Chapter 7
Enzymatic Conversion of First- and Second-Generation Sugars

Roger A. Sheldon

Abstract Processes for the hydrolytic conversion of polysaccharides to fermentable sugars as feedstocks for biofuels and commodity chemicals are discussed. The production of first-generation biofuels, for example, bioethanol, involves the conversion of sucrose or starch; the latter requires initial enzymatic hydrolysis of the starch to glucose in a two-step process catalyzed by α-amylase and glucoamylase. These methods are established industrial processes that are conducted on an enormous scale. Although the enzymes involved are relatively inexpensive, they are used on a single-use, throw-away basis, and substantial cost savings can be achieved by immobilization of the enzymes to enable their recycling. In particular, immobilization of the enzymes as magnetic cross-linked enzyme aggregates (mCLEAs), in combination with magnetic separation using commercially available equipment, offers possibilities for achieving substantial cost reductions.

The production of second-generation biofuels involves, in the long term, more sustainable conversion of waste lignocellulose to fermentable sugars, a much more complicated process requiring multiple enzymes. The hydrolytic step is preceded by a pretreatment step that opens the structure of the recalcitrant lignocellulose to make it accessible for the hydrolytic enzymes. This step is usually conducted in water, in which the lignocellulose is insoluble, but there is currently much interest in the use of ionic liquids or deep eutectic solvents in combination with water. Subsequent hydrolysis of the cellulose and hemicellulose to fermentable sugars involves a complex cocktail of enzymes referred to as "cellulase." In this case the percentage cost contribution of the enzymes to the biofuel is even higher than with first-generation biofuels. Consequently, it is even more important to reduce the costs of enzyme usage by immobilization, and magnetic separation of magnetic immobilized enzymes, such as magnetic CLEAs, is a potentially attractive way to achieve this.

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7.1 Introduction

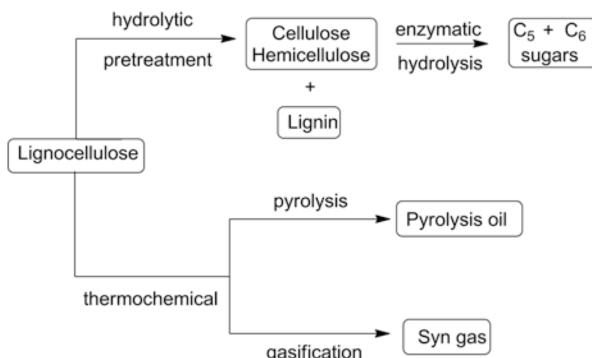
One of the grand challenges in chemistry and biology, motivated by the pressing need to mitigate climate change, is to devise green and sustainable technologies for the conversion of renewable biomass to fuels, commodity chemicals, and bio-based materials such as bioplastics in integrated biorefineries (Imhof and van der Waal 2013; Yang et al. 2013; Gallezot 2012; De Jong et al. 2012) The majority (60–80%) of all biomass consists of carbohydrates, which can be divided into storage carbohydrates—starch, inulin, and sucrose—and structural polysaccharides, such as cellulose, hemicelluloses, and chitin. In particular, lignocellulose, a fibrous material that constitutes the cell walls of plants, is available in very large quantities. In addition, aquatic carbohydrates derived from micro- and macroalgae consist of a variety of polysaccharides that differ in structure from those of terrestrial biomass. The remainder of biomass comprises triglycerides (from fats and oils), proteins, and terpene hydrocarbons.

The use of first-generation (1G) biomass feedstocks, comprising sucrose, starch, and triglycerides from edible oil seeds, is not perceived as a sustainable option in the long term because it competes, directly or indirectly, with food production. Second-generation (2G) feedstocks, in contrast, comprise lignocellulose and triglycerides produced by the deliberate cultivation of fast-growing, nonedible crops or, in a more attractive option, by the valorization of waste triglycerides (oils and fats) and, in particular, the enormous amounts of waste lignocellulose generated in the harvesting, processing, and use of agricultural products, including foods and beverages. Pertinent examples include sugarcane bagasse, corn stover, wheat straw, rice husks, and orange peel (Tuck et al. 2012). Indeed, the so-called bio-based economy is founded on the full utilization of agricultural biomass by using green and sustainable chemistry.

The first step in the conversion of polysaccharide feedstocks to, for example, biofuels and commodity chemicals is generally hydrolytic depolymerization to fermentable sugars, mainly glucose. The preferred method, from both economic and environmental aspects, is enzymatic hydrolysis. The hydrolysis of starch, for example, consists of two steps: liquefaction and saccharification. The former is conducted at 90 °C and pH 7 and is catalyzed by α-amylase (E.C. 3.2.1.1), which hydrolyzes α-(1-4) glycosidic bonds, affording a mixture of glucose oligomers (maltodextrins). The latter is conducted at 60 °C and pH 5 and involves hydrolysis of both α-(1-4) and α-(1-6) glycosidic bonds catalyzed by glucoamylase (E.C. 3.2.1.3).

Lignocellulose is much more difficult to process than starch. It consists of three major polymeric components: lignin (~20%), cellulose (~40%), and hemicellu-

Fig. 7.1 Methods for depolymerization of lignocellulosic biomass



lose (~25%) in weight composition. Lignin is a three-dimensional polyphenolic biopolymer having a nonuniform structure that imparts rigidity and recalcitrance to plant cell walls. In volume, it is the second largest biopolymer after cellulose and the only one composed entirely of aromatic subunits. Basically, depolymerization and (partial) deoxygenation of lignocellulose can be performed in two ways: hydrolytic and thermochemical (Fig. 7.1). The latter involves pyrolysis to a mixture of charcoal and pyrolysis oil or gasification to afford syngas (a mixture of carbon monoxide and hydrogen), analogous to syngas from coal gasification (Sheldon 1983). The syngas can be subsequently converted to liquid fuels or platform chemicals using established technologies such as the well-known Fischer–Tropsch process or methanol synthesis, respectively. Alternatively, it can be used as a feedstock for the microbial production of biofuels and platform chemicals (Munasinghe and Khanal 2007; Henstra et al. 2007). In this chapter, we are concerned with the hydrolytic conversion of the polysaccharide feedstock, starch or lignocellulose, to monosaccharides (primarily glucose) for subsequent conversion to 1G and 2G biofuels and commodity chemicals, respectively.

7.2 Enzymatic Hydrolysis of Starch to Glucose

Although lignocellulosic biofuels are seen as the long-term option, in the short term biofuels consist primarily of corn- or sugar-based bioethanol. The United States (USA) is the largest producer, with a production of 14.7 billion gallons in 2015 (Renewable Fuels Association 2016), from cornstarch as the feedstock. This process includes the enzymatic hydrolysis of the starch to glucose (Fig. 7.2), followed by fermentation of the latter to ethanol; it can be conducted in a separate hydrolysis and fermentation (SHF) mode or, to be more cost-effective, in a combined simultaneous saccharification and fermentation (SSF) process (Olofsson et al. 2008). An SSF process has the added advantage that the glucose is immediately consumed by the fermenting organism, thus circumventing possible inhibition by increasing concentrations of glucose.

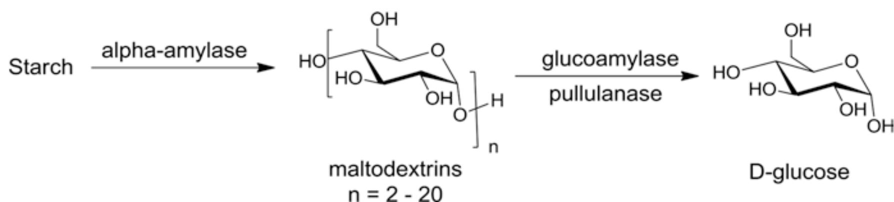


Fig. 7.2 Enzymatic hydrolysis of starch

7.3 Enzyme Immobilization

The enzyme costs per kilogram of product are a crucial factor in determining the economic viability of these processes, particularly in the case of lignocellulosic feedstocks (see later). Enzyme manufacturers have achieved remarkable results, in recent years, in reducing enzyme costs by optimizing the production of the enzymes involved, but there is still room for improving the enzyme usage. The enzyme(s) used in the hydrolysis step are dissolved in the aqueous reaction mixture and, consequently, are discarded with the wash water; that is, they are employed on a single-use, throw-away basis. Multiple recycling of the enzymes represents a clear opportunity to substantially reduce the enzyme costs and environmental footprint to drive competitiveness and sustainability. It can be achieved by using the enzyme in a solid, immobilized form, which can be easily recovered and reused. A further benefit of immobilization is that the resulting decrease in flexibility leads to increasing operational stability by suppressing the propensity of enzymes to unfold (denature) under the influence of heat or organic solvents.

Immobilization typically involves binding the enzyme to a prefabricated carrier (support), such as an organic resin or silica, or entrapment in a polymeric inorganic or organic matrix that is formed in the presence of the enzyme. Binding to a prefabricated carrier can involve simple adsorption, such as via hydrophobic or ionic interactions or the formation of covalent bonds (Cao 2005). Typical supports are synthetic resins (Cantone et al. 2013), biopolymers such as polysaccharides, or inorganic solids such as (mesoporous) silicas (Hartmann and Kostrov 2013; Zhou and Hartmann 2012; Magner 2013). Entrapment involves inclusion of an enzyme in an organic or inorganic polymer matrix, such as polyacrylamide and silica sol-gel, respectively, or a membrane device such as a hollow fiber or a microcapsule (Reetz 2013). The use of a carrier inevitably leads to 'dilution of activity,' owing to the introduction of a large portion of noncatalytic ballast, ranging from 90% to more than 99%, giving rise to lower space–time yields and catalyst productivities (Cao et al. 2003).

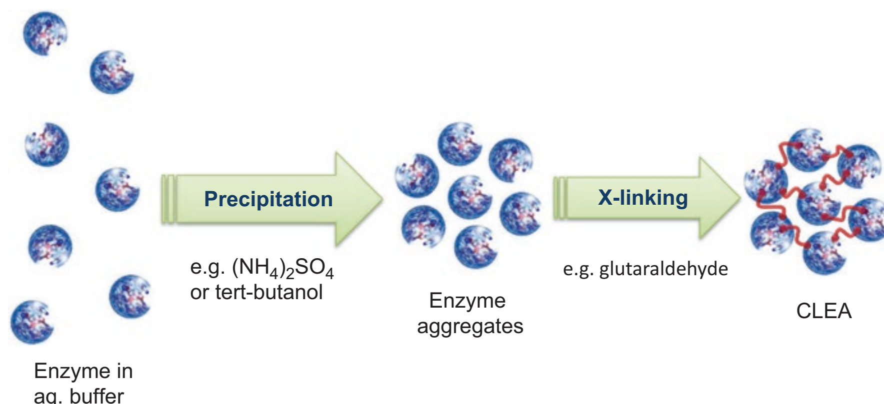


Fig. 7.3 Enzyme immobilization as cross-linked enzyme aggregates (CLEAs)

7.3.1 Immobilization Via Cross-Linking: CLEAs

In contrast, immobilization by cross-linking of enzyme molecules affords carrier-free immobilized enzymes with high productivities and avoids the extra costs of a carrier. For example, cross-linked enzyme aggregates (CLEAs) are formed by precipitation of the enzyme from aqueous buffer, as physical aggregates held together by noncovalent bonding without perturbation of their tertiary structure, followed by cross-linking with a bifunctional reagent, such as glutaraldehyde (Fig. 7.3) (Cao et al. 2000). The method is simple and inexpensive as it does not require highly pure enzymes. Indeed, because selective precipitation with ammonium sulfate is commonly used to purify enzymes, the CLEA methodology essentially combines two processes, purification and immobilization, into a single unit operation. The CLEA technology has subsequently been applied to the immobilization of a broad spectrum of enzymes and forms the subject of several reviews (Sheldon 2011, 2013; Talekar et al. 2013a; Cui and Ja 2015; Valesco-Lozano et al. 2015; Sheldon et al. 2013).

Multipurpose CLEAs can be prepared from crude enzyme extracts consisting of multiple enzymes (Dalal et al. 2007a, 2007b). Interestingly, enzymes that would not be compatible in solution can function effectively when co-immobilized. For example, 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 novel lipase/protease multi-CLEA (Mahmod et al. 2015).

The term combi-CLEA is generally used when two or more enzymes are deliberately co-immobilized in a single CLEA for the purpose of performing two or more sequential biotransformations in a multi-enzyme cascade process. Catalytic cascade processes have several advantages compared with classical multistep syntheses: fewer unit operations, less solvent, smaller reactor volume, shorter cycle times, higher volumetric and space–time yields, and less waste. Furthermore, coupling of

reactions can be used to drive equilibria toward the product, thus avoiding the need for excess reagents. Because biocatalytic processes generally proceed under roughly the same conditions—in water at ambient temperature and pressure—they can be readily integrated into cascade processes. These processes have become a focus of attention in recent years, largely motivated by these envisaged environmental and economic benefits (Lopez-Gallego and Schmidt-Dannert 2010; Schrittweiser et al. 2011; Santacoloma et al. 2011; Xue and Woodley 2012; Muschiol et al. 2015; Land et al. 2016). However, different enzymes can be incompatible, and Nature solves this problem by compartmentalizing enzymes in different parts of the cell. Hence, compartmentalization via immobilization is a possible solution in enzymatic cascade processes.

The rates of sequential biocatalytic cascades can be substantially increased by simulating the close proximity of the enzymes extant in microbial cells by co-immobilization of the respective enzymes in, for example, combi-CLEAs. For example, combi-CLEAs of glucose oxidase or galactose oxidase with catalase exhibited significantly better activities and stabilities than corresponding mixtures of the two separate CLEAs (Schoevaart et al. 2004). Moreover, the combi-CLEAs could be recycled without significant loss of activity. 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.

Combi-CLEAs have also been widely used in carbohydrate conversions. As already mentioned, glucoamylase catalyzes the hydrolysis (saccharification) of α-(1,4)- and α-(1,6)-glycosidic bonds in starch. Because the hydrolysis of the α-(1,6) branches is relatively slow, a second enzyme, pullulanase (E.C. 3.2.1.41), is sometimes added to facilitate this hydrolysis. Co-immobilization of the two enzymes in a combi-CLEA results in a shift in optimum pH values (from 5 to 7) and temperature (from 60 °C to 70 °C) (Talekar et al. 2013b). In a batch mode hydrolysis of starch, the combi-CLEA gave 100% conversion after 3 h compared with 30% with the free enzyme. A mixture of the two separate CLEAs gave 80% conversion. The combi-CLEA had good stability, retaining 90% and 85% of the glucoamylase and pullulanase activity, respectively, after eight recycles.

More recently, the same group prepared a tri-enzyme combi-CLEA containing α-amylase, glucoamylase, and pullulanase from commercially available enzyme preparations (Talekar et al. 2013c). In a one-pot starch hydrolysis (Fig. 7.4) in batch mode, 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. Co-immobilization increased the thermal stability of all three enzymes, and the catalytic performance was maintained for up to five cycles.

Similarly, combi-CLEAs have been used with success in the hydrolysis of lignocellulose, which has become the focus of attention in connection with second-generation biofuels production from waste lignocellulose streams (see Sect. 7.5).

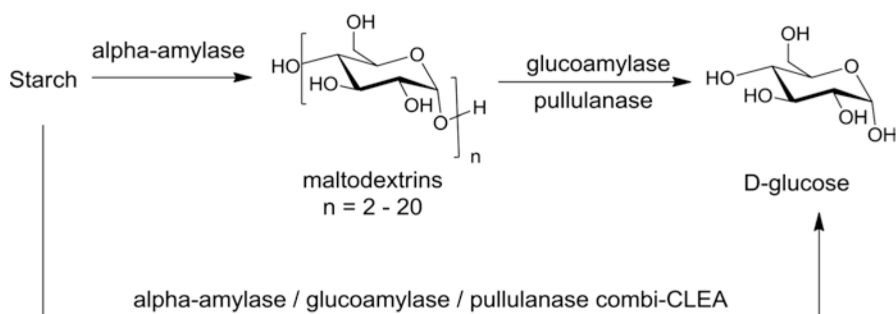


Fig. 7.4 One-pot starch hydrolysis with a combi-CLEA

7.3.2 Advantages and Limitations of CLEAs

Immobilization of enzymes as cross-linked enzyme aggregates can lead to dramatic increases in storage and operational stability (van Pelt et al. 2008). Moreover, because the enzyme molecules in CLEAs are bound by covalent bonds, no leaching of enzyme is observed in aqueous media, even under drastic conditions such as in the presence of surfactants.

An important advantage from a cost-effectiveness aspect is their ease of recovery and reuse, as heterogeneous catalysts, by filtration, centrifugation, or, alternatively, in a fixed-bed reactor. In practice this translates to simpler, less expensive downstream processing. Another important cost advantage is the fact that CLEAs can be prepared from enzyme samples of low purity, including crude cell lysate obtained from fermentation broth. Furthermore, because they consist mainly of active enzyme, CLEAs exhibit high catalyst productivities (kilograms per product per kilograms of enzyme) compared to carrier-bound enzymes, and the costs of the carrier ballast are avoided.

A limitation of the technique is that, because every enzyme is a different molecule, the protocol has to be optimized for every enzyme. However, in practice optimizing the aggregation plus cross-linking protocol is not a lengthy procedure and it can be readily automated. Another limitation is the relatively small particle size, as already mentioned, which can be an issue for processes conducted in a fixed-bed reactor, for example. The problem can be alleviated by mixing the CLEA with an inert, less compressible solid, such as controlled microporous glass or perlite (Hickey et al. 2007).

7.4 Magnetically Separable Immobilized Enzymes

Applications in processes involving suspensions of other water-insoluble solids, such as fibers or yeasts in SSF conversions of 1G and 2G biomass, is a challenge for standard immobilized enzymes. However, industrially viable separation on a large

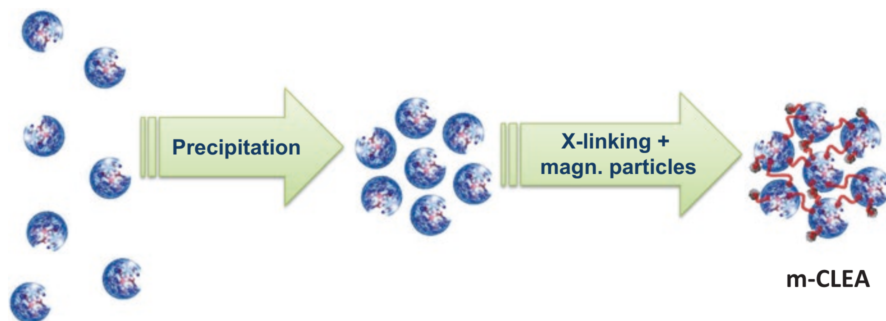


Fig. 7.5 Formation of magnetic cross-linked enzyme aggregates (mCLEAs)

scale, using standard commercial equipment, can be readily achieved using enzyme-ferromagnetic particle composites, affording novel combinations of biocatalysis with downstream processing. Indeed, recently increasing attention has been devoted to the design of magnetically recoverable catalysts, based on ferromagnetic magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) (nano)particles, in chemocatalytic (Rossi et al. 2014; Ranganath and Glorius 2011; Gawande et al. 2013; Zamani and Hosseini 2014) and biocatalytic (Ansari and Husain 2012; Johnson et al. 2008; Liu et al. 2011; Yiu and Keane 2012; Netto et al. 2013) processes. The magnetic separation of ferromagnetic heterogeneous catalysts, such as Raney nickel, has long been known (Whitesides et al. 1976; Lindley 1982) and is practiced on an industrial scale. In combination with commercially available magnetic separation equipment (e.g., see www.eclipsemagnetics.com), very high recoveries can be obtained at industrially acceptable flow rates. Magnetic separation can also be used to alleviate problems encountered with separation of relatively small particles by filtration or centrifugation. Ideally, one would like to have the high activity of small particles while maintaining the ease of processing of large particles, and this goal can be achieved with magnetically separable immobilized enzymes.

Similarly, the CLEA technology has been raised to a new level of sophistication and industrial relevance with the invention of robust, cost-effective ferromagnetic CLEAs (mCLEAs), produced by conducting the cross-linking in the presence of ferromagnetic (nano)particles (Fig. 7.5) (Sheldon et al. 2012). Using the latest methodology (Janssen et al. 2016), conversion to mCLEAs adds little cost to regular CLEAs and it does not require many recycles to achieve cost reductions. Hence, mCLEAs are expected to find applications in a variety of processes, including 1G and 2G biofuels.

mCLEAs have been prepared from a variety of enzymes, including lipases (Cruz-Izquierdo et al. 2014; Zhang et al. 2015a; Cui et al. 2016; Tudorache et al. 2016), penicillin G amidase (Kopp et al. 2014), phenyl ammonia lyase (PAL, EC. 4.3.1.24) (Cui et al. 2014), laccase (Kumar et al. 2014,) and even a combi-mCLEA of horseradish peroxidase and glucose oxidase for use in dye decolorization (Zhou et al. 2016). However, industrial interest is primarily in carbohydrate conversions,

particularly in the conversion of polysaccharides such as starch and lignocellulose in connection with 1G and 2G biofuels and food and beverage processing.

For example, Talekar and coworkers (Talekar et al. 2012) described the preparation of mCLEAs of α-amylase, with an activity recovery of 100%, for use in starch hydrolysis. Thermal and storage stability was improved compared to the free enzyme, and the mCLEA retained 100% of its activity after six recycles. A mCLEA of α-amylase was also prepared with pectin dialdehyde as the cross-linker and exhibited 95% activity recovery compared to 85% using glutaraldehyde as the cross-linker (Nadar and Rathod 2016). The authors attributed the higher activity recovery to better mass transfer with macromolecular substrates in the more open porous structure. mCLEAs of glucoamylase from *Aspergillus niger* were prepared by Gupta and coworkers (Gupta et al. 2013) with 93% activity recovery and showed enhanced thermal and storage stability and reusability.

Probably the most exciting and challenging application of the mCLEA technology is in the complex chemistry of lignocellulose conversion, which is discussed in the following section.

7.5 Enzymatic Depolymerization of Lignocellulose

Conversion of lignocellulose to biofuels and commodity chemicals basically involves three steps: pretreatment, enzymatic hydrolysis to fermentable sugars (saccharification), and fermentation, for example, to ethanol, or chemocatalytic conversion. Alternatively, so-called consolidated bioprocessing (CBP) involves the use of cellulolytic enzyme-producing microbes—bacteria, fungi, or yeasts—in a single-step process in which enzyme production, enzymatic hydrolysis, and fermentation proceed simultaneously (Jouzani and Taherzadeh 2015). Although CBP is potentially very attractive, it is still in its infancy, and a detailed discussion falls outside the scope of this review, which focuses on the more established classical approach of using separately produced enzyme cocktails.

7.5.1 Pretreatment of Lignocellulose

Some form of pretreatment, such as a steam explosion, ammonia fiber expansion (AFEX), or lime treatment, is generally necessary to open up the recalcitrant lignocellulose structure and render it accessible to the enzyme cocktail (Rabemanolontsoa and Saka 2016; Kumar et al. 2009; Alvira et al. 2010). The pretreatment generally accounts for a large fraction of the total energy requirements of lignocellulose processing (Menon and Rao 2012.) It is generally conducted in water in which the cellulose, hemicellulose, and lignin are present as suspended solids. The use of alternative reaction media, which (partially) dissolve these polymeric substrates, could have processing advantages. However, to be economically and

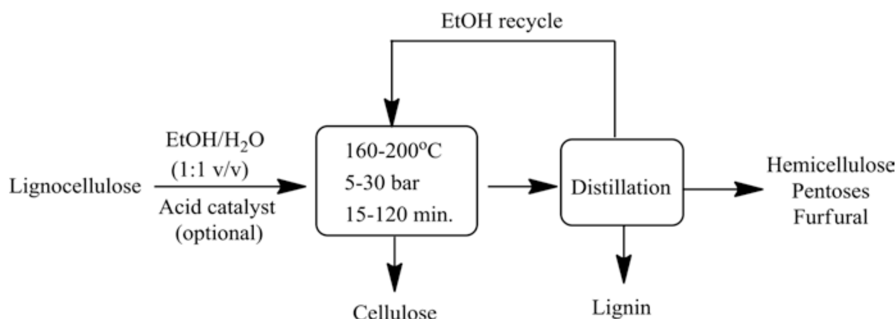


Fig. 7.6 The Organosolv process with aqueous ethanol

environmentally viable, the solvent should be inexpensive, nontoxic, biodegradable, recyclable, and preferably derived from renewable resources.

In the Organosolv process (Fig. 7.6) (Wildschut et al. 2013), for example, lignocellulose is subjected to elevated temperatures (185–210°C) in water/organic solvent (e.g., 50% v/v aqueous ethanol) mixtures, in the presence (Zhao et al. 2009) or absence of an acid catalyst (Viell et al. 2013a). This reaction results in hydrolysis of the hemicellulose and dissolution of the lignin. The remaining cellulose is separated, and the dissolved lignin is precipitated by water addition or ethanol evaporation. Overall the products comprise cellulose, solid lignin, and an aqueous stream containing hemicellulose, C_5 sugars, and derivatives thereof, such as furfural.

In a variation on this theme, ionic liquids (ILs), which are known to dissolve polysaccharides, are being considered as potential alternatives for the deconstruction of lignocellulosic biomass in a so-called Ionosolv process (Brandt et al. 2013). Rogers and coworkers (Swatloski et al. 2002) showed that the room temperature IL, [bmim] [Cl], can dissolve 100 g l^{-1} cellulose at 100 °C. Currently, much attention is focused on the use of ILs (Bogel-Lukasic 2016; Fang et al. 2014; Dominguez de Maria 2014; Vancov et al. 2012; Zavrel et al. 2009) as reaction media for the saccharification of lignocellulose. A variety of waste lignocellulosic biomass, such as wood chips (Sun et al. 2009; Xu et al. 2014) and wheat straw (Li et al. 2009; Magalhaes da Silva et al. 2013), has been shown to dissolve in ILs, and the latter could be used as reaction media for subsequent chemocatalytic (Rinaldi 2014; Morales-de la Rosa et al. 2012; Long et al. 2012) or enzymatic hydrolysis. For example, [emim] [OAc] was used as the solvent for the fractionation of wheat straw into lignin and carbohydrate fractions (da Costa Lopes et al. 2013) and for the combined pretreatment and enzymatic hydrolysis of wood chips (Viell et al. 2013b), switchgrass (*Miscanthus giganteus*) (Shi et al. 2013), and sugarcane bagasse (Qiu et al. 2012). Cholinium salts of amino acids, such as cholinium lysinate [Ch] [Lys], are examples of ILs derived from renewable resources. A 20:80 mixture (v/v) of [Ch] [Lys] and water was used for the pretreatment and subsequent enzymatic hydrolysis of rice straw (Hou et al. 2013) and switchgrass (Sun et al. 2014).

Ultimately, the goal is to develop an integrated process for IL pretreatment of lignocellulose and enzymatic hydrolysis, with efficient recycling of both the IL and

the (immobilized) enzyme (Ungerean et al. 2014). The commercial viability of ILs in lignocellulose pretreatment and saccharification depends on the biomass loading, the cost of the IL, its environmental acceptability and stability under operating conditions, and its recycling efficiency. Protic ionic liquids (PILs) are interesting from a cost and availability aspect as they are readily prepared by mixing commodity amines with inexpensive acids. Welton and coworkers (Verdía et al. 2014; Brandt et al. 2011) used a mixture of the PIL, 1-butylimidazolium hydrogen sulfate, [bhim][HSO_4], and water (80:20 v/v) for dissolution and subsequent cellulase-catalyzed saccharification of switchgrass. In another study (George et al. 2015), a range of PILs containing the hydrogen sulfate anion, the cost of which is primarily determined by the price of the amine, was prepared by simply mixing the corresponding amines with sulfuric acid. The best results were obtained with triethylammonium hydrogen sulfate, [Et_3NH] [HSO_4]. Similarly, Henderson and coworkers (Achinivu et al. 2014) used pyrrolidinium acetate for extracting lignin, with little or no cellulose extraction, from lignocellulosic biomass. The PIL was recovered by vacuum distillation.

Deep eutectic solvents (DESs), formed by mixing a salt with a hydrogen bond donor and gently heating, constitute inexpensive alternatives to ILs and PILs for lignocellulose pretreatment (see Fig. 7.7 for structures of Ils, PILs, and DESs). An example of a DES derived from renewable raw materials is the 1:2 mixture of choline chloride and glycerol (Ch/Gly). Aqueous ChCl/Gly was effective as the reaction medium for the pretreatment and saccharification of lignocellulose from switchgrass (Xia et al. 2014). Similarly, ChCl-based DESs were successfully used in the pretreatment and saccharification of lignocellulose from empty fruit bunches of the oil palm (Nor et al. 2016), corncob residues (Procentese et al. 2015), and wheat straw (Jablonski et al. 2015). Kroon and coworkers (Francisco et al. 2012; Kroon et al. 2013) screened a range of natural deep eutectic solvents (NADES), derived from mixtures of CHCl or natural amino acids as hydrogen bond acceptor and natural carboxylic acids as hydrogen bond donor, as solvents for biopolymers (lignin, cellulose, and starch). Most combinations exhibited high lignin solubilities and negligible solubility for cellulose, and, hence, are potential candidates for lignocellulose fractionation. Similarly, Kumar and coworkers (Kumar et al. 2016) recently reported the use of NADES, such as CHCl/lactic acid, in the pretreatment of rice straw lignocellulose, with separation of high-quality lignin and holocellulose in a single step. Chitin was also shown to dissolve in DESs such as CHCl/Gly (Sharma et al. 2013).

7.5.2 Enzymatic Hydrolysis of Cellulose and Hemicellulose

Hydrolysis of cellulose and hemicellulose to fermentable sugars requires the involvement of a complex cocktail of cellulolytic and hemicellulolytic enzymes (Bhattachariya et al. 2015) in a process referred to as saccharification (Bornscheuer et al. 2014). The hydrolysis of cellulose involves catalysis by at least five enzymes:

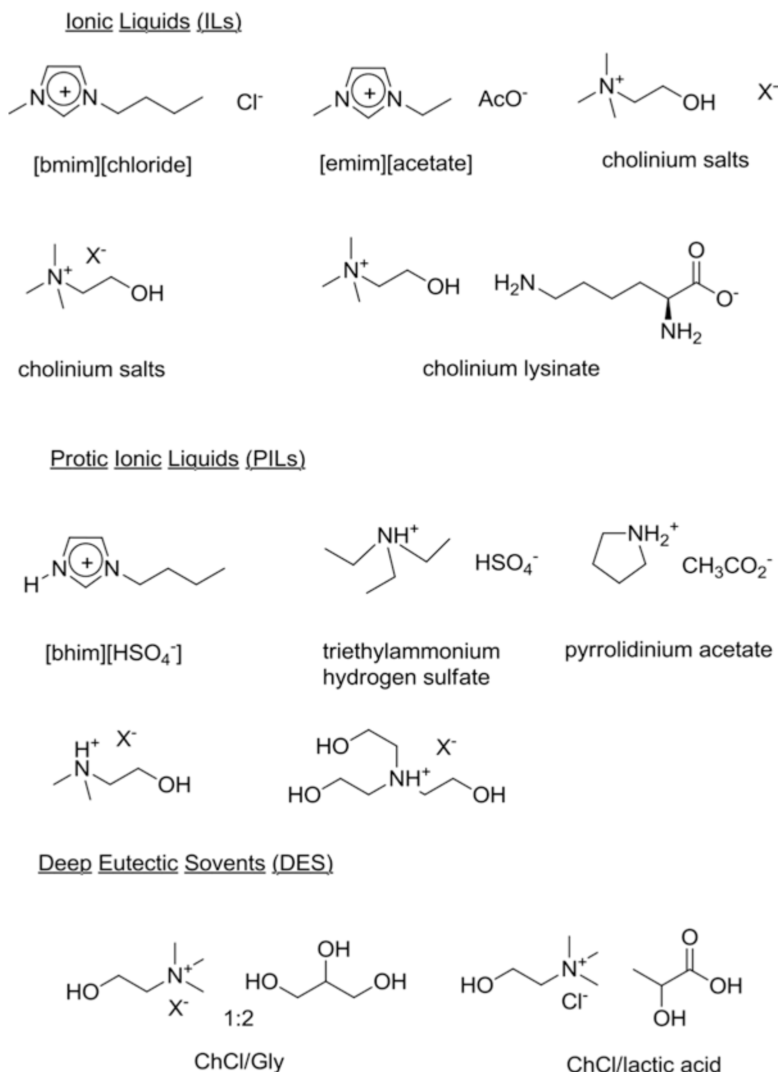


Fig. 7.7 Structures of ionic liquids (ILs), protic ionic liquids (PILs), and deep eutectic solvents (DESs)

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 (LPMO) (Johansen 2016; Hemsworth et al. 2015; Walton and Davies 2016), which catalyze the oxidative cleavage of polysaccharides. Hemicellulose has a more complicated structure than cellulose and requires a diverse suite of enzymes to effect its hydrolysis to its constituent sugars, mainly xylose and mannose. These enzymes can be

divided into two groups: core enzymes that catalyze cleavage of the polysaccharide backbone and ancillary enzymes that perform the removal of functional groups. Examples of the core enzymes 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). Ancillary enzymes include β-glucuronidase (EC 3.2.1.139), acetyl xylan esterase (EC 3.2.1.55), ferulic acid esterase (EC 3.1.1.73), and *p*-coumaroyl acid esterase (EC 3.1.1.B10).

In vivo these enzymes are contained in multi-enzyme complexes, so-called cellulosomes (Artzi et al. 2017) produced by many cellulolytic fungi and bacteria. Cellulosomes have a distinct advantage compared to simple mixtures of the free enzymes owing to the close proximity of the enzymes in the former. This advantageous close proximity of the individual enzymes can be mimicked in combi-CLEAs without many of the disadvantages of cellulosomes.

For example, a xylanase-mannanase combi-CLEA was prepared and successfully applied in the conversion of lime-pretreated sugarcane bagasse and milled corn stover (Bhattacharya and Pletschke 2015). The authors concluded 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 that 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 (Periyasamy et al. 2016). The combi-CLEA was successfully used in the hydrolysis of ammonia-cooked sugarcane bagasse and could be recycled six times. Various groups have reported the successful immobilization of a cellulase cocktail as cross-linked enzyme aggregates (CLEAs) (Dalal et al. 2007b; Perzon et al. 2017; Jones and Vasudevan 2010; Li et al. 2012; Jamwal et al. 2016). Interestingly, hybrid cellulase CLEAs containing a silica core, prepared by physical adsorption of cellulase CLEAs on a highly porous silica support, exhibited twice as much activity as the regular CLEA, and settled better after the hydrolysis, thus facilitating its separation (Sutarlie and Yang 2013).

7.5.3 Magnetic Immobilized Enzymes in Lignocellulose Conversion

Probably the most exciting and challenging development in the processing of lignocellulosic biomass is the use of magnetic immobilized enzymes, to provide the possibility of achieving substantial cost reductions by magnetic separation and recycling of the enzymes (Asgher et al. 2014). Immobilization of the cellulase enzyme cocktail on magnetic particles has been extensively studied, either on prefabricated magnetic (nano)carriers (Alftren and Hobley 2014; Abraham et al. 2014; Zhang et al. 2015b, 2016; Jordan et al. 2011; Khoshnevisan et al. 2011; Zang et al. 2014; Roth et al. 2016) or as magnetic CLEAs (Khorshidi et al. 2016; Xie et al. 2012; Jia et al. 2017), but activities were generally measured only in the hydrolysis of the

water-soluble carboxymethyl cellulose as a model for the complex mixture derived from lignocellulose. Immobilization of β-glucosidase, one of the enzymes contained in the cellulase cocktail, on magnetic silica-based particles has also been described (Alftren and Hobley 2013). It is also worth noting that it may not be essential to immobilize all the enzymes in the cellulase cocktail. Immobilization and recycling of a selection of the enzymes present could lead to significant cost reductions.

Bhattachariya and Pletschke (2014) prepared mCLEAs of a bacterial xylanase and observed that incorporation of Ca^{2+} ions in the CLEA led to increased thermal stability. The Ca-mCLEA exhibited 35% more activity than the free enzyme and a ninefold higher sugar release from ammonia pretreated sugarcane bagasse. Similarly, Illias and coworkers (Shaarani et al. 2016) prepared mCLEAs of a recombinant xylanase from *Trichoderma reesei* using maghemite (γ-Fe_2O_3) rather than the more usual magnetite (Fe_3O_4) nanoparticles.

As discussed in Sect. 7.5.1, much attention is currently being devoted to lignocellulose pretreatment in ILs and DESs or their mixtures with water. Obviously, it would be attractive to conduct subsequent enzymatic hydrolysis in the same medium, which requires that the cellulase enzyme cocktail be active and stable in the presence of ILs and/or DESs, which has been demonstrated with [emim] [OAc] (Datta et al. 2010). However, to our knowledge, such magnetic cellulase CLEAs have not yet been used in IL- or DES-containing media. Xu and coworkers (Xu et al. 2015) used a cellulase from *Trichoderma aureoviride*, encapsulated in alginate beads, in the enzymatic in situ saccharification of rice straw pretreated in aqueous [emim] [$(MeO)_2PO_2$]. Interestingly, Ogino and coworkers (Nakashima et al. 2011) combined pretreatment and saccharification with fermentation in a direct SSF production of bioethanol by a cellulase-displaying, ionic liquid-resistant yeast. The cellulase enzyme cocktail is known to be active and stable in designer ILs and DESs (Nakashima et al. 2011). For example, cellulase was engineered to improve its stability in mixtures of CHCl/Gly and concentrated seawater as a low-cost reaction medium for enzymatic hydrolysis of cellulose (Lehmann et al. 2012).

7.6 Conclusions and Future Outlook

The costs of the hydrolytic conversion of first- and second-generation biomass to fermentable sugars can be optimized by reducing the costs of the enzymes involved, which are currently used on a single-use, throw-away basis. This change can be achieved by enabling the recycling of the enzymes via immobilization. However, this move can be challenging because of the heterogeneous nature of the reaction mixture, which contains various suspended solids. A potentially attractive methodology is to produce magnetic immobilized enzymes, such as mCLEAs, which can be separated from other suspended solids on an industrial scale using commercially available equipment. Second-generation biofuels are still at the beginning of the learning curve, and significant future cost reductions are still needed to achieve

commercial viability. In this context, cost-effective immobilization and recycling of the complex enzyme cocktail involved can make an important contribution. In addition, we expect that such technologies will, in the future, be widely applied to conversions of polysaccharides in other areas such as food and beverage processing (Sojitra et al. 2016; Dal Magro et al. 2016).

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