We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



11

Competing Plant Cell Wall Digestion Recalcitrance by Using Fungal Substrate- Adapted Enzyme Cocktails

Vincent Phalip, Philippe Debeire and Jean-Marc Jeltsch
University of Strasbourg
France

1. Introduction

1.1 General needs for energy

General needs for energy are still increasing. In 2000, the energy provided worldwide was 10 Gt of oil equivalent (Gtoe) and the demand is forecasted to be around 15 Gtoe for 2020 (source: Energy Information Administration [EIA], 2002, as cited in Scragg, 2005). During the 20th century, coal proportion in energy supply decreased whereas oil and gas increased drastically. First after the 1973 oil crisis and afterwards periodically depending on oil prices, developments for producing energy by new ways were considered. In the last decade, the depletion of fossil energy sources appeared as a reality although exhaustion time remains highly controversial. Currently, it is clear that considerable efforts to promote alternative sources of energy are driven by both environmental concern (limiting fuel by-products emissions) and economic necessity linked to the fossil fuel depletion.

1.2 Bioethanol among other alternative sources

Ethanol from biomass (Bioethanol) is one of these alternative sources. Despite polemics for biomass uses i.e. biofuels vs food and some alarmist politicians' declarations, this alternative is really promising considering (i) the ability to satisfy a significant part of the demand for energy and (ii) biomass renewability. Polemics and limitations could have been almost rational when first generation of biofuels was concerned, "noble parts" of plants, the same used for food, being transformed. It is not the case anymore for any modern project. Another problem raised is the part of the cultivated surfaces to be reserved to biofuels, but in fact, realistic scenario is not to replace all fossil fuels volumes, but only part of them by using wastes preferentially.

1.3 "Biomass to ethanol" process and review of improvements

The general scheme of "Biomass to ethanol" process is presented elsewhere in this book. Our purpose in this section is to highlight the numerous and various ways to optimize the whole process from biomass to ethanol at different steps: choice of the biomass, pretreatment, enzyme productions, enzymatic hydrolysis, and ethanol fermentation. First of all, as discussed earlier, in the second generation of biofuels, biomass collection should not compete with food plants. Biomass should be abundant and cultural practices as

sustainable as possible. Interest was recently focused on plants providing good yields of biomass for a given surface as the tall Miscanthus. Reduction of lignin cell wall content is another interesting approach to enhance sugar recovery from biomass, lignin being an abundant and resistant polymer limiting the digestion of biomass in biofuels processes. With anti-sense technology, tobacco plants lines were obtained with 20% lower lignin content (Kavousi et al., 2010). The modified lines displayed a threefold increase of saccharification efficiency compared to wild type. Of course, the application of such studies in larger scales depends on the acceptance of transgenic plants by the society. Decision to use these plants has to be supported by studies of environmental risks and potential benefits (Talukder, 2006). Literature about pretreatment is very abundant, describing various methods: physical, chemical or combination of both (Soccol et al. 2010). Fine optimization of conditions should be performed individually depending on biomass. Among innovative method proposed, dry wheat straw has been treated successfully with supercritical CO_2. After treatment, 1kg biomass yields to 149 g sugars (Alinia et al., 2010). Another currently emerging feature for bioethanol process amelioration is protein engineering. For instance, a cellulase from the filamentous bacterium *Thermobifida fusca* has been modified both in its catalytic domain and in its carbohydrate binding module (Li et al., 2010). A mutant enzyme displays a two fold increase activity, and a better synergy with other enzymes, leading it to be very useful for biomass digestion. At the next step, i.e. sugar fermentation to ethanol, many efforts have been run to allow yeast to perform both hexoses and pentoses fermentations. Industrial yeast *Saccharomyces cerevisiae* strains, fermenting only hexoses have been modified by addition of xylose degradation enzymes (Hector et al., 2010). Finally the outcome of engineering could be the use of synthetic biology, which is creating cell systems able to convert biomass to sugars and also to ferment them to ethanol. This strategy needs better fundamental knowledge to be developed (Elkins et al., 2010).

As discussed above, the step following the pretreatment of the biomass could be performed via the enzymatic hydrolysis of the cell wall polysaccharides into fermentescible, monomeric sugars. Unfortunately, it is well known that recalcitrance of plant cell wall to enzymatic digestion impairs the process. The behavior and the efficiency of the cell wall degrading enzymes (CWDE) *in situ* and *in vitro* with isolated polysaccharides are completely different. The properties of the CWDE, as conformation, hydrophobicity, capacity of adsorption onto the cell wall, interaction with the lignins, and catalytic efficiency in heterogeneous catalysis, are major parameters which should be considered and studied.

This chapter focuses on biomass degradation enzymes. What is the best strategy to produce the most efficient enzymes? What is the best choice depending of up- and downstream steps: commercial enzyme cocktail, enzymes produced by a given microorganism or heterologous production of individual enzymes? Efficiencies and cost of enzymes, two bottlenecks in the process, will be discussed. For some authors, the improvements of the conversion of biomass to sugar offer larger cost-saving potential than those concerning the step from sugar to biofuels (Lynd et al., 2008). These authors evaluated two scenarios; the first based on current technology and the second one including advanced nonbiological steps. In both cases, conversion of polysaccharides from biomass could be improved by increasing polysaccharides hydrolysis yields combined by lowered enzyme inputs. On-site enzyme production was also identified as beneficial for cost of the whole ethanol production process.

2. Diversity of plant cell wall structures

The plant cell wall structures are highly diverse. Various lignocellulosic species have been used for biofuels production, woods, crop by-products, herbaceous plants, beet pulp, municipal and paper industry wastes. Although all these different biomasses contain typically four major components (i.e. cellulose, hemicelluloses, pectin and lignin), the architecture of the cell wall, the fine biochemical structures of these components and their interactions into the cell wall could be quite different. Nevertheless, cellulose and hemicelluloses leading, with lignins, to the formation of an insoluble, tridimensional network is a constant behavior. A schematic drawing of the plant cell wall polysaccharides is shown in Fig. 1.

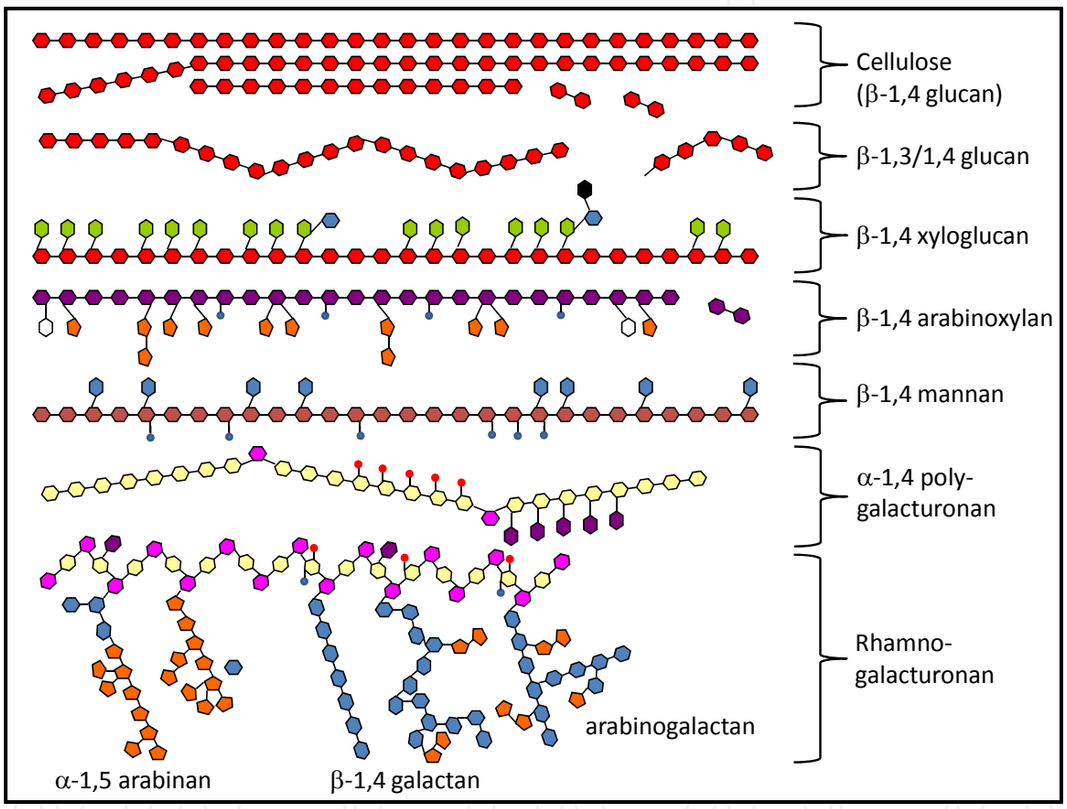


Fig. 1. Schematic representation of plant cell wall polysaccharides. Cellulose and β-1,3/1,4-glucan are composed of glucose residues (red). β-1,4-xyloglucan is a glucose-based polymer substituted by xylose residues (green) themselves possibly linked to galactose (blue) and fucose (black). β-1,4-arabinoxylan is a xylose backbone linked with arabinose (orange), and/or with glucuronic acid acid (white). Xylose could be substituted by acetyl groups (blue circle). β-1,4-mannan is a mannose polymer (dark red) linked to some galactose residues and sometimes acetylated. α-1,4-polygalacturonan is formed of linear chains of galacturonic acid (pale yellow) linked by rhamnose (pink). Galacturonic residues could be either methylated (red circle) or linked to xylose residues. Rhamnogalacturonan is highly ramified and is also called "hairy region" for this reason. The basic backbone is a rhamnose-galacturonic acid motif. Side chain of arabinose, galactose, mixed or not, linear or not, forms a very complex and variable structure. Adapted from Dalboge (1997).

As another example of variable composition of plant cell wall, lignins and sugars in cell walls of different origins were quantified (Table 1). Although this study is not exhaustive, it is clear that every plant has its own characteristic. These biomasses have been used as growth substrates for *Fusarium graminearum* (see paragraph 4).
Regarding cell wall composition variation, it could be postulated that cell wall degradation recalcitrance could be related to cell wall structure and ultra structure variability.

	Hop	Wheat bran	Corn cops	Birch
Lignin	30.9	17.3	6.5	18.1
Total neutral sugars	35.2	50.1	63.5	57.6
Glucose (i.e. glucans)	20.1	14.8	28.8	40.0
Xylose (i.e. xylans)	2.7	15.0	21.0	15.6

Table 1. Lignin, glucose and xylose contents of hop, destarched wheat bran, corn cops and birch. Results are expressed as % of dry matter (unpublished results from the laboratory). Klason lignin was quantified as the acid-soluble residue after sulfuric acid hydrolysis (Rémond et al. 2010) and sugar contents were estimated by enzymatic methods as described in Phalip et al. (2009).

3. A strategy for improving biomass hydrolysis: Studying (and using afterwards) fungi able to degrade plant cell wall components

3.1 Introduction on phytopathogenicity, saprophytism

The primary choice of a microorganism (fungus) potentially providing cell wall degrading enzymes should be directed toward one naturally present in plant environment i.e. a phytopathogen or a saprophyte. Considering the ecology, fungi are qualified as "decomposers" in the opposite to plants, the producers and animals, the consumers. Some fungi, called saprotrophs, get nutrients from dead organisms, especially plants. Some other are pathogen, attacking living organisms. Invasive growth thanks to hyphae gets fungi very adapted to penetrate plants. Hyphae diameter (2–10 μm) permit cell penetration and their hyphal growth in several directions allow them to colonize quickly the plant material with very close contact. Many saprotrophs, phytopathogens and other fungi living in plant environment developed tools for gaining energy from plants during their evolution. Cell wall degrading enzymes (CWDE) are one of these tools which are also efficient for bioethanol production. This is the reason why this chapter focuses on CWDE produced and secreted by some fungi.

3.2 Genome level studies

After the completion of the project of the backer's yeast *Saccharomyces cerevisiae* genome sequencing in 1996, genomes of some fungi have been decrypted. Many reasons drive the decision to sequence one genome and not the other one: some fungi being for a long time scientific models, others displaying industrial relevance, and others acting as saprophytes or pathogens. Backer et al. (2008) propose an interesting concept: let's change our way of thinking and let's consider microorganisms (especially fungi) as reservoirs for sustainable answers to environmental concerns. This point of view fits well with the directing idea of this chapter. To improve the "biomass to ethanol" process, we have to consider many options and enlarge the

fields to be prospected rather than being focalized on a single system. As an example for biomass degradation, many efforts have been directed though *Trichoderma reesei* due to historical reasons (discovered during World War II because it degraded uniforms and cotton tents) and to its capacity to produce cell wall degrading enzymes, specially cellulases. But, as it will be described later in this section, this fungus is not –by far for some categories of enzymes- the most equipped in CWDE. Other fungi have to be considered then.

Genome sequence availability offers the scientific community the opportunity to analyze them, deciphering their metabolism, in order to find response to fundamental or applied questions. As valorization of plant biomass arise as an important question to be addressed, several studies attempt to describe the fungal polysaccharide degradation potential. An extensive and complete work leads to a comparison of the genome of 13 fungi (Martinez et al., 2008). The first observation is that the yeast model *Saccharomyces cerevisiae* is poorer in CWDE than filamentous fungi (Fig. 2). This is not surprising regarding their respective lifestyles; all the filamentous fungi shown in Fig. 2 are saprotrophs or pathogens in the opposite of *S. cerevisiae*. This is a first argument for considering the natural habitat of a fungus when examining it for a peculiar application. Here, clearly, fungi living in plant environment displayed many more genes encoding CWDE or associated activities. Note that the model for plant polysaccharide degradation, *Trichoderma reesei*, displays fewer putative glycosyl hydrolases (200) than the pathogens *Magnaporthe grisea* (231) and *Fusarium graminearum* (243). Perhaps even more important is the number of cellulose binding modules (CBM), allowing a better enzyme-substrate binding and then a better efficiency in natural cellulose hydrolysis. *T. reesei* was predicted to have half CBM than the two pathogens (Fig. 2). In the same study, *T. reesei* is shown to be poorer than *M. grisea* and *F. graminearum* in cellulases, hemicellulases and pectinases (Martinez et al., 2008).

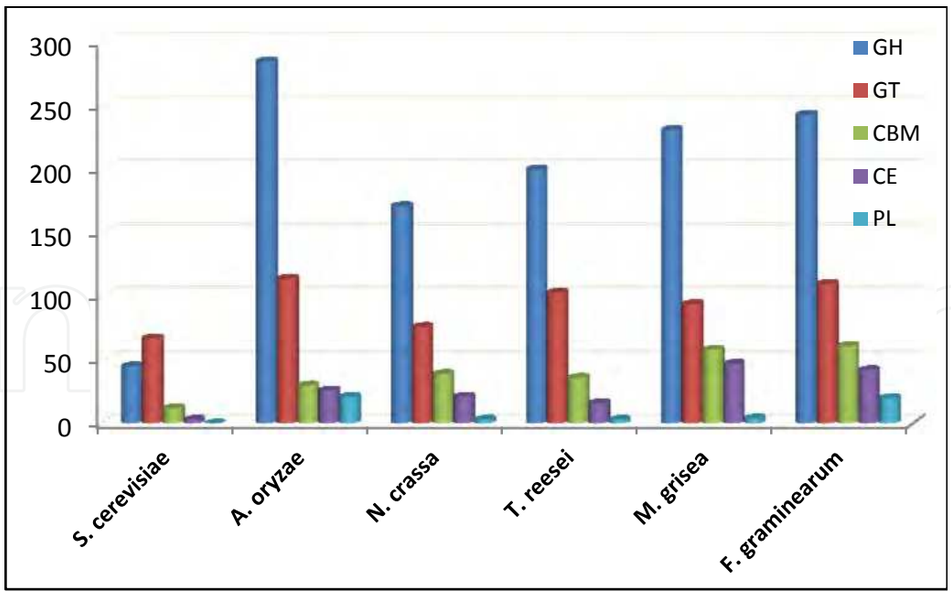


Fig. 2. Number of predicted glycosyl hydrolases (GH), glucosyl transferase (GT), cellulose binding modules (CBM), carbohydrate esterase (CE) and polysaccharide lyases (PL) in the genome of *Saccharomyces cerevisiae*, *Aspergillus oryzae*, *Neurospora crassa*, *Trichoderma reesei*, : *Magnaporthe grisea* and *Fusarium graminearum* (data from Martinez et al., 2008).

The main idea driving genome study is that evolution leads to genomes remodeling: i.e. leading to CWDE diversity for fungi dealing with plants. But through the example of *T. reesei*, it could be concluded that genome study - if available- is useful but not sufficient. Furthermore, obviously, a gene is not a protein; it has to be transcribed and mRNA has to be translated and modified to yield to mature and functional proteins.

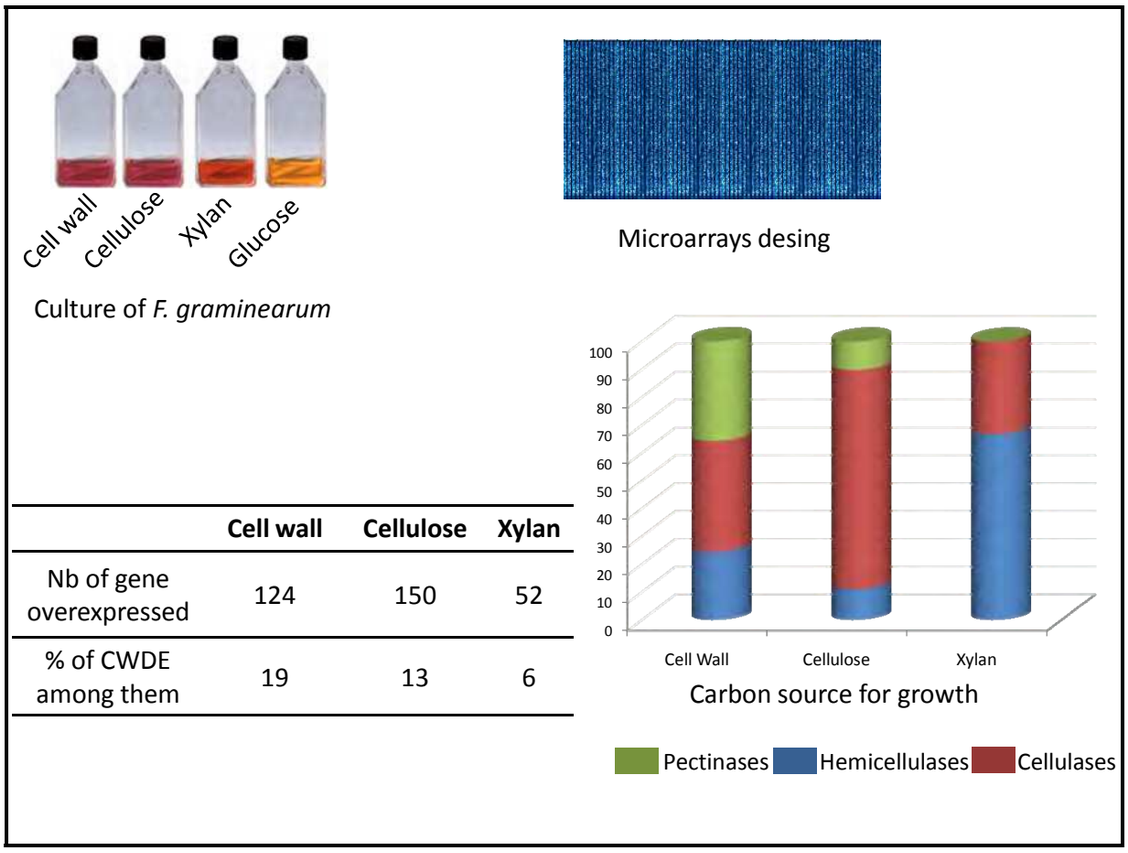
3.3 Transcriptome studies

In order to appreciate if the information provided by genome analysis is pertinent, transcriptome studies should also be performed. Our purpose is not to describe the regulation of CWDE in fungi, but it is essential to determinate efficiency of CWDE transcription depending on growth conditions. The goal is of course to optimize conditions leading to high transcription of the required enzymes. This regulation is rather complex, variable and well described in reviews (as an example see Aro et al., 2005). However, global characteristics leading to transcription of hydrolases genes are interesting to point out, since they could be a rational strategy basis for ethanol production. First, and for a long time, CWDE genes were considered generally as being repressed by glucose (catabolic repression) and by other released monosaccharides upon polysaccharide hydrolysis (de Vries & Visser, 2001). On the opposite, CWDE are massively expressed when fungi are grown in presence of polysaccharides and plant material (de Vries & Visser, 2001; Foreman et al., 2003 ; Aro et al., 2005). However, the view of a strict co-regulation of all CWDE is wrong. Induction of a given hydrolase goes on as a function of the polysaccharide in contact with the fungus. An interesting illustration is found in the pea pathogen *Nectria hematococca*. Two pectate lyases were found to be involved in pathology (Rogers et al., 2000). The first one was induced by pectin and repressed *in planta*, whereas the other was induced *in planta* but repressed by pectin. This means that CWDE transcription could be individual and precise. In *Fusarium graminearum*, well known as pathogen of cereals, we performed microarray experiments to test the expression on the whole genome on glucose, cellulose, xylan and hop cell wall (Carapito et al., 2008). Methods and essential findings are summarized in Fig. 3.

First, some genes were actually found to be over-expressed on polysaccharides comparatively to their expression on glucose (Fig. 3.). Their number varies depending on carbon source. CWDE represent also a variable part of overexpressed genes. It is particularly interesting to note that the largest proportion of CWDE was observed when the fungus was grown on plant cell wall (19% of overexpressed genes) i.e. the most diverse substrate. It denotes a strong re-orientation of the metabolism towards cell wall degradation since CWDE correspond to approximately 0.5% of the genome only. Furthermore, cellulases, hemicellulases and pectinases encoding genes are quite equally represented as overexpressed ones when the fungus is grown on plant cell wall, whereas mostly cellulases were shown to be overexpressed on cellulose and mostly hemicellulases were overexpressed on xylan. This data suggest that there is no global response to the presence of plant cell wall, but that the different polysaccharides sent specific signals which are recognized by the fungus and induce various responses.

3.4 Proteomics

As the number of fungal genome sequenced increase, the number of proteomics studies increase the same way. The studies are driven for various reasons, but some of them are devoted to the identification of proteins produced (and most frequently secreted) by fungi



	Cell wall	Cellulose	Xylan
Nb of gene overexpressed	124	150	52
% of CWDE among them	19	13	6

Fig. 3. Expression of the genome of *Fusarium graminearum* when grown on different carbon sources. Microarrays were analyzed and the number of genes overexpressed ($p<0.02$, fold change >2) comparatively that after growth on glucose where determined (Table). The graph represents the repartition of the CWDE (cellulases in yellow, hemicellulases in pink and pectinases in green) as a function of the substrate used for growth.

in response to plant material. This fact fits well with the purpose of this chapter and is perfectly summarized by the title of a recent paper: "Plant-pathogen interactions: what is proteomics telling us" (Mehta et al., 2008). In this paper, is shown that when pathogens are in the presence of plants, their metabolism is changed to secrete proteins, including CWDE, potentially involved in plant cell wall degradation. These findings are in perfect accordance with the conclusion of the transcriptomics studies (see previous section).

We performed a proteomics study with the plant pathogen *Fusarium graminearum* grown either on glucose or on a preparation of plant cell wall as the sole carbon source (Phalip et al., 2005). When it is grown on glucose (Fig. 4.), the fungus secretes a few proteins in small quantities. When the more complex and diverse plant cell wall is used, the fungus reacts by secreting a much higher amount of more diverse proteins. Approximately half (45%) of these are putative CWDE. Furthermore, CWDE identified are able to take in charge the three cell wall layers: cellulose (11 proteins are putative cellulases), hemicellulose (25) and pectin (19). These results are also perfectly correlated with transcriptome studies. The fungus clearly responds to cell wall diversity by enzyme diversity. It could be a good point to keep in mind when looking for an enzyme cocktail for biomass valorization.

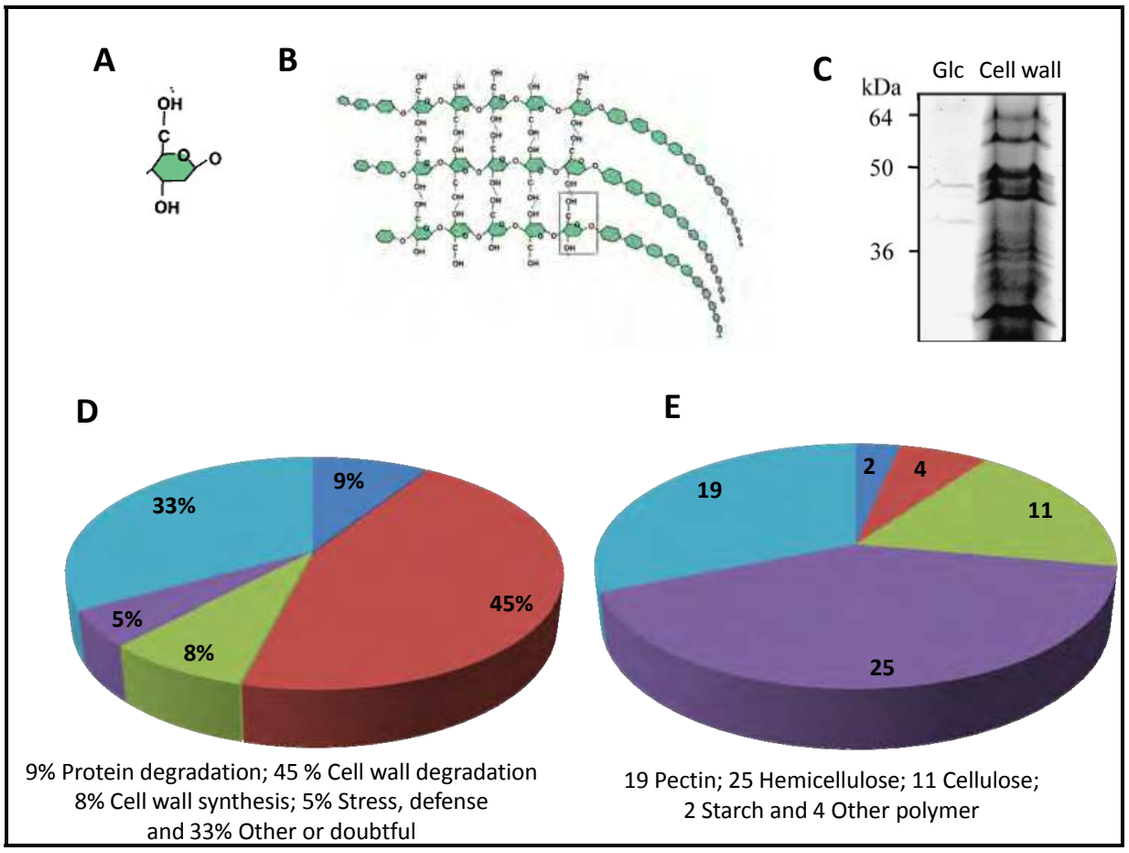


Fig. 4. Proteomics studies of *Fusarium graminearum* grown on glucose (A) and plant cell wall (B). Culture supernatants were concentrated and the equivalent of the same volume of supernatant was loaded on SDS-PAGE (C). The corresponding proteins were by identified by Mass Spectrometry and classified thanks to their homologies with protein in databases (D). The CWDE were further considered as a function of the cell wall layer they degrade (E).

3.5 Enzymatic measurements

Genome, transcriptome and proteome studies lead to interesting but perturbing questions. Several studies indicate that fungi could secrete up to 50 different CWDE in order to degrade cellulose, hemicellulose, pectin and, for some of them, lignin. Among these enzymes, some display the same EC number and/or belong to the same glycosyl hydrolase family (GH). Why fungi use up so much energy to secrete enzymes with quite similar activities? Is it true or apparent redundancy?

Several clues indicate that this is apparent redundancy. Most of the CWDE, still putative, wait for potent substrate specificities characterization. By analogy with the enzymes already characterized, it means that slightly different specificities are likely to be discovered and could be essential to complete plant cell wall degradation. Furthermore, quantitative studies performed on *Fusarium* hemicellulases demonstrate that on hop cell wall, the expression level of the 30 putative enzymes varies greatly from 1 (the less abundant) to 1500 (Hatsch et al., 2006). When another biomass is used for growth, the pattern of secreted enzymes is different, clearly indicating that there is no "general response" to the presence of plant material but specific responses to a given biomass. Taken together these studies mean that

the fungus exhibits a large flexibility in its response. Then it could be thought that the observed redundancy actually reflects the multitude of different structures of plant cell wall. Consequently, it is of primary importance that *in silico* studies should be carefully validated by enzymatic measurements. For example, an exponential increase of putative CAZY (Carbohydrate-Active enZYmes) described is observed, but unfortunately only a small proportion of them are biochemically characterized yet (Cantarel et al., 2009). Whereas entries in CAZY database increased exponentially from 1999 to 2007 (a 14-fold increase), less than 10% of them have been enzymatically characterized and less than 1% of the enzyme structures have been solved. This means that there is a real lack in enzyme knowledge regarding to the huge potential of new activities undiscovered yet. It should be noticed that the increasing difference between the number of putative enzymes and well characterized ones also lead to the possibility of mis-annotation and/or false identification. This phenomenon has been known for a long time by molecular biologists and correction of errors and inconsistencies in data bases became an authentic research area (Ghisalberti et al., 2010). In order to thoroughly characterize the enzyme activities and their capacity to degrade the complex structures found in plant cell wall, CWDE substrate specificity should be determined with both artificial and natural substrates. This absolute necessity drives us to perform the characterization of the enzyme cocktail produced by *F. graminearum* on hop cell wall. We used 29 different substrates, poly-or oligosaccharides, natural or artificial (Phalip et al., 2009). Enzyme activities were evaluated by assays of the products (monomers) or by their visualization by polysaccharide analysis using carbohydrate gel electrophoresis (PACE). The conclusion of this study is that the enzymes constituting this cocktail are no more putative but active on each layer of the plant cell wall. On the opposite, the enzyme cocktail produced on glucose displays very tiny activities, furthermore on a small number of substrates. The proof is then provided that to get a large diversity of cell wall degrading enzymes, it is very important to choose the right substrate for a given fungus to grow.

3.6 Synergy

A relevant feature of CWDE activities is the synergy observed between them. Typically an endo-enzyme acts randomly on the polysaccharide to yield oligomers. These oligomers are numerous for a single starting polysaccharide and their extremities are hydrolyzed to di- or monosaccharides by exo-acting enzymes. Accessory enzymes (debranching or desubstituting) proceed if necessary and all three kinds of enzymes work together. Synergy leads to the concept of "Minimal enzyme cocktail", i.e. a few selected enzymes (Sorensen et al., 2007), supposed to be sufficient for the complete digestion of plant cell wall. This paper described efforts performed to digest the more efficiently wheat arabinoxylan to arabinose and xylose. Starting from an "enzymatic base" (a β-xylosidase, and three endo-β-xylanases), they screened three different arabinofuranosidases to enhance substrates digestion. AbfIII was found to be the best enzyme when used alone (Fig. 5). Addition of AbfI significantly increased hydrolysis yields, although in different extent depending on the substrate used.
Addition of the three arabinofuranosidases together did not improve the yields further. The strategy used and the results obtained clearly support the view of the necessity of a rational design of the process leading to ethanol. In other words, such a study has to be repeated when another biomass is concerned. Would the same enzyme be as efficient with the other substrate? Only experiments could address this question. Nevertheless, this study indicates that the starting biomass influence the choice of the enzymes to be used. For the complete

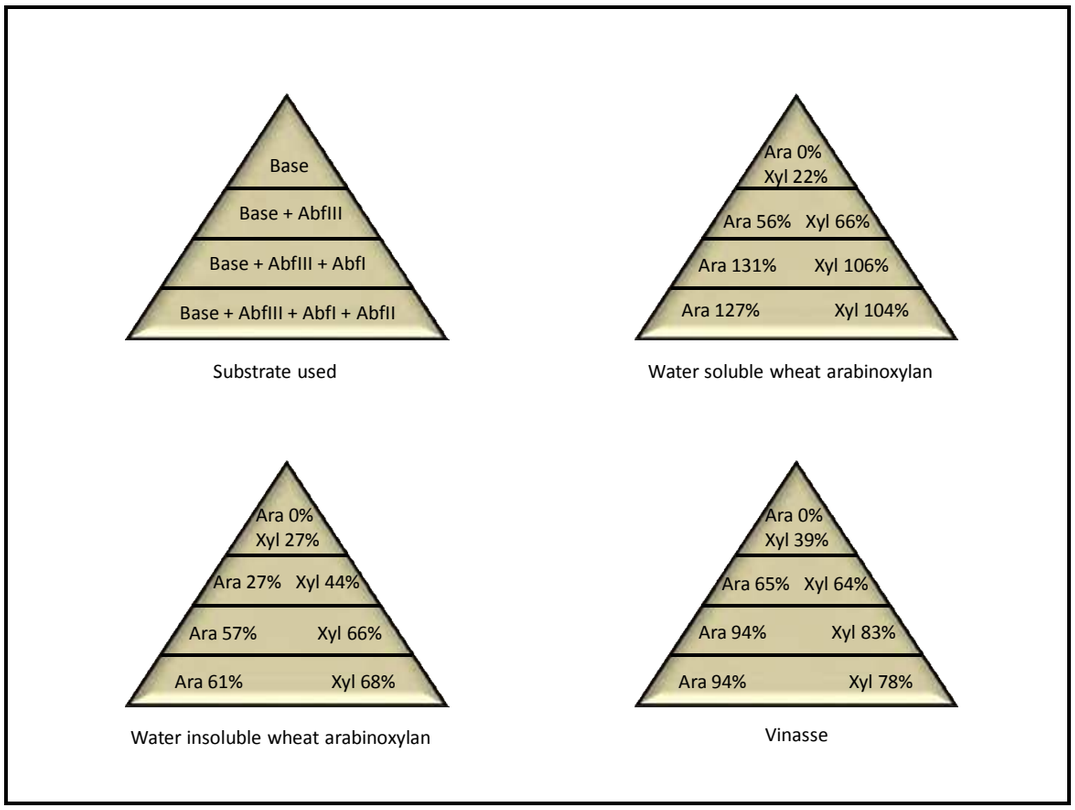


Fig. 5. Yields of arabinose and xylose released from different substrates (substrates are indicated below each pyramid). Base mix of enzyme (top of each pyramid) is constituted of a xylosidase and three endo-β-xylanases. ArfI, AfrI and ArfIII are three arabinofuranosidases belonging to GH (glycosyl hydrolase) families 51, 43 and 51, respectively. As described in the top left pyramid, the more different enzymes were added, the more the result is shown at the bottom of the pyramid. Yield superior to 100% was due to pretreatment. Adapted from Sorensen et al., 2007.

digestion of a plant biomass, minimal enzyme cocktails for all kind of polysaccharide are required. Note that Section2 of this chapter concludes to a great diversity of cell wall fine structures leading to design of much larger and much diverse cocktails.

4. Conclusions and future prospects

Studies of cell wall and CWDE presented in this chapter could be summarized by the same word: diversity. But how the previously mentioned studies inform us about the right strategy driving efficiently from biomass to ethanol? This will be discussed below.

We demonstrated for the first time that the exoproteome of *Fusarium graminearum* grown in presence of plant material was rich in various CWDE: more than 80 different proteins, half of them being putatively involved in cell wall digestion were recovered from culture supernatant (Phalip et al., 2005). It is noticeable that later, rather the same number of proteins was found to be secreted by *Trichoderma reesei* grown on corn cell wall (Nagendran et al., 2009). Commercial preparation Spezyme® used for biomass hydrolysis contains also more than 80 different proteins. All these data corroborate the concept of using complex

enzyme cocktails for complete biomass hydrolysis. A thorough analysis of *Fusarium* and *Trichoderma* proteomes reveals some differences between them. For instance, *Fusarium* secretes enzymes belonging to 29 different GH families and 6 pectate lyases whereas *Trichoderma*'s exoproteome exhibits 22 GH families but no pectate lyase. Furthermore, only 14 GH families are present in both proteomes (Fig 6), 15 are recovered in *Fusarium* but not in *Trichoderma* and 8 are only found in *Trichoderma*. These are convincing arguments that in a strategy meant to produce bioethanol from various sources, the design of a biomass to ethanol process should be optimized for each couple biomass/fungus.

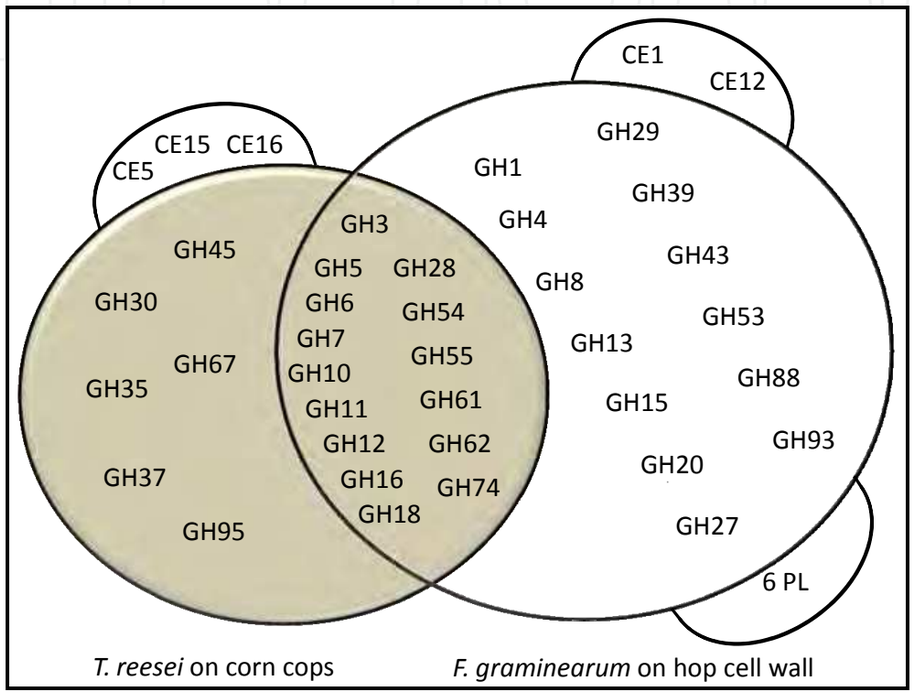


Fig. 6. Nature of putative secreted CWDE found in *Trichoderma reesei* grown on corn cops (left; Nagendran et al., 2009) and in *Fusarium graminearum* grown on hop (right; Phalip et al., 2005).

We studied the patterns of enzymes produced by *F. graminearum* on four different lignocellulosic biomasses, two poorly lignified (wheat bran and corn cobs) and two highly lignified (hop and birch). Each enzyme cocktail was thereafter used for long-term hydrolyses of the ammonia pretreated four biomasses (16 combinations). The oligo- and mono-saccharides (end products) have been characterized. Their patterns showed variations depending on the nature of the biomass used for growth. Accordingly, different enzyme activities were measured on different culture supernatants. For example, enzymes produced when grown on birch, were efficient with pretreated birch and hop in a lesser extent but were also the most efficient for poorly lignified biomasses. Furthermore, the proteins produced in each condition were identified by mass spectrometry. As shown in Fig. 7, the number of unique proteins recovered in supernatants varies greatly from 25 on birch to 72 on hop (14 for the monosaccharide glucose). More interesting is that 80% (20/25) of the proteins recovered on birch corresponds to CWDE whereas only 19% of the proteins are putatively active on polysaccharides with corn cops for growth (0 on glucose). Finally,

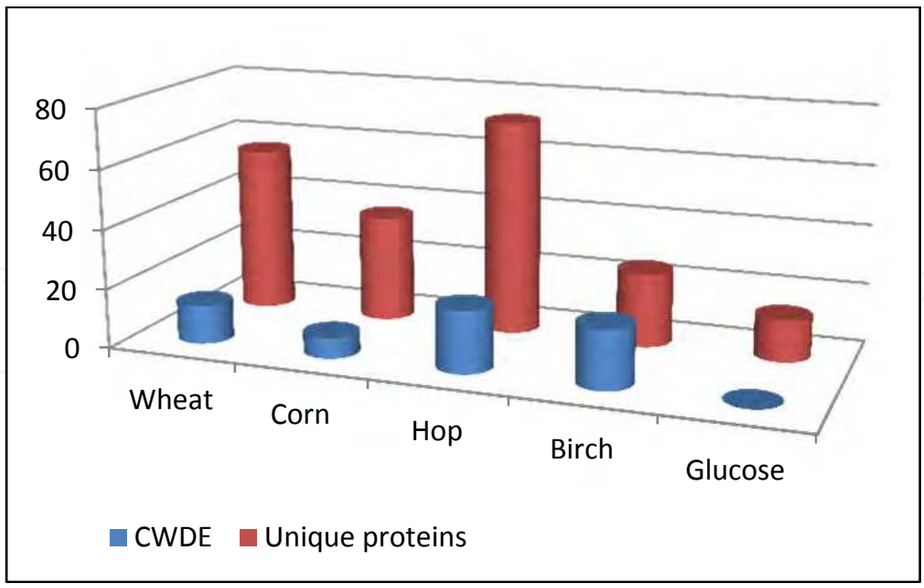


Fig. 7. Number of unique proteins and CWDE identified by mass spectrometry in culture supernatants after growth on wheat bran, corn cops, hop, birch and glucose.

among the 43 CWDE identified during this experiment, not less than 32 were recovered in one culture condition only, 11 were produced in at least two conditions and only 2 were present for each growth conditions (hop, birch, corn cops and wheat bran). Therefore, the fine specificities of the CWDE towards polysaccharides are directly induced by the different lignocellulosic biomasses used for growth.

Of course, even if the couple biomass/fungus is rationally chosen, one could not exclude that the microorganism does not produce a set of keys enzyme(s) for the peculiar biomass to be completely split up. In this case, mixing two (or more) enzymes crude cocktails could be a very good alternative as illustrated by Gottschalk et al. (2010). Enzymes from *Trichoderma reesei* grown on corn steep liquor and *Aspergillus awamori* grown of wheat bran have been obtained. The cocktails display different hydrolytic activities and blends of both led to enhancement of some synergic activities up to a 2-fold factor. It is also observed that their association improved glucose release from steam-treated sugarcane. The authors notice that *T. reesei* cocktail was better for cellulose hydrolysis and *A. awamori* was better for xylan hydrolysis. This is obviously a very good argument for a fungal specific response towards a given biomass. The same kind of concept could also be extended by using one of the rare lignin-degrading fungi, *Phanerochaete chrysosporium* (Kersten & Cullen, 2007) in association with another one providing great amount of CWDE. Lynd et al. (2008) suggest that replacing chemical pretreatment by enzymatic one could be a way to explore to improve the process. Furthermore, proceeding this way is mimicking nature diversity since fungi are often associated in communities acting in synergy for the degradation of plants. Furthermore, Wei et al. (2009) claims rightly that "the plant-microbe-enzyme relationship is the foundation of plant biomass degradation in natural environments". They mean that plant cell wall is naturally degraded by a community of microorganisms. Efficient processes from "biomass to ethanol" could advantageously use this property, of course by means of accelerating the natural process.

Many studies have been performed aiming to get huge quantities of individual enzymes, mostly cellulases. Actually, high quantity of enzyme input is not the panacea since

saturation of sugar yields due to increasing enzyme charge is often observed (see Sorensen et al., 2007 as an exemple). After reaching the plateau, adding more enzymes did not improve yields anymore. A real diversity of enzyme responding to that of cell wall is better to overcome cell wall recalcitrance for full degradation. Although fungi naturally secrete small amounts of enzymes in liquid cultures, solid state fermentation (SSF) is preferable as an accurate solution for increasing enzyme production yield at the industrial level. Furthermore, a lot of different biomasses, including wastes, have been proved to support fungal growth and to promote CWDE production. The "waste-to-energy" technology was recently reviewed (Bemirbas et al., 2011) and the authors underlined that as population and urbanization increase, the amount of wastes increased regularly. As described elsewhere in this volume (Verardi et al., 2011; Xavier et al., 2011), wastes included municipal solid waste, paper wastes and also agricultural and forestry by-products, all containing lignocellulosic material.

For sustainable development, we strongly encourage the concept of local small units of bioethanol production. Gnansounou & Dauriat (2010) evoke the necessity of "low-risk biorefineries" in opposition to "complex schemes" production units. Therefore biomass should be easily available, preferentially composed of wastes, transports as limited as possible, wastes almost totally used and co-products fully valorized.

As a conclusion, seeking a universal process for total hydrolysis of all kind of biomasses is utopian. Rather, there is an appropriate methodology to follow, described in this chapter, considering the biomass to be treated and the co-products to be valorized in the respect to sustainable development. This point is perfectly illustrated by Saxena et al., (2009). For every step of biomass conversion, starting by the biomass choice, there are multiple routes for hydrolysis technology, monomers produced, microorganism used for fermentation and by-product formed. In this volume, Xavier and al., 2011 underline the necessity of a specific

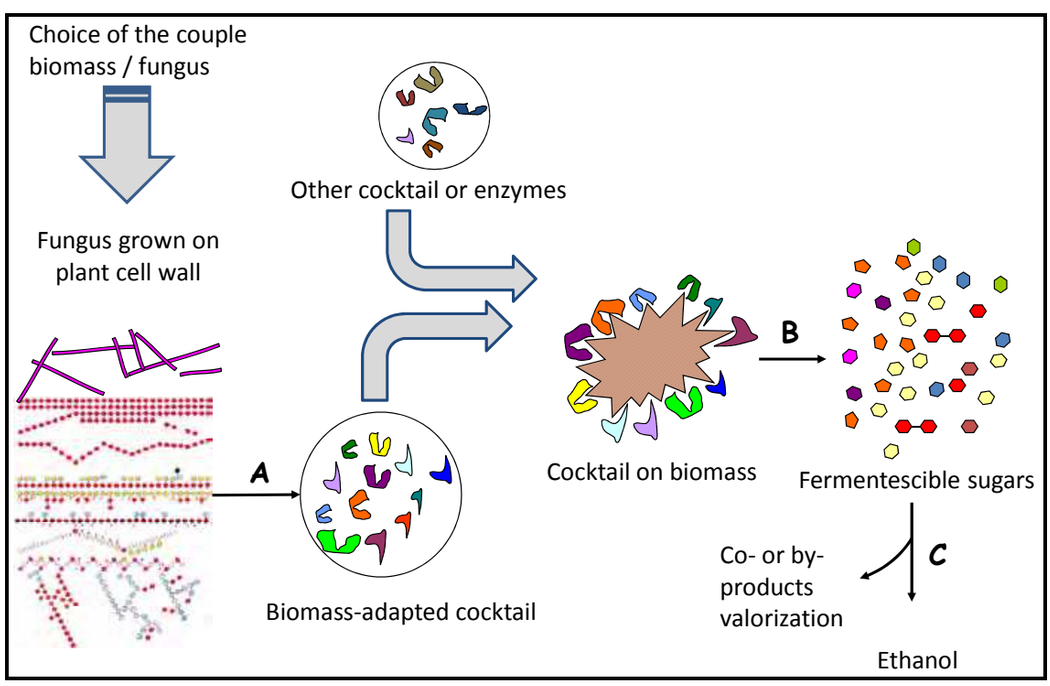


Fig. 8. Schematic representation of the whole process from "biomass to ethanol".

pretreatment for each biomass to be digested. This means that an industrial process should be developed by taking into account the nature of biomass, and consequently the enzymes necessary for its digestion and the down-stream processes.
Fig. 8 summarizes the views developed in this chapter. Taking into account fundamental research, a couple biomass / fungus is chosen. The fungus is grown on the biomass and produced a cocktail rich in CWDE (A). After adding maybe another cocktail (or individual enzymes; see also Verardi et al., 2011), the enzymes are used to digest the pretreated biomass (in a first approach, the same biomass that the one used for growth), yielding diverse fermentescible sugars in quantity (B). The latter are taken in charge by microorganisms to produce ethanol (C).

5. Acknowledgment

We are grateful to Marie-Laurence Phalip for language revision.

6. References

Alinia, R.; Zabihi, S.; Esmaeilzadeh, F. & Kalajahi, J.F. (2010) Pretreatment of wheat straw by supercritical CO_2 and its enzymatic hydrolysis for sugar production. *Biosystems Engineering*, Vol.107, No.1, pp. 61-66, ISSN 1537-5110

Aro, N.; Pakula, T. & Penttilä M. (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiology Reviews*, Vol.29, No.4, pp. 719-739, ISSN 1574-6976

Baker, E.; Thykaer, J.; Adney, W.S.; Brettin, T.S.; Brockman, F.J.; D'haeseleer, P. et al. (2008) Fungal genome sequencing and bioenergy. *Fungal Biology Reviews*, Vol.22, No.1, pp. 1-5, ISSN 1749-4613

Cantarel, B.L.; Coutinho, P.M.; Rancurel, C.; Bernard, T.; Lombard, V. & Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Research*, Vol.37, pp. D233-D238, ISSN 0305-1048

Carapito, R.; Hatsch, D.; Vorwerk, S.; Petkovski, E.; Jeltsch, J.-M. & Phalip, V. (2008) Gene expression in *Fusarium graminearum* grown on plant cell wall. *Fungal Genetics and Biology*, Vol.45, No.5, pp. 738-748, ISSN 1087-1845

Dalbøge, H. (1997) Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance. *FEMS Microbiology Reviews*, Vol.21, No.1, pp. 29-42, ISSN 0168-6445

Demirbas, M.F.; Balat, M. & Balat, H. (2011) Biowastes-to-biofuels. *Energy Conversion and Management,* Vol.52, No.4, pp. 1815-1828, ISSN 0196-8904

De Vries, R.P. & Visser, J. (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews,* Vol.65, No.4, pp. 497-522, ISSN 1092-2172

Elkins, J.G.; Raman, B. & Keller, M. (2010) Engineered microbial systems for enhanced conversion of lignocellulosic biomass. *Current Opinion in Biotechnology*, Vol.21, No.5, pp. 657-662, ISSN 0958-1669

Foreman, P.K.; Brown, D.; Dankmeyer, L.; Dean, R.; Diener, S.; Dunn-Coleman, N.S.; Goedegebuur, F. et al. (2003) Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. *Journal of Biological Chemistry*, Vol.278, No.34, pp. 31988-31997, ISSN 0021-9258

Ghisalberti, G.; Masseroli, M. & Tettamanti, L. (2010) Quality controls in integrative approaches to detect errors and inconsistencies in biological databases. *Journal of Integrative Bioinformatics*, Vol.7, No.3, pp. 119-132, ISSN 1613-4516

Gnansounou, E. & Dauriat, A. Techno-economic analysis of lignocellulosic ethanol: A review. *Bioresource Technology*, Vol.101, No.13, pp. 4980-4991, ISSN 0960-8524

Gottschalk, L.M.F.; Oliveira, P.A. & da Silva Bon, E.P. (2010) Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. *Biochemical Engineering Journal*, Vol.51, No.1-2, pp. 72-78, ISSN 1369-703X

Hatsch, D.; Phalip, V.; Petkowski, E. & Jeltsch, J.-M. (2006) *Fusarium graminearum* on plant cell wall: no fewer than 30 xylanase genes transcribed. *Biochemical and Biophysical Research Communications*. Vol.345, No.3, pp. 959-966, ISSN 0006-291X

Hector, R.E.; Dien, B.S.; Cotta, M.A. & Qureshi, N. (2010) Engineering industrial *Saccharomyces cerevisiae* strains for xylose fermentation and comparison for switchgrass conversion. *Journal of Industrial Microbiology and Biotechnology*, Epub ahead of print, pp. 1-10, ISSN 1367-5435

Kavousi, B.; Daudi, A.; Cook, C.M.; Joseleau, J.-P.; Ruel, K.; Bolwell, G.P. & Blee, K.A. (2010) Consequences of antisense down-regulation of a lignification-specific peroxidase on leaf and vascular tissue in tobacco lines demonstrating enhanced enzymic saccharification. *Phytochemistry*, Vol.71, No.5-6, pp. 531-542, ISSN 0031-9422

Kersten, P. & Cullen, D. (2007) Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. *Fungal Genetics and Biology*, Vol.44, No. 2, pp. 77-87, ISSN 1087-1845

Li, Y.; Irwin, D.C. & Wilson, D.B. (2010) Increased crystalline cellulose activity via combinations of amino acid changes in the family 9 catalytic domain and family 3c cellulose binding module of *Thermobifida fusca* cel19a. *Applied and Environmental Microbiology*, Vol.76, No.8, pp. 2582-2588, ISSN 0099-2240

Lynd, L.R.; Laser, M.S.; Bransby, D.; Dale, B.E.; Davison, B.; Hamilton, R.; Himmel, M.; Keller, M.; McMillan, J.D.; Sheehan, J. & Wyman, C.E. (2008) How biotech can transform biofuels. *Nature Biotechnology*, Vol.26, pp. 169-172, ISSN 1087-0156

Martinez, D.; Berka, R.M.; Henrissat, B.; Saloheimo, M.; Arvas, M. et al. (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nature Technology*, Vol.26, No.5, pp. 553-560, ISSN 1087-0156

Mehta, A.; Brasileiro, A.C.M.; Souza, D.S.L.; Romano, E.; Campos, M.A.; Grossi-De-Sá, M.F.; Silva, M.S.; et al. (2008) Plant-pathogen interactions: What is proteomics telling us? *FEBS Journal*, Vol.275, No.15, pp. 3731-3746, ISSN 1742-464X

Nagendran, S.; Hallen-Adams, H.E.; Paper, J.M.; Aslam, N. & Walton J.D. (2009) Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus *Amanita bisporigera*, based on the secretome of *Trichoderma reesei*. *Fungal Genetics and Biology*, Vol.46, No.5, pp. 427-435, ISSN 1087-1845

Phalip, V.; Delalande, F.; Carapito, C.; Goubet, F.; Hatsch, D.; Dupree, P.; VanDorsselaer, A. & Jeltsch, J.-M. (2005) Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. *Current Genetics*, Vol.48, pp. 366-379, ISSN 0172-8083

Phalip, V.; Goubet, F.; Carapito, R. & Jeltsch, J.-M. (2009) Plant cell wall degradation with a powerful *Fusarium graminearum* enzymatic arsenal. *Journal of Microbiology and Biotechnology*, Vol.19, No.6, pp. 573-581, ISSN 1017-7825

Rémond, C.; Aubry, N.; Crônier, D.; Noël, S.; Martel, F.; Roge, B.; Rakotoarivonina, H.; Debeire, P. & Chabbert, B. (2010) Combination of ammonia and xylanase pretreatments: Impact on enzymatic xylan and cellulose recovery from wheat straw. *Bioresource Technology*, Vol.101, No.17, pp. 6712-6717, ISSN 0960-8524

Rogers, L.M.; Kim, Y.-K.; Guo, W.; González-Candelas, L.; Li, D. & Kolattukudy, P.E. (2000) Requirement for either a host- or pectin-induced pectate lyase for infection of *Pisum sativum* by *Nectria hematococca*. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.97, No.17, pp. 9813-9818, ISSN 0027-8424

Saxena, R.C.; Adhikari, D.K. & Goyal, H.B. (2009) Biomass-based energy fuel through biochemical routes: A review. *Renewable and Sustainable Energy Reviews*, Vol.13, No.1, pp. 167-178, ISSN 1364-0321

Scragg, A. (2005) *Environmental Biotechnology* (2nd Edition), Oxford University Press, ISBN 978-0-19-926867-2, Oxford, UK

Soccol, C.R.; Vandenberghe, L.P.S.; Medeiros, A.B.P.; (…) Araújo, J.A. & Torres, F.A.G. (2010) Bioethanol from lignocelluloses: Status and perspectives in Brazil. *Bioresource Technology*, Vol.101, pp. 4820-4825, ISSN 0960-8524

Sørensen, H.R.; Pedersen, S.; Jørgensen, C.T. & Meyer, A.S. (2007) Enzymatic hydrolysis of wheat arabinoxylan by a recombinant "minimal" enzyme cocktail containing β-xylosidase and novel endo-1,4-β-xylanase and α-L-arabinofuranosidase activities. *Biotechnology Progress*, Vol.23, No.1, pp. 100-107, ISSN 8756-7938

Talukder, K. (2006) Low-lignin wood - A case study. *Nature Biotechnology*, Vol.24, No.4, pp. 395-396, ISSN 1087-0156

Xavier, A.; Fernandes, D.L.A.; Pereira, R.R.; Serafim, L.S. & Evtyugin, D. (2011). Second generation bioethanol, In: *Bioethanol*, InTech - Open Access Publisher, ISBN: 978-953-307-346-0, Rijeka, Croatia

Verardi, A.; De Bari, I.; Ricca, E. & Calabro, V. (2011) Hydrolysis of lignocellulosic biomass: current status of processes and technologies and future perspectives, In: *Bioethanol*, InTech - Open Access Publisher, ISBN: 978-953-307-346-0, Rijeka, Croatia

Wei, H.; Xu, Q.; Taylor, L.E., Baker, J.O.; Tucker, M.P. & Ding, S.-Y. (2009) Natural paradigms of plant cell wall degradation. *Current Opinion in Biotechnology*, Vol.20, No.3, pp. 330-338, ISSN 0958-1669

IntechOpen



Bioethanol

Edited by Prof. Marco Aurelio Pinheiro Lima

ISBN 978-953-51-0008-9
Hard cover, 290 pages
Publisher InTech
Published online 01, February, 2012
Published in print edition February, 2012

Recent studies have shown strong evidence of human activity impact on the climate of the planet. Higher temperatures and intensification of extreme weather events such as hurricanes are among the consequences. This scenario opens up several possibilities for what is now called "green" or low carbon economy. We are talking about creating new businesses and industries geared to develop products and services with low consumption of natural resources and reduced greenhouse gases emission. Within this category of business, biofuels is a highlight and the central theme of this book. The first section presents some research results for first generation ethanol production from starch and sugar raw materials. Chapters in the second section present results on some efforts around the world to develop an efficient technology for producing second-generation ethanol from different types of lignocellulosic materials. While these production technologies are being developed, different uses for ethanol could also be studied. The chapter in the third section points to the use of hydrogen in fuel cells, where this hydrogen could be produced from ethanol.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Vincent Phalip, Philippe Debeire and Jean-Marc Jeltsch (2012). Competing Plant Cell Wall Digestion Recalcitrance by Using Fungal Substrate – Adapted Enzyme Cocktails, Bioethanol, Prof. Marco Aurelio Pinheiro Lima (Ed.), ISBN: 978-953-51-0008-9, InTech, Available from: http://www.intechopen.com/books/bioethanol/competing-plant-cell-wall-digestion-recalcitrance-by-using-

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen