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Microbial glycoside hydrolases for biomass utilization in biofuels applications

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Renewable biomass is predicted to have the potential to meet at least a quarter of the world demand for transportation fuel, but to do so both terrestrial lignocellulosic as well as marine algal resources need to be efficiently utilized. In the processes where these biomasses are converted to different types of energy-carriers (for example fuel alcohols *e.g.* ethanol or butanol) microbial glycoside hydrolases have a role in the saccharification process. During saccharification polymeric carbohydrate resources (*e.g.* starch, cellulose or hemicellulose) are hydrolysed into mono and oligosaccharides that can be utilized by the organism selected to ferment these carbohydrates into the desired energy-carrier. This chapter aims to shed light on different processing alternatives for the conversion of lignocellulose or algal starch into mono or oligosaccharides, and what roles the microbial glycoside hydrolases have as processing aids in these conversions.

1.Introduction to biofuels

With the depletion of crude oil, attention has gone towards use of natural recoverable resources for production of biofuels. Public and scientific attention is also driven by factors such as the price, concern over greenhouse gas emissions, as well as support from government subsidies. In 2010 worldwide biofuel production reached 105 billion liters and provided 2.7% of the fuels for road transport (Shrank and Farahmand 2011). Moreover, it is predicted that biofuels have the potential to meet more than a quarter of world demand for transportation fuels by 2050. (Platts 2011)

A biofuel is by definition a fuel, whose energy is derived from biological carbon fixation. This includes fuels derived directly from solid biomass or fuels obtained by conversion of biomass into energy-carrying compounds such as fuel-alcohols (e.g. methanol, ethanol, butanol), biodiesel, hydrogen or biogas (Chandra et al 2012). These biofuels are liquid or gaseous, and with this they meet the requirements of a) being portable, b) being easy to handle (they can be pumped) and c) to burn cleanly. Bioethanol is the biofuel that today is

produced in largest amounts. Ethanol production accounted for more than 80% of the biofuel production volume (86 billion litres in 2010, of which almost 90% was produced in US and Brazil) (Shrank and Farahmand 2011).

Biofuels are defined as first, second or third generation, based on the type of biomass and technology used for its production (Figure 1). First generation biofuels are made from sugar, starch, and vegetable oil by established technologies, and include mainly ethanol, biogas and biodiesel. The use of first generation technology has however been the subject of considerable media attention and political debate to draw attention to the environmental and social impacts of producing biofuels from food crops (European Biofuels Technology Platform 2009). Second generation biofuels, include the ethanol and biogas as above, but in this case the fuels are produced from cellulosic materials (lignocellulosic feedstocks) which are more challenging to degrade into fermentable sugars for further conversion. Second generation biofuels also include other types of fuels e.g. hydrogen, other bio-alcohols and mixed compounds (Figure 1).

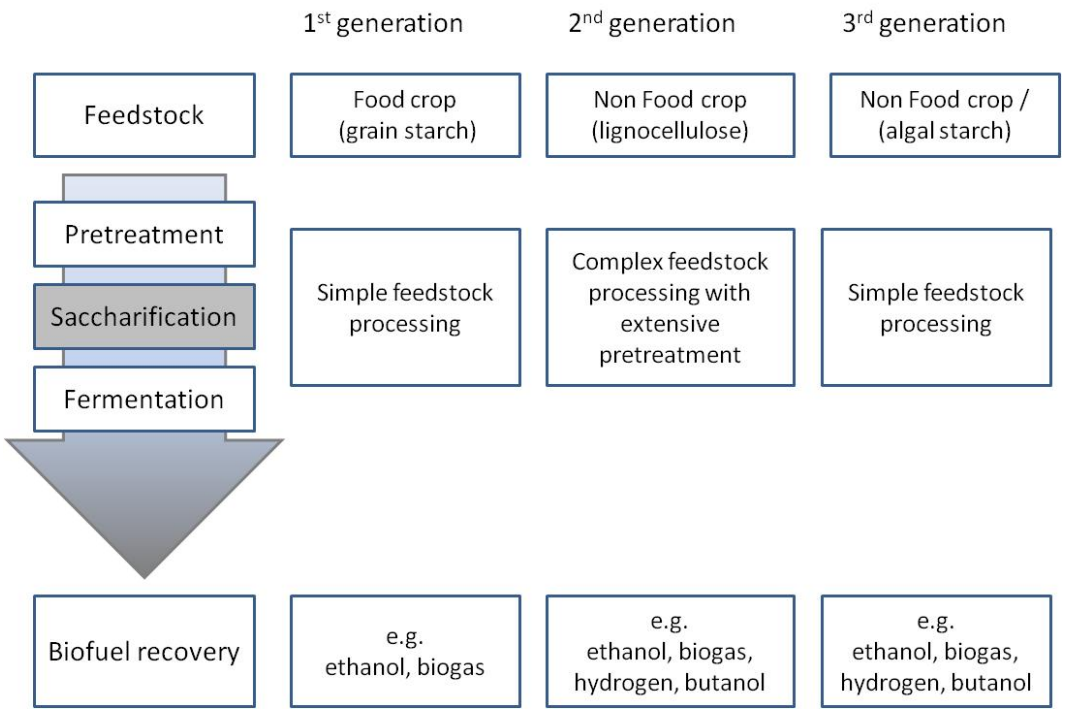

Figure 1. A simple division of 1st, 2nd and 3rd generation biofuels from carbohydrates, based on feedstock and processing. Glycoside hydrolases have a role in the saccharification step, which is the degradation of polymeric carbohydrates into smaller oligo or monosaccharides allowing fermentation into the desired biofuel. Biodiesel production is excluded from the scheme as it is based on oil and has a different overall processing scheme.

The lignocellulosic biomasses are not food crops or are the non-edible parts of the food crops, and demands technology developments for efficient processing. Moreover, the feedstocks should be defined as sustainable, and sustainability is for instance judged based on the availability of the feedstock, the impact on green house gas emissions and the impact on biodiversity and land use (European Biofuels Technology Platform 2009). Recently a third generation of biofuels has also been suggested implying the use of (macro and micro) algae as biomass (An et al, 2011). Algae can be cultured on sea or waste-water and do not require the same use of land area. Algae has to date mainly been considered for biodiesel, hydrogen and biogas production (Demirba 2011, Aitken and Antizar-Ladislao 2012), but an emerging interest has also risen for their use in ethanol production (Demirba 2010, Aitken and Antizar-Ladislao 2012, Harun et al 2010). In this chapter, our focus is on the second and third generation biomass resources, and what possibilities the microbial glycoside hydrolases give us to access and degrade the polymeric carbohydrate fibers into shorter oligo and monosaccharides fermentable by microorganisms for conversion into metabolites, which are the energy carriers of biofuels. The oil fractions used for biodiesel production, have been reviewed elsewhere (see for example Stuart et al 2010) and are not considered in this chapter.

2. Biomass for second and third generation biofuels

2.1. Lignocellulosic biomass – raw material for second generation biofuel

Lignocellulosic feedstocks consist of mainly cellulose, hemicellulose and lignin and can be found in the cell walls of almost all plant-derived materials, such as wood and grass, agricultural residues and municipal solid wastes. The relative composition of the lignocellulosic material however varies greatly, depending on source (Chandel and Singh 2011, Garrote et al., 1999, Mosier et al 2005) and for an overview the weight percentage of dry biomass of representative lignocellulosic materials are listed (Table 1).

Table 1. Percent dry weight composition of some lignocellulosic feed stocks and paper wastes (extracted from Mosier et al 2005, Chandel and Singh, 2011)

Feedstock	Cellulose	Hemicellulose	Lignin
Corn stover	37.5	22.4	17.6
Corn fiber	14.28	16.8	8.4
Pine wood	46.4	8.8	29.4
Poplar	49.9	17.4	18.1
Wheat straw	38.2	21.2	23.4
Switch grass	31.0	20.4	17.6
Office paper	68.6	12.4	11.3
Newspaper	61	16	21

Cellulose (β-1-4-glucan), a linear polymer of glucose units, is the major component of the lignocellulose (accounting up to 50% of the total plant dry weight), the most abundant form of biologically fixed carbon in the biosphere, and a primary target for biofuels that are metabolites from microbial conversions (as in bioethanol production). It is hence a material of high interest to utilize well, but also a very recalcitrant material, making its utilization difficult. A major challenge is still to manage to convert lignocellulose in high yields to fermentable sugars (see also section 3. Lignocellulosics requires pretreatment for degradation) and to follow this with an efficient process that reduces the oxygenated carbohydrates to fuel molecules (Chundawat et al, 2011). In the process to obtain fermentable sugars, microbial glycoside hydrolases (GHs) are used as catalysts to obtain saccharification (hydrolysis) of different polysaccharides in the biomass (explained more in the sections below). The microbial GHs are catalysts designed to degrade complex carbohydrate polymers into mono or oligosaccharides, that allow uptake and metabolism by the microorganism selected as cell-factory for the conversion into the desired biofuel, even if the microorganism on its own is not capable to degrade the polymeric carbohydrate-forms.

It has been predicted that based on available land, the energy potential of lignocellulosics worldwide allows an energy outtake of approximately 100 EJ/ annum (1 EJ = 1×10^{18} J,

covering woody biomass, straw and energy crops) (Parikka 2004), which is to be compared to the global energy demand (425 EJ in 2001) (Lewis and Nocera 2006) showing that approximately one quarter of the current demand can be obtained, and thus additional resources are needed to cover a shift from fossil to renewable resources. A means to increase the possible overall energy outtake is to also turn to biomasses from marine environments.

2.2. Algal biomass –marine resources as rawmaterial for third generation biofuels

Algae has the possibility to provide a high-yield source of biofuels without compromising food supplies, forests or arable land (Subhadra and Edwards 2011, John et al, 2011, An et al, 2011), thereby being an interesting complement to the lignocellulosic second generation agricultural feedstocks. Marine environments are predicted to supply approximately 50% of global biomass (Carlsson et al, 2007, John et al, 2011) thereby significantly increasing the potential of biomass as a source of transportation fuel. Algae represent a large number of different photosynthetic species (both heterotrophic and autotrophic). The autotrophic species can fix inorganic carbon from CO_2 which is assimilated into for example carbohydrates (John et al, 2011), which can be converted into fermentable sugars for further conversion into selected energy carriers (Figure 2). The heterotrophic species take up organic molecules and convert into mainly lipids and protein, of which the lipid fraction is of interest for biodiesel production (Figure 2). Some species, called mixotrophic algae, can utilize both processes (John et al, 2011). Through these processes, carbohydrates, lipids and proteins can be produced in a very short time, allowing as frequent harvests as in 1-10 days for some microalgae (Harun et al, 2010).

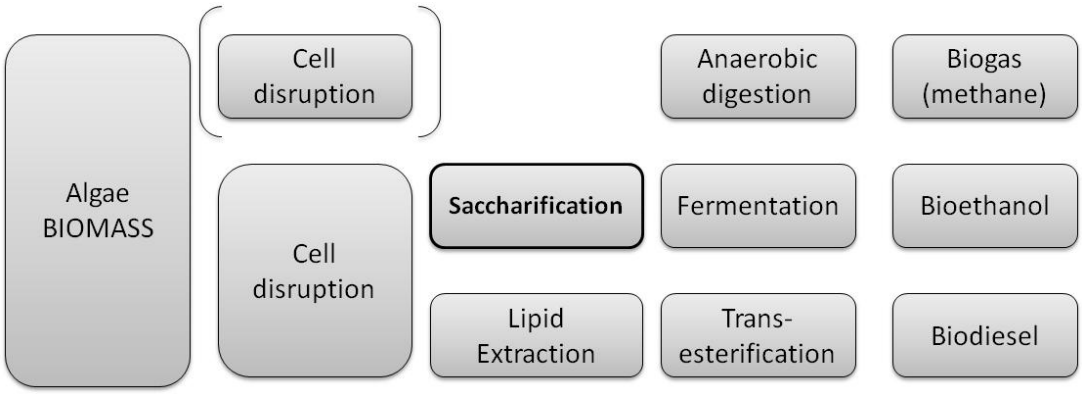

Figure 2. Schematic process steps for the three fuel types that are currently considered as most suitable for energy production from algal biomass (adapted from Aitken and Antizar-Ladislao 2012) . Enzymatic processing using GH is primarily predicted in the saccharification (or polysaccharide hydrolysis step, in bold) to boost fermentable sugars in the bioethanol production process.

Based on size and morphology, algae are roughly grouped as macro- or microalgae. As the name implies, microalgae are microscopic frequently unicellular organisms. Macroalgae are multicellular, and are composed of structures resembling higher plants, with the difference that they are buoyant and don´t need the lignin-containing structural polymer complexes that are necessary for terrestrial plants. This makes their polysaccharides easier to degrade into fermentable sugars (John et al 2011, Chen et al 2009). The types of polysaccharides available in the algae differ dependent on the species, but there are species shown to have both high cellulose and high starch content (Table 2). Use of organisms with high starch content, would allow the same type of processing of the starch as for first generation biofuels, involving use of starch degrading GHs.

Table 2. Carbohydrate and starch content in some selected algae (from Aitken and Antizar-Ladislao 2012, John et al 2011, Rodjaroen et al, 2007)

Algal source	Protein (%)	Lipid (%)	Carbohydrate (%)	Starch (% of biomass after oil extraction)
Chlamydomonas *C . reinhardtii*	48	21	17	53 (strain UTEX90) 45 (strain UTEX2247)
Chlorella *C. vulgaris* *C. pyrenidosa*	51-58 57	14-22 2	12-17 26	12-37 n.d.
Dunaliella *D. salina*	57	6	32	n.d.
Scenedesmus *S. obliquus*	50-56	12-14	10-17	23 (strain TISTR85446)
Spirulina *S. fusiforma* *S. maxima* *S. platensis*	n.d. 60-71 46-63	n.d. 6-7 4-9	n.d. 13-16 8-14	37-56 n.d. n.d.

n.d. = not determined

3. Lignocellulose requires pretreatment for degradation

In plant biomass, cell wall models predict cellulose microfibrils (polymers of beta-1,4-linked glucose packed by hydrogen bonds) surrounded by a matrix of hemicellulose (e.g. xylans,

mannans, glucans and xyloglucans), pectin (polymers of mainly galacturonic residues, common in the middle lamella of the cell wall) and lignin (phenyl-propanoid polymers). Unbranched hemicelluloses form hydrogen bonds with the surface of cellulose microfibrils, while branched hemicellulose form bonds (mainly ester linkages) with the phenolic acids in lignin (Chundawat et al, 2011; Sjostrom 1993). This association of cellulose, hemicellulose, pectin and lignin results in bundles or macrofibrils formation.

Due to this physicochemical, structural and compositional complexity cellulose is resistant to microbial as well as enzymatic digestion. Many microorganisms also lack the enzyme systems necessary for efficient degradation of the lignocellulosic material, and this is especially evident in cases, such as the conventional way of ethanol production, where a single non-cellulolytic microorganism (typically *Saccharomyces cerevisiae*) is used for the conversion from carbohydrate to biofuel. Thus, it is necessary to start with a pre-treatment step which reduces the crystallinity of the cellulose, removes lignin and hemicellulose, and improves the porosity of the biomass. This enhances the accessibility of cellulose to enzymes, which in turn leads to a more efficient conversion of cellulose to fermentable sugars. Over the years, an impressive number of pre-treatment methods have been developed that breaks down the intertwined interaction, among others, between lignin, cellulose and hemicelluloses, and a summary of the common pretreatment methods is given in Table 3. The efficiency of the treatment methods vary from method to method, and depends on the type and source of the biomass treated.

For a given biomass, among the available methods, a suitable pre-treatment can be selected based on: (a) process cost, (b) susceptibility of the treatment product to enzymatic hydrolysis, (c) effect of pretreatment on hemicelluloses and cellulose, (d) presence or absence of by products that inhibit enzyme activity and fermentation processes, and (e) amount and type of chemical consumption.

Table 3. Summary of lignocellulosic pretreatment methods.

Pre-treatment	Process	Principles	Notable remarks	References
Physical	Milling, chipping, grinding	Easier handling and increased surface area to volume ratio which facilitates reactivity. Decreased degree of lignocellulose crystallinity.	First step in multiple pre-treatment processes. High power consumption.	Tassinari and Macy 1977, Cadoche and Lopez 1989, Galbe and Zacchi 2007
	Irradiation	The energy breaks the hydrogen bonds of the cellulose crystalline structure and makes it prone to enzymatic digestion	Efficient in the presence of lignin. Expensive and not convenient for large scale application	Kumakura and Kaetsu 1983, Kumakura et al 1982
	Hydro-thermal	Uses water at high temperature and pressure that dissolves most of the lignin and hemicelluloses which in turn facilitates the hydrolysis of the cellulosic fraction	Generates acetic and other organic acids	Mosier et al. 2005, Negro et al. 2003
	Pyrolysis	Use of high temperature to disrupt the lignocelluloses	Efficient when carried out in the presence of oxygen	Shafizadeh and Bradbury 1979
Physio-chemical	Explosion e.g. steam explosion, ammonia fiber explosion, CO2 explosion	Alters the structure of cellulosic biomass to make it more accessible. Exposes biomass to high temperature and pressure followed by a sudden pressure fall to make an explosive decomposition	Steam explosion is the most commonly used method for the pretreatment of lignocellulosic biomass	Grous et al 1986, Brownell et al 1986, Emmel et al. 2003, Kumar et al 2009
Chemical	Acid	Increases the biomass porosity by removing the hemicelluloses and altering the lignin structure, and this facilitates enzymatic digestibility	Accompanied with aldehyde formation. Consumes significant energy for pretreatment and product recovery	Mosier et al 2005, Kumar et al. 2009
	Alkali	Removes lignin, acetyl and various uronic acid substitutions from the biomass by saponification that breaks the intermolecular ester bonds. This improves enzymatic digestibility of the biomass	utilize lower temperatures and pressures compared to other pretreatment methods	Kassim, and El-Shahed 1986, Fox et al. 1989, MacDonald et al. 1983
	Wet oxidation	Treatment of biomass in the presence oxygen/air and water at high temperature and pressure opens the crystalline structure of cellulose	All biomass fractions are affected. Hemicelluloses degrade substantially	Palonen et al 2004, Varga et al 2004, Martin et al 2007
	Ozonolysis	Targets lignin degradation by attacking and cleavage of aromatic rings structures	The cellulose and hemicellulose fractions remain intact	Neely 1984, Euphrosine-Moy et al 1991
	Solvent extraction	Use solvents to remove lignin and some hemicelluloses to facilitate enzymatic hydrolysis of lignocellulosic biomass. Often accomplished at moderately high temperature	Requires removal of the solvent from the treated biomass	Pan et al 2005, Pan et al 2006, Araque et al 2007
Biological	Micro-biological	Micoorganisms (often fungi) degrades lignin and hemicelluloses	Long process but low in energy consumption and requirement.	Kurakake et al 2007

4. Enzymatic hydrolysis of lignocellulose

4.1 Hydrolysis of cellulose to fermentable sugar

Cellulose is one of the most important resources for production of the biofuel ethanol. However, the common organisms used in the production of bioethanol, *Saccharomyces cerevisiae* and *Zymomonas mobilis* cannot utilize cellulose. Therefore, cellulose has to be depolymerized to a fermentable sugar (glucose) that can be utilized by these organisms to produce bioethanol. Although there are alternative chemical methods of cellulose depolymerization, the enzymatic hydrolysis is a preferred process as it results in high quality hydrolysate (no side products) and uses mild reagents (enzymes), which is beneficial from a sustainability perspective.

The major cellulose degrading enzymes belong to the glycoside hydrolases. These cellulose degrading enzymes are sub-categorized as i) endo-glucanases (E.C. 3.2.1.4) enzymes that randomly attack the β-1,4-linkages within the polymer chain and release oligosaccharides, ii) exo-glucanases or cellobiohydrolases that cleave off cellobiose either from the reducing (E.C. 3.2.1.176) or non-reducing ends (E.C. 3.2.1.91) of the chains, and iii) β-glucosidases (E.C. 3.2.1.21) which degrade smaller chain oligosaccharides releasing the terminal, non-reducing β-D-glucosyl residue (Figure 3). All the cell wall degrading enzymes are classified under multiple glycoside hydrolase families (which are based on similarities in sequence and structure), showing examples of convergent evolution. Endo-glucanases are for example classified under many different GH-families, with different folds and with both retaining (GH5, 7, 12, 44, 51) and inverting (GH6, 8, 9, 45, 48, 74, 124) reaction mechanisms (see: http://www.cazy.org). The cellobiohydrolases are structurally related to endo-glucanases and the enzymes acting from the reducing end are mainly classified under GH7 and 48, while those acting from the non-reducing end are predominantly found in the inverting GH6 and 9. The β-glucosidases are classified under GH1, 3, 9, 30 and 116, of which the inverting GH9 also harbour structurally related endo-glucanases and cellobiohydrolases.

The architecture of the plant cell wall degrading enzymes varies and many of the microbial enzymes are composed of a number of modules (Mba Medie et al, 2012). The ancillary modules are often carbohydrate binding modules (CBMs), which are believed to target the enzyme (catalytic module) towards certain parts of the cell wall.

The use of endo-glucanases, exo-glucanases and β-glucosidases in combination exhibit a synergistic effect on depolymerisation of both crystalline and amorphous cellulose to the fermentable sugar glucose (van Dyk and Pletschke 2012). The commercially available glycoside hydrolases which are currently in use by the sector are in principle originated from fungi and cellulases account about 20% of the total enzyme market which is estimated to be 6 billion dollar in 2012 (Mathew et al 2008). However, a vast number of alternative enzymes exist, and many efforts have for example been put in developing methods utilizing thermostable enzymes (Turner et al, 2007), allowing processing at higher temperatures. The cost of cellulases is still high (Cheng and Timilsina 2011) and it needs a concerted effort to bring it down to a comfortable price floor.

In addition to glycoside hydrolases, it is recently shown that oxidative enzymes such as cellobiose dehydrogenase and polysaccharide monooxygenases also cleave the glycosidic bonds in cellulose and play a role in its degradation (Phillips et al 2011, Mba Medie et al 2012). Some of these enzymes, such as the chitin-binding protein (Cbp21) or the oxidative enzymes classified under GH61 (Vaaje-Kolstad et al 2010, Harris et al 2010), have been shown to open up structures in crystalline polysaccharides (like cellulose and chitin) that are inaccessible by other glycoside hydrolases.

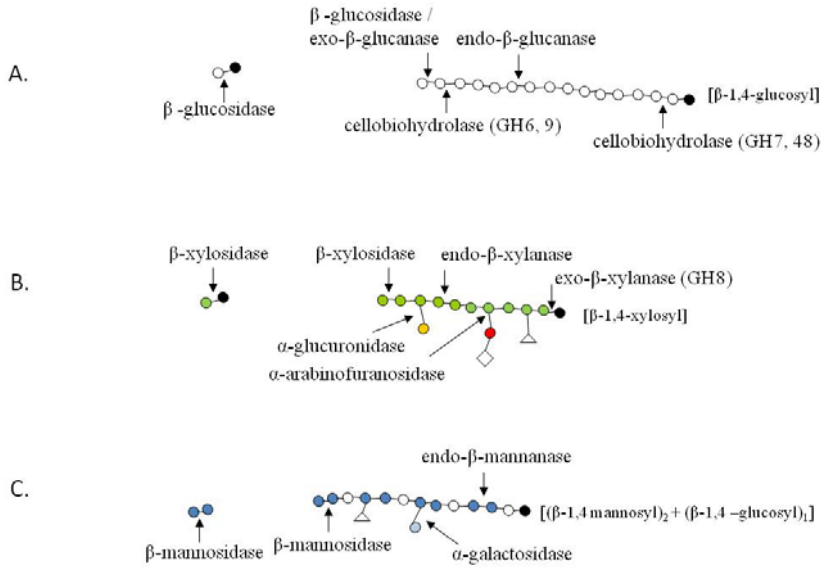

Figure 3. Simplified structures and sites of enzymatic attack on polymers from lignocellulose. A cellulose chain fragment (A) is shown, along with hypothetical fragments of the hemicelluloses xylan (B) and glucomannan (C). Sites of attack of some of the major enzymes acting on the respective material are indicated by arrows. The glycosidic bond type of the main-chain is indicated in brackets to the right of each polymer fragment. Carbohydrates are indicated as circles, and the redicing end of each main chain is shown as a black circle. White = glucose, green = xylose, yellow = glucuronic acid, red = arabinose, dark blue = mannose, light blue = galactose, Acetate groups are shown as triangles, phenolic groups as diagonals, and methyl groups as rombs. Adapted from Turner et al, 2007

4.2. Hydrolysis of hemicelluloses

The thin profit margin of ethanol production from lignocellulose is partly due to the utilization of only the cellulose fraction of the biomass (Gowen and Fong 2010). Thus, the utilization of hemicelluloses is expected to increase the profitability of the process, and this has initiated a remarkable degree of research activity. As for cellulose, the utilization of hemicelluloses requires hydrolysis of the polymers into oligomeric and monomeric units. However, unlike cellulose, hemicelluloses are structurally and chemically heterogenous and in general vary from source to source (Beg et al 2001). Both chemical and enzymatic hydrolysis processes exist that can depolymerize hemicelluloses; however, from a sustainability perspective, the enzymatic hydrolysis is preferable over the chemical route. Xylans are the most common type of hemicellulose in plants, and are heteropolysaccharides with homopolymeric backbone chains of 1,4- linked β-D- xylopyranose units (Saha, 2003, Garrote et al., 1999, Koukiekolo et al. 2005).

Xylanases that degrade this fraction of the biomass are often used in the hydrolysis of lignocellulosic biomass. Like the cellulolytic enzymes, xylanases can be divided into endo-acting xylanases (E.C. 3.2.1.8, available in GH5, 8, 10, 11, 43), exo-acting xylanase (E.C. 3.2.1.156, found in GH8) acting from the reducing end and complemented with xylosidases (E.C. 3.2.1.37, for example in GH1, 3, 39, 43, 52, 54, 116, 120) acting from the non-reducing end (Shallom and Shoham 2003). The enzymes acting on hemicellulose are like the cellulolytic enzymes also frequently modular, composed of catalytic as well as ancillary domains (Shallom and Shoham 2003). It can also be noted that a single GH-family often include enzymes of many different specificities. Mutagenesis studies have shown that exchange of only a few residues in the glycone binding site will change the binding preference of one monosaccharide for another (Corbett et al 2001) and in GH-family 1 and 3 many of the glycosidases can for example hydrolyse gluco- as well as xylo-oligosaccharides (Yernool et al 2000, Zhou et al 2012).

Use of other hemicellulose degrading enzymes such as mannanases (EC 3.2.1.78, acting on the different mannan containing hemicelluloses, mainly classified under GH5, 26 and 113), mannosidases (EC 3.2.1.25, GH1, 2 and 5), galactosidases (EC 3.2.1.23, GH1, 2, 3, 35, 42) and arabinofuranosidases (EC 3.2.1.55, GH3, 43, 51, 54, 62) (Figure 3) can together with xylanases (dependent on the biomass used) further increase the monosaccharide yield of certain materials, to obtain better conversion of hemicelluloses to their monomeric units.

Most of this work has been implemented to improve the production of ethanol, and in this field a sizable portion of the research has also been related to engineering fermentative organisms to make them utilize the monomeric pentoses and produce ethanol (Hahn-Hägerdal et al 2007). The fermentation of pentoses to ethanol undoubtedly improves the overall production of ethanol from lignocellulosic biomass. However, the inability of naturally existing commercial ethanol producer strains has hindered its implementation. (*S. cerevisiae*, does not naturally utilize pentoses). Today, there are many metabolically engineered yeast strains that successfully produce ethanol from xylose (Matushika et al 2008, Matushika et al 2009, Kuhad et al 2011) and this will in the future improve the ethanol yield per unit mass of the lignocelluloses used and improves the profit margin of companies involved in production of ethanol. Another field of engineering involves introduction of cellulolytic enzymes to enable cellulose degradation or use of cellulolytic microorganisms followed by modification of their ethanol production pathway, described more in the following section.

5. Exogenous or endogenous enzymes for saccharification of lignocellulose

The production of ethanol from biomass can be accomplished in any of three known processes. In the **conventional process**, the hydrolysate which is obtained from a separate hydrolysis of pre-treated biomass, is used to formulate fermentation media for production of ethanol often using *S. cerevisiae* or *Z. mobilis* (Figure 4). This method is the most common process. However, it is believed that the separate processing steps made the process relatively expensive and hence, alternative methods have been developed. The process known as **simultaneous saccharification and fermentation** (SSF) or simultaneous saccharification and co-fermentation (SSCF) is one of the alternative approaches. In this process, the hydrolysis of the pre-treated biomass (with addition of exogenously produced cellulolose degrading enzymes) and the fermentation process for production of ethanol performed simultaneously in the same reactor. However, this process requires biomass feedstock which is extensively pre-treated (Carere et al. 2008). Although extensive pre-treatment is necessary to ensure easy handling and efficient enzymatic degradation of the cellulose, it is expensive. The third alternative process of ethanol production from lignocellulosic biomass is **consolidated bioprocessing** (CBP), a system in which cellulase production, substrate hydrolysis, and fermentation are accomplished in a single process step by cellulolytic microorganisms (with endogenous cellulose degrading enzymes) (Carere et al. 2008,

Hasunuma and Kondo 2012). Combining these three processes in a single step is expected to substantially cut down the ethanol production cost. However, despite this great promise, so far, there is not even a single microorganism that fulfils all the required traits of substrate hydrolysis and ethanol production at commercial level. This has led to the option of metabolically engineering selected microorganisms that can potentially be used for production of ethanol from biomass through consolidated bioprocessing system (Hasunuma and Kondo 2012).

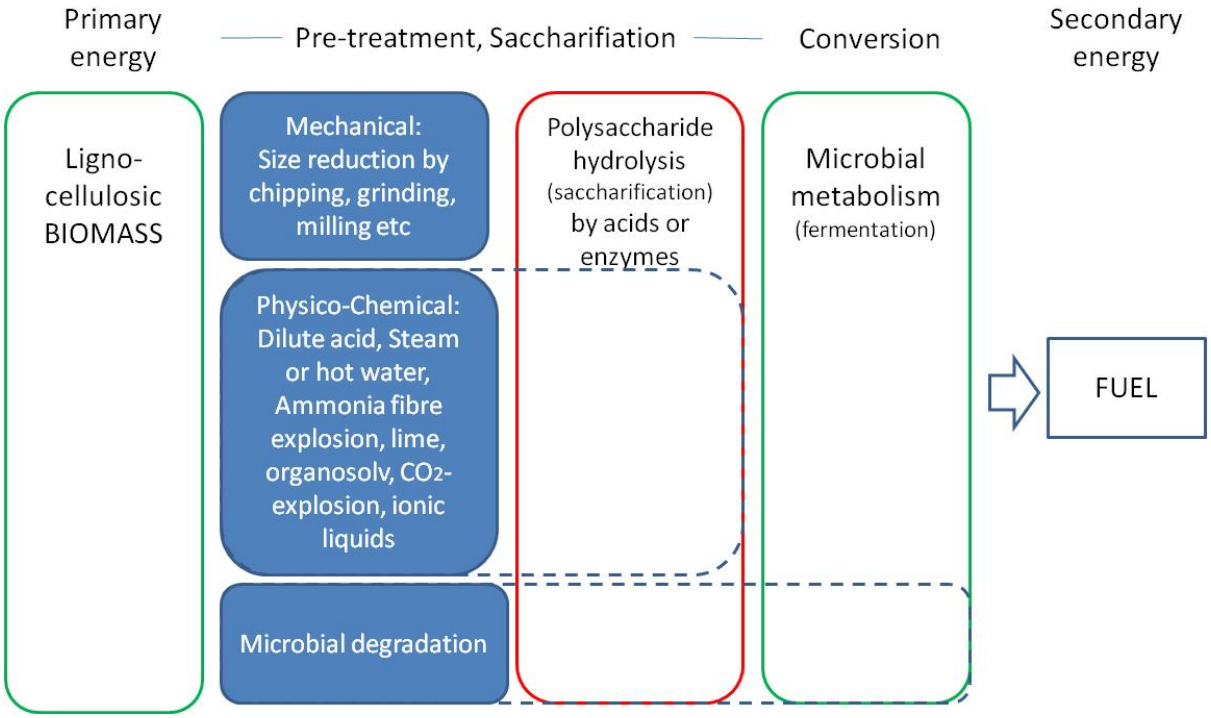

Figure 4. For the conversion of recalcitrant lignocellulosic biomass the pretreatment is a necessity to gain enough efficiency in the following conversion. The different methods range from low to high pH, and novel methods are still under development. The possibilities of microorganisms to take up polymeric sugars are often limited, and hence the final pretreatment step is frequently a hydrolysis step, allowing uptake of mono and oligosaccharides in the organism (s) selected for the conversion into the energy carrier. In this step, microbial glycoside hydrolases have a potential.

There are two possible approaches to engineer organisms for the CBP system, i.e. to recombinantly express the necessary cellulose degrading enzyme in a commercial ethanol producer strain such as *S. cerevisiae* (van Zyl et al. 2007, van Wyk et al. 2010) or enhance the ethanol producing capabilities of known cellulolytic microbes such as *Geobacillus*, *Clostridium* or *Fusarium*. For example *Fusarium oxysporum* is known to produce several cellulose and hemicellulose degrading enzymes and ferment both hexose (glucose) and pentose (xylose) into ethanol with reasonably good yield (1.8 mole ethanol/mole of glucose

and 1 mole ethanol/mole of xylose) (Panagiotou and Christakopoulos 2004, Xiros and Christakopoulos 2009). Thus, if the ethanol yield is increased through metabolic engineering, this organism is attractive for CBP of ethanol production. To date, attemps in this direction has been made in the cellulolytic moderate thermophile *Geobacillus thermoglucosidasius*, by elimination of the lactate dehydrogenase and pyruvate formate lyase pathways together with up-regulation of the expression of pyruvate dehydrogenase (Cripps et al, 2009) resulting in improved ethanol production. Unlike aerobic microorganisms which generally produce non-complexed extracellular cellulases, some anaerobic cellulolytic microorganisms such as *Clostridium* spp are also of interest, as these organisms degrade cellulose using large extracellular complex of enzymes that act in a consortium and known as cellulosome (Carere et al. 2008). Cellulosomes often contain glycoside hydrolases, polysaccharide lyases and carboxyl esterases which are arranged around the non-catalytic protein scaffoldins which consist of cohesins linked to enzymes and carbohydrate binding modules using dockerins (Ding et al 2008, Gilbert 2007, Bayer et al 2007, Fontes and Gilbert 2010). This complex consortium degrades not only cellulose, but also other plant biomass fractions such as hemicelluloses and pectin which makes it attractive for bioethanol production.

6. Use of algal starch for production of ethanol

A wide range of research aiming to utilize algae for production of energy is ongoing. Like plant biomass, the algal biomass contains carbohydrates that can be used for production of ethanol through fermentation (Goh and Lee 2010, Brennan and Owende 2010). Starch has been the most ideal substrate for production of ethanol. However, the starch used in conventional fermentation comes from grains and this created a competing demand between food and energy production and hence not favoured from social, economical and political stands. The use of starch from non food sources alleviates the problem emerged from the fierce competition between food and energy production. Different algal genera such as *Chlorella, Glacilaria*, *Spirulina*, *Prymnesium*, *Ulva* etc. are known to accumulate starch (Zemke-White and Clements 1999) (Table 2) which can be used for bioethanol production the same way grain starch is being used. For hydrolysis of the starch, different enzymes from the α-amylase superfamily (Figure 5) are utilized. The family consists of sequence related retaining enzymes (classified under GH13, 70 and 77).

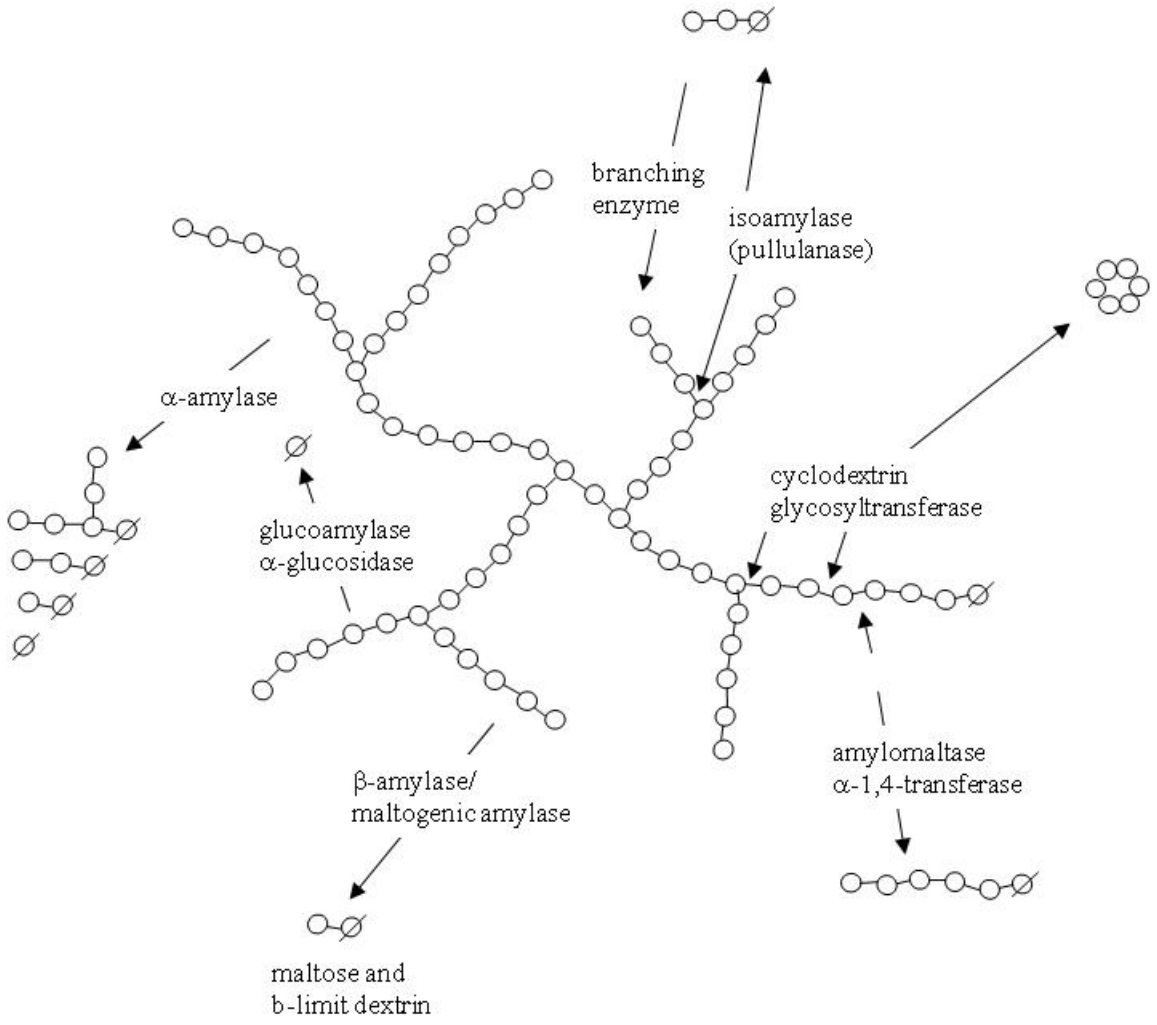

Figure 5. Enzymatic attack on part of a hypothetical amylopectin molecule. Circles are glucose molecules and circles with a line through represents a reducing glucose molecule. Adapted from Turner et al, 2007

If the algal starch is considered for ethanol production, it needs to be gelatinized, and enzymatically liquefied and saccharified as in the conventional grain starch processing for ethanol production (Turner et al, 2007). In liquefaction, thermostable α-amylase (EC3.2.1.1) is used to obtain oligosaccharides, followed by saccharification using using β-amylase (EC3.2.1.2) to obtain maltose or glucoamylase (EC3.2.1.3) to obtain glucose. The efficiency of the saccharification can also be increased by adding debranching enzyme (or pullulanase, EC3.2.1.41). Recently, it has also been shown that presence of cyclodextrins (produced by cyclodextrin glycosyltransferase (CGTases EC2.4.1.19)) can increase the ethanol tolerance of e.g. *S. cerevisiae* in the following fermentation step (Liang et al 2011).

However, gelatinization of starch is an energy intensive process and there has been a growing interest to decrease the energy consumption of starch processing. The high temperature

cooking of starch (at 140 to 180 °C) which is necessary to disrupt the starch granules increases the ethanol production cost. Direct grain raw starch saccharification is one possible alternative to achieve a reduction in the energy consumption of the process (Robertson et al. 2006). Indeed, the use of low temperature-cooking fermentation systems has been tried and it succeeded in reducing the energy consumption significantly (Matsumoto et al 1982, Shigechi et al 2000). However, raw starch is known to be resistant to enzymatic hydrolysis and this has limited its application. Interestingly, algal raw starch is known to degrade efficiently compared to many raw starch coming food grade cultivars.

7. Conclusion

Biofuel production from renewables require use of lignocellulosic biomass as well as algal biomass to allow large production volumes. This requires hydrolysis of lignocellulose as well as algal starch. For starch processing, enzymatic technologies are developed and in addition a number of enzyme mixes are available.. Lignocellulose hydrolysis is more complex, but the understanding is progressing and current research show that hydrolysis is stimulated by interplay with hydrolysing enzymes (like the microbial glycoside hydrolases) and oxidizing enzymes. A question for the future is if use of exogenous enzymes is economically feasible, or if endogenous enzymes in engineered organisms for high production are more desired for future biofuel production systems. The advantage using exogenous enzymes is that saccharification is general and can in a following step be used for different fermentation processes (using different microorganisms, and for production of different types of energy carriers). Endogenous enzymes can however be a promising alternative when developing efficient organisms for a single process. In the future, it is likely that both strategies will be in use for different purposes.

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