Introduction section or others.

3. Fusion enzymes in biocatalysis

Because of the many potential advantages described above, fusion enzymes have been included as the solution of choice for some of the problems of biocatalysis. Herein, some examples of genetically fused enzymes and their application are discussed.

3.1. Fusion enzymes for C—C bond formation

Carbon-carbon bond formation reactions can be considered the essence of organic synthesis, as they allow the creation of the carbon skeleton of any organic molecule by connecting smaller substructures to obtain more complex molecules (Corey and Cheng, 1995). Most enzymes capable of catalyzing this reaction belong to the subclass of lyases (EC 4.1), ligases (EC 6.4) or oxidoreductases (E.C. 1.14).

3.1.1. Aldol formation

The aldol addition reaction has long been recognized as one of the most useful tools that synthetic chemist has for the formation of new C—C bonds (Alcaide and Almendros, 2003; Palomo et al., 2004). The process of carbon-carbon bond formation is accompanied by the formation of one or two new stereocenters, which allows access to a wide range of natural and novel compounds. Aldolases are among the most powerful tools for enantioselective C—C bond formation (Brovetto et al., 2011; Sukumaran and Hanefeld, 2005) and nature uses them to build carbohydrates, amino acids, hydroxy acids, and other important molecules because they can accept a wide range of acceptor substrates (Gastaldi et al., 2021; Hélaine et al., 2022; Lee et al., 2022). However, it is also known that their major drawback is their strict specificity for the donor substrate, which can be chemically unstable and/or expensive, as in the case of dihydroxyacetone phosphate (DHAP)-dependent aldolases (Iturrate and García-Junceda, 2008). In this sense, our research group designed a bifunctional enzyme named DLF with aldolase and kinase activity in the same polypeptide chain for one-pot C—C bond formation using commercially available dihydroxyacetone (DHA) as the starting substrate (Iturrate et al., 2009). Since dihidroxyacetone kinase (DHAK) from *Citrobacter freundii* is a dimeric enzyme (Siebold et al., 2003), fructose-1,6-bisphosphate (FBP) aldolase from *Staphylococcus carnosus* was chosen as the aldolase partner due to its monomeric structure, resulting in a dimeric fusion protein. Both enzymes were fused via a five amino acid linker. A 20-fold increase in the aldol reaction rate of the bifunctional enzyme was observed compared to that of the multi-enzyme system. By using a third enzyme, glycerol-3-phosphate dehydrogenase (GDH), as a trapping enzyme that competes with aldolase for DHAP, a proximity channeling effect was proven (Fig. 3; Iturrate et al.,

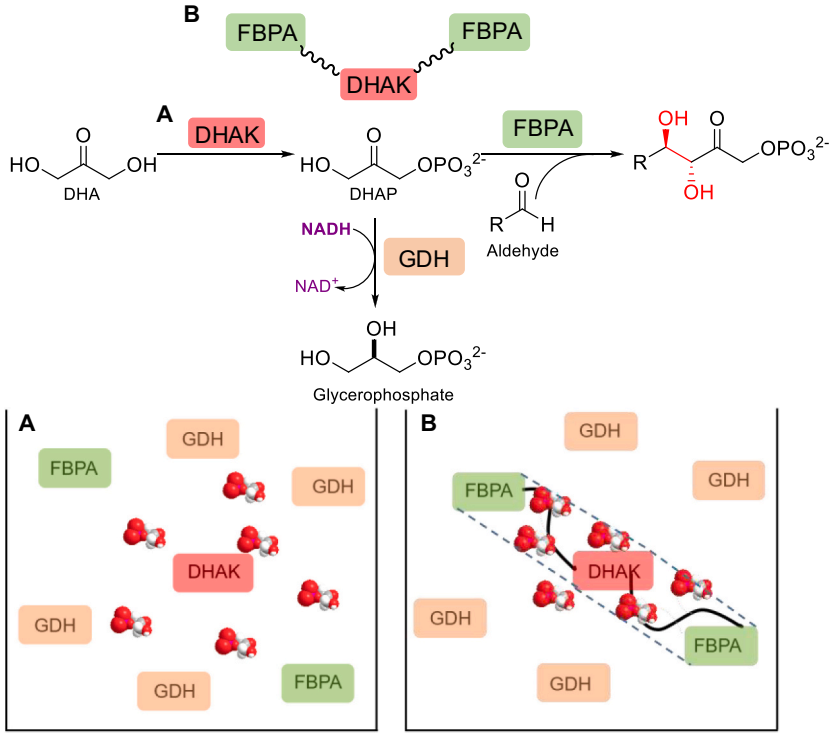

Fig. 3. Schematic representation of the experiment performed to detect the channeling effect in the fused DLF enzyme. When using the multi-enzyme system (A), DHAP can freely diffuse into the bulk solution and the GDH activity measured was almost twice than that measured when the reaction was catalysed by the fused enzyme (B) in which DHAP is directly transferred to the aldolase without exposure to the bulk solution.

2010).

Recently, Hartley et al. (2019) described a novel and generalizable approach that provides a solution to one of the main problems in the application of continuous flow biocatalysis, namely the need for co-factors, as it allows their regeneration and retention. This rather elegant method was inspired by enzymes that retain their substrates during a reaction cascade through covalent bonds. The fused multi-enzyme biocatalyst for the conversion of glycerol by regiospecific phosphorylation and oxidation via ATP and NAD^+ dependent steps consisted of three modules: (i)a catalytic module that drove the desired synthesis reaction, (ii) a module for regeneration of the cofactor, and (iii) a conjugation module that enabled site-specific covalent immobilization on an activated surface. The cofactor was covalently attached between the catalytic and regeneration modules via a flexible swinging arm that ensured regeneration of the cofactor and prevented its diffusion (Fig. 4).

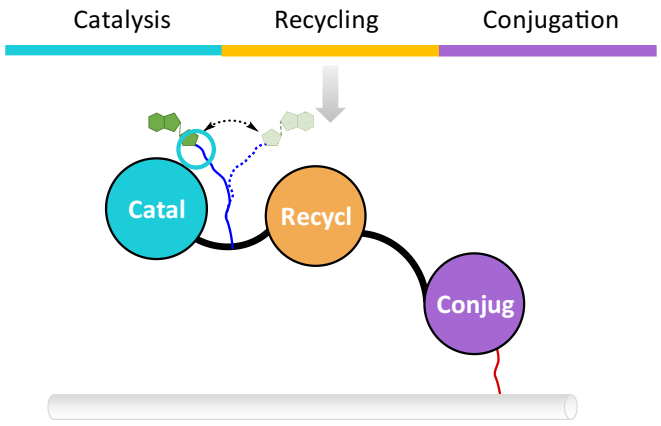

Fig. 4. Schematic representation of the modular multi-enzyme fusion biocatalyst able of retaining and recycling a bound cofactor (Hartley et al., 2019).

As proof of concept, the authors tackled the synthesis of a chiral D-fagomine precursor from glycerol, sequentially coupling three catalytic steps (Fig. 5). Each of these catalytic steps was performed using a bioreactor designed as outlined in Fig. 4. In the first bioreactor, phosphorylation of glycerol to glycerol-3P is performed with regeneration of ATP. In the second, the NAD^+ dependent oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) was catalysed, with the concomitant oxidation of NADH to NAD^+. Finally, the FBPA-catalysed aldol addition reaction was performed in the third bioreactor (Fig. 5). This approach to continuous flow reactor design provided a highly flexible platform for the construction of complex cascade reactions that could incorporate cofactor-dependent enzymes and could be extended to the development of artificial metabolic networks.

3-Hexulosa-6-phosphate synthase (Hps) and 6-phospho-3-hexuloisomerase (Phi) genes from *Mycobacterium gastri* catalysed the sequential reactions for formaldehyde assimilation in the ribulose monophosphate pathway (Kato et al., 2006). A fusion enzyme (HPS-PHI) created from these enzymes was able to fix formaldehyde to ribulose-5-phosphate to form D-arabino-3-hexulose-6-phosphate, which was then isomerized more efficiently (about twofold) to fructose-6-phosphate than the mixture of isolated enzymes (Orita et al., 2007). The *E. coli* strains expressing this bifunctional enzyme showed resistance to formaldehyde in vivo. Since formaldehyde is an important pollutant, the possibility of creating formaldehyde-resistant detoxifying microorganisms is very interesting from an environmental point of view.

Methanol has acquired high popularity for biotechnological applications, since it can be obtained from very abundant low-priced non-food feedstock. Natural or synthetic methylotrophic microorganisms have gained great interest for the production of high-value chemicals using methanol as the sole carbon and energy source or as a co-substrate with carbohydrates (Zhang et al., 2018). This bioprocess is hindered by the formation of formaldehyde, which affects the efficiency of methanol bioconversion. To overcome this drawback, Fan et al. (2018), fused a NADH-dependent methanol dehydrogenase (Mdh) with Hps and Phi

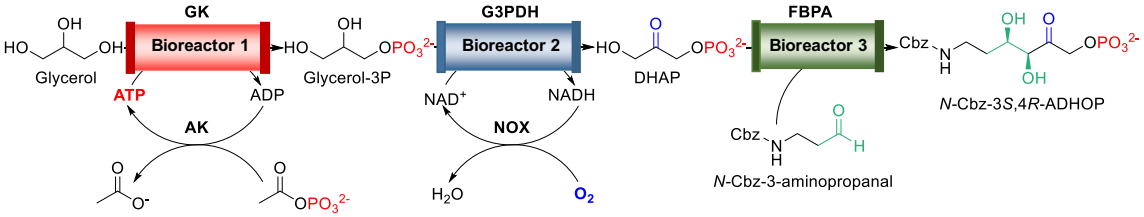

Fig. 5. Biocatalityc cascade for the synthesis of the D-fagomine precursor *N*-Cbz-3*S*,4*R*-amino-3,4-dihydroxy-2-oxyhexyl phosphate (*N*-Cbz-3*S*,4*R*-ADHOP). Bioreactor 1: GK = glycerol kinase from *Thermococcus kodakarensis*; AK = acetate kinase from *Mycobacterium smegmatis*; Bioreactor 2: G3PDH = glycerol-3-phosphate dehydrogenase from *E. coli*; NOX = NADH oxidase from *Clostridium aminovalericum*; Bioreactor 3: FBPA = fructose aldolase from *Staphylococcus carnosus*.

enzymes (Fig. 6). The efficiency in forming D-fructose-6-phosphate (F6P) was used to evaluate the performance of the fusion proteins in methanol bioconversion. Although fusion decreased formaldehyde assimilation activity, methanol conversion to F6P was increased by 30% when Mdh was fused with Hps or HPS-Phi. This approach has a remarkable implication for the biotechnological use of methanol, as these multifunctional enzymes could be assembled with other metabolic pathways and the enzymes could be engineered to improve their efficiency.

3.1.2. *Fusion enzymes for production of terpenes*

Terpenes are a chemically diverse group of metabolites classified by the number of isoprene units: monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}). These compounds are widely used in industry as agrochemicals, fragrances, nutraceuticals, and pharmaceuticals (Mewalal et al., 2017). Deng et al. (2016), designed a bifunctional enzyme fusing two different genes of (*S*)-linalool synthase with a farnesyl diphosphate synthase (FPPS) to improve the (*S*)-linalool production in *S. cerevisiae* from geranyl diphosphate (GPP), the universal precursor of monoterpenes. The production of (*S*)-linalool achieved 67.9% conversion, more than when the individual enzymes were used. Recently, Navale et al. (2019), linked an FPPS from *Santalum album* (*Sa*FFPS) to an *epi*-cedrol synthase from *Artemisa annua* (*Aa*ECS) via a glycine-rich linker (GSGGS) to synthesize a high valuable sesquiterpene, *epi*-cedrol from isopentyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Fig. 7). *Epi*-cedrol is a precursor of cedrol, which is widely used as a flavor/odour enhancer in cosmetics, food, medicine, fine fragrances, etc. (Luo et al., 2019). They successfully expressed the fused protein in *E. coli* and tested its biocatalytic efficiency. The fused enzyme was able to produce an amount of 8-*epi*-cedrol from IPP 1.66 fold higher than when using a mixture of the individual parental enzymes.

3.1.3. *C—C bond formation with fused P450*

Cytochromes P450 have the potential to be valuable catalysts for C—C bond formation, but their application is hampered by their poor solubility, and their requirement for auxiliary redox-transport proteins for the delivery of reducing equivalents from NAD(*P*)H. Numerous attempts have been made to overcome this drawback (see Section 3.2.2. *Self-sufficient Cytochrome P450 monooxygenases*). For instance, an example of a fusion P450 capable of cleaving and forming C—C bonds was reported by Schückel et al. (2012). In this work, the authors took advantage of their previously developed LICRED vector for the expression of plant P450s genetically fused to reductase proteins to fuse P450 isoflavone synthase (IFS) from *Glycine* max with the bacterial P450 reductase domain (Rhf-RED) from *Rhodococcus* sp., to create the first active bacterial-plant fusion P450 enzyme (Sabbadin et al., 2010). They also created the ACRyLIC vector for fusion of the desired P450 enzyme with the P450 reductase ATR2 from from *Arabidopsis thaliana*. The usefulness of both systems was confirmed for the in vivo conversion of naringenin to genistein.

3.1.4. *Fusion enzymes for enantioselective Michael reactions*

An interesting application of the gene fusion strategy that has only recently come into use is its use to break the symmetry of oligomeric proteins at the level of the quaternary structure (Studer et al., 2018; Wu et al., 2019; Xu et al., 2021a). In the work by Xu et al., 2021a, this strategy was used to evolve a promiscuous homohexameric 4-oxalocrotonate tautomerase (4-OT) into an efficient biocatalyst for enantioselective Michael reactions. The 4-OT consists of a trimer of dimers, with two active sites per adjacent pair of subunits. Each dimer is formed by a strongly interacting pair of β-α-β monomers (62 amino acids; Fig. 8). This particular structure entails that any point mutation occurs simultaneously in all six subunits, which drastically reduces the chances of their optimization by directed evolution techniques (DE). To extend the potential of genetic optimization of 4-OT by allowing independent

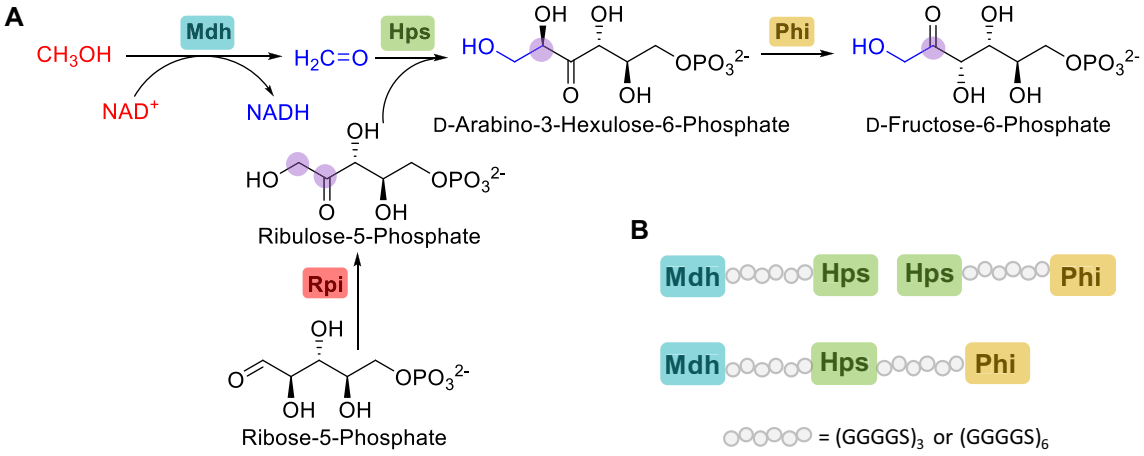

Fig. 6. (A) Scheme of fructose-6P formation from methanol and ribose-5P. (B) Schematic representation of the prepared bifunctional and trifunctional fusion proteins (Fan et al., 2018).

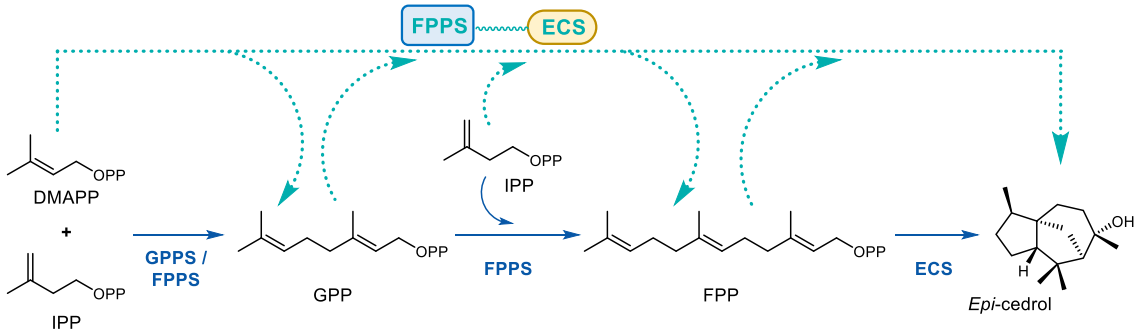

Fig. 7. Scheme representing the *epi*-cedrol biosynthetic pathway in recombinant *E. coli* (blue arrows) and of sesquiterpene 8-*epi*-cedrol by the fusion enzyme (dashed green arrows). Abbreviations: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl diphosphate; FPP, farnesyl pyrophosphate; GPPS, geranyl pyrophosphate synthase; FPPS, farnesyl pyrophosphate synthase; ECS, *epi*-cedrol synthase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

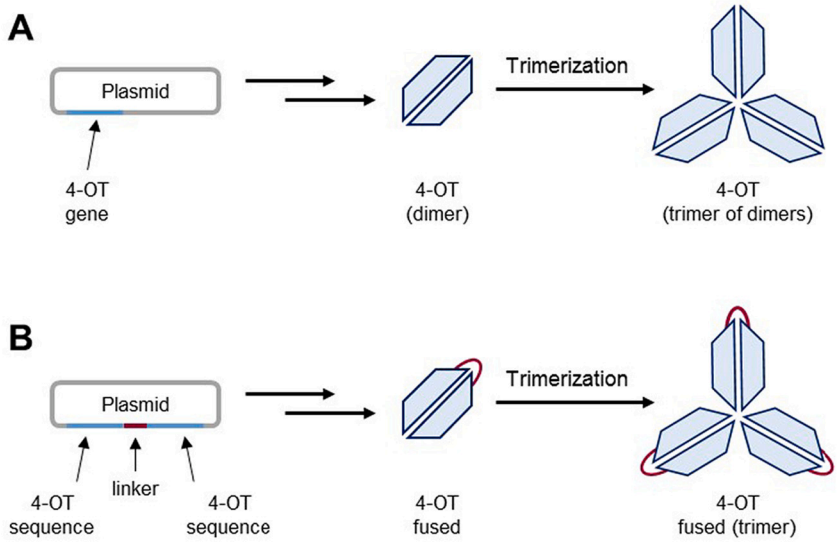

Fig. 8. Schematic representation of the tandem fused 4-OT design; each β-α-β unit (62 amino acids) is represented by a blue triangle and the linker is shown in red (Xu et al., 2021a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mutagenesis of two adjacent subunits, a tandem fusion between the C-t of the first monomer and the N-t of the second monomer was performed using a short and flexible linker of 5 amino acids (GGGAG) designed considering the distance between the endings in the crystal structure of 4-OT. The activity of this fused 4-OT (Fu-WT) was assayed on the Michael addition of nitromethane to cinnamaldehyde.

To optimize and improve the Michael addition activity, the fused enzyme was subjected to 11 rounds of directed evolution combining iterative focused saturation mutagenesis (ISM), shuffling by staggered and overlap extension PCR and error-prone PCR. After 11 rounds of evolution, the most active variant, 4-OT (F11), showed a 320-fold increase in catalytic activity compared with the wild-type enzyme in the Michael addition of nitromethane to cinnamaldehyde. Interestingly, the crystal structure of the 4-OT(F11) variant showed that the designed linker and the seventeen cumulative mutations did not significantly affect the folding of the 4-OT backbone, but the trimer of 4-OT(F11) had a reduced symmetry compared with the wild-type 4-OT. Thus, gene fusion allowed independent diversification of adjacent subunit sequences and the expansion of the space of protein sequences that can be optimized by directed evolution (Xu et al., 2021a).

3.2. Fusion enzymes for oxidative biocatalytic reactions

Oxidoreductases are a broad group of enzymes widely distributed in microbial, plant, and animal organisms that catalyze the electron transfer, or redox equivalents, from one substrate (oxidized substrate/electron donor) to another (reduced substrate/electron acceptor) (Demarche et al., 2012). Fused enzymes for oxidative reactions have been recently reviewed (Aalbers and Fraaije, 2019), so we will limit ourselves here to discussing a few selected recent examples.

The industrial application of oxidoreductases is hampered by the fact that they require stoichiometric amounts of expensive and unstable cofactors, such as NAD(*P*)H/NAD(P)$^+$ (Martínez et al., 2017). This problem has been addressed in several ways: i) using whole cells as biocatalysts to take advantage of the host metabolic machinery for cofactor biosynthesis (Mihovilovic et al., 2008); ii) replacing the natural cofactors NAD(*P*)H with synthetic, low-cost biomimetics (Josa-Culleré et al., 2019; Knaus et al., 2016); iii) replacement of NADPH in flavin-dependent enzymatic reactions by using light as an energy source and EDTA as an electron reservoir (Hollmann et al., 2007); and iv) in situ enzymatic regeneration of the cofactor (Hummel and Gröger, 2014; van der Donk and Zhao, 2003). In this sense, enzyme fusion is an attractive alternative to the latter strategy, i.e., cofactor regeneration, as shown by the following examples.

3.2.1. Self-sufficient Baeyer-Villiger monooxygenases

Baeyer-Villiger monooxygenases (BVMOs) are highly versatile biocatalysts that catalyze the regio-, chemo-, and enantioselective

oxygenation of a wide range of substrates. They require an electron donor (usually NADPH) to complete the catalytic cycle by reducing the flavin bound to the protein responsible of the oxidation of the substrate (Torres Pazmiño et al., 2010). A study involving a BVMO investigated the possibility of synthesizing lactones starting from unsaturated alcohols as substrates through the fusion of three enzymes, i.e., an alcohol dehydrogenase, an ene-reductase (ERED), and a BVMO (Peters et al., 2017). In particular, the original design of this multifunctional enzyme used xenobiotic reductase B (XenB) from *Pseudomonas putida*, a thermostable variant of the cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. and for the ADH domain either the corresponding enzymes from *Lactobacillus kefir* (Lk-ADH) or from *Rhodococcus ruber* (ADH-A). Unfortunately, the fusion of the three enzymes showed no production of caprolactone when starting from cyclohexanol. However, this setback was overcome by simultaneous overexpression of a CHMO-XenB (CHMO-QC_G_XenB) fusion protein in *E. coli* together with Lk-ADH, to perform whole-cell biocatalysis using cyclohexanol as the initial substrate. The results showed that product formation increased from 1.23 ± 0.03 mM upon biotransformation with the three individual enzymes to 2.02 ± 0.03 mM with the CHMO-XenB fusion enzyme plus ADH system.

Recently, following the pioneering works of Fraaije's group (Torres Pazmiño et al., 2009, 2008) in which they developed BVMOs capable of recycling their own coenzyme, Mourelle-Insua et al. (2019) developed a collection of fusion enzymes between a BVMO (cyclohexanone monooxygenase from *Thermocrispum municipal*; *Tm*CHMO) or an alcohol dehydrogenase (alcohol dehydrogenase from *Lactobacillus brevis*: *Lb*ADH) with three different cofactor regenerating enzymes —with different sacrificial substrates—, that can be used in self-sufficient oxygenation and ketone reduction (Fig. 9). These new biocatalysts showed good tolerance to various organic co-solvents, including deep eutectic solvents. The versatility of the fused enzymes was demonstrated with a variety of substrates such as methylcyclohexanone (converted into (*S*)-4-methyl-ε-caprolactone), thioanisole or butyrophenone, which were processed with conversion rate close to 99% and enantiomeric excess >99% in almost all cases.

3.2.2. *Self-sufficient cytochrome P450 monooxygenases*

Another family of biocatalysts of great interest for the production of pharmaceutical intermediates, aromatic compounds, and fine chemicals are the cytochrome P450 monooxygenases. They are able to introduce oxygen into activated and non-activated carbon-hydrogen bonds, with good control over the regio- and the stereochemistry of the process. Completion of the catalytic cycle requires the sequential transfer of two electrons from a reducing cofactor to the heme group (Meunier et al., 2004). Electron transfer is accomplished by transfer proteins, which may be domains fused to the monooxygenase component or independent proteins that transfer electrons in transient assemblies (McLean et al., 2005). The need for redox partners, coupled with the need for a constant supply of NAD(*P*)H, hampers the large-scale application of these enzymes (Grogan, 2011; O'Reilly et al., 2011). In this sense, the catalytically self-sufficient flavocytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* circumvents the first of these limitations because it is a single polypeptide consisting of a reductase domain (BMR) containing FAD and FMN fused to the C-terminus of the heme-containing monooxygenase domain (BMP) (Fig. 10; Miles et al., 1992).

Fusion enzymes between P450 BM3 and phosphite dehydrogenase (PTDH; Fig. 9; Beyer et al., 2017) and with formate dehydrogenases (FDH; Fig. 10; Kokorin et al., 2021) were prepared for the regeneration of NADPH. PTDH is one of the most cost-effective options because it uses the oxidation of phosphite, which is not expensive, to phosphate to regenerate NADPH. The major advantage of FDH, in addition to the low cost of the substrate formate, is the ease of removal of CO_2 produced during formate oxidation (Maurer et al., 2003). Kokorin et al. (2021) investigated the effect of gene order on fusion as well as the type of enzyme-binding linker with respect to substrate conversion, stability, and kinetic parameters to test whether fusion leads to improvements in essential enzymatic properties. In this sense, they found that the catalytic efficiency and stability of FDH at high concentrations of formate were not change compared to the parent enzyme when the order of fusion was FDH/BM3. However, when a more rigid or longer linker was used while maintaining this order of fusion, a 1.1- to 1.3-fold increase in FDH activity was observed. This was probably because in these cases it facilitated the adoption of the conformations required for HCOO-

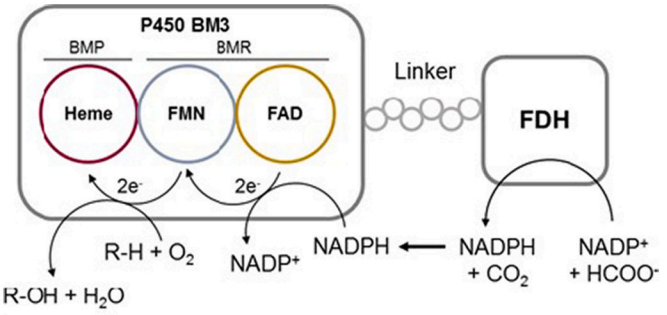

Fig. 10. Schematic representation of the structure of the P450 BM3/FDH fusion enzyme (Kokorin et al., 2021).

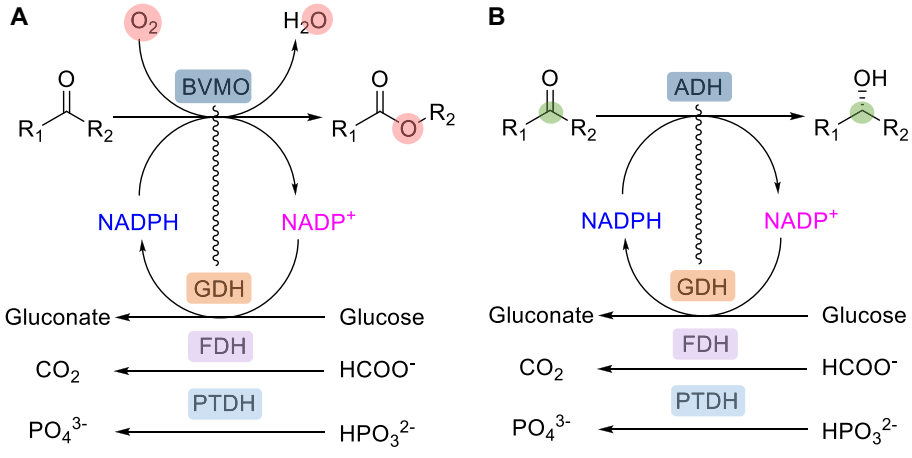

Fig. 9. Schematic representation of the self-sufficient (A) BVMOs and (B) ADHs, with their fusion partner and the different sacrificial substrates to recycle the NADPH cofactor.

binding at the corresponding FDH domain. In contrast, the BM3/FDH construct with the short glycine linker showed a two- to threefold higher FDH activity. These results demonstrate the importance of the order of the fused genes (N- and C-terminal orientation) to obtain a biocatalyst with catalytic behavior superior to that of the parent enzymes acting independently.

Other self-sufficient cytochromes P450 were developed to catalyze different biotransformations. Thus, $OleT_{JE}$ is a cytochrome P450 able to catalyze the oxidative decarboxylation of saturated fatty acids to terminal olefins using hydrogen peroxide as oxidant. Unfortunately, H_2O_2 deactivates the enzyme at millimolar concentrations. To achieve an H_2O_2 supply compatible with an efficient conversion of fatty acids by $OleT_{JE}$ while maintaining its concentration at subinhibitory levels, Matthews et al. (2017) linked cytochrome $OleT_{JE}$ to alditol oxidase (AldO) from *Streptomyces coelicolor*. AldO is a flavoprotein that catalyzes the oxidation of the primary alcohol moiety of various polyols, including glycerol, using molecular oxygen as the oxidizing agent, yielding aldose and H_2O_2 as a byproduct. Since the activity of AldO depends on the concentration of polyol, the OleTJE-AldO system could be regulated through alditol substrate additions to achieve a trade-off between efficient substrate conversion and minimizing the destruction of the heme group of OleTJE. In another approach, Lu et al. (2018) developed a fusion enzyme to achieve an H_2O_2-independent $OleT_{JE}$. In this fused enzyme, the heme domain of cytochrome P450BM3 was replaced with $OleT_{JE}$, so that it could use O_2 as oxidizing agent (Fig. 10). The OleT-BM3R fusion enzyme showed high decarboxylation activity in the presence of NADPH toward both long- and medium-chain saturated FAs and cyclic and acyclic α-branched carboxylic acids. Compared with other redox systems for fatty acid decarboxylation based on the use of $OleT_{JE}$ (Dennig et al., 2015), the OleT-BM3R fused enzyme offers higher product titer (1.40 g L^{-1} vs 0.93 g L^{-1}) and volumetric productivity (116.7 mg L^{-1} h^{-1} vs 42.5 mg L^{-1} h^{-1}), presumably because of the greater TOF (472 h^{-1} vs 53 h^{-1}) and NADPH coupling efficiency (61% vs 25%).

Another self-sufficient cytochrome P450 was developed by linking class I cytochrome P450, TxtE, with the reductase domains of BM3 (Zuo et al., 2016). TxtE is the only enzyme known to catalyze the direct indole nitration reaction of L-tryptophan. Moreover, it exhibits a considerable tolerance for small modifications in the indole ring (Dodani et al., 2014), which gives it a special interest as biocatalyst since aromatic nitration is a very important industrial process to produce chemical products for various applications. The fused enzyme TxtE-BM3R was comparable to wild-type TxtE in terms of nitration yield and other biochemical characteristics. To demonstrate the synthetic applicability of the fusion enzyme TxtE-BM3R, large-scale nitration of the two unnatural substrates 4-F-DL-tryptophan and 5-fluoro-L-tryptophan was performed, resulting in 4-fluoro-7-NO_2-L-tryptophan and 4-NO_2–5-fluoro-L-tryptophan, respectively. Interestingly, although nitration was performed in principle at the C4 of the indole ring of L-tryptophan, TxtE-BM3R performed nitration at the C7 when the C4 was replaced with a fluorine atom (Zuo et al., 2016).

3.2.3. Oxidase–peroxidase/peroxygenase fusion enzymes

Although oxidase-peroxidase fusion enzymes have not been observed in nature, they are often co-expressed because they are interconnected since oxidases produce hydrogen peroxide, which in turn is a substrate for peroxidases (Colpa et al., 2017).

A well-known example of this interaction between oxidases and peroxidases is the ligninolytic enzyme consortium produced by white rot fungi (Gonzalez-Perez and Alcalde, 2014). Classified according to their oxidative behavior, the main ligninases are the high redox potential peroxidases and laccases. Among other enzymes involved in the modification of lignin at different stages are: nonspecific peroxygenases (UPO) and hydrogen peroxide supplier oxidases (aryl alcohol oxidases, AAOs) (Alcalde, 2015). In this sense, UPOs can provide a wide range of reactions for the synthesis of pharmaceutical products and are becoming the most promising alternative to the already known methods based on chemical, enzymatic —P450— or whole cell transformations (Hofrichter and Ullrich, 2014). However, UPOs are rapidly and irreversibly inactivated by catalytic concentrations of H_2O_2. This key problem has been addressed with various strategies combining peroxygenases with photocatalysis, electrocatalysis, and chemocatalysis, as well as with biocatalytic cascades, all aimed at controlling the supply of H_2O_2 in situ (Bormann et al., 2015). To this end, a novel self-sufficient peroxygenase-oxidase was recently developed by fusing a fungal unspecific peroxygenases (UPO) from *Pleurotus eryngii* and an aryl-alcohol oxidase from *Agrocybe aegerita* (AAO) (Gomez De Santos et al., 2020). The individual enzymes of this fused biocatalyst were previously evolved through several rounds of directed evolution (Gomez de Santos et al., 2019; Molina-Espeja et al., 2014; Viña-Gonzalez et al., 2018; Viña-Gonzalez et al., 2019). The efficacy of the developed fusion enzyme was tested in the preparative synthesis of dextrorphan, a human drug metabolite (HDM) of the cough suppressant dextromethorphan (Fig. 11). This reaction system was optimized to control the conversion rate of the aromatic alcohol and thus theH_2O_2 supply, to achieve a total turnover number (TTN) of 62,000 by using a fed-batch strategy with a dosage rate of the aromatic alcohol of 0.5 mM h^{-1}. This value is the maximum reported so far for the biocatalytic synthesis of dextrorphan and is significantly higher than the 7500 TTN achieved with the mutant UPO without the H_2O_2 cascade (Gomez de Santos et al., 2019) or the 10,540 TTN obtained with a sulfite oxidase-peroxygenase cascade system (Schie et al., 2020).

3.3. Carbohydrate-active fused enzymes

Carbohydrates, glycoconjugates, and their derivatives play key biological roles in physiological processes such as cell recognition, signalling, infection, adhesion, inflammation, and normal or abnormal cellular development. In this regard, access to defined molecules of this class of compounds to determine their precise biological roles is of paramount importance, not only to understand the physiological processes in which they are involved, but also to exploit their potential use for medical therapeutic purposes, such as the development of carbohydrate-based drugs, vaccines, adjuvants, as well as novel drug delivery systems. Unlike peptides or nucleotides, and despite their high medicinal and biological potential, the development of efficient and general synthetic routes remains a major challenge due to the complexity of their structures. Therefore, enzymatic methods have already become a viable alternative to catalyze the degradation, biosynthesis, or modification of carbohydrates and glycoconjugates, and several fusion enzymes have been developed for this purpose.

3.3.1. Fusion enzymes for oligosaccharide synthesis

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranose) is a non-reducing disaccharide ubiquitously distributed in various living organisms. This compound is involved in numerous functions. It is an essential component for maintaining cell viability, an energy reservoir, a protective agent against a variety of physical and chemical stresses, and a protein stabiliser. Due to its physical and chemical properties, trehalose is used in various industries as a preservative for food, cosmetics, and drugs (Ohtake and Wang, 2011). At least three different fusion enzymes have been developed for the industrial production of trehalose. The first bifunctional fusion enzyme was produced by fusing the *E. coli* genes for trehalose-6-phosphate (T6P) synthetase (TPS) and trehalose-6-phosphate phosphatase (TPP) (Seo et al., 2000). The fused TPSP catalysed the successive reactions in which T6P was formed and then dephosphorylated, resulting in the synthesis of trehalose. Trehalose synthesis by TPSP was 65% faster than that by the equimolar mixture of TPS and TPP, and the catalytic efficiency was 3.5 to 4.0 times higher. In a second approach, maltooligosyltrehalose synthase (BvMTS) and maltooligosyltrehalose trehalohydrolase (BvMTH) from *Brevibacterium helvolum*, were fused (Kim et al., 2000). The bifunctional BvMTSH enzyme

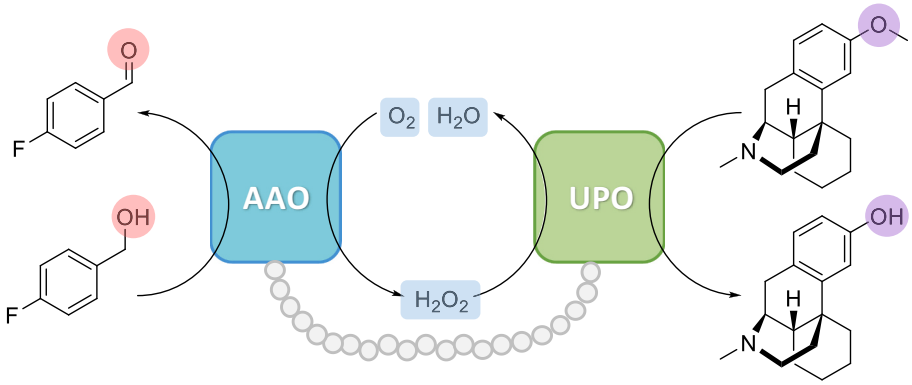

Fig. 11. Schematic representation of the dextromethorphan transformation into dextrorphan catalysed by the fused AAO-UPO (Gomez De Santos et al., 2020).

catalysed the conversion of maltooligosaccharides to maltotriosyltrehalose. In the subsequent hydrolysis, the maltooligosaccharides were shortened by two glucose units per cycle of the successive reactions to produce trehalose and maltotriose (Fig. 12). In the third approach, a thermophilic bifunctional enzyme combining the genes for maltooligosyltrehalose synthase (MhMTS) and maltooligosyltrehalose trehalohydrolase (MhMTH) from the thermophilic microorganism *Metallosphaera hakonensis* (Seo et al., 2008) was produced. This enzyme could overcome the limitations imposed by the low thermal stability of enzymes from mesophilic organisms in industrial processes that often require high temperatures. In this case, maximum trehalose production was reached at about 70 °C and about 80% of the initial activity was maintained at this temperature for >48 h.

In a pioneering work, biomedically important Galα1,3Gal-terminated oligosaccharides could be synthesized using the chimeric proteins built by Peng Wang group (Chen et al., 2000). These fusion enzymes consisted of a uridine diphospho-galactose 4-epimerase (UDP-galactose 4-epimerase) bound to either the C-terminus (f1) or the N-terminus (f2) of a truncated bovine α-1,3-galactosyltransferase gene. These enzymes, f1 and f2, showed an increase of 300% and 50%, respectively, over the overall reaction rate for the formation of Galα1,3Lac of the multienzyme system (Fig. 13).

The results of these authors suggest that these increases in the overall catalytic activity were due to the proximity of the active sites, but they also clearly show that the proximity effect is not the only cause of this increase and that other factors may be crucial, such as in this case the order of enzymes in the final construct. Fusion with epimerase changed the donor requirement of the α-1,3-galactosyltransferase from UDP-galactose to UDP-glucose without changing the acceptor specificity of the transferase. The change in donor requirement resulted in a reduction in the cost of the synthesis of the oligosaccharide by >40-fold.

3.3.2. *Fusion enzymes for plant biomass degradation*

Plant biomass can serve as an abundant and inexpensive source of fermentable sugars for the production of various chemicals and biofuels. The plant cell wall is a complex structure in which cellulose, hemicellulose, and lignin are the main components. Cleavage of the bonds present in the cellulose chains requires the synergistic action of three enzymes: endoglucanases, which cleave within the chain, exoglucanases, which cleave at the end of the chain, and β-glucosidases, which cleave oligomers into monomers. In fungi and bacteria, the cell wall-degrading enzymes occur as a free enzyme system, but anaerobic bacteria produce a large extracellular enzyme complex called the cellulosome, which consists of a noncatalytic protein, scaffoldin, and various enzymes bound to scaffoldin via the specific recognition and interaction of cohesins and dockerins. In many cases, the enzymes that degrade the

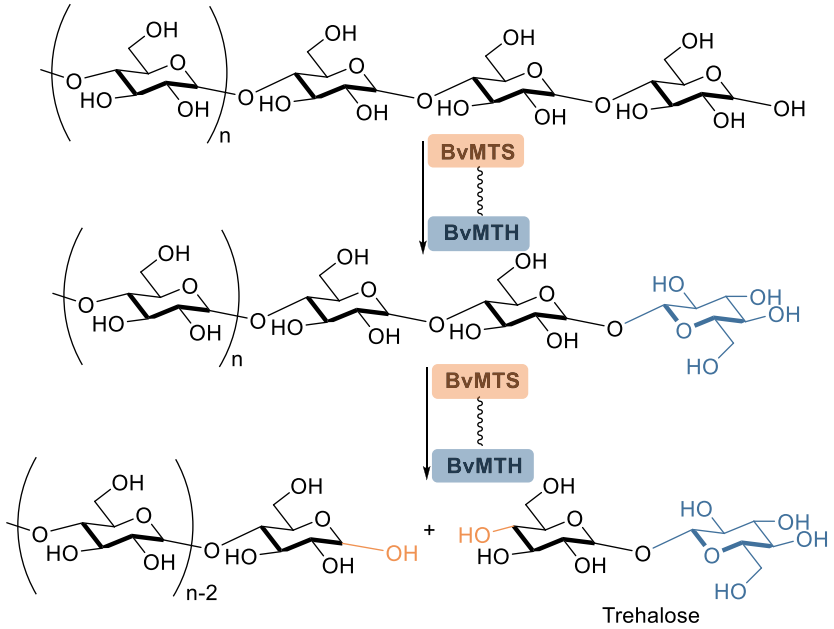

Fig. 12. Synthesis of trehalose from maltooligosaccharides using BvMTS-BvMTH fusion enzyme.

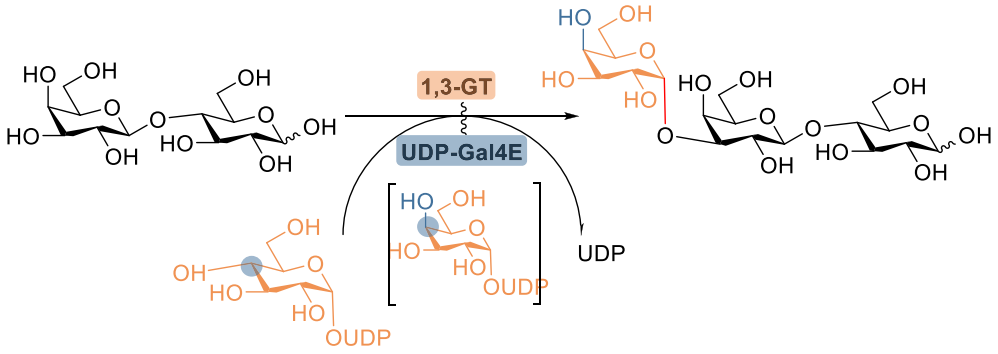

Fig. 13. Reaction catalysed by the UDP-galactose-4-epimerase/α-1,3-galactosyltranferase fused enzyme.

plant cell wall are multimodular enzymes that may contain one or more catalytic modules and other modules, such as the carbohydrate binding module (CBM), that bind the enzyme to the substrate to allow more efficient degradation (Xu et al., 2011). From a biotechnological point of view, the development of a multifunctional enzyme capable of hydrolyzing multiple types of glycosidic bonds has a high potential to break down polysaccharides into usable oligo- or monosaccharides for biotechnological applications (Martins et al., 2020; Sarai et al., 2019). Moreover, since hydrolysis of cellulosic biomass requires a consortium of enzymes, reducing the number of proteins to be expressed and purified would reduce production costs. Considering the large application potential, a large number of bifunctional enzymes have been described in this field (Fan et al., 2009a, 2009b, 2009c; Fan and Yuan, 2010). Xylan is the most abundant component of hemicelluloses. Therefore, various combinations of xylanase with other endoglycosidases such as xylosidase (Adelsberger et al., 2004), arabinofuranosidase (Adelsberger et al., 2004) or mannanase (Guo et al., 2013) have been described and tested. In Section 2.3, an interesting example of the design and optimization of fusion enzymes for the degradation of crystalline cellulose has already been discussed (Nakashima et al., 2014).

Although fungi such as *Trichoderma reesei* and *Aspergillus niger* are the most common sources of plant cell wall hydrolyzing enzymes, the use of new glycoside hydrolases from bacteria is now widely used due to their easier production, high specific activity, and less stringent pH requirements (Demain et al., 2005). In this sense, Yazdani and co-workers cloned and expressed endoglucanase (Endo5A), endoxylanase (Xyl11D) and β-glucosidase (Gluc1C) from a *Paenibacillus* strain isolated from the gut of cotton bollworm (Adlakha et al., 2012, 2011). The authors constructed six fusion enzymes between Endo5A and Gluc1C by varying their relative positions and the length of the glycine-serine linkers. Interestingly, one of the constructs, EG5 (with the structure Endo5A-$(G_4S)_3$-Gluc1C), showed specific activities that were 3.2 and 2 times higher than those of the corresponding enzymes individually for β-glucosidase and endoglucanase, respectively. When the fused EG5 enzyme was incubated with carboxymethyl cellulose, a 10% increase in the amount of reducing sugars released was observed compared with a mixture containing the same amount and ratio of Endo5A and Gluc1C. These results suggest that the proximity of the active sites in the fused enzyme favors cellulolytic activity. In addition, fusion of the enzymes resulted in an increase in thermostability of 5 °C for Gluc1C and 9 °C for Endo5A (Adlakha et al., 2012).

In another study, the cellulase system from the highly efficient cellulolytic bacterium *Thermobifida fusca* was used as a model to compare free and cellulosomal enzyme systems by artificial fusion of their enzymes (Caspi et al., 2008). The exoglucanase Cel6B, which contains native CBM2, is one of the key enzymes of the cellulase system of *T. fusca*, which is important to achieve maximum activity of synergistic mixtures of free enzymes. Therefore, Setter-Lamed et al. (2017) studied the effects of its incorporation into artificial pseudo-cellulosomes on enzymatic activity. Pseudo-cellulosomes containing Cel6B derivatives achieved better cellulose substrate degradation compared to the corresponding mixture of free parent enzymes. The composition of the linker had a greater effect on cellulolytic activity than its length. Surprisingly, both shifting the CBM from the N-terminus to the C-terminus of Cel6B and reducing the linker length resulted in increased cellulose activity compared with wild-type Cel6B. This result suggests some plasticity in the relationship between the CBM and the catalytic module of this exoglucanase (Caspi et al., 2009).

3.4. Fusion enzymes for the generation of chiral amines

Chiral amines are important chemical building blocks for the synthesis of a wide range of pharmaceutical, agrochemical, and bioactive products. Among other enzymes, transaminases (TAs) have proven to be powerful tools for the production of chiral amines (Gomm and O'Reilly, 2018; Schrittwieser et al., 2015). TAs reversibly catalyze the formation of (*R*) or (*S*)-configured amines by transferring an amino group from a suitable donor to a carbonyl acceptor. To avoid the addition of the amine donor in excess, an alternative strategy to shift the equilibrium of the reaction is to remove the resulting carbonyl coproduct to prevent the reverse reaction from competing, which compromises the yield. An interesting example of the fusion of TAs was reported for the obtaining of isosorbide amino alcohol from the biotechnologically accessible dicyclic dialcohol isosorbide (Lerchner et al., 2016). In this case, a fusion protein was designed between the levodione reductase (LR) from *Leifsonia aquatica* and the previously engineered variant L417M of the ω-aminotransferase from *Paracoccus denitrificans* (PDωAT(L417M)). It is noteworthy that both parent enzymes have a homooligomeric structure, with the LR being a tetrameric enzyme and the PDωAT(L417M) being a dimer. Therefore, to ensure sufficient steric flexibility for efficient oligomerization of each enzyme moiety with its characteristic quaternary structure, different linkers with different chain lengths were tested. The linkers used were 20, 40, or 60 residues in length and consisted of three small amino acids Pro, Ala, and Ser. Interestingly, although both ADH and TA activities were decreased compared to the individual enzymes, all fused enzymes had higher turnover rates —2-fold, 1.6-fold, and 1.2-fold depending on spacer length—, in the synthesis of desired amino alcohol. This effect was more favoured the lower the initial substrate concentration was (by up to a factor of 7 at 75 mM substrate), hinting at the possible existence of substrate channeling due to the proximity effect.

3.5. Fusion enzymes in the synthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS)

Sulfation is a fundamental reaction in living organisms and plays a key role in the functionality of proteins, steroids, carbohydrates, and other metabolites, either in triggering biological reactions or in regulating their function at the cellular level (Günal et al., 2019). Because of this great biological importance, there is a growing interest in the

synthesis of sulfated molecules (Correia-da-Silva et al., 2014; Kauffman, 2004; Manlusoc et al., 2019). In nature, sulfation is catalysed by sulfotransferases (EC 2.8.2). These enzymes (with the exception of the arylsulfate sulfotransferases EC 2.8.2.22) use the 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the donor of the sulfuryl group. Unfortunately, large-scale synthetic application of PAPS dependent sulfotransferases is hampered by the high cost and instability of PAPS, and by their strong inhibition by the desulfated donor compound, adenosine 3′,5′-diphosphate (PAP), which is formed after sulfation (Chapman et al., 2004).

The synthesis of PAPS occurs from sulfate and two molecules of ATP in a two-step process catalysed by an ATP sulfurylase (ATPS), which catalyzes the formation of adenosine 5′-phosphosulfate (APS), and an APS kinase (APSK), which catalyzes the phosphorylation of APS to form PAPS (Datta et al., 2020). For this reason, it is necessary to develop efficient strategies not only for the regeneration of PAPS from PAP, but also for the de novo synthesis of PAPS (Liu et al., 2021). In this sense, Xu et al., 2021b developed a biocatalytic cascade that enables equimolar conversion of ATP to PAPS and can potentially be used for industrial applications (Fig. 14). This cascade was based on the use of an artificial bifunctional PAPS synthase constructed from an ATPS from *S. cerevisiae* and an APSK from *E. coli*, previously selected for their high catalytic efficiency.

The performance of this bifunctional enzyme was optimized by analysing the effects of rigid (EAAAK) or flexible (GGGGS) linkers with different lengths on activity. The optimal construct proved to be the one that linked the C-t of ATPS to the N-t of APSK using a flexible linker with six copies of the sequence GGGGS. This artificial bifunctional PAPS synthase produced a higher conversion of PAPS than the ATPS + APSK-catalysed two-step reaction, and 1.93 ± 0.05 g L^{-1} PAPS was synthesized within 60 h. Moreover, the accumulation of the intermediate APS was much lower in the reaction catalysed by the bifunctional enzyme, indicating that a direct transfer of the intermediate between the enzymes occurred in the fused enzyme without its release into the bulk solution. To complete the biocatalytic cascade, the authors implemented an ATP regeneration system based on the use of polyphosphate (polyP) kinases (PPK) that consume polyP, an inexpensive and readily available phosphate donor (Tavanti et al., 2021; Fig. 14). In this way, this biocatalytic cascade enabled a theoretically equimolar conversion of ATP to PAPS.

3.6. Fusion enzymes for plastics depolymerization

Plastic pollution is one of the biggest environmental problems facing the world today. In this sense, microbial biodegradation and depolymerization mediated by free enzymes are emerging as an efficient and sustainable alternative for plastic treatment and recycling (Magnin et al., 2020; Priya et al., 2022; Wei et al., 2020). So far, several microbial enzymes have been discovered that degrade hydrolyzable plastics and mainly belong to the carboxylic ester hydrolase families, such as cutinases, lipases (EC 3.1.1.3), and carboxylesterases (Gricajeva et al., 2022; Urbanek et al., 2020). Fusion of a plastic-degrading enzyme has been used to create bifunctional biocatalysts for improved depolymerization efficiency (Zhu et al., 2022). Recently, a two-enzyme system for the depolymerization of polyethylene terephthalate (PET) was discovered (Yoshida et al., 2016) by screening natural microbial communities exposed to PET in the environment. The system uses a PETase enzyme —which is a cutinase-like serine hydrolase— to convert the polymer into soluble intermediates, including mono-(2-hydroxyethyl) terephthalate (MHET), which is cleaved by the second enzyme, an MHETase, to yield terephthalic acid (TPA) and ethylene glycol (EG) (Fig. 15). Knott et al. (2020), performed a thorough and complete study combining structural, computational, biochemical, and bioinformatic approaches on the mechanism of action and structure of MHETase and to the engineering of the two-enzyme PETase/MHETase system for PET depolymerization. The authors designed three fused enzymes by covalently linking the C-t end of MHETase to the N-t end of PETase using flexible glycine-serine linkers with a total of 8, 12, and 20 residues (Fig. 15). Their activity was assayed using amorphous PET as substrate. In all three cases, the depolymerization yield of the fusion enzymes was better than that of PETase alone, MHETase alone, and an equimolar mixture of free PETase and MHETase in solution, with no major differences observed between the performance of the three fusion enzymes. Moreover, the activity of MHETase in the fused enzymes, mainly in MP12, was about 3-fold higher than that of MHETase alone.

In recent years, biodegradable plastics have been developed and marketed to reduce the environmental impact of synthetic plastics (Geyer et al., 2017). Among these, poly-(ε-caprolactone) (PCL) has attracted particular interest for commodities and biomedical applications due to its physicochemical properties and compatibility with other polymers (Malikmammadov et al., 2018). Recently, Liu et al. (2018, 2019) developed a bifunctional lipase/cutinase (Lip/Cut) that was superior to the two original enzymes or their mixture in degrading PCL films. The weight loss of PCL films by Lip/Cut was 14.35, 12.77, and 6.67 times higher than that achieved with lipase and cutinase alone or with a mixture of lipase and cutinase, respectively. Moreover, PCL could be completely degraded to hydroxyhexanoic acid monomers, being the recovery of monomers one of the most suitable recycling methods for the environment (Liu et al., 2019).

These studies indicate that the synergistic action in the hydrolysis of plastics has been improved in the previously discussed fusion enzymes, so this strategy seems promising to obtain efficient biocatalysts for their degradation and recycling.

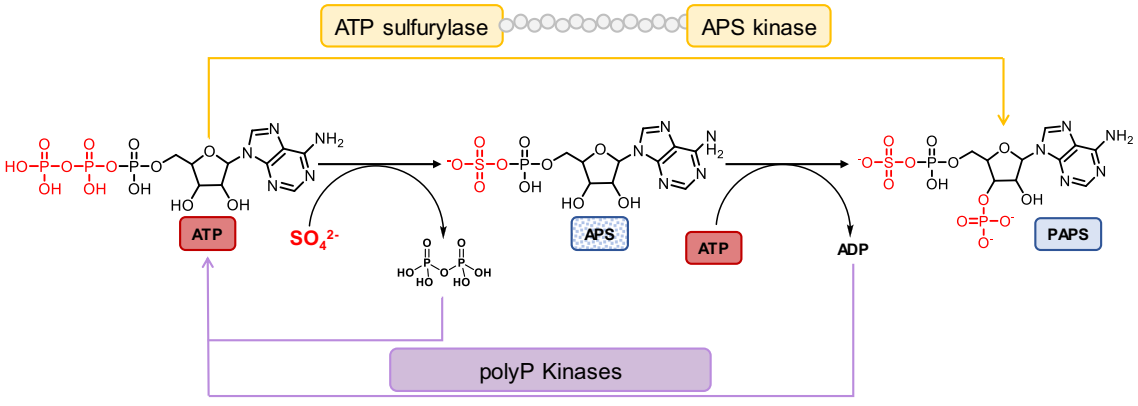

Fig. 14. Schematic representation of the PAPS synthesis by the artificial bifunctional PAPS synthase coupling with ATP regeneration driven by polyP kinases (Xu et al., 2021b).

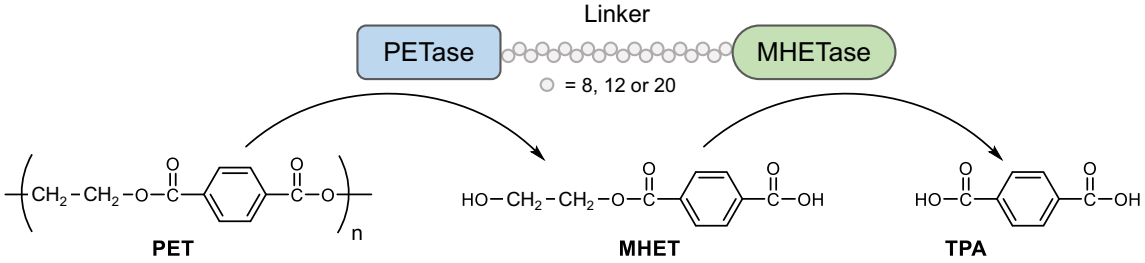

Fig. 15. Degradation of PET to TPA by a PETase/MHETase fusion enzyme. Depolymerization performance was tested with constructions bearing linkers of different lengths, i.e. 8, 12 and 20 amino acid residues.

4. Conclusions

Nature's strategy for evolution from monofunctional enzymes to bi- or multifunctional enzymes has proven to be an efficient method for catalyzing chemical transformations in the crowded environment of the cell. Multifunctional enzymes offer numerous advantages for cellular metabolism such as increased catalytic efficiency, control of metabolic flux, circumvention of unfavorable equilibria and kinetics, protection of unstable intermediates and/or stabilization of labile cofactors, protection of the cell from reactive or toxic intermediates. In recent decades, chemists have begun to exploit these advantages by applying Nature's approach to obtain fused multifunctional enzymes with desired catalytic activities to improve the rate and efficiency of biocatalytic reactions. In addition, the use of fused enzymes is very attractive because both protein purification and protein immobilization are greatly simplified, reducing the cost and manufacturing requirements for catalysts in bioprocess development.

The examples given in this review show that the development of new multifunctional fused enzymes with optimized properties is a complex and multidisciplinary problem that involves aspects of biophysics and biochemistry as well as protein engineering and demonstrates the power of genetically fused enzymes as synthetic tools in biocatalysis. Moreover, in the coming years, we expect to see increased use of fused enzymes in combination with metabolic engineering and synthetic biology to create new opportunities for the development and production of new compounds of practical interest in areas such as drug discovery, materials science, fine chemistry, etc.

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CRediT authorship contribution statement

Dianelis T. Monterrey: Visualization, Writing – original draft, Writing – review & editing. **Iván Ayuso-Fernández:** Visualization, Writing – original draft, Writing – review & editing. **Isabel Oroz-Guinea:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Eduardo García-Junceda:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interest.

Data availability

No data was used for the research described in the article.

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