Chapter

Enzymes – Key Elements of the Future Biorefineries

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Abstract

The biorefinery concept in its modern meaning has emerged after it has become apparent that biofuel production from non-food biomass is struggling for economic viability. Lignocellulosic biomass is more recalcitrant and more complex than the starch-based feedstocks used for food. The former, therefore, calls for a more complex approach to its utilization. This chapter reflects MetGen's vision of the future development of biorefineries. We will discuss the zero-waste approach to lignocellulosic biomass utilization and various ways to valorize the resulting streams to boost the economic viability of the biorefinery. We will mostly explore the relevant enzyme-based approaches and will make a special focus on lignin valorization. Enzymatic and cell-based approaches to sugar valorization will be discussed as well.

Keywords: enzyme, lignocellulosic biomass, lignin, cellulose, hemicellulose, laccase

1. Introduction: from bioethanol to biorefineries

The progenitor of the modern biorefinery concept was bioethanol production. In the 1970s, Brazil and the United States started mass production of bioethanol grown from sugarcane and corn respectively. The most common usage of bioethanol is to power automobiles by mixing it with petrol. The sugar yield from these feedstocks is very high and the biomass processing is rather simple, thus fueling the transportation this way was economically viable, especially in the countries with scarce fossil fuel resources, like Brasil.

Bioethanol is generally CO_2 neutral because the released during the burning of ethanol is compensated by the absorption of the CO_2 by growing the feedstock biomass. This however does not consider the CO_2 generated by the logistics of the biomass production and processing. Besides, blending bioethanol with gasoline helps to reduce greenhouse gases (GHG) emissions by oxygenating the fuel mixture which makes it burn more completely. Thus bioethanol was considered to be an environmentally friendly alternative to petrol.

In the future, with improved efficiency, utilization of non-agricultural feedstocks and use of renewable energy, the respective life cycle GHG emissions could be cut by up to 86 percent relative to gasoline as reported in EPA's Emission Facts [EPA (2007) Emission Facts; Greenhouse Gas Impacts of Expanded Renewable and Alternative Fuels Use. Emission Facts Report (EPA420-F-07-035). Office of Transportation and Air Quality, EPA, US].

Thus the agenda of bioethanol production was shifted to the products derived from lignocellulosic biomass to avoid competition with food and limit the use of agricultural land.

In the brink of the 21st century a considerable public and private effort to implement the so-called second-generation bioethanol industry based on lignocellulosic, non-edible feedstock was undertaken, and eventually faded away due to economic inefficiency. The frustrating experience of lignocellulosic bioethanol hype of the past years triggered the formation of a broader view of the biorefinery concept. It grew with the understanding that if only a part of the biomass, namely the cellulose, is used to make a product, moreover, not a high-value product such as bioethanol, the economics of such an undertaking does not work [1].

This notion coincided with the growing understanding that biomass is not merely a quick fix for a deficit of fossil resources in some countries, but a fundamental raw material for bioeconomy. Consequently, a biorefinery concept was forming with the term borrowed from the petroleum oil refinery, which goes beyond the exhaustion of biomass into a spectrum of products. Biorefineries are based on four principles [2], namely principles of sustainability, cascading, non-conflict with food, and neutral carbon footprint.

Thus to implement the biorefineries as fundamental units of bioeconomy, all biomass components cellulose, hemicellulose, and lignin need to be utilized. Moreover, the general approach should be similar to the oil refinery concept - the raw material needs to be fractionated to result in a range of intermediates leading to a variety of products from high to low-value. Ideally, there has to be in-built flexibility allowing to change the product portfolio according to the current market demands.

One of the fundamental differences of biorefineries from oil refineries is the repertoire of tools that can be used, where enzymes - the natural catalysts play an important role.

Nature is using biocatalysts – the protein molecules called enzymes - for performing virtually all biochemical reactions happening inside organisms, and often outside as well.

It is logical to assume that a bio-based economy would be largely relying on biocatalysis. Extremely high specificity and selectivity as well eco-friendliness make enzymes potentially very attractive in many industrial applications.

2. Biorefinery as an industry sector

Following the concept of Biorefineries, the society has to make a leap from biofuel factories using local agricultural feedstocks to produce bioethanol for fulfilling local demand for automobile fuel to the biorefineries providing raw materials for various industries from energy to chemicals and materials producers. From this perspective, wood seems to be the most likely feedstock to be able to fulfill the industry demand.

It has to be noted that biomass alone, wood or other types, is unlikely to fulfill the energy demand of modern society from the volume point of view. Other energy-providing technologies, like solar and wind energy need to fill in the gap. However, biomass is suited to assume other roles in a circular economy, related to materials and chemicals. This notion provides only more motivation for diversifying the biorefineries' product range.

Wood is one of the most abundant, sustainable raw materials on Earth, which is available around the year. It requires no roof for storage and has a high density, which is favorable for logistics and handling. Furthermore, it requires no additional field space and has no agricultural or nutritional use.

If wood is the most likely feedstock of the rising bioeconomy, then the pulp and paper industry is the most likely first block in the value chains of the biobased products. This industry has many years of experience in maintaining and working forests, as well as harvesting, transporting, and processing wood. It is also noteworthy that in the Nordic countries, the volume of sustainably harvested forests is growing faster than the current consumption: regulation and standardized systems are in place to allow forests to be harvested sustainably to meet significant industrial demand.

The drawback of this industry being at the foundation of the biobased economy is that this industry has highly refined processes focusing on cellulose fibers only, the industry is highly conservative due to low-margin economic positioning and besides this industry in its current state is used to offering a very narrow product portfolio which is marketed through distributors.

Diversifying the product offering of the pulp and paper industry may require changes in the processes or the addition of parallel process lines and more intimate interaction with various markets. This in turn requires investments and a change in attitude.

Biorefineries can be positioned on the interface of pulp and paper/forestry industry and chemicals and materials industry. And it has to be admitted that this interface is yet to be created. For example, it can be implemented with a third party operating a biorefinery with the over-the-fence supply of raw materials (feedstocks) and possibly even utilities from a pulp and paper mill. These feedstocks may comprise lignin, zero fibers (short fibers disposed of with the wastewater), pulp products depending on the demand of both markets. This could be set up as a joint venture so that both organizations can benefit from this model. Alternatively, a joint venture with the end-user of the biorefinery products can be envisaged as well.

Enzymes as an important part of the economics and the technology of the biorefineries can also be considered as part of the production process. On-site manufacturing of enzymes allows saving costs on concentration, formulation, storage, and shipping of the enzymes. Some companies embracing biorefineries, develop their own enzymatic solutions to be implemented in their biorefineries, and can set up on-site manufacturing at their will. Whereas other types of biorefinery owners, like pulp and paper companies, usually rely on an external enzyme supplier. Usually, enzyme suppliers are not open to providing their production strains to third parties for on-site manufacturing. In this respect, MetGen has a more flexible business model towards supplying enzymatic solutions for biorefineries, including a possibility of on-site manufacturing.

3. Enzymes—ultimate tools for biobased industries

Many if not most industrial chemical processes are dependent on catalysts - substances that accelerate chemical reactions without themselves being consumed in the catalyzed reaction and can continue to act repeatedly. Because of this, only very small amounts of catalyst are required to have a dramatic effect on the reaction rate. The development of affordable durable and efficient catalysts was vital for the establishment and economic viability of fossil-based chemistry and material science.

It is equally important for the biobased economy to adapt and further develop nature's catalytic tools.

The historic concern about enzymes is that they are vulnerable to industrial conditions and often could not be applied to existing industrial processes. Modern molecular biology and bioengineering pave the way to much wider use of enzymes

in the industry by making it possible to adapt enzymes to performing in unnatural harsh conditions. The development time for new enzymes was further reduced with the development of bioinformatics tools and genome editing.

Especially as new bio-based processes are being developed it is a good time to consider making them more enzyme-adaptable by assuming somewhat longer retention times while transitioning to lower temperatures and pressures, as compared to currently common conditions.

Enzymatic processes are truly similar to chemical catalysis. They can be run as homogeneous catalysis with a soluble enzyme added and disposed of with every production batch, or as heterogeneous catalysis, where the enzyme is used in the immobilized form and reused from batch to batch or used in a continuous process with a column set up.

Importantly, the enzymes present also a third option not applicable with the chemical catalysts - a continuous membrane bioreactor. Sometimes this technology is called "enzymes immobilized by perfusion". This setup exploits the best of the previous two - affordability of the soluble enzyme and reusability of the immobilized one. In this setup, the enzyme is trapped in a bioreactor connected to a tangential flow micro-filtration membrane unit allowing the low-molecular-weight product to penetrate through the membrane but retaining the enzyme inside.

This setup allows not only an efficient use of the enzyme but can also provide product fractionation and more complex designs with parallel processes. Ultra and nano-filtration is also a very useful and economical water removal tool. We will further discuss this setup in the section dedicated to lignin valorization.

3.1 Bioconversion—enzyme or whole cell?

One important aspect of enzyme-dependent catalysis is the necessity of a cofactor for some enzymes. Cofactors are important accessories to biochemical processes. They are small organic compounds or metal ions empowering enzymes to function at maximal catalytic effectiveness or endurance. Cofactors may aid in substrate binding, catalysis, stabilizing the transition state, or contributing to the overall stability of the enzyme's structure. In some cases cofactors are modified during the reaction, for example, providing or accepting an electron in reduction–oxidation reactions, or providing energy through a high energy bond braking. In this case, in order to be reused, cofactors need to be regenerated during the reaction - oxidized/reduced/phosphorylated respectively. Regeneration requires another enzyme and a co-substrate to be oxidized or reduced. With the cofactor regeneration in place, the reaction can proceed continuously with only a small amount of the cofactor present. The chemistry of cofactor regeneration is well known nowadays [3]. The challenge is mostly regarding how to achieve the regeneration with immobilized enzyme systems which are preferred for industrial processes to facilitate the recovery and continuous use of the catalysts. This has become a great hurdle for the industrialization of many promising enzymatic processes. Once again, recent advances in membrane technologies led to the development of sustainable methods based on membrane entrapment [4].

Nevertheless, the necessity of a cofactor complicates the enzymatic process and increases the cost. Thus most of the bio-transformations involving cofactors have been traditionally performed in the industry with living cells often referred to as microbial cell factories [5].

Whole-cell biotransformation has advantages and disadvantages as compared to the enzymatic process. As mentioned before it solves the problem with the cofactors as they are widely used in cell metabolism and regeneration routes are in place. The balance of cellular metabolic fluxes can be further genetically adjusted

for the increased level of the components necessary for the product synthesis. Another advantage of the microbial cell factories is that multistep reactions can be carried out, and it is often possible to use simple and affordable raw materials such as glucose because the cell has a metabolic pathway in place to convert it to a large variety of precursor and eventually to the final product. Among the shortcomings of the cell factories, one should mention a very narrow operational space due to microorganisms viability constraints. While some individual enzymes can tolerate high temperatures close to water boiling point and a wide range of pH, industrial microorganisms are usually performing only in ambient conditions. Besides, there are often multiple pathways in the cell to convert the starting material, which leads to the formation of side products. In more detail, the cons and pros of enzymatic and whole-cell bioconversions are listed in **Table 1**. In conclusion, as opposed to whole microorganism bio-conversions, more common in the past, enzymes provide faster and safer processes with a broader operational range.

Enzymes are also attractive in industrial use from the safety point of view: enzymes are not living organisms and they cannot breed (as opposed to the whole-cell factories) and can be considered environmentally safe. Additionally, being proteins, enzymes do not create toxic waste and decompose naturally over time. It should be noted that enzymes – as is the case with all proteins – may cause allergenic irritation. Therefore, the use of highly concentrated industrial enzymes should always be done according to handling instructions and material safety documentation.

Thus, with all the pros and cons in mind, the preferred type of bioconversion needs to be identified for each particular process.

Respective sectors of molecular biology dealing with enzymatic processes and whole-cell bioconversions are Enzyme engineering [6] and Metabolic engineering [7] respectively. Where enzyme engineering provides tools for optimizing protein structure for better performance; and metabolic engineering provides tools for optimizing the microbial genome to redistribute metabolic pathways in favor of the desired product formation. It has to be noticed that industrial process engineering is extremely important to go hand in hand with molecular engineering.

3.2 Enzyme resources of nature

Enzymes are extremely abundant in nature and exist in all living organisms from bacteria to humans. All industrial enzymes have their origin and prototypes in nature, where wood is decomposed by rot microorganisms, the most efficient

	Enzymatic process	**Whole call process**
Temperature, pH range	Wide	Narrow
Substrate/product load	High	Low
Tolerance to solvents	Moderate	Low
External cofactors/cofactor regeneration system	Needed	Not needed
Multistep processes	Difficult	Natural
Control over the reaction speed	By increasing the enzyme concentration	Only by increasing the size of the vessel
Side products	No	Yes

Table 1.
Enzymatic process vs. whole-cell bioconversion.

of which are fungi. It is thus natural that most enzymes used in industry for wood and other biomass applications are of fungal origin. Robust industrial strains and processes for fungal enzyme production have been developed through decades of optimization.

One of the major problems of fungal enzymes is that fungi are not known to live in extreme environments, such as elevated temperatures and extreme pHs, and their enzymes are usually not tolerant to harsh industrial conditions, which is sometimes limiting their application. In contrast, bacteria populate such environments as hot springs, salt lakes, and ocean depths. Some bacteria also possess individual enzymes with relevant catalytic activities for industrial biomass applications. Recent advances in molecular biology, genome sequencing, and genetic engineering made bacterial enzymes an attractive alternative to their fungal counterparts. Bacteria offer more diverse natural prototypes, and there are better-developed tools for genetic engineering in bacteria, allowing further optimization of the enzymes to required conditions.

Some unique industrial enzymes of bacterial origin have been developed in the past years breaking the boundaries of industrial enzyme applications. Nevertheless, when multiple enzymes are required in one process, such as cellulases, fungal production is usually a preferred option.

4. Biomass hydrolysis

4.1 Biomass pretreatment

The biorefinery platform requires pretreatment of lignocellulosic materials, which can be very recalcitrant, to improve further processing through enzymatic hydrolysis, and for other downstream unit operations.

Pretreatment employs a combination of chemical and physical elements such as temperature, pressure, and acid or alkali. This partially separates biomass components such as cellulose, hemicellulose, and lignin from each other resulting in a paste-like rather than a solid substance. This level of destruction allows access of enzymes to all the biomass components and further separation and hydrolysis.

Many pretreatment methods and unit operations were inherited from the bioethanol-oriented processes, where the target product was a fermentable sugar mix, and the ultimate goal to reduce the cost of the process. Now, when the focus of the biorefinery concept has shifted from the design of more or less energy-driven biorefineries to much more versatile facilities where chemicals and other raw materials can be produced apart from energy carriers, the view to the pretreatment has been transforming as well. In some cases, a pretreatment with a higher cost, but also better separation of the biomass components and higher quality streams are preferred. For example, organosol or chemical treatment employing ionic liquids and deep eutectic solvents. In the end, the choice of pretreatment must be based on a thorough techno-economic evaluation considering the proposed applications and the source of the biomass. This topic is reviewed in detail elsewhere [8, 9].

4.2 Cellulases for biomass hydrolysis

It is well known that wood is efficiently decomposed in nature by filamentous fungi. In their natural habitat, these microorganisms live on a solid substrate like wood and secrete a number of hydrolytic enzymes degrading all wood components down to low molecular weight substances that can be used as nutrients. In industry, the enzyme preparations were traditionally obtained by the propagation of the

fungal strains in a liquid medium, and such production method resulted in a cocktail of different enzymatic activities, often generally referred to as cellulase [10]. Fungal metabolism has a complex regulation in order to be able to produce the set of enzymes relevant to the available type of biomass [11]. Thus, for example, cellulase production by the fungal cells is induced by certain compounds generated in wood hydrolysis. The enzymatic cocktail produced by a fungus depends on the fungal strain properties and is not always optimal for a particular industrial application.

The most noticeable hurdle for the industrial application of natural fungal cellulase cocktails is the mechanism preventing glucose accumulation in the environment of the fungal cell. Such accumulation could provide a favorable environment for competing microbes such as bacteria, which cannot degrade wood themselves. To achieve this regulation, all of the enzymes of the cellulase cocktail are inhibited by their reaction products [12]. As seen in **Figure 1**, cellulose is initially attacked by a number of enzymes most prominent one - exoglucanase (also known as cellobiohydrolase or CBH) comprising more than 50% of total protein in the cocktail, which is assisted by accessory enzymes endoglucanase, oxidative cellulase lytic polysaccharide monooxygenase (LPMO), and indirectly by other enzymes. The concerted action of these enzymes results in the formation of glucose dimer, cellobiose. This is followed by the last step of the cellulose hydrolysis, splitting cellobiose to two glucose, performed by beta-glucosidase. Glucose is further absorbed by the cell and metabolized. If the hydrolysis proceeds faster than glucose is consumed causing glucose accumulation, beta-glucosidase is inhibited by glucose and slows down, this, in turn, results in cellobiose accumulation slowing down exoglucanase, and thus the entire chain of the reactions is regulated by the feedback response from the last step (as shown with red arrows in **Figure 1**). However, in industrial biomass hydrolysis, glucose accumulation is the ultimate goal. Thus, this feedback loop needs to be overruled. This is usually done by artificially increasing the amount of beta-glucosidase in the cocktail. This can be done by inserting additional genes for beta-glucosidase into the fungal strain. Besides, some beta-glucosidases are less inhibited by glucose (or more glucose-tolerant) than others, and this can also be exploited in composing industrial cellulase cocktails. It has to be noted that glucose tolerance of beta-glucosidases is poorly understood and occurs more often in bacterial enzymes than in fungal ones. Elucidating the molecular mechanisms of glucose tolerance is a very important aspect of cellulose biotechnology research of glucose tolerance [13].

Apart from providing a feedback regulation of hydrolysis speed, beta-glucosidase has another important function - generating the inducers of cellulase gene expression and ultimately the cellulase production. Those inducers are unusual glucose dimers of which sophorose, a glucose dimer with β-1,2 bond, is the most efficient one. In nature, these compounds appear in small amounts in the presence of cellobiose as a result of a side-activity of some beta-glucosidases. This activity is referred to as trans-glycosylation [14]. The molecular mechanism underlying the ability of the beta-glucosidase to perform trans-glycosylation is obscure. There are

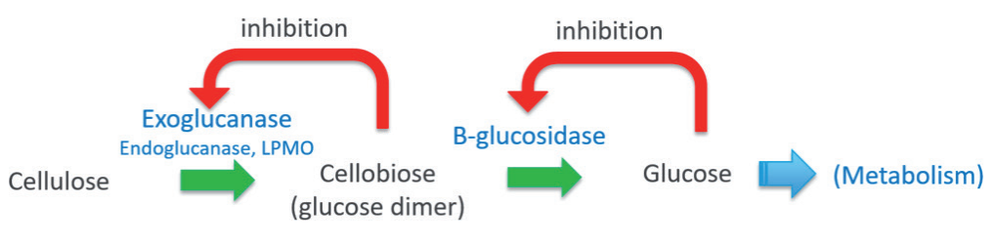

Figure 1.
Natural regulation of cellulose hydrolysis.

several different glucosidases in the fungal cell, extracellular as well as intracellular. Most probably it is the intra-cellular beta-glucosidases that are responsible for the inducer generation in nature [15].

In the industrial hydrolysis process, the inducer sugar is produced artificially from glucose either using random chemical dimerization catalyzed by phosphoric acid under elevated temperature and pressure or using beta-glucosidase with transglucosylase activity.

Thus in cellulose hydrolysis, CBH is the most prominent enzyme comprising more than a half of the total protein content of the cocktail, however, beta-glucanase is the most important enzymatic tool to providing cellulase efficiency in industrial hydrolysis applications.

Biochemists and molecular biologists have been studying the components of fungal cellulases and identified specific proteins responsible for the degradation of various plant polymers such as cellulose, other glucans, pectin, xylan, mannan, lignin, etc.

The native cocktails were improved after discovering that certain additional activities could enhance the conversion rates of specific biomass feedstock types [16–19]. For example, feedstocks could comprise different plants, pretreatments, or combinations of both. These augmented cellulases were produced by blending different secretomes containing the desired activities. More recently, some required activities have been genetically engineered into production strains.

Despite the considerable improvements in general-purpose cellulases available from the main enzyme-producing companies, a "one-size-fits-all" cellulase does not effectively address the wide range of biomass type-pretreatment chemistry combinations. However, customization of the cellulase cocktail is not commonly offered by enzyme producers. On the contrary, MetGen offers customization of the hydrolysis solution MetZyme® SUNO™ to the client's specific substrate/pretreatment as well as an option for on-site enzyme manufacturing.

4.3 Special enzymes for biomass degradation and valorization

Biomass is mostly comprised of polymers. The main structural components of plant biomass are polysaccharides (cellulose, hemicellulose, starch, pectin, and other plant polymers), and polyphenols (lignin). All these polymers apart from cellulose are branched and diverse in structure. In the plant cell, they are interlaced to form a complex and often recalcitrant structure.

Natural fungal cocktails are instrumental in the full hydrolysis of the biomass to monomers or low molecular weight compounds. This was the mainstream strategy of non-food biomass processing in the biofuel era. When we think of wider and wiser use of the biomass in a range of industrial applications it may appear sensible to preserve the polymeric structure of certain components. This can be partly achieved by choosing the right pretreatment method and other chemical and physical methods of processing the streams. Further, the process of biomass fractionation can be tuned by enzymes.

Generally speaking, the same enzyme toolbox is applicable both in the biorefinery industry and in Pulp and Paper sector. Notably, however, while in the P and P industry enzymes are minor and optional components of the process, in the biorefinery concept, enzymatic processes play a major role and represent a major cost, thus also opening a major market.

Let us consider how various product streams from biomass can be tuned by specific enzymes. An overview of the individual enzyme activities used in enzymatic solutions provided by MetGen is given in **Table 2**.

Generic activity	Application	Reaction conditions	MetZyme® family
Cellulase (cocktail)	Biomass hydrolysis	55 C, pH 5.0	MetZyme® SUNO™
Endoglucanase	Cellulose fiber modification, nanocellulose production; biomass hydrolysis;	High/ambient temperature, acidic/alkaline pH,	MetZyme® BRILA™ components
Xylanase	Hydrolysis of xylan for fiber modification; customization of hydrolysis cocktail	High/medium temperature, acidic/alkaline pH	MetZyme® BRILA™ optional components MetZyme® Suno optional components
Mannanase	Hydrolysis of mannan for fiber modification; customization of hydrolysis cocktail	High/ medium temperature, acidic pH	MetZyme® BRILA™ optional components MetZyme® SUNO™ optional components
Pectinase	Hydrolysis of pectin for fiber modification; customization of hydrolysis cocktail; R&D – pectin valorization	High/ medium temperature, acidic pH/highly alkaline pH	MetZyme® BRILA™ optional components MetZyme® SUNO™ optional components
Glucose isomerase	Low purity glucose to fructose conversion for platform chemicals production (especially suited for lignocellulosic glucose).	High temperature Acidic to moderately alkaline pH	MetZyme® PURECO™-GI
Pyranose oxidase	Glucosone from Glucose	Medium temperature Acidic to moderately alkaline pH	MetZyme® PURECO™-Pyranose oxidase
Lytic polysaccharide monoxygenase (LPMO)	Fiber modification (adding charge to cellulose fibers)		Not commercially available. Lab samples are available from MetGen for joint product development.
Laccase	Phenol/Polyphenol oxidation		METNIN™ – lignin refining technology MetZyme® BRILA™ optional components MetZyme® SUNO™ optional components

Table 2.
MetGen's product families and respective enzymatic activities.

4.3.1 Lignin valorization

In the earlier biorefinery concepts, lignin was often mostly regarded as a recalcitrance factor, fermentation inhibitor, sugar stream contaminant, etc. A broader view of the biorefinery, however, considers the valorization of lignin as a vital component of the economics of the entire concept. This is why it is one of the fastest-growing research and development areas in the biomass valorization field [20–23].

The main hurdles of lignin valorization are its diverse structure and poor solubility. Liquefaction of lignin would allow its use as fuel, as it is reached in high-energy chemical structures. More precise depolymerization or fragmentation

of lignin may enable higher-value products for various industries from construction to high-performance materials. Even though lignin-based replacement products have already been reported [20, 24–26] to be useful as binders, coatings, and fillers, and others, these applications are not yet widely industrially implemented. The main challenges for the full valorization of lignin are the economical production of suitable lignin and maintaining consistent quality throughout different batches. In order to achieve desirable properties for the industrial application, lignin usually needs to be fragmented and refined to a lower molecular weight and often chemically modified as well.

Enzymatic degradation of lignin, which occurs in nature, was speculated for a long time to be applicable in the industry [27]. This approach seems attractive because the catalysis takes place in water and under mild conditions avoiding high pressure, temperatures, and hazardous and expensive chemical catalysts, thus saving CAPEX and lowering environmental impact.

Research efforts for enzymatic lignin depolymerization were especially focusing on laccases (copper oxidases), as these enzymes require no cofactors, or co-substrates (such as hydrogen peroxide), they use oxygen as an electron acceptor and produce water as the only by-product. Prospective and challenges of laccase application in biotechnology were recently reviewed [28, 29]. The vast majority of industrially available laccases are fungal enzymes. These enzymes, however efficient, work in acidic-to-neutral pH [30], at which lignin is hardly soluble in water. This prevents their industrialization in this area.

Recently METGEN has developed and brought to the market a proprietary lignin refining technology METNIN™. This technology is based on combining enzyme-catalyzed lignin oxidation and cascading membrane fractionation. The enzymatic element of this technology is a proprietary artificially evolved enzyme MetZyme® METNIN™ laccase able to function under extremely alkaline conditions (typical process pH 10.5) [31, 32]. METNIN™ process is outlined in **Figure 2**. Membrane-based separation of lignin by molecular size provides useful fractions of various molecular weights.

Lignin preparations of different molecular weights can be further valorized and utilized in various industrial applications [20, 25] as long as chemical/physical properties are matching the requirements [33]. Thus, the target of lignin refining is to create lignin fractions that are bioequivalent, for example, to oil-based compounds used as resins, adhesives, composites and foams (**Table 3**).

Importantly, the absence of organic solvents in the reaction mixture allows for utilization of polymer-based ultrafiltration membranes widely used in the food industry, making this technology scalable and economically feasible. Ultrafiltration membranes of different cut-off are available and widely used in industry. The choice of membranes can be customized and adds flexibility to the technology. By adjusting process parameters, outcoming lignin properties and mass distribution between the fractions can be changed.

Demethylation is a desirable process in lignin upgrade, as it increases the number of hydroxyls and thus results in activation of lignin. Demethylation can be monitored by measuring MeOH in the reaction mixture after depolymerization using Purpald-method [34]. This is a fast and convenient method to monitor the oxidation process, however, it does not give the full picture of the chemical modification of the lignin, as some of the resulting hydroxyls can end up in new ester bonds or be further oxidized. For further characterization, titration methods and NMR need to be used. Using these methods, we observed an increased number of hydroxyls and sometimes carboxylic groups per gram of dry matter, especially towards lower Mw fractions.

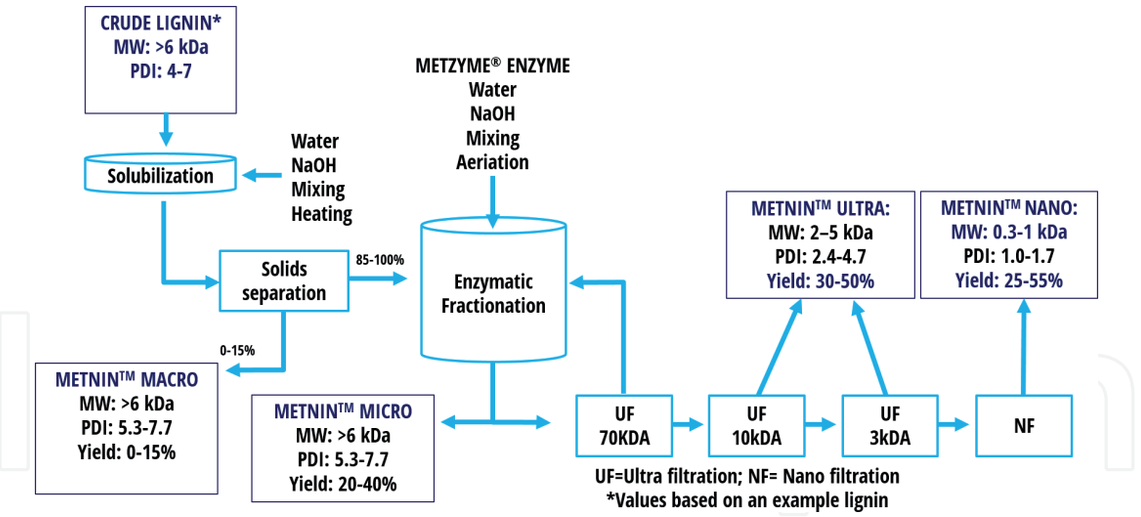

Figure 2.
METNIN™ process, schematic representation.

Lignin type	Application areas examples	Bio-equivalent of	Indicative price of the oil-based chemical
Crude Lignin	Fuel	Oil/Electricity	50–100 €/ton
METNIN™ MACRO	Bitumen, Fillers (Market established products, agro)	SBS Polymer, Inorganic fillers	400–600 €/ton
METNIN™ MICRO	Coatings & Surface treatment (Sizing value chain, Carbon fibers, agro)	Phenol resins, AKD, ASA, Wax, Latex	1000–2000 €/ton
METNIN™ ULTRA	Composites (Toy value chain)	Polyols	1500–3000 €/ton
METNIN™ NANO	Carbon Fibers (re-polymerized version), new materials	Specialty Chemicals, aromatics & phenols	> 4000 €/ton

Table 3.
METNIN™ products.

METNIN™ process allows tuning the resulting fractions in several ways: by choosing membranes with different cut-offs according to the desired molecular weight, by adjusting the extent of oxidation to tune other properties such as the content of OH-groups (phenolic aliphatic and carboxylic), and controlled polymerization, which affects reactivity and solubility of the resulting fractions. Thus variance in the starting material can be compensated by the process adjustment and the refining process results in more homogeneous fractions with a less batch-to-batch variation. Post-fractionation processing of the fractions can further tune the properties – purity and solubility in water or solvents.

Refining of lignin in METNIN™ process is accompanied by chemical activation via demethylation and benzylic oxidation as well as increased solubility in neutral and acidic pH and altered colloidal behavior. The process parameters largely depend on the starting lignin itself. In practice, each new lignin needs to be investigated to understand its behavior in the process.

One of the biggest challenges of lignin valorization is that lignin's structure is highly dependent not only on the species of wood but also on the treatment and extraction method. Therefore, the process parameters of lignin oxidation and

fractionation need to be optimized experimentally. MetGen not only provides the licensing of the technology but also offers customer-specific projects for demonstration of the impact of METNIN™ process on customer's lignin.

METNIN™ process has been demonstrated and is routinely run at a pilot-scale (400 Liters reactor vessel). In addition, technology transfer to a ton (1000 kg) scale batch production has been completed and an engineering package for the industrial scale is developed and available for licensing from MetGen. Lignin fractions were tested in various applications.

Oligomeric fraction METNIN™ ULTRA was used as lignopolyol to completely replace a commercial polyol in polyurethane rigid foam formulations [35]. The specifications of the obtained foams such as closed cell count, water uptake, and compression characteristics, were all within industry standards for rigid foam applications.

METNIN™ MICRO showed excellent potential in paper coating application and the respective product is being developed together with the pulp and paper industry. Other applications are being tested with industry partners.

4.3.2 Cellulose fibers modification

Cellulose is the most traditional product from non-food biomass. Cellulose fibers further turned into paper were produced for centuries by the pulp and paper industry. However, if the printing and writing paper used to be the main product of this industry, the recent changes in the consumer market and the digitalization of the information market shifted the focus of the pulp and paper industry to hygiene and packaging products, which are much more diverse in terms of the required properties of the fibers (strength, softness, odor, water absorption/resistance etc). Changing the fiber properties can be achieved by adjusting the wood refining and chemical treatment, however, it can also be enhanced by enzymatic treatment [36]. For this purpose, individual enzymes or a set of enzymes are needed rather than a natural hydrolytic cocktail.

The main component of fiber strength improvement cocktail is endoglucanase, an enzyme that introduces individual brakes in a cellulose strand [37]. It attacks amorphous regions of cellulose fiber, where the crystalline structure was distorted by refining. These brakes make fibers more "hairy" and improve fibrillation (incorporation of the fibers into paper webbing), which eventually translates into improved strength properties of the paper [38, 39]. This enzymatic activity can also be used to even further cleave the amorphous region and help to create nano-cellulose [40], which is widely used in various applications from packaging to electronics and health. The conventional mechanical process of obtaining nanocellulose is highly energy demanding and enzymes can considerably reduce the required refining energy. Another cellulose base product with growing demand is the so-called dissolving pulp, which is used for viscose production. The process of liquefying the pulp by separating the fibrils (the strands of cellulose) is also highly energy demanding and chemically polluting. Viscose production can be more eco-friendly and economic by using enzymes, specifically xylanases and cellulases to selectively remove hemicelluloses and improve pulp reactivity, respectively [41].

These cellulose products are usually not considered to be in the scope of biorefineries but rather pulp and paper industry, however, some fiber-based products could be introduced into the biorefineries offering. The MetZyme®BRILA™ product family of MetGen's portfolio is dedicated to fiber modification solutions (see **Table 2**).

4.3.3 Glucose conversion

The main outcome of cellulose processing in the biorefineries is currently glucose. Glucose is the central nutrient in the microbial world. Almost all microorganisms can be cultivated on glucose with some supplement of nitrogen-containing compounds and microelements. Thus the demand for glucose will grow as the bioeconomy develops. And glucose can be a starting material for bioconversion to practically any natural compound by a whole-cell microbial factory.

Apart from being a central nutrient for microbial production glucose can also serve as a starting material for platform chemicals [42]. By acid catalysis, sugar molecules can be converted to platform chemicals such as hydroxy-methyl furfural (HMF), furfuryl alcohol (FAL), and levulinic acid (LA) which can be further used for polymer synthesis [43].

HMF is an important emerging platform chemical that can be further converted to 2,5-furan dicarboxylic acid (FDCA) by chemical [44] or enzymatic [45] oxidation. In turn, FDCA is a precursor for a new to the world polymer polyethylene 2,5-furandicarboxylate (PEF) which provides an alternative to the oil-based plastics polyethylene terephthalates (PET) used for the majority of disposable plastic bottles. Remarkably, PEF represents not only a biobased alternative to PET but also provides a technical advantage in gas retention, which is extremely important for carbonated drinks' shelf life. Apart from PEF, other polyesters and various polyamides and polyurethanes containing FDCA have been described in the literature [46].

HMF can be obtained by dehydration of carbohydrates. The preferred substrate for dehydration is fructose, which can be obtained by the chemical or enzymatic isomerization of glucose (**Figure 3**).

The respective enzyme is called glucose isomerase, although biochemically speaking it is xylose isomerase with a side activity of glucose isomerization [47]. These enzymes are widely available: they are one of the largest in volume in the industrial enzyme market for their production of widely-used High Fructose Syrups (HFS) for food applications. Isomerization is a reversible reaction; enzymes bring the mixture of glucose and fructose to the equilibrium ratio of about 1:1 and the reaction stops. Fructose provides sweetness for food and beverages, but can also be used as an intermediate for bioplastics.

The currently available commercial enzymes are highly sensitive to substrate sugar impurities. This is acceptable for food industry applications, where sugar has to be pure anyway. Typically, even sugar produced from starch requires activated carbon filtration, ion-exchange chromatography, and degasification before it can proceed to isomerization reaction as described by the technology providers, see, for example https://www.myandegroup.com/starch-syrup-process-technology.html. Sugars produced from 2nd generation biorefinery (especially from wood) have much more impurities than starch-derived sugar, including lignin, extractives, etc. It would need a lot of purification to enable utilization of currently available glucose isomerase, and the required level of purity is not justified for the technical sugar. MetGen has developed a proprietary industrially relevant recombinant bacterial glucose isomerase with high tolerance to substrate impurities. The enzyme can work directly in biomass hydrolysate. Lower requirements for purification lead to reduced process costs. This enzyme was further engineered to be much less sensitive to the presence of xylose - a preferred substrate of all of the natural glucose isomerases, and thus a potent competitive inhibitor in the reaction with glucose.

Proposed enzymatic solutions for biorefinery and especially for sugars-to-biochemicals pathways are numerous, however, they are still mostly in the research and development stage [48], and the takeoff of the bio-based economy

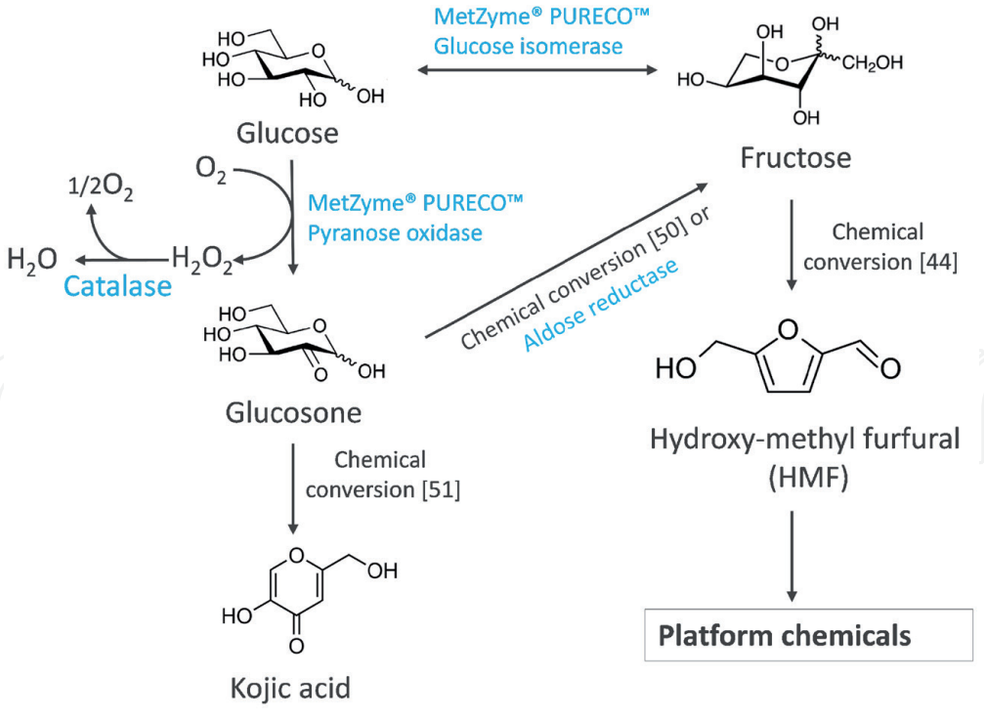

Figure 3.
Bioconversion of glucose using MetZymes® and related processes.

largely depends on the success of this effort. MetZyme® PURECO™ product family – to which previously mentioned glucose isomerase also belongs – is dedicated to next-generation enzymes allowing specific conversions towards high value-addition renewable chemicals, which are beyond sugars. One of these enzymes MetZyme® PURECO™Pyranose oxidase opens an economical route to previously commercially unavailable above a gram scale compound glucosone (2-Keto-D-glucose). While until now the use of d-glucosone has been limited due to its high price and limited availability, it has been envisaged for a long time to provide an alternative route to fructose [49]. As opposed to isomerization reaction, oxidation of glucose to glucosone can be driven to completion, and glucoson's aldehyde group can be further reduced with high specificity to a hydroxyl leading to fructose (**Figure 3**). The second step can be performed either by chemical hydrogenation [50] or enzymatically by an aldose reductase [49]. Recently, more applications of glucosone started to be developed, for example, it has been shown, that certain fine chemicals such as kojic acid could potentially be produced from this source (**Figure 3**) [51].

MetZyme®PURECO™ Pyranose oxidase and glucose isomerase are commercially available proprietary enzymes developed by MetGen in the course of the Horizon 2020 research and innovation program, funded by the European Union's Bio-Based Industries Joint Undertaking.

5. Concluding remarks

MetGens philosophy in serving biobased industries is to provide a full solution rather than on-shelf enzymes to the customers, and where possible an engineering package. Therefore, MetGen embraces all stages of enzyme technology development from enzyme discovery and molecular biology to application testing, streamlined efficient production, and integration into an industrial process. We call this ENZYNE platform.

Another vastly important principle for us is to take an active part in open innovation to combine forces with industry players to build the new value chains in the bioeconomy.

Building consumer awareness is also key for the expansion of the bioeconomy. Society needs to gain a common understanding of the importance of bio-based solutions and their impact on sustainability and circularity.

Acknowledgements

This research recieved funding from the Bio Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation program under grant agreement No: 792061 SWEETWOODS is acknowledged.

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