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Thermostable Glycoside Hydrolases in Biorefinery Technologies

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Abstract: Glycoside hydrolases, which are responsible for the degradation of the major fraction of biomass, the polymeric carbohydrates in starch and lignocellulose, are predicted to gain increasing roles as catalysts in biorefining applications in the future bioeconomy. In this context, thermostable variants will be important, as the recalcitrance of these biomass-components to degradation often motivates thermal treatments. The traditional focus on degradation is also predicted to be changed into more versatile roles of the enzymes, also involving specific conversions to defined products. In addition, integration of genes encoding interesting target activities opens the possibilities for whole cell applications, in organisms allowing processing at elevated temperatures for production of defined metabolic products.

In this review, we overview the application of glycoside hydrolases related to the biorefining context (for production of food, chemicals, and fuels). Use of thermostable enzymes in processing of biomass is highlighted, moving from the activities required to act on different types of polymers, to specific examples in today's processing. Examples given involve (i) monosaccharide production for food applications as well as use as carbon source for microbial conversions (to metabolites such as fuels and chemical intermediates), (ii) oligosaccharide production for prebiotics applications (iii) treatment for plant metabolite product release, and (iv) production of surfactants of the alkyl glycoside class. Finally future possibilities in whole cell biorefining are shown.

Keywords: Amylase, antioxidant, biofuels, biomass, cellulase, glucanase, glucosidase, hemicellulose, lignocellulose, mannan, pectin, prebiotics, starch, surfactant, xylan, xylanase.

1. INTRODUCTION

A competitive biobased society is built upon efficient utilization of renewable raw materials instead of fossil resources for production of chemicals, materials and energy in sustainable interplay with food and feed production. The major feedstock for the industry will gradually shift to a plethora of biobased raw materials, including waste streams from related industries. To date, the political initiatives to support a transformation into a fossil free society have exclusively focused on the production of biobased energy. Examples are the 20-20-20 goals (20% greenhouse gas reduction, 20% of energy from renewables and 20% increase in energy efficiency in 2020) set up by the EU [1] and a number of supportive initiatives on national level [2]. Moreover, the U.S. DOE (Department of Energy) Energy Independence and Security Act (EISA) mandates a national production level of 36 billion gallons of biofuels (with 21 billion gallons derived from renewable/sustainable feedstocks) by 2022 [3], and China aims at generating 15% of its energy consumption from nonfossil fuel sources by 2020 [4]. However, the production of value added chemicals and materials will help to drive the development of the new bioeconomy, due to their higher profit margins. Hence, co-production of chemicals, materials and energy in biomass refineries will be economical and allow for a resource efficient utilization of the biomass.

Use of biomass for production is nothing new. In the early 20th century, the major part of chemicals and materials were produced from biomass. Along with increasing use of fossil raw materials for energy and transport, biomass was gradually out-competed for chemicals production by cheap and increasingly available fossil hydrocarbons [5]. About one century later, and in the light of climate change and exhausted fossil resources, biomass is getting renewed attention as raw material for process industry. Today, the chemical industry meets about 8% of its demand for raw materials with renewable feedstock and about 5% of all fine chemicals are produced with enzymatic and microbial processes [6]. To increase those numbers, the process industry will need to adapt their processes and value chains to be successful in replacing the well incorporated fossil based production methods with new technology platforms that are tailored for conversion of biomass. Such technology platforms will form the core of a biorefinery where biomass is refined into chemicals, materials and energy by the use of chemical, biotechnical and thermal processes.

A key enabling technology that by the European Commission has been identified to play an important role in replacing non-renewable materials with renewable resources is industrial biotechnology [7] which involves the use of microorganisms and their enzymes for production. These technologies are together with chemical synthesis also considered when potential top value added chemicals from biomass were identified at Pacific Northwest National Laboratory (PNNL) and National Renewable Energy Laboratory (NREL) in the US. Identification of top candidates was in this work made using an iterative review-

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process based on current petrochemical building blocks, chemical data, properties and known market data [8]. Microbial enzymes perform a number of important reactions needed to process biomass into products that will meet the consumer needs. While carbohydrates are the most abundant components of biomass, enzymes capable of using polysaccharides as substrate will be important in order to perform a number of reactions leading to useful products. This review highlights the use of one important group of enzymes in a biorefinery context, the thermostable glycoside hydrolases. Glycoside hydrolases are enzymes capable of degrading carbohydrates in the form of both starch and lignocellulose, and thus have a natural role in biorefining. Processing of starch and lignocellulose is often involving thermal treatments to facilitate hydrolysis, hence giving thermostable variants of the glycoside hydrolases a role in this context. However, other transformations performed by these enzymes may be even more important in the future, and the purpose of this review is to give an overall view of biorefining, and on the roles of glycoside hydrolases in current and future processing of biomass.

2. BIOREFINERIES: CONCEPTS AND DEFINITIONS

In a biorefinery, raw materials from forest, agriculture, marine sources and various waste streams will be converted into chemicals, materials and energy hand-in-hand with sustainable production of food and feed (Fig. 1). According to IEA (International Energy Agency), biorefining is the sustainable processing of biomass into a spectrum of marketable biobased products and bioenergy [9]. They distinguish between Energy Driven biorefineries and Products Driven biorefineries (www.iea-bioenergy.task42-biorefineries.com/activities/classification/). Energy driven biorefineries mainly produce energy carriers from biomass with a concomitant valorisation of residues to biobased products to maximize the value of the biomass. Likewise, product based biorefineries focus on the production of chemicals, materials, food and feed from biomass where remaining residues from the process are used for value-added bioenergy production, either for internal or external use. In an ideal biorefinery, the production takes place with as low impact on the environment as possible and in an energy saving and carbon dioxide neutral manner. Moreover, the products will easily be reabsorbed in the rotation without any negative environmental impact. Hence, it is important to design new processes and value chains from a system analysis perspective.

There are different opinions about how complex a process plant has to be before it is considered a biorefinery. Traditionally, a starch factory can be described as a biorefinery, since a refining of biomass takes place. However, for a transition into a higher degree of biomass utilization, more complex biorefining strategies will be needed. Kamm & Kamm [10] grouped biorefineries according to their complexity, which fits the continuous evolvement of the concept towards a higher utilization of several types of biomass for the production of several different kinds of products (Fig. 2).

The starch factory referred to above can be classified as a phase I biorefinery, from which one type of biomass, in a

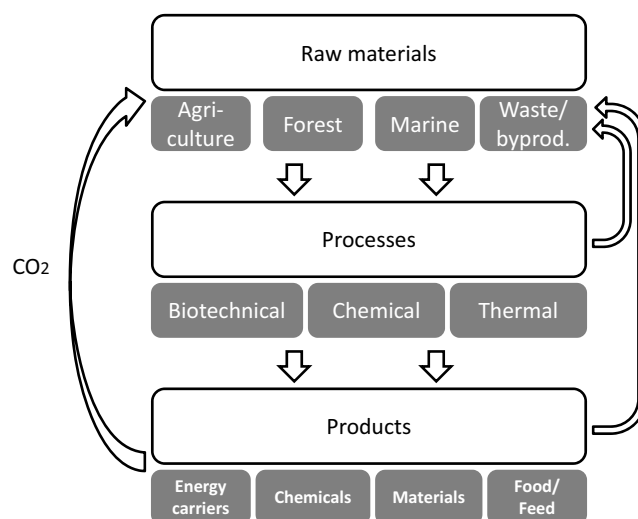


Fig. (1). The biorefinery concept for a sustainable production.

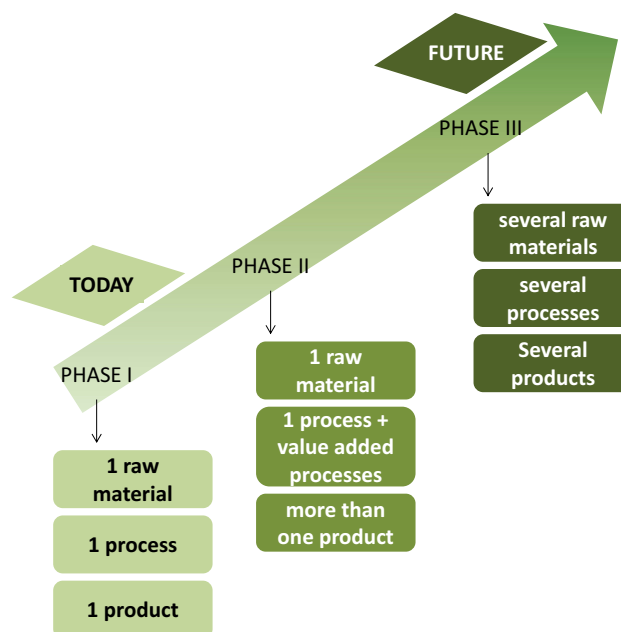


Fig. (2). Classification into phase I, II and III biorefineries according to Kamm & Kamm [10].

rather non-flexible process, gives one product. In a phase II biorefinery, new process technology is used in combination with traditional processes to produce several end products from the biomass, making use of side streams and byproducts. One example of a phase II biorefinery is Domsjö factories in Sweden, where forest raw materials are refined into specialty cellulose, lignosulphonate and bioethanol (www.domsjo.adityabirla.com). The concept also includes energy supply, a purification plant and other infrastructure. In Norway, Borregaards plant in Sarpsborg is another example where the goal is to use as much as possible of the forest raw materials to produce a range of products (www.borregaard.com). In Denmark, Dong Energy and Novozymes and others are collaborating around building a phase II biorefinery for the co-production of bioethanol, biogas, electricity and district heating, using straw as raw

material (www.maabjergenergyconcept.eu). Other examples can be found in many European countries, the US and Brazil. Further development of the concept leads to a phase III biorefinery where not only a range of different products are produced, but also a range of different raw materials and processes are utilized. Phase III biorefineries are characterized by a raw material flexibility which will have a large impact on the development of cost efficient industrial processes (Fig. 2).

There is no generally accepted classification principle of biorefineries, and in literature a number of different naming strategies can be found. Biorefineries can be classified: (i) according to the feedstock used, such as forest-based biorefineries, marine biorefineries, whole crop biorefineries, lignocellulosic biorefineries, or (ii) according to the technology platform used with examples such as the thermochemical biorefinery or biochemical biorefinery. Another classification strategy is based on which product that is produced such as the syngas platform, biodiesel platform or the oleochemical biorefinery [9].

As stated above, industrial biotechnology is defined as the utilization of microorganisms and their enzymes in industrial applications. It is thus one of the technology platforms that will have a great importance in a biorefinery for the development of processes that are suitable for the conversion of biomass into products. Microorganisms and their enzymes are biotechnical tools that nature has designed to utilize biomass that is present in the habitat around them. Hence, nature offers a vast diversity and a large pool of tools evolved to process a wide range of raw materials. Enzymes that are capable of degrading and modifying carbohydrates will play an important role for conversion of starch and lignocellulosic raw materials in a biorefinery. To show the role of glycoside hydrolases in this context, the main carbohydrate fractions available in biomass are described below, highlighting the types of glycoside hydrolases that are required for their degradation.

3. BIOMASS FEEDSTOCKS

Biomass is an abundant carbon-neutral renewable source for the production of energy, platform chemicals and biomaterials, and it is the most promising substitute of crude oil [11-13]. Biomass feedstocks can be grouped into two categories: oleaginous and carbohydrate rich [14]. The main components of oleaginous feedstock are triglycerides and free fatty acids. Current use of oleaginous feedstock in biorefining is mainly the production of biodiesel by esterification with alcohols such as methanol [15]. The carbohydrates are, by far, the most abundant components of biomass, and are mainly polysaccharides. The carbohydrate fraction of the feedstocks can be roughly grouped into two main categories: starch and lignocellulose. In the biorefinery context, polysaccharides in these two categories can either be used in polymeric form (e.g. cellulose fibers in textiles or paper) or be degraded into oligo- and monosaccharides. The oligo- and monosaccharides can in turn either be used in food/feed applications or be further converted in the biorefinery. Irrespective of the source of biomass, a first step in biorefining procedures is fractionation [16]. After this step (which may include additional pretreatments), selective degradation can be obtained by enzymatic hydrolysis using

glycoside hydrolases, and the action of major enzyme types on starch and lignocellulose fractions of biomass are shown below.

3.1. Starch and its Enzymatic Degradation

Starch is a ubiquitous and easily accessible source of energy and it is usually stored as large starch granules in the cytoplasm of plant cells, seeds or tubers. Starch is synthesized by plants as energy store. Cultivated plants, such as corn, potatoes, cassava, wheat, rice and other species rich in starch are thus also a main source of energy in food and feed. Starch is composed of two high-molecular-weight polymers: amylose (30%), a linear chain of α -1,4-linked glucopyranose residues, and amylopectin (70%), a branched chain of α -1,4-linked glucopyranose residues with α -1,6-linked branch points every 20-25 glucose units. Amylose molecular weight is between 5×10^5 and 10^6 g/mol, while the molecular weight of amylopectin is several millions [17]. Starch granules are insoluble in cold water, which makes the first extraction from plants relatively easy. To further process the granules, the water-starch slurry is heated (gelatinization), until a point where the granules break apart into a viscous colloidal solution, which upon cooling (retrogradation) forms an elastic gel [18].

Starch degradation (which ideally starts before retrogradation) requires a set of hydrolases with different enzyme activities (Fig. 3). As heating is part of the processing, thermostable enzymes are generally used. The enzymes can be roughly grouped into three categories: endo-, exo-acting and debranching enzymes [19-21]. Endo-acting enzymes hydrolyze linkages in the interior of the starch in a random fashion, involving mainly α -amylase (EC 3.2.1.1, in GH13, 14, 57 and 119), which yields linear and branched oligosaccharides. Exo-acting enzymes hydrolyze starch from the non-reducing end, yielding small and well-defined sugars. Thus, β -amylases (EC 3.2.1.2, GH13 and 14) produce maltose, and γ -amylases (also termed glucoamylases, EC 3.2.1.3, GH15) release glucose. The α -glucosidases (EC 3.2.1.20, GH4, 13, 31, 63, 97 and 122) hydrolyze α -1,4-linkages, but unlike γ -amylase, liberate glucose with α -anomeric configuration. In addition, α -glucosidases do not have activity against high-molecular-weight substrates such as starch (or pullulan a related polymer of bacterial origin, Fig. 3), and participate in the last step of starch degradation.

Some enzymes, acting on starch, are reported as raw-starch degrading enzymes, i.e. enzymes acting directly on the starch granules. These enzymes have carbohydrate binding modules (CBMs) appended, which are noncatalytic entities aiding in binding and digestion of raw starch [22]. Their use in starch degradation, can somewhat reduce the need of high temperatures, but certain heating is still needed to improve amylose solubility.

Finally, debranching enzymes (also denominated pullulanases based on their action on the major bond-type in pullulan) (EC 3.2.1.41, GH13 and 57) hydrolyze α -1,6-glycosidic bonds in both amylopectin and pullulan, while isoamylases, acting on the same bond type (EC 3.2.1.68, GH13) are unable to hydrolyze pullulan. Pullulanases (EC 3.2.1.41) hydrolyze α -1,6-linkages in linear as well as in

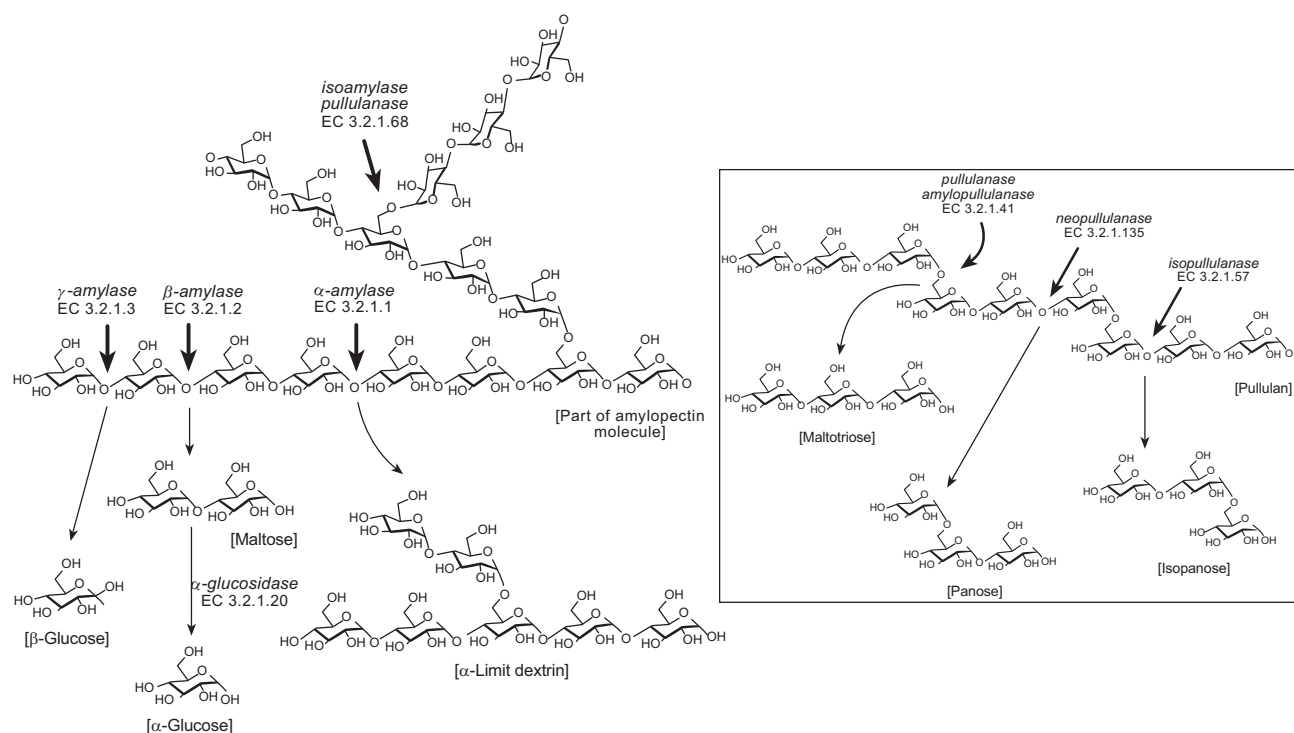


Fig. (3). Hypothetical fragment of amylopectin molecule (left) showing the enzymatic attack of glycoside hydrolases. The inserted box (right), shows glycoside hydrolases acting on the related bacterial polymer pullulan.

branched polysaccharides, while isopullulanase (EC 3.2.1.57, GH49) is able to attack both α -1,6- and α -1,4-glycosidic linkages, but is virtually inactive on starch. In addition, neopullulanase (EC 3.2.1.135, GH13) act on both α -1,6- and α -1,4-glycosidic linkages hydrolyzing pullulan to the final product panose, while isopullulanase (EC 3.2.1.57) hydrolyze pullulan to isopanose (Fig. 3). A number of reports in literature have also shown that enzymes classified as neopullulanases (EC 3.2.1.135) are indistinguishable from maltogenic amylases (EC 3.2.1.133, GH13) and cyclodextrinases (EC 3.2.1.54, GH13) [23, 24].

3.2. Lignocellulose, its Carbohydrate Components and Carbohydrate Degrading Enzymes

Lignocellulose is a complex mixture of polysaccharides and lignin. The polysaccharide fraction includes cellulose (40-50 wt%) as main component, high amounts of hemicelluloses (25-40 wt%) and pectins in a significant lower proportion. Lignin is a multi-linked heterogeneous polymer comprised of oxygenated phenylpropane units and represent 10-25wt% of the lignocellulosic biomass [14, 25, 26]. The cellulose content in different types of lignocellulosic raw materials (including wood, pulp, cane bagasse, cane straw, maize straw, rice straw, palm, corncobs, barley, oat straw, cotton straw and others) is variable. Despite this, cellulose is considered to be our most inexhaustible feedstock for the increasing demand of environmentally friendly and biocompatible products [27, 28]. Due to its abundance (predicted to allow a yearly energy outtake of approximately 100×10^{18} J [29], corresponding to approximately one quarter of current need) its utilization is desired, but due to its recalcitrance, its degradation involves

several challenges. Hydrolysis of native lignocellulose is a slow process, much slower than the hydrolysis of starch. Biorefinery applications of lignocellulose therefore rely on a combination of “pretreatments” (both mechanical and thermal) to decrease the recalcitrance of the lignocellulose, prior to enzymatic hydrolysis.

Microbial glycoside hydrolases (GHs) act on cellulose and hemicellulose components and have a great potential to degrade these materials [16, 30]. Due to the necessity of thermal pretreatments, thermostable variants of GHs are of particular interest. Different cellulose degrading enzymes are often grouped together and are simply called cellulases, a term that reflects at least three different types of activities. The major cellulose degrading enzymes are sub-categorized as endoglucanases, exoglucanases (mainly cellobiohydrolases) and β -glucosidases (Fig. 4A). Endo-glucanases (E.C. 3.2.1.4) are classified under more than 15 different GH-families (with either retaining or inverting reaction mechanism) and randomly attack β -1,4-linkages in cellulose-polymers [38]. Cellobiohydrolases cleave off cellobiose either from the reducing end (E.C. 3.2.1.176, GH7 and 48) or non-reducing end (E.C. 3.2.1.91, GH6 and 9) of the chains. The β -glucosidases (E.C. 3.2.1.21, GH1, 3, 9, 30 and 116) degrade smaller chain oligosaccharides releasing the terminal non-reducing β -D-glucosyl residue (Fig. 4A). CBMs also play a role in lignocellulose degradation and can enhance the action of for example cellobiohydrolases. In fact, it should be noted that CBMs of different binding specificities are found in many GH-families, attached to catalytic modules of varying specificity.

The above, generally accepted, view on enzymes important in lignocellulose hydrolysis was recently

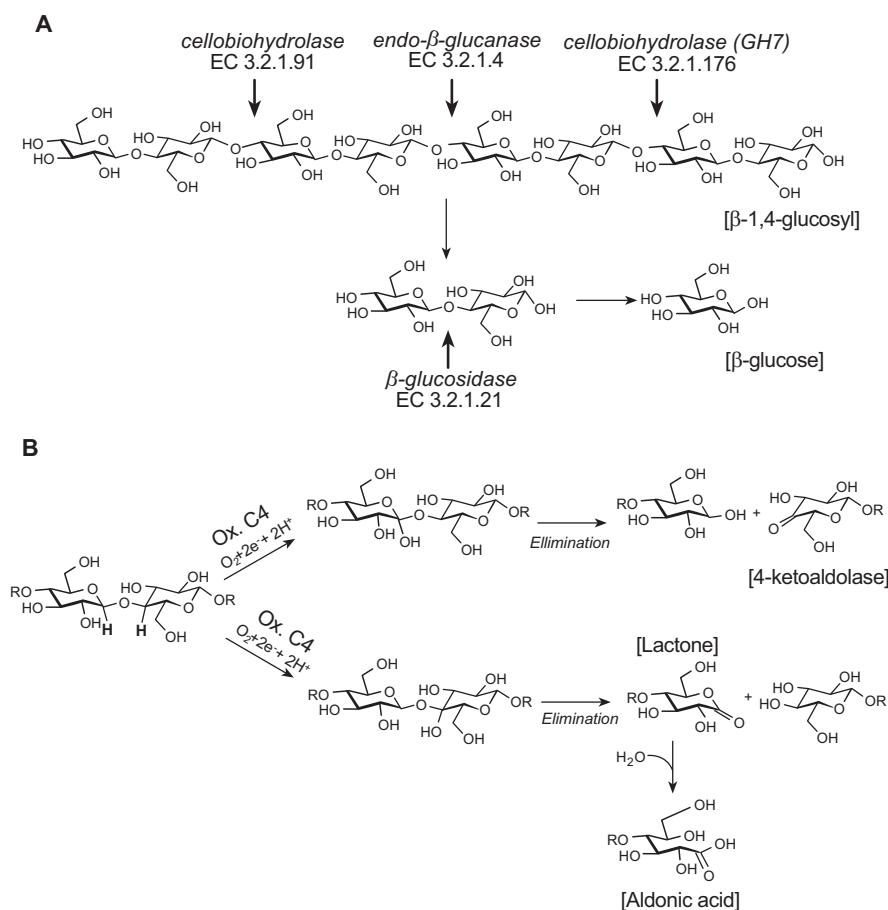


Fig. (4). Enzymatic activities acting on a hypothetical fragment of cellulose. In panel (A) the action of endoglucanase, exoglucanase of the cellobiohydrolase type, and β -glucosidase on the cellulose fragment are shown. Panel (B) shows the proposed reactions for oxidative cleavage of cellulose by polysaccharide monooxygenase family AA9 (formerly family 61, www.cazy.org) [86]. The first step of the reaction is the enzymatic oxidation of carbon C1 or C4, followed by spontaneous non-reversible eliminations yielding lactone and 4-ketoaldolase respectively. The lactone can be hydrolyzed to aldonic acid both spontaneously or enzymatically by lactonases [87].

complemented with polysaccharide monooxygenases. These enzymes use copper-dependent oxidative pathways for the cleavage of glycosidic linkages and are shown to enhance cellulose (and chitin) degradation, when added to GH cocktails [31-33]. To correctly display these enzymes in the database for carbohydrate active enzymes (www.cazy.org), a new class of auxiliary activities (AA) has been implemented in which copper dependent polysaccharide monooxygenases acting on cellulose (previous GH61 [34]) are classified under AA9 (Fig. 4B), and those mainly acting on chitin under AA10 (former CBM33 [32]).

In native lignocellulosic materials, the cellulose is embedded in hemicelluloses and lignin. Enzymes acting on the hemicellulose fraction are thus a necessity for decomposition. The two main types of hemicelluloses are xylans and mannans (Fig. 5A, B) and unlike cellulose, these polymers are heterogenous and their compositions vary dependent on the source. It has been shown that different hemicellulases, e.g. xylanases and mannanases act in synergy in the decomposition [35]. Also in hemicellulose degradation, CBMs carried by these enzymes play a role, and either enhance the action of the hemicellulases or reduce non-productive binding onto lignin [36, 37].

Xylan is the most common type of hemicellulose, and in Fig. (5A), enzymes acting on the backbone and on common substituents are shown. Endo-acting xylanases (E.C. 3.2.1.8, available mainly in GH5, 8, 10, 11, 43), dominate over exo-acting xylanases (E.C. 3.2.1.156, GH8) found in a few microbial species, while xylosidases (E.C. 3.2.1.37, in for example in GH1, 3, 39, 43, 52, 54, 116, 120) also are widespread [16, 38]. Mannan, on the other hand is the dominating hemicellulose in softwood. The enzymatic hydrolysis of (galacto)glucomannan into monomers requires the action of β -mannanases (endo-1,4- β -mannanases, EC 3.2.1.78, GH5, 26 and 113), β -mannosidases (EC 3.2.1.25, GH1, 2 and 5), and α -galactosidases (EC 3.2.1.22, GH4, 27, 36, 57, 97 and 110) (Fig. 5B) complemented with the action of *O*-acetyl(mannan) esterases when the polymer needs deacetylation [39].

Pectin is a minor part of lignocellulosic biomass, but is abundant in fruits, e.g. citrus fruit and apple, where it can form up to half of the polymeric content of the cell wall [40]. Pectin is a complex group of polysaccharides with a composition and branching that is highly variable and dependent on the source. The pectin backbone consists of (sometimes methylated) homo-galacturonic acid regions (Fig. 6), and regions with both rhamnose and galacturonic

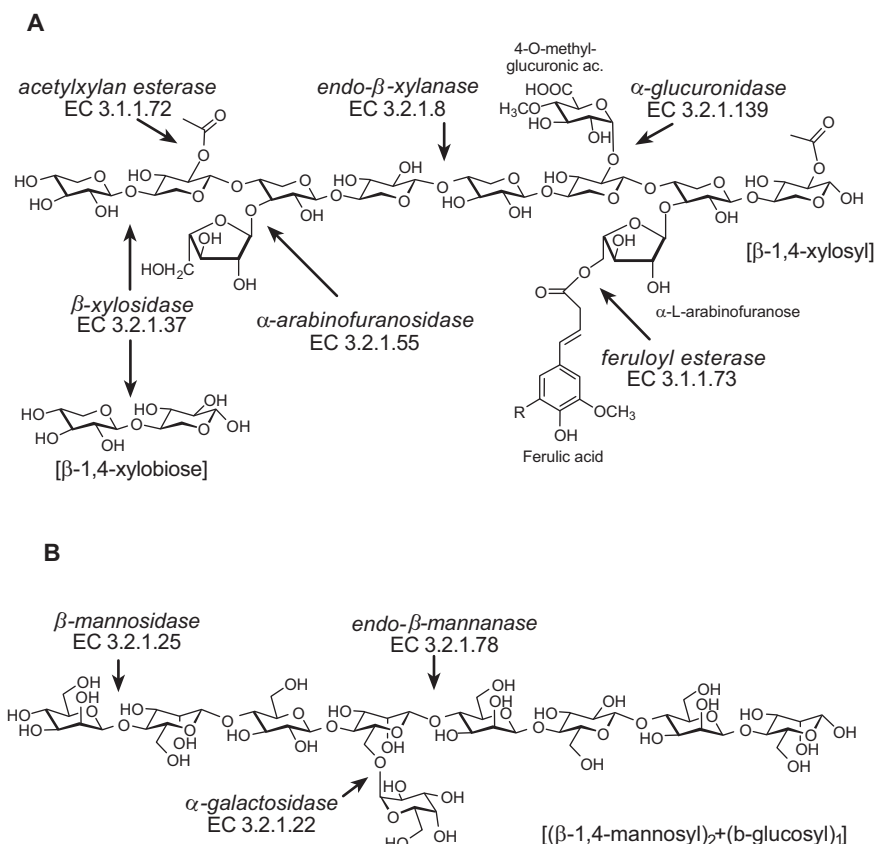


Fig. (5). Glycoside hydrolases acting on hypothetical fragments of hemicellulose: **(A)** Xylans are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β -D-xylopyranose units. In plants, like cereals where arabinose content is significant, they are termed arabinoxylans. **(B)** Galactoglucomannan is built of alternating 1,4-linked β -D-mannose and glucose residues, and when galactose content is substantial they are termed galactoglucomannans.

acid, and the polymer has neutral sugar side-chains made up from rhamnose, arabinose, galactose and xylose and single xylogalacturonan side chains [41]. Pectin has found widespread commercial use, especially in food industry e.g. as thickener, emulsifier, stabilizer, filler in confections, dairy products, and bakery products etc. The pectin polymer can however also hinder release of other types of natural products and for this purpose pretreatment with pectin degrading enzymes can be useful.

Pectin degrading enzymes act by hydrolysis or trans-elimination, the latter by lyases [16]. Endo-polygalacturonase (EC 3.2.1.15) (Fig. 6), exopolygalacturonase (EC 3.2.1.67), and exopolygalacturanosidase (EC 3.2.1.82) all classified under GH28, are along with α -L-rhamnosidases (EC 3.2.1.40, in GH family 28, 78 and 106) acting on different parts of the pectin backbone. Polysaccharide lyases (PL), e.g. pectin lyase (EC 4.2.2.10), pectate lyase (EC 4.2.2.2), and pectate disaccharide-lyase (EC 4.2.2.9) [42-44] also cleave the galacturonic acid part of the backbone. Side-chain acting enzymes include endo-arabinase (EC 3.2.1.99, GH43) that hydrolyze arabinan side chain, and α -L-arabinofuranosidases along with a number of other glycosidases hydrolyzing monosaccharide substituents and pectinesterase (EC 3.1.1.11) de-esterify the methyl ester linkages to the pectin backbone [43].

4. GLOBAL MARKETS FOR GLYCOSIDE HYDROLASES: PRODUCTION AND PROSPECTING

Glycoside hydrolases are already today applied in industrial scale in technical industry, food manufacturing, animal nutrition, and cosmetics industry and the enzymes applied are frequently thermostable (Table 1). Even though numerous enzymes are known it is, according to Li and coworkers, only about 20 enzymes that are yet produced on a truly industrial scale [4]. Li *et al.* have recently reviewed the international enzyme production structure, and showed that nearly 70% of total enzymes sales were produced by two enzyme companies: Denmark-based Novozymes, and US-based DuPont (through the acquisition of Denmark-based Danisco). The remaining sales were shared between several other companies including DSM (Netherlands), BASF (Germany), Roche (Switzerland) and Amano (Japan).

Hydrolytic enzymes (glycoside hydrolases, proteases and lipases) dominate the enzyme market, accounting for more than 70% of all enzyme sales. The global enzymes market is still dominated by the food and beverage industries, and is based on expansion of the middle class population in developing countries, estimated to rise to reach \$4.4 billion in 2015 [45]. Technical enzymes are typically used as commodities in detergents, textile, pulp and paper, organic

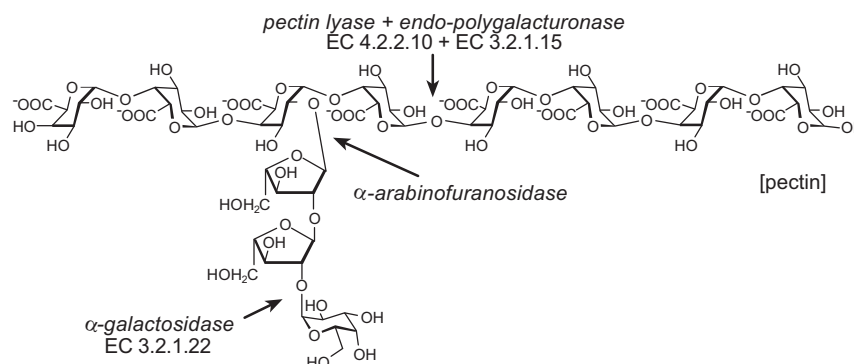


Fig. (6). A simplified view on a hypothetical fragment of pectin, showing a few examples of commonly characterized glycoside hydrolase acting on these types of polymers.

synthesis and biofuels industry (Table 1). These enzymes have an estimated value of approximately \$1 billion in 2010 which is expected to reach \$1.5 billion in 2015, with highest sales in the bioethanol market. In this field, there is also large potential in creating building blocks for platform and speciality chemicals (Table 2) making it an interesting sector for process development in the biorefinery context. The GHs sold for feed processing are foremost in use for swine and poultry, while a rise can be predicted in aqua culture as well as for ruminant nutrition, and this sector is expected to reach \$727 million in 2015 [46]. Moreover, cosmetics is a sector that by marketing institutes has been predicted to increase its use of enzymes, with a growth of 5% per year up to 2015 predicted, driven by both technological development of enzymes and increased consumer awareness [4].

As stated in the introduction, many worldwide institutes and corporations have recognized bio-based technologies as a key driver of sustainable growth, but a major bottleneck is that biocatalytic processes are often considered only when traditional chemistry fails in synthesis of the target molecule (Fig. 7). This is believed to be a consequence of a combination of the relatively few commercially available enzymes (opposed to the plethora of enzymes reported in scientific literature) and difficulties to choose the best candidate (among variants with the same EC-number) for the specific target reaction.

Recent success of genome and metagenome sequencing has resulted in an explosion of information available from sequence databases. By accessing extreme habitats such as hot springs and volcanic vents, these methods undoubtedly aid in finding genes coding for enzymes functioning at a variety of physicochemical conditions (pHs, temperatures, solvents), and the potential to find novel biocatalysts is enormous. This enormous sequence-based information however needs to be accessed in a way that allows selection of promising candidates. Development of bioinformatic tools promoting biocatalyst selection is thus a bottleneck, and needs to be combined with better availability of enzymes for potential users (Fig. 7). In this field it is inevitable, that biochemically characterized enzymes of known sequence and structure are necessary, in order to be able to develop prediction tools. Without such knowledge, the remaining option is to operate *via* high throughput methods for screening and development, which will limit process

development, due to the demand of laborious and time consuming procedures to find a suitable biocatalyst for each individual process. Selection possibilities in this field will likely expand the interest in thermostable glycoside hydrolases, allowing their application in relevant sectors.

5. WHY AND WHEN ARE THERMOSTABLE GLYCOSIDE HYDROLASES USEFUL?

The stability of the catalyst and the possibility to use it repeatedly, have always been major challenges in development of biocatalytic reactions (Fig. 8). Temperature, along with different chemical agents, has always been a factor that can promote enzyme inactivation. Hence, in the beginning of the exploitation of thermophiles and thermostable enzymes there was a great belief in that these enzymes, due to their robustness, would be major biocatalytic tools in numerous applications. Several advantages were obvious such as better storage stability, better solubility of substrates/products, lower viscosity, as well as a more favourable equilibrium in endothermic reactions [47, 48]. The very optimistic belief on the commercialization of thermostable enzymes has with time been replaced by the more sober realization that the strength of these enzymes lies in applications where their activity at high temperatures, and not only their thermostability, is a true advantage. The high temperature optimum for activity (normally in the range 55- 110 °C), may actually be a drawback in some applications (Fig. 8), e.g. in detergents for laundry where the temperature demand will result in increased energy consumption. It is also evident that thermostable enzymes, along with all other enzymes, suffer from the drawback that their production costs are often significant.

Storage stability, as well as stability at the reaction conditions are however advantages of general importance, and are valid for most biocatalytic reactions, irrespective of the temperature conditions. This has resulted in trials, where thermoactive enzymes are run at conditions below their optimum temperature for activity, with lower specific activity, instead taking advantage of the increased stability of the enzyme-scaffold [49, 50]. Other listed advantages (Fig. 8) are coupled to benefits of actually running reactions at higher temperature. Hence, interest in thermostable glycoside hydrolases has increased with increasing need to

Table 1. Examples of glycoside hydrolases with established use in industrial processes. Commercial enzymes in these processes are commonly thermostable glycoside hydrolases. (information collected from Ekman *et al.* [50], Li *et al.* [4], Svensson *et al.* [141] and Turner *et al.* [16].

Field	Branch	GH-Activity	Function
Technical industry	Pulp & paper processing	α -amylase	Starch hydrolysis to reduce viscosity. For surface sizing in coatings
		cellulase	Cellulose fiber modification (hydrolysis), to improve softness, making fibers flexible.
		xylanase, mannanase	Hydrolysis/removal of hemicellulose (xylan, glucomannan). Due to co-removal of lignin enhancing brightness and bleaching efficiency
	Textile manufacture	α -amylase	Starch hydrolysis, for desizing without harmful effects on the fabric.
		cellulase	Cellulose fiber modification for removal of fuzz and microfibers gives a smoother/glossier appearance to the fabric. Loosening indigo dye on denim for a slightly worn look.
		pectinase	Pectin hydrolysis. Destabilizing the outer cell layer to improve fiber extraction
	Detergent production	α -amylase	Additive to laundry detergent. Removing resistant starch residues.
		cellulase	Additive to laundry detergent. Modifying the cellulose fiber to increase color brightness and soften cotton.
	Chemicals production	GH with transferase activity	Synthesis of compounds with glycosidic bonds utilizing e.g. α -fucosidases, sialidases, glucosidases, CGTases
Food industry	Dairy industry	α -amylase, glucoamylase	Degradation of starch to monomers for fermentation by ethanol producing microorganisms.
		cellulase, xylanase (hemicellulases)	Degradation of cellulose and hemicellulose fibers to monomers for fermentation by ethanol producing microorganisms.
	Baking industry	amyloglucosidase	Additive to toothpastes, mouthwashes and skin conditioning products.
		galactosidase (lactase)	Hydrolysis of lactose in milk to glucose and galactose to avoid lactose intolerance.
	Juice industry	xylanase	Improving dough stability
		α -amylase	Degrading starch in flours and controlling the volume and crumb structure of bread.
		amylases, glucoamylases	Starch degradation (to glucose). Clarifying cloudy juice (especially apple juice).
		pectinase	Degrading pectins which are structural polysaccharides present in the cell wall, to increase the overall juice production.
		(cellulase, hemicellulase)	Lowering viscosity and affecting texture in juice. (due to legislations, not applicable in the EU).
		naringinase, limoninase	Acting on polyphenolic compounds that cause bitterness in citrus juices
	Starch processing	α -amylase	Cleaving α -1,4-glycosidic bonds, decreasing molecular weight and viscosity of polymers in starch.
		pullulanase	Attacking α -1,6-linkages, liberating straight-chain oligosaccharides linked by α -1,4-bonds.
		neopullulanase (amylopullulanase)	Acting on both α -1,6- and α -1,4-linkages.
		β -amylase	Cleaving α -1,4-linkages (from non-reducing end). Producing low-molecular weight carbohydrates, such as maltose and β -limit dextrin.
		glucoamylase	Attacking α -1,4-linkages and α -1,6-linkages from the non-reducing ends to release β -D-glucose
		isoamylase	Hydrolyzing α -1,6-linkages.
		GH with glycosyltransferase activity	Transferring a 1,4- α -D-glucan segment to a primary hydroxy group in another glucan chain. (can for example be used to change functional properties such as solubility and viscosity.
Animal feed Industry	Brewing Industry	α -amylase	Hydrolyzing starch to reduce viscosity, and to increase maltose and glucose content in starchy materials (e.g. wheat, barley).
		pullulanase	Securing maximum fermentability of the wort (by hydrolyzing α -1,6 branch points).
		β -glucanase	Hydrolyzing glucans to decrease viscosity and improve filterability.
		amyloglycosidase	Increasing glucose content, and thus fermentable sugars in beer.
		xylanolytic enzymes	Hydrolyzing xylans of malt, barley and wheat to improve extraction and filtration
		xylanase	Degrading fibres in viscous diets.
		α -amylase	Digesting starch
		α -galactosidase, glucanase, polygalacturonase	Nutrition improvement in feed for swine and poultry

Table 2. Selected building blocks (products) from the sugar platform (produced by hydrolysis of lignocellulose or starch from biomass) identified as top value products. The products are from the top 50 list, based on current petrochemical building blocks, chemical data, properties and known market data. Use of combined biocatalysis fermentation, and chemical synthesis in the production routes is considered. Selection from Aden *et al.* [8].

Product	Rationale and Route
Ethanol	Major use as fuel. Limited use as building block. Produced <i>via</i> microbial fermentation of glucose (originating mainly from starch or cellulose).
Ethylene glycol	Antifreeze product, building block for polymer production. Produced by hydrogenolysis of xylitol (below).
Lactic acid	Building block for polymers. Produced <i>via</i> microbial fermentation (fermentation by microorganisms, growing on monosugars from cellulose or starch).
Arabinitol	Production of anhydro-sugars, unsaturated polyester resins. The procedure is not established, but difficulties are not anticipated as hydrogenation of glucose to sorbitol is straight forward. Arabinitol is produced <i>via</i> hydrogenation of arabinose (in turn originating from arabinose in hemicellulose and pectin).
Furfural	Produced at large scale for application as solvent or building block for resins. Applied as flavour compounds and in the manufacture of pharmaceuticals. Furfural can be formed during lignocellulose pretreatment. It is e.g. formed abiotically by threefold dehydration of xylose (can be catalyzed by mineral acid). (Origin: hemicellulose).
Xylitol	Used as non-nutritive sweeteners, and in production of anhydro-sugars and unsaturated polyester resins. Produced by hydrogenation of xylose to xylitol, <i>via</i> chemical synthesis or biocatalysis. (Origin: hemicellulose).
Xylonic acid	Selective oxidation of alcohols (ROH) such as xylitol to acids (RCOOH). New field. (From hemicellulose).
2,5-furan dicarboxylic acid (FDCA)	Produced <i>via</i> biotransformation of hydroxymethyl-furfural (originating from hexoses). FDCA has a large potential as a replacement for terephthalic acid, a widely used component in various polyesters, such as polyethylene terephthalate (PET) and polybutyleneterephthalate (PBT).
Sorbitol	Used as chemical. Produced <i>via</i> hydrogenation of glucose, which has been described as straight forward. (The origin of glucose is mainly cellulose or starch).

MAJOR BOTTLENECKS

- Biocatalytic processes considered first when traditional chemistry fails
- Relatively few commercially available enzymes as opposed to the number of published enzymes
- Methods to select enzymes that catalyze the desired reaction in an efficient manner are underdeveloped

Fig. (7). Major bottlenecks for the implementation of biocatalytic processes.

utilize lignocellulose as feedstock, as this material is recalcitrant to hydrolysis and requires thermal treatment for degradation. A simple search in Google patent, using only the search terms “thermostable” and “glycoside hydrolase”, for example showed that the number of patents issued yearly has more than three-doubled (from approximately 30 to more than 100) during the last 5-year period. In the first half of 2013, approximately 50% of the issued patents were related to degradation of lignocellulosic biomass or bioenergy production and the holders included more than 20 different companies and organizations (although Novozymes A/S and DuPont dominated as patent holders).

Below some examples are given where thermostable enzymes have established use or has expanded the use of

biocatalysis, leading to advantages or increases in efficiency of the processing. These applications range from established large scale processing (like the treatment of starch in liquefaction and saccharification) and pretreatment and hydrolysis of lignocellulosics, to less established use as extraction and conversion aids in novel types of processes.

5.1. Enzymatic Starch Processing

The most common uses of processed starch are in glucose, maltose, and oligosaccharide production, utilized for food (Table 1), but also as carbon source in microbial production of chemical intermediates and energy carriers from primary and secondary metabolites, such as biofuels ethanol (in phase I biorefining) [51]. A number of products/intermediates can also be produced *via* cyclodextrins produced from starch by CGTases (cyclomaltoextrin glucanotransferases, EC 2.4.1.19, GH13) [16]. Starch processing is performed in a two-step hydrolysis process of liquefaction and saccharification, and these steps are performed at temperatures suitable for thermostable enzymes.

Liquefaction is the conversion of granular starch into soluble dextrans and in this process, starch is gelatinized by thermal treatment. Heating is necessary both to solubilise the starch granules, and the amylose contained. During cooling, a thermo-irreversible gel is formed during which amylose chains interact by hydrogen bonding (retrogradation), resulting in loss of crystallinity as the starch granules swell

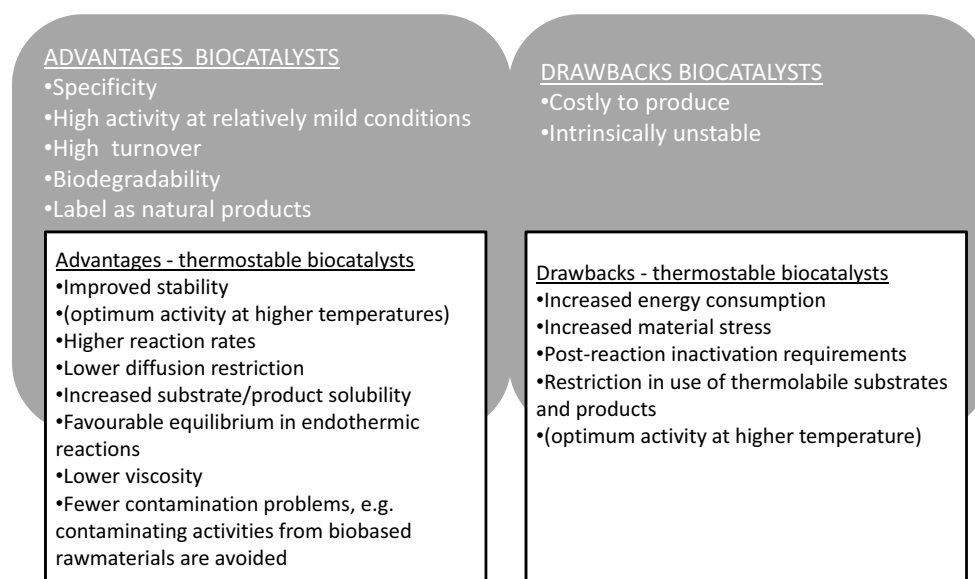


Fig. (8). Advantages and drawbacks on the use of biocatalysts, as well as on the use of thermostable biocatalysts in industrial processes.

during hydration of amylose and amylopectin chains [21, 52, 53]. An α -amylase is typically added before the heat treatment, which is frequently reaching temperatures of 105–110 °C for a few minutes followed by cooling to 95 °C and incubation at that temperature for one or two hours to complete enzymatic liquefaction [54, 55]. Consequently, a highly thermostable enzyme is required which will be active during the whole procedure. Many thermostable endo-acting amylases have been characterized (examples are listed in Turner *et al.* [16]). Enzymes from hyperthermophilic bacteria include α -amylases from *Desulfurococcus mucosus*, *Pyrodictium abyssi*, *Pyrococcus woesei*, *Pyrococcus furiosus*, *Thermococcus profundus*, *Thermococcus hydrothermalis* [19] that all have optimal activity at 100 °C. One of the most characterized α -amylases originates from *Bacillus licheniformis*, with optimal activity between 85 and 90 °C [56], and in addition several engineered variants are developed and commercialized by Dupont (previously Genencor) such as *Multifect AA 21L*[®], and Novozymes (such as *Termamyl*[®] and *Liquozyme*[®]) [16].

The liquefaction step is followed by saccharification, involving further hydrolysis of the produced maltodextrins into either maltose syrup by β -amylase or glucose/glucose syrups by glucoamylase (γ -amylase) [57]. Also this step is run at high temperature (avoiding costs of cooling as well as viscosity problems). To increase the efficiency in saccharification, a debranching enzyme, such as pullulanase can be added. Enzymes acting on pullulan are broadly present in thermophilic bacteria and archaea, and have been divided into different specificity groups, dependent on the linkage hydrolyzed (Table 3). Thermostable exo-acting β -amylases and glucoamylases are also available. β -amylases have for example been isolated from *Thermotoga maritima* [58] and *Clostridium thermosulfurigenes* [59, 60]. The *T. maritima* enzyme has optimal activity at 95 °C and pH 5, while the *C. thermosulfurigenes* is optimally active at 75 °C, pH 5 although its stability is enhanced by Ca^{2+} . Glucoamylases (γ -amylases) have been studied from anaerobic species such as *Clostridium thermosaccharoly-*

ticum with optimal activity at 70 °C [61], *Thermoanaerobacterium thermosaccharolyticum* optimally active up to 65 °C [62], and more recently from *Thermoanaerobacter tengcongensis*, showing maximum activity at 75 °C and pH 5, also hydrolyzing α -1,6-linkages [63], which is common for glucoamylases when the bond is next to the α -1,4-linkage. Thermophilic archaea such as *Thermoplasma acidophilum*, *Picrophilus torridus* and *Picrophilus oshimae* also produce both thermostable as well as acid-stable glucoamylases, active at 90 °C and pH 2 [19]. The glucose, produced from saccharification by glucoamylase, can for food applications be converted to high-fructose syrups, crystalline dextrose and dextrose syrups [21], but is as stated above nowadays also used as a carbon source in microbial fermentations for production of selected metabolites, like ethanol. Conversion of glucose to high-fructose syrup by glucose isomerase (EC 5.3.1.5) is usually run at 55–60 °C and pH 7–8.5 [21], again requiring a thermostable enzyme.

Maltose can be converted to glucose by α -glucosidases, and these enzymes have been isolated from thermophilic archaea and bacteria. One interesting species is *Sulfolobus solfataricus*, which grows at 80 °C, pH 3 using starch as sole source of carbon and energy [64, 65]. *S. solfataricus* produces α -glucosidase with optimal activity over 120 °C at pH 4.5, and also secretes α -amylase into the supernatant of the culture medium. Other examples of α -glucosidases originate from *Thermococcus hydrothermalis* with optimal activity at 120 °C, *Pyrococcus furiosus* (105–115 °C), *Pyrococcus woesei* (100 °C) and *Thermoanaerobacter ethanolicus* (75 °C) [19]. An unusual α -glucosidase, active at 90 °C, pH 7.5 has been found in *T. maritima*, but this enzyme requires NAD^+ (an expensive additive) as well as Mn^{2+} , Co^{2+} or Ni^{2+} [66], and these requirements may reduce its industrial applicability.

5.2. Enzymatic Hydrolysis of Lignocellulosic Polysaccharides

Bioethanol production (Tables 1 and 2) from sucrose and starch, available today, is a robust process, but a change to biotransformation of lignocellulosic biomass into biofuels

Table 3. Examples of microbial thermostable enzymes acting on pullulan.

Pullulanase	Organism	Optimal Temp. (°C)	Optimal pH	Mw (kDa)	Reference
EC 3.2.1.41	<i>Thermotoga maritima</i> MSB8	90	6.0	93	[19]
	<i>Fervidobacterium pennavorans</i> Ven5	80	6.0	190	[91]
	<i>Thermotoga neapolitana</i>	80	5.0-7.0	93	[92]
	<i>Bacillus flavocaldarius</i> KP 1228	75-80	7.0	55	[93]
	<i>Geococcus thermoleovorans</i> US 105	70	6	80	[94]
	<i>Thermus caldophilus</i> GK24	75	5.5	65	[95]
	<i>Anaerobranca gottschalkii</i>	70	8.0	70	[96]
(amylopullulanase) EC 3.2.1.1/41	<i>Desulfurococcus mucosus</i>	100	5.5	66	[97]
	<i>Pyrodictium abyssi</i>	100	9.0	n.d	[19]
	<i>Thermococcus</i> strain TY	100	6.5	n.d	[98]
	<i>Pyrococcus woesei</i>	100	6.0	90	[99]
	<i>Thermococcus sicuti</i>	100	5.0-6.0	148.6	[100]
	<i>Pyrococcus furiosus</i>	98	5.5	110	[101]
	<i>Thermococcus litoralis</i>	98	5.5	119	[101]
	<i>Thermococcus hydrothermalis</i>	95	5.5	128	[102]
	<i>Thermococcus celer</i>	90	5.5	n.d	[98]
	<i>Thermoanaerobacter ethanolicus</i>	90	5.5	n.d	[19]
	<i>Thermotoga maritima</i>	90	7.5	58	[19]
	<i>Thermoanaerobacter</i> strain B6A	75	5.0	450	[103]
	<i>Thermoanaerobacterium saccharolyticum</i> NTOU1	70	5.0-6.0	n.d	[104]
	<i>Clostridium thermosulfurigenes</i> EM1	60-65	5.5-6	102	[105]
(neopullulanase) EC 3.2.1.135	<i>Bacillus stearothermophilus</i> TRS	60-65	6.0	62	[106]
(isopullulanase) EC 3.2.1.57	<i>Bacillus</i> sp. US 149	60	5.0	200	[107]
EC 3.2.1.-	<i>Thermococcus aggregans</i>	100	6.5	83	[108]

will be a necessity when a higher volume demand from biomass, as well as a larger spectrum of products from microbial fermentations, are required. Lignocellulose is our most abundant renewable raw material, frequently leaving a significant unutilized fraction in waste products. Hardwood and softwood from forestry, and straw, husks and bran from agriculture are examples of materials with significant lignocellulose content. Application of this feedstock in a biorefinery will due to its slow degradation however rely on development of good combinations of pretreatments (disrupting structures to decrease its recalcitrance and enhance enzyme accessibility) and hydrolysis (saccharification) procedures [67, 68].

Pretreatment of the lignocellulosic materials reduces crystallinity of the cellulose and aids in removal of lignin and hemicellulose. Alvira *et al.* [67] grouped different pretreatment technologies as follows: (i) biological pretreatment - using lignin and cellulose degrading microorganisms, (ii) chemical pretreatment - using alkali, acids, ozone, organic solvents or ionic liquids and (iii) physico-chemical pretreatment - consisting of SO₂ steam explosion, ammonia fiber explosion, CO₂ explosion, wet

oxidation, microwaves, ultrasound and liquid hot water. Combinations of pretreatment methods can enhance the disaggregation and disruption of the lignocellulosic material. There is, for several reasons, a strong drive towards less severe pretreatment procedures, preferably without added acid or base. This is due to a strive towards: (i) reduced consumption of chemicals, (ii) reduced demands on construction steel in process plants, and (iii) minimized amounts of toxic derivatives in the downstream bioconversion steps (see also section 6). Less harsh pretreatment normally leads to a material that is more difficult to degrade enzymatically due to that: (i) more hemicellulose is left, shielding the cellulose and (ii) the polysaccharide structures will be differently modified. Even less harsh pretreatment, will require high temperatures, and will increase the need to use of robust thermostable enzyme cocktails, acting on both the cellulose and hemicellulose fractions. Thus, there is high interest in thermostable enzymes that hydrolyze polymeric carbohydrates in lignocellulose into metabolizable intermediates (oligomeric and monomeric), as this is predicted to improve utilization of lignocellulosic carbohydrate fractions. Production of

oligomeric and monomeric forms of the carbohydrates will both facilitate direct use in food and feed products (Table 1), and facilitate microbial uptake for fermentation to products e.g. biofuels and chemical intermediates (Table 2). Thermostable enzymes are excellent to combine with thermal and even thermochemical pretreatment technologies, as the higher temperature during hydrolysis promotes penetration of the enzymes in the lignocellulosics, and the stability of the enzymes reduces the need of cooling from the previous step.

Feed processing of lignocellulose in agricultural products (Table 1) is normally also performed at high temperatures [69], so use and development of stable and robust enzymes has been imperative. In the production of pellets, the material is treated with moist heat (70-90 °C), followed by mechanical pressing. High process temperatures are also used to reduce the risk of pathogen transfer (e.g. salmonella) [70]. Different types of cellulases and hemicellulases are of interest to increase the fraction of digestible carbohydrates, which is an especially pronounced need in feeds for non-ruminant animals (e.g. poultry and pig).

Cellulose hydrolysis is one of the main targets for the enzymatic reactions since cellulose is the most abundant biomass component. Many cellulolytic enzymes have been isolated and cloned from mesophilic or moderately thermophilic fungi, such as *Talaromyces*, *Thermoascus*, *Chaetomium*, and from thermophilic bacteria such as the anaerobes *Thermotoga*, *Anaerocellum* and the aerobe *Rhodothermus* (Table 4) and researchers are constantly working on finding more efficient candidates and enzyme cocktails.

It is also inevitable that hemicellulases are required to remove the hemicellulose fraction, and exposing the cellulose fiber to enzymatic hydrolysis. The most applied hemicellulases are xylanases and mannanases, and thermostable candidates of these enzymes have previously been listed by Turner *et al.* [16]. The products of hemicellulose degradation are also of interest for further use in the biorefinery perspective. Extraction of oligomeric forms of hemicellulose components from agricultural lignocellulosic byproducts for food purposes (non-digestible by humans), can for example be a source to obtain oligosaccharides with prebiotic properties [71]. In this case, process intensification is of special interest, using residues obtained after milling (such as husks or bran), or residues that remain underutilized after a first processing step, such as **Distillers Dried Grains with Solubles (DDGS)**, which is a byproduct from ethanol production. For this purpose, endo-xylanases are of interest, as xylooligosaccharides and arabinoxylooligosaccharides are shown to have a prebiotic potential, stimulating growth of probiotic bacteria (such as bifidobacteria) in the human gut. The thermostable endo-xylanase *RmXyn10A* from *Rhodothermus marinus* [72], has for example proven useful for this purpose [49].

The most established, larger scale degradation processing of lignocellulose, takes into account both the cellulose and hemicellulose fraction, and its main purpose is to obtain monosaccharides for use in microbial fermentation processes e.g. biofuels production. The need of monosaccharides is a consequence of the lack of efficient enzymes for cellulose degradation in many of the established fermentative

microorganisms used (i.e. *Saccharomyces cerevisiae* in ethanol production) [73]. In fact, only few microorganisms can directly utilize such a complex carbon-source, and hence efficient degradation is fundamental for this resource to be used for production of many different types of compounds (e.g. fuels, additives, chemical intermediates) *via* microbial metabolism.

5.3. Glycoside Hydrolases to Promote Release of Natural Products

Enzymatic hydrolysis of the polymeric carbohydrates can also facilitate release of other desirable products from the materials. Dependent on their specificity, glycoside hydrolases can be used both in pretreatment of the raw materials - acting on the polysaccharide fibres to simplify release of secondary metabolites (such as antioxidants or antimicrobial compounds) in a following extraction [74]. Pre-treatment with polysaccharide-degrading glycoside hydrolases [cellulases, hemicellulases (e.g. xylanases and mannanases), and pectinases] before the extraction have for example been reported to promote release of the desired secondary metabolite flavonoids from matrices of different sources containing complex polysaccharides. Sources investigated include fruits and berries e.g. apples [75] and black currants [76], other agricultural products, such as pigeon peas [77] or products from forestry, such as pine [78].

Extraction methods with environmentally sound, non-toxic solvents, such as pressurized hot water extractions can successfully be combined with thermostable glycoside hydrolases, not only to promote extraction, but also to modify the glycosylation of the secondary metabolite using thermostable glycoside hydrolases. This has for example been applied using thermostable glucosidases to obtain quercetin aglycone from onion waste [50, 79].

5.4. Enzymatic Synthesis of Chemicals from Biomass, Exemplified by Alkyl Glucoside Production

Enzymatic synthesis of specific target compounds from renewables often starts with refined rawmaterials. This pinpoints the importance of developing techniques to both hydrolyze and purify components from the polymeric carbohydrate fraction in carbohydrate rich biomass, as well as the extraction and separation of the oil fraction from oleaginous biomass. Hence, selective hydrolysis and fractionation are prerequisites in order to facilitate more detailed processing or synthesis of desired chemicals.

One important class of chemicals, with large and widespread use in consumer products, is surfactants. They are used to provide function in a vast array of consumer products such as food products, soaps, detergents, skin lotions, cosmetics, cleaning products. With 70% of the world's consumption of surfactants today being produced from petrochemicals, there is substantial room for improvement in terms of replacement with bio-based varieties. An alternative among bio-based surfactants are alkyl-glycosides. Alkyl-glycosides are produced from carbohydrates and fatty alcohols, thus taking into account products from both oleaginous and carbohydrate based feed stocks.

Table 4. Examples of thermostable cellulases.

Cellulases	Organism	Optimal Temp. (°C)	Optimal pH	Mw (kDa)	Reference
Cellobiohydrolase EC 3.2.1.91					
	<i>Thermotoga sp.</i> strain FjSS3-B.1	105	7.0	36	[109]
CBH IA	<i>Talaromyces emersonii</i>	78	3.6	66.1	[110]
CBH IB	<i>Talaromyces emersonii</i>	66-69	4.1	56.3	[110]
CBH II	<i>Talaromyces emersonii</i>	68	3.8	56.24	[110]
Cel7A	<i>Thermoascus aurantiacus</i>	65	5.0	46.9	[111]
Cel7A	<i>Chaetomium thermophilum</i>	65	4.0	54.6	[111]
Cel7A	<i>Acremonium thermophilum</i>	60	5.0	53.7	[111]
Cel7A	<i>Trichoderma reesei</i>	60	5.0	67	[112]
CBH3	<i>Chaetomium thermophilum</i>	60	5.0	48	[113]
	<i>Clostridium thermocellum</i>	60	6.5	78	[114]
	<i>Fomitopsis pinicola</i>	50	5.0	64	[115]
Cel48A	<i>Thermobifida fusca</i>	40-60	4.0-6.0	104	[116]
	<i>Thermomonospora fusca</i>	n.d	7.0-8.0	61.2	[117]
CBH I	<i>Chrysosporium lucknowense</i>	n.d	5.0-5.5	52	[118]
CBH I	<i>Chrysosporium lucknowense</i>	n.d	5.0-5.5	65	[118]
Endo-β-glucanase EC 3.2.1.4					
CelB	<i>Thermotoga neapolitana</i>	106	6.0-6.6	30	[119]
	<i>Anaerocellum thermophilum</i>	95-100	5.0-6.0	230	[120]
EglA	<i>Pyrococcus furiosus</i>	100	6.0	35.9	[121]
CelA	<i>Thermotoga neapolitana</i>	95	6.0	29	[119]
	<i>Rhodotermus marinus</i>	95	7.0	49	[122]
	<i>Thermotoga maritima</i>	95	6.0-7.5	27	[123]
	<i>Opuntia vulgaris</i>	90	4.5	36	[124]
	<i>Clostridium stercorarium</i>	90	6.0-6.5	100	[125]
Endoglucanase I	<i>Acidothermus cellulolyticus</i>	85	5.1	86	[126]
	<i>Thermoascus aurantiacus</i>	75	4.5	n.d	[127]
	<i>Thermomonospora curvata</i>	70-73	6.0-6.5	100	[128]
	<i>Sporotrichum sp.</i>	70	4.5-5	33	[129]
	<i>Clostridium thermocellum</i>	70	6.6	83	[130]
	<i>Clostridium thermocellum</i>	70	7.0	76	[131]
EgG5	<i>Phialophora sp.</i> G5	70	4.0-5.0	42.8	[132]
	<i>Opuntia vulgaris</i>	70	7.0	66	[124]
CelA	<i>Geobacillus sp.</i> 70PC53	65	5.0	43	[133]
	<i>Bacillus halodurans</i>	60	9.0	44	[134]
MtEG7	<i>Myceliophthora thermophila</i>	60	5.0	65	[135]
	<i>Chaetomium thermophilum</i>	60	4.0	68	[136]
	<i>Cladosporium sp.</i>	60	4.0-6.0	n.d	[137]
β-Glucosidase EC 3.2.1.21					
	<i>Pyrococcus furiosus</i>	102-105	5.0	230±20	[138]
Bgl3B	<i>Thermotoga neapolitana</i> DSM4359	90	5.0-6.0	81.1	[139]
BglB	<i>Thermotoga neapolitana</i>	90	6.0-7.0	81	[140]
Bgl1A	<i>Thermotoga neapolitana</i> DSM4359	90	5.0-6.0	52	[79]

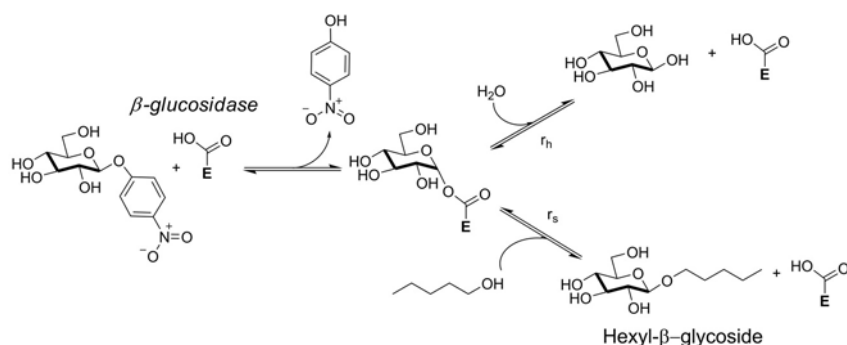


Fig. (9). Reaction for the production of hexyl- β -glucoside from p -nitrophenyl- β -D-glucoside (transglycosylation) with hydrolysis to glucose as the side reaction. The enzyme is an engineered β -glucosidase from *Thermotoga neapolitana* [88].

In an enzymatic process, retaining glycoside hydrolases can be used as catalysts for their production, and most commonly glucosidases have been utilized. The reactions proceed *via* a glycosyl-enzyme intermediate, which can be deglycosylated either by water, in the normal hydrolytic reaction, or by other nucleophiles, such as alcohols, yielding alkyl glycosides (Fig. 9).

In both cases, these reactions are hampered by the low solubility of hydrophobic alcohols in the enzyme containing aqueous phase, and the yield decreases with increasing chain length of the alcohol [80] which is a main factor of concern as surfactants with long length are often desired. Thermostable enzymes can be quite beneficial in these reactions, due to the increased solubility of the fatty-alcohol in the water phase at elevated temperatures, which is increasing the possibilities to obtain better yields.

6. FUTURE SYSTEMS WITH ENGINEERED MICROBES AND SYNTHETIC MICROBIAL PLATFORM

It is not only individual enzymes that are of interest, in the biorefining perspective. New and emerging technologies allow redesign and construction of metabolic pathways in microorganisms [81]. Therefore, the development of whole-cell biocatalysts, containing relevant genes encoding glycoside hydrolases combined with the whole metabolic pathway set-up offered by a microorganism, is a promising technology for efficient production of fuels and platform-chemicals in a biorefinery. This approach is somewhat different, not only utilizing prehydrolysis of the polymeric carbohydrates to obtain generally applicable monosaccharides, as described above (section 5.1 and 5.2). Instead, it can also aim for specialized production organisms in a “single pot” reaction from the complex carbohydrate substrate (ideally lignocellulose) to one product. Such an approach is of course most interesting if the product of interest is produced in a large scale, and typically biofuels production (e.g. microbial production of ethanol) is again one of the most researched examples.

There are, however, many challenges in this field. Pretreatment of lignocellulose (section 5.2) leads to production of side products such as furfural and its derivatives, which (despite interest as platform chemicals, Table 2) are toxic for many types of microorganisms, thus

affecting the yield in for example bioethanol production [73]. The hydrolysis of cellulose, hemicelluloses and pectins also require a number of different additional enzymes (see section 3). For hydrolysis of cellulose, many anaerobic cellulolytic bacteria have developed a complex multiprotein structure called the cellulosome for efficient degradation [82]. Therefore, a one-pot conversion of lignocellulosic feedstock into ethanol requires the development of a whole-cell biocatalyst able to thrive in the harshest environment generated by the pre-treatment of biomass and able to hydrolyze many types of polysaccharides and finally ferment these to a relatively high concentration of the desired metabolite.

A conceptual optimal whole-cell biocatalyst for producing liquid transport fuels was proposed recently [83]. The most important characteristics of this highly engineered system would involve the (i) expression of a synergistic mixture of highly active exo- and endoglucanases, glucosidases, and xylanases; (ii) highly, robust and balanced fermentation of hexoses and pentoses; (iii) high tolerance to toxic from pretreated biomass and (iv) production of next-generation biofuels or advanced biofuels through synthetic pathways (Fig. 10). The host cell proposed by Elkins and coworkers was the yeast *Saccharomyces cerevisiae* because of its widespread use in ethanol industry and its potential to express recombinant proteins both from fungi as well as bacteria.

Another important characteristic is however thermostability (missing from the proposed *S. cerevisiae* system), and the production of ethanol at higher temperatures would facilitate the process design. Thereby alternative host cells would be thermophiles such as *Geobacillus thermoglucosidasius*, *Thermoanaerobacterium saccharolyticum* or *Thermoanaerobacter mathranii*, currently used in several new biotechnology companies [84]. These bacteria have catabolic flexibility and capacity for enhancing the ethanol production, and they are able to ferment both hexoses and pentoses. In addition, many thermophiles already have the capacity to hydrolyze cellulose and other components of the lignocellulosic biomass, and some of them such as the thermophilic anaerobe *Clostridium thermocellum*, produce cellulosomes [85]. However, it is necessary to develop more robust techniques and tools for genetic engineering of thermophilic strains, and work in this field is ongoing.

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