

Review

Recent Advances in Enzymatic and Chemoenzymatic Cascade Processes

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Abstract: Cascade reactions have been described as efficient and universal tools, and are of substantial interest in synthetic organic chemistry. This review article provides an overview of the novel and recent achievements in enzyme cascade processes catalyzed by multi-enzymatic or chemoenzymatic systems. The examples here selected collect the advances related to the application of the sequential use of enzymes in natural or genetically modified combination; second, the important combination of enzymes and metal complex systems, and finally we described the application of biocatalytic biohybrid systems on in situ catalytic solid-phase as a novel strategy. Examples of efficient and interesting enzymatic catalytic cascade processes in organic chemistry, in the production of important industrial products, such as the designing of novel biosensors or bio-chemocatalytic systems for medicinal chemistry application, are discussed

Keywords: enzymes; cascade catalysis; organometallic; biocatalysis; chemoenzymatic processes

1. Introduction

Cascade reactions, typically defined as a consecutive series of chemical reactions proceeding in a concurrent fashion, have attracted the research community's attention in the last few years.

One of the main areas where this strategy plays a pivotal role is in nature, with the biosynthesis of natural products [1,2].

Systems where different enzymes, homogeneous organometallic complexes, are combined successively in a one-pot or tandem processes, in different manners, have been described as successful catalysts in different chemical applications [3,4].

These processes present advantages when compared to the typical single reaction, such as atom economy, step-saving, and therefore high yield and efficiency of the chemical process [5,6]. Additionally, the use of enzymes as catalysts, conforming the cascade system, leads to a more sustainable and environmentally benign process. However, these systems have some disadvantages, such as the fact that the enzymes have different pH and optimum temperatures, so it is necessary to develop strategies that exceed these requirements.

The application of molecular biology techniques for the creation of improved enzymes [7], solid-support technology in order to obtain heterogeneous more efficient cascades [8,9], or the combination of enzymes with novel nanozymes systems, with enzyme-like activities [10,11], have made more efficient cascade systems.

Hence, we focus this review article on the most recent advances achieved in the development and application of multi-enzymatic systems, based on three different approaches: enzymatic cascade systems, chemoenzymatic cascades, or a third strategy based on multi-enzymatic cascade on solid phase (Figure 1).

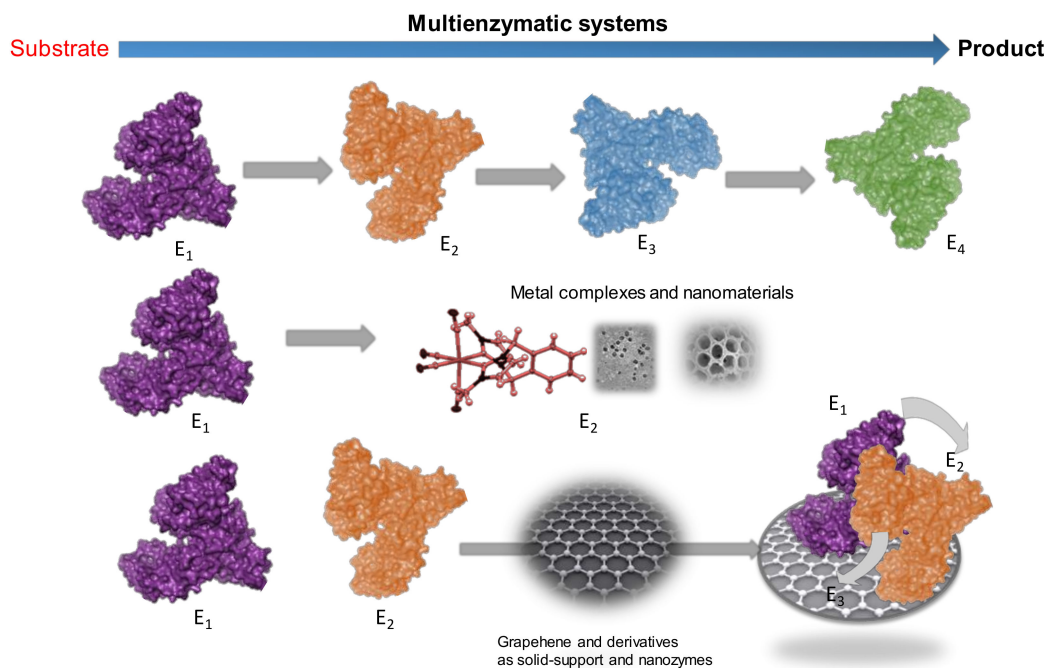


Figure 1. Different concepts of multi-enzymatic cascade processes. E: Enzyme.

2. Enzymatic Cascade Processes

The application of multiple enzymes as a single system in a one-pot process has important advantages in the development of cascade processes [12].

Multi-enzymatic reactions have the benefit of being biodegradable, highly selective, and compatible with each other within certain operating conditions. The application of multiple enzymes as a single system in a one-pot process has the important advantages of improving overall synthetic efficiency, and avoiding intermediate purification steps, with the consequent reduction of waste generation [13]. Further benefits include handling unstable intermediates, and the favorable shift of reaction equilibrium [14].

Despite the advantages associated with the use of multi-enzymatic reactions, their application in industry require strategies for reducing the costs of biocatalyst generation, improving biocatalyst stability, use of low-cost substrates, and enabling economically sustainable continuous cascade operation [15]. Developments in protein engineering provide excellent opportunities for designing and constructing novel industrial biocatalysts, with improved functional properties to match process demands. Among these properties are higher catalytic activity, stability, substrate specificity, improved expression levels, and additionally, the creation of non-natural activities [16]. On the other hand, the improvement of enzyme stability, through their co-immobilization is also gaining scientific popularity, as reflected in several recent reviews, especially because the immobilization of cascade enzymes in a co-localized form offers the advantage that the product of one enzyme reaction is promptly transported to the next biocatalyst, improving overall performance [17,18].

Since a major part of multi-enzymatic cascade reactions incorporate the requirement of stoichiometric amounts of expensive cofactors, which are easily degraded under certain conditions (presence of oxygen, extremes of pH, or high temperature), the achievement of efficient regeneration and reuse of these compounds is a key factor in the development of an economically viable process [19]. One traditional approach to regenerating the cofactor is the use of a second enzyme;

however, the necessity for sacrificial co-substrates, as well as an additional enzyme, is unfavorable. Consequently, self-sufficient one-pot reactions that show high atom economy and high molecular selectivity are pursued [20].

Cascade enzyme reactions have been utilized with different purposes, especially in the development of a more-efficient synthesis of chemical compounds, such as bioactive oligosaccharides, glycosylated molecules, or amoniacid derivatives [21–26].

2.1. Glyco-Enzymes

Chemical synthesis of rare sugars (monosaccharides and their derivatives that rarely exist in nature) usually results in low yield, complicated steps, or/and toxic reagents, and enzymatic processes have represented an alternative way. The production of ketoses by an aldolase-catalyzed reaction of dihydroxyacetone phosphate (DHAP) and L-/D-glyceraldehyde, and the further dephosphatation with acid phosphatase, has been described [27]. DHAP may be produced from glycerol 3-phosphate through oxidation by glycerol phosphate oxidase, and the by-product hydrogen peroxide must be degraded into water and oxygen by a catalase [28,29]. Considering that glycerol 3-phosphate and L-/D-glyceraldehyde are still either expensive and/or relatively unstable, their production from glycerol has been investigated [29,30].

Recently, Li et al. [30] introduced the one-pot synthesis of a series of rare ketohexoses, using glycerol as the sole carbon source, by a four-enzyme cascade reaction (Figure 2). Glycerol was initially phosphorylated by the acid phosphatase from *Shigella flexneri* (PhoN-Sf), and further oxidized by a recombinant glycerol phosphate oxidase (GPO). Oxidation of glycerol to D- and L-glyceraldehyde was evaluated using alditol oxidase (AldO). For the C–C bonding formation, different aldolases from different sources (L-rhamnulose-1-phosphate aldolase (RhaD), L-fuculose-1-phosphate aldolase (FucA), D-fructose-1,6-bisphosphate aldolase (FruA)/L-rhamnulose-1-phosphate) were tested. Each aldolase was evaluated using AldO or a dehydrogenase, obtaining six systems. The same PhoN-Sf removed the phosphate group in the aldol adduct and phosphate was recycled in the system. The reaction conditions and multienzyme ratios were extensively studied and optimized. According to the enzymes utilized, D-sorbose/D-allulose, L-fructose, L-tagatose/L-fructose, and L-sorbose were obtained. This study provides a useful method for rare ketose synthesis on a multi-milligram to gram scale, starting from relatively inexpensive materials [30].

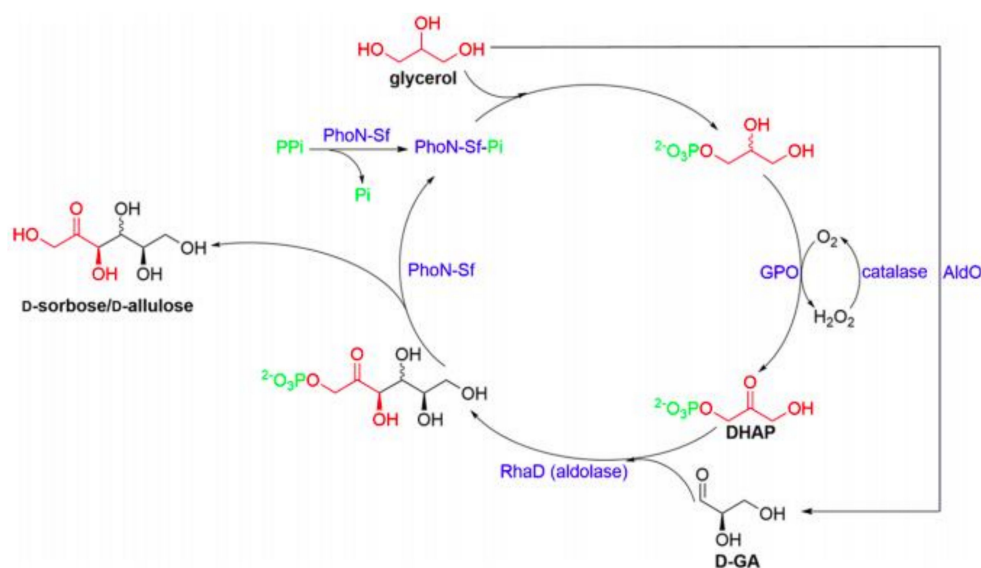


Figure 2. Multi-enzyme synthesis of D-sorbose/D-allulose from glycerol. PPi: pyrophosphate, Pi: inorganic phosphate. Reprinted with permission from Ref. [30]. Copyright © 2020 American Chemical Society.

D-allulose was also synthesized from inulin using a one-pot two-enzyme cascade system formed by an exo-inulinase and D-allulose 3-epimerase [31]. A fructose syrup containing D-glucose, D-fructose, and D-allulose in a 1:3:1 proportion at 67% yield was obtained.

The asymmetric synthesis of ketoses and related acyloin compounds in one step by transketolases (TK) is another interesting process [32]. These enzymes transfer an α -hydroxy carbonyl (ketol) group from a donor to an aldehyde acceptor, where the irreversible release of carbon dioxide from hydroxypyruvate (HPA) as donor kinetically drives the conversion. A challenge for the application of this reaction is the instability of HPA and the cost of expensive aldehyde precursors. In this sense, Lorilliere et al. [33] recently optimized the synthesis of natural (3S,4S)-ketoses (L-ribulose, D-tagatose, and L-psicose) from (2S)-hydroxyaldehyde using TK from *Geobacillus stearothermophilus*; the donor HPA as well as the acceptor substrates were generated in situ, in a one-pot procedure. HPA was obtained from L-serine and pyruvate by a transaminase-catalyzed reaction. Additionally, three different (2S)- α -hydroxylated aldehydes, L-glyceraldehyde, D-threose, and L-erythrose, were generated from the simple achiral compounds, glycolaldehyde and formaldehyde, by D-fructose-6-phosphate aldolase catalysis.

For the synthesis of more complex sugar structures, cellodextrins are linear β -1,4-gluco-oligosaccharides ($DP \leq 6$) with attractive properties [34], which can be produced by depolymerization of cellulose [35] or bottom-up synthesis [36]. Recently, Nidetzky and coworkers [36] developed a three-enzyme glycoside phosphorylase cascade for the synthesis of soluble cellodextrins, with a degree of polymerization between three and six, using sucrose and glucose as substrates (Figure 3). The reactions involved iterative β -1,4-glucosylation of glucose from α -glucose 1-phosphate (α Glc1-P) donor that is formed in situ from sucrose and phosphate. Sucrose phosphorylase (ScP), cellobiose phosphorylase (CbP), and cellodextrin phosphorylase (CdP) were used.

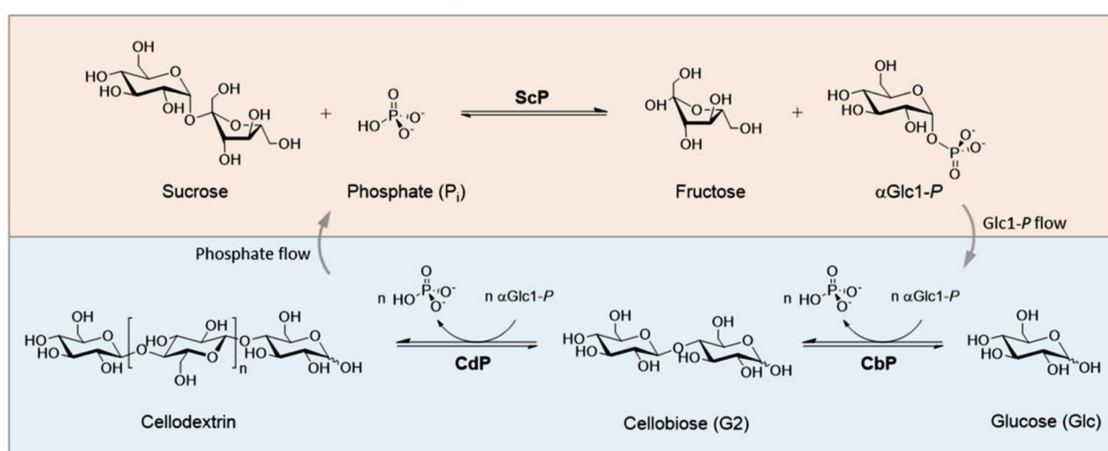


Figure 3. Synthesis of cellodextrin from sucrose by a three-enzyme cascade reaction [36].

The synthesis of cellodextrins was performed by two approaches, by two sequential steps and in simultaneous reaction. In the two-sequential steps, first in the sucrose ScP catalysis, a phosphate concentration equal to, or higher than, that of the sucrose was supplied to limit the phosphate/ α Glc1-P shuttle to only one single operational cycle. In the second step, cellodextrin synthesis was carried out, and then the precipitation of the magnesium salt of the phosphate, released from α Glc1-P, allowed pulling the CbP-CdP phosphorylases reaction toward completion. In the case of simultaneous reaction, analysis of critical process factors for cellodextrin synthesis showed that in order to maximize soluble product formation, careful balancing of the individual phosphorylase activities is key for process efficiency. The increase of glucose concentration did not significantly affect sucrose utilization but benefited the formation of soluble cellodextrins. This study establishes a basis for the integrated production of soluble cellodextrins.

The application of a cascade of five glycoenzymes (sucrose synthase (SUS), UDP-glucose 4-epimerase (GalE), galactinol synthase (GS), raffinose synthase (RS), and stachyose synthase (STS)) has been performed in the synthesis of bioactive oligosaccharides, raffinose (Gal-Glu-Fru) and stachyose (Gal-Gal-Glu-Fru) [37], the major bioactive components of soybean oligosaccharides.

Another interesting property of glycoenzyme cascade has been focused on for the development of glycosylated natural products. This has been described as an interesting strategy to improve the solubility, stability, and bioactivity of bioactive molecules [38].

In this regards, nucleoside diphosphate (NDP)-sugar dependent Leloir glycosyltransferases (GT) have received considerable attention in recent years offering excellent control over the reactivity and selectivity of glycosylation reactions with unprotected carbohydrates [39]. Due to the high cost of NDP-sugars, cascade enzymatic systems for their regeneration have been developed in order to efficiently obtain natural and non-natural polyphenol saccharides [40–42].

Sohng and co-workers [42] have reported the glycosylation of naringenin, a flavonoid found in citrus fruits, with different bioactive properties such as being antioxidant, a free radical scavenger, and anti-inflammatory [43].

A cascade enzymatic system was used based on the combination of the glucosyltransferase (Yjic), for transferring glucose from UDP- α -D-glucose to naringenin, and the sucrose synthase AtSUS1, for regenerating UDP- α -D-glucose from sucrose (Figure 4) [42].

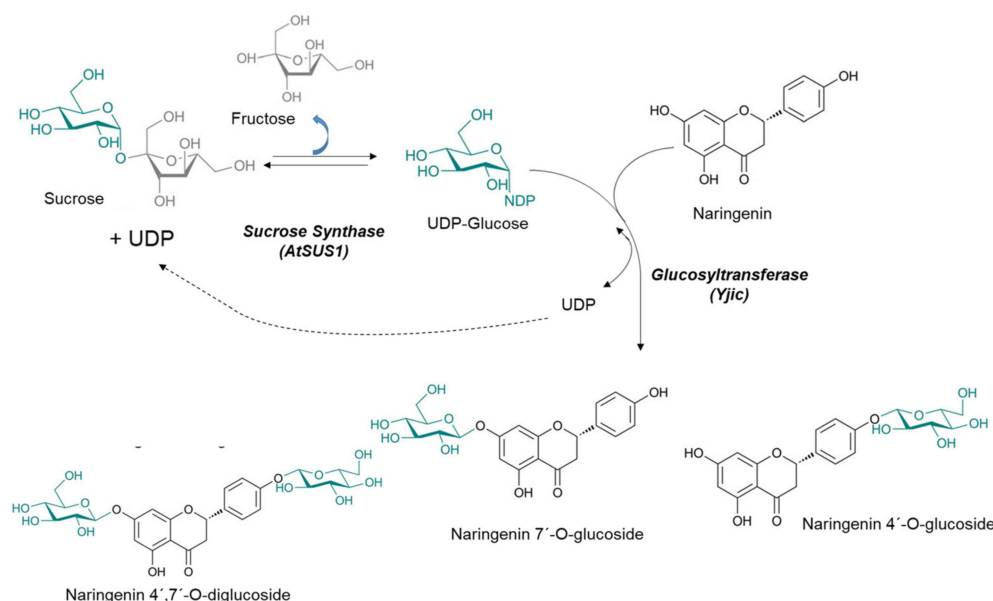


Figure 4. Synthesis of Naringenin-glycosylated flavonoids by multi-enzymatic cascade processes.

Optimization of the cascade processes was developed in order to produce a highly feasible system for large-scale production of different derivatives of naringenin. Three different naringenin glucosides were synthesized, 4'-O- β -D-glucoside, 7-O- β -D-glucoside, and 4',7-O- β -D-diglucoside derivatives (Figure 4).

A similar strategy was used for the synthesis of glycosylated quercetin derivatives [42]. In this case a three-enzyme cascade process was developed. Rhamnose synthase (AtRHM) was used to transform UDP- α -D-glucose to UDP-rhamnose, which was selectively incorporated on the 7-O position of quercetin by rhamnosyltransferase UGT89C1. The sucrose synthase was again used to regenerate UDP-glucose from sucrose.

The cascade reaction of glucosyltransferase (Yjic) and sucrose synthase was also applied in the synthesis of Ginsenoside Rh2 (3b,12b-Di-O-Glc-protopanaxadiol), with anticancer, anti-inflammatory, and antiallergic pharmaceutical activities [44], from protopanaxadiol (PPD) (Figure 5) [45].

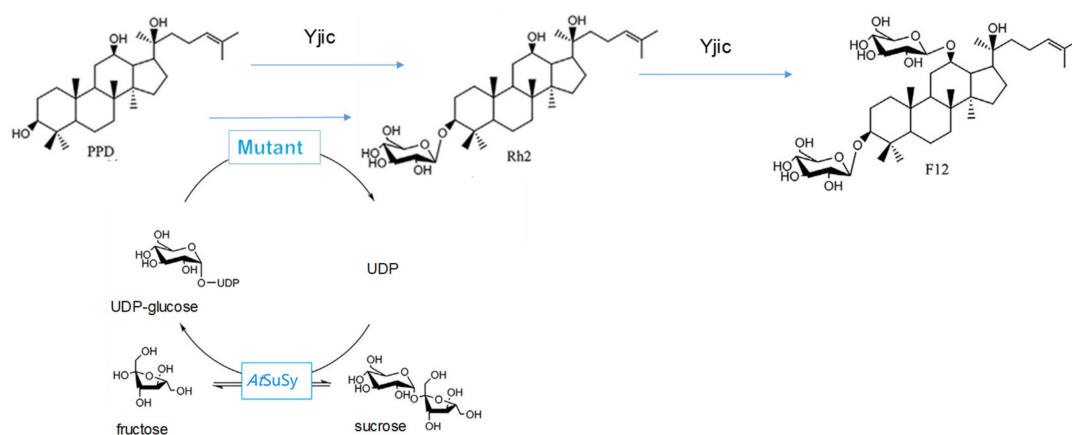


Figure 5. Cascade enzymatic process for Ginsenoside Rh2 synthesis. Figure was reprinted from Ref. [45]. Copyright © 2020, Elsevier.

A second glycosylation by Yjic was observed, producing finally, mainly F12 (3b,12b-di-O-Glc-protopanaxadiol) in a ratio of 7:3 compared to Rh2. Therefore, the authors used genetic engineering of the Yjic to improve the selectivity. Mutant M315F efficiently synthesized Rh2 (~99%) (Figure 5).

Following the idea described previously of preparation of sugars from small molecules, the ability to apply a multi enzymatic cascade for preparation of complex molecules, such as nucleoside derivatives has been recently described [46]. An outstanding method has been developed in the synthesis of islatravir, a drug for the treatment of HIV, by a multi-enzymatic cascade process [46]. Five of the nine enzymes utilized in this synthesis were developed through directed evolution in order to favor their action on unnatural substrates. Islatravir was synthesized with a 51% overall yield, using the alcohol ethynyl glycerol as the starting substrate (Figure 6). The atom economy far exceeded that of previous syntheses of this target, and the number of steps was less than half. The entire sequence takes place under mild conditions in a single aqueous solution, without the isolation of intermediates.

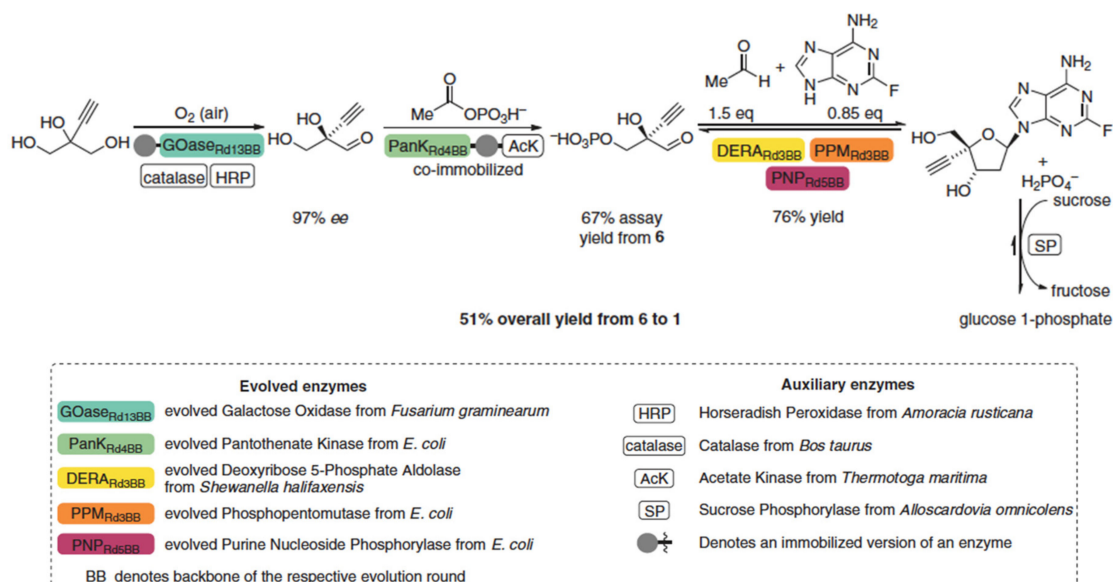


Figure 6. Multi-enzymatic synthesis of islatravir. Figure printed from Ref. [46]. Copyright © 2020, American Association for the Advancement of Science.

The application of evolved enzymes together with immobilization strategies, especially in the first two steps (Figure 6), selective oxidation of ethynyl glycerol by galactose oxidase, together with peroxidase and catalase, and posterior selective phosphorylation by the two kinases, makes the process

feasible with a yield of 67% of the phosphate product at this point (Figure 6). The second step of combining an aldolase (DERA), a phosphopentomutase (PPM), and purine nucleoside phosphorylase (PNP) finally produced the bioactive compound.

2.2. Cascade Enzymes in Aminoacid Chemistry

Amino acids and their derivatives represent a sustainable approach for producing various high added value compounds [47]. Recent works in the area of amino acid synthesis through cascade reactions have focused mainly on the incorporation of lower cost starting materials and/or enzymes with genetically improved properties into the synthetic route [48].

By this premise, semi-scale synthesis of L-alanine was performed by a biocatalytic cascade process starting from glucose [49]. The process involved a multi-enzymatic cascade with an optimized combination of six biocatalysts (a glucose dehydrogenase, two dihydroxy acid dehydratases, a keto-deoxy-aldolase, an aldehyde dehydrogenase, and an L-alanine dehydrogenase) to produce the aminoacid with more than 95% yield. The production level of L-alanine was $0.17 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, which although being below the current industrial production values ($13.4 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$) [50], the proof of concept that has been developed is still of great interest, as it confirms the feasibility of carrying out multistep in vivo routes outside the cell.

Another interesting example is the efficient synthesis of L-tyrosine derivatives by a one-pot biocascade using L-lactate as a starting material (Figure 7) [51].

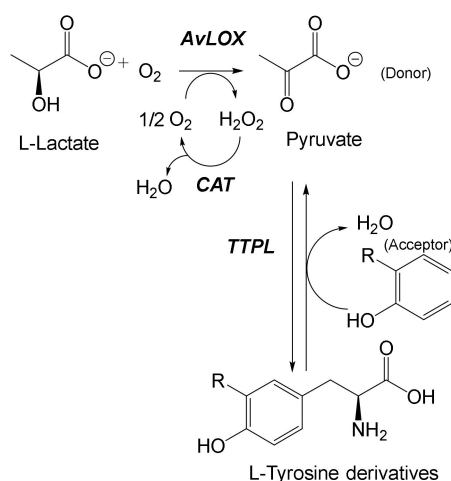


Figure 7. Enzymatic cascade synthesis of L-tyrosine derivatives.

The combination of a L-lactate oxidase (AvLOX), a catalase (CAT), and a tyrosine phenol-lyase (TTPL) permitted obtaining different tyrosine derivatives in high yields, between 81 and 98%, while the enantiomeric excess (e.e) was higher than 99% (Figure 7). A first step combined the use of a high active oxidase to produce pyruvate from lactate and catalase to degrade the formed hydrogen peroxide. Then, tyrosine phenol-lyase catalyzed the C–C bond reaction with different phenol derivatives as acceptor. This environmentally friendly procedure provides a real alternative to the conventional synthesis of L-tyrosine derivatives, since by using L-lactate as a starting material it is possible to avoid the high cost and easy decomposition of pyruvate.

β -Methyl- α -amino acids represent a very attractive synthetic challenge because of their high potential as chiral building blocks [52]; however, limitations in stereoselective synthesis still remain.

Very recently, Seebeck and coworkers [53] described a four-enzyme cascade system for the efficient asymmetric synthesis of different β -Methyl amino acids, in high yields (>80%) and stereoselectivity (>90%) (Figure 8).

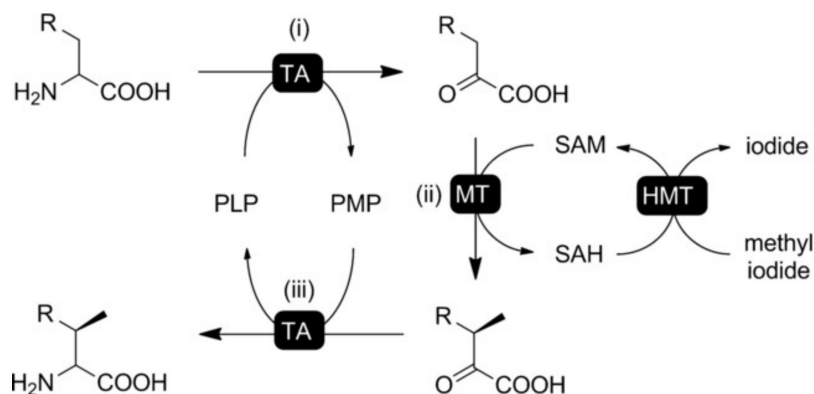


Figure 8. Enzymatic cascade synthesis of β-methyl-α-amino acids. TA: transaminase, MT: methyltransferase, HMT: halide methyltransferase.

The cascade was composed of a PLP-dependent L-amino acid or D-amino acid transaminase from *Escherichia coli* (*E. coli*) (IlvE or D-TA) that catalyzes the oxidation of the α-amino acid substrate to the corresponding α-keto acid. The α-keto acid is methylated by an S-adenosylmethionine (SAM) dependent α-keto acid methyltransferase from *Streptomyces griseoviridis* (SgvM). The methylated R-α-keto acid obtained recovers its amino group from pyridoxamine (PMP) contained in the enzymes L-TA (IlvE) or D-TA to form L-β-Me-α-amino acids or D-β-Me-α-amino acids, respectively. The third enzyme in the cascade is a halide methyltransferase from *Burkholderia xenovorans* (HMT), used for SAM regeneration by stereoselective S-methylation of S-adenosylhomocysteine (SAH), through the use of methyl iodide as a methyl donor (Figure 8).

Moreover, strategies for enzymatic production of high added value alcohols from amino acids have also been developed [54,55].

3. Chemo-Enzymatic Cascades

Chemoenzymatic cascades enable reactions with a high productivity of chemocatalysts and a high selectivity of enzymes. Nevertheless, combining chemo- and biocatalysts is extremely challenging, since the chosen chemocatalysts need to tolerate the presence of the enzyme generally in aqueous media [56].

To combine the advantages of both catalysts, chemoenzymatic C–C coupling reactions are often combined with enzymatic oxidations or reduction reactions [57].

One interesting example, where enzymes and organometallic complexes have been successfully combined, was the synthesis of cycloalkanes and cycloalkenes, not being reported via enzymatic pathways. Ward and coworkers [58] described a multi-enzymatic cascade system combined with (Hoveyda)-Grubbs ruthenium (II) catalysts. They combined a ruthenium-catalyzed ring closing metathesis reaction as the last step of one-pot chemo-enzymatic cascades that included (up to) nine enzymatic steps for production of cyclopentene, cyclohexene, and cycloheptene from olive oil derived intermediates, hosted by whole cells of *E. coli* in a single reaction vessel (Figure 9). Different routes were purposed where, in one, the enzymatic cascade was involved in the selective decarboxylation of oleic acid using *E. coli* decarboxylases (UndB), previously obtained from hydrolysis of olive oil using *E. coli* *Thermomyces lanuginosus* lipase (TLL), whereas a final ring closed-metathesis (RCM) catalyzed by the Ru catalyst gave the final cycloheptene product in 44% yield. Another route went through an enzymatic cascade *E. coli* (C9) (by hydroxylation, oxidation, hydrolysis) to finally produce the corresponding dicarboxylic acid. Then successive chemoenzymatic cascades by decarboxylases, in *E. coli* UndB and Ru catalysis, produced cyclohexane and cyclopentane, in 22% or 65% yield, respectively (Figure 9).

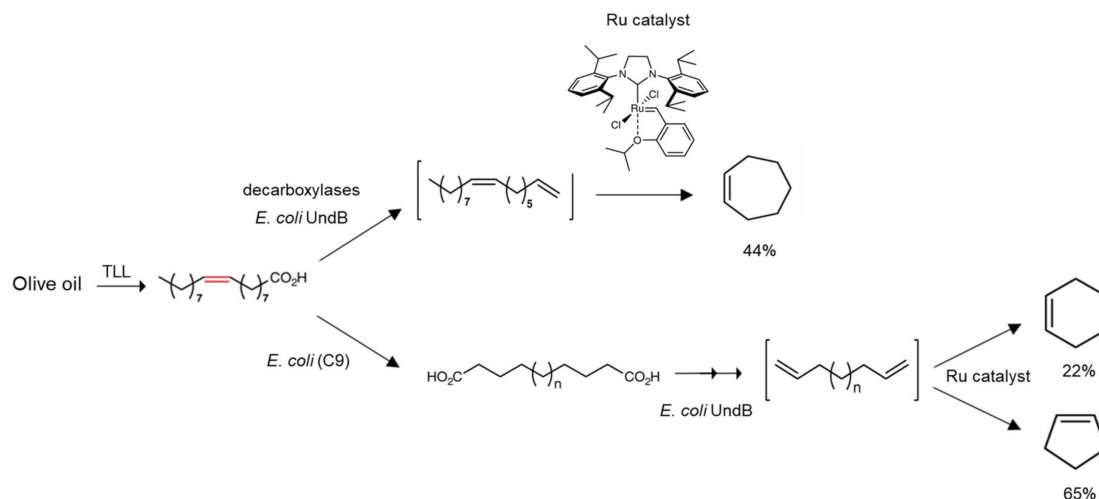


Figure 9. Chemo-enzymatic cascades to produce cycloalkenes from olive oil.

Catalytic selective oxidative C–H activation of hydrocarbon substrates remains a challenge today, especially due to the low regioselectivity of the catalysts and the overoxidation proneness of the substrate [59].

In order to prevent diminishing yields due to these phenomena, a vast number of enzymes have been characterized that do not suffer these shortcomings [60]. Among these are unspecific peroxygenases (UPOs), and extracellular fungal heme-thiolate enzymes that only use hydrogen peroxide (H_2O_2), as both oxygen donor and final acceptor of electrons, to generate the activated oxygen species at the active site [61]. In addition, they are relatively stable with high efficiency toward H_2O_2 , and do not require ancillary flavoproteins and cofactors that need regeneration [62].

However, these enzymes are not exempt from limitations, because they can be easily deactivated by modest concentrations of H_2O_2 , requiring a constant supply at low concentrations [61].

In this regard, a successfully chemo-enzymatic strategy has been developed for the selective hydroxylation of cyclohexane.

Freakley and coworkers [63] developed a tandem catalytic system, combining the use of peroxygenases for the C–H activation with AuPd/TiO₂ for the in situ supply of H_2O_2 at mild conditions (Figure 10).

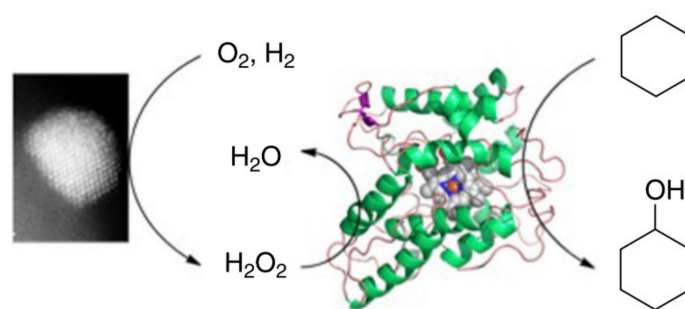


Figure 10. Chemoenzymatic cascade for selective oxidation of cyclohexane using in situ produced H_2O_2 . Figure adapted from Ref. [63].

This cascade system produced cyclohexanol with high yields (87%) and total turnover numbers (TTN) of 25,300, with a minimal over oxidation to cyclohexanone, meaning that the bimetallic AuPd catalyst was not facilitating further overoxidation reactions.

In order to expand this chemoenzymatic strategy, the enzyme–metal tandem was applied in the C–H activation of ethylbenzene and isophorone [63]. In the case of using ethylbenzene as a substrate, selective production of enantiopure *R*-phenyl ethanol (>99%) with a TTN value of 25,900 was obtained.

The hydroxylation of isophorone resulted in two products, 4-hydroxyisophorone and 7-hydroxyisophorone, at 75% yield in roughly equal amounts.

Another interesting application of chemoenzymatic cascades has been described in the synthesis of benzofuran derivatives, which are known to be pharmacologically active against breast cancer cell lines, and which show antimicrobial activity against diverse pathogenic fungi and bacteria [64].

From the different approaches, one interesting strategy has focused on the development of these bioactive molecules from short starting materials, combining C–C bonding and oxidation reactions. For that purpose, the application of Pd free Sonogashira coupling with regio- and stereoselective enzymatic catalysis (C–H activation, dehydration) properties described by cytochrome P450 BM3 [19], was attempted [65].

Schwaneberg and coworkers [65] described a one-pot two-step catalytic cascade reaction for the synthesis of a bis (2-substituted benzofuran) derivative, combining an organometallic Cu catalyst and P450 BM3 (Figure 11).

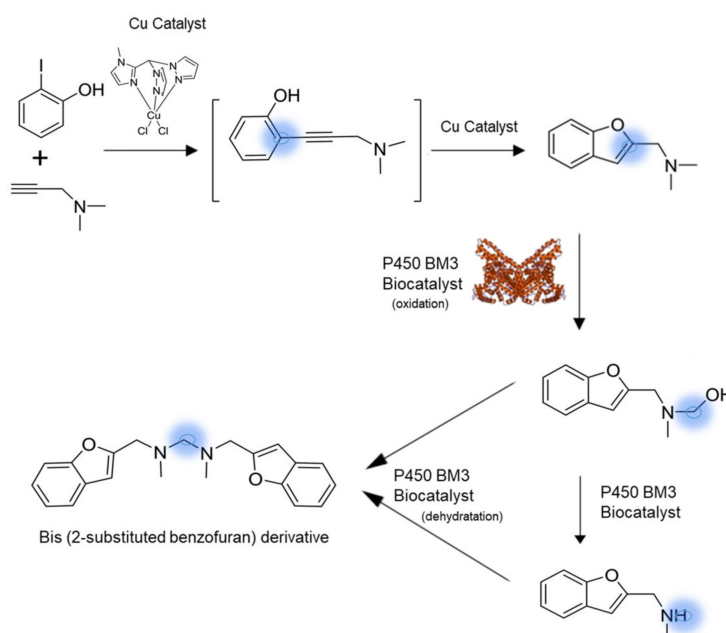


Figure 11. Chemoenzymatic cascade synthesis of a bis (2-substituted benzofuran) derivative [65].

In the first reaction step, a copper scorpionate catalyst (bis(pyrazol) (methyl imidazolyl)-methane copper (II) chloride complex) Cu scorpionate catalyst was used to synthesize a 2-substituted benzofuran from 2-iodophenol to perform a cascade Sonogashira coupling/cyclation reaction. In the next step, enzymatic hydroxylation via P450 BM3, followed by formaldehyde elimination of the benzofuran produced a novel bis (2-substituted benzofuran) derivative in a 33% yield.

This seems to be a moderate yield, considering that the first step after Cu catalyst, the yield of the benzofuran was 88%.

Therefore, conditions were optimized, an excess of EDTA was supplemented to reactions with the crude reaction mixture after completion of the first cascade step, acting as a chelating agent and capturing the complex metal ions. Using this simple additive, final yield of the product increased up to 84% yield.

This is a further promising approach, since it is a sequential two-step catalytic cascade reaction and moreover, it was the first time in this area that a Pd-free Sonogashira coupling could be combined with an enzyme.

Chemo-enzymatic one-pot syntheses offer the replenishment of the versatility of traditional chemical catalysis with the unbeatable selectivity and mild reaction conditions of enzymes. However, they also pose several challenges, like the compatibility of the considerable differences of ideal reaction

conditions for the individual process steps [66]. Alternative solvents, such as deep eutectic solvents (DESs) can help to overcome this obstacle of solvent compatibility [67], and also, can give alternative solutions for issues such as low substrate solubility, enzyme activity, and stability. On the other hand, DESs bring along some drawbacks, including occasional toxicity and high viscosity, depending on the starting materials.

Following this line, Grabner, Kourist, and Gruber-Woelfler and coworkers [68] have developed a chemoenzymatic process by a fully integrated two-step flow setup for the asymmetric synthesis of stilbenes in DESs [68].

The process consists of the production of bio-based stilbenes from p-coumaric acid and iodobenzene (low price and easily available substrates) by combining a first enzymatic decarboxylation with a phenolic acid decarboxylase, followed by a Heck reaction catalyzed by a Pd-substituted Ce–Sn-oxide (Figure 12).

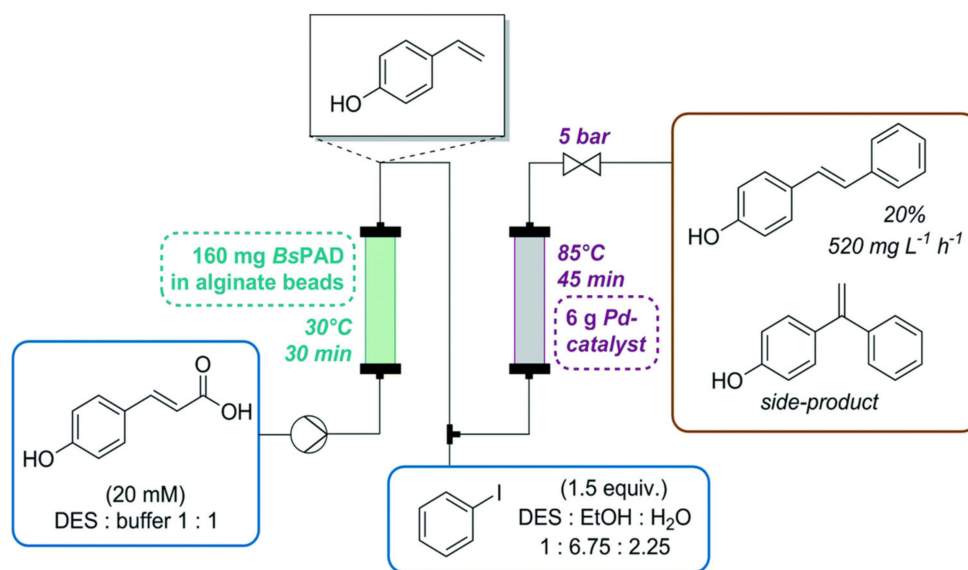


Figure 12. General scheme for asymmetric chemoenzymatic synthesis of stilbenes [68]. Published by The Royal Society of Chemistry.

For the development of the flow system, both enzymatic and metallic catalysts were previously immobilized on different supports and optimized at the best conditions. The decarboxylated product from coumaric was obtained in a 90% yield in DESs: buffer mixture using immobilized decarboxylase, whereas a full conversion in Heck reaction producing (*E*)-4-hydroxy stilbene was possible using immobilized Pd catalyst in this solvent mixture, with ethanol: water 1:1 (*v/v*) containing iodobenzene. The residence time in the reactors was determined to be 30 min for the decarboxylation, and 45 min for the Heck coupling resulting in a space–time yield of 4.8 g·L⁻¹·h⁻¹ for decarboxylation, and 0.52 g·L⁻¹·h⁻¹ for Heck coupling (Figure 13).

4. Solid-Phase Multi-Chemoenzymatic Cascade Reactions

In the development of cascade processes combining enzymatic and chemo reactions, nanomaterials mimicking enzymatic activity (nanozymes) have emerged in the last years. They show interesting advantages, such as high stability, long-term storage, ease of modification, and also a multimodal platform, interfacing complex biologic environments [69–72].

Benefiting from the combination of enzymes and nanozymes, the activity of the cascade catalysis and the stability of the enzyme can be significantly improved. However, the overall performance of multi-enzyme systems is affected not only by specific interactions between catalysts, substrates, and the reactor, but also by other factors, such as substrate channeling, kinetic coincidence, and spatial

distribution in the reactor [73]. Therefore, the immobilization on supports of the multienzyme system is the key to efficient cascade catalysis and high stability.

From the different approaches described in the literature, recently the application of graphene or derivatives has gained special attention, because of their unique properties; they can be used as material for selective immobilization of enzymes but also show enzyme-like activity, acting at the same time as nanozymes, being a very interesting system for chemo-enzymatic cascade processes [74–77].

The conversion of CO₂ in high-added value compounds is very attractive, particularly in terms of sustainable chemical processes [78].

From the different strategies, the enzymatic conversion has been described as a very interesting alternative. Formate dehydrogenase (FateDH) catalyzes CO₂ reduction into formic acid in the presence of nicotinamide adenine dinucleotide (NADH) as electron and proton donor [79]. This enzyme, in combination with formaldehyde dehydrogenase (FaldDH), and alcohol dehydrogenase (ADH), has been described as an excellent enzymatic cascade to transform CO₂ into methanol [80]. However, although the NADH cofactor is a highly efficient proton and electron donor for CO₂ reduction, it is irreversibly oxidized to NAD⁺ (i.e., it is sacrificial), limiting its potential [81].

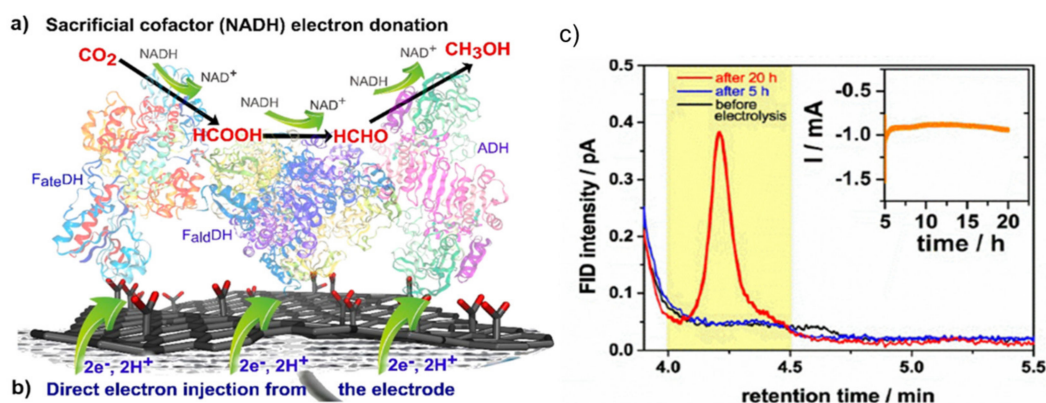


Figure 13. Multi-enzymatic cascade reduction of CO₂ to methanol. (a) NADH sacrificial cofactor approach; (b) NADH-free approach; (c) Faradaic efficiency of the cascade. Figure adapted from Ref. [82].

In this regard, Sariciftci and coworkers [82] have developed a novel heterogeneous cascade system formed by three dehydrogenases (FateDH, FaldDH, ADH) homogeneously covalent immobilized on carboxyl-functionalized graphene (~15% degree of functionalization) as three graphene-based dual-function biocatalysts for evaluation of the conversion of CO₂ to methanol in two approaches: chemical reduction using NADH as cofactor (Figure 13a), and the NADH-free cascade electroreduction (Figure 13b).

The reduction of CO₂ to methanol by this cascade resulted in about 20 ppm of methanol production (by GC), corresponding to a Faradaic efficiency of 12% (Figure 13c). As a control, electrolysis of the modified electrode was carried out under N₂ saturated conditions. This experiment did not reveal methanol formation after 20 h of electrolysis, which confirms the conversion of CO₂ to methanol through the catalysts. The direct electron injection mechanism for the reduction of CO₂ to methanol, without immobilization of the enzymes, showed a concentration of 0.1 ppm (corresponding to around 10% faradaic efficiency). In the present case, graphene modified with three dehydrogenases showed absolute currents of one order of magnitude higher delivered to the reaction sites, suggesting a much more efficient transport of electrons from the electrode to the active sites of the enzymes, through the conductive graphene support, as well as a higher production rate (around 0.6 μmol·h^{−1}).

Another example of cascade reaction of graphene-supported enzymes is a simple and effective strategy for the detection of organophosphate (OP) pesticides using an enzyme-based assay; such as acetylcholinesterase (AChE) that was inhibited by OP, whose concentration could be measured colorimetrically by enzymatic activity through a decreased color reaction, described by Chu et al. [83].

This method shows a fast response, but has the problems of instability and sensitivity, because enzymes are susceptible to environmental influences. Recently, advances in nanotechnology have improved this analysis. In most cases, AChE was immobilized onto nanomaterials for developing biosensors with a low detection limit, a rapid response, and long storage stability [84]. For instance, the AChE/TiO₂/reduced GO-based electrode increased the catalytic activity of AChE and could detect OPs at nanomolar levels with high reliability [85].

This method required a chemoenzymatic cascade combining two enzymes, AChE, choline oxidase (CHO) and graphene derivative (GO) acting in sequence, in which AChE/CHO catalyzed acetylcholine (ACh) to betaine aldehyde (BA), forming H₂O₂ that was detected by the peroxidase-like activity of GO, by converting 3,3',5,5'-tetramethylbenzidine (TMB) into the oxidized blue product (Figure 14a).

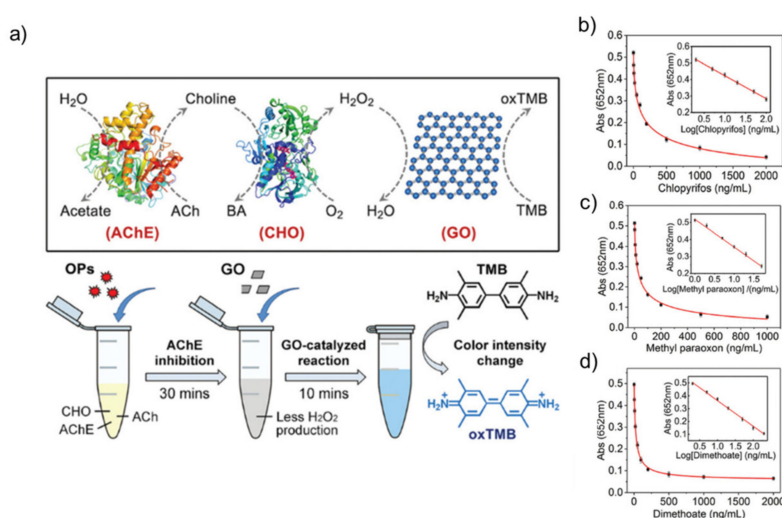


Figure 14. Chemoenzymatic cascade process as a pesticide biosensor. (a) General scheme of the strategy. (b–d) Dose–response curve and semilogarithmic plot (inset) for OP detection: chlorpyrifos, methyl paraoxon and dimethoate. Figure adapted from Ref. [83].

This chemoenzymatic cascade was applied for the detection of different pesticides, dimethoate, methyl paraoxon, and chlorpyrifos at low levels (Figure 14b–d). As a result, the inhibition of AChE by OPs resulted in a decrease in color intensity, which is linearly related to the concentration of OPs. The corresponding limit of detection (LOD) was measured to be lower than 2 ppb. In addition, the detection could be completed within 40 min, and provided a linear response for OP concentration levels from 1–200 ng mL⁻¹, revealing its great potential for quantitative analysis of pesticide residues.

Recently, metal-organic frameworks (MOFs) with regular pores or channels have garnered considerable research interest due to their excellent potential as a universal platform for the immobilization of various functional materials, including enzymes and nanozymes [86,87]. High flexibility and tunability of MOFs, in terms of their pore size and shape allow the encapsulation of catalysts with different sizes and functions in specific spaces, for efficient cascade reaction.

Thus, Zheng et al. [88] have developed a novel sensitive and stable electrochemical biosensor for glucose detection, using a chemo-enzymatic cascade system (Figure 15). In this multienzyme system, glucose oxidase (GOx) was immobilized within Cu-based MOF layers grown on the surface of a three-dimensional (3D) porous conducting copper foam (CF) electrode (GOx@Cu-MOF/CF) via a facile electrochemical assisted biomimetic mineralization (Figure 15a). This system was compared with the simple GOx immobilized on copper foam electrode.

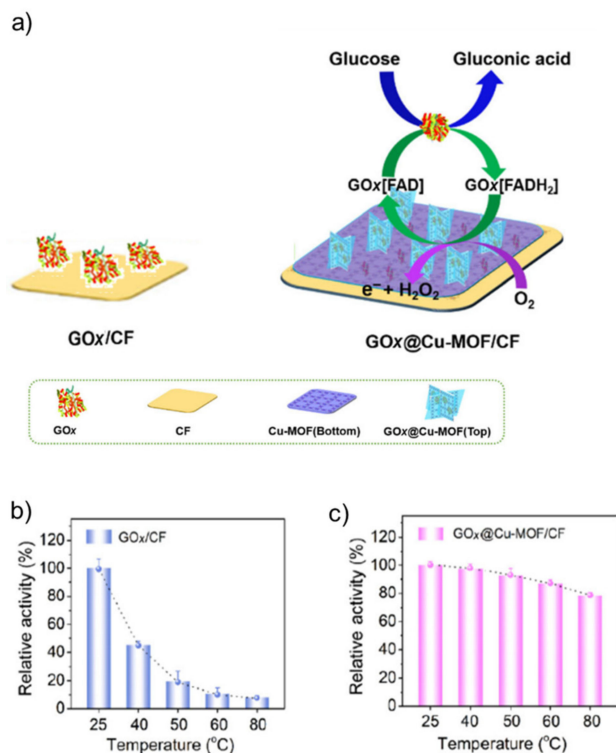


Figure 15. Chemoenzymatic cascade process as a new glucose biosensor. (a) Scheme of the bio-electrocatalytic cascade reaction; (b) Thermostability GOx/CF; (c) Thermostability GOx@MOF/CF. Figure adapted from Ref. [88].

The chemoenzymatic cascade in the detection process of glucose was based on, first, enzymatic glucose oxidation to gluconic acid (producing H_2O_2) catalyzed by GOx embedded within the Cu-MOF layer, and then, the electro-catalyzed reduction of H_2O_2 immediately by the Cu-MOF/CF (Figure 15a).

This system presents the advantage over a normal immobilization of the enzyme on the electrode, that the open and porous 3D feature of the copper foam provides enhanced specific surface area for enzyme immobilization, and facilitates the substrate transport and charge collection. Another advantage of the architecture is that the encapsulation structure provides good protection for GOx. Due to the bio-electrocatalytic cascade reaction mechanism, this well-designed GOx@Cu-MOF modified electrode exhibited superior catalytic activity and thermal stability for glucose sensing.

Notably, this chemoenzymatic system GOx@Cu-MOF/CF retained more than 80% activity after being incubated at 80 °C (Figure 15b). In sharp contrast, the activity of the unprotected electrode was reduced to the original 10% after the same treatment (Figure 15c).

Therefore, this work offers an interesting cascade system with potential application for efficient photo-thermal therapy, and other platforms subjected to harsh operating conditions.

5. Concluding Remarks

Recent enzyme-based cascade processes have been discussed. In this article we have approached the cascade system in terms of applicability of the enzyme as a synthetic tool, with advantages for a possible industrial implementation. Three different approaches have been proven as excellent tools, and as advanced technology in enzyme applications.

First, multi-enzyme cascades, from two to nine different enzymes working in one-pot to obtain a final product in a more effective way. These systems have been successfully tested for different important sugars, oligosaccharides, glycosylated natural products, amino acid derivatives, or very importantly, demonstrating the value of these systems in the synthesis of an anti-HIV drug.

Furthermore, in many cases the combination of enzymes with organometallic complexes has made it possible to solve some basic problems formed by enzymes. For example, chemoenzymatic success has been achieved in the synthesis of new biofuels, the regeneration of green oxidants, and the synthesis of complex molecules with high added value.

As a last point, examples have been developed of the application of a cascade system that involves the use of combined hybrid systems, in solid phase, where the support material of the enzymes acts in the process, acting as catalyst. In this case, these advantageous systems that use graphene or derivatives have made it possible to design new pesticide biosensors, or to solve environmental problems (such as the transformation of CO₂ into high added value compounds). Solid phase metal enzyme systems have also been described as novel glucose biosensors.

Therefore, these systems open the possibility of combining catalytically supported materials, enzymes and metals, with new trends such as flow (micro) reactors, as a novel approach to develop new cascade routes for biopharmaceuticals, but also in the development of environmentally friendly biomedical or electronic (new-bio batteries) devices.

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References

1. Carlson, J.C.; Li, S.; Gunatilleke, S.S.; Anzai, Y.; Burr, D.A.; Podust, L.M.; Sherman, D.H. Tirandamycin biosynthesis is mediated by co-dependent oxidative enzymes. *Nat. Chem.* **2011**, *3*, 628–633. [[CrossRef](#)] [[PubMed](#)]
2. Galanie, S.; Entwistle, D.; Lalonde, J. Engineering biosynthetic enzymes for industrial natural product synthesis. *Nat. Prod. Rep.* **2020**, *37*, 1122–1143. [[CrossRef](#)] [[PubMed](#)]
3. Reetz, M.T. Biocatalysis in organic chemistry and biotechnology: Past, present, and future. *J. Am. Chem. Soc.* **2013**, *135*, 12480–12496. [[CrossRef](#)] [[PubMed](#)]
4. Shihoodi, R.K.; Gevorgyan, V. Metal-catalyzed double migratory cascade reactions of propargylic esters and phosphates. *Chem. Soc. Rev.* **2013**, *42*, 4991–5001. [[CrossRef](#)] [[PubMed](#)]
5. Zhao, C.; Lercher, J.A. Upgrading pyrolysis oil over Ni/HZSM-5 by cascade reactions. *Angew. Chem. Int. Ed.* **2012**, *51*, 5935–5940. [[CrossRef](#)] [[PubMed](#)]
6. Grondal, C.; Jeanty, M.; Enders, D. Organocatalytic cascade reactions as a new tool in total synthesis. *Nat. Chem.* **2010**, *2*, 167–178. [[CrossRef](#)] [[PubMed](#)]
7. Arnold, F.H. Innovation by evolution: Bringing new chemistry to life (Nobel lecture). *Angew. Chem. Int. Ed.* **2019**, *58*, 14420–14426. [[CrossRef](#)] [[PubMed](#)]
8. Shylesh, S.; Wagener, A.; Seifert, A.; Ernst, S.; Thiel, W.R. Mesoporous organosilicas with acidic frameworks and basic sites in the pores: An approach to cooperative catalytic reactions. *Angew. Chem. Int. Ed.* **2010**, *49*, 184–187. [[CrossRef](#)]
9. Palo-Nieto, C.; Afewerki, S.; Anderson, M.; Tai, C.W.; Berglund, P.; Córdova, A. Integrated heterogeneous metal/enzymatic multiple relay catalysis for eco-friendly and asymmetric synthesis. *ACS Catal.* **2016**, *6*, 3932–3940. [[CrossRef](#)]
10. Zhou, Y.B.; Liu, B.W.; Yang, R.H.; Liu, J.W. Filling in the gaps between nanozymes and enzymes: Challenges and opportunities. *Bioconjugate Chem.* **2017**, *28*, 2903–2909. [[CrossRef](#)]
11. Singh, S. Nanomaterials Exhibiting Enzyme-Like Properties (Nanozymes): Current Advances and Future Perspectives. *Front Chem.* **2019**, *7*, 46. [[CrossRef](#)]

12. Ricca, E.; Brucher, B.; Schrittwieser, J.H. Multi-enzymatic cascade reactions: Overview and perspectives. *Adv. Synth. Catal.* **2011**, *353*, 2239–2262. [\[CrossRef\]](#)
13. Wang, Y.; Ren, H.; Zhao, H. Expanding the boundary of biocatalysis: Design and optimization of in vitro tandem catalytic reactions for biochemical production. *Crit. Rev. Biochem. Mol. Biol.* **2018**, *53*, 115–129. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Muschiol, J.; Peters, C.; Oberleitner, N.; Mihovilovic, M.D.; Bornscheuer, U.T.; Rudroff, F. Cascade catalysis-strategies and challenges en route to preparative synthetic biology. *Chem. Commun.* **2015**, *51*, 5798–5811. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Wilding, K.M.; Schinn, S.M.; Long, E.A.; Bundy, B.C. The emerging impact of cell-free chemical biosynthesis. *Curr. Opin. Biotech.* **2018**, *53*, 115–121. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Wilttschi, B.; Cernava, T.; Dennig, A.; Casas, M.G.; Geier, M.; Gruber, S.; Haberbauer, M.; Heidinger, P.; Herrero Acero, E.; Kratzer, R.; et al. Enzymes revolutionize the bioproduction of value-added compounds: From enzyme discovery to special applications. *Biotechnol. Adv.* **2020**, *40*, 107520. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Arana-Peña, S.; Carballares, D.; Morellon-Sterling, R.; Berenguer-Murcia, Á.; Alcántara, A.R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Enzyme co-immobilization: Always the biocatalyst designers' choice ... or not? *Biotechnol. Adv.* **2020**, 107584. [\[CrossRef\]](#)
18. Hwang, E.T.; Lee, S. Multienzymatic Cascade Reactions via Enzyme Complex by Immobilization. *ACS Catal.* **2019**, *9*, 4402–4425. [\[CrossRef\]](#)
19. Cutlan, R.; De Rose, S.; Isupov, M.N.; Littlechild, J.A.; Harmer, N.J. Using enzyme cascades in biocatalysis: Highlight on transaminases and carboxylic acid reductases. *Biochim. Biophys. Acta. Proteins. Proteom.* **2020**, *1868*, 140322. [\[CrossRef\]](#)
20. Perl, J.M.; Sieber, V. Multienzyme Cascade Reactions—Status and Recent Advances. *ACS Catal.* **2018**, *8*, 2385–2396.
21. Cheng, C.; Zuo, X.; Tu, D.; Wan, B.; Zhang, Y. Synthesis of 3,4-Fused Tricyclic Indoles through Cascade Carbopalladation and C-H Amination: Development and Total Synthesis of Rucaparib. *Org. Lett.* **2020**, *22*, 4985–4989. [\[CrossRef\]](#)
22. Mekheimer, R.A.; Al-Sheikh, M.A.; Medrasi, H.Y.; Sadek, K.U. Advancements in the synthesis of fused tetracyclic quinoline derivatives. *RSC Adv.* **2020**, *10*, 19867–19935. [\[CrossRef\]](#)
23. Schmaltz, R.M.; Hanson, S.R.; Wong, C.-H. Enzymes in the Synthesis of Glycoconjugates. *Chem. Rev.* **2011**, *111*, 4259–4307. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Wen, L.; Edmunds, G.; Gibbons, C.; Zhang, J.; Gadi, M.R.; Zhu, H.; Fang, J.; Liu, X.; Kong, Y.; Wang, P.G. Toward Automated Enzymatic Synthesis of Oligosaccharides. *Chem. Rev.* **2018**, *118*, 8151–8187. [\[CrossRef\]](#)
25. Krasnova, L.; Wong, C.-H. Oligosaccharide Synthesis and Translational Innovation. *J. Am. Chem. Soc.* **2019**, *141*, 3735–3754. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Yang, J.; Zhang, T.; Tian, C.; Zhu, Y.; Zeng, Y.; Men, Y.; Chen, P.; Sun, Y.; Ma, Y. Multi-enzyme systems and recombinant cells for synthesis of valuable saccharides: Advances and perspectives. *Biotechnol. Adv.* **2019**, *37*, 107406. [\[CrossRef\]](#)
27. Li, Z.; Cai, L.; Qi, Q.; Wang, P.G. Enzymatic synthesis of D-sorbose and D-psicose with aldolase RhaD: Effect of acceptor configuration on enzyme stereoselectivity. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 7081–7084. [\[CrossRef\]](#)
28. Fessner, W.-D.; Sinerius, G. Synthesis of Dihydroxyacetone Phosphate (and Isosteric Analogues) by Enzymatic Oxidation; Sugars from Glycerol. *Angew. Chem. Int. Ed.* **1994**, *33*, 209–212. [\[CrossRef\]](#)
29. Burek, B.O.; Bormann, S.; Hollmann, F.; Bloh, J.Z.; Holtmann, D. Hydrogen peroxide driven biocatalysis. *Green Chem.* **2019**, *21*, 3232–3249. [\[CrossRef\]](#)
30. Li, Z.; Li, F.; Cai, L.; Chen, Z.; Qin, L.; Gao, X.-D. One-Pot Multienzyme Synthesis of Rare Ketoses from Glycerol. *J. Agric. Food Chem.* **2020**, *68*, 1347–1353. [\[CrossRef\]](#)
31. Zhu, P.; Zeng, Y.; Chen, P.; Men, Y.; Yang, J.; Yue, X.; Zhang, J.; Zhu, Y.; Sun, Y. A one-pot two-enzyme system on the production of high value-added D-allulose from Jerusalem artichoke tubers. *Process Biochem.* **2020**, *88*, 90–96. [\[CrossRef\]](#)
32. Lorillière, M.; Dumoulin, R.; L'Enfant, M.; Rambourdin, A.; Thery, V.; Nauton, L.; Fessner, W.-D.; Charmantray, F.; Hecquet, L. Evolved Thermostable Transketolase for Stereoselective Two-Carbon Elongation of Non-Phosphorylated Aldoses to Naturally Rare Ketoses. *ACS Catal.* **2019**, *9*, 4754–4763. [\[CrossRef\]](#)

33. Lorilli re, M.; Gu rard-H elaine, C.; Gefflaut, T.; Fessner, W.D.; Clap s, P.; Charmantray, F.; Hecquet, L. Convergent in situ Generation of Both Transketolase Substrates via Transaminase and Aldolase Reactions for Sequential One-Pot, Three-Step Cascade Synthesis of Ketoses. *ChemCatChem* **2020**, *12*, 812–817. [[CrossRef](#)]
34. Sybesma, W.; Kort, R.; Lee, Y.K. Locally sourced probiotics, the next opportunity for developing countries? *Trends Biotechnol.* **2015**, *33*, 197–200. [[CrossRef](#)] [[PubMed](#)]
35. Chen, P.; Shrotri, A.; Fukuoka, A. Soluble Cello-Oligosaccharides Produced by Carbon-Catalyzed Hydrolysis of Cellulose. *ChemSusChem* **2019**, *12*, 2576–2580. [[CrossRef](#)] [[PubMed](#)]
36. Zhong, C.; Nidetzky, B. Three-Enzyme Phosphorylase Cascade for Integrated Production of Short-Chain Cellodextrins. *Biotechnol. J.* **2020**, *15*, 1900349–1900358. [[CrossRef](#)]
37. Tian, C.; Yang, J.; Zeng, Y.; Zhang, T.; Zhou, Y.; Men, Y.; You, C.; Zhu, Y.; Sun, Y. Biosynthesis of Raffinose and Stachyose from Sucrose via an In Vitro Multienzyme System. *Appl. Environ. Microbiol.* **2019**, *85*, 02306–18. [[CrossRef](#)]
38. Hofer, B. Recent developments in the enzymatic O-glycosylation of flavonoids. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 4269–4281. [[CrossRef](#)]
39. Tegl, G.; Nidetzky, B. Leloir glycosyltransferases of natural product C-glycosylation: Structure, mechanism and specificity. *Biochem. Soc. Trans.* **2020**, *48*, 1583–1598. [[CrossRef](#)]
40. Hu, Y.; Min, J.; Qu, Y.; Zhang, X.; Zhang, J.; Yu, X.; Dai, L. Biocatalytic Synthesis of Calycosin-7-O- β -D-Glucoside with Uridine Diphosphate–Glucose Regeneration System. *Catalysts* **2020**, *10*, 258–269. [[CrossRef](#)]
41. Huang, F.-C.; Hinkelmann, J.; Hermenau, A.; Schwab, W. Enhanced production of β -glucosides by in-situ UDP-glucose regeneration. *J. Biotechnol.* **2016**, *224*, 35–44. [[CrossRef](#)] [[PubMed](#)]
42. Thapa, S.B.; Pandey, R.P.; Bashyal, P.; Yamaguchi, T.; Sohng, J.K. Cascade biocatalysis systems for bioactive naringenin glucosides and quercetin rhamnoside production from sucrose. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 7953–7969. [[CrossRef](#)] [[PubMed](#)]
43. Salehi, B.; Fokou, P.V.T.; Sharifi-Rad, M.; Zucca, P.; Pezzani, R.; Martins, N.; Sharifi-Rad, J. The therapeutic potential of naringenin: A review of clinical trials. *Pharmaceuticals* **2019**, *12*, 11. [[CrossRef](#)] [[PubMed](#)]
44. Kim, Y.-J.; Zhang, D.; Yang, D.-C. Biosynthesis and biotechnological production of ginsenosides. *Biotechnol. Adv.* **2015**, *33*, 717–735. [[CrossRef](#)]
45. Ma, W.; Zhao, L.; Ma, Y.; Li, Y.; Qin, S.; He, B. Oriented efficient biosynthesis of rare ginsenoside Rh2 from PPD by compiling UGT-Yjic mutant with sucrose synthase. *Int. J. Biol. Macromol.* **2020**, *146*, 853–859. [[CrossRef](#)]
46. Huffman, M.A.; Fryszkowska, A.; Alvizo, O.; Borra-Garske, M.; Campos, K.R.; Canada, K.A.; Devine, P.N.; Duan, D.; Forstater, J.H.; Grosser, S.T.; et al. Design of an in vitro biocatalytic cascade for the manufacture of islatravir. *Science* **2020**, *368*, 1255–1259. [[CrossRef](#)]
47. Song, W.; Chen, X.; Wu, J.; Xu, J.; Zhang, W.; Liu, J.; Chen, J.; Liu, L. Biocatalytic derivatization of proteinogenic amino acids for fine chemicals. *Biotechnol. Adv.* **2020**, *40*, 107496. [[CrossRef](#)]
48. Laurent, V.; Goubeyre, L.; Uzel, A.; H elaine, V.; Nauton, L.; Tra kia, M.; Salanoubat, M.; Gefflaut, T.; Lemaire, M.; Gu rard-H elaine, C. Pyruvate Aldolases Catalyze Cross-Aldol Reactions between Ketones: Highly Selective Access to Multi-Functionalized Tertiary Alcohols. *ACS Catal.* **2020**, *10*, 2538–2543. [[CrossRef](#)]
49. Gmelch, T.J.; Sperl, J.M.; Sieber, V. Optimization of a reduced enzymatic reaction cascade for the production of L-alanine. *Sci. Rep.* **2019**, *9*, 1–9. [[CrossRef](#)]
50. Takamatsu, S.; Tosa, T.; Chibata, I. Production of L-alanine from ammonium fumarate using two microbial cells immobilized with κ -carrageenan. *J. Chem. Eng. Jpn.* **1985**, *18*, 66–70. [[CrossRef](#)]
51. Li, G.; Lian, J.; Xue, H.; Jiang, Y.; Ju, S.; Wu, M.; Lin, J.; Yang, L. Biocascade Synthesis of L-Tyrosine Derivatives by Coupling a Thermophilic Tyrosine Phenol-Lyase and L-Lactate Oxidase. *Eur. J. Org. Chem.* **2020**, *2020*, 1050–1054. [[CrossRef](#)]
52. Fern andez-Tejada, A.; Corzana, F.; Busto, J.H.; Avenoza, A.; Peregrina, J.M. Conformational effects of the non-natural α -methylserine on small peptides and glycopeptides. *J. Org. Chem.* **2009**, *74*, 9305–9313. [[CrossRef](#)] [[PubMed](#)]
53. Liao, C.; Seebeck, F.P. Asymmetric β -Methylation of l- and d- α -Amino Acids by a Self-Contained Enzyme Cascade. *Angew. Chem. Int. Ed.* **2020**, *59*, 7184–7187. [[CrossRef](#)] [[PubMed](#)]
54. Contente, M.L.; Paradisi, F. Self-sustaining closed-loop multienzyme-mediated conversion of amines into alcohols in continuous reactions. *Nat. Catal.* **2018**, *1*, 452–459. [[CrossRef](#)]

55. Burgener, S.; Cortina, N.S.; Erb, T.J. Oxalyl-CoA Decarboxylase Enables Nucleophilic One-Carbon Extension of Aldehydes to Chiral α -Hydroxy Acids. *Angew. Chem. Int. Ed.* **2020**, *59*, 5526–5530. [\[CrossRef\]](#)
56. Groeger, H.; Hummel, W. Combining the ‘two worlds’ of chemocatalysis and biocatalysis towards multi-step one-pot processes in aqueous media. *Curr. Opin. Chem. Biol.* **2014**, *19*, 171–179. [\[CrossRef\]](#)
57. Ríos-Lombardía, N.; García-Álvarez, J.; González-Sabín, J. One-pot combination of metal-and bio-catalysis in water for the synthesis of chiral molecules. *Catalysts* **2018**, *8*, 75. [\[CrossRef\]](#)
58. Wu, S.; Zhou, Y.; Gerngross, D.; Jeschek, M.; Ward, T.R. Chemo-enzymatic cascades to produce cycloalkenes from bio-based resources. *Nat. Commun.* **2019**, *10*, 1–10. [\[CrossRef\]](#)
59. Schuchardt, U.; Cardoso, D.; Seercheli, R.; Pereira, R.; da Cruz, R.S.; Guerreiro, M.C.; Mandelli, D.; Spinacé, E.V.; Pires, E.L. Cyclohexane oxidation continues to be a challenge. *Appl. Catal. A Gen.* **2001**, *97*, 1–17. [\[CrossRef\]](#)
60. Soussan, L.; Pen, N.; Belleville, M.P.; Marciano, J.S.; Paolucci-Jeanjean, D. Alkane biohydroxylation: Interests, constraints and future developments. *J. Biotechnol.* **2016**, *2022*, 117–142. [\[CrossRef\]](#)
61. Hrycay, E.G.; Bandiera, S.M. The monooxygenase, peroxidase, and peroxygenase properties of cytochrome P450. *Arch. Biochem. Biophys.* **2012**, *522*, 71–89. [\[CrossRef\]](#)
62. Wang, Y.; Lan, D.; Durrani, R.; Hollmann, F. Peroxygenases en route to becoming dream catalysts. What are the opportunities and challenges? *Curr. Opin. Chem. Biol.* **2017**, *37*, 1–9. [\[CrossRef\]](#)
63. Freakeley, S.J.; Kochius, S.; Van Marwijk, J.; Fenner, C.; Lewis, R.J.; Baldenius, K.; Marais, S.S.; Opperman, D.J.; Harrison, S.T.L.; Alcalde, M.; et al. A chemo-enzymatic oxidation cascade to activate C–H bonds with in situ generated H₂O₂. *Nat. Commun.* **2019**, *10*, 1–8. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Khanam, H. Bioactive Benzofuran derivatives: A review. *Eur. J. Med. Chem.* **2015**, *97*, 483–504. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Mertens, M.S.; Thomas, F.; Nöth, M.; Moegling, J.; El-Awaad, I.; Sauer, D.F.; Dhoke, G.V.; Xu, W.; Pich, A.; Schwaneberg, U. One-Pot Two-Step Chemoenzymatic Cascade for the Synthesis of a Bis-benzofuran Derivative. *Eur. J. Org. Chem.* **2019**, *2019*, 6341–6346. [\[CrossRef\]](#)
66. Schmidt, S.; Castiglione, K.; Kourist, R. Frontispiece: Overcoming the Incompatibility Challenge in Chemoenzymatic and Multi-Catalytic Cascade Reactions. *Chem. Eur. J.* **2018**, *24*. [\[CrossRef\]](#)
67. Cicco, L.; Ríos-Lombardía, N.; Rodríguez-Álvarez, M.J.; Moris, F.; Perna, F.M.; Capriati, V.; García-Álvarez, J.; González-Sabín, J. Programming cascade reactions interfacing biocatalysis with transition-metal catalysis in Deep Eutectic Solvents as biorenewable reaction media. *Green Chem.* **2018**, *20*, 3468–3475. [\[CrossRef\]](#)
68. Grabner, B.; Schweiger, A.K.; Gavric, K.; Kourist, R.; Gruber-Woelfler, H. A chemo-enzymatic tandem reaction in a mixture of deep eutectic solvent and water in continuous flow. *React. Chem. Eng.* **2020**, *5*, 263–269. [\[CrossRef\]](#)
69. Geng, P.B.; Zheng, S.S.; Tang, H.; Zhu, R.M.; Zhang, L.; Cao, S.; Xue, H.G.; Pang, H. Transition metal sulfides based on graphene for electrochemical energy storage. *Adv. Energy Mater.* **2018**, *8*, 1703259. [\[CrossRef\]](#)
70. Losada-García, N.; Rodríguez-Otero, A.; Palomo, J.M. Tailorable synthesis of heterogeneous enzyme–copper nanobiohybrids and their application in the selective oxidation of benzene to phenol. *Catal. Sci. Technol.* **2020**, *10*, 196–206. [\[CrossRef\]](#)
71. Palomo, J.M. Nanobiohybrids: A new concept for metal nanoparticles synthesis. *Chem. Commun.* **2019**, *55*, 9583–9589. [\[CrossRef\]](#)
72. Wei, H.; Wang, E.K. Nanomaterials with enzyme-like characteristics (nanozymes): Next-generation artificial enzymes. *Chem. Soc. Rev.* **2013**, *42*, 6060–6093. [\[CrossRef\]](#)
73. Zhang, Y.F.; Ge, J.; Liu, Z. Enhanced activity of immobilized or chemically modified enzymes. *ACS Catal.* **2015**, *5*, 4503–4513. [\[CrossRef\]](#)
74. Wang, Q.; Zhang, X.; Huang, L.; Zhang, Z.; Dong, S. One-pot synthesis of Fe₃O₄ nanoparticle loaded 3D porous graphene nanocomposites with enhanced nanozyme activity for glucose detection. *ACS Appl. Mater. Interfaces* **2017**, *9*, 7465–7471. [\[CrossRef\]](#)
75. Ruan, X.; Liu, D.; Niu, X.; Wang, Y.; Simpson, C.D.; Cheng, N.; Du, D.; Lin, Y. 2D Graphene oxide/Fe-MOF nanozyme nest with superior peroxidase-like activity and its application for detection of woodsmoke exposure biomarker. *Anal. Chem.* **2019**, *91*, 13847–13854. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Losada-García, N.; Berenguer-Murcia, A.; Cazorla-Amorós, D.; Palomo, J.M. Efficient production of multi-layer graphene from graphite flakes in water by lipase-graphene sheets conjugation. *Nanomaterials* **2020**, *10*, 7. [\[CrossRef\]](#)

77. Losada-Garcia, N.; Rodriguez-Oliva, I.; Simovic, M.; Bezbradica, D.I.; Palomo, J.M. New Advances in Fabrication of Graphene Glyconanomaterials for Application in Therapy and Diagnosis. *ACS Omega* **2020**, *5*, 4362–4369. [[CrossRef](#)]
78. Malkhandi, S.; Yeo, B.S. Electrochemical conversion of carbon dioxide to high value chemicals using gas-diffusion electrodes. *Curr. Opin. Chem. Eng.* **2019**, *26*, 112–121. [[CrossRef](#)]
79. Seelbach, K.; Riebel, B.; Hummel, W.; Kula, M.-R.; Tishkov, V.I.; Egorov, A.M.; Wandrey, C.; Kragl, U. A Novel, Efficient Regenerating Method of NADPH Using a New Formate Dehydrogenase. *Tetrahedron Lett.* **1996**, *37*, 1377–1380. [[CrossRef](#)]
80. Alissandratos, A.; Easton, C.J. Biocatalysis for the application of CO₂ as a chemical feedstock. *Beilstein J. Org. Chem.* **2015**, *11*, 2370–2387. [[CrossRef](#)]
81. Schlager, S.; Neugebauer, H.; Haberbauer, M.; Hinterberger, G.; Sariciftci, N.S. Direct Electrochemical Addressing of Immobilized Alcohol Dehydrogenase for the Heterogeneous Bioelectrocatalytic Reduction of Butyraldehyde to Butanol. *ChemCatChem* **2015**, *7*, 967–971. [[CrossRef](#)]
82. Seelajaroen, H.; Bakandritsos, A.; Otyepka, M.; Zbořil, R.; Sariciftci, N.S. Immobilized Enzymes on Graphene as Nanobiocatalyst. *ACS Appl. Mater. Interfaces* **2019**, *12*, 250–259. [[CrossRef](#)]
83. Chu, S.; Huang, W.; Shen, F.; Li, T.; Li, S.; Xu, W.; Lv, C.; Luo, Q.; Liu, J. Graphene oxide-based colorimetric detection of organophosphorus pesticides via a multi-enzyme cascade reaction. *Nanoscale* **2020**, *12*, 5829–5833. [[CrossRef](#)]
84. Periasamy, A.P.; Umasankar, Y.; Chen, S.M. Nanomaterials-acetylcholinesterase enzyme matrices for organophosphorus pesticides electrochemical sensors: A review. *Sensors* **2009**, *9*, 4034. [[CrossRef](#)] [[PubMed](#)]
85. Cui, H.F.; Wu, W.W.; Li, M.M.; Song, X.; Lv, Y.; Zhang, T.T. A highly stable acetylcholinesterase biosensor based on chitosan-TiO₂-graphene nanocomposites for detection of organophosphate pesticides. *Biosens. Bioelectron.* **2018**, *99*, 223–229. [[CrossRef](#)] [[PubMed](#)]
86. Wang, Q.Q.; Zhang, X.P.; Huang, L.; Zhang, Z.Q.; Dong, S.J. GOx@ZIF-8 (NiPd) nanoflower: An artificial enzyme system for tandem catalysis. *Angew. Chem. Int. Ed.* **2017**, *56*, 16082–16085. [[CrossRef](#)] [[PubMed](#)]
87. Chen, G.S.; Huang, S.M.; Kou, X.X.; Wei, S.B.; Huang, S.Y.; Jiang, S.Q.; Shen, J.; Zhu, F.; Ouyang, G.F. A convenient and versatile aminoacid-boosted biomimetic strategy for the nondestructive encapsulation of biomacromolecules within metal–organic frameworks. *Angew. Chem. Int. Ed.* **2019**, *58*, 1463–1467. [[CrossRef](#)] [[PubMed](#)]
88. Cheng, X.; Zhou, J.; Chen, J.; Xie, Z.; Kuang, Q.; Zheng, L. One-step synthesis of thermally stable artificial multienzyme cascade system for efficient enzymatic electrochemical detection. *Nano Res.* **2019**, *12*, 3031–3036. [[CrossRef](#)]

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