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Cascade Biocatalytic Processes through Combined Cross-Linked Enzyme Aggregates

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Abstract: Cascade reactions catalyzed by immobilized multienzymatic systems are emerging as a tool to address the increasing need for green and sustainable chemistry. In this regard, the carrier-free immobilization strategy of combined cross-linked enzymes aggregates (combi-CLEAs) is of great interest. In a combi-CLEA two or more enzymes are co-immobilized into a single unit where they are accomodated in close proximity in order to minimize the intermediates diffusion in the reaction medium, at the same time allowing the access of substrates and coenzymes to the active sites. The use of combi-CLEAs provides a simple and low cost methodology which enhances operational and storage enzyme stability and facilitates the biocatalyst recovery and recycle. Some significant applications of cascade reactions catalyzed by combi-CLEAs are reported in this mini-review. These examples have been applied on a multigram scale and have furnished practical steps towards the implementation of enzymes on an industrial scale.

Keywords: CLEA; Combi-CLEAs; Enzyme cascades; Biotransformation; Biocatalysis; Immobilized enzymes; Green chemistry; Multienzymatic system.

1. INTRODUCTION

Biocatalysis, namely the use of enzymes as catalysts for the transformation of unnatural compounds [1], is increasingly attesting as a competitive and cost-effective alternative for the manufacturing of fine chemicals, agrochemical derivatives and active pharmaceutical intermediates.

The major value of biocatalysis relies on the enzymes unique stereo-structure in the active site capable to promote highly selective interactions with chiral or prochiral substrates. The resulting diastereoisomeric substrate-enzyme transition state complexes differ in transition state ($\Delta G^{\#}$) energy thus favouring stereoselective reactions.

High turnover number, excellent stereo- regio- and chemo-selectivities, environmental friendliness are only few of the indisputable advantages of biocatalysts. The use of biocatalytic methods has led to numerous economic and environmental improvements.

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Indeed, the biocatalysts derive from readily available, renewable resources, are biodegradable, not toxic or dangerous. The use of enzymes allows to avoid metals, thus saving the consequent costs needed to remove metal traces from the final products.

Notwithstanding, the application of enzymes is expected to increase in the next future as a tool to develop more sustainable chemistry [2,3,4].

The use of enzymes on a large scale appears to be reflected in the market growth trend. In 2016, the world market for enzymes was over USD 5 billion, and in 2024 it is expected to reach USD 17.5 billion [5]. Relevant advances in biotechnology over the last two decades have deeply expanded the field of application of biocatalysis.

DNA sequencing has made it possible to sequence more than 2000 bacterial and fungal genomes and these data has been made available on databases. Currently, genes can be identified in silico by "extracting the genome" from databases. Genes can be synthesized in a week, cloned into a host organism and produced on scale at an acceptable price by recombinant DNA technology. Twenty years ago it was necessary to modify the process to make the available enzyme work, whereas nowadays enzymes can be optimized to fit a predefined process.

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Directed evolution artificially accelerate the evolutionary process of enzyme through mutation and recombination addressed to the optimization of one or more features of interest [6,7]. Today evolution in vitro allows to engineer enzymes in order to obtain certain parameters such as activity, specificity and stability for a target process. When structural data and knowledge of mechanism/function are known, it is possible to finely tune the catalytic properties through key mutations in the active site or in the binding pocket. Computational tools support the experimental techniques enhancing the accuracy of predictions and allowing to generate more sophisticated protein de novo designed enzymes [8,9,10].

Simultaneously to enzyme engineering, process engineering has been developed in order to meet the requirements for large scale applications of biotransformations.

~~Indeed,~~ Free-state enzymes have limitations such as sensitivity to organic solvents, low operational and storage stability and denaturation at high temperature. Indeed, enzymes are subjected to self-digestion, auto-oxidation and shear stresses causing the loss of catalytic activity. Enzymes are usually employed under diluted conditions since they often suffer from substrate and product inhibition, thus hampering the productivity of industrial processes. Moreover, they display the highest activitity in water, an unsuitable solvent for most of the organic molecules.

Immobilization techniques are good alternatives to overcome these problems and can enhance enzymes performances mainly by extending their lifetime. The simplification of the biocatalyst recycling procedure and downstream processing can also be achieved as additional benefits [11,12].

Biocatalytic cascade reactions are synthetic methodologies which can benefit most from immobilized enzymes. Since enzymes work under similar experimental conditions, it is easy to integrate multiple reactions into one-pot cascade processes which are both cost-effective and environmentally beneficial [13,14,15,16].

We report in this review some illustrative cascades catalyzed by immobilized enzymes [17,18]. In particular, we cover cascade reactions catalyzed by combined cross-linked enzyme aggregates (combi-CLEAs) which are ideally suited for preparative transformations and applications [19,20]. The target compounds discussed herein such as hydroxy acids, nucleotides, halogenated hetero aromatic compounds and carbohydrates, represent important building blocks and biological active compounds.
Our review aims to complete the picture of modern biocatalysis by presenting the most recent applications of cascade synthesis by combi-CLEAs and highlighting the progress of research with regards to the typical issues related to the need of promoting enzyme stability and recyclability in order to develop large-scale one pot biotransformations.

2. CARRIER-FREE ENZYME IMMOBILIZATION BY CROSS-LINKING

Enzyme immobilization has proven to be an advantageous solution to circumvent stability problems and it also facilitates the recovery and recycling of the biocatalyst [21]. However, the binding of an enzyme to a solid support (carrier) inevitably leads to a "dilution of the activity", due to the large non-catalytic dead weight used, ranging from 90% to > 99%. As a consequence, lower space time yield is observed. For this reason, in the 90' the interest of industries was focused on carrier-free immobilized enzymes, the cross-linked enzyme crystals (CLECs) [22]. CLECs are prepared by crystallization of an enzyme in aqueous buffer solution, at optimal pH, followed by the addition of a bifunctional reagent, such as glutaraldehyde, which aims to join together the crystals. The resulting CLECs consist of uniform particles exhibiting outstanding benefits in terms of operational stability and activity.

Unfortunately, the crystallization procedure is not only laborious but also expensive since it requires the use of extremely pure enzymes. The simpler and cheaper alternative to crystallization is the precipitation of proteins from the aqueous solution in the form of protein aggregates held together by non-covalent bonds. The tertiary structure of proteins is thus retained and therefore enzymes do not undergo denaturation.

When protein aggregates are subjected to cross-linking, they become permanently insoluble, thus originating cross-linked enzyme aggregates (CLEAs) which maintain the native enzyme structure and therefore the catalytic activity [23].The crucial advantage given by the use of CLEAs is the possibility to combine purification and immobilization in a single step, which also makes this approach viable for crude protein extracts. Other advantages are high productivities due to high enzyme loading, good filterability and centrifugability, mechanical stability, excellent operational stability to heat and organic solvents, extreme pH and proteolysis (autolysis) and storage stability.

Finally, cost savings are achievable by avoiding additional costs related to the use of carriers (Figure 1) [24,25].

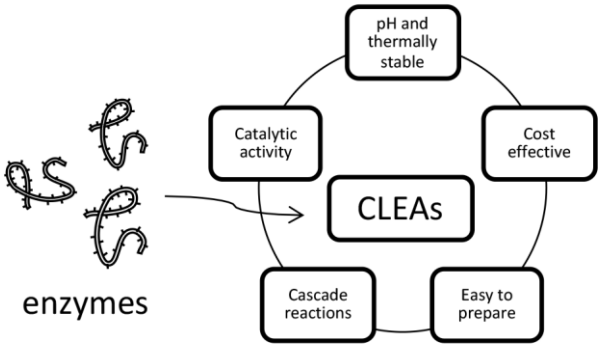


Figure 1. CLEAs advantages.

Moreover, CLEAs can be modified in order to modulate their physical properties or to simplify their handling. For example CLEA-polymer composites with tuneable hydrophobic/hydrophilic properties have been prepared through cross-linking in the presence of a $RSi(OCH_3)_3$ siloxane monomer undergoing copolymerization [26]. Furthermore, when the cross linking step has been carried out in the presence of functionalized magnetic Fe_3O_4@SiO_2-

NH_2 nanoparticles, a biocatalyst easily recoverable through magnetic decantation could be generated [27,28].

The mutual interactions among neighboring proteins in the aggregates are of non-covalent nature. Thus, the catalyst activity is mainly retained as a consequence of unmodified tertiary structure [29].

The ability to stabilize the quaternary structures of oligomeric enzymes, a structural characteristic of many enzymes used at industrial level, such as alcohol dehydrogenases, oxidases, peroxidases and nitrilases represents another advantage of CLEAs [30,31].

The observed immobilized catalyst activity with respect to the free enzyme activity can be highly dependent on physical properties such as particle and pore size, hydrophobicity/hydrophilicity balance, which affects the mass transfer limitations of substrates and/or products in the immobilization matrix.

Also, the possibility of recovering the biocatalyst deserves particular attention. The particle size of CLEAs, generally between 5-50 μm, allows them to be filtered or centrifugated. In addition, if necessary, the particle size can be changed by the enzyme/cross-linker ratio and/or the cross-link reaction time [32]. Moreover, the porous nature of CLEA particles does not reduce the mass transfer during the biotransformation, a shortcoming which is often observed in other immobilization methodologies [33].

CLEA technology is widely applied, especially to an increasingly broad selection of enzymes such as hydrolases, oxidoreductases and lyases [34,35,36,37,38,39,40,41]. Proteases, lipases, esterases, amidases, nitrilases and glycosidases, are the most used hydrolases enzymes in industries because they are the simpler enzymes to handle [21,42]. In fact, in addition to the advantages in common with other enzymes, hydrolases do not need cofactors, accept a broad range of substrates and are mostly commercially available and stable in organic solvents. This means that hydrolases can be used not only for breaking bonds through hydrolysis but also to generate new bonds thanks to their ability to operate in "reverse mode" in organic solvents.

Parameters of paramount importance to develop CLEAs include the careful choice of the precipitant and the cross-linking agent, the addition of co-aggregants such as bovine serum albumin (BSA), the optimization of the concentration of the cross linking agent and the modification of cross-linking reaction time. The optimization of such parameters designed to maximize the recovered activity of CLEAs is described herein.

2.1 Optimization of CLEAs preparation

The physical aggregation and precipitation of soluble free proteins can be obtained by the addition of neutral salts, such as ammonium sulphate, or organic hydrophilic solvents such as acetone, ethanol, 2-propanol and *tert*-butanol, or non-ionic polymers such as polyethylene glycol. The subsequent change of the hydration state of the enzymatic molecule and the dielectric constant of the solution facilitates the aggregation and precipitation of supramolecular structures held together by non-covalent bonds. Each enzyme has its own primary sequence and a quaternary structure, so the optimal precipitation conditions vary from one enzyme to another. The different precipitants induce the formation of enzyme aggregates with distinct conformations which, in turn, influence the catalytic properties of the CLEAs. The efficiency of the precipitation might be easily evaluated as the amount of precipitate enzyme generated. Then, the aggregates are redissolved in an appropriate buffer to measure their activity [43]. The precipitation parameters are chosen to generate CLEAs with the highest recovery of the enzymatic activity. Furthermore, the precipitant must be water soluble and must not react with enzymes and buffer. Inexpensive and commercially available precipitants are commonly used.

The second key element, the cross-linker, is a bifunctional agent capable to covalently bind amino groups located on the enzymatic surface. Glutaraldehyde has been widely used for years as a cross-linker in the preparation of CLEAs, because it is very advantageous from an economic point of view [44]. The cross-linking step is based on the formation of imine covalent bonds between the aldehyde group of the cross-linkers and the lysine residues of the enzymes. However, it is supposed that the mechanism is more complex and not completely elucidated until now and also depends on alkaline or acidic experimental conditions. Glutaraldehyde does not react as a monomer, but it is subjected to autocondensation reactions giving polymeric α,β-unsaturated carbonyl structures undergoing nucleophilic 1,4 or 1,2 addition by the lysine amino groups [45].

Glutaraldehyde is not always the optimal reagent, since it is a relatively small molecule that can fit substrate binding sites and consequently causing enzymes inactivation. Larger cross-linkers such as dextran polyaldehyde have been studied to overcome this problem [46].

Also, carboxylic groups of bicarboxylic amino acids such as glutamic and aspartic acid can be involved in the cross-linking with polymers containing amino groups with the consequent formation of amide bonds. For this purpose, several cross-linking agents containing amino groups have been successfully developed. Polyethylenimines were used for the preparation of lipase-CLEAs from rough *Candida* by cross-linking of carboxylic groups activated with carbodiimide [47]. Laccase-CLEAs were also prepared, by crosslinking the carboxylic groups activated with carbodiimide and amine groups of chitosan [48].

The number of cross-links influences the properties and the activity of the resulting CLEAs, therefore it is essential to adjust the amount of cross-linker. Lower concentrations lead to the formation of an insufficient number of bonds. On the other hand, higher concentrations reduce the activity of CLEAs preventing the substrate from reaching the active sites and limiting the internal mass transfer [49].

Since the enzymes are sensitive to high concentrations of glutaraldehyde, a second protein with a high amount of amino groups (the so called proteic feeder) such as BSA can be added to act as a spacer, thus reducing excessive cross-linking of the enzyme [50,51].

The temperature of the cross-linking step should also be considered. Higher temperatures decrease the time of cross linking, however causing an irreversible denaturation of the enzyme, which is avoided in the case of lower temperatures. The partial unfolding of the three-dimensional structure

along with the intramolecular bond breaking in the tertiary and quaternary structure induce the loss of activity in the denaturated enzyme [52].

2.2 Combined CLEAs approach to catalytic cascade reactions

Catalytic cascade reactions are excellent both from an economic and environmentally point of view as they combine several catalytic steps into a single multi-stage process. Thus, common issues related to intermediates stability and their isolation and purification might be circumvented. Competitive pathways are minimized as subsequent reactions drive equilibria in favor of the desired product. This involves other advantages such as the efficient space and resource management, the shorter time taken to complete a process, the lower amount of waste produced.

The idea of assembling the different enzymes needed to catalyze a cascade process into the same aggregate has represented the turning point which has transformed simple CLEAs to combined-CLEAs (combi-CLEAs, Figure 2).

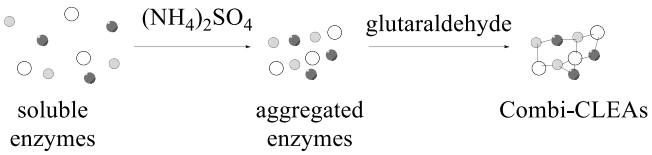


Figure 2. Combi-CLEAs preparation.

Since combi-CLEAs are prepared by co-precipitation and subsequent crosslinking of two or more different enzymes, the optimization of the experimental conditions must take into account different needs typical of each enzyme involved. The optimum pH should be considered first, since the enzyme activity after immobilization can be strongly influenced by pH.

The quantitative ratio of each enzyme taking part in the catalytic process is a further fundamental parameter to be considered in combi-CLEAs. In order to improve the catalytic efficiency of the multi-enzymatic system, the catalytic rate of each enzyme should be kept equal, under ideal conditions (Figure 3).

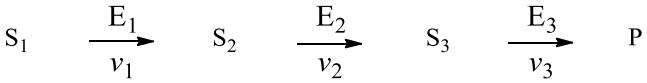
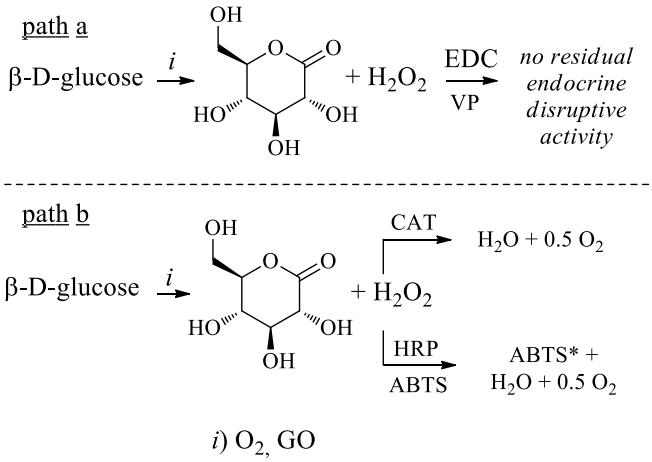


Figure 3. General scheme of a cascade combi-CLEAs made of 3 enzymes E ($v_1 = v_2 = v_3$).

The optimal amount of each enzyme is dependent on the reaction rate and the stability of the catalyst. These are the limiting parameters for the total turnover number of the system (TTN, determined as the moles of product/amount of catalyst during its lifetime). Therefore, the proportion of each enzyme in the immobilized multienzymatic system should be carefully determined. Moreover, the effect of product accumulation and inhibition should not be neglected.

Indeed, in a process where a combi-CLEA formed from glucose oxidase (GOD) and versatile peroxidase (VP) is the biocatalyst, glucose is oxidized by GOD to D-glucone-δ-lactone with the formation of hydrogen peroxide, which is the substrate of VP. This biocatalyst has been tested to oxidatively eliminate endocrine disrupting chemicals (EDC) such as bisphenol A, nonyl phenol and triclosan (Scheme 1, path a) [53]. An excess of GOD leads to high concentration of hydrogen peroxide which causes the peroxidase inactivation. Conversely, too low an amount limits the catalysis rate of combi-CLEA. When VP and GOD are in the optimal ratio of 10:7, the maximum apparent rate of the biocatalyst was observed. Under this reaction conditions hydrogen peroxide is completely consumed by VP as soon as it is formed.



Scheme 1. GOD/HRP combi-CLEAs.

The rates of multienzyme cascade reactions can be increased by simulating the close proximity of enzymes present in microbial cells [54]. For example, a cascade process where hydrogen peroxide is formed by GOD during the oxidation of glucose to D-glucono-δ–lactone and used by horseradish peroxidase (HRP) for the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to ABTS* was successfully carried out with free GOD and HRP (Scheme 1, path b) [55]. However, when catalase was added as a third free enzyme hydrogen peroxide was quickly decomposed thus strongly inhibiting ABTS oxidation as clearly shown by the lighter green color due to a lower ABTS* concentration. In the cascade reaction, if both reactions have similar reaction rates, then the overall reaction rate is determined by the speed at which the intermediate hydrogen peroxide can reach HRP. Thus, it is expected that the overall reaction rate increases when the distance between GOD and HRP is reduced. In order to enhance the close proximity of the two enzymes, GOD and HRP were co-immobilized into a combi-CLEA. Under the optimal reaction conditions, the reaction rate of the first reaction is the same as the other one, and there is no accumulation of hydrogen

peroxide. On the other hand, when free catalase was added to the GOD/HRP combi-CLEA, only minor amounts of the generated H_2O_2 was decomposed and no clear change in the green color was observed confirming the high ABTS* concentration. This result shows that the intermediate H_2O_2 is not decomposed by catalase due to a short distance between GOD and HRP in the combi-CLEA.

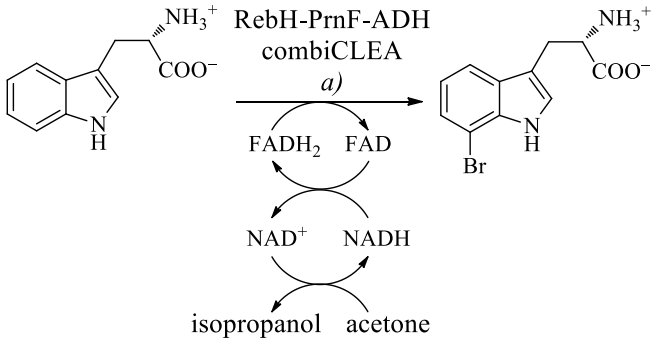
3. CASCADE REACTIONS CATALYZED BY combi-CLEAs

The cascade mentioned herein are particularly meaningful because they are ideally suited for preparative scale, or allow access to important building blocks and intermediates or enable the successful combination of a high number of enzymes. In any case they provide an inspiration for new cascade design.

3.1 Tryptophan regioselective halogenation

The halogenation reaction is an unfavorable process from the environmental point of view since requires chlorine or bromine as reagents, often in combination with Lewis acids. In addition, it is affected by the formation of halogenated by-products due to poor regioselectivity. In nature, the enzymatic strategies for performing these reactions use halide salts, oxygen at 25 °C and pH 7. The main enzymatic class responsible for highly regioselective halogenations includes FAD dependent halogenases. Tryptophan 7-halogenase PrnA and the related Tryptophan 7-halogenase RebH derived from *Lechevalieria aerocolonigenes* are the main representatives of this class. L-Tryptophan is regioselectively chlorinated at the disadvantaged C7 position of the indole ring through a N-haloamine intermediate generated by hypoalogenous acid produced in situ. Up to now halogenases have always been used only for analytical studies due to low kinetic constants and TTN below 200, making synthetic application wasteful.

The halogenation of L-tryptophan catalyzed by FAD dependent L-tryptophan-7-halogenase RebH has been studied in order to develop a practical synthetic method overriding the kinetical constraint and the need for high volumes [56].



a) NaBr, O_2, pH 7.4, 25 °C, 6-8 d

Scheme 2. Combi-CLEA promoted L-Tryptophan regioselective bromination.

The project involved the simultaneous immobilization of over expressed L-tryptophan-7-halogenase RebH from crude *Escherichia coli* lysate with the necessary auxiliary enzymes, a flavin-reductase from *Pseudomonas fluorescens* (PrnF), and an alcohol dehydrogenase from *Rhodococcus sp.* (ADH). The combi-CLEA has been prepared through precipitation with ammonium sulfate followed by crosslinking with glutaraldehyde (Scheme 2). Under optimized conditions, the authors achieved an immobilization yield of 99% of all three enzymes with a 30% activity recovery. The CLEAs, obtained from a culture of 6 L of *Escherichia coli*, with over-expressed L-tryptophan-7-halogenase RebH, was used for the halogenation of 1 g of L-tryptophan in a final volume of 5 L. Within 8 days, the reaction took place with complete conversion leading to the formation of 1.813 g of L-7-bromotryptophan trifluoro acetate in a 92% yield. The combi-CLEA was stable for more than 4 months at 4 ºC, while purified, free-form RebH showed a noticeable loss of activity after a 12-week period. Furthermore, the combi-CLEA could be recycled 10 times, although a significant reduction of conversion was observed after 5/6 cycles.

Due to the dimeric structure of RebH, the formation of CLEAs may be the cause of the stabilization of the quaternary structure of the enzyme making it more active.

In addition, the RebH combiCLEAs was also applied for the bromination of D-tryptophan and chlorination of L-5-hydroxytryptophan. Even for these substrates, despite the lower yields, halogenation works on a preparative scale. A 3:1 inseparable mixture of L-7-chloro-5-hydroxytryptophan and L-6-chloro-5-hydroxytryptophan was recovered in the chlorination of L-5-hydroxytryptophan.

Although enzymatic halogenation is still in its infancy, due to application on limited substrates, the use of immobilized RebH for regioselective bromination of L-tryptophan, on a gram scale, has laid the foundation for efficient biocatalytic halogenations.

3.2 Biocatalytic route to (*S*)-mandelic acid

Optically active 2-hydroxycarboxylic acids are interesting building blocks in organic synthesis and the hydrolysis of cyanohydrins is a particularly efficient synthetic pathway for their preparation.

Dynamic kinetic resolution of racemic cyanohydrins can be used on an industrial scale to obtain 2-hydroxy carboxylic acids by means of enantioselective nitrilases (NLases). This synthetic route is the basis of the large scale industrial synthesis of (*R*)-mandelic acid, a precursor in the synthesis of numerous compounds with biological activity such as semi-synthetic penicillins, cephalosporins, anti-obesity drugs, anti-tumor agents and important resolving reagents for the resolution of racemic alcohols and amines.

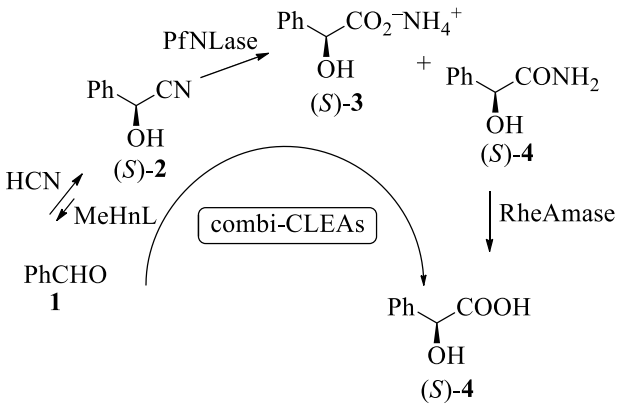
However, the lack of nitrilases selective for cyanohydrins with absolute (*S*) configuration is a weakness of this strategy.

On the other hand, (*S*)-mandelic acid can be generated through the enantioselective synthesis of (*S*)-cyanohydrin by

means of an hydroxynitrile lyase (HnL, oxynitrilase) selective for configuration (*S*), followed by hydrolysis with concentrated hydrochloric acid at reflux. This synthetic route is advantageous thanks to the availability of (*S*)-hydroxynitrile lyases which, alongside a high stereoselectivity, show a wide substrate tolerability. However, the drastic conditions that must be adopted for the hydrolysis of cyanohydrins are incompatible with the presence of other labile functional groups. Furthermore, large quantities of salts are generated during neutralization of the strongly acidic solution.

These drawbacks can be circumvented combining the benzaldehyde hydrocyanation with the hydrolysis in a bis-enzymatic cascade process (Scheme 3) [57].

In order to inhibit the background reaction favoured at pH 7 in the aqueous phase and leading to racemic cyanohydrin, the use of hydroxynitrile lyase occurs preferably at pH less ≤ 5 in biphasic water/diisopropyl ether mixtures. The selective



Scheme 3. Combi-CLEAs route to (*S*)-mandelic acid (4).

enzyme chosen for the formation of the corresponding (*S*)-cyanohydrin is the hydroxynitrile lyase from *Manihot esculenta* (MeHnL) both because it is easily available at low costs and for its stability.

Pseudomonas fluorescens PfNLase was chosen to hydrolyze the cyanohydrin since it is exceptionally active up to pH 5.5 and converts (*R*)- and (*S*)-mandelonitrile at comparable rates. Other NLases are intolerant to organic solvents and show maximum activities at pH 7-9 being inactivated by the high HCN concentrations used to shift the equilibrium towards the cyanohydrin formation. However, PfNLase produces significant amounts of (*S*)-mandelic amide (**4**) byproduct. It was therefore necessary to add amidase from *Rhodococcus erythopolis* (RheAMase) as a third enzyme in the combi-CLEA in order to convert (*S*)-mandelic amide to the desired (*S*)-mandelic acid. Thus a combi-CLEA containing MeHnL, PfNLase and RheAMase could be prepared with an activity recovery of 45% (MeHnL), 58% (PfNLase) and 57% (RheAMase) and used in the cascade conversion of benzaldehyde (**1**) to (*S*)-mandelic acid (**4**) (Scheme 3). The enantiomeric excess (ee) of (*S*)-mandelic acid obtained with this procedure was 99%, improving the previous 94% ee obtained by a tandem process catalyzed by MeHnL-CLEA and PfNLase-CLEA [58].

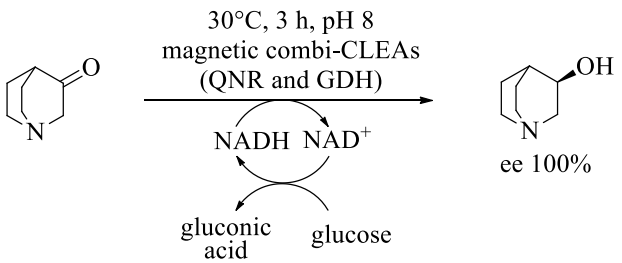
The increased ee of the obtained (*S*)-mandelic acid has been ascribed by the authors to the immediate hydrolysis into the combi-CLEAs particles of cyanohydrin (*S*)-**2** as it is formed, without having time to diffuse in the aqueous phase where it could racemize.

3.3 Synthesis of (*R*)-3-quinuclidinol

(*R*)-3-quinuclidinol is a key chiral intermediate of many drugs such as Aclidinium bromide, Solifenacin succinate and Revatropate and can be obtained by resolution of (dl)-3-quinuclidinone or by stereoselective synthesis. One of the most common synthetic pathways is the asymmetric hydrogenation of the parent ketone which leads to (*R*)-3-quinuclidinol with up to 90% ee. However, this method suffers from a number of limitations such as the cost and toxicity of the catalyst as well as the contamination of the product with transition metals. The biocatalytic reduction was achieved with various biocatalysts such as those isolated from *Nocardia sp.* and *Rhodococcus erythropolis*, a screened fungal system belonging to *Mucor piriformis* or by means of a recombinant ketoreductase.

A new approach has been developed by co-immobilizing 3-quinuclidinone reductase (QNR) and glucose dehydrogenase (GDH) into a new magnetic combi-CLEAs for the enantioselective synthesis of (*R*)-3-quinuclidinol with regeneration of coenzymes in situ [59]. The preparation of magnetic combi-CLEA of QNR-GDH obtained from *Escherichia coli BL*21 cell lysate consists of two physico-chemical phases: the initial co-precipitation by ammonium sulfate of QNR, GDH and amino functionalized silica coated Fe_3O_4@SiO_2-NH_2 magnetic nanoparticles (MNPs), followed by cross-linking with glutaraldehyde. The magnetic combi-CLEAs can be separated from the reaction mixture by external magnet without the need for filtration or centrifugation steps.

The biotransformation of 3-quinuclidinone into (*R*)-3-quinuclidinol has been investigated first using the free enzymes at 25 °C and pH 7 and then the magnetic combi-CLEAs at 30 °C and pH 8 (Scheme 4). In both cases glucose has been used as a substrate for the regeneration of the cofactor. The reaction with the immobilized enzymes was found to be about twice as fast as that with the free enzyme.



Scheme 4. Reduction of 3-quiniclidinone by magnetic combi-CLEAs.

The biocatalyst was able to run up to 10 cycles with increasing reaction times to achieve a complete conversion. Quantitative conversions were observed at each cycle. The biocatalyst showed also good storage stability. This cheap and efficient magnetic combi-CLEA allows to open a new horizon for the quantitative synthesis of enantiomerical pure (*R*)-3-quinuclidinol.

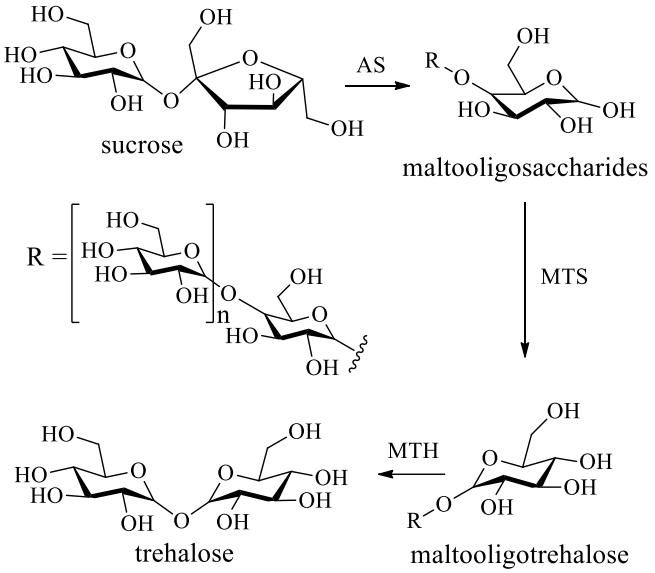
3.4 Sucrose to trehalose conversion

Trehalose is a non-reducing disaccharide in which two glucose molecules are linked by a α-1,1-glycosidic bond. Trehalose is of interest in the industrial field, because of its greater stability and lower sweetness than sucrose. Additionally, it has a moderate glycemic index with a low insulin response. Finally, it has low cariogenicity and high capacity to retain water and for these reasons it has an important role in the food, cosmetic and pharmaceutical industries.

Among several synthetic strategies studied for the synthesis of trehalose, the biocatalytic one starting from sucrose has aroused considerable interest [60]. The biocatalysts involved in this process are amylosucrase (ASase), maltooligosyl trehalose synthase (MTS) and maltooligosyltrehalose trealohydrolase (MTH). ASase belongs to the glycoside hydrolase family capable to catalyze various reactions, such as hydrolysis, trans-glycosylations and isomerizations. In particular it can react with sucrose, releasing glucose and fructose by means of a hydrolysis reaction. At the same time it can transfer glucose donor to another glucose molecule or another acceptor. Thus, ASase can form polymers by means of α-1,4 bonds, or other trans-glycol products using sucrose as starting product, instead of an expensive activated sugar, such as Adenosine- Uridine diphosphate glucose (ADP- or UDP-glucose).

ASase from *Deinococcus geothermalis* (DGAS) is an enzyme with high thermal stability and was chosen for the first step of this process. DGAS synthesizes maltooligo saccharides from sucrose thanks to its transglycosylation activity. The other steps are mediated by maltooligosyltrehalose synthase (MTS) and maltooligosyltrehalose trehalohydrolase (MTH). The two enzymes and the corresponding genes were isolated from the non-pathogenic bacterium *Brevibacterium helvolum* (Bv) and subsequently expressed in *Escherichia coli*. MTS converts the α-1,4 glycosidic bond to α-1,1 glycosidic bond, producing maltooligotrehalose. Finally, MTH hydrolyzes the second α-1,4 glycosidic bond of the intermediate releasing trehalose (Scheme 5).

In order to carry out the sucrose to trehalose conversion in a single step, a combi-CLEA has been prepared by using the optimal amount of AS, BvMTS and MTH [61]. It turned out that a wide range of MTS/MTH ratios gave the same amount of trehalose. Therefore, the two enzymes did not appear to be the rate limiting catalysts in the cascade reaction. On the other hand a 8:1:1 ratio of AS/MTS/MTH provided 13 times higher yield with respect to the yield obtained with a 1:1:1 ratio.



Scheme 5. Sucrose to trehalose bioconversion through combi-CLEA promoted cascade reaction.

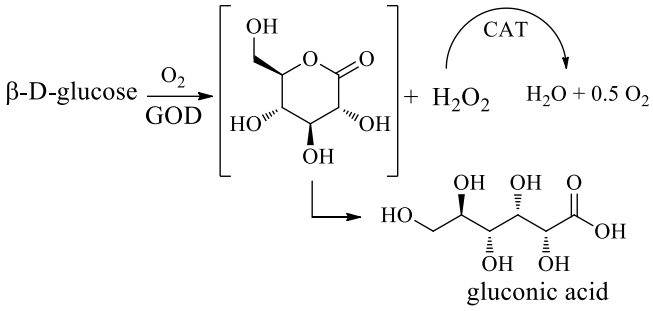
It can be concluded that under these conditions the AS enzyme catalyzed the formation of enough maltooligo saccharide to be gradually and efficiently converted to trehalose by MTS and MTH.

In order to prepare the combi-CLEAs, acetone was selected as the better precipitating agent. The evaluation of the optimal amount of glutaraldehyde was rather laborious. For low concentrations of aldehyde the cross-linking was not effective and the aggregates were dispersed after washing. However, larger amounts of aldehyde decreased trehalose production. The problem could be circumvented by adding BSA bearing a large number of amino groups which prevented excessive cross-linking of the enzymes.

The activity of the biocatalyst remained similar until the fifth cycle and started to significantly decrease in the next cycles. This results have been ascribed to a thermostability limitation as evidenced by stability tests of the combi-CLEAs at 37 °C for 140 h.

3.5 Synthesis of gluconic acid

Recently, combi-CLEAs of glucose-oxidase (GOD) from *Aspergillus niger* and catalase (CAT) from bovine liver have been used for gluconic acid production in a bubble column reactor. GOD catalyzes the oxidation of β-D-glucose to the corresponding β-D-glucono-δ-lactone using molecular oxygen and releasing H_2O_2. The desired gluconic acid is produced by spontaneous hydrolysis of β-D-glucono-δ-lactone (Scheme 6) [62].



Scheme 6. Oxidation of β-D-glucose to gluconic acid through in situ hydrolysis of β-D-glucono-δ-lactone.

Hydrogen peroxide is known to deactivate GOD and therefore has been removed from the reaction medium by CAT promoted decomposition.

The bubble column bioreactor has been chosen to ensure the efficient supplement and mass transfer of oxygen for the saturation of GOD.

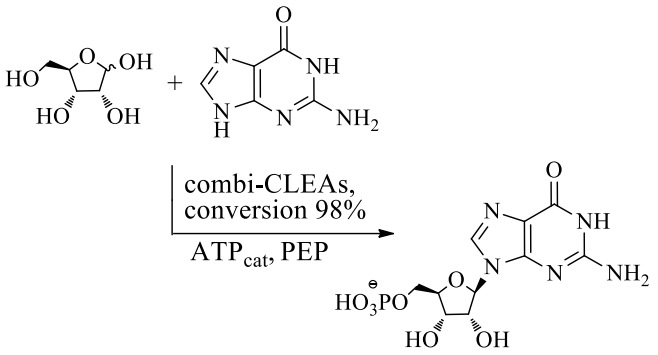
Both enzymes are multimeric (GOD is a dimeric protein and CAT is a tetramer) and the immobilization as a combi-CLEAs was expected to prevent subunit dissociation. The optimized conditions for the preparation of combi-CLEAs envisage the use of dimethoxyethane as precipitant and glutaraldehyde as cross-linker. BSA was also added as a feeder agent. The conversion of glucose to gluconic acid obtained in the first batch was around 96%. Combi-CLEAs of glucose oxidase and catalase were shown to be a robust catalyst which maintains its efficiency over 5 cycles.

3.6 Synthesis of nucleotides analogues

Nucleoside and nucleotide analogues are promising small molecule chemotherapeutic agents against various infectious DNA and RNA viruses. Nucleoside preparation is not devoid of synthetic challenges to control the stereochemistry during introduction of nucleobases. The purification of these highly polar, water soluble compounds is not straightforward. Nucleosides are then converted to nucleotides by selective phosphorylation of 5'-hydroxy group, often taking advantage of protective group chemistry.

An enzymatic alternative to the chemical synthesis of nucleotide analogues has recently been developed by using an engineered hypoxanthine phosphoryl transferase (8B3PRT) enabling to generate a variety of nucleotides through purine bases addition to phosphoribosyl pyrophosphate (PRPP) [63]. The low stability of the enzyme, which was not isolated, and PRPP were significant limitations of this approach.

To overcome these problems, an immobilized multienzyme aggregate in the form of combi-CLEAs which contains the enzymes needed for the synthesis of the purine nucleotide analogue and to recycle the auxiliary adenosine triphosphate (ATP) has been designed [64]. A total of 5 enzymes have been immobilized in the same aggregate (Scheme 7).

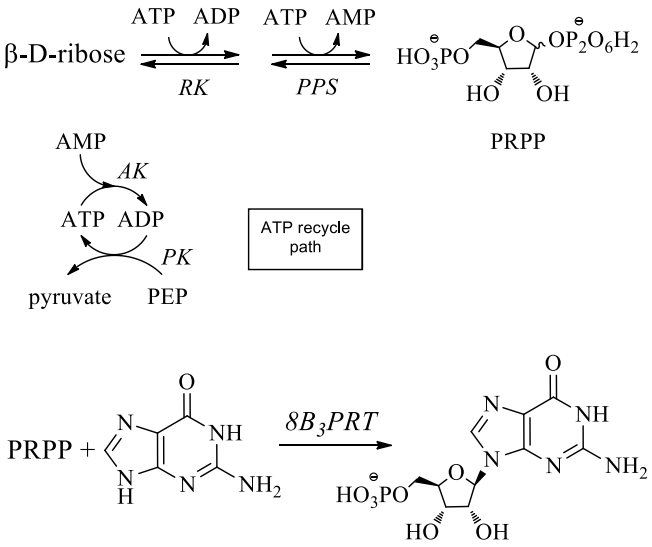


Scheme 7. Nucleotide synthesis through 5-enzymes combi-CLEAs.

The combi-CLEAs has been prepared by ammonium sulfate precipitation of a mixture of five enzyme preparations and BSA. The aggregated enzyme suspension thus obtained was later subjected to crosslinking with glutaraldehyde.

The combi-CLEAs cascade process proceeded through regioselective D-ribose 5'-phosphorylation by ribokinase (RK), followed by pyrophosphorylation on the anomeric carbon by phosphoribosyl pyrophosphate synthetase (PPS). The phosphoribosyl pyrophosphate (PRPP) thus obtained could provide the desired nucleotide analogues upon purine base addition by 8B3PRT transferase.

Two ATP equivalents, converted to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in the phosphorylation steps, could be recycled to ATP by adenylate cyclase (AK) and pyruvate kinase (PK), using phosphoenol pyruvate (PEP) as a source of activated phosphate which have also been co-immobilized in the combi-CLEAs (Scheme 8).



Scheme 8. Regeneration pathway for ATP in the nucleotide synthesis.

The combi-CLEAs was found to be 30% less active with respect to the reaction using the same amount of the free enzymes. However, the combi-CLEAs retained 50% activity after incubation at 37 °C for 7 days whereas the soluble enzyme mixture showed no residual activity after 1 day. The biocatalyst was recovered by centrifugation, followed by washing, and was shown to be reusable with a limited reduction of activity. When particles were generated in the presence of BSA, the biocatalyst retained up to 60% activity after 7 cycles. BSA has been proposed to favor the formation of reticulate preventing excessive crosslinking. The authors ascribed the loss of activity mainly to the pellets disintegration caused by repeated handling throughout cycles.

3.7 New hybrid catalyst

A chemo-enzymatic heterogeneous catalyst has been prepared through the loading of enzymes into an on-purpose designed titanium silicalite TS-1 [65] catalyst modified in order to increase the pore dimension needed to accommodate enzymes [66]. When glucose oxidase (GOD) has been loaded on modified TS-1 through an impregnation-precipitation-cross-linking method using ethyl lactate as precipitating agent and glutaraldehyde as cross-linker, the resulting CLEAs showed a 50% activity with respect to the free enzyme. However, the hybrid catalyst maintained a similar activity after incubation at 45 °C, whereas the free GOD activity was found to decrease with an estimated half-time of around 8 h. In addition, more than 99% of the enzymatic activity could be ascribed to the solid, whereas no leaching was detected even after prolonged storage.

The new catalyst has been tested in the allylic alcohol to glycidol epoxidation by hydrogen peroxide, produced in situ by glucose oxidase using molecular oxygen and β-D-glucose as the substrates.

Moreover, horseradish peroxidase (HRP) has also been entrapped in TS-1 maintaining its activity as proved by the colorimetric assay with 2,2'-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) and H_2O_2 as substrates. It is also worth noting that a combi-CLEAs made of both GOD and HRP could be successfully assembled inside the microspheres producing the same ABTS oxidation without addition of H_2O_2. Indeed, HRP uses H_2O_2 generated in situ by GOD to oxidize ABTS.

Thus, these multifunctionalized hybrid solids proved to be ideally suited to promote cascade reactions.

4. CONCLUSION

CLEAs are useful industrial biocatalysts with numerous advantages such as their easy preparation from raw enzyme extracts, and the possibility to save costs deriving from carriers used in enzyme immobilization. In addition, they are robust biocatalysts with improved storage stability and resistant to autolysis, heat and organic solvents. They are characterized by a high productivity and can be recovered and reused.

The possibility to co-immobilize two or more enzymes in the form of combi-CLEAs capable to catalyze cascade processes enlarge their field of application for synthetic purposes.

In the last twenty years, protein engineering and directed evolution techniques have deeply improved biocatalysis through enzyme performance optimization, providing new pathways for biocatalytic applications on scale.

Recently, biocatalysis is greatly expanding as a useful tool to develop more sustainable protocols. The combi-CLEAs cascades open the way to the possibility to carry out complex chemical processes under mild conditions with negligible waste generation at competitive prices.

The advances of research in this area strengthen the primary role of biocatalysis in the growth and success of green chemistry. The examples illustrated in this review prove this trend and we may imagine that in the next future artificial cascades could imitate more and more the complex metabolic network of chemically processes that takes place in nature.

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