CLEAs, Combi-CLEAs and 'Smart' Magnetic CLEAs: Biocatalysis in a Bio-Based Economy

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Abstract: Biocatalysis has emerged in the last decade as a pre-eminent technology for enabling the envisaged transition to a more sustainable bio-based economy. For industrial viability it is essential that enzymes can be readily recovered and recycled by immobilization as solid, recyclable catalysts. One method to achieve this is via carrier-free immobilization as cross-linked enzyme aggregates (CLEAs). This methodology proved to be very effective with a broad selection of enzymes, in particular carbohydrate-converting enzymes. Methods for optimizing CLEA preparations by, for example, adding proteic feeders to promote cross-linking, and strategies for making the pores accessible for macromolecular substrates are critically reviewed and compared. Co-immobilization of two or more enzymes in combi-CLEAs enables the cost-effective use of multiple enzymes in biocatalytic cascade processes and the use of "smart" magnetic CLEAs to separate the immobilized enzyme from other solids has raised the CLEA technology to a new level of industrial and environmental relevance. Magnetic-CLEAs of polysaccharide-converting enzymes, for example, are eminently suitable for use in the conversion of first and second generation biomass.

Keywords: enzymes; immobilization; biocatalysis; enzymatic cascades; cross-linking; CLEAs; combi-CLEAs; magnetic CLEAs; biomass conversion; polysaccharides

1. Introduction

One of the great technological challenges of the 21st century is to implement the transition from an unsustainable fossil resources-based economy to a greener and more sustainable one based on renewable biomass and utilizing manufacturing processes that minimize, or preferably avoid, the generation of waste and the use of toxic and/or hazardous materials. Biocatalysis has the right credentials to achieve this [1].

- Enzymes are produced from raw materials that are inexpensive, readily available and renewable, which means that prices are not subject to large fluctuations.
- Enzymes are essentially non-hazardous, non-toxic, biocompatible and biodegradable. In contrast, serious environmental costs are associated with mining precious metal catalysts and large price fluctuations are the norm. Moreover, significant costs are associated with the removal of traces of noble metals from end-products as is required by regulatory agencies in, for example, the pharmaceutical industry.
- Enzymatic reactions can be performed in conventional reactors, without any need for specialized equipment, under mild conditions (ambient temperature and pressure, physiological pH) in an environmentally acceptable solvent (water).
- Enzymatic reactions involving multifunctional molecules can proceed with high regio- and stereoselectivity and generally without the need for functional group activation and protection

and deprotection steps. This affords synthetic routes that, compared with conventional organic syntheses, are more step economic, more energy efficient, generate less waste and provide products in exquisite stereochemical purities that are difficult to compete with.

Consequently, in the last two decades biocatalysis has emerged as an important technology for meeting the growing demand for green and sustainable processing [2–4]. This embraces the whole gamut of chemicals manufacture, from the enantioselective synthesis of chiral drugs [5–12] to the conversion of renewable biomass to liquid fuels and commodity chemicals [13–15]. This raises the perennial question: if biocatalysis is so superior why has it not been extensively used in the past? The answer is simple: thanks to advances in molecular biology and biotechnology, biocatalysis has undergone a spectacular leap forward in the last two decades:

- Many more enzymes have been identified through (meta)genome mining, that is the in silico analysis of publicly accessible genome sequence data bases that have been generated as a result of next generation genome sequencing [16].
- Advances in gene synthesis have enabled the synthesis of identified genes, ready for cloning into a host production organism, in a few weeks at relatively low cost. This has significantly reduced the cost of development and subsequent production of enzymes at industrial scale.
- Advances in protein engineering, using directed evolution techniques [17,18], have enabled optimization of enzyme performance under the challenging conditions encountered in industrial-scale processes, namely, high (stereo)selectivities, activities and space-time yields with non-natural substrates at high substrate concentrations, in the presence of organic solvents.

In short, more enzymes are available, the enzymes are better, and, thanks to recombinant DNA technology, they can be economically produced on an industrial scale, thus enabling the design of improved industrial biocatalysts [19]. Notwithstanding these numerous benefits of using enzymes, their industrial application is often hampered by a lack of long-term stability and difficult recovery and re-use. However, for both economic and environmental viability, these valuable catalysts need to be efficiently recovered and recycled and this is accomplished using immobilization technologies [20,21].

2. Enzyme Immobilization

The fact that enzymes are soluble in water means that recovery from aqueous waste streams is challenging. This hurdle can be overcome by immobilizing the enzyme as a solid, heterogeneous catalyst that can be separated by filtration or centrifugation. In addition to facilitating the recovery and re-use of enzymes, immobilization generally results in an increase in their storage and operational stability since it decreases the flexibility of the enzyme, reducing its propensity to unfold and denature under the influence of heat, organic solvents or by autolysis. Improved performance is reflected in a higher catalyst productivity (kg product/kg enzyme) and a lower enzyme cost contribution. Furthermore, immobilization can facilitate their use in continuous flow processing [22,23]. In short, immobilization can enable applications that would not be economically viable with the free enzyme [24–31]. Nonetheless, the majority of biocatalyst applications still involve a single use of water-soluble enzymes, i.e., on a throw away basis, which is not cost effective and not in line with the concept of a circular economy.

Since most biocatalytic processes are performed under roughly the same conditions of (ambient) temperature and pressure, multiple biocatalytic steps can be integrated into enzymatic cascade processes [32] and co-immobilization of two or more enzymes affords multifunctional solid catalysts capable of catalyzing such cascade processes [33].

Methods for immobilization can be conveniently divided into three types: (i) immobilization on a prefabricated support (carrier) typically an organic resin or silica, (ii) entrapment in a polymeric matrix that is generated in the presence of the enzyme and (iii) cross-linking of enzyme molecules. Binding to a carrier can involve simple adsorption, e.g., via hydrophobic or ionic interactions or actual

covalent bonding [34]. The simplest method is to physically adsorb the enzyme on a carrier and can be successful in organic media [35]. However, the enzyme will readily be leached from the surface in the presence of water [35]. A serious drawback of carrier-bound enzymes [36] is their low productivities (kgs product per kg enzyme) owing to the large amount of non-catalytic ballast (generally >90 to >99% of the total mass). Moreover, the cost contribution of the carrier is often substantially higher than that of the enzyme. In contrast, immobilization by cross linking of enzyme molecules affords carrier-free immobilized enzymes with high productivities and avoids the costs of a carrier.

Usually some loss of activity is observed on immobilization but the increase in stability coupled with reusability, affording dramatic cost reductions compared with the soluble enzyme, more than compensates for this. Two of the most important industrial enzymes in use today are glucose isomerase and penicillin G amidase (PGA) [27]. Roughly 500 tons of immobilized glucose isomerase (EC 5.3.1.18) are used annually to produce ca. 10^6 tons of high fructose corn syrup (HFCS), corresponding to catalyst productivities of 10,000 kgs per kg. Similarly, PGA is used in the production of more than 20,000 tons per annum of 6-amino penicillanic acid (6-APA), by enzymatic hydrolysis of penicillin G, and the manufacture of more than 10,000 tons of semi-synthetic penicillin and cephalosporin antibiotics annually [37]. Its use in an immobilized form is an indispensable condition for commercial viability and productivities of 600 kg per kg or more are the norm [38]. Interestingly, the production of both immobilized enzymes involves a cross-linking step with glutaraldehyde. The latter is generally the cross-linker of choice as it is inexpensive, readily available and its use in cross-linking of proteins has GRAS (generally regarded as safe) status for use in, among other things, food and beverages processing [39].

Turner and coworkers [40] recently outlined the requirements that an immobilized enzyme should meet for commercial viability of a process in the pharmaceutical industry: (i) high enzyme loading (>10 wt%), (ii) high activity recovery (>50%), (iii) no leaching under reaction conditions, (iv) tolerance to organic solvents, (v) recyclable (>20 cycles), (vi) good substrate mass transport and (vii) mechanically stable in batch and flow reactors and (viii) last but not least, it must result in a lower cost of goods compared with alternatives. The authors also pointed out that a method should have broad applicability over numerous enzyme classes and, as an absolute minimum, generality within the same enzyme class. However, we note that while generality may be a positive attribute it has no influence on the cost-effectiveness of a method.

3. Cross-Linked Enzyme Aggregates (CLEAs)

Cross-linked enzymes (CLEs) were already known in the 1960s. They were formed by mixing a solution of an enzyme in aqueous buffer with an aqueous solution of glutaraldehyde. They were difficult to handle, gelatinous materials exhibiting low activity, poor reproducibility and low stability and shelf life. Consequently, carrier-bound enzymes became the norm for the next three decades. In the early 1990s Altus Biologics introduced the use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts [41]. The methodology was broadly applicable and CLECs exhibited excellent operational stability and controllable particle size coupled with high productivity and facile recovery and re-use, making them ideally suited for applications as industrial biocatalysts. However, they had one inherent limitation: the need to crystallize the enzyme, a laborious and costly procedure requiring enzyme of high purity.

We surmised that crystallization could be replaced by precipitation of the enzyme from aqueous buffer, a simpler and less expensive method not requiring high purity enzymes. It was well-known that the addition of salts, or water miscible organic solvents or non-ionic polymers, to aqueous solutions of proteins leads to their precipitation as physical aggregates that are held together by non-covalent bonding, without perturbation of their tertiary structure, and redissolve on the addition of water. We showed that addition of a bifunctional cross-linker rendered them permanently insoluble, as what is essentially a cross-linked polymer, while maintaining their pre-organized superstructure, and, hence their catalytic activity. The result was a new class of immobilized enzymes for which we

coined the term cross-linked enzyme aggregates (CLEAs). The first publication [42] appeared in 2000 and the technology was commercialized by the industrial biotech company, CLEA Technologies B.V., founded in 2002 as a spin-out of the Biocatalysis and Organic Chemistry Group at Delft University of Technology.

Since selective precipitation with ammonium sulfate is commonly used to purify enzymes, the CLEA methodology essentially combines enzyme purification and immobilization into a single unit operation. This enables cost effective production of the CLEA from crude cell lysate obtained from fermentation broth, without the need for intermediate work-up or purification. The CLEA technology has very broad scope. It has been successfully applied to the immobilization of countless enzymes from the five enzyme classes—hydrolases, oxidoreductases, lyases, transferases and isomerases—used in chemicals manufacture and forms the subject of an increasing number of reviews [43–46].

3.1. Cross-Linked Enzyme Aggregate (CLEA) Preparation

The preparation of CLEAs involves (a) precipitation as physical aggregates from a solution in aqueous buffer and (b) cross-linking of the physical aggregates with a bifunctional cross-linking agent (Figure 1). The precipitation step is instantaneous and much faster than the cross-linking step. Therefore, the precipitant (aggregant) and the cross-linker can be added simultaneously, combining the two steps into one operation.
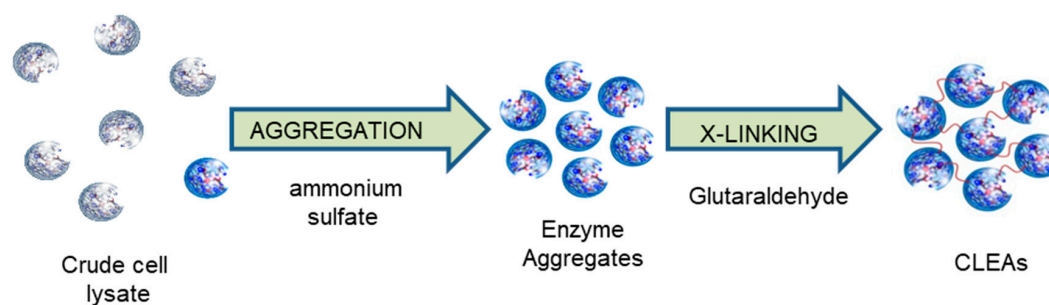
Figure 1. Cross-linked enzyme aggregate (CLEA) preparation.

The precipitant can be a salt, such as ammonium sulfate or sodium sulfate, a water-miscible organic solvent or a non-ionic polymer, such as polyethylene glycol (PEG). In practice, ammonium sulfate works well with almost all enzymes and is generally the precipitant of choice. Water miscible organic solvents, on the other hand, are generally less environmentally attractive.

As with cross-linking of proteins in general, glutaraldehyde is generally the cross-linker of choice. Although the chemistry is complex and not fully understood, cross-linking involves reaction of free amino groups on the surface of neighbouring enzyme molecules, with oligomers or polymers of glutaraldehyde, and is usually performed at pH 7.0 to 9.0. With some enzymes, e.g., nitrilases, low or no activity recovery was observed with glutaraldehyde cross-linking. We reasoned that it could be caused by glutaraldehyde penetrating the interior of the protein and reacting with amino groups crucial for the activity of the enzyme. We found that cross-linking with dextran polyaldehyde, a bulky polyaldehyde that is not able to penetrate the interior of the enzyme, followed by reduction of the Schiff's base moieties to form irreversible amine linkages, could be used in such cases [47]. Other cross-linkers, derived from readily available polysaccharides, have also been used, e.g., pectin dialdehyde [48]. More recently, the use of a readily available bis-epoxide, glycerol diglycidyl ether as a cross-linker was described [49].

The molar ratio of cross-linker to enzyme influences the activity, stability and particle size of the resulting CLEA. If the ratio is too low sufficient cross linking doesn't occur, affording a CLEA that is too flexible and unstable towards leaching in water. If it is too high, excessive cross linking can cause a complete loss of the enzyme's flexibility resulting in a low activity recovery. The rate of addition of the cross-linker and the cross-linking time also influence the activity recovery and particle

size and morphology of CLEAs. The particle size of a CLEA can also increase on ageing [50] strongly suggesting that the cross-linking process was not complete.

3.2. Effect of Additives

As would be expected, the efficacy of cross-linking is influenced by the availability of free amino groups, usually of Lys residues, on the surface of the enzyme and is problematical with electronegative enzymes containing a paucity of free amino groups. CLEAs derived from such enzymes tend to be mechanically fragile and suffer from facile enzyme leaching [51]. The problem can generally be overcome by co-aggregation with polymers containing numerous amino groups. For example, co-aggregation with polyethyleneimine (PEI) affords CLEAs with high activity recoveries and excellent operational stabilities [52–54]. Similarly, pentaethylene-hexamine [55], and polylysine [56] have been used to good effect. Alternatively, co-aggregation with so-called proteic feeders rich in Lys residues, such as bovine serum albumin (BSA), led to remakable improvements in activity recovery and operational stabilities of a lipase and penicillin amidase [57,58]. Hen egg white was also an effective proteic feeder [59] and, more recently, feather meal, an inexpensive poultry byproduct rich in protein, was used as a novel proteic feeder in the preparation of combi-CLEAs of a mixture of pectinase, pectin lyase, pectine methyl esterase and polygalacturonase, for use in grape juice clarification [60].

4. Scope of CLEA Technology

CLEA technology has broad scope and, over the last two decades, an increasingly wide selection of hydrolases, oxidoreductases, lyases, transferases and isomerases has been successfully used in organic synthesis in general [61], and asymmetric synthesis in particular [62].

4.1. Hydrolase CLEAs

The majority of CLEAs described in the literature involve hydrolases, probably because they are the enzymes that have the most industrial applications and are the simplest enzymes to work with. These include CLEAs from lipases [63], proteases [64], esterases [65,66], amidases [67], nitrilases [68], epoxide hydrolases [69], and glycosidases.

CLEAs have been prepared from numerous lipases, including *Candida Antarctica* lipase B (CaLB) [70,71], *Thermomyces lanuginose* [72], *Penicillium expansum* [73], *Penicillium notatum* [74] and *Candida rugosa* [75], to name but a few. A CLEA was also produced from a perhydrolase [76] and used to catalyze the in situ generation of a peracid in a Baeyer–Villiger oxidation.

CLEAs have been prepared from a variety of proteases. In particular, the CLEA of alcalase (E.C. 3.4.21.62), the inexpensive alkaline protease from *Bacillus licheniformis* (also known as subtilisin Carlsberg) used in laundry detergents, has been widely used in resolutions of amino acid esters and amines and peptide synthesis [77]. Penicillin G amidase (PGA) CLEAs were shown to be extremely effective catalysts, with high volumetric productivities, for the hydrolysis of penicillin G and the synthesis of semi-synthetic penicillins [78] and cephalosporins [79] such as ampicillin and cephalexin, respectively.

In our experience, over almost two decades CLEAs have been exceptionally suited to carbohydrate conversion processes. The number of enzymes acting on carbohydrates is vast with numerous commercially important applications, not only in food and beverages processing [80] but also in pharmaceuticals, nutraceuticals, cosmetic ingredients, and biomass conversion. The latter is of increasing importance in the context of the bio-based economy (see later).

CLEAs have been successfully prepared from a variety of glycosidases (E.C.3.2.1.) which catalyze the formation of ether bonds between two carbohydrate molecules or a carbohydrate and an aglycon. For example, an *Aspergillus oryzae* β-galactosidase CLEA was applied successfully to the synthesis of galacto-oligosaccharides (GOS) [81], commercially important prebiotic food additives. More recently, a CLEA of a novel β-galactosidase from a marine metagenomic library was described [82]. In comparison with the free enzyme, the CLEA displayed a slightly higher GOS yield, a much higher

thermostability, good storage stability, better galactose tolerance and 82% activity retention after 10 cycles.

Glycoside CLEAs have also been used in the hydrolysis and synthesis of glycosides with aglycons. Thus, a CLEA of *Penicillium decumbens* naringinase (E.C. 3.2.1.40) catalyzed the hydrolysis of the glycoside naringin into naringenin (Figure 2 [83]). A CLEA of the β-glucosidase (E.C. 3.2.1.20) from *Prunus domestica* seeds catalyzed the synthesis of *p*-hydroxyphenethyl α-D-glucopyranoside, salidroside that is used in Chinese traditional medicine. The CLEA had a much higher productivity and improved thermal stability compared to the free enzyme (Figure 2) [84].
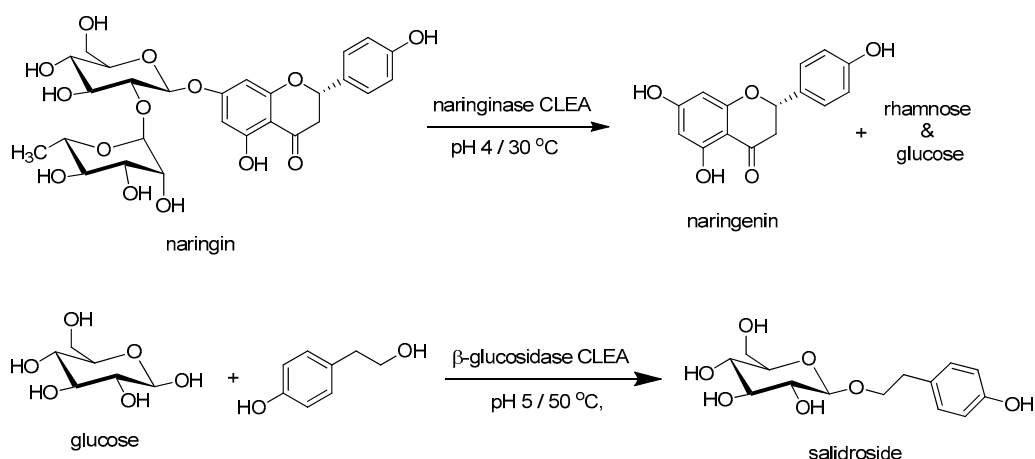
Figure 2. Hydrolysis of naringin and synthesis of salidroside with glycoside CLEAs.

Levansucrase (E.C. 2.4.1.10) catalyzes the synthesis of levan from sucrose and the transfer of fructosyl moieties from sucrose to other acceptor molecules such as xylose (Figure 3a). A levansucrase-CLEA catalyzed the synthesis of the oligofructosides and transfructosylations as efficiently as the free enzyme and showed the highest reported specific activity for fructosyl- and glucosyl-transferases [85].
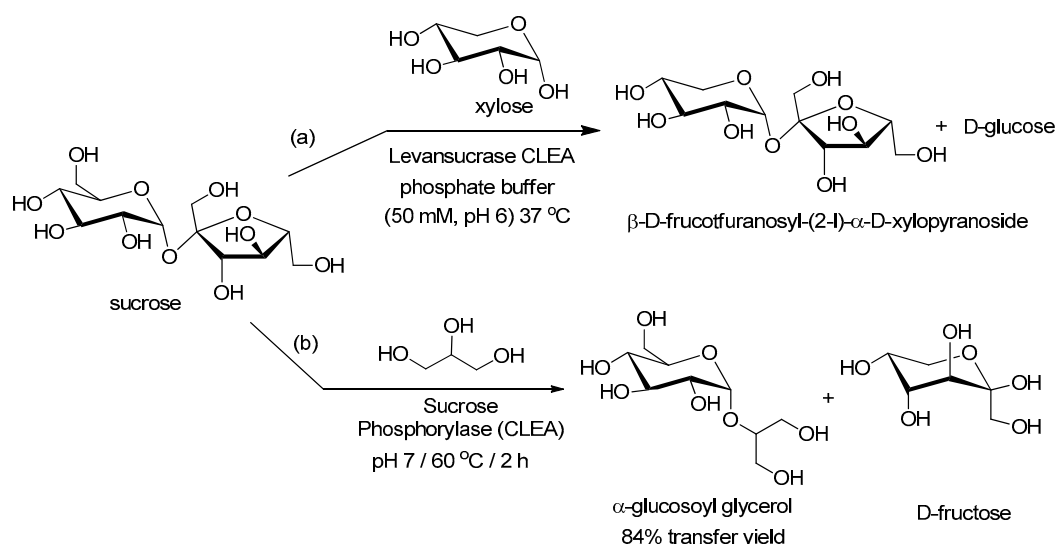
Figure 3. CLEA-catalyzed transglycosidations.

Sucrose phosphorylase (E.C. 2.4.1.7) catalyzes the reversible phosphorylation of sucrose into a mixture of fructose and D-glucose-1-phosphate (G1P). Soetaert and coworkers [86] showed that a CLEA of sucrose phosphorylase from *Bifidobacterium adolescentis* had a temperature optimum of 75 °C compared with 58 °C for the free enzyme. This was accompanied by an increase in the operational

stability of the CLEA which retained full activity after 1 week at 60 °C compared with 20% loss of activity in 16 h with the free enzyme. Furthermore, the CLEA was recycled at least 5–10 times without loss of activity. Sucrose phosphorylase also catalyzes transglucosylations of carbohydrates and non-carbohydrate molecules such as glycerol (Figure 3b) [87]. The CLEA was extremely stable and eminently suitable for operation at 60 °C.

Similarly, a *Saccharomyces cerevisiae* invertase-CLEA exhibited an optimal operational temperature of 70 °C, compared with 40 °C for the free enzyme, in the hydrolysis of sucrose to a mixture of fructose and glucose (inverted sugar syrup) [88].

4.2. Oxidoreductase and Lyase CLEAs

CLEAs have been prepared from a variety of oxidoreductases. These include oxidases such as glucose oxidase (GOX), galactose oxidase (GOase), laccase [89,90], monoamine oxidase (MAO) [91] peroxidases [55,92–94] and dehydrogenases such as alcohol dehydrogenases/ketoreductases (KREDs), and formate dehydrogenase [95]. Laccase-CLEAs have been extensively studied because of the interest in using them for the removal of dyes, pharmaceutical residues and endocrine disruptors such as bisphenol A, from waste water [96–98].

Hydroxynitrile lyases (HNLs; EC 4.1.2.10) [99] are good examples of lyases that have been successfully 'cleated'. HNL-CLEAs are robust, highly effective catalysts for enantioselective hydrocyanation of aldehydes under microaqueous conditions and can be recycled several times without loss of activity. They have been prepared from inter alia, *Prunus amygdalis* [47], *Prunus dulcis* [100,101], *Hevea brasiliensis* [102], *Manihot esculenta* [103] and *Linus usitatissimum* (LuHNL, E.C. 4.1.2.46) [104]. HNL-CLEAs perform exceptionally well in organic solvents, essentially completely suppressing competing non-enzymatic hydrocyanation and can afford enantioselectivities that are not possible with the free enzyme (Figure 4) [105].
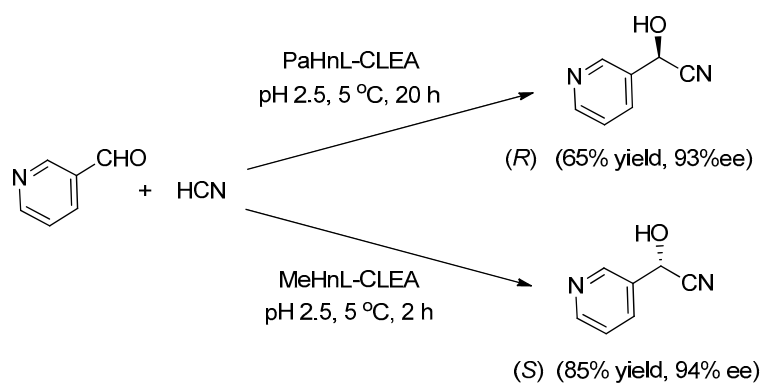
Figure 4. Asymmetric synthesis of cyanohydrins with hydroxynitrile lyase (HNL)-CLEAs.

Because of the industrial importance of HNLs, they are in constant demand and when new HNLs are discovered they are usually 'cleated' to render them suitable for operation in organic solvents. For example, new HNLs from *Davallia tyermannii* [106] and *Baliospernum montanum* [107] were converted to CLEAs to afford robust, recyclable catalysts for the synthesis of chiral cyanohydrins.

N-acetyl-D-neuraminic acid aldolase (NAL, E.C. 4.1.3.3) is used industrially at basic pH for the condensation of pyruvate and *N*-acetyl-D-mannosamine (ManNAc) into *N*-acetyl-D-neuraminic acid (Neu5Ac), an advanced intermediate for GSK's antiviral drug Relenza® (zanamivir). Garcia-Garcia et al. [108,109] prepared a robust CLEA from the *N*-acetyl-D-neuraminic acid aldolase from *Lactobacillus plantarum* (LPNAL, E.C. 4.1.3.3) by ammonium sulfate co-precipitation with BSA and cross-linking with glutaraldehyde and applied it in the chemo-enzymatic synthesis of Neu5Ac (Figure 5), which could ultimately be made from the inexpensive *N*-acetyl-D-glucosamine. The CLEA exhibited good activity and operational stability at alkaline pH and at least 10 cycles were possible without any significant loss of activity.
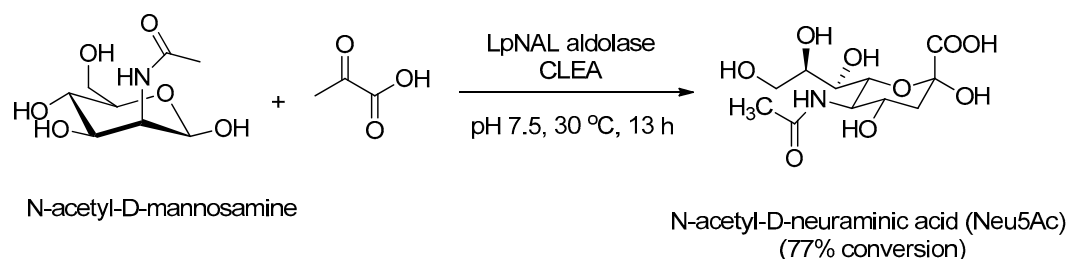
Figure 5. *N*-acetyl-D-neuraminic acid synthesis using a *L. planatarum N*-acetyl-D-neuraminic acid aldolase (NAL)-CLEA.

Cui and coworkers [110] prepared CLEAs of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) from *R. glutinis* which catalyzes the industrially important formation of L-phenylalanine by asymmetric addition of ammonia to cinnamic acid (Figure 6). Compared to the free enzyme the PAL-CLEA had increased storage and operational stability and was recycled 12 times without appreciable loss of activity. More recently, the same group [111] prepared PAL-CLEA by coaggregation with bovine serum albumin and obtained CLEAs that were even more stable towards heat and organic solvents.
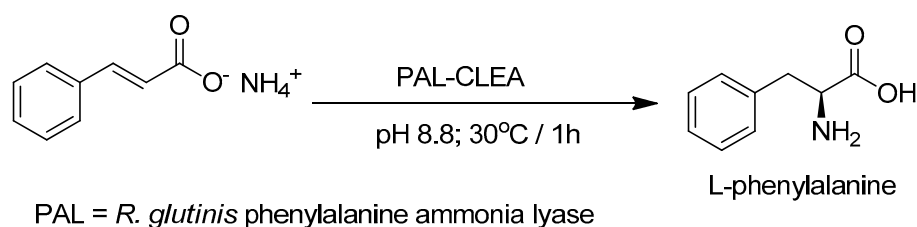
Figure 6. Phenylalanine ammonia lyase (PAL)-CLEA catalyzed synthesis of L-phenylalanine.

Nitrile hydratases (NHases) are used in various industrially important processes [112] but they are used as whole cell biocatalysts because of very limited stability of the free enzyme. In contrast, NHase CLEAs exhibited excellent storage and operational stability (see next section) [113].

5. Advantages, Limitations and Optimization of CLEAs

An important advantage of immobilized enzymes in general, certainly from a cost-effectiveness viewpoint, is their ease of recovery and re-use, as heterogeneous catalysts, by filtration, centrifugation or, alternatively, in a fixed bed reactor. CLEAs are eminently recyclable. For example, the (*R*)-hydroxynitrile lyase (HNL)-CLEA from *Prunus amygdalis* which catalyzed the enantioselective hydrocyanation of o-chlorobenzaldehyde was recycled 10 times with neglible loss in activity [114].

In addition to facilitating its recovery and reuse, another important reason for immobilizing an enzyme is the significant enhancement of operational stability towards heat and organic solvents that is generally observed. Indeed, immobilization of enzymes as cross-linked enzyme aggregates can lead to dramatic increases in storage and operational stability. For example, the operational stability of a papain-CLEA, was dramatically enhanced compared to the limited thermal stability of free papain [45].

Many enzymes consist of multimers that are characterized by limited thermal stability outside the cell owing to their facile dissociation with concomitant loss of activity. CLEA formation can stabilize multimers by suppressing this dissociation through cross-linking. For example, CLEA preparation was used successfully to stabilize two tetrameric catalases [115]. The industrially important nitrile hydratases (NHases) are also multimers and notoriously unstable outside the cell. Consequently, NHase mediated industrial biotransformations, such as acrylamide and nicotinamide manufacture from the corresponding nitriles, are conducted as whole cell processes. In contrast, immobilization of a nitrile hydratase from an alkaliphilic bacterium, *Nitriliruptor akaliphilus*, as a CLEA led to a dramatic increase in storage stability compared to the free enzyme [113]. In the hydration of n-hexyl cyanide, the NHase-CLEA was recycled 36 times with a minor loss in activity that was largely attributed

to mechanical losses that inevitably occurred on pipetting the supernatant from the solid CLEA. The industrially important enzyme, halohydrin dehalogenase (HHDH) is a homotetramer and is active only as the tetramer. It was recently shown that immobilization of HHDH as a CLEA occurred with 90% activity recovery and the resulting HHDH-CLEA exhibited a strong tolerance to organic solvents and retained 70% activity after 10 recycles [116].

Since the enzyme molecules in CLEAs are bound by covalent bonds, there is no leaching of enzyme observed in aqueous media, even under drastic conditions such as in the presence of surfactants. A further advantage, of immobilized enzymes in general, and CLEAs in particular, is that they exhibit no or low allergenicity since they cannot easily penetrate the skin.

Another important cost benefit is the fact that CLEAs can be prepared from enzyme samples of low purity such as crude cell lysate, obtained from fermentation broth. Furthermore, because they consist mainly of active enzyme, CLEAs exhibit high catalyst productivities (kg product per kg of enzyme) compared to carrier-bound enzymes and the costs of the carrier ballast are avoided.

The CLEA technology is broadly applicable but, since every enzyme is a different molecule, the protocol has to be optimized for every enzyme. This can be seen as a limitation but, in practice, optimizing the aggregation plus cross-linking protocol is not a lengthy procedure and can be readily automated [117]. The response surface methodology (RSM) has been successfully used, for example, in the optimization of the preparation of a CaLB-CLEA 68, a lipase CLEA from cocoa pod husk [118] and a protease CLEA [64]. The optimization of CLEA preparation has been reviewed by several authors [119–121].

Particle size is an important property of any heterogeneous catalyst since it directly affects mass transfer and filterability under operational conditions. Obviously, the rate of diffusion is influenced by the particle size and this is determined by, inter alia, the cross linker/enzyme ratio. A typical particle size of CLEAs is 5–50 μm and their filterability is generally sufficient for batch operation. CLEAs are highly porous materials and diffusional limitations are generally not observed when using them in biocatalytic transformations commonly used in organic synthesis. Optimum rates are observed with smaller particles but practical considerations, e.g., ease of filtration, necessitate the use of larger particles and in practice a compromise between good activity and good filterability has to be accepted.

During CLEA formation the enzyme molecules are packed together in a small volume which results in a relatively small pore size that can cause diffusion limitations with macromolecular substrates, such as polysaccharides and proteins. Moreover, washing and centrifugation can lead to further compression of the particles. Consequently, so-called porous CLEAs (p-CLEAs), with increased porosity, were developed and shown to have better internal mass transfer [122–124]. It involved adding starch to the enzyme solution prior to precipitation and cross-linking. After glutaraldehyde cross-linking α-amylase is added to hydrolyze the starch. The resulting p-CLEAs exhibited improved mass transfer. In a variation on this theme, porous β-mannanase CLEAs were prepared by using linear dextran polyaldehyde as a macromolecular cross-linker [125]. An alternative approach for circumventing compression of CLEAs during centrifugation or filtration is to use 'smart' magnetic CLEAs and separate them with a permanent magnet (see later). Moreover, the use of magnetic CLEAs enables the use of small, highly active particles without compromising the ability to recover and reuse the CLEA (see later).

In a novel refinement on the cross-linking theme, Brady and coworkers [126,127] prepared Spherezymes by adding cross-linking agents to a water-in-oil emulsion of an aqueous solution of *P. fluorescens* lipase. The spherezyme particles exhibited good activity in organic solvents.

5.1. Bioimprinting

In the early days of the CLEA technology we showed [128] that the addition of crown ethers or surfactants to solutions of lipases, prior to aggregation and cross-linking, could result in the formation of CLEAs with a higher activity than the free enzyme. This was consistent with the idea that the surfactant or crown ether induced the lipase to adopt a different conformation that could be more

favourable. Subsequent cross-linking could then 'lock' the enzyme in this favourable conformation and the additive could simply be removed by washing. This additive effect was later extended to the idea of using molecular "bioimprinting" to influence the activity and/or substrate specificity of CLEAs. For example, addition of butanone to an HNL prior to aggregation and cross-linking led to the formation of an HNL-CLEA with improved synthetic activity towards butanone as substrate [104]. More recently, it was shown that the specificity of of sucrose phosphorylase towards α-glucosyl glycerol as substrate increased two-fold by prior imprinting with the substrate [87].

5.2. Reactor Configurations

The choice of reactor configuration is intimately connected with downstream processing and is largely influenced by the industry segment and characteristic product volumes. In the pharmaceutical and fine chemical industries product volumes are relatively small and processes are generally conducted in batch in multi-purpose stirred tank reactors (STRs), equipped with a propeller stirrer. The catalyst is recovered by filtration or centrifugation. A problem that is generally encountered in the use of STRs is mechanical attrition of the immobilized enzyme, resulting from shear forces caused by the propeller stirrer, leading to the formation of difficult to handle pulverized particles.

Several concepts have been employed to address the attrition problem. For example, in a membrane slurry reactor (MSR) the immobilized enzyme is retained inside the reactor because it is too large to pass through the pores of a membrane patch in the reactor wall. This enables the use of a broad range of catalyst particle sizes including the relatively small particles of CLEAs. The reaction and biocatalyst separation are combined into a single operation. High catalyst loadings, longer catalyst life-times owing to reduced mechanical stress, and higher volumetric and catalyst productivities are some of the many advantages of an MSR. Its practical utility was demonstrated in the industrially important PGA-CLEA catalyzed hydrolysis of penicillin G to 6-APA [129].

The perfusion basket reactor (BR) is a refinement of the 'tea bag' concept, whereby the immobilized biocatalyst is contained in a filtration membrane-like module suspended in the reactor. It was used successfully, for example, in the degradation of endocrine-disrupting chemicals in aqueous effluents catalyzed by laccase-CLEAs [130]. The rotating flow cell reactor is a novel variation on the basket reactor, developed by SpinChem (Umeå, Sweden). It involves a catalyst-containing compartment attached to a propeller stirrer. In addition to protecting the biocatalyst from shear forces caused by the stirrer, rates of mass transfer are significantly increased thus enabling process intensification. Yet another alternative is to use a bubble column reactor (BCR). This was used, for example, in the production of highly viscous fatty acid esters of polyols which was not feasible in conventional STRs or fixed bed reactors (FBRs) [131]. The water formed in the reaction was removed by entrainment with pressurized air and the latter also serves to mix the reactants without causing any significant attrition of the catalyst.

Continuous processing with immobilized enzymes in fixed bed reactors (FBRs) is the norm in the oils and fats processing industry. The relatively small particle size of CLEAs is an issue as it can lead to large pressure drops over the column. The problem can be alleviated by mixing the CLEA with an inert, less compressible solid, such as controlled microporous glass or perlite. Littlechild and coworkers [132], for example, prepared CLEAs from a thermophilic L-aminoacylase from *Thermococcus litorali* and subsequently mixed them with controlled pore glass before packing them in a capillary reactor fitted with a silica frit to contain them in the reactor. Much attention has been focused in recent years on process intensification through the use of microchannel reactors because of the many advantages compared with traditional batch processing: rapid mass and heat transfer and large surface area to volume ratios.

Yet another alternative is to use magnetic CLEAs that are contained in a column reactor with a magnetic field. This is discussed in Section 6.

5.3. Multi- and Combi-CLEAs

The union of the CLEA technology with multipurpose and cascade biocatalysis has proven to be particularly attractive, producing many interesting progeny [133]. Multipurpose CLEAs can be prepared from crude enzyme extracts containing multiple enzymes. For example, Gupta and coworkers [134] prepared a multi-CLEA exhibiting lipase, phospholipase and α-amylase activity from porcine pancreatic acetone powder. Similarly, the same group prepared a multi-CLEA from a commercial enzyme preparation, Pectinex™ Ultra SP-L, exhibiting pectinase, xylanase and cellulase activity [135]. More recently, Rodrigues and coworkers [136] prepared multi-CLEAs, with and without BSA as a feeder, from pectinases and cellulases in crude enzyme preparations and demonstrated their efficacy in grape juice clarification. The multi-CLEA and multi-CLEA-BSA were recycled 4 and 6 times, respectively, with 100% retention of activity.

A multi-oxidoreductase CLEA, containing laccase, versatile peroxidase and glucose oxidase was used for the elimination of pharmaceuticals from aqueous waste streams [137]. Multi-CLEAs can also be produced by combining different enzymes from the same (sub)class. For example, a multi-CLEA containing a fungal laccase having an acidic pH optimum and a bacterial laccase having an alkaline pH optimum afforded a robust biocatalyst, active at both acidic and alkaline pH, for removal of phenolic compounds contaminating wastewater [138]. This concept of creating multi CLEAs with broader activities can also be applied to other enzymes. For example, it has been successfully applied to commercial preparations containing mixtures of ferulic acid esterases (FAEs) to afford a multi-FAE-CLEA with activity and stability superior to the individual enzymes in the preparations [139]. Similarly, one can easily envisage application of this concept to lipases, where individual enzymes often have very different specificities.

Recently, a novel lipase/protease multi-CLEA was reported [140]. Conducting reactions with a mixture of a protease and a lipase is not feasible with the free enzymes as the protease would break down the lipase but it is possible when the two enzymes are co-immobilized in a multi-CLEA.

We generally use the term combi-CLEA for deliberate co-immobilization of two or more enzymes in a single CLEA for the sole purpose of performing two or more sequential biotransformations. Multi-enzyme cascade processes [141–145] that telescope the individual reactions into one-pot cascade processes have several economic and environmental benefits: fewer unit operations, less solvent, and reactor, shorter cycle times, higher volumetric and space time yields and less waste. Furthermore, coupling of reactions can be used to drive equilibria towards product and, since individual biocatalytic steps generally proceed in water at ambient temperature and pressure, they can be readily integrated into one-pot processes. However, different enzymes are not always compatible and nature solves this problem by compartmentalizing enzymes in different parts of the cell. Hence, compartmentalization via immobilization can be the solution in enzymatic cascades.

In addition, co-immobilization of enzymes in combi-CLEAs can significantly increase the rates of sequential biocatalytic cascades by simulating the close proximity extant in microbial cells, what has become known as sequential channeling of substrates [146]. In our initial studies of combi-CLEAs we prepared combi-CLEAs of glucose oxidase or galactose oxidase with catalase [117]. Oxidases catalyze the aerobic oxidations of various substrates, generally with concomitant production of an equivalent of hydrogen peroxide. The latter can cause oxidative degradation of the enzyme and, in vivo, oxidases generally occur together with catalase which catalyzes the spontaneous decomposition of hydrogen peroxide to oxygen and water. We found that combi-CLEAs, containing co-immobilized oxidase with catalase, could not only be recycled without significant loss of activity but also exhibited significantly better activities and stabilities compared to a mixture of the two separate CLEAs.

A combi-CLEA consisting of glucose oxidase and horse radish peroxidase (HRP) can be used for the in situ production of hydrogen peroxide by aerobic oxidation of glucose followed by HRP catalyzed oxidations with the hydrogen peroxide [147]. Similarly, a combi-CLEA consisting of glucose oxidase and versatile peroxide (VP), with added glucose, catalyzed the elimination of endocrine disruptors from industrial wastewater streams [148].

The combi-CLEA concept has also been used to advantage in oxidation reactions requiring cofactor regeneration. A pertinent example is the ketoreductase (KRED)/glucose dehydrogenase (GDH) combi-CLEA that was used in the enantioselective reduction of a prochiral ketone with cofactor regeneration with glucose/GDH [149,150]. The combi-CLEA exhibited increased thermal and pH stability and high substrate tolerance compared with the free enzymes, coupled with high volumetric productivity, good operational stability and reusability. The authors predicted wide application of this combi-CLEA strategy in the production of various chiral alcohols on the basis of its high efficiency coupled with low cost.

Another example involving GDH for cofactor recycle is the enoate reductase (ERED)/GDH combi-CLEA. The combi-CLEA exhibited improved thermal stability and 110% retention of the initial activity after 14 recycles (Figure 7) [151].
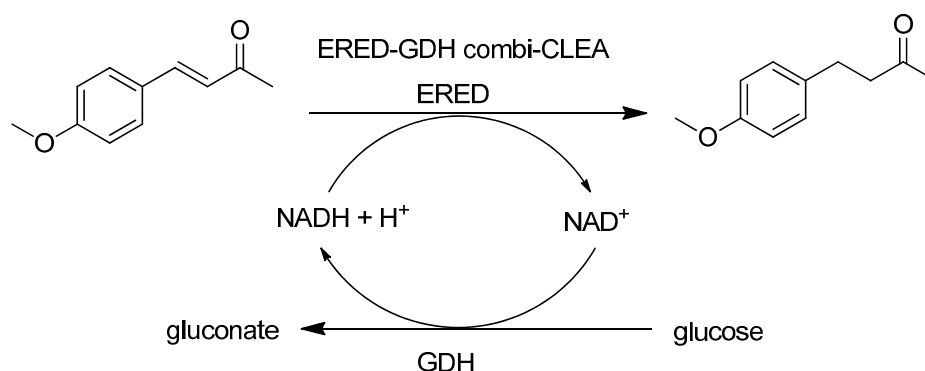

Figure 7. Enoate reductase/glucose dehydrogenase (ERED/GDH)-combi-CLEA.

A trienzymatic combi-CLEA, consisting of a (*S*)-hydroxynitrile lyase from *Manihot esculenta*, a non-selective nitrilase from *Pseudomonas fluotrescens* EBC 191 and an amidase from *Rhodococcus erythropolis*, catalyzed the enantioselective conversion of benzaldehyde to (*S*)-mandelic acid in 90% yield and >99% ee (Figure 8) [152,153]. It was originally developed [33] as a hydroxynitrile lyase/nitrilase bienzymatic combi-CLEA but large amounts of the corresponding amide were formed as a byproduct of the nitrilase-catalyzed hydrolysis and an amidase was needed to convert the amide side product to the acid. Rates were higher than with mixtures of the respective CLEAs. Similarly, a combi CLEA comprising the *manihot esculenta* HNL and the alkaliphilic NHase from *Nitriliruptor akaliphilus* (EC 4.2.1.84) catalyzed the one-pot conversion of aldehydes to (*S*)-α-hydroxycarboxylic acid amides (Figure 8) [154].

Proline-specific X-prolyl-dipeptidyl aminopeptidase (PepX) and aminopeptidase N (PepN) are used in cheese-making, baking and meat tenderization. Enzymatic hydrolysis of casein, for example, can cause an undesirable bitter taste but this can be significantly reduced using a combination of PepX and PepN. Fischer and coworkers [155] showed that by using a PepX/PepN combi-CLEA the degree of hydrolysis of casein was increased by 52%.

An area where combi-CLEAs have made significant inroads is in conversion of carbohydrates in general, and polysaccharides in particular. A pertinent example is the use of a trienzymatic combi-CLEA of amylosucrase (AS; EC 2.4.1.4), maltooligosyltrehalose synthase (MTS; EC 5.4.99.15) and maltooligosyltrehalose terhalohydrolase (MTH; EC 3.2.1.141) in a one-pot conversion of sucrose to trehalose (Figure 9) [156]. The CLEA was recycled five times.
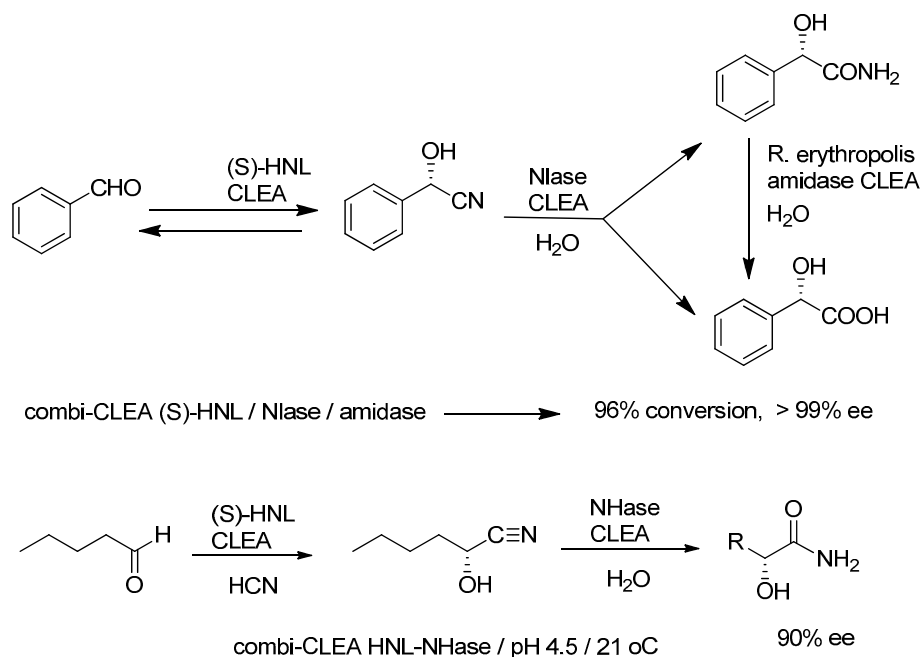
Figure 8. HNL-Nlase-Amidase combi-CLEAand HNL-NHase combi-CLEA.
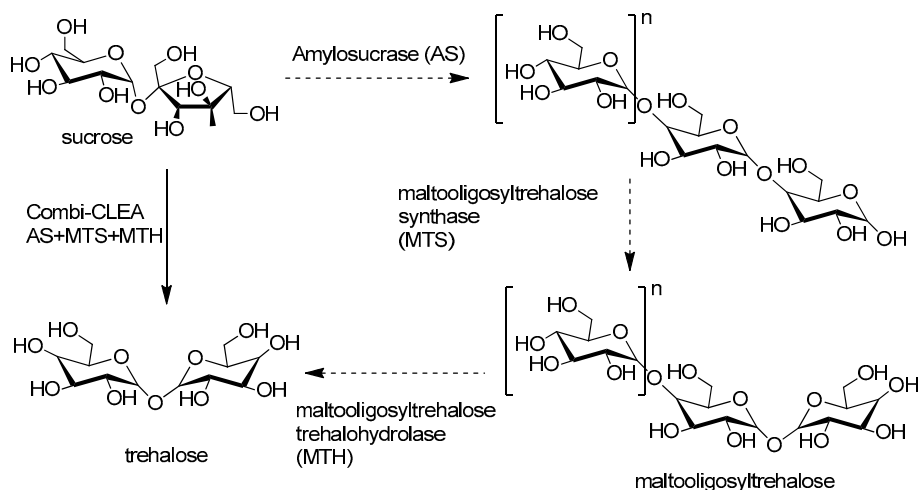
Figure 9. One-pot conversion of sucrose to trehalose with a combi-CLEA.

A combi-CLEA of α-L-arabinose (ARA) and β-glucosidase (βG) prepared from a commercial enzyme preparation, Rapidase AR 2000, was used for aroma enhancement in wine [157]. ARA catalyzes the formation of terpenyl-β-glucosides and βG catalyzes their subsequent hydrolysis to release terpenyl alcohols that enhance the wine aroma (Figure 10). All grape varieties comprise such precursors but Muscat grapes in particular have the highest concentrations. Wine treated with the combi-CLEA exhibited the highest concentration of total terpenes (18% higher) and of the individual terpenes—linalool (20% higher), nerol (20% higher) and geraniol (100% higher)—which determine the typical Muscat aroma. The stability of the enzymes in the combi-CLEA was an order of magnitude higher than that of the free enzymes, thus facilitating their reuse and reducing the impact of enzyme costs on the total costs of the winemaking.
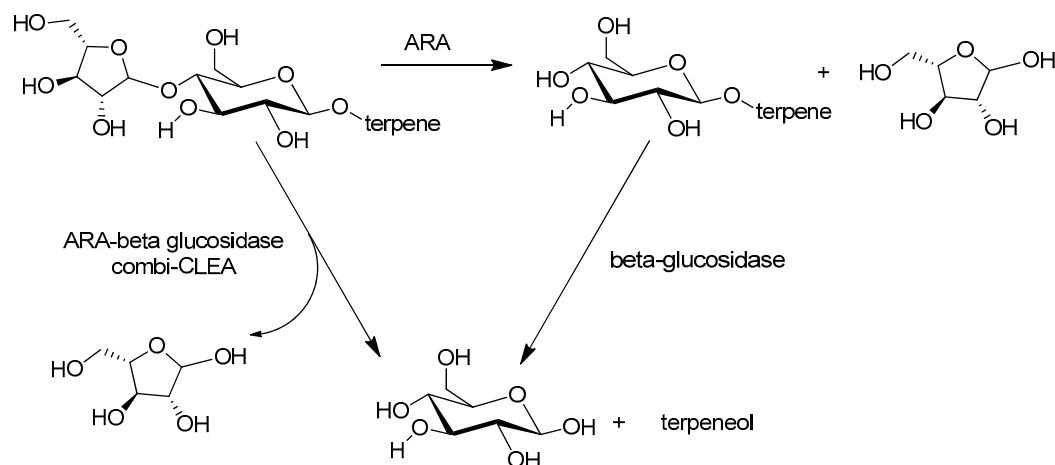
**Figure 10.** Wine aroma enhancement catalyzed by a combi-CLEA.

Hydrolysis of starch, cellulose and other polysaccharides are important reactions in many industries including food and beverages processing. Furthermore, their industrial importance is rapidly increasing with the impending transition to a biobased economy, in which oil refining will, be largely superseded by polysaccharide refining for the production of basic chemicals and liquid fuels. In this scenario the enzymatic conversion of starch and waste lignocellulose, as first and second generation renewable biomass, respectively, to fermentable sugars will take centre stage [14].

Enzymatic hydrolysis of starch involves two consecutive steps: liquefaction and saccharification. Liquefaction is catalyzed by α-amylase (E.C. 3.2.1.1) at 90 °C and pH 7 and hydrolyzes α-(1-4) glycosidic bonds. This is followed by glucoamylase (E.C. 3.2.1.3) catalyzed saccharification at 60 °C and pH 5, involving hydrolysis of both α-(1-4) and α-(1-6) glycosidic bonds. CLEAs have been successfully prepared from both α-amylase [158–160] and glucoamylase [161]. In some cases [161], macromolecular cross-linkers were used to produce more porous CLEAs with better activities.

The glucoamylase catalyzed hydrolysis of α-(1,6) branches is relatively slow and pullulanase (E.C. 3.2.1.41), is sometimes added to facilitate this process. Co-immobilization of the two enzymes in a combi-CLEA resulted in a shift in optimal pH (from 5 to 7) and temperature (from 60 to 70 °C) [162]. The combi-CLEA gave 100% conversion after 3 h compared with 30% with the free enzymes and 80% with a mixture of the two individual CLEAs. The combi-CLEA exhibited 90% and 85% retention of the glucoamylase and pullulanase activity, respectively, after 8 recycles.

More recently, the same group prepared a tri-enzyme combi-CLEA containing α-amylase, glucoamylase and pullulanase from commercially available enzyme preparations [163]. The thermal stability of all three enzymes was increased compared to the free enzymes. In a one-pot starch hydrolysis (Figure 11) 100%, 60% and 40% conversions were observed with the combi-CLEA, a mixture of the separate CLEAs and a mixture of the free enzymes, respectively, and the catalytic performance was maintained for up to 5 cycles.

The hydrolysis of lignocellulose is currently the focus of attention in connection with second generation biofuels production from waste lignocellulose streams. Lignocellulose has a complex structure consisting of lignin (15–25%), cellulose (30–45%) and hemi-cellulose (25–40%) that requires a complex cocktail of cellulolytic and hemi-cellulolytic enzymes to catalyze hydrolysis to its constituent sugars [164]. Cellulose hydrolysis involves at least five enzymes: exo-1,4-β-glucanase (EC 3.2.1.91), endo-,1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.176) and β-glucosidase (EC 3.2.1.21) and the more recently discovered, copper-dependent lytic polysaccharide monooxygenases (LPMOs).
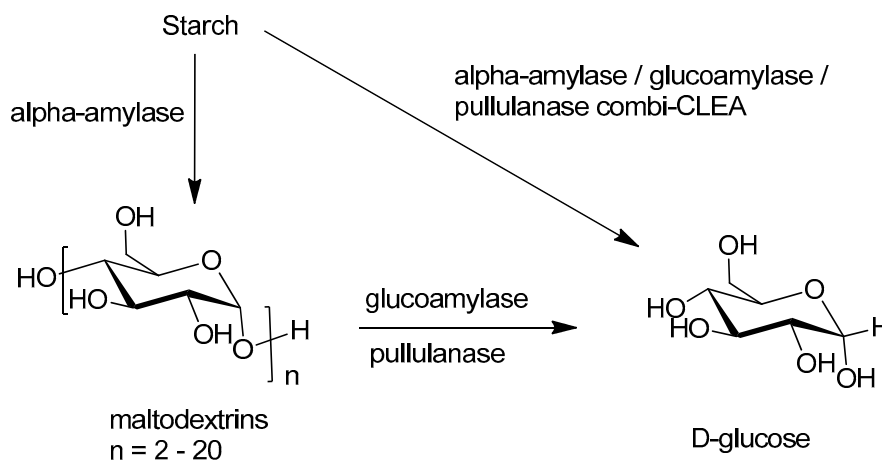
Figure 11. One-pot starch hydrolysis with a combi-CLEA.

Hemi-cellulose has a more complicated structure than cellulose and requires a diverse suite of enzymes to affect its hydrolysis to its constituent sugars, mainly xylose and mannose. They can be divided into two groups: core enzymes catalyzing cleavage of the polysaccharide backbone and ancilliary enzymes involved in the removal of functional groups. Examples of the former are endo-β-1,4-xylanase (EC 3.2.1.8), xylan-1,4-β-xylosidase (EC 3.2.1.37), endo-1,4-β-mannanase (EC 3.2.1.78) and β-1,4-mannosidase (EC 3.2.1.25). Ancilliary enzymes include β-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.2.1.55), ferulic acid esterase (EC 3.1.1.73) and p-coumaric acid esterase (EC 3.1.1). Cellulolytic fungi and bacteria produce so-called cellulosomes, containing multi-enzyme complexes that, because of the close proximity of the enzymes, are more effective than simple mixtures of the free enzymes. One can envisage that this proximity effect can be mimicked in combi-CLEAs without having many of the disadvantages of cellulosomes [165].

For example, a xylanase-mannanase combi-CLEA was successfully used in the conversion of lime-treated sugar cane bagasse and milled corn stover [166] leading the authors to conclude that the efficiency of combi-CLEAs makes them ideal candidates for achieving cost-effective application of lignocellulytic enzymes. Similarly, co-immobilization of xylanase, β-1,3-glucanase and cellulase gave a combi-CLEA which was more thermally stable than the free enzymes and retained more than 97% of its activity on storing at 4 °C for 11 weeks, compared to 65% for the free enzymes [167]. The combi-CLEA was successfully used in the hydrolysis of ammonia-cooked sugar cane bagasse and could be recycled 6 times.

6. Magnetic CLEAs: The New Frontier

Notwithstanding the many advantages of CLEAs, the relatively small particle size and lack of mechanical robustness remain issues in many cases. The particle size can be increased by, for example, modifying the cross-linking protocol but inevitably a compromise has to be reached between the ease of processing of large particles and the lower mass transfer limitations and, hence, higher activities of small particles. Moreover, the processing of polysaccharides, in particular lignocellulosic feedstocks, is fraught with another difficulty: recovery and reuse of the solid catalyst often requires its separation from other solid materials present in the crude mixtures. In order to rectify these shortcomings, the CLEA technology has been raised to a new level of sophistication and industrial relevance with the invention of "smart" magnetically recoverable CLEAs (m-CLEAs) by conducting the cross-linking in the presence of magnetic (nano)particles (Figure 12).
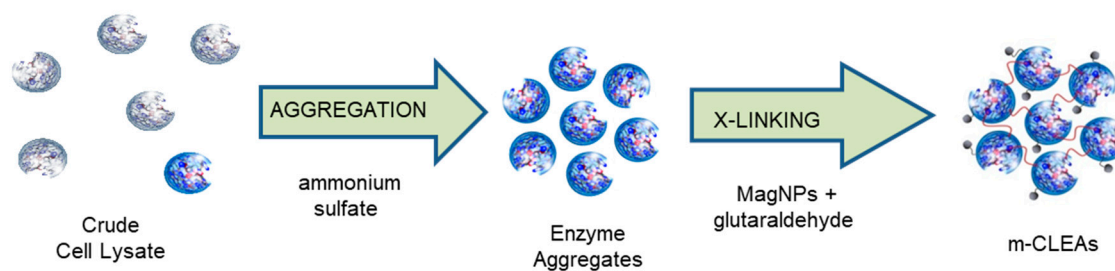
Figure 12. Preparation of magnetic CLEAs.

m-CLEAs have a relatively small particle size, and high activity, combined with ease of separation on an industrial scale using standard commercial equipment for magnetic separation with permanent magnets, affording novel combinations of bioconversions and downstream processing. The m-CLEAs are particularly useful for processes in which they have to be separated from other solids, e.g., in slurry processes.

6.1. Preparation of m-CLEAs

Our original development of m-CLEAs [168] involved conducting the cross-linking step in the presence of amino functionalized particles of magnetite (Fe_3O_4) obtained by reaction of (3-aminopropyl)triethoxysilane (APTES) with surface hydroxyl groups. The magnetic particles are incorporated in the CLEA superstructure through reaction of the cross-linker with both the enzyme aggregates and the NH_2 groups on the surface of the magnetic particles. The use of ferromagnetic catalysts, such as Raney nickel, on an industrial scale is well established [169]. In combination with commercially available magnetic separation equipment, e.g., from the Eclipse Magnets Automag series (see www.eclipsemagnetics.com), very high recoveries can be obtained at flow rates that are acceptable for industrial scale production. Subsequently, we and others [170] found that robust, stable mCLEAs could be produced in a more cost-effective way using non-functionalized magnetite partcles.

However, we found that leaching of iron occurs at acidic pH and is, moreover, accelerated in the presence of free carboxylic acids. These are precisely the conditions encountered in the hydrolysis of starch and, especially lignocellulose. We attribute the fact that iron leaching has not been observed by other authors to the fact that it becomes apparent only after long residence times and several recovery cycles. This obviously poses serious problems for their use in industrial processes. Another limitation of the conventional magnetite-based mCLEAs is the relatively low saturation magnetizability of magnetite which makes magnetic recovery in large scale processes challenging. In order to overcome these shortcomings of the conventional magnetite-based m-CLEAs, we developed mCLEAs based on non-functionalized magnetizable particles of zerovalent iron, so-called carbonyl iron particles (CIP) produced by thermal decomposition of iron pentacrabonyl [171]. This well-established method produces spherical iron particles with particle sizes of a few micrometers. These particles were then covered by a protected coating of silica with a thickness of a few nanometers silica in order to maintain their physical properties and protect them from corrosion under reaction conditions.

The m-CLEAs typically have particle sizes in the range 1–15 µm. Iron leaching was not observed with these m-CLEAs, even under relatively harsh conditions. The saturation magnetizations of these CIP-m-CLEAs were surprisingly high—up to 200 emu·g^{-1}—compared to the range of 10–40 emu·g^{-1} observed with magnetite-based m-CLEAs. Using this new cost-effective methodology to prepare m-CLEAs with very high magnetic susceptibility adds little cost to regular CLEAs and not many recycles are required to achieve cost reductions. The technique was demonstrated with glucoamylase and glutaminase.

6.2. Applications of m-CLEAs and m-Combi-CLEAs

m-CLEAs have been prepared from a variety of enzymes. However, interest in industrial application of m-CLEAs and m-combi-CLEAs has primarily been motivated by the possibility of using them in the complex conversions of polysaccharides such as starch and, particularly, lignocellulose in connection with first- and second-generation biofuels and food and beverages.

For example, Talekar and coworkers [172] prepared m-CLEAs of α-amylase, for use in starch hydrolysis. The activity recovery was 100% with improved thermal and storage stability compared to the free enzyme and 100% activity retention after 6 recycles. A m-CLEA of glucoamylase was prepared using pectin dialdehyde as the cross linker with 95% activity recovery compared to 85% using glutaraldehyde as the cross linker [173]. The authors attributed the higher activity recovery to better mass transfer with macromolecular substrates in the more open porous structure. m-CLEAs of glucoamylase from *Aspergillus niger* with enhanced thermal and storage stability and reusability and 93% activity recovery were prepared by Gupta and coworkers [174]. More recently, Tardioli and coworkers [175] prepared m-glucoamylase CLEAs by co-aggregation with amino functionalized magnetic nanoparticles and using PEI as an aid in the cross-linking with glutaraldehyde. The m-CLEA exhibited similar behavior to the free enzyme in starch saccharification under typical industrial conditions (55 °C and pH 4.5). We found (see above) that m-glucoamylase CLEAs based on magnetite were not stable towards leaching at acidic pH.

Probably the most exciting and challenging development is the application of the m-CLEA technology to the complex chemistry of lignocellulose hydrolysis in connection with the production of second-generation biofuels and commodity chemicals from waste biomass [176,177]. Various groups have reported [178–180] the preparation of m-CLEAs with improved stability from a cellulase cocktail. However, its activity was measured only in the hydrolysis of the water soluble carboxymethyl cellulose. Bhattachariya and Pletschke [166] prepared m-CLEAs of a bacterial xylanase and observed that incorporation of Ca^{2+} ions in the CLEA led to increased thermal stability. The Ca-m-CLEA exhibited 35% more activity than the free enzyme and a 9 fold higher sugar release from ammonia pretreated sugar cane bagasse. Illias and coworkers [181] prepared m-CLEAs of a recombinant xylanase from *Trichoderma reesei* using maghemite (γ-Fe_2O_3) rather than the more usual magnetite (Fe_3O_4) nanoparticles. Second-generation biofuels are an obvious application for m-CLEAs of lignocellulolytic enzymes but applications in food and beverages processing are also envisaged.

The enzyme costs per kg product are crucial for the economic viability of these processes, particularly in the case of lignocellulosic feedstocks. In both 1G and 2G biofuels and commodity chemicals production, in order to be more cost-effective the enzyme catalyzed hydrolysis of the feedstock—starch or lignocellulose—is often combined with the subsequent fermentation step in so-called simultaneous saccharification and fermentation (SSF) processes [182]. Multiple recycling of the enzyme(s), using an immobilized, solid enzyme which can be easily recovered and re-used, represents a clear opportunity to reduce the enzyme costs and drive competitiveness and sustainability. However, the need to separate the solid immobilized enzyme from the vast amount of other solids such as fibers and/or yeasts (in SSF processes) suspended in the reaction mixture precludes the use of standard immobilized enzymes.

Up till now, attention has been focused on starch and lignocellulose hydrolysis but the m-CLEA technology can also be applied in the hydrolysis of other polysaccharides in processes for their conversion to biofuels and commodity chemicals. For example, pectins (galacturonans) and inulins (fructans) can be hydrolyzed using pectinase and inulinase, respectively. For example, a trienzyme magnetic combi-CLEA consisting of an α-amylase, pectinase and cellulase was used for clarification of fruit juices [183].

The growing trend towards the utilization of food supply chain waste (FSCW) [184], most of which goes to landfill and has a negative value, is also stimulating the application of biocatalysis, e.g., proteases, carbohydrases, and lipases, in the processing of waste proteins, carbohydrates and oils and fats, respectively.

6.3. Lipase-m-CLEAs

Lipase-m-CLEAs have significant commercial potential in the processing of non-edible and waste oils and fats to 2G biodiesel. Conventional chemical processes generally involve catalysis by bases such as sodium methoxide and are energy-intensive operations with high waste treatment costs. Moreover, the feedstock must have a low free fatty acid content since any free fatty acids will neutralize the basic catalyst, resulting in catalyst losses and the formation of soap emulsions. In contrast, enzymatic transesterification is characterized by mild reaction conditions, low energy demand, low waste treatment costs and more flexibility with regard to free fatty acids in the feedstock, as they are also esterified to biodiesel. However, current lipase-based methods suffer from high enzyme cost contributions and inactivation of the lipase by the methanol and/or impurities in the feedstock. The use of recyclable m-CLEAs of the lipase catalysts could provide the necessary reduction in enzyme costs to enable commercial viability.

m-CLEAs of CaLB were used in the production of biodiesel by transesterification of sunflower oil [185], non-edible vegetable oils, such as jatropha and cameline, and waste frying oils [186] and microalgae oil [187]. Similarly, surfactant activated m-CLEAs of *Thermomyces lanuginosus* lipase (TLL) [188] and *Rhizomucor miehei* lipase (RML) [189] were used in the production of biodiesel from jatropha oil. The m-CLEAs could be readily recovered and recycled for several cycles.

A m-CLEA of a *Candida* sp. lipase was used to catalyze the epoxidation of oleic acid with hydrogen peroxide, via in situ lipase catalyzed formation of peroleic acid (Figure 13) [190]. The m-CLEA exhibited higher thermal and storage stability and better tolerance of hydrogen peroxide compared to the free enzyme and the standard CLEA. Similarly, a m-CLEA of an *Aspergillus niger* lipase was used [191] to catalyze the epoxidation of α-pinene with hydrogen peroxide in ethyl acetate as solvent and acetate-source (Figure 13) and the synthesis of glycerol carbonate by transesterification with dimethyl carbonate [192].
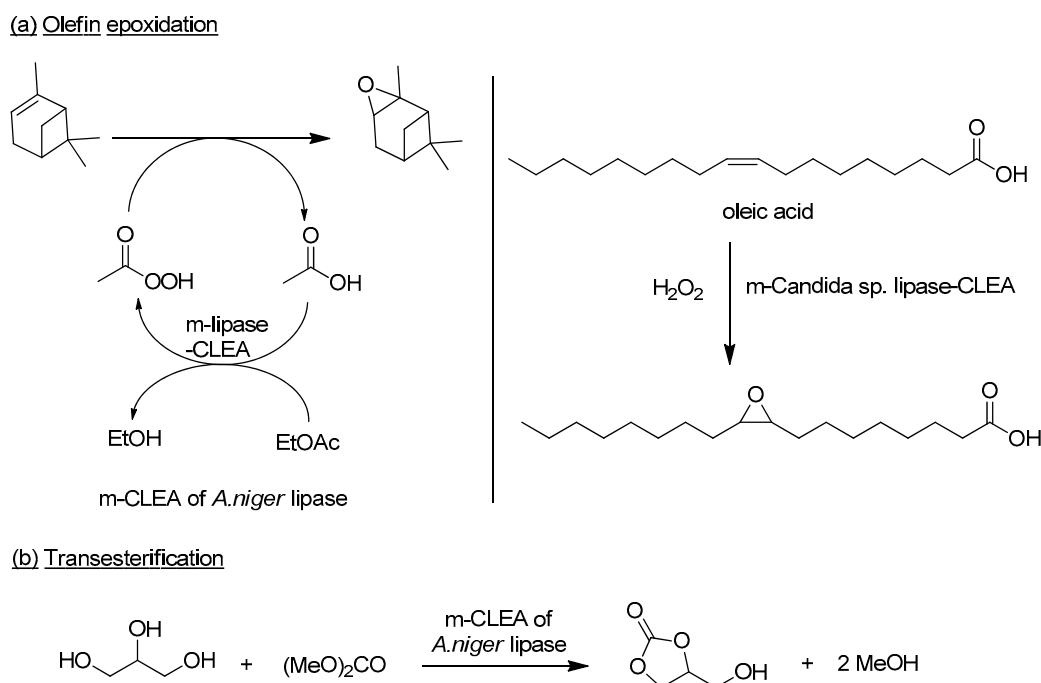
Figure 13. Lipase-m-CLEA catalyzed epoxidation and transesterification.

m-CLEAs of *Rhizopus arrhizus* lipase were used for the production of butyl oleate, a widely used diesel additive, in a novel microreactor consisting of a column containing the m-CLEA, and sieve plates through which solvents and products can pass, and surrounded by a rotating magnetic field [193]. Reactants were fed from the bottom of the column as in a fluidized bed.

Lipase-m-CLEAs could presumably also be used to advantage in the lipase catalyzed synthesis of biobased polyesters and polyamides [194–196].

6.4. Ligninolytic Enzymes and Waste Water Treatment

White rot fungi produce a range of extracellular enzymes—laccases, lignin peroxidases and manganese peroxidases—which are involved in the recycling of organic material in vivo and have broad applications in bioremediation. Immobilization of these enzymes, for example as CLEAs or combi-CLEAs is necessary in order to be cost-effective in waste water treatment. Although it is difficult to achieve the very low enzyme costs required for commercial viability in waste water treatment in general there may very well be niche opportunities in the case of emerging micropollutants that are difficult to remove by other means For example, laccase-m-CLEAs [197,198] and a combi-m-CLEA of horseradish peroxidase and glucose oxidase [199] have been used for the decolorization of recalcitritant dyes in waste water and various reactor concepts have been considered. A laccase-m-CLEA was also used for removal of pharmaceuticals from waste water [200] and an acrylamidase m-CLEA was used to catalyze the biodegradation of acrylamide in industrial waste water [201].

6.5. Synthesis of Semi-Synthetic Penicillin and Cephalosporin Antibiotics

Semi-synthetic penicillins and cephalosporins are produced on an industrial scale by PGA catalyzed coupling of the side-chain with the 6-aminopenicillanic acid (6-APA) or 7-aminodesacetoxycephalosporanic acid (7-ADCA) nucleus (Figure 14). The coupling is conducted in water and the product crystallizes out of the reaction mixture. This is essential as otherwise the enzyme would catalyze the thermodynamically favored hydrolysis of the product. However, the enzyme needs to be immobilized to enable its reuse but then it has to be separated from the solid product. This can be achieved using a sieve bottom reactor in which the crystals of product pass through the sieve while the larger particles of immobilized enzyme are retained in the reactor [202]. Alternatively, PGA m-CLEA was shown to catalyze the hydrolysis of penicillin G [203] and it could be used to produce semi-synthetic penicillins [204] and cephalosporins [205].
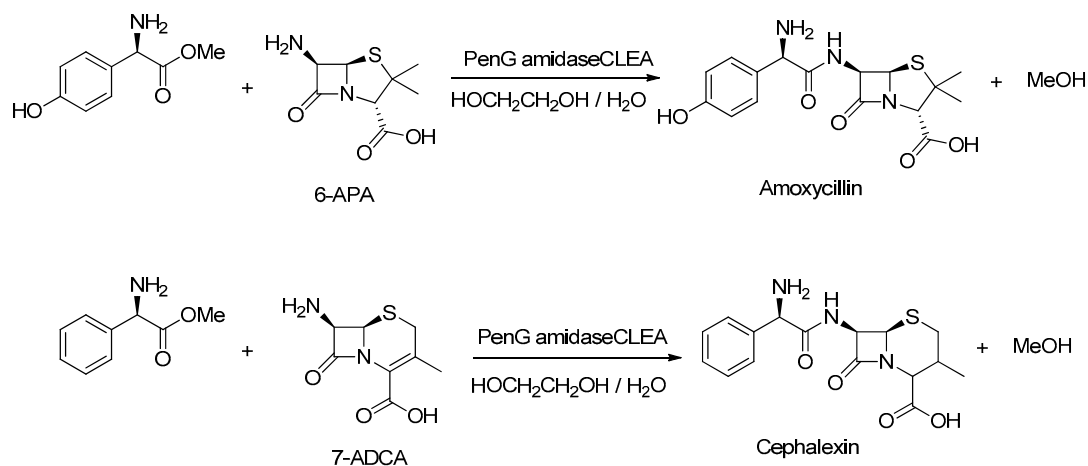

Figure 14. Enzymatic synthesis of penicillins and cephalosporins.

6.6. Other Potential Applications

Magnetic CLEAs of other enzymes have been described. For example, m-combi-CLEAs have been prepared from ketoreductases (KREDs) and glucose dehydrogenase for cofactor regeneration with glucose and used in the synthesis of chiral alcohols [149,206,207]. Cui and coworkers [170] prepared m-CLEAs of phenyl alanine ammonia lyase (PAL EC 4.3.1.24) and showed that storage and operational stabilities were superior to those of the simple CLEA. Bovine carbonic anhydrase, of interest in connection with processes for CO_2 capture, was also successfully immobilized as a

m-CLEA [208]. Finally, glucose isomerase, the largest volume immobilized enzyme (see earlier) has been immobilized as a m-CLEA [209]. The temperature and pH optima shifted in the m-CLEA and a significant increase in activity with 80% retention after 6 recycles was observed which led the authors to conclude that the "m-CLEAs of glucose isomerase clearly have practical utility in HFCS production".

7. Conclusions and Outlook

In the preceding decade the efficacy and cost-effectiveness of the cross-linked enzyme aggregates (CLEA) methodology in the immobilization of a wide variety of enzymes, thereby enabling many applications of biocatalysis, has been amply demonstrated. With regard to the eight requirements that Turner and coworkers [40] recently outlined for the commercial viability of an immobilized enzyme in the pharmaceutical industry, CLEAs exhibit (i) high enzyme loading (>10 wt%); (ii) high activity recovery (>50%); (iii) no leaching under reaction conditions; (iv) good tolerance to organic solvents; (v) are eminently recyclable (>20 cycles); and (vi) result in a lower cost of good compared with alternatives. Mass transport of substrates is sometimes an issue, especially with macromolecular substrates, but techniques have been developed to alleviate this problem. Mechanical stability in batch and flow reactors is also an issue with CLEAs but methods have been developed to address these problems. In contrast, carrier-bound enzymes have low enzyme loadings, often low activity recoveries and, generally higher cost contributions. Depending on the method of binding to the carrier, leaching can also be an issue but they tend to have better mechanical stability than CLEAs. Both mass transport and mechanical stability issues can be circumvented by employing smart m-CLEAs with small particle size in conjunction with recovery using permanent magnets in commercially available separation equipment.

In short, the CLEA technology has proven its practical utility and industrial viability. It has been particularly successful in the immobilization of a variety of enzymes for use in the conversion of carbohydrates, ranging from mono- to polysaccharides, in aqueous media, thus enabling multiple recycling of the enzymes. Applications in industrial biocatalysis are currently being further stimulated by the ongoing transition to a more sustainable bio-based, circular economy in which there is a growing trend towards the use of carbohydrates as a substitute for hydrocarbon feedstocks. We expect this trend to continue in the future and that combi-CLEAs and "smart" mCLEAs will be increasingly utilized in carbohydrate conversion processes in both traditional food and beverages processing and in the production of biofuels, commodity chemicals and bio-based polyesters and polyamides from waste renewable biomass, including food supply chain waste.

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