States and outlook of second-generation biofuel production

CBS KNAW

Fungal Physiology

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Acknowledgment:

My special thanks goes to Dr. de Vries for his kind supervision and support.

Overview

Introduction

Bioethanol as a substitute for petroleum can prevent to a great extent the global, environmental and political conflicts reliance on fossil fuels. Although burning ethanol produces emission gas, the net effect does not result in increasing CO_2 concentrations in the atmosphere (Schlamadinger, B. et al. 1997). The abundant and renewable supply of biomass makes it an outstanding candidate for bioethanol production. Ongoing research trails have set up a number of pilot plants and predominant routes for second generation bioethanol production yet to be produced commercially.

There are several obstacles for cost effective bioethanol production. Next to high costs of cellulases the most important one is the pre-treatment process of biomass to enable efficient enzymatic hydrolysis to monomers. Also lignin removal to allow access of cellulases to biomass cellulose is quite challenging. Plant genetic engineering provides potential solutions to reduce bioethanol production costs. In this essay we try to summarize difficulties facing cost effective second-generation biofuel production. Moreover, the predominant lignocellulosic candidates for bioethanol production and commercial enzymes being used for their saccharification are introduced.

Biomass structure as bioethanol production building blocks

Photosynthesis is the process that captures solar energy and stores its energy in the form of cell wall polymers. Plant cell wall is the source of lignocellulosic biomass. Plant cell wall is involved in determining a solid structure of plant and defense against pathogens and insects (Carpita, N. & McCann, M. 2002). **The primary** cell wall is comprised of a scaffold of cellulose with cross-linking glycans. Dicotyledonous plants have cell walls with equal amounts of glucan and xyloglucan imbedded in pectin. In contrast, cereals and other grasses contain glucuronoarabinoxylans and lack pectin. Polysaccharides present in plant cell walls can be hydrolyzed to provide fermentable sugars for bioethanol production (Carpita, N. & McCann, M. 2002). The s**econdary** cell wall contains cellulose, hemicellulose and lignin. Tree trunks have three layers of secondary cell walls called from the outermost side inward S1, S2 and S3, as is shown in detail in figure 1.

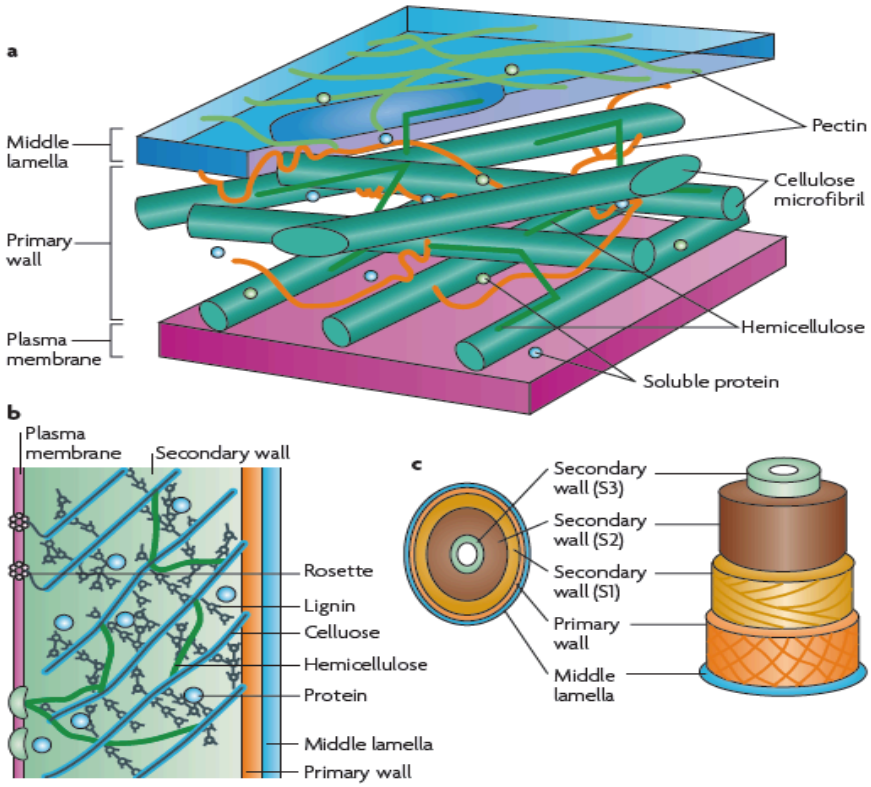

Figure 1: Cell-wall structure of a plant. A) Different compartments making cell wall structure: Cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. B) Rosette complexes are floating cellulose synthesis enzymes. C) Lignification occurs in all three layers of secondary cell wall. Adopted from *Nature Reviews Genetics* 9, 433-443 (June 2008).

Biomass degradation (pretreatment)

Biomass degradation calls for opening up the structure to make the long chain polysaccharides (such as cellulose and hemicellulose) accessible and subsequent hydrolysis into their components (pentose and hexose sugars). Industrial degradation involves heat and acidic conditions and tends to be expensive, slow and relatively inefficient (Mosier, N. et al. 2005). Moreover pretreatment procedures produce inhibitors that decrease fermentation efficiency, like furfural and 5-hydroxymethylfurfural. Cellulases have a major role in biofuel production and their high expenses prevent cost effective biofuel production. Therefore, optimization of saccharification is a crucial part for economical production of biofuel (Himmel, M. E. et al. 2007). Table 1 gives a list of plant biomass degrading enzymes being used in different combinations for potential commercial enzyme production.

Table 1: Examples of cell wall hydrolyzing enzymes and their products (Warren, R. et al. 1996; D'Souza et al.1999)

Cellulases	Endoglucanase (EC 3.2.1.4) Exoglucanase or cellobiohydrolase (EC 3.2.1.91) β-glucosidase (EC 3.2.1.21)	Chain ends Cellobiose unit Glucose monomer
Hemicellulases	Xylanase (with endo- and exo-activity)	β-1,4-xylan
Ligninases	Laccases Manganese peroxidases Lignin peroxidases	

The bioethanol production process

Lignocellulosic biofuel production involves collection of biomass, deconstruction of cell wall polymers into component sugars (pretreatment and saccharification), and conversion of the sugars to biofuels (fermentation). Lignocellulosic biomass degradation products contain large amounts of pentose sugars (D-xylose and L-arabinose), present in hemicellulose part of biomass. Wild type *Saccharomyces cerevisiae* cannot use pentose sugars in contrast with hexoses (van Maris, A. J. et al. 2006).

The fermentation product is toxic for the fermenting host. For instance, *S. cerevisiae* cannot tolerate ethanol concentrations exceeding 25% (v/v) (Wang, M. et al. 2007). As a result ethanol yield will be limited and the obtained product must be concentrated by distillation, which is an expensive step. Despite vast ongoing research most of the organisms are not as tolerant to ethanol or other inhibitors as *Saccharomyces cerevisiae* (Olsson, L. et al. 1993). Additionally, there is no report of a microorganism capable of fermenting D-xylose at high concentration and productivity (Fischer, C. R. et al. 2008). Among the numerous organisms that naturally metabolizing D-xylose in which some also fermenting it to ethanol, D-glucose fermentation is happening in much more lower degrees than *S. cerevisiae*. Characteristics such as high ethanol yield and high productivity under anaerobic conditions make *S. cerevisiae* a preferred organism for biomass conversion into bioethanol. Yet, the catabolic versatility of *S. cerevisiae* could be improved by the functional expression of a variety of foreign genes that are associated with D-xylose and L-arabinose assimilation and catabolism (Jeffries, T.W. 2006). However, *Pichia stipitis* represents one yeast species, which is able to use the pentose breakdown products of lignocellulosic biomass (Jeffries, T. W. et al. 2007), which creates opportunities to optimize pentose pathway and construct systems for heterologous expression of this pathway.

Table 2: An overview of technological status of each sub processes in conversion of lignocellulose to ethanol adopted from IEA Bioenergy 2008.

Sub-process	Objectives	State of development
Pretreatment	Properly size the material. Produce ideal bulk density. Remove dirt and ash. Rapid depressurisation to explode fibre. Open the fibre structure.	Demonstration/commercial - but needs optimisation for different feedstocks and downstream processing.
Fractionation	Cyclone to separate solids from vapours.	R&D.
Enzyme production	Cost and processing rate are key factors.	Commercial -but needs further cost reductions to reach USD 0.02-0.03 /litre of ethanol.
Enzymatic hydrolysis	Produce C6 and C5 sugars. Reduce viscosity.	Early demonstration.
Hexose fermentation	Standard yeast	Commercial.
Pentose fermentation	Standard yeast is not suitable. New micro-organisms dictate yield and rate. This affects feedstock demand / unit of product and capital expenditure on plant.	Research/pilot plant moving towards commercialisation.
Ethanol recovery	Distillation to obtain 99.5% ethanol.	Commercial.
Lignin recovery and applications	Separate lignin and other solids. Combust for heat and power or to produce biomaterial co-products.	Research/pilot plant -co-products to improve economic performance.
Waste treatment		Research/commercial

Genetic manipulation

Cell wall polysaccharides can be converted to fermentable sugars by hydrolysis enzymes such as cellulases and hemicellulases. The main difficulty in conversion procedure is caused by lignin as it blocks access of enzymes to polysaccharides (Mosier, N. et al. 2005; Somerville, C. S. 2006). Pre-treating biomass with heat and/or chemicals is a progressing procedure, which opens up the cell wall and removes lignin in order to expose more polysaccharides such as cellulose to enzymes. Pre-treatment conditions with heat and chemicals, requires the development of enzymes that are resistant to higher temperature and a broader pH range. Additionally, including more enzymes to commercially produced mixtures is required, as for instance hemicellulases increase the output of five and six carbon fermentable sugars. The fact that in last few years' cellulase production efficiency has been increased more than 10 fold, makes enzymatic saccharification a much more economical process than before (Himmel, M. E et al. 2008). Table 3 summarizes already used or potential feed stock's genome sequencing development status.

Table 3: Feedstock and feedstock models adopted from Edward M. Rubin, nature reviews 2008.

Organism	Genome bp x 10^6	Status	Reference
Populus trichocarpa (poplar)	480	Complete	Tuskan, G. A. et al. (2006)
Chlamydomonas reinhardtii	120	Complete	Merchant, S. S. et al. (2007)
Glycine max (soya bean)	1,200	Draft	-
Manihot esculenta (cassava)	770	In progress	-
Sorghum bicolor	760	In progress	-
Eucalyptus globulus	600	In progress	-
Brachypodium distachyon	355	In progress	-
Zea mays (maize)	2,500	In progress	-
Elaeis guineensis (oil palm)	3,400	In progress	http://www.checkbiotech.org/green_News_Biofuels.aspx?infoId515100
Panicum virgatum (switchgrass)	5,600	In progress	-
Setaria italica (foxtail millet)	515	In progress	-

In a recent study Gao L et al. (2012) analyzed the effect of cellobiohydrolase (CBH) *N*-glycosylation on cellulase activity. Fungal CBH are one of key factors in lignocellulosic biomass hydrolysis. *CBH1* represents different glycoforms most likely modified by posttranslational modification (García, R et al. 2001). Mutagenesis in *Penicillium decumbens* shows whether or not CBH glycosylation affects its activity, together with glycosylation site and structure. More genetic studies for revealing glycosylation effect on cellulase activity could easily accelerate cost effective production of bioethanol form lignocellulosic biomass (Gao L et al. 2012).

Production of hydrolyzing enzymes in plants

Many enzymes and other proteins, carbohydrates, lipids, industrial polymers and pharmaceuticals are already industrially produced in plants (Sticklen, M. B, 2006; Howard, J. A., & Hood, E, 2005). Heterologous expression of plant cell wall hydrolyzing enzymes in plants is a new challenge in order to produce cost effective enzymes for use in cellulosic hydrolysis. Growing transgenic plants in the field requires less energy input than other ways like bacterial production of the enzymes. However, efficient expression needs codon alteration of the coding region, which is widely used for the heterologous expression of microbial proteins in eukaryotes. Moreover, accumulation of hydrolysis enzymes is preferred in sub-cellular compartments to prevent misfolding in the new environment (Sticklen, M. B, 2006; Horn, M. E., Woodard, S. L. & Howard, J. A. 2004). An oxidizing environment and the presence of numerous chaperons with few proteases make the endoplasmic reticulum (ER) a potential compartment for targeting enzymes, which cause more stable enzymes with 2-10 fold more activity than enzymes translocated to cytosol (Schillberg, S. et al. 2003; Fischer, R. et al. 2004). These enzymes can be extracted as part of plant total soluble protein from fresh or dry transgenic crop biomass. However, stability determination of the biological activity of enzymes is required in case of storage (Oraby, H. et al. 2007). To this end, increasing biological activity and the levels of production of the heterologous enzymes has a great value. One way to increase level of enzymatic production is to engineer chloroplast genome instead of nuclear genome. Chloroplast genome in most flowering plants is inherited maternally, which allows transgene containment. Cereal crops cannot be regenerated from leaf or cotyledon explants, which make a difficult task to achieve homoplasmic chloroplast transformation (in which every chloroplast carries the transgene) except for poplar (National Research Council, 2004; Okumura, S. et al. 2006). Furthermore, it has already been shown that targeting xylanase to both chloroplast and peroxisomes results in higher levels of production in compare to targeting to either of them (Hyunjong, B., Lee, D. S. & Hwang, I. 2006). Therefore targeting to several compartments simultaneously might increase the level of enzyme production. A recent study by Zhang Q et al. (2012) reports expressing high level of endo-1,4-β-glucanase from *Acidothermus cellulolyticus* (E1) in rice seeds (*Oryza sativa* L. ssp*. japonica*). The activity of obtained enzyme without purification and enrichment is already close to some commercially produced enzymes (830 U/g). The results demonstrate potentiality of producing hydrolyzing enzymes especially cellulase in plants with low production costs.

Reduce necessity of pretreatment

Genetic modification of lignin has been a matter of interest in industry to decrease the bleaching need in paper production and also to increase digestibility (Boudet, A. 2000; Ralph, J. et al. 2006). Modifying lignin content,

composition, hydrophobicity and cross-linking can improve the enzymatic hydrolysis of cell walls. Lignin is composed of *para*-coumaryl, coniferyl and sinapyl alcohols. Down-regulation of any of lignin biosynthetic pathway enzymes would create a potential way to decrease pretreatment costs (Ragauskas, A. J. et al. 2006). Second strategy to this end is to prevent plant carbon sources to be consumed in lignin production. Moreover the latter will also help plant biomass content improvement. As an example, in aspen (P. tremuloides) shifting energy from lignin biosynthesis to polysaccharide synthesis by down regulation of 4CL and CAld5H, generated 52% decrease in lignin and 30% increase in cellulose content (Li, Y. et al. 2007). Modifying monomeric composition of lignin even without the need to reduce its entire content helps to improve biomass digestibility. As an example, over expression of ferulate 5-hydroxylase gene in poplar results in higher content of syringyl lignin and easier digestion in compare with wild type plant (Stewart et al. 2009). On the other hand crucial observation is necessary to insure that lignin manipulation does not disturb plants defense system and integrity (Sticklen, M. B. 2007).

Bioenergy crops

Certain features of wild plants millenniums ago made them desirable for domestication so they became today's food crops. Crops properties such as cell wall composition, growth rate, suitability for growth in different geographical regions and source use efficiencies are used to characterize future bioenergy crops (Sanderson, K. 2006). Different crops would be adopted in separate locations because of soil and climate influence on plant species performance. There are two kind of CO_2 assimilation namely, C_3 and C_4. Plants using **C_4** photosynthesis considered to be the most productive, having higher maximum efficiencies of light, nitrogen and water use in carbon uptake. Therefore C_4 group is potential energy crop and includes perennial grasses such as *switchgrass* and *Miscanthus*. Additionally these grasses don't require replanting after a yearly harvest and have rapid growth, low nutrient and water needs which, facilitates their growth on marginal lands. However they are rare in cold climates and can't grow below 10 ^{o}C. In cold environments **C_3** plants including trees are the choice. Potential energy crops among them are poplar and eucalyptus, which relatively grow fast in difficult to plough areas. The efficiency of conversion of total solar energy to biomass in a C4 plant is approximately 3.7% while in a C3 cereal this value is only 2.4% (Zhu et al. 2010). Optimization of bioenergy crops as a source for biofuel production is developing and genomic information and resources will be essential for accelerating their domestication. Most of these efforts are targeted to improve growth on low quality lands to minimize competition with food crops over land. Moreover, maximizing yield of biomass per unit of land area is outstanding since it minimizes the overall agricultural land use. Combination of plant genomes with gene functions and expression studies has identified potential gene candidates (Kalluri, U. et al. 2007; Busov, V. B. et al. 2008). These genes are involved in cellulose and hemicellulose synthesis as well as those participates in morphological growth characteristics such as height and branch thickness (Ragauskas, A. J. et al. 2006). *Agrobacterium tumefaciens* or gene-gun-mediated gene transfer are used to efficiently transform many crops such as rice, maize, sorghum, poplar and switchgrass (Somleva, M. N.,

Tomaszewski, Z. & Cong, B. V, 2002). Table below summarizes potential bioenergy crops and their characteristics while dividing them up on their priority for bioenergy production.

Table 4: Characteristics of potential lignocellulosic feedstock for 2nd generation bioethanol production (adopted from © OECD/IEA 2010)

	Second-Generation Biofuel Feedstock	Technical requirements for harvesting/collection	Potential advantages	Constraints	Availability
Dedicated Energy Crops					
Short-rotation coppice	**Poplar** (*Populus spec.*), **willow** (*Salix spec.*), **eucalyptus** (*Eucalyptus spec.*), **locust** (*Robinia spec.*)	Manual harvest possible but labour intensive; specialist machines needed to harvest efficiently (*e.g.* modified forage harvester)	Relatively fast growing; can reduce soil erosion; can increase soil carbon and soil fertility in poor soils	Potentially invasive; usually planted on arable land; relatively low energy density means not suitable for long transportation	All year round, though harvest for deciduous trees is best done in winter
Perennial cultivation	**Miscanthus** (*Miscanthus sinensis*), **switchgrass** (*Panicum virgatum*), **reed canary grass** (*Phalaris arundinacea*), other **Grasses**	Existing pasture machinery (mower, baler)	Can be grown on degraded land; can mitigate soil erosion; can increase soil carbon and soil fertility in poor soils	Potentially invasive; usually planted on arable land; relatively low energy density means not suitable for long transportation	Harvest during autumn and winter
Primary Residues					
Agriculture	**Straw, stover**	Existing pasture machinery (*e.g.* baler)	No competition with food; no additional land required; collection can prevent pests	Other uses: nutrient cycling, animal feed, heating; low energy density means not suitable for long transportation distances	During crop harvesting season
Forestry & logging	**Treetops, branches, stumps**	Specialist machines to collect residues efficiently	Relatively cheap; no additional land required; removal can help to prevent forest-fires	Other uses: fuel wood demand, heat/ electricity production; removal can cause loss of organic matter, soil carbon and reductions in habitat for biodiversity; not suitable for long transportation distances	Year-round (if residue mat is not needed to protect soils during rainy season)

Secondary Residues					
Crop processing	**Coffee, rice, corn, cacao (shells, husks, cob)**	**No additional technical equipment; no additional infrastructure**	**By-product - no food competition; no additional land required; concentrated at processing site; avoids disposal costs**	**Competition with heat and electricity generation**	**Year-round**
Sugar and first-generation bioethanol Production	**Sugar cane, sweet sorghum, sugar beet (bagasse, pulp)**	**No additional technical equipment; no additional infrastructure**	**No food competition; concentrated at ethanol-plant; no additional land required; avoids disposal costs**	**Competition with heat and electricity generation; animal feed**	**During feedstock harvesting season**
Vegetable oil production	**Canola, oil palm, jatropha (presscake, shells, fruit bunch)**	**No additional technical equipment; no additional infrastructure**	**Concentrated at oil mills; currently very cheap; no additional land required; avoids disposal costs**	**Competition with heat and electricity generation. Press cake provides a valuable animal fodder.**	**During crop harvesting season**
Forestry processing	**Sawdust, bark**	**No additional technical requirements**	**Concentrated at saw and paper mills; no additional land required; avoids disposal costs**	**Competition with heat and electricity generation**	**Year-round**
Tertiary Residues					
Municipal solid waste	**Palettes, furniture, demolition timber**	**Separation from other waste might be required**	**Concentrated at landfill site; no additional land required; avoids disposal costs**	**Competition with heat and electricity generation**	**Year-round**

Next to this, aside from research and innovation prospective, at the moment there is a rational need for practical system that will enable us to generate and harvest enough energy from crops to replace some of energy obtained from fossil resources. As is shown below bioethanol production increases drastically especially in last years. Yet, production of 2nd generation bioethanol is still in its infancy, despite advantages of 2nd generation biofuel over 1st generation. Estimated higher production costs and lack of commercially proven manufacturing technology are the main reasons why 2nd generation biofuel is not commercially taken up at the moment.

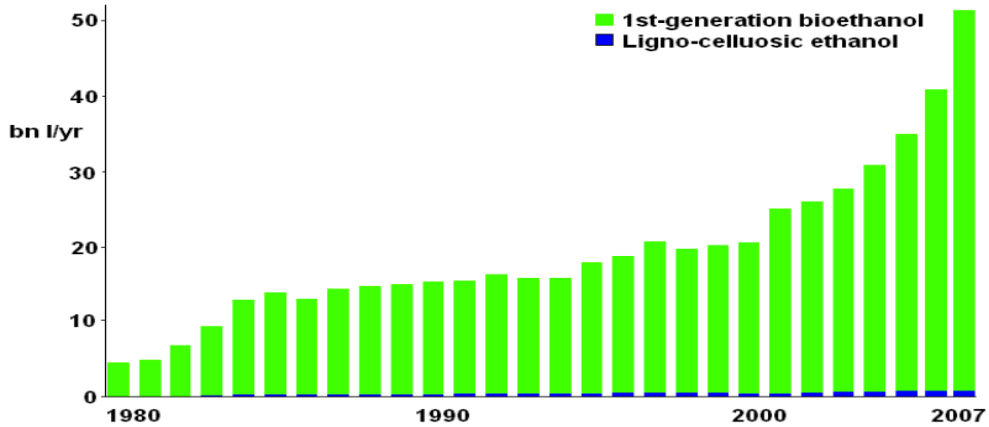

Figure 2: World bioethanol production comparison originated from 1st generation and lingocellulose (Mabee and saddler, 2007).

Aleman grass (*Echinochloa polystachya*), **Elephant** grass (*Pennisetum purpureum*), **foxtail** millet (*Setaria italica*), **miscanthus** (*Miscanthus giganteus*), **sweet sorghum** (*Sorghum bicolor*), **sugarcane** and **switchgrass** (*Panicum virgatum*) are grass species with C_4 photosynthesis. Their characteristics make

them ideal energy crops (Taylor et al. 2010). Figures below compare final dry harvestable biomass for number of C_3 and C_4 species and draw attention to importance of length of growing season. In warmer areas it is possible to harvest two crops per year if crop duration is short.

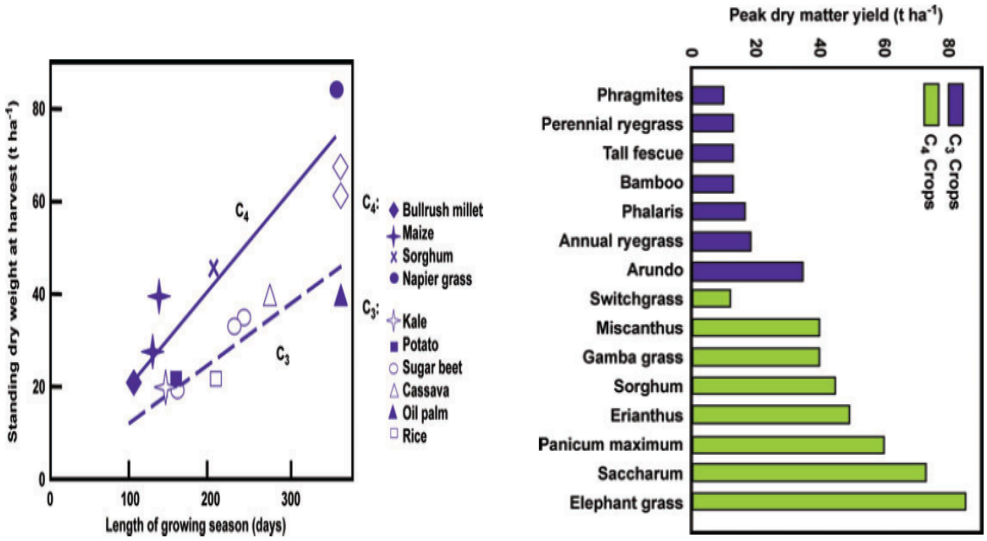

Figure 3: Left, Standing dry weight at harvest for a variety of C3 and C4 crops plotted against length of growing season. Adapted from Monteith (1978). Right, Peak dry matter yields of field grown C3 and C4 potential biofuel species grown under optimal conditions in the field (El Bassam 1998).

At the moment, biofuel production by processing lignocellulosic biomass is more expensive than processing sugar cane and maize. This originates from high costs involved in separating cellulose from lignin and its hydrolysis costs. Optimistically the energy used to process lignocellulosic biomass should be significantly lower than yielded energy. Yet, in terms of energy out verses energy in, swichgrass with 10.8–11.3 outperforms corn with 1.4–2.3 (Vadas et al. 2008). This number for sugarcane and sugar beet respectively is 8.1-10 and 2 (Boddey et al. 2008; Sanchez and Cardona 2008; Goldenberg and Guardabassi 2010). Transgenic approaches may be used to increase sugar content of the biomass, by producing sugars that cannot metabolized by the plant, which eventually increases ethanol yield. For instance, introducing bacterial isomerase to sugar cane that converts sucrose into isomaltose increased sugar yield up to twice more than regular plant (Wu and Birch, 2007).

At present miscanthus, (Heaton et al. 2004; Dohleman and Long 2009) (Schmer et al. 2008) switchgrass, corn stover and willow are already being used in lignocellulosic bioethanol trail as feedstocks (Li et al. 2010; Van Hulle et al. 2010). Yet, there are many promising lignocellulosic biomass feedstock nominates to be tested such as *Echinochloa polystachya*. Furthermore, *Setaria viridis* is a C_4 grass species, which its characteristics such as small genome, compact structure and short life cycle makes it predominant candidate for biofuel feedstock model plant (Brutnell et al. 2010).

There is a growing attempt toward using waste and residues as feedstock. Recently Saucedo-Luna J et al. (2010) show that Bagasse of *Agave tequilana* (BAT), remaining residue of tequila production process has the potential to be used as ligno-cellulosic material for ethanol production. This process by using *Pichia caribbica* UM-5 for fermentation resulted in 56.75% of theoretical ethanol

yield (w/w). Moreover, this is a relief for waste problem generated during the tequila production procedure and also eliminates the transportation costs. Interestingly in another study J.H. Ha et al. (2011) showed beer remnants potential as lignocellulosic biomass for bioethanol production. They use waste from beer fermentation broth (WBFB) in simultaneous saccharification and fermentation procedure for bioethanol production. Interestingly because WBFB contains high amounts of carbon, nitrogen and other substances including saccharifying enzymes from malt, entire procedure is accomplished without addition of any extra hydrolyzing enzymes or fermenting organisms, which turns it to a cost effective procedure. Ethanol concentration in cultivations of WBFB supernatant within 7 days increases to 103.8 g/l in shaking cultures in 30 ^{o}C. Using rDNA sequencing and fatty acid methods they confirmed presence of *Saccharomyces cerevisiae, Candida krusei, Pediococcus dextrinicus* and *Brevibacterium verniforme* in WBFB. This fact emphasizes WBFB potential for bioethanol production as it contains two important fermentation yeast strain. However, further studies are required to optimize stock time and depress other bacterial contaminants. Moreover another study by Kapu NU et al. (2012) reports spent mushroom compost (SMC) potential as lignocellulosic feedstock. SMC is a byproduct of commercial mushroom (*Agaricus bisporus*) industry, which 1 kg mushroom production generates 5 kg of it Finney et al. (2009). However, SMC is considered an environmental problem and upon field storage contaminates water sources by leaching nitrates and phosphorous, which is a limiting factor in mushroom growing industry Finney et al. (2009). Therefore using SMC as a lignocellulosic feedstock is a viable strategy for utilization of this waste product. Additionally, their results show using surfactants such as PEG 6000 improve saccharification of SMC and provides up to 77% saving in hydrolyzing enzymes.

Hydrolyzing enzymes

Filamentous fungi generally provide their nutrition supply by decomposing biomass via secretion of mixture of plant biomass degrading enzymes such as cellulases, hemicellulases, ligninases and pectinases (Van den Brink and de Vries, 2011). These enzymes are used in production of second-generation bioethanol for saccharification and include a large amount of overall cost. Yet, innovation in cost effective and efficient commercial saccharication step is an ongoing trail (Margeot et al., 2009). Table 4 shows a list of potential organisms that could be used as hydrolysis enzyme factories.

Table 5: Hydrolyzing enzymes producers and their genomic status.

Organism	Genome size bp x 10^6	Status	Reference
Acidothermus cellulolyticus 11B	2.4	Complete	http://www.checkbiotech.org/green_News_Biofuels.aspx?infoId515100
Bacillus pumilis SAFR-032	3.7	Complete	Gioia, J. et al. (2007)
Caldicellulosiruptor saccharolyticus DSM	3.0	Complete	-

8903			
Clostridium phytofermentans ISDg	4.8	Complete	-
Clostridium thermocellum ATCC 27405	3.8	Complete	-
Cytophaga hutchinsonii ATCC 33406	4.4	Complete	-
Flavobacterium johnsoniae UW101	6.1	Complete	-
Rubrobacter xylanophilus DSM9941	3.2	Complete	-
Saccharophagus degradans	5.1	Complete	Taylor, L. E. et al. (2006)
Thermobifida fusca strain YX	3.6	Complete	Lykidis, A. et al. (2007)
Clostridium cellulolyticum H10	4.0	Draft	-
Elusimicrobium minutum Pei191	1.6	Draft	-
Nectria haematococca/Fusarium solani	51	Completed	-
Phanerochaete chrysosporium	35.1	Completed	-
Postia placenta	33	Completed	-
Sagittula stellata E-37	5.3	Draft	-
Trichoderma reesei/Hypocrea jecorina	33	Completed	-
Cellulomonas flavigena DSM 20109	Almost 4.0	In progress	-
Cellvibrio japonicus Ueda107	Almost 6.0	In progress	-
Fibrobacter succinogenes subsp. succinogenes S85	Almost 3.8	In progress	-
Ruminococcus albus	4.0	In progress	-
Teredinibacter turnerae T7902	Almost 2	In progress	-
Termite hindgut community	NA	Complete	Warnecke, F. et al. (2007)
Poplar biomass degrading community	NA	In progress	http://www.jgi.doe.gov/sequencing/lspssseqplans2007.html
Asian longhorned beetle (*Anoplophora glabripennis*) gut community	NA	In progress	http://www.jgi.doe.gov/sequencing/DOEmicrobes2007.html
Bovine rumen community transcriptome	NA	In progress	http://www.energybiosciencesinstitute.org/ index.php?option5com_content&task5view&id5159&Itemid520

Among the organisms listed above *Trichoderma reesei* is a filamentous fungus broadly used for production of commercial cellulases, which clearly stated in table (6). *T. reesei* lacks a lot of hemicellulases as well as pectinases and secretes three types of cellulases. It has already been shown by Couturier et al., 2011; Gao et al., 2011 that, addition of hemicellulases or other polysaccharide and ligninmodifying enzymes to cellulases provide more cellulose accessible to hydrolyzing enzymes. One step towards more efficient commercial hydrolysis mixtures is to add up the lacking enzymes produced by other fungi to them. Ravalason H. et al. 2012 shows that addition of *Fusarium verticillioides* secreted enzymes to *T. reesei* cellulase increases release of glucose, xylose and arabinose from wheat straw up to 24%, 88% and 68% respectively.

Table 6: Common commercial enzymes for biomass hydrolysis.

Enzyme complex	Producing Company	Production host	Mentioned Ref& Components
ACCELLERASE® 1500	GENENCOR www.genencor.com	*Trichoderma reesei*	1-2-18 exoglucanase, endoglucanase, hemi-cellulase and beta-glucosidase
SPEZYME CP	GENENCOR	*Trichoderma reesei*	3-4-6-10
Novozyme 188	SIGMA-ALDRICH sigma-aldrich.com	Cellobiase, Aspergillus niger	3-4-6-9-12-19-20 β-Glucosidase
Multifect xylanase	GENENCOR		3-4-6-10
Multifect pectinase	GENENCOR		3-4-6
Spirizyme Plus	**Novozymes**		5 amyloglucosidase
Acremonium cellulase	Meiji Seika, Tokyo, Japan	Acremonium cellulolyticus	8
Cellic CTec2	Novozymes www.bioenergy.novozymes.com		5-12 cellulases β-glucosidases Hemicellulase
cellulases GC220	GENENCOR	*T. reesei*	7 cellulases
	Heilongjiang Zhaodong Enzyme Product Co. Ltd, Zhaodong, China	*Trichoderma reesei*	14
Novozyme SP188	Novozymes, Bagsvaerd, Danemark	Aspergillus niger	7
Acremozyme	Kyowa Kasei, Osaka, Japan	*Acremonium cellulolyticum*	13
Meycellase (Kyowa Kasei)	Wako Pure Chemicals, Osaka, Japan	*Trichoderma viride*	13
Optimash BG	Genencor International, Palo Alto, CA	*T. reesei*	8
celluclast 1.5L	Novozymes	*T. reesei*	9-20 cellulase
β-Glucosidase from almonds	SIGMA-ALDRICH		10
CPN cellulase	Iogen Corp		11
NS50013, Novozymes	Novozymes North America Inc., Franklinton, NC)		15 cellulase
Novozymes NS50010	Novozymes		15 βglucosidase
NS 50012	Novozymes	Aspergillus aculeatus	16
NS 50013	Novozymes	*T. reesei*	16-17

Jan-A	DSM, Holland	16
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1: Klement T. et al. (2012), 2: Luo X, Zhu JY. (2011), 3: Jin M et al. (2010), 4: Sills DL et al. (2010), 5: Van Eylen D et al. (2011), 6: Jin M et al. (2012), 7: Ravalason H. et al. (2012), 8: Buaban B et al. (2010), 9: Saucedo-Luna J et al. (2011), 10: Wang D et al. (2011), 11: Smith BT et al. (2010), 12: Sørensen A et al. (2011), 13:Yasuda M et al. (2012), 14: Sun Q et al. (2012), 15: Kapu NU et al. (2012), 16: Gao L et al. (2012), 17: Balsan G et al. (2012), 18: Zhao J et al. (2012), 19: Barr CJ et al. (2012), 20: Sipos B et al. (2011).

Table 7 shows a comparison of hydrolyzing enzyme activity produced by two filamentous fungi *Acremonium cellulolyticus* and *Trichoderma reesei*, both potential cellulase producers and commercial enzymes. Better understanding of hydrolyzing enzymes produced by potential fungi would result in more efficient saccharification and greater performance in bioethanol production. Glucan hydrolyzing activity of *A. cellulolyticus* is higher than *T. reesei*. However the xylan hydrolyzing activity is greater in *T. reesei* culture supernatant than *A. cellulolyticus*. However an optimize mixture of this two enzymatic pools can complement hydrolysis efficiency of each other.

Table 7: Specific activities of cellulases and hemicellulases derived from *A. cellulolyticus* CF-2612 (SCF-2612) and *T. reesei* CDU-11 (SCDU-11). Obtained from Fujii T. et al. 2009.

Enzyme		Specific activity (U mg^{-1} protein)							
		FPase	Avicelase	CMCase	β-glucosidase	Xylanase	β-xylosidase	Mannanase	β-mannosidase
culture supernatant	SCF-2612	0.66 ± 0.13	0.26 ± 0.02	4.52 ± 1.32	1.20 ± 0.02	12.4 ± 0.15	0.011 ± 0.001	1.10 ± 0.15	0.00045 ± 0.0001
	SCDU-11	0.25 ± 0.05	0.11 ± 0.03	3.55 ± 1.01	0.072 ± 0.003	25.4 ± 2.32	0.049 ± 0.001	1.20 ± 0.06	0.0078 ± 0.0004
commercial enzymes	Accellerase 1000	0.44 ± 0.01	0.25 ± 0.01	6.75 ± 0.34	2.85 ± 0.06	8.86 ± 0.85	0.023 ± 0.001	0.29 ± 0.03	0.00084 ± 0.0001
	Acremonium cellulase	0.41 ± 0.02	0.19 ± 0.05	4.44 ± 0.87	2.09 ± 0.24	6.21 ± 0.21	0.0074 ± 0.0001	4.88 ± 0.87	0.051 ± 0.001

Additionally, in agreement to this, Wang D et al. (2011) significantly improve hydrolysis efficiency of commercial enzyme Genencor Spezyme CP from *Trichoderma reesei* for corn stover hydrolysis. *Aspergillus fumigatus* ECU0811 has various cellulases and a β-glucosidase with higher activity than *T. reesei.* As shown in figure 4 interestingly, supplementing Spezyme CP with *A. fumigatus* cellulase improves glucan to glucose conversion ratio from 25.6% up to 99.5%. Further more, it reduces the amount of commercial enzyme requirement as much as 10 times.

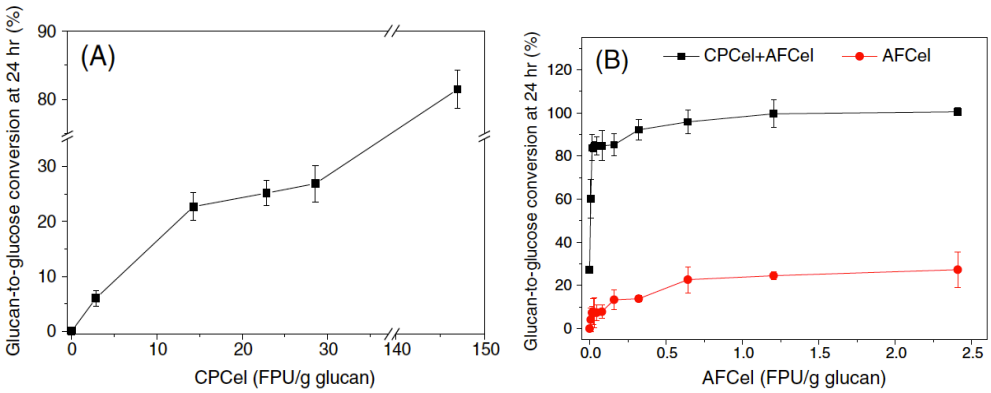

Figure 4: Hydrolytic profile of pretreated dried corn sover in graphs within 24 h. time scale. A, Treated with increasing amount (filter paper units per gram of glucan) of Spezyme CP (CPCel). B, Treated with fixed dosage of CPCel (14.2 FPU/g glucan) complemented with increasing amount of *A. fumigatus* cellulase (AFCel) starting from 0 to 2.5 FPU/g glucan.

Furthermore, efficient hydrolyzing needs optimization of temperature and pH range for hydrolyzing enzymes. Balsan G et al. (2012) lately have characterized NS 50013 commercial enzyme's features in terms of optimum temperature and pH with respect to activity and stability. In temperature between 30 to 50 ^{o}C 15% reduction in enzyme (NS 50013) activity is reported after 150 h of reaction. While in temperature above 60, within few minutes the enzyme activity is entirely lost. Regarding to pH range, the enzyme is very stable between 5.0 and 5.5, whereas pH 4.5 results in 40% loss of activity. However, one should also take into account the optimum combination of in used enzymes activities.

Conclusions

Fulfilling the argent need to substitute fossil fuels with clean and sustainable resource requires acceleration in research and development of biofuel. Moreover this product must be economically competitive with fossil fuels. Some aspects of difficulties for cost effective bioethanol production has mentioned earlier. Upgrading transgenic microorganisms, degrading enzymes and improving efficiency of C5 sugars use for fermentation, seem to be promising for economical saccharification and fermentation procedure. However, determination and/or developing potential feedstock crop and efficient land, which wouldn't compete with agriculture or forestry purposes is another necessity for sustainable bioethanol production. Furthermore, enhanced technology and development exchange and global scientific collaborations are obligatory to decrease bottlenecks facing economical bioethanol production.

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