Perspective

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Development of heterogeneous enzymatic cascades with a case study for a separable and recyclable system using a combination of magnetic and nonmagnetic supports

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Abstract

In recent years, energy and environmental issues have become more topical as the public have become more conscious about the fragility of the planet we live in. Biocatalysis can have a strong impact on these issues as most enzymes are operated at low energy and solvent-free environments with often high conversion, activity and selectivity. Enzymatic cascades are multistep reactions that can produce more complex chemical products and mimic *in vivo* reactions in nature. Therefore, they can provide valuable solutions for many environmental problems. However, enzymes can be expensive, difficult to recycle and wasteful, as seen in other homogeneous systems. For heterogenized biocatalytic systems, enzyme immobilization has been considered to enhance reuse and recycling of enzymes but immobilized enzymes are still underdeveloped for use in cascade systems. Hence, there is an opportunity for advancement. The difficulties for implementing immobilized enzymes in cascades mainly concern enzyme compatibility, compartmentalization and optimization. In this perspective, we have highlighted key examples in using enzymatic cascades using immobilized enzymes, in particular those systems related to the energy and environment sectors. We also demonstrate use of a combination of a magnetic and a nonmagnetic support for enhancing enzyme reuse and recycling in a cascade system, and more importantly, separation of enzymes from each other postreaction. We can foresee that, with significant effort spent on the development, enzyme immobilization could play a significant role in cascades for the green synthesis of fine chemicals.

Supporting information may be found in the online version of this article.

Keywords: enzymatic cascade; magnetic nanoparticles; nickel(II) ferrite $NiFe_2O_4$; transaminases; glucose dehydrogenase; alcohol dehydrogenase

INTRODUCTION: ENZYMATIC CATALYSIS AND IMMOBILIZED ENZYMES

Enzymes can be considered as environmentally friendly catalysts as a result of their high activities at low temperatures and derivation from renewable resources.[1] Generally, they are active under milder conditions compared to those of equivalent synthetic systems and, in some cases, have shown higher catalytic activity and conversion efficiency than synthetic catalysts for the same reaction.[2,3] They also often associate with high chemo-, regio- and enantioselectivities,[4] which enhance downstream processes for separation and purification, a reduction in waste and overall energy efficiency.

However, enzymes can be sensitive to the reaction environment and can denature when used beyond their normal operating range – pH 7 and 37 °C. To mitigate this, immobilization of enzymes on a solid support can improve operational stability against denaturation caused by heat or changes in pH. It also allows for other benefits including the possibility of adapting continuous processing,

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ease of separation and enhanced recyclability.[5,6] There are four main protocols for enzyme immobilization: covalent binding, physical absorption, encapsulation and cross-linked aggregates, which are well-documented in the literature.[7-10] Immobilization also has been used for developing enzymatic cascade systems, which involve multistep reactions to achieve a final product through multiple enzymes. Cascades can avoid accumulation of intermediates, which could inhibit the reaction, as well as reduce the energy requirement to synthesize more complex products.[11] Enzymatic cascades occur within whole cells (*in vivo*) in nature, but much research work also has been directed towards developing *in vitro* immobilized systems as alternatives.[12]

IMMOBILIZED ENZYMES FOR ENERGY PRODUCTION AND ENVIRONMENTAL USES

Enzymes have been used for environmental applications for several decades, notably in remediation. However, in recent years, research in using enzymes (free or immobilized) for the productions of biofuels[13] and bioethanol[14] has also been expanded as a consequence of the increasing demand for these products. In other areas of the energy sector, enzymes also play their role. For example, enzymes have been shown capabilities in hydrogen (H_2) production from carbohydrates and glycerol in *in vivo* systems. Multi-enzyme systems also have been reported to be used in CO_2 utilization, through reduction to form formate and, ultimately, methanol [see Fig. 1(a)].[15] Although examples are plentiful in these areas, free enzymes are used in most of these systems. While immobilized enzymes show clear advantages in catalyst recycling and reduction in operational cost, challenges do exist in large-scale use of immobilized enzymes. For typical biodiesel productions, one key step is to use transesterification of fat molecules (from animal, vegetable or waste fat) to form long-chain esters and glycerol, followed by a decarboxylation of esters, as examples of two-step cascades. Enzymes, notably lipases, have been widely used as catalysts for this transesterification reaction.[16] Decarboxylation of fatty acids to form hydrocarbons also may be carried out using other enzymes.[17] Immobilized enzymes also have been used for biodiesel[18] and bioethanol production,[19] enabling enzyme recycling and enhancing sustainability of these cascades. Various supports have been tested for these applications, including magnetic[20,21] and nonmagnetic supports.[22] This also suggests that development of support materials can play some critical role in enzymatic process for future energy production. Moreover, immobilized enzymes also have been used in various applications for the environment, notably remediation.[23]

ENZYME CASCADES

An enzymatic cascade depicts a process that requires two or more enzymatic steps to produce the target chemical compound.[24] One of the aims of cascades is to mimic biological systems at a cellular and organismal level through their spatial distributions of functional modules in cells and how cells are compartmentalized to form their own multi-enzyme systems; these are often difficult to emulate.[24] Compartmentalization sometimes has a stabilizing effect on the enzyme, especially for enzymes that require a cofactor for reactions.When free enzymes are used in processes/reactions, compartmentalization would not occur and quite often yields in a loss of certain catalytic properties. To produce a successful enzymatic cascade, three key parameters need to be satisfied: (i) overall thermodynamics of the cascade must be

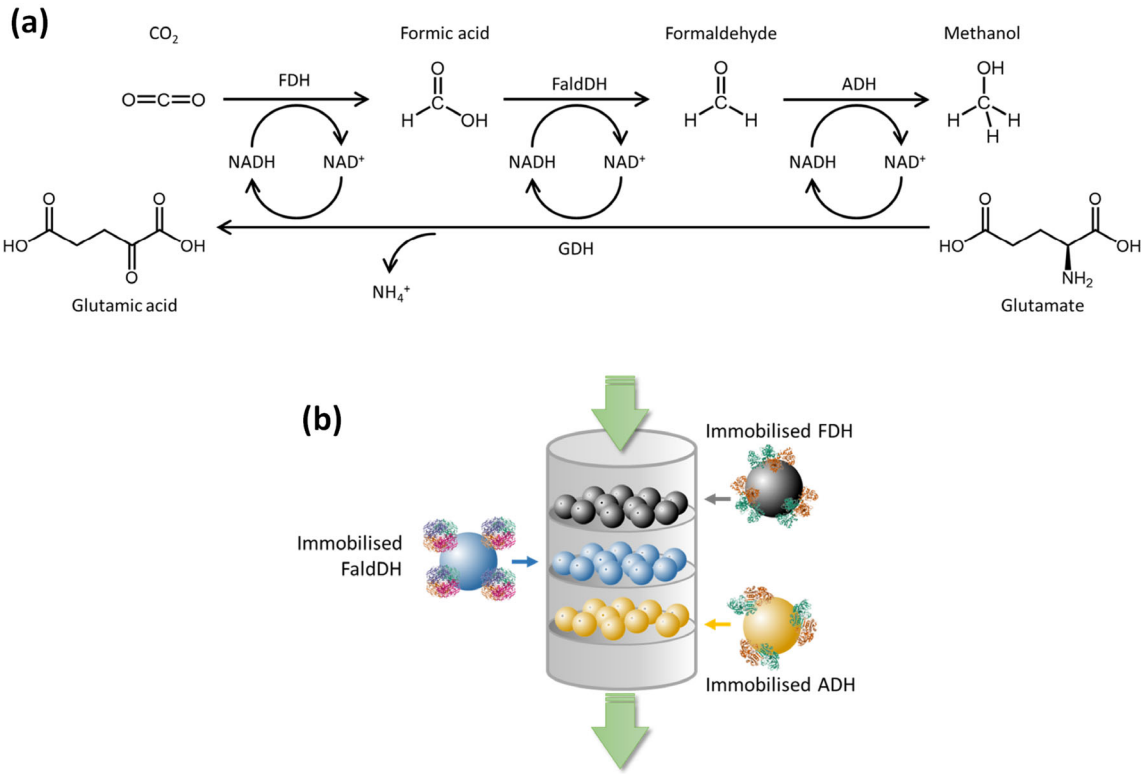

Figure 1. (a) The multistep cascade process for CO_2 transformation to methanol with NADH regeneration regime using GDH and glutamate as the sacrificial hydrogen donor, (b) Illustration of sequential Immobilization of enzymes to form methanol from CO_2.

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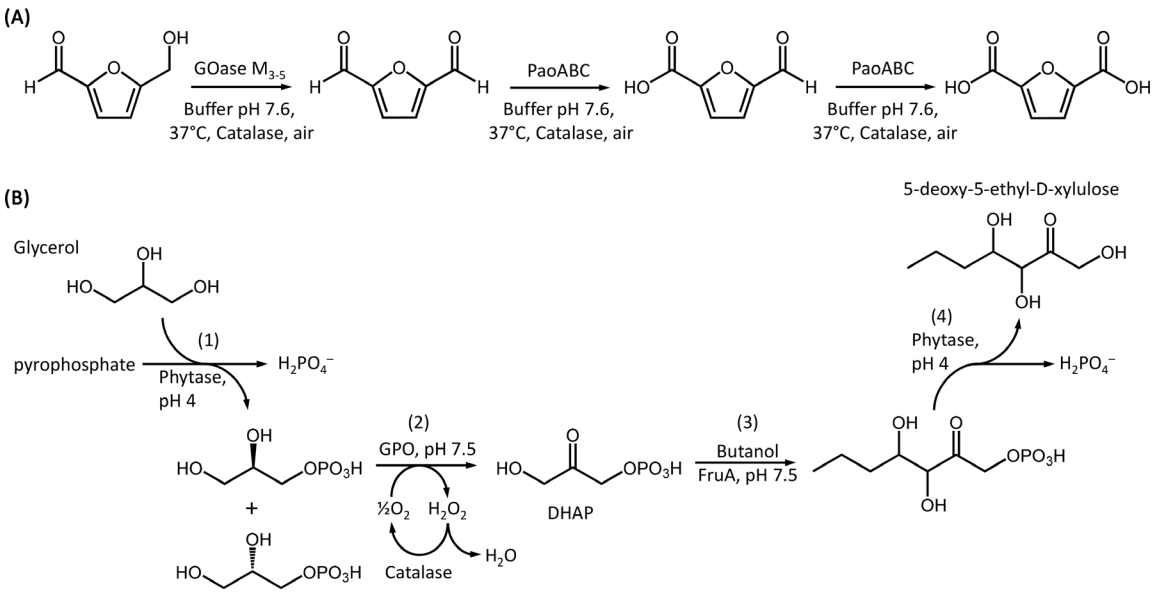
favourable,[25] (ii) cross-reactions between different substrates must be avoided by choosing an enzyme that exhibits high selectivity[26,27] and (iii) the activities of the enzymes have to control the reaction kinetics to ensure that there is reaction flux.[28,29] Implementing immobilized enzymes in a cascade-style system could make large-scale, multistep processing more attainable.[30]

There are three types of enzymatic cascades: *in vitro*, *in vivo* and hybrid. Selection of enzymes in a cascade depends on numerous key factors, including the availability of gene sequences, heterologous enzymes, cofactor requirements, uptake and release of substrates and products, and their metabolic stability.[31] Cascade systems for industrial applications are less common owing to the need for optimization of process variables, such as operational cost, product purity and concentration, stability and recyclability of enzymes, and their solvent tolerance.[32] For *in vitro* cascades, some of these issues can be solved with enzyme immobilization. For issues regarding enzyme stability and solvent tolerance, immobilization of enzymes can help as it induces small changes to the enzymes' secondary structure (α-helixes and β-pleated sheets) and prevents the enzyme from unfolding (i.e. denaturing).[33] However, combining enzyme immobilization with cascade reactions is still underdeveloped.[25] To implement enzymatic cascades, a system is required to be able to immobilize a broad range of enzymes, and therefore to mitigate the need for screening a wide range of platforms for each biocatalyst individually. As a cascade system increases in the number of steps, or the number of enzymes, involved, each step must be optimized to achieve the highest conversion to desired products possible.[34]

In vitro cascades can employ free, purified enzymes, cell lysates, cell-free extracts or freeze-dried whole cells, which allow for easier optimization on the amount of enzyme used in each step for a maximum flux and product yield.[35] Purified enzymes have advantages over crude enzymes as complications as a consequence of complex metabolic pathways in living cells can be eliminated, hence eliminating unwanted side reactions. In general though, enzyme purification is an expensive and time-consuming process. Moreover, if the process is cofactor-dependent, the cost will be escalated considerably as cofactors also can be expensive and are needed to be added in stoichiometric amounts.[36] In these cases, a cofactor recycling system needs to be implemented in order to make the cascade viable.

The simplest type of *in vitro* cascades are free enzymatic cascades (a series of reactions catalyzed by free enzymes). These systems involve no immobilization of enzymes and typically they are carried in a 'one-pot' style. Compared to immobilized cascades, the steps may be harder to optimize using free enzymes, which also are difficult to recover and recycle, adding obstacles for implementation in industries. For example, McKenna *et al.* demonstrated a synthesis pathway for 2,5-furandicarboxylic acid (FDCA) from hydroxymethylfurfural (HMF) using galactose oxidase (GOase) and aldehyde oxidase (PaoABC)[37] [see Scheme 1(a)]. Their results suggested that using both enzymes together in one pot only produced an overall conversion of 60% for FDCA. When the cascade was tuned by sequentially enzyme introduction by first adding GOase then followed by PaoABC to convert the intermediates to FDCA, the final conversion was increased to 90%. This shows that tuning each step of the cascade individually is important to achieve a high product yield. HMF can be easily derived from biomass[39] whereas FDCA can be used as a sustainable alternative to terephthalic acid in plastic production for biodegradable plastics replacing polyethylene terephthalate (PET),[40] which is one of the top five commodity plastics currently being produced.

Schoevaart *et al.* reported a larger enzyme cascade system involving four enzymes for the synthesis of non-natural carbohydrates from glycerol.[38] This cascade was carried out sequentially in a one-pot system because each step required different reaction conditions for the enzymes to produce a high product yield. The steps of the reaction include: (1) phytase catalyzed phosphorylation of glycerol to racemic glycerol-3-phosphate, (2) glycerolphosphate oxidase (GPO) and catalase converting the L-glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) and (3) finally the aldol reaction of DHAP and butanal catalyzed by aldolase to form fructose-1,6-biphosphate [see



Scheme 1. Examples of two multistep cascades: (a) 3-step synthesis for 2,5-furandicarboxylic acid (FDCA) from hydroxymethylfurfural and (b) 4-step transformation of glycerol to a non-natural carbohydrate, 5-deoxy-5-ethyl-d-xylulose. Adapted from McKenna *et al.* and Schoevaart *et al.*[37,38]

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Scheme 1(b)]. Notably, these steps require changes in pH from 4 to 7.5 and then back to 4 to achieve an overall yield of 79%. Glycerol is a known by-product from biodiesel production and is an abundant organic waste.[41] Making use of an abundant chemical for the synthesis of valuable product such as non-natural carbohydrates would undoubtedly be beneficial to societies.

CASCADES USING IMMOBILIZED ENZYMES

In order to increase the efficiency of *in vitro* free enzyme cascades, enzyme immobilization can be considered as it provides a pathway for a heterogeneous biocatalytic system. When enzymes are immobilized on a support, a microenvironment is created, as seen in both eukaryotic and bacterial cells.[35] As immobilized cascades attempt to mimic metabolism processes, the fundamental concept of compartmentalization is introduced into the enzymatic cascades. Compartmentalization of a cascade should improve the kinetics and the stability of the system as a result of the co-localization of the enzymes. It also would allow for the reaction intermediates to diffuse more effectively between the enzymes.[42] By using either chemical (covalent) binding or physical absorption methods, immobilization provides a solution for establishing heterogeneous cascades. These cascades could be developed from either co-immobilization of enzymes onto the surface of one single carrier or sequential immobilization onto different carriers. Table 1 summarizes some recent examples of *in vitro* co-immobilized and sequential cascade systems, the reactions being achieved and supports being used.

When considering co-immobilization of enzymes on the same carrier, the most important factor is to choose a suitable carrier that is compatible with all of the enzymes involved in the cascade system. The parameters for carriers (e.g. surface chemistry/functionalities, surface area, pore size) and the reaction conditions required for immobilization will affect the product yield, loading capacity and activity of the enzymes. As mentioned previously, there is no universal procedure or carrier for enzyme immobilization, hence the design and the fabrication of multifunctional carriers may be necessary for a cascade system with several enzymes of very different characters. Multifunctional carriers may be used but they are not without problems, depending on the properties of the enzymes of interest. Immobilization techniques also may vary widely and therefore could lead to the deactivation of other enzymes involved in the system.

For simplicity, compatible enzymes in a cascade system can be co-immobilized via the same chemistry. Examples of cascades using co-immobilized enzymes can be found in the literature.

Table 1. Recent examples of *in vitro* heterogenized enzymatic cascade systems

	Cascade Reaction	Multi-enzyme system	Support(s)	Type of immobilization	Ref
1	Oxidation of phenolic compounds	Formate dehydrogenase (FDH) and NADPH oxidase (NOX) with Horseradish Peroxidase (HRP)	Agarose beads activated with glyoxyl groups and NOX Agarose beads activated with boronate groups	Co-immobilized	43
2	Conversion of glucose to gluconic acid	GOx (Glucose Oxidase) and CAT (Catalase) GOx and HRP	Fe_3O_4 magnetic nanoparticles	Co-immobilized	44
3	Production of L-amino acids	D,L-hydantoinase, hydantoin racemase, L-carbamoylase and carbamoyl racemase	Agarose beads	Co-immobilized	45
4	Degradation of biogenic amines for the production of aldehydes and hydrogen peroxide	Amine oxidase and catalase	Glyoxyl agarose gel	Co-immobilized	46
5	Reduction of 4-(4-methoxyphenyl)-3-buten-2-one	Enoate reductases (ERs) and glucose dehydrogenase (GDH)	CLEAs and silica particles	Co-immobilized	47
6	Treatment of starch pectin and protein in waste water	α-amylase, protease and pectinase	Sodium alginate beads and Glutaraldehyde activated chitosan beads	Co-immobilized	47
7	Quantifying lactose in buffer and in milk	β-galactosidase (β-gal), glucose oxidase (GOD), HRP	Glass microfluidic channel	Sequential	48
8	Oxidative reaction of sodium laurate	Monooxygenase (P450 BM3) and GDH	Amino-agarose Epoxy agarose Polyvinyl alcohol Lentikats	Co-immobilized	49
9	Production of (R)-1-phenyl-1,2-ethanediol	FDH, (2R,3R)-2,3-butanediol hydrogenase (BDH)	Silicon dioxide nanoparticles with glutaraldehyde	Sequential and co-immobilized	30
10	Methanol production from CO_2	FDH, formaldehyde dehydrogenase (FaldDH), alcohol dehydrogenase (ADH), GDH	Polystyrene microparticles (500 nm)	Co-immobilized	50
11	Methanol production from CO_2	FDH, FaldDH, ADH	UF membranes, with regenerated cellulose layer on a PP support	Sequential and co-immobilized	51

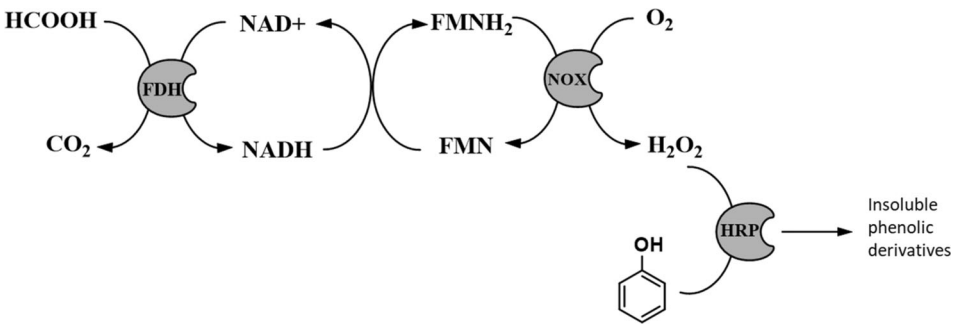
For instance, Yong *et al*. (entry 2, Table 1) demonstrated a co-immobilized enzymes system glucose oxidase (GOx) and catalase (CAT), as well as a combined GOx-HRP (horseradish peroxidase) systems. Both systems were co-immobilized using concanavalin A (ConA) and glutaraldehyde via glycosyl groups on ConA.[44] This cascade has shown a high relative activity and improved conversions of glucose to gluconic acid compared to the free enzyme systems (5% increase). This work demonstrated that two compatible model enzymes can be co-immobilized on one support. ConA has a nonreducing terminal α-D-mannosyl or α-D-glucosyl moieties,[52] ready to react with either GOx, HRP or CAT as they are considered as glycosylate enzymes and can be agglutinated by ConA. Ideally a support with multiple functional groups on the surface is needed to overcome the problems for multi-enzyme systems. Another example of co-immobilization has been reported by Garcia-Garcia *et al*. (entry 4, Table 1), where diamine oxidase (DAO) and CAT are co-immobilized on glyoxyl agarose gel via covalent attachment.[46] This cascade is set up for the degradation of biogenic amines present in wine to produce aldehydes and hydrogen peroxide (H_2O_2). Two different systems were tested: a co-localized and a delocalized system. Co-localization involves DAO and CAT immobilized one support sequentially, whereas delocalized is a random distribution of the two enzymes. Although the enzymes were immobilized successfully in both systems, they showed different reaction profiles. These results also were supported by the degradation profile of biogenic amines from both systems. The co-localized system reached 100% degradation in 2 h while the delocalized system reached only 60% over the same period. Also, the residual activity profile for the co-localized system stayed at 100% after 100 h compared to the delocalized system. It was concluded that the enzyme arrangement on the support is crucial for the initial rate for the degradation of the biogenic amines, and compartmentalization improves mass transfer of the substrate molecules.

Examples of heterogeneous cascades for the environment

Regarding cascades for the environment, Rocha-Martin *et al*. demonstrated a heterogenous cascade for the oxidation of phenolic compounds by co-immobilized three different enzymes, formate dehydrogenase (FDH), NADH-oxidase (NOX) and HRP on to agarose beads (entry 4, Table 1),[43] illustrated in Scheme 2. The initial test on this tri-enzymatic system for phenol degradation in an aqueous medium through *in situ* H_2O_2 production using free enzyme was not successful with little oxidation recorded. It was speculated that enzyme inhibition occurred due to formation of free radicals during oxidation or from the H_2O_2 product generated *in situ*. Immobilized enzymes were then proposed as an alternative but HRP was found to be incompatible with the other two enzymes during immobilization. Two different functional groups on the surface of the support materials were required for effective immobilization: FDH and NOX were co-immobilized on agarose beads functionalized with glyoxyl groups, whereas HRP was on agarose beads with boronate groups. This dual-carrier system was able to achieve 100% conversion of phenolic substrates due to the co-localization of FDH and NOX. This, in turn, gave a higher H_2O_2 production enough for HRP to catalyze at a maximum rate without inhibition. This example shows that a cascade system with three immobilized enzymes can be implemented. However, owing to the differences in immobilization chemistry of the enzymes and the conditions that they required, a dual-carrier system may be necessary. This also suggests that multifunctional carriers could have an important role to play in immobilized cascade reactions. Phenolic compounds, including phenol and cresols, are known pollutants from the oil and gas industry as they can contaminate soils and possess strong threat to ground water.[53] Degradation of these pollutants via biocatalytic pathways will be welcomed.

As mentioned before, an enzymatic cascade has been used in CO_2 reduction to produce methanol [Fig. 1(a)]. Heterogenizing this multi-enzyme system also has been demonstrated (entry 10, Table 1).[50] El-Zahab *et al*. co-immobilized all four enzymes (FDH, FaldDH, ADH and GDH) on polystyrene beads to enhance enzyme recycling. Moreover, the cofactor NADH also was immobilized on the beads in order to further improve the sustainability of the system. This heterogeneous cascade was found to lose only 20% of the activity after 11 cycles. However, in a 20-mL reaction, the overall methanol production was only 0.02 μmol h^{-1} $g_{enzyme}{}^{-1}$, which is comparable to the equivalent free enzyme cascade (at 0.04 μmol h^{-1} $g_{enzyme}{}^{-1}$). Although scaling up may be possible, such a low conversion is unlikely to have any impact in research on CO_2 utilization. Another problem presented from this system is that the enzymes cannot be separated from each other due to co-immobilization.

In order to overcome issues of enzyme separation postcascade, Luo *et al*. reported a heterogeneous cascade system by immobilizing these enzymes in layers of commercial membranes (entry 11, Table 1),[51] as illustrated in Fig. 1(b). This flow system achieved a maximum methanol production of 0.7 mmol L^{-1} at a high NADH concentration of 50 mmol L^{-1}. Because this system is rather different from El-Zahab's heterogeneous cascade (e.g. flow *versus* bath processes), it is difficult to make direct comparison. Although this multimembrane system allowed separation of individual enzymes, cofactor regeneration became impossible. The high NADH concentration (50 mmol L^{-1}) being used suggested that this system is not attainable economically as the NADH cofactor is far more valuable than methanol.



Scheme 2. Reaction scheme for *in situ* H_2O_2 production and phenolic removal using HRP.[43]

There are other challenges in using heterogeneous cascades for CO_2 reduction which remain to be overcome before large-scale implementation is possible. First, CO_2 is a thermodynamically stable compound (enthalpy of formation, $\Delta H_f^{\circ} = -393.5$ kJ mol^{-1}) and activation also requires overcoming a high energy barrier (240 kJ mol^{-1}). This is always a challenge for all CO_2 utilization processes. Moreover, although successful as a demonstrator, these two examples provide a low rate of methanol production (e.g. at 0.02 µmol s^{-1} mg_{enzyme}^{-1} reported by El-Zahab *et al.*[50]). For a large-scale operation such as CO_2 utilization, such a rate may not be sufficiently high to make economic sense, or indeed any impact on the environment. Regarding economic aspects, four enzymes (including GDH for cofactor regeneration) are required for this cascade, and their substantial operational cost must be considered. In addition, the cost for NADH cofactor needs to be considered, even with an efficient regeneration system. More importantly, from the first system, for every mole of CO_2 to be converted to methanol, three moles of glutamate are needed as the sacrificial H donor for NADH regeneration. The cost for glutamate is around 10-fold more expensive than methanol per kg. Cofactor regeneration is not compatible with the second membrane system. All of these factors make this process unattractive. Therefore, scientists still need to search for processes using heterogeneous cascades in order to make CO_2 utilization feasible for large-scale operations.

Advantages and disadvantages of *in vitro* heterogeneous cascades using immobilized enzymes

Regarding heterogeneous cascades, the enzymes are immobilized on a support and the assumption is that they can be recovered and recycled for further reactions/uses. Recycling exists in most immobilized enzyme system, with the majority being reused in numerous cycles. There are a wide variety of supports that can be used for immobilization, depending on the reaction and immobilization conditions. Common supports include inorganic materials and synthetic and natural polymers, including silicas, MNPs, alginate and poly(carboxybetaine methacrylate) (PCBMA).[54] Recovering these supports can be carried out by filtration or centrifugation, but these techniques are not without their disadvantages.[55] A trade-off between particle size and loss of activity is the main issue with both filtration and centrifugation. Increasing the particle size can enhance separation via filtration and centrifugation, but could reduce activity owing to slower diffusion of the substrate through larger particles.[45] Success in *in vitro* cascades relies on mimicking the microenvironments which cause compartmentalization to ensure that the substrates can diffuse between the enzymes. This also would be applied to integrated immobilized cofactor and enzyme systems. Scaling up immobilized enzymes for use in continuous processes also could encounter its own problems with larger particle sizes used in industrial equipment. For example, most bioreactors are based on packed bed columns or fluidized bed reactors. A larger particle size could increase the pressure drop across the column while in the fluidized bed the particle size would have to be on the micro-/nanoscale.[56] To ensure an adequate fluidization, the particles must be dense enough to avoid them escaping the reactor as a consequence of high velocities inside. In order to mitigate some of these problems, enzymes can be immobilized onto magnetic nanoparticles (MNPs). This involves immobilizing the enzymes on a magnetic support, typically iron oxide (Fe_3O_4) MNPs and instead of recovery through centrifugation or filtration MNPs can be recovered using an external magnetic field.[5,57]

SEPARABLE AND RECOVERABLE ENZYMES VIA MAGNETIC SEPARATION: A CASE STUDY

Although compatible enzymes can be co-immobilized in a cascade for recovery and recycling, they cannot be separated from each other postreaction. For expensive enzymes, co-immobilization may not be as useful for recycling. For those systems in which enzymes are immobilized separately, recovery of these immobilized enzymes is still problematic via separation of different particle types (supports) from each other with standard methods (e.g. filtration and centrifugation). In order to separate and recycle immobilized enzymes from a cascade system postreaction, innovative methods need to be developed.

To demonstrate the idea of separating and recovering different enzymes individually using magnetic separation, an enzymatic cascade was selected. In this cascade, (S)- methylbenzylamine was converted to acetophenone then 1-phenylethanol using ω-transaminase from *Halomonas elongata* (HeωT) and alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* respectively while glucose dehydrogenase from *Bacillus subtilis* (*Bs*-GDH) was also used for the regeneration of NADH cofactor that was required for the ADH step. 1-Phenylethanol is a chiral secondary alcohol and is commonly used as drug precursors[58] or as toxophores in agroindustry.[59] The interplay of these three enzymes in this cascade system is illustrated in Fig. 2(a).[60] The magnetic support used here is $NiFe_2O_4$ MNPs (average diameter = 10 nm, as seen from TEM in Fig. 3(a)) while NiO/SiO_2 was used as the nonmagnetic support. Both supports has free nickel sites on surface for direct binding of his-tagged enzymes (HeωT and *Bs*-GDH) without further functionalization. The magnetic property measured using SQUID magnetometry and the powder x-rays diffraction patterns (XRD) are shown in Fig. 3(b), (c). The synthesis protocol of $NiFe_2O_4$ MNPs can be found in Supporting Information SI. Since HeωT was immobilized on $NiFe_2O_4$ MNPs while *Bs*-GDH was supported on nonmagnetic NiO/SiO_2, these two enzymes can be separated using an external magnetic field postreaction, as shown in Fig. 2(b). The third enzyme ADH was used as an unsupported free enzyme, which can be separated from the other two immobilized enzymes via simple filtration.

Separation of $NiFe_2O_4$ MNPs and NiO/SiO_2 particles from a mixed suspension

The main purpose for using a combination of magnetic and nonmagnetic carriers in tandem is the option for separation and recovery of the immobilized enzymes individually after reaction. Separation of a mixture of solid particles can be challenging and magnetic separation offer a unique pathway to achieve that. To attain the separation efficiency between the nickel ferrite ($NiFe_2O_4$) MNPs and the nonmagentic nickel/silicon oxide (NiO/SiO_2) carriers in the immobilized combined enzyme cascade, an experiment on magnetic separation of the two carriers was performed. In this experiment, a mixed suspension of $NiFe_2O_4$ MNPs and NiO/SiO_2 particles was prepared in potassium phosphate buffer (see Scheme I). After the magnetic separation using a neodymium (NdFeB) magnet, the iron content in the supernatant was then analyzed using atomic absorption spectroscopy (AAS) to assess the separation efficiency. The magnetic properties of these two carriers also were measured using SQUID magnetometry and shown in Fig. 3(a). $NiFe_2O_4$ MNPs showed a typical superparamagnetic character with a saturation of 53.0 emu g^{-1} at H = 15 kOe whereas NiO/SiO_2 particles did not show any significant magnetization at the measured range. The AAS result suggested that only 17.6 µg Fe was found in the supernatant.

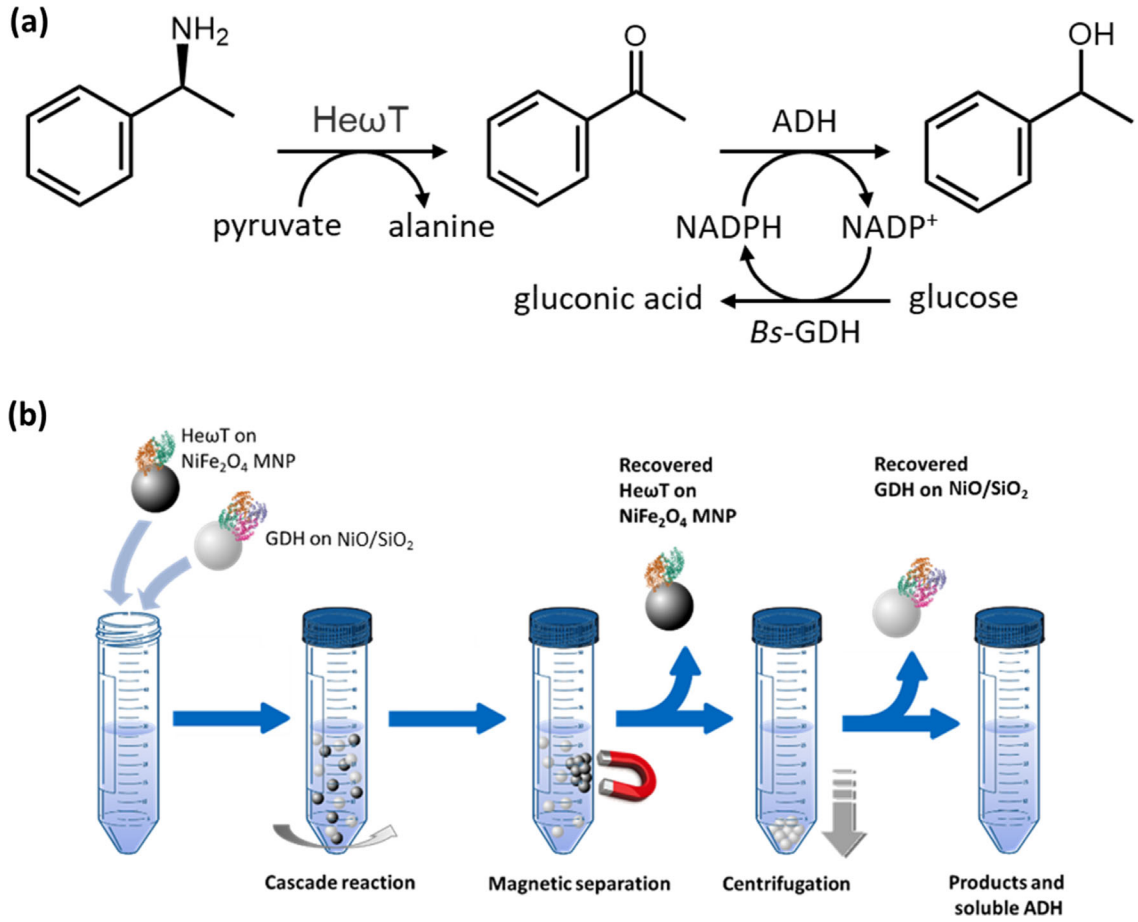


Figure 2. (a) Cascade system using Halomonas elongata (HeωT) and alcohol dehydrogenase (ADH) for the transformation of *s*-methylbenzylamine to acetophenone then to 1-phenylethanol. Glucose dehydrogenase from *Bacillus subtilis* (*Bs*-GDH) was used for regenerating the NADH cofactor using glucose as a sacrificial hydrogen donor; (b) illustration of how this three-enzyme system can be separated using a combination of magnetic and nonmagnetic support.

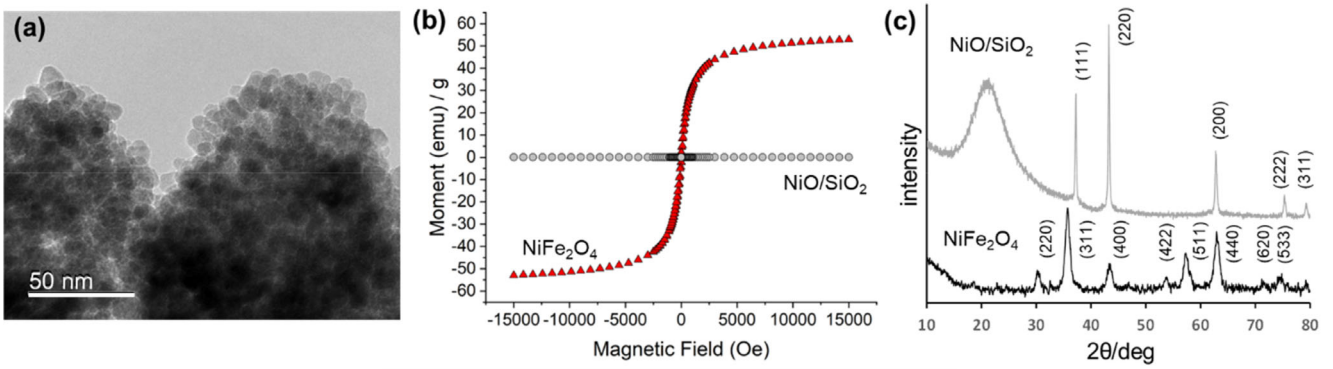


Figure 3. Characterization of $NiFe_2O_4$ MNPs and NiO/SiO_2 particles. (a) TEM image for $NiFe_2O_4$ MNPs; (b) plots of M *versus* H; (c) XRD pattern with indexes (JCPDS card no. 10-0325 for $NiFe_2O_4$ MNPs and 73-1523 NiO/SiO_2).

Because the initial Fe content in the mixed particle suspension was 4.7 mg (10 mg $NiFe_2O_4$ MNPs), only an equivalent to 0.37% Fe was found in the supernatant after magnetic separation. This indicates a successful separation of a magnetic and nonmagnetic carrier from a mixed suspension.

A model cascade reaction of three enzymes on different supports

Table 2 shows the yield of 1-phenylethanol after 1 h for both free and immobilized cascade reactions. From the data we can draw a preliminary conclusion that the free enzyme cascade reaction has produced the target alcohol product, 1-phenylethanol. Moreover, the yield for 1-phenylethanol was higher than that from a free enzyme system, which seemed to produce acetophenone for the first step but failed to convert it to 1-phenylethanol. Experimental details can be found in Scheme I.

The low yield of 1-phenylethanol could be attributed to the poor acceptance of acetophenone in the active site of ADH. This was seen previously in the free ADH activity test and it may be one of the causes of a low production of 1-phenylethanol even

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Table 2. Free enzyme cascade system concentrations of substrate, intermediate and product after 1 h

Cascade system	*s*-Methylbenzyl amine concentration (mmol L^{-1})	Acetophenone concentration (mmol L^{-1})	1-Phenylethanol concentration (mmol L^{-1})
Free enzymes	62.206	19.256	0.095
Immobilized cascade system	82.159	0.406	0.264

after 2 h (0.166 μmol mg_{enzyme}^{-1}). Screening a range of ADHs could be beneficial to optimize both reactions by identifying an ADH enzyme that could better work with acetophenone as a substrate to increase the yield of product in both cascade systems. To further validate the production of 1-phenylethanol in both systems (free and immobilized enzymes), there is an increase in gluconic acid over time, suggesting that the *Bs*-GDH was functioning normally. According to Fig. 2(a), *Bs*-GDH's primary function is to recycle $NADP^+$ back to NADPH as the cofactor for ADH by converting glucose (the sacrificial H donor) to gluconic acid. In theory if there was an increase in gluconic acid over time, *Bs*-GDH was converting $NADP^+$ to NADPH for the ADH to consume in its reaction. Both enzymes in the cascade are intrinsically reliant on each other, meaning that one reaction would not be able to proceed without the other.

Other observations of the two cascades include a much lower production of the products acetophenone and gluconic acid from immobilized HeωT and *Bs*-GDH over the same period. This is not unexpected as both immobilized enzymes are less active than the free enzymes due to mass transfer constraints for heterogeneous catalysts in general. This also would account for a lower concentration of acetophenone available for the second step in this cascade. The same observation on the production of gluconic acid over the same time was also featured, with a lower productivity observed in the immobilized cascade than its free cascade counterpart. This emphasises that both cascades require much more effort in optimization in terms of screening ADHs for better compatibility with the intermediates of the reaction and to also fine-tune each enzymatic stage to ensure that the cascades can work at optimum efficiency.

The $NiFe_2O_4$ MNPs used in this case study have a magnetization value (53.0 emu g^{-1}), which is comparable to the Fe_3O_4 MNPs (usually 40–80 emu g^{-1}) widely used as a benchmark for MNP research. As discussed, magnetic separation offers advantages such as a high recovery of immobilized enzyme on the magnetic support, especially if an enzyme is hard to purify. One clear advantage of using magnetic separation in a multi-enzyme system is that the enzymes can be separated from each other postreaction as demonstrated here. In this case study, the three enzymes all can be separated individually, using MNP-supported, nonmagnetic-supported and unsupported enzymes. In general, nonmagnetic supports are difficult to separate from each other and co-immobilization is to be implement without the prospect of separation of enzymes postreaction. The system demonstrated here also would be useful in cascade reactions with one enzyme that is more valuable than the others, and it is essential to recover that particular enzyme. This system also could enhance the practicality of some heterogeneous cascade systems and reduces the operational cost by recycling the expensive enzymes.

This case study demonstrated that a separable tri-enzyme system using a combination of magnetic and nonmagnetic supports is feasible despite the low yield recorded for the target product. As the results from the free enzyme system suggested, this combination of enzymes has not been optimized and this is a typical obstacle for an artificial enzymatic cascade. In nature, the cascade systems in cells were optimized and that can be difficult to mimic in laboratories. In this particular system, use of the most compatible ADH could be critical. As suggested earlier, screening a range of ADHs is necessary in order to optimize all three in tandem reactions and maximize the production of 1-phenylethanol. However, even an optimized multi-enzyme system may behave very differently once immobilized, as demonstrated here. Optimization is key for a successful cascade, no matter if it is an immobilized system of free-enzyme system, and this can involve intensive studies.

CONCLUSIONS

In nature, many complex compounds are made from multi-enzyme cascade systems *in vivo*. We can exploit these systems in simulated environments and scale them up for the production of fine chemicals as well as valuable natural products. Enzymatic systems are generally of low energy consumption and low waste emission, and thus are considered to be environmentally friendly. Indeed, several enzymatic cascades also have been demonstrated for the production of biodiesel and bioethanol, as well as CO_2 conversion to methanol. The challenges in developing enzymatic cascades include enzyme recycling and compatibility among enzymes. Use of immobilized enzymes can mitigate the first challenge but the issues of enzyme compatibility will need lots of research to address. Regarding immobilized systems, separation between enzymes postreaction can be problematic, even if they are immobilized on supports. We have demonstrated a separable system of three enzymes using magnetic and nonmagnetic supports. Such a system can open up many opportunities for industries to consider enzyme cascades because recycling enzymes can reduce operational costs.

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DATA AVAILABILITY STATEMENT

Upon reasonable request from corresponding author.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Sheldon RA and van Pelt S, Enzyme immobilisation in biocatalysis: why, what and how. *Chem Soc Rev* **42**:6223–6235 (2013).
2 Sanchez S and Demain AL, Useful microbial enzymes—an introduction, in *Biotechnology of Microbial Enzymes*, ed. by Brahmachari G. Elsevier, Amsterdam, pp. 1–11 (2017).
3 Koeller KM and Wong CH, Enzymes for chemical synthesis. *Nature* **409**: 232–240 (2001).
4 Hanefeld U, Gardossi L and Magner E, Understanding enzyme immobilisation. *Chem Soc Rev* **38**:453–468 (2009).
5 Liese A and Hilterhaus L, Evaluation of immobilized enzymes for industrial applications. *Chem Soc Rev* **42**:6236–6249 (2013).
6 Velasco-Lozano S, Benítez-Mateos AI and López-Gallego F, Co-immobilized phosphorylated cofactors and enzymes as self-sufficient heterogeneous biocatalysts for chemical processes. *Angew Chem, Int Ed* **56**:771–775 (2017).
7 Yiu HHP and Wright PA, Enzymes supported on ordered mesoporous solids: a special case of an inorganic-organic hybrid. *J Mater Chem* **15**:3690–3700 (2005).
8 Santos JCSD, Barbosa O, Ortiz C, Berenguer-Murcia A, Rodrigues RC and Fernandez-Lafuente R, Importance of the support properties for immobilization or purification of enzymes. *ChemCatChem* **7**: 2413–2432 (2015).
9 Zhang Y, Ge J and Liu Z, Enhanced activity of immobilized or chemically modified enzymes. *ACS Catal* **5**:4503–4513 (2015).
10 Sheldon RA and Pereira PC, Biocatalysis engineering: the big picture. *Chem Soc Rev* **46**:2678–2691 (2017).
11 Schrittwieser JH, Velikogne S, Hall M and Kroutil W, Artificial biocatalytic linear cascades for preparation of organic molecules. *Chem Rev* **118**:270–348 (2018).
12 Küchler A, Yoshimoto M, Luginbühl S, Mavelli F and Walde P, Enzymatic reactions in confined environments. *Nat Nanotechnol* **11**:409–420 (2016).
13 Andrade TA, Martín M, Errico M and Christensen KV, Biodiesel production catalyzed by liquid and immobilized enzymes: optimization and economic analysis. *Chem Eng Res Des* **141**:1–14 (2019).
14 Vaz RP, de Souza Moreira LR and Ferreira Filho EX, An overview of holocellulose-degrading enzyme immobilization for use in bioethanol production. *J Mol Catal B: Enzym* **133**:127–135 (2016).
15 Chen H, Huang Y, Sha C, Moradian JM, Yong Y-C and Fang Z, Enzymatic carbon dioxide to formate: mechanisms, challenges and opportunities. *Renewable Sustainable Energy Rev* **178**:113271 (2023).
16 Altinok F, Albayrak S, Arslan NP, Taskin M, Aygun E, Sisecioglu M *et al.*, Application of Anoxybacillus gonensins UF7 lipase as a catalyst for biodiesel production from waste frying oils. *Fuel* **334**:26672 (2023).
17 Guo X, Xia A, Zhang W, Huang Y, Zhu X, Zhu X *et al.*, Photoenzymatic decarboxylation: A promising way to produce sustainable aviation fuels and fine chemicals. *Bioresource Technol* **367**:128232 (2023).
18 Abdulmalek SA and Yan Y, Recent developments of lipase immobilization technology and application of immobilized lipase mixtures for biodiesel production. *Biofuels, Bioprod Biorefin* **16**:1062–1094 (2022).
19 Partovinia A, Salimi Koochi M, Talaeian M and Rasooly Garmaroody E, Comparative study of bioethanol production from bagasse pith by Pichia stipitis in free cell system and immobilized electrosprayed micro-beads: SSF and combined hydrolysates fermentation. *Sustain Energy Technol Assess* **54**:102855 (2022).
20 Alikhani N, Shahedi M, Habibi Z, Yousefi M, Ghasemi S and Mohammadi M, A multi-component approach for co-immobilization of lipases on silica-coated magnetic nanoparticles: improving biodiesel production from waste cooking oil. *Bioprocess Biosyst Eng* **45**:2043–2060 (2022).
21 Domingues O, Remonatto D, dos Santos LK, Martínez Galán JP, Flumignan DL and de Paula AV, Evaluation of Candida rugosa lipase immobilized on magnetic nanoparticles in enzymatic/chemical hydroesterification for biodiesel production. *Appl Biochem Biotechnol* **194**:5419–5442 (2022).
22 Zhang B, Weng Y, Xu H and Mao Z, Enzyme immobilization for biodiesel production. *Appl Microbiol Biotechnol* **93**:61–70 (2012).
23 Chen J, Guo Z, Xin Y, Gu Z, Zhang L and Guo X, Effective remediation and decontamination of organophosphorus compounds using enzymes: from rational design to potential applications. *Sci Total Environ* **867**:161510 (2023).
24 Dubey NC and Tripathi BP, Nature inspired multienzyme immobilization: strategies and concepts. *ACS Appl Bio Mater* **4**:1077–1114 (2021).
25 France SP, Hepworth LJ, Turner NJ and Flitsch SL, Constructing biocatalytic cascades: in vitro and in vivo approaches to de novo multienzyme pathways. *ACS Catal* **7**:710–724 (2017).
26 Valikhani D, Bolivar JM, Dennig A and Nidetzky B, A tailor-made, self-sufficient and recyclable monooxygenase catalyst based on coimmobilized cytochrome P450 BM3 and glucose dehydrogenase. *Biotechnol Bioeng* **115**:2416–2425 (2018).
27 Polakovič M, Švitel J, Bučko M, Filip J, Neděla V, Ansorge-Schumacher MB *et al.*, Progress in biocatalysis with immobilized viable whole cells: systems development, reaction engineering and applications. *Biotechnol Lett* **39**:667–683 (2017).
28 Betancor L and Luckarift HR, Co-immobilized coupled enzyme systems in biotechnology. *Biotechnol Genet Eng Rev* **27**:95–114 (2010).
29 El-Zahab B, Jia H and Wang P, Enabling multienzyme biocatalysis using nanoporous materials. *Biotechnol Bioeng* **87**:178–183 (2004).
30 Metzger KE, Moyer MM and Trewyn BG, Tandem catalytic systems integrating biocatalysts and inorganic catalysts using functionalized porous materials. *ACS Catal* **11**:110–122 (2021).
31 Ji Q, Wang B, Tan J, Zhu L and Li L, Immobilized multienzymatic systems for catalysis of cascade reactions. *Process Biochem* **51**:1193–1203 (2016).
32 Virgen-Ortíz JJ, dos Santos JCS, Berenguer-Murcia Á, Barbosa O, Rodrigues RC and Fernandez-Lafuente R, Polyethylenimine: a very useful ionic polymer in the design of immobilized enzyme biocatalysts. *J Mater Chem B* **5**:7461–7490 (2017).
33 Secundo F, Conformational changes of enzymes upon immobilisation. *Chem Soc Rev* **42**:6250–6261 (2013).
34 Kazenwadel F, Franzreb M and Rapp BE, Synthetic enzyme supercomplexes: Co-immobilization of enzyme cascades. *Anal Methods* **7**: 4030–4037 (2015).
35 Schmidt S, Castiglione K and Kourist R, Overcoming the incompatibility challenge in chemoenzymatic and multi-catalytic Cascade reactions. *Chem A Eur J* **24**:1755–1768 (2018).
36 Oroz-Guinea I and García-Junceda E, Enzyme catalysed tandem reactions. *Curr Opin Chem Biol* **17**:236–249 (2013).
37 McKenna SM, Leimkühler S, Herter S, Turner NJ and Carnell AJ, Enzyme cascade reactions: synthesis of furandicarboxylic acid (FDCA) and carboxylic acids using oxidases in tandem. *Green Chem* **17**:3271–3275 (2015).
38 Schoevaart R, van Rantwijk F and Sheldon RA, A four-step enzymatic cascade for the one-pot synthesis of non-natural carbohydrates from glycerol. *J Org Chem* **65**:6940–6943 (2000).
39 Soukup-Carne D, Fan X and Esteban J, An overview and analysis of the thermodynamic and kinetic models used in the production of 5-hydroxymethylfurfural and furfural. *Chem Eng J* **442**:136313 (2022).
40 Heo JB, Lee YS and Chung CH, Conversion of inulin-rich raw plant biomass to 2,5-furandicarboxylic acid (FDCA): Progress and challenge towards biorenewable plastics. *Biotechnol Adv* **53**:107838 (2021).
41 Saini M, Wang ZM, Chiang CJ and Chao YP, Metabolic engineering of Escherichia coli for production of n-butanol from crude glycerol. *Biotechnol Biofuels* **10**:173 (2017).
42 Velasco-Lozano S and López-Gallego F, Wiring step-wise reactions with immobilized multi-enzyme systems. *Biocatal Biotransformation* **36**: 184–194 (2018).
43 Rocha-Martin J, Velasco-Lozano S, Guisán JM and López-Gallego F, Oxidation of phenolic compounds catalyzed by immobilized multienzyme systems with integrated hydrogen peroxide production. *Green Chem* **16**:303–311 (2014).
44 Yong Y, Su R, Liu X, Xu W, Zhang Y, Wang R *et al.*, Lectin corona enhances enzymatic catalysis on the surface of magnetic nanoparticles. *Biochem Eng J* **129**:26–32 (2018).
45 Rodríguez-Alonso MJ, Rodríguez-Vico F, Las Heras-Vázquez FJ and Clemente-Jiménez JM, Immobilization of a multi-enzyme system for L-amino acids production. *J Chem Technol Biotechnol* **91**:1972–1981 (2016).
46 García-García P, Rocha-Martin J, Fernandez-Lorente G and Guisan JM, Co-localization of oxidase and catalase inside a porous support to improve the elimination of hydrogen peroxide: oxidation of

biogenic amines by amino oxidase from Pisum sativum. *Enzyme Microb Technol* **115**:73–80 (2018).
47 Li H, Xiao W, Xie P and Zheng L, Co-immobilization of enoate reductase with a cofactor-recycling partner enzyme. *Enzyme Microb Technol* **109**:66–73 (2018).
48 Fornera S, Kuhn P, Lombardi D, Schlüter AD, Dittrich PS and Walde P, Sequential immobilization of enzymes in microfluidic channels for cascade reactions. *ChemPlusChem* **77**:98–101 (2012).
49 Solé J, Caminal G, Schürmann M, Álvaro G and Guillén M, Co-immobilization of P450 BM3 and glucose dehydrogenase on different supports for application as a self-sufficient oxidative biocatalyst. *J Chem Technol Biotechnol* **94**:244–255 (2019).
50 El-Zahab B, Donnelly D and Wang P, Particle-tethered NADH for production of methanol from CO_2 catalyzed by coimmobilized enzymes. *Biotechnol Bioeng* **99**:508–514 (2008).
51 Luo J, Meyer AS, Mateiu RV and Pinelo M, Cascade catalysis in membranes with enzyme immobilization for multi-enzymatic conversion of CO_2 to methanol. *New Biotechnol* **32**:319–327 (2015).
52 Goldstein IJ, Winter HC and Poretz RD, Chapter 12 plant lectins: Tools for the study of complex carbohydrates, in *New Comprehensive Biochemistry 29 (PART B)*, ed. by Montreuil J, Vliegenthart JFG and Schachter H. Elsevier Science Publishers, Amsterdam, pp. 403–474 (1997).
53 Panigrahy N, Priyadarshini A, Sahoo MM, Verma AK, Daverey A and Sahoo NK, A comprehensive review on eco-toxicity and biodegradation of phenolics: recent progress and future outlook. *Environ Technol Innov* **27**:102423 (2022).
54 Nussbaumer MG, Nguyen PQ, Tay PKR, Naydich A, Hysi E, Botyanszki Z *et al.*, Bootstrapped biocatalysis: biofilm-derived materials as reversibly Functionalizable multienzyme surfaces. *ChemCatChem* **9**:4328–4333 (2017).
55 Sigurdardóttir SB, Lehmann Ovtar S, Grivel JC, Negra MD, Kaiser A and Pinelo M, Enzyme immobilization on inorganic surfaces for membrane reactor applications: mass transfer challenges, enzyme leakage and reuse of materials. *Adv Synth Catal* **360**:2578–2607 (2018).
56 Fahad MK, Prakash R, Majumder SK and Ghosh P, Frictional pressure drop in a flotation column: An experimental investigation in continuous mode and its prediction by a general model. *Multiph Sci Technol* **31**:235–254 (2019).
57 Yiu HHP and Keane MA, Enzyme–magnetic nanoparticle hybrids: new effective catalysts for the production of high value chemicals. *J Chem Technol Biotechnol* **87**:583–594 (2012).
58 Ötvös SB and Kappe CO, Continuous flow asymmetric synthesis of chiral active pharmaceutical ingredients and their advanced intermediates. *Green Chem* **23**:6117–6138 (2021).
59 Garrison AW, Probing the enantioselectivity of chiral pesticides. *Environ Sci Technol* **40**:16–23 (2006).
60 Yun H, Yang YH, Cho BK, Hwang BY and Kim BG, Simultaneous synthesis of enantiomerically pure (R)-1-phenylethanol and (R)-α-methylbenzylamine from racemic α-methylbenzylamine using ω-transaminase/alcohol dehydrogenase/glucose dehydrogenase coupling reaction. *Biotechnol Lett* **25**:809–814 (2003).

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