LIGNOCELLULOSE AS A SUSTAINABLE OPTION FOR BIOETHANOL PRODUCTION BY FUNGAL LIGNOCELLULOSOMES – A REVIEW

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This review presents current advances and future perspectives for bioethanol production by fungal lignocellulosomes, based on studies conducted in the last few decades. The key steps for obtaining fermentable sugars for bioethanol production from lignocellulose are its pretreatment and enzymatic saccharification. Lignocellulosics are abundant and cost-effective sources as a potential "green" substitution for fossil fuels. Therefore, it is not surprising that the powerful fungal lignocellulolytic enzymes have been intensively studied, especially in terms of environmentally friendly economic and social development. The effects of fungal co-cultivation on the capacity of their lignocellulosomes are also discussed. As bioethanol production has not yet developed on a large scale, the future of this field lies in redesigning enzyme cocktails and reducing limitations in the conversion process. Lignocellulose is definitely a promising source for biofuel production, but on the way to its successful transformation are obstacles that will be overcome by future research.

Keywords: lignocellulosome, fungal pretreatment, enzymatic hydrolysis, bioethanol

INTRODUCTION

Population growth, increasing industrialization and urbanization have led to a rise in global energy demand, resulting in a 17-fold increase in energy consumption in the 20th century. According to UN estimates, the current world population is expected to reach 9.8 billion people by 2050, which could increase the energy demand to at least 9 million barrels of oil per day and 24 billion tons of coal equivalents per year, double the current consumption (Fig. 1). Energy sources can be divided into primary (including non-renewable and renewable) and secondary, which are obtained by converting some primary energy sources providing electrical, thermal or chemical energy. In recent decades, fossil fuel use in large urban areas has led to numerous harmful impacts on the environment, but still provides more than 88% of the energy needed, although the annual global oil production is expected to decline in the near future.[1-5]

To overcome the problems caused by the limitations of conventional non-renewable fossil fuels, it is necessary to incorporate their rational use and find alternatives. In this scenario, renewable energy s ources such as wind, water, biomass, solar and geothermal energy could serve as alternatives becoming increasingly important.[6] It is estimated that the share of these sources will be more than 30% in 2030 and more than 40% in 2040, while global energy consumption will increase up to 80% by 2100.[4,7] Today, countries around the world are focusing on the use of biomass as a future energy source to meet the Kyoto Protocol targets and reduce CO_2 emissions and dependence on fossil fuels. For example, CO_2 emissions can be reduced by up to 80% by using bioethanol instead of gasoline, which could contribute significantly to a cleaner future.[8] Considering that lignocellulose accounts for about 60% of the total biomass on earth, it represents promising feedstock for bioethanol production and other industrial processes.[9,10] It has been reported that global bioethanol production increased from 31 billion liters in 2012 to 100 billion liters in 2015.[11] However, renewable energy sources still do not play a major role in the global energy balance, which means that achieving sustainable development is still a long way off.

Currently, there are several countries where the production of first-generation bioethanol is well developed to meet the needs of the transportation sector. However, the edibility of some feedstocks raises questions about the sustainability of this approach, as it focuses on the transportation sector rather than the solving the problem of the world's undernourished population. Due to the unfavorable characteristics of first-generation feedstocks, Aditiya *et al*.[8] predict that second-generation bioethanol production will surpass that of first-generation and will dominate the global biofuel market in the next decade. Currently, bioethanol is more expensive than gasoline, but as feedstock availability determines overall production costs, bioethanol could be expected to be cheaper than fossil fuels in the future.[8]

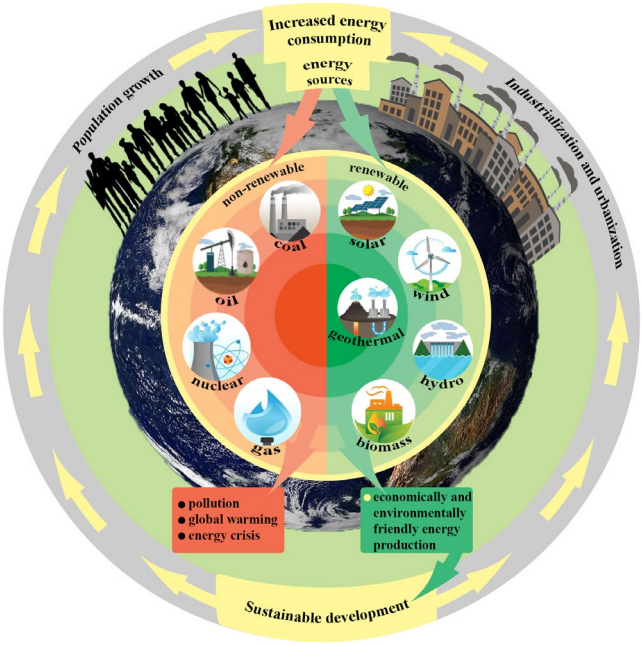

Figure 1: The significance of renewable energy sources in modern society

BIOMASS: A SUSTAINABLE ENERGY SOURCE FOR THE FUTURE?

Plant biomass is a renewable, cheap and, above all, remarkably abundant raw material, which is an important factor in the global bioeconomy based on utilization of "green" sustainable sources to meet the needs of society.[12] A huge amount of this material is produced worldwide every year (150 billion tons), mainly in agricultural and forestry countries, but the predominant biomass differs from region to region.[9,13] However, that enormous amount of generated waste lignocellulosics could be either significant environmental ballast or promising ecofriendly resource for the production of biofuels, chemicals, biofibers, enzymes and other products, and in such a way contributes to a circular economy. One of the challenges in extensive ethanol production is to improve the management of ballast biomass due to insufficient utilization and inadequate deposition.[9] Wheat straw, rice straw, corn stalks and sugarcane bagasse are the most important agricultural wastes in terms of the amount of biomass available.[14] However, the efficiency of bioenergy production also depends on the chemical composition of raw materials, *i.e*. Binod *et al*.[15] reported that rice straw has an advantage over other raw materials due to its low total alkali content. The fact that the global annual production of rice straw ranks second after wheat straw shows its enormous potential for bioethanol production.[16]

All the above-mentioned leads us to the conclusion that the improvement of agricultural production can affect the sustainable development and particularly the energy sector in different regions. Definitely, certain parts of the world have a huge potential for sustainable development based on agriculture and waste biomass left behind it. For example, although Serbia is the leader in blackberry production in Europe (27558 tons), the fourth largest producer of raspberries in the world (68500 tons) and a major producer of

apples (378644 tons), plums (330582 tons) and grapes (165568 tons) (FAO - 2013), a small proportion of the resulting waste is reused. The FAO has also reported that cereals are the most important group of crops produced in 2020, followed by sugar crops (23%), vegetables and oil crops (12% each), while fruit accounts for 9–10% of total world production. Also, the annual production of forestry residues, whether from natural or cultivated forests, is about 4.6 Gt worldwide. Added to this is the biomass of grasses and/or weeds, municipal waste and waste from the food industry.[17] Thus, the same authors reported that forestry residues after removal of roots, branches and foliage are a rich source of fuel. Among renewable energy sources, biomass has the greatest potential as it is a unique carbon source that can be converted into solid, liquid and gaseous fuels through various conversion processes.[10] Biomass includes residues from agriculture and forestry, waste from some industries and biodegradable parts of municipal waste.[4,16,18] According to Cuong *et al*.,[7] about 40% of the world's population (~3.1 billion) traditionally use biomass as an energy source for heating and/or food preparation, especially in rural areas of developing countries. It is estimated that more than 90% of the world's population will live in these regions by 2050, so the utilization of biomass potential will become increasingly important.[19]

According to Rastogi and Shrivastava[10] and Edeh,[20] biofuels can be categorized into first, second, third and fourth generation biofuels based on their biomass feedstock: (i) first-generation biofuel – produced from agricultural residues based on starch, sugar or oil; (ii) second-generation biofuel – produced from lignocellulosic biomass, with the possibility of using some industrial by-products; (iii) third-generation biofuels – produced from the biomass of microalgae and microorganisms; (iv) fourth-generation biofuel – produced by genetically modified microalgae and is still the subject of extensive research.

Lignocellulosic biomass as alternative for fossil fuels

Lignocellulose has the advantage over other biomass sources in that it is a novel abundant raw material that can replace fossil ones.[5] Nowadays, global warming, along with the resulting "greenhouse effect", is an urgent environmental problem on a global scale caused by burning of fossil fuels and the release of CO_2 and NOx, while the use of lignocellulose instead of fossil fuels brings benefits, such as the elimination of PAH pollutants and the reducing CO_2, NOx and SOx emissions, thus helping to improve the environment and people's quality of life.[10] In contrast to fossil fuels, the overall balance of CO_2 emissions from the combustion of bioethanol is zero, regardless of the type of raw material from which it was derived.[21] For example, Adytiya *et al*.[8] reported that replacing gasoline with bioethanol could reduce CO_2 emissions by up to 80%, while bioethanol burning releases virtually no sulfur, but produces a significant amount of heat energy. Although its energy equivalent is 68% lower than gasoline, the combustion process is cleaner due to high oxygen content of bioethanol and therefore releases fewer toxic compounds.[22] However, since conventional engines are not able to completely combine fossil fuels with bioethanol, Enguídanos *et al*.[23] proposed a solution using a mixture of fossil fuel and bioethanol, which avoids the need to modify the engine.

The predominant lignocellulose used in biotechnological processes varies from region to region.[24,25] In addition to biofuels production, lignocellulosic biomass has enormous potential as a raw material for the production of chemicals, enzymes, paper, *etc*., and therefore justifies the environmental acceptability and "green" prices for this type of products.[26,27] However, despite its plethora of advantages, lignocellulose has some limitations. Namely, lignocellulose has a very complex structure, so its preparation for the biotechnological processes increases the cost of the potential products.[28]

Physical and chemical characteristics of lignocellulosic biomass

Lignocellulose consists of two polysaccharides – cellulose and hemicellulose –, and a recalcitrant polyphenolic compound – lignin, which acts as a protective shield for the polysaccharides and prevents their decomposition, as well as a small number of other components.[26,29,30] The main structural components of the primary plant cell wall account for about 80% of the dry weight of lignocellulose, and the composition varies depending on the plant species, climatic and other

growth conditions and developmental stages of the plant.[31] The high lignin content impairs the digestibility of lignocellulosic residues and reduces their nutritional value.[32] Moreover, the enzymatic degradation of plant residues is very slow due to the lignin nature, so the use of lignocellulose in industrial processes requires effective pretreatment.[33] Many other factors, such as the crystalline structure of cellulose, the degree of polymerization, the particle size of the biomass, *etc*., limit the digestibility of cellulose and hemicelluloses.[30] In general, lignocellulosic wastes contain low amounts of ash, cyclic hydrocarbons, proteins, vitamins and other compounds.[34]

Lignin, cellulose and hemicelluloses

Lignin is a natural phenolic polymer with a high molecular weight that forms a matrix with cellulose and hemicelluloses, and protects them from attack by hydrolytic enzymes.[35,36] Valorization of lignin is an important part of the circular economy concept for lignocellulosic biomass to increase the profitability of biorefineries, one fifth of which, in Europe, are based on this material.[37] In lignin, the main components – p-coumaryl, coniferyl and sinapyl alcohol – are present in the form of phenylpropanoids, such as p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S).[36] Enzymes, such as laccases or peroxidases, catalyze oxidation reactions to form phenoxy radicals, which then polymerize into various configurations.[35,38-40,41] Based on physicochemical properties, three types of lignin are distinguished: (i) G-lignin – lignin from softwood or gymnosperms, containing only residues of coniferyl alcohol; (ii) GS-lignin – lignin from hardwood or angiosperms, containing residues of coniferyl and sinapyl alcohol; (iii) HGS-lignin – lignin from grasses and herbaceous plants, containing all three lignin components. Due to its physical and chemical properties, lignin from hardwoods is more susceptible to transformation than lignin from softwood.[32]

Cellulose is the most abundant biopolymer, with an estimated annual production of 1.5×10^{12} tons, and is considered an unlimited source of raw material for energy production. In general, the proportion of cellulose in plant cells is between 23% and 53% of the dry weight.[42] Multiple parallel glucose chains form fibrils that are grouped in bundles to form a microfilament structure, while multiple microfilaments form a macrofilament bundle. In the regions between the fibrils, the cellulose forms a crystalline structure, while the rest of the segment is amorphous.[42-45] Cellulose is insoluble in water and dilute acids, which gives it chemical and mechanical stability and resistance.[46] In addition, cellulose is resistant to the action of various chemicals and enzymes due to the resistance of the crystal structure and binding to other components, which has a negative effect on the enzymatic hydrolysis efficiency.[45]

Hemicelluloses are the second most abundant biopolymer in nature after cellulose.[47,48] Due to their branched structure, there is no crystalline formation. For this reason, substituents with a low molecular mass and a low degree of polymerization (80-200 units) are located on the main chain or at the branches. The role of hemicelluloses is to organize the cellulose in the cell wall and ensure its rigidity thanks to its interaction with each other and with lignin.[28] Hemicelluloses comprise four groups: (i) xylans – very important and widely used polymers; (ii) xyloglucans – present in the primary cell wall of higher plants and bound to cellulose; (iii) mannans – present in the secondary cell wall of softwoods; (iv) a group of differently linked β-glucans, mainly in the cell wall of grasses.[47-49]

FUNGI AND FUNGAL ENZYMES INVOLVED IN LIGNOCELLULOSE DEGRADATION

The advantage of some fungi over bacteria in the effective decomposition of lignocellulose with higher lignin content is that they are better producers of ligninases. It has been shown that white-rot fungi and their strong ligninolytic enzymes are able to powerfully convert and/or degrade lignin, which is necessary for efficient biomass pretreatment. In contrast to white-rot fungi, brown-rot fungi only partially modify lignin. Soft-rot fungi, on the other hand, show only initial signs of degradation.[38]

Ligninolytic mechanisms of white rot fungi

Due to the persistence of lignin, white-rot fungi degrade it by specific mechanisms, which have been the subject of extensive research. These organisms can synthesize one or more types of enzymes from the group of lignin-modifying enzymes. For this group of fungi, Kirk and Cullen[38] have described two main mechanisms of lignin degradation:

(i) cellulose, hemicelluloses and lignin are decomposed almost simultaneously

(*Phanerochaete chrysosporium*, *Trametes versicolor*, *Xylaria hypoxylon* are some of the species that degrade wood in this way);

(ii) selective degradation – lignin and hemicelluloses are degraded before cellulose (some representatives are *Dichomitus squalens*, *Ganoderma australe*, *etc.*).[50]

There are different modes of lignin degradation both between different fungal species and between strains of the same species. Interestingly, in some cases, the same species can cause different types of degradation on different parts of the same plant.[38] Several different mechanisms lead to the formation of unstable lignin radicals, which is why they undergo a series of spontaneous cleavage reactions.[35] Fungi that degrade lignin face several problems: (i) it is a large and highly branched polymer, so the ligninolytic mechanism must be extracellular; (ii) the decomposition mechanism is oxidative, as there are stable ether and carbon bonds between the subunits; (iii) lignin is a combination of stereo-irregular units, so ligninolytic enzymes must be characterized by a broad specificity in mineralization of different substrates; (iv) the insolubility of lignin in water makes the degradation process slow.[38]

According to Janusz *et al.*,[51] the mechanism of lignin degradation was first described in *P. chrysosporium*, which synthesizes lignin- and Mn-oxidizing peroxidases, but no laccase. Ligninolysis in this species requires conditions with a reduced amount of nitrogen in the medium, whereas this element is necessary for the same processes in *Bjerkandera* sp. and *Pleurotus* sp.[35,52]

Fungal enzyme systems

The degradation of lignocellulose by fungi is enabled by a variety of enzymes whose mechanisms of action can be divided into oxidative and hydrolytic. According to the studies of Sánchez[30] and Leonowicz *et al.*,[53] the enzymes of white-rot fungi are divided into:

(i) enzymes that degrade lignin, cellulose and hemicelluloses, *i.e.* lignocellulolytic enzymes, which include ligninases, cellulases and hemicellulases;

(ii) enzymes that cooperate with the enzymes of the first group, but do not function independently;

(iii) enzymes indirectly involved in delignification and directly involved in cellulose degradation.

In general, enzymes involved in lignin degradation can be divided into heme peroxidases, which include lignin- and Mn-oxidizing peroxidases, and polyphenol oxidases, *e.g.* laccases.[54] An important group are "auxiliary" enzymes, which cannot act on their own, but are necessary for the completion of the process through the formation of H_2O_2.

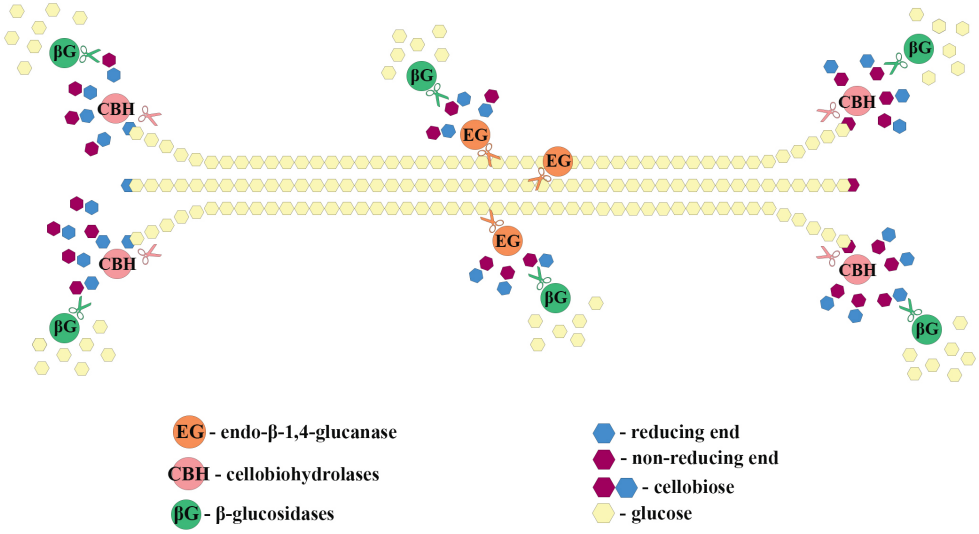

Figure 2: Synergistic mechanism of cellulases activity

Fungal metabolites, such as aromatic compounds, low molecular weight peptides, metal ions, *etc.*, actively participate in the ligninolytic process as mediators.[55-57] The complex of cellulolytic enzymes consists of three enzyme groups that act synergistically (Fig. 2):

(i) endocellulases or endo-β-1,4-glucanases, which can cleave the internal β-(1-4) or β-(1-3)

bonds within the glucose chains and generate a reducing and a non-reducing ends;

(ii) exocellulases or cellobiohydrolases, which "attack" these ends to generate mainly cellobiose;

(iii) β-glucosidases, which catalyze the degradation of cellobiose into two glucose molecules.[8,58]

During hydrolysis, the hemicellulases help the cellulases to access the substrate, but also are necessary for the complete degradation of hemicelluloses to monomeric sugars and acetic acid.[30] This group of enzymes includes endo-1,4-β-xylanases, which catalyze the breaking of bonds in xylan and the formation of oligosaccharides, and xylan-1,4-β-xylosidases, which hydrolyze oligosaccharides to xylose. The synergistic action of auxiliary enzymes is also necessary to complete the process.

Ligninolytic enzymes

Lignin-peroxidases

Lignin peroxidases (LiP) were the first lignin-modifying enzymes discovered in *P. chrysosporium*, while they were later discovered in *Trametes versicolor*, *Bjerkandera* sp. *etc*. However, species such as *D. squalens*, *Ceriporiopsis subvermispora*, and *Pleurotus* spp. have been shown not to synthesize these enzymes.[38,59,60] Due to its high redox potential, LiPs are a strong oxidizing agent, oxidizing common peroxidase substrates and a number of non-phenolic compounds whose structure resembles lignin units, such as veratryl alcohol.[38,61]

Mn-oxidizing peroxidases

This group of enzymes includes Mn-dependent peroxidases (MnP) and Mn-independent/versatile peroxidases (MnIP/VP). MnPs are important lignin-modifying enzymes that were first identified in *P. chrysosporium* and later in most white-rot fungal species.[38,51] Their molecular structure is similar to that of LiP, but they are weaker oxidizing agents because they lack electrons in the porphyrin ring. These enzymes are H_2O_2-dependent heme-glycoproteins with an iron protoporphyrin IX prosthetic group, whose main function is the oxidation of Mn^{2+} to highly reactive Mn^{3+} as a mediator in the oxidation of organic substrates similar to lignin, phenol, *etc*.[62] MnIP/VP were for the first isolated by Martínez *et al*.[63] in *P. eryngii* after liquid cultivation in glucose/peptone/yeast extract medium. Later, these enzymes were detected in species of the genera *Bjerkandera*, *Ganoderma*, *Trametes* and others.[56,64,65] According to the reports of Martínez *et al*.[63] and Guardina *et al*.,[66] MnIP were considered as MnP isoenzymes, since they catalyze oxidative reactions characteristic for them (oxidation of Mn^{2+} to Mn^{3+}). However, they also catalyze oxidative reactions characteristic for LiP, which is why Caramelo *et al*.[67] referred to them as LiP-MnP hybrids. To date, a large number of studies have been carried out on the activity of these enzymes, especially on the dependence on the cultivation substrate, and it has also been the subject of our own investigations. For example, in the most recent studies, Galić *et al*.[68] detected a very high level of Mn-peroxidases by different strains of *P. eryngii* and *P. pulmonarius*, while Ćilerdzić *et al*.[69] measured lower activities of these enzymes by the same *Pleurotus* species depending on the three different lignocellulosics. Furthermore, these enzymes reached high activities after cultivation of *G. lucidum* and *G. tsugae* on various substrates.[70] Similar studies were conducted with species such as *Grifola frondosa* and *Auricularia auricula-judae*, where significant activities were also observed on different agro-forestry residues.[71,72]

Laccase

According to Dashtban *et al*.[54] and Thurston,[73] white-rot fungi, such as *Cerrena unicolor*, *P. chrysosporium*, *P. ostreatus*, *Trametes* spp., are the best producers of laccases. In addition, some brown-rot fungi such as *Chaetomium thermophile*, *Coniophora puteana*, *Neurospora crassa*, *etc*. synthesize these enzymes. Fungal laccases have been found to play a physiological role in pigmentation, fruiting body formation and pathogenicity.[74] They catalyze four successive one-electron oxidation reactions of numerous organic and inorganic substrates, including phenolic and non-phenolic aromatic compounds to the corresponding radicals using molecular oxygen as electron acceptor being reduced to H_2O.[73,75] Oxidation of the substrate can occur either directly by interaction with the catalytic center of the enzyme or indirectly by chemical mediators (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydroxybenzotriazole, *etc*.).[76,77,78] Laccases contain one to four copper atoms, which are reduced during substrate oxidation and play a key role in the catalytic cycle. According to Alcade *et al*.,[79] the Cu atom in the active center is also the primary electron acceptor. On the other hand, laccases in certain

fungal species can bind other metals (Zn, Fe or Mn), but then are not classified as so-called "blue" laccases.[80,81] This was the case for the isoform synthesized by *P. ostreatus* – POXA1, which contains one Cu atom, one Zn atom and two Fe atoms and therefore is classified as a "white" laccase due to the lack of the characteristic blue color. A broad spectrum of substrates and the use of O_2 as the final electron acceptor give them an advantage for application in various biotechnological processes, including bioethanol production.[54] Similar to Mn-peroxidases, we have so far performed numerous measurements of laccase activity in a large number of species and strains of white-rot fungi during fermentation of different lignocellulosics. In most cases, the results showed the extraordinary activity of this enzyme in various species, many times higher than that of other ligninolytic enzymes.[33,68,69,70] Therefore, Stajic *et al*.[70] proved that Lac was dominant in the enzyme cocktail, with a peak of even 42480 U/L on plum sawdust with *G. lucidum*, while Galic *et al*.[68] detected slightly weaker Lac activity, of 36052 U/L, after the fermentation of oak sawdust by *Pleurotus* species.

Cellulolytic enzymes

In addition to the ligninolytic enzymes, which are responsible for the modification and degradation of lignin, fungi produce hydrolytic enzymes consisting of cellulases and hemicellulases, responsible for the degradation of polysaccharides.[82] Since they hydrolyze delignified material to sugars, they play an important role in numerous processes.[58,83] What distinguishes cellulases from other enzyme classes is their ability to hydrolyze an insoluble substrate. Sweeney *et al*.[84] emphasized the importance of the synergism of endo- and exocellulases in biomass transformation processes. The active center of these enzymes has the form of a cleft in which the enzyme binds and degrades cellulose chains. However, the cellulose-binding domain may be absent in fungal endocellulases.[85,86] CBH (cellobiohydrolases) contain a tunnel-like active center through which the ends of the cellulose chains can be pulled and release cellobiose. The exocellulases then slide further down the cellulose chain, where they initiate the next hydrolysis step.[87,88] *Trichoderma reesei* is one of the best cellulase and hemicellulase producers and is widely used in industry.[89] In our study, we analyzed the potential of *T. viridae* for the synthesis of cellulolytic enzymes during solid-state fermentation of wheat straw pretreated with a potent delignificator – *Pleurotus pulmonarius*. The results clearly showed that this strain has exceptional cellulolytic potential, and it is expected that many more species and strains will be found in future studies that have the potential to synthesize highly active cellulases, which is important for future large-scale industrial application.[90] Of the total cellulases, exocellulases account for 80% in this species (CBH I: CBH II = 60%: 20%), while endocellulases and β-glucosidases account for only 15.5% (15%: 0.5%).[91,92]

After the action of endo- and exocellulases, β-glucosidases hydrolyze cellobiose to glucose. These enzymes play an important role in regulating the process, which is the limiting factor of the entire cellulose hydrolysis process.[84,93] It is known that the accumulation of glucose and cellobiose as hydrolysis end-products inhibits the cellulases and thus reduces the glucose yield. Among the cellulolytic enzymes involved in the hydrolysis process of lignocellulosic substrates, endocellulases and cellobiohydrolases are most inhibited, whereas β-glucosidases are less sensitive to high concentrations of monosaccharides.[94] Therefore, β-glucosidases play a crucial role in preventing a drastic slowdown of hydrolysis.[92] In general, cellulases have a cellulose-binding domain, which is necessary for the enzyme to adhere to the substrate so that the catalytic domain can fulfill its function.[95] Although the cellulose-binding domain is not involved in the process, Bayer *et al*.[96] have shown that its removal would significantly reduce the effectiveness of hydrolysis. However, the available genomic data show that many cellulases lack the binding domains.[85] Furthermore, recent results have confirmed that their presence is not necessary for the action of cellulases, but they can still influence the increase in enzyme concentration on the substrate surface.

Hemicellulolytic enzymes

Xylan is the most abundant polysaccharide in hemicelluloses and accounts for more than 30% of the dry mass of the cell wall of vascular plants, so its degradation is of great importance.[93] Sánchez[30] reported that the efficient hydrolysis of hemicelluloses requires the synergistic action of several enzymes, not only because of its structure, but also because of its association with other components of the cell wall. The most important

are endo-1,4-β-xylanases, which hydrolyze β-1,4-xylan chains, releasing oligosaccharides, and exo-1,4-β-xylosidases, which cleave xylobiose and xylo-oligosaccharides, releasing xylose. The hemicellulolytic enzymes also include a group of "auxiliary" enzymes that are required to complete the process.[97] For example, the complete degradation of arabinoxylan, one of the components of the cell wall of wheat straw, requires the action of some "auxiliary" enzymes that lead to the breaking of the covalent bonds between α-arabinose and D-xylose and the removal of xylose residues.[98]

BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

Sánchez[30] divided the process of obtaining bioethanol from lignocellulosic residues into three phases:

(i) pretreatment, *i.e.* delignification of the lignocellulose;

(ii) enzymatic hydrolysis, *i.e.* saccharification of the pretreated biomass;

(iii) fermentation of the hydrolysate.

Importance of pretreatment for bioethanol production

The process of transforming lignocellulosic waste into fermentable sugars requires an expensive and complex pretreatment necessary to remove lignin, which increases the availability of cellulose to the enzymes and enables its efficient hydrolysis. Due to the complexity of the lignocellulosic material, this process is the most important step in the conversion to ethanol.[99,100] The efficiency of saccharification, *i.e*. the conversion of polysaccharides into simple sugars, and the yield of ethanol depend on the pretreatment. Although pretreatment is a costly step in bioethanol production (up to 33% of total costs), it has been shown that effective pretreatment can increase ethanol yields by up to 70%. This justifies efforts to develop new methods for this process in order to make bioethanol production profitable.[92,101]

There are different chemical, physical, and physico-chemical procedures for delignification, but the major characteristics of these pretreatments are their high cost, large energy consumption, and the release of numerous harmful byproducts. A different approach is provided by biological pretreatment, which is characterized by extremely low energy consumption, minimal waste accumulation, and no negative environmental effects. However, this method also has some disadvantages that limit its application, such as a long period (several weeks to several months) and a large space required for its realization.[71] Additionaly, for economic growth, the ideal pretreatment method is the one that provides higher sugar yield with lower inhibitor production.[25] Since the biological pretreatment is characterized by lower yield of the final product, its application on an industrial scale is still limited.[26,102,105-107] Overall, based on our own research and many other studies, it can be concluded that white-rot fungi are the most promising agents in biological pretreatment due to synthesis of an efficient enzyme cocktail.[68,69,103,104]

Factors affecting lignocellulosic pretreatment

The efficient transformation of lignocellulosic waste by white-rot fungi depends on a number of factors, of which the fungal species/strain is one of the most important.[33,52,66,108-110] Fungi differ in the selectivity of lignocellulose degradation, *i.e*. their tendency to degrade lignin and hemicellulose to a greater extent than cellulose.[111] These authors showed that *P. chrysosporium* strongly delignified the substrate after only a few days of cultivation, but showed no selectivity, whereas *P. ostreatus* as a selective and efficient delignifier required several weeks for this process. In addition to the fungal species and strain, the effectiveness of the pretreatment also depends on the physical and chemical properties of the lignocellulosic material and the conditions of its fermentation.[9,26,52,110,111] For example, some *Pleurotus* species efficiently degrade lignin from straw, but not lignin from some hardwood or softwood species.[26]

Numerous studies have also shown that the delignification process is much more efficient under solid fermentation conditions than under liquid fermentation conditions due to: *(i)* a higher oxygen content that stimulates the delignification process, which is completely oxidative; *(ii)* a relatively low humidity that favors the synthesis of specific compounds; *(iii)* the similarity of solid fermentation conditions to those of natural fungal habitats.[65,110,112,113] However, the main disadvantage of solid-state fermentation is the absence of free water, which limits the transfer of nutrients and enzymes, and the formation of a temperature gradient within the substrate due to the metabolic activity of the fungi.[111]

An important factor influencing the production of highly active forms of enzymes and thus the efficiency of lignocellulose degradation is the nitrogen source and its concentration.[27,114] For example, Mikiashvili *et al*.[115] showed that *P. ostreatus* synthesized highly active forms of enzymes in a medium containing organic nitrogen sources, but not in the presence of inorganic compounds. Carbon source and its concentration in the medium also influence the production of ligninolytic enzymes.[52,116,117] Mikiashvili *et al*.[116] showed that cellobiose and mannitol are good carbon sources for the synthesis of laccase in *T. versicolor*, but much weaker compared to mandarin peels, which induced the production of a seven times more active form of this enzyme. Similar results were obtained by Stajić *et al*.[52] and Ćilerdžić *et al*.,[65] who showed that mandarin peels and grapevine sawdust were significantly more effective in inducing laccase activity in *Pleurotus* spp., while the optimal substrate for the synthesis of highly active forms of MnP in *T. hirsuta* was wheat straw. The addition of microelements such as Mn^{2+}, Cu^{2+}, *etc*., and inducers, such as aromatic compounds, can also stimulate the production of ligninolytic enzymes and biomass degradation.[26,109,111,114,118] For example, in the studies of Palmieri *et al*.[80], Bonnarme and Jeffris[118] and Baldrian and Gabriel[119], the addition of Mn^{2+} induced MnP synthesis in *P. chrysosporium*, *Phlebia* spp., *Lentinula edodes*, and *Phellinus pini*, but inhibited LiP production, while the addition of Cu^{2+} stimulated laccase activity. In addition, Stajić *et al*.[109] showed that Cu^{2+} and Mn^{2+} have different effects on the production of laccases and peroxidases in *Pleurotus* species, depending on the species/strain, cultivation conditions and concentration of these microelements.

Certainly, the degree of aeration is an important parameter for the degradation of lignocellulose. Indeed, Zadražil *et al*.[120] stimulated the delignification of straw during solid-state fermentation by *Stropharia rugosso-anulata* with a high oxygen content. However, the oxygen concentration was not shown to correlate positively with the selectivity of degradation.[121] An average humidity of 60% to 80%, a slightly acidic pH and a temperature between 15 °C and 35 °C are the optimal conditions for efficient pretreatment of lignocellulosic biomass.[26,111,121-124] Given the great potential of fungal ligninolytic enzymes, special emphasis is now placed on optimizing delignification conditions to convert the most abundant lignocellulosic residues into food, feed, paper and biofuels.[104]

Promotion of enzymatic hydrolysis of low-cost lignocellulosic residues

Cellulases are relatively expensive enzymes, so a significant reduction in their production costs is crucial if the price of bioethanol from lignocellulose is to compete with the price of fossil fuels or bioethanol from starchy biomass. The production of these enzymes on cheap substrates, increasing their productivity and thermal stability are necessary to make the hydrolysis process as competitive as possible on the market.[125,126]

Enzymatic hydrolysis of cellulose has numerous advantages compared to acidic or basic hydrolysis: (*i*) mild reaction conditions (45-50 °C, pH 4.8); (*ii*) high yield of reducing sugars; (*iii*) high selectivity; (*iv*) lower costs; (*v*) no corrosive influences.[18,126]

The interaction of cellulases with the substrate and the identification of rate-limiting factors for their action are the most important parameters for understanding the kinetics as a way to optimize hydrolysis. Enzymatic hydrolysis occurs through two processes: (*i*) the primary one, which generally occurs at the surface of the substrate and forms intermediates, *i.e*. soluble sugars with a degree of polymerization below 6; (*ii*) the secondary one during the hydrolysis of intermediates into smaller molecules that are further hydrolyzed to glucose by β-glucosidases.[18]

Numerous studies have highlighted the lignocellulosic pretreatment as a key step, but the process of saccharification, whose efficiency affects fermentation yield, is not far behind. Although excellent producers of cellulolytic enzymes, micromycetes have unjustifiably received much less scientific attention compared to macromycetes.[125] Since *Trichoderma* and *Aspergillus* species are the best-studied cellulase-producing micromycetes, their enzymes are commercially available for various industrial applications. Future studies should focus on the other effective producers of these enzymes as cellulases are currently the third most important industrial enzymes and even industrial scale enzymes.[125]

The efficiency of the hydrolysis process depends on several factors, including: (*i*) enzyme stability; (*ii*) ability of the enzyme to bind to the substrate; (*iii*) amount of inhibitory compounds

formed; (*iv*) physicochemical properties of the lignocellulose; (*v*) synergism within the enzyme complex.[127,128]

A synergistic hydrolysis mechanism was well investigated in a most studied cellulolytic agents – *Trichoderma* spp., but species of the genera *Chaetomium*, *Helotium*, *Coriolus*, *Phanerochaete*, *Schizophyllum*, *Serpula*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Myrothecium*, *Paecilomyces*, *Penicillium* are also important producers of these enzymes.[90,129,130]

To increase the yield of cellulolytic enzymes on an industrial scale, many parameters of this process must be optimized, including the selection of substrate, since the nature of the plant waste (crystalline structure of cellulose, degree of polymerization, lignin content) are important factors affecting enzyme production.[131,132] Cellulases are not only used in the conversion of lignocellulosic biomass, but are also widely used in the food, paper, textile and chemical industries.[133] Although a considerable amount of fermentable sugars can be obtained by the degradation of hemicelluloses, their hydrolysis is much more complex compared to that of cellulose due to their structure and the need for the involvement of several enzymes, which makes it economically unviable.[134] In addition, the products of hemicellulose hydrolysis are strong inhibitors of cellulases and β-glucosidases.[8]

Fungal co-cultivation, a new powerful tool for the complete degradation of lignocellulosic material

According to Sharma *et al*.,[3] a single organism can rarely synthesize all the enzymes required for the complete degradation of lignocellulosic material. For example, most species of the phylum Ascomycota are unable to delignify plant waste, but have evolved a cellulolytic enzyme system, unlike fungi causing white-rot, which are generally very good lignin degraders. Therefore, the trend in modern research is to cultivate two or more species from the above fungal groups together (Fig. 3) and find an optimal system with a synergistic or additive effect on lignocellulose mineralization.[135] As reported by Qi-He *et al*.,[136] the application of such systems would not only enable the mineralization of lignocellulose, but would also be very effective in the processes of bioremediation, production of pharmaceuticals, numerous chemicals, *etc*.

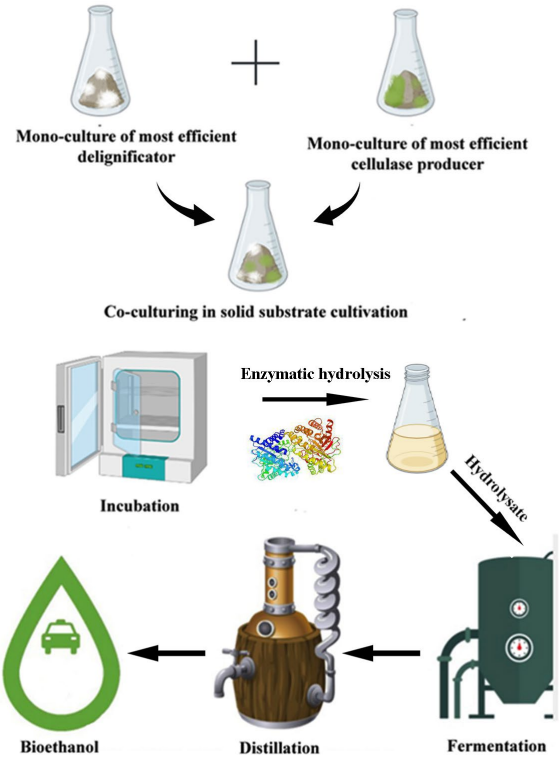

Figure 3: Model of fungal co-cultivation for bioethanol production

Although co-cultivation has been studied and applied in biotechnology for several decades, the main challenges for an optimal combination of organisms are the differences between their genotypes, the types of enzymes and their production, and the ecological niches.[3] In natural habitats, most organisms interact with each other, which can be positive, such as symbiosis and commensalism, or negative, such as parasitism and competition.[137] As Sperandio and Ferreira Filho[135] reported, to understand the interactions of different fungal species under controlled conditions, *i.e*. how gene expression, enzyme production and their activity influence each other, it is important to know their natural relationships. Species compatibility and the amount of inoculum used are the main factors affecting the efficiency of co-cultivation, so they should be given great attention in future studies.[137,138]

An important characteristic of fungi that also affects the success of co-cultivation is the growth rate, which varies from species to species and depends on the cultivation conditions. The difference in growth rate is a serious problem in co-cultivation because the species with the higher growth rate can completely consume the resources necessary for the other species and even inhibit its growth by releasing certain metabolites.[135] For this reason, it is necessary to optimize the time of inoculation of the species, as well as the amount of inoculum.[139] Rabelo *et al*.[137] recorded a significantly higher activity of endoglucanases during solid co-cultivation of *A. niger* and *T. reesei* when the inoculum share was 3:1, while for the synthesis of β-glucosidases the optimal inoculum ratio was 1:3. In general, the degradation of lignocellulosic material by co-cultivation of two or more fungal species whose metabolic interaction has not yet been fully elucidated is a complex process. However, the co-cultivation of two compatible species is profitable as the efficiency of such a system is similar or even superior to some commercial products.[140] Reducing the cost of enzyme production would have an impact on the price of bioethanol and thus increase the potential of biorefineries as an important link in the new bioeconomy.

Previous research has shown significant potential for the application of co-cultures in biotechnological processes, which is why research in this area will be even more valuable in the future.

Fungal fermentation of lignocellulosic hydrolyzate for improved bioethanol production

Fermentation is the metabolic process in which sugar is converted into alcohol, acids, CO_2 and water. In addition to glucose, lignocellulose hydrolysates also contain mannose, galactose, xylose, arabinose and some oligosaccharides, which are often fermentation inhibitors or indigestible components.[8] The fermentation of these monomeric sugars (hexoses and pentoses) produced during saccharification is carried out by various microorganisms. With the aim of improving the efficiency of industrial bioethanol production, the fermentation process can be carried out either in parallel with hydrolysis, the so-called simultaneous saccharification and fermentation using co-cultures, such as *Saccharomyces cereviaise/Fusarium oxisporum*, or independently of hydrolysis.[141] According to Vohra *et al*.[141] and Jambo *et al*.,[142] the advantage of the two-step process is that both take place under optimal conditions, while the disadvantage is the accumulation of sugars that inhibit enzymes activity, which in turn has a negative effect on ethanol yield. In simultaneous saccharification and fermentation, the processes take place in the same bioreactor, and the main feature of this process is the rapid conversion of sugars into alcohol, which prevents inhibition by the substrate or other components of the hydrolysate.[141,144,145] However, the main problem in this process is the optimization of the various parameters on which the efficiency of both the microorganisms and the enzymes depends.[145] As an example, these authors mentioned the optimization of temperature as the optimal temperature for the activity of cellulolytic enzymes is 50 °C and for fermentation between 28 °C and 37 °C. Lowering the optimal enzyme temperature by protein engineering methods is a challenging process, so it is necessary to use thermotolerant strains that effectively convert sugars to ethanol at high temperatures. From the point of view of economic justification, it should be a consolidated bioprocess.

However, fungal fermentation of lignocellulosic hydrolysate for bioethanol production also has disadvantages, such as low ethanol yield and the fact that an efficient organism with all the required physiological properties has not yet been found.[10,146]

The fermentation of hydrolysate to bioethanol is mainly carried out by *S. cerevisiae*, which can only ferment certain mono- and disaccharides such as glucose, maltose and sucrose, but is unable to assimilate pentoses (such as xylose).[8] One way to solve this problem is to use other microorganisms, such as *Pichia stipitis* and *Candida shehatae*, which can assimilate pentoses. However, their use leads to a five times lower ethanol yield compared to *S. cerevisiae*. In addition, these species require the presence of oxygen and are sensitive to lower pH. They are also 2-4 times less tolerant to ethanol compared to *S. cerevisiae*.[126,147] Genetic engineering can overcome this problem with a modified strain of *P. stipitis* BCC15191 that successfully ferments both glucose and xylose, in contrast to the natural strain that only ferments xylose.[148] *Mucor indicus* has also demonstrated the ability to assimilate pentoses and hexoses, as well as inhibitors found in hydrolysates.[126] Theoretically, 0.51 kg of bioethanol and 0.49 kg of CO_2 are obtained from 1 kg of glucose. However, since the microorganisms use part of the glucose for their growth and the process depends on factors such as the type of sugar fermented, the type of organism and the fermentation conditions, the yield of bioethanol is lower, ranging from 90 to 95% of the theoretical value.[21,149]

CONCLUSION

This review clearly shows that the abundant, but insufficiently investigated lignocellulosic residues could be a valuable substrate for use in numerous biotechnological processes. It is therefore expected that fungal lignocellulases will become the leading enzymes for the industrial scale production of bioethanol and other value-added compounds. Current and future research should focus on optimizing the synthesis and application of lignocellulases and on introducing new technologies for successful conversion of lignocellulosic waste into bioethanol. Fungi are promising agents in all phases of lignocellulose transformation to bioethanol, but also there are many unsolved problems and obstacles to be passed by future studies. After that, we could expect the development of an efficient enzyme cocktail that would enable the full utilization of lignocellulosic residues for bioethanol production, in the interest of ecological and economic sustainability.

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