1 – FROM FIRST TO SECOND GENERATION BIOFUELS

1.1 – Generality on Biofuels

The conversion of biomass into useful energy is done through various types of processes. The choice of the type of conversion process depends on the type, quantity, raw material properties, but also by the end user, the latter being able to put constraints of cost, environmental, or related to a specific design project.

Biomass has always been a reliable source of energy, from the first man-made fire up to the utilization of pelletized wood as a feed for thermal plants. Although the use of lignocellulosic feedstock as a solid biofuel is a well-known concept, conversion of biomass into liquid fuel is a considerable challenge, and the more complex the biomass gets (in terms of chemical composition) the more complicated and generally expensive the conversion process becomes. The concurring phenomena of world energy need increasing and oil stocks decreasing have generated an increased interest toward biofuels in the last 10 to 20 years, although for most of the 20th century, research on biofuel closely depended on the price trend of petroleum.

Biofuels can be defined are fuels produced from biological material, a definition that can also be applied to renewable sources of carbon. Use of ethanol for lamp oil and cooking has been reported for decades (called spirit oil at the time) before Samuel Morey first tested it in an internal combustion engine in early 19th century[2]. Ethanol then replaced whale oil before being replaced by petroleum distillate (starting with kerosene for lighting). By the end of the 19th century, ethanol was used in farm machinery and introduced in the automobile market. Oil-derived products replaced ethanol for most of the 20th century before being introduced again during the Arab oil embargo in the 1970s when the price of petroleum and its derivatives peaked[2].

Biofuels can provide a potentially favorable alternative in lowering the carbon intensity of the transport sector and increasing energy security concerns. Demand for 1st-generation biofuels continues to grow strongly, but it is increasingly understood that first–generation biofuels (produced primarily from food crops such as grains, sugar beet and oil seeds) are limited in their ability to achieve targets for oil-product substitution, climate change mitigation, and economic growth. How really sustainable is their production is under review, as is the possibility of creating undue competition for land and water used for food and fiber production; some biofuels have been deeply criticized in reason of:

- rising food prices;

- relatively low greenhouse gas (GHG) abatement;

- high marginal carbon abatement costs ($/t C avoided);

- essentiality of government support and subsidies to achieve economical viability;

- direct and indirect impacts on land use change and the related greenhouse gas emissions.

Not to all 1G biofuels can be pointed out the inconveniences above; sugarcane ethanol is the exception: it is already being successfully produced in several African and South American countries based on the Brazilian model. This 1G biofuel presents few of the problems identified for others and, at least where good conditions and suitable available land exist, can be cost competitive with gasoline without needing any government subsidies. Several developing countries are therefore proceeding to produce their own ethanol, driven by high oil prices and the promise of sustainable development. They do not need to wait for 2nd-generation biofuels to become commercially viable, but could benefit further when they do[2].

In this scenario, biofuels that doesn't need and edible biomass to be produced, became then the center of the scientific attention, with the hope that their technology conversion will be soon economical and for large production scale; be cost competitive with first-generation and oil-based fuels; and resolve many of the other issues often raised concerning some first-generation.
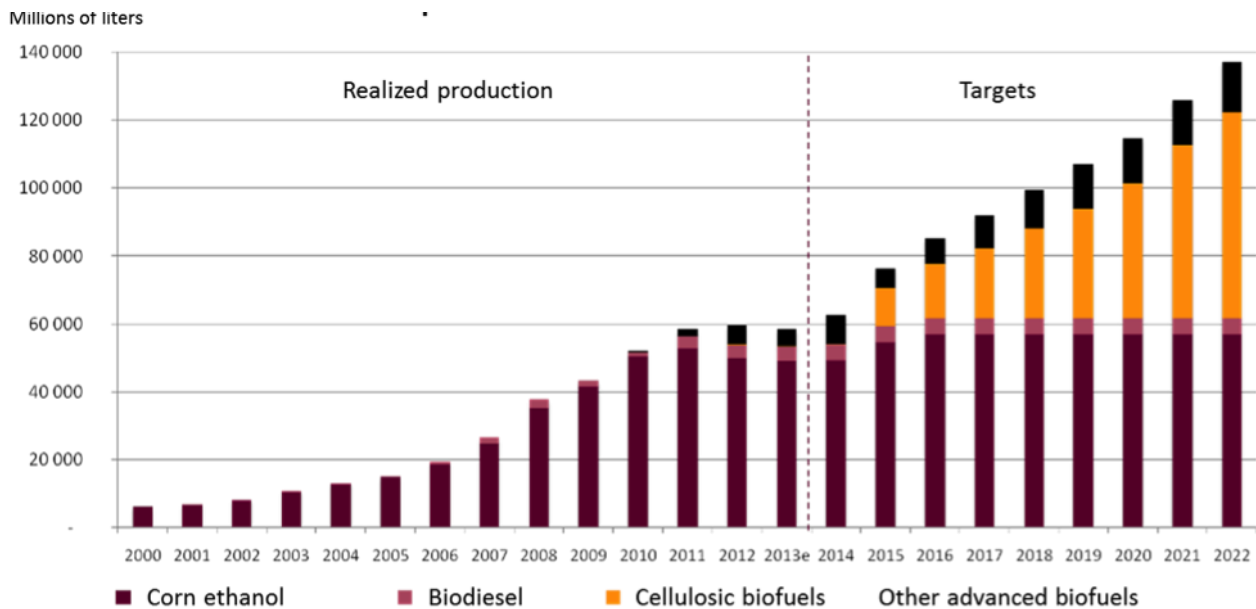

Fig 1.1 – U.S. biofuels production 2000-2013 and estimates[25].

To overcome the only rely on food crops towards the conversion of lignocellulosic biomass to bioethanol, biodiesel and aviation fuels, the necessary transition from 1G to 2G biofuels will require major technology improvements - but the pathways and timelines are unclear. It is recognized that 2G biofuels generally have several advantages over both fossil fuels and many 1G biofuels. These include reduced GHG emissions, a more positive energy balance, and better access to sustainable biomass feedstocks all year-round in order to keep the conversion plant operating and hence spread the annual overhead costs over a greater number of liters of biofuel produced[24]. The challenge for a project developer is to procure sufficient feedstock from within a reasonable transport radius of the plant over the long term.

The commercialization of second-generation biofuels will have consequences for many developing countries that are actual or potential biofuel producers, consumers and exporters. If carefully managed, development of these technologies offers the promise of sustainable development, rural revenue generation, and mitigation of the impacts of environmental changes worldwide[24].

The cumulative impacts of these concerns have increased the interest in developing biofuels produced from non-food biomass. Feedstocks from ligno-cellulosic materials include cereal straw, bagasse, forest residues, and purpose-grown energy crops such as vegetative grasses and short rotation forests. These second-generation biofuels could avoid many of the concerns facing first-generation biofuels and potentially offer greater cost reduction potential in the longer term.

1.2 – First Generation (1G) Biofuels

Bioethanol and biodiesel are considered the classic 1G biofuels and are directly related to a biomass that is more than often edible; the demand for first-generation biofuels, produced mainly from agricultural crops traditionally grown for food and animal feed purposes, has continued to increase significantly during the past few years. The main liquid and gaseous biofuels on the market today are:

1. Bioethanol - produced from sugar-containing plants or cereal (grain) crops used as a gasoline substitute mainly as blends in spark ignition engines and providing 2% of total gasoline fuel supply (although research continues on also using ethanol in compression ignition engines);

2. Biodiesel - produced from vegetable oils or animal fats, usually after conversion into a range of fatty acid methyl (or ethyl) esters, although at times consumed as untreated raw oils, which when used as a mineral diesel fuel substitute in compression ignition engines provides around 0.2% of total diesel fuel supply;

3. Biomethane - as landfill gas or biogas, produced by the anaerobic fermentation of organic wastes including animal manures. The raw gases can be scrubbed (cleaned and purified) to produce a high quality methane-rich fuel, similar to commercial natural gas. This can then be compressed and used in vehicle engines using technology proven when fuelling with compressed natural gas (CNG). Due to a lack of compatible vehicles and infrastructure, gaseous biofuels are far less popular than liquid biofuels.

All together, biofuels currently provide over 5 % of the european total transport fuels[26] and the crops grown for biomass feedstock take up less than 2% of the world's arable land (WWI, 2007). The US has become the largest producer, having recently overtaken Brazil; since it also imports large volumes, mainly ethanol from Brazil, it is also the largest consumer.

1.2.1 - Bioethanol

Ethanol is generally produced from the fermentation of C_6 sugars (mostly glucose) using classical or GMO yeast strains such as *Saccharomyces cerevisiae*. Only a few different feedstocks, mostly sugarcane or corn, are actually used for the production of first-generation bioethanol; other more marginal feedstocks that are used or considered to produce first-generation bioethanol include but are not limited to whey, barley, potato wastes, and sugarbeets. Sugarcane is a common feedstock for biofuel production, Brazil being one of the leading countries for its use.

Fig 1.2 - Sugarcane (a) is a common feedstock used for biofuel production. Once harvested (b), it can be crushed (c) and purified in a mill (d) to produce raw sugar or ethanol.

The traditional biological conversion routes for bioethanol production are well established, since the process that allows the production of ethanol out of sugarcane is rather simple; the sugarcane is crushed in water to remove sucrose, which is then purified either to produce raw sugar or ethanol. The main raw materials needing to be extracted are sucrose or starch. For sucrose from sugarcane or sugar beet crops, the juices are first mechanically pressed from the cooked biomass followed by fractionation. The sucrose is metabolized by yeast cells fermenting the hexoses and the ethanol is then recovered by distillation. Starch crops must first be hydrolyzed into glucose before the yeast cells can convert the carbohydrates into ethanol. Pre-treatment consists of milling the grains of corn, wheat or barley followed by liquefaction and fractionation. Acidic or enzymatic hydrolysis then occurs prior to fermentation of the resulting hexoses. Although highly efficient, the starch grain-based route consumes more energy (and thus potentially emits more CO_2 into the atmosphere depending on the energy sources used), than the sucrose-based route. From the fermentation process onwards, both routes are almost identical. Overall using either sugar or starch is a mature technology to which few significant improvements have been made in recent years.
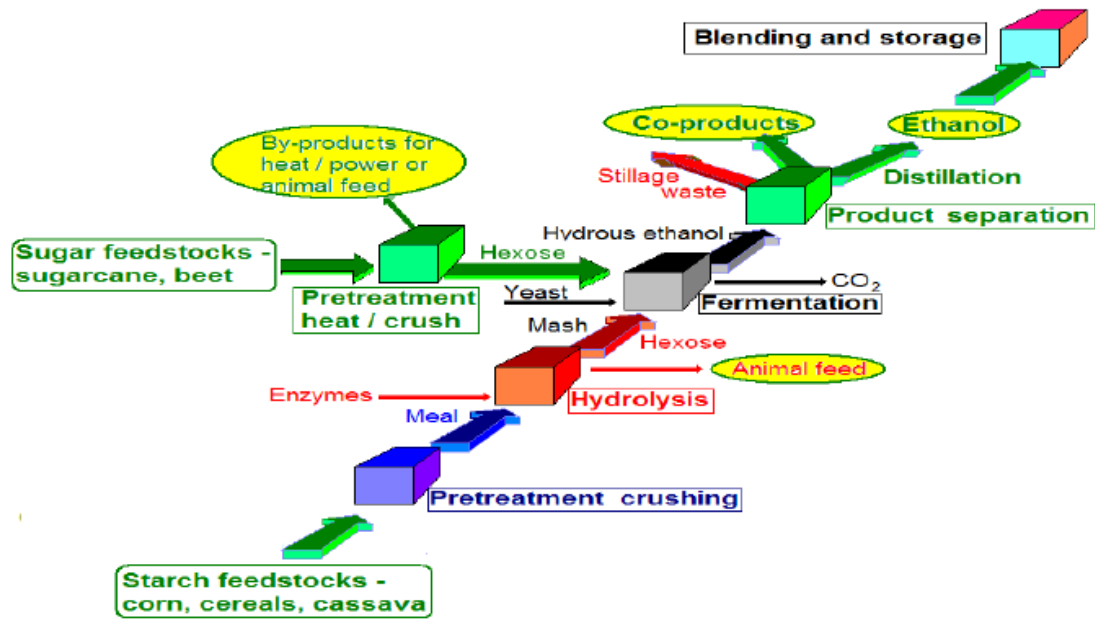
Fig. 1.3 - Conversion routes for sugar or starch feedstocks to ethanol and co-products.

Although very advantageous for the producers, increases in the sugar price are a problem for the bioethanol business. In August 2012, the price of raw sugar was close to US$0.20 per pound while the price for ethanol was US$2.59 per gallon (US$0.68/L). Production of 1 L of ethanol out of raw sugar should cost around US$0.30 to US$0.35, and therefore, the market favored production of raw sugar instead of ethanol[2].
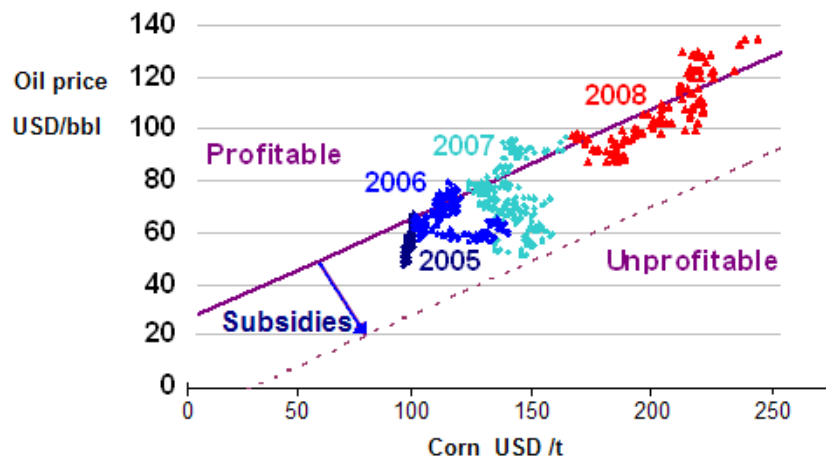

Fig. 1.4 - Corn ethanol profitability depends largely on feedstock costs, competing oil prices and government subsidies[24].

Corn is the other major source of carbohydrates for production of ethanol, although unlike sugarcane, as said before corn requires a preliminary hydrolysis of starch to liberate the sugars that can then be fermented to ethanol. The enzyme generally used for hydrolysis of starch, *a-amylase*, is rather inexpensive at US$0.04 per gallon of ethanol produced. Corn market value in August 2012 was close to US$338/t, leading to the production of 400 to 450 L of ethanol, depending on the process efficiency. Moreover, the value of the by-

products, like post-distillation spent grain used for livestock feed, is a net asset for the whole economical balance of the process.

1.2.2 - Biodiesel

Biodiesel is the only other biofuel produced on an industrial scale. The production process of this biofuel is very different from ethanol because it could be considered as a chemical process. Of course, it uses biomass (oily plants and seeds), but the process itself relies on extracting the oils and converting them into biodiesel by breaking the bonds linking the long chain fatty acids to glycerol, replacing it with methanol in a process called transesterification. A simplified version of the lipids used for the production of biodiesel is presented in Fig 1.2. Oil price on the international market varies among vegetable sources. As an example, in August 2012, soybean oil market value was US\$1,230/t while palm oil was US\$931/t. Canola oil, another common feedstock for the production of biodiesel, had a market value of US\$1,180/t. Based on the latter, it can be roughly estimated that each ton of oil will produce between 1,000 and 1,200 L of biodiesel with a market price estimated from diesel at US\$3.2077/gallon (US\$0.85/L). Production of biodiesel also requires methanol (typically between 125 and 150 L/t of oil converted) at an approximated market price of US\$0.35/L. The price for feedstock is the most crucial factor affecting biodiesel production. Therefore, use of other less expensive sources such as used oils (US\$331/ton) and oil from non-edible plants like jatropha (estimated between US\$350 and US\$500/ton) is gaining interest. Algal biomass is also considered as a source of lipids for production of biodiesel, although it is generally related to third-generation biofuels. Production of ethanol from sugarcane or corn and biodiesel from edible oils depends on the prices dictated by the international market, whereas the prices of used cooking oil and jatropha are presently not influenced by such market, which is a good incentive for their use for biodiesel production. The availability of such oils is somehow less than the classical canola or soybean oil. Moreover, residual cooking oil requires additional processes for purification, whereas production of jatropha could be limited by its low market value which would not be appealing for production on good arable land, making it economically viable only on marginal land.
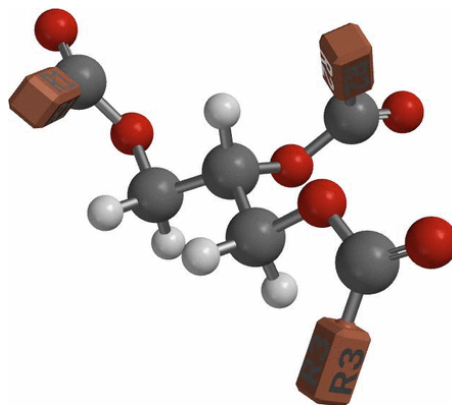

Fig 1.5 - Simplified 3D structure of the lipids (feedstock for biodiesel), where R1, R2, and R3 represent alkane chains with 5 to 17 carbons.

1.3 – Second Generation (2G) Biofuels

Second-generation biofuels are defined as fuels produced from a wide array of different feedstocks, especially but not limited to non-edible lignocellulosic biomass. Biomass used for production of second-generation biofuels is usually separated in three main categories: homogeneous, such as white wood chips with a price value of US$100 to US$120/t; quasi-homogeneous, such as agricultural and forest residues pricing between US$60 and US$80/t; and non-homogeneous, including low value feedstock as municipal solid wastes (between US$0 and US$60/ton) as reported by Lavoie et al. (2011). The price for this biomass is significantly less than the price for vegetable oil, corn, and sugarcane, which is an incentive.

On the other hand, such biomass is generally more complex to convert and its production is dependent on new technologies[2]. To be acceptable, biofuel feedstocks must be sustainably produced in terms of agricultural practices, forest management, protection of bio-diverse ecosystems, responsible and efficient use of water, and free of exploitation of landowners. For 2nd-generation they do not compete with food and fibre. Many developing countries could theoretically benefit from strategic partnerships with public and private sector organizations from both industrial countries and the more advanced developing countries such as Brazil, which have knowledge and experience in the production, distribution and consumption of biofuels[24].

1.3.1 – Biomass Feedstock

Lignocellulosic biomass is an abundant and renewable feedstock, with an estimated annual worldwide production of 10-50 billion dry tons though only a small portion of this could be utilized in practice. This includes cereal straw, wheat chaff, rice husks, corn cobs, corn stoves, sugarcane bagasse, nut shells, forest harvest residues, wood process residues. The technical potential from available annual supplies has been estimated in energy terms at over 100 EJ per year7, with costs in the range of $ 2-3/GJ annual[24].

Low-cost crop and forest residues, wood process wastes, and the organic fraction of municipal solid wastes can all be used as lignocellulosic feedstocks. Where these materials are available, it should be possible to produce biofuels with virtually no additional land requirements or impacts on food and fiber crop production. However in many regions these residue and waste feedstocks may have limited supplies, so the growing of vegetative grasses or short rotation forest crops will be necessary as supplements. Where potential energy crops can be grown on marginal and degraded land, these would not compete directly with growing food and fibre crops which require better quality arable land.

Relatively high annual energy yields from dedicated energy crops, in terms of GJ/ha/yr, can be achieved from these crops compared with many of the traditional food crops currently grown for 1st-generation biofuels. Also their yields could increase significantly over time since breeding research (including genetic modification) is at an early phase compared with the breeding of varieties of food crops. New varieties of energy crops may lead to increased yields, reduced water demand, and lower dependency on agro-chemical

inputs. In some regions where low intensity farming is currently practiced, improved management of existing crops grown on arable land could result in higher yields per hectare. This would enable energy crops to also be grown without the need for increased deforestation or reduction in food and fiber supplies.

The lignocellulosic materials are the most abundant organic compounds in the biosphere, participating in approximately 50% of the terrestrial biomass. The term lignocellulose structure is related to the part of the plant which forms the cellular wall (half-lamella, primary e secondary walls), composed of fibrous structures, basically constituted of polysaccharides: cellulose (40-60%) and hemicellulose (20-40%); these components are associated to a macromolecular structure containing aromatic substances, denominated lignin (15-25%). In a general way, it can be affirmed that those materials possess in their compositions approximately, 60-70% of polysaccharides (in a dry basis), which contain in their monomeric units valuable glycosides (sugars)[27].
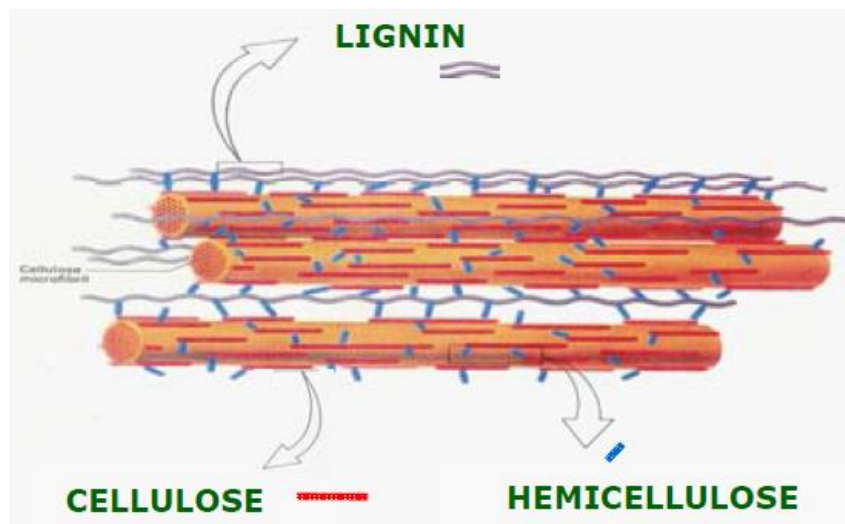

Fig. 1.6 – Lignocellulosic biomass structure.

Cellulose is a polysaccharide, polymer of D-glucose, forming chains of β-1,4 bonds, and maintaining a linear and plane structure. Cellobiose, disaccharide 4-0-(β-D-glycopyranosil-D-glucopyranose), is the repeated polymer unit. In natural celluloses, the chains are aligned in a way of forming complex organized fibrils, whether in crystalline or amorphous structures. These fibrils are established amongst them with inter and intra hydrogen bonds, which individually are weak, but collectively, they result in a great binding strength, giving to the cellulose a high resistance to the hydrolysis attack.

Hemicelluloses are closely associated with cellulose in plant tissues and together with cellulose they are the most abundant carbonic material in plants. These macromolecules, contrarily to cellulose, present heteropolysacchric nature and a considerable degree of ramification, consequently not presenting crystalline regions. They are constituted, in their great majority, of a mixture of polysaccharides with a low molecular mass, as follows: xylans, arabinans, arabinoxylans, mannans and galactomannans. The fundamental units (monomers) are, basically, molecules of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, D-

glucuronic acid, D-galacturonic acid, α-D-4-O-methylglucuronic acid and also some oxidation products, as for example, acetates. The main advantage of hemicelluloses is that, due to their highly ramified structure, they can be hydrolyzed easily using water at high temperatures or a very diluted aqueous mixture of acids. The key problem is that C5 sugars do not ferment with classical yeast strains and require genetically modified organisms to produce ethanol. Furthermore, acids (both acetic and formic) may inhibit the fermentation process, requiring an additional operation for detoxification.

CELLULOSE	HEMICELLULOSES
Consists of glucose units	Consist of various units of pentoses and hexoses
High degree of polymerization (2,000 a 18,000)	Low degree of polymerization (50 a 300)
Forms fibrous arrangement	Do not form fibrous arrangement
Presents crystalline and amorphous regions	Present only amorphous regions
Slowly attacked by diluted inorganic acid in hot conditions	Rapidly attacked by inorganic acid diluted in hot conditions
Insoluble in alkalis	Soluble in alkalis

Table 1.1 – Differences between cellulose and hemicellulose.

Lignin is a natural macromolecule composed by p-propylphenolic units with methoxyl substituents on the aromatic ring and, between these units, exist principally ether-type bounds. Lignin presents a highly complex structure, formed by polymerization of three different monomers: coumaric alcohol, coniferyl alcohol and synapyl alcohol, which differ from one another by possessing different substituents in their aromatic ring. This structure is also responsible for the hardness of the cell wall, constituting in a binding material ("glue-like substance"), which holds the cellulosic fibers. Lignin possesses high molecular mass and presents about 25% of the photosynthesis biomass produced yearly on earth, retaining 50% more carbon than cellulose. The macromolecule is highly energetic and has been used for cogeneration or as a fuel by the pulp and paper industry.

1.3.2 – Conversion to 2G Ethanol

The production of biofuels from ligno-cellulosic feedstocks can be achieved through two very different processing routes. They are:

- Biochemical – in which enzymes and other micro-organisms are used to convert cellulose and hemicellulose components of the feedstocks to sugars prior to their fermentation to produceethanol;

- Thermochemical – where pyrolysis/gasification technologies produce a synthesis gas ($CO + H_2$) from which a wide range of long carbon chain biofuels, such as synthetic diesel or aviation fuel, can be reformed.
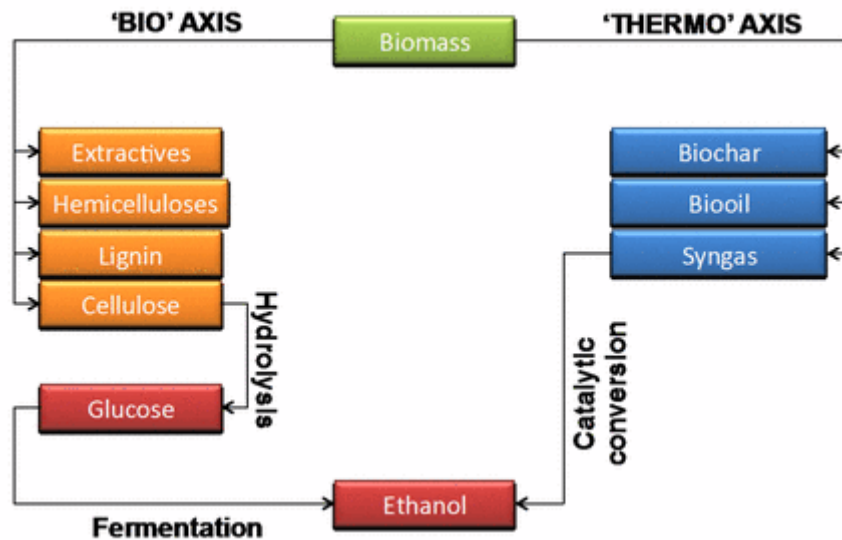
Fig 1.5 - Simplified scheme for the "bio" and "thermo" pathways for conversion of lignocellulosic biomass into biofuels.

These are not the only 2nd generation biofuels pathways, and several variations and alternatives are under evaluation in research laboratories and pilot-plants. They can produce biofuel products either similar to those produced from the two main routes or several other types including dimethylether, methanol, or synthetic natural gas. However, at this stage these alternatives do not represent the main thrust of RD&D investment.

In this work, we will focus on the "bio axis" to show the conversion into ethanol of a lignocellulosic feedstock such as spelt straw harvested from the region of Garfagnana, Italy.

In principle, the key steps involved are similar to those for current ethanol production that converts grain starch to ethanol. However, each step presents a significant technical challenge when lignocellulosic feedstocks are used, because:

- the strong bonds in lignocellulosic feedstock require pre-treatment so that the polysaccharides can be accessed for conversion;

- cellulose, unlike starch, is not hydrolyzed by conventional enzymes and requires the application of sophisticated (expensive) cellulase enzymes;

- novel micro-organisms are required to ferment the xylose sugars extracted from the hemicelluloses since common yeasts will not work;

It is only with recent advancements in biotechnology that improved micro-organisms and enzymes have been designed with sufficient activity for commercial cellulose hydrolysis to be considered credible.

Bio-chemical conversion uses biological agents, specifically enzymes or micro-organisms, to carry out a structured deconstruction of the ligno-cellulose into its base polymers and to further break down cellulose and hemicellulose into monomeric sugars including glucose and xylose.

These sugars can then be fermented into ethanol. Feedstocks are based upon agricultural and forest biomass (either residues or dedicated crops) but could also include the potential recovery of biomass from urban municipal solid waste (MSW) streams.

The bio-chemical platform consists of three main process elements:

1. pretreatment

2. enzymatic hydrolysis

3. fermentation

The effectiveness of the first 2 phases is currently the main subject of research to make 2G technologies ready to commercial take-off. Process steps also include feedstock harvesting, handling, recovery and transport; comminution of the biomass to give small and homogeneous particles; fractionation of the polymers; separation of the solid lignin component; and end product recovery. The cellulose undergoes enzymatic hydrolysis to produce hexoses such as glucose. Pentoses, mainly xylose, are produced from the hemicellulose, thereby fully utilizing the feedstock.
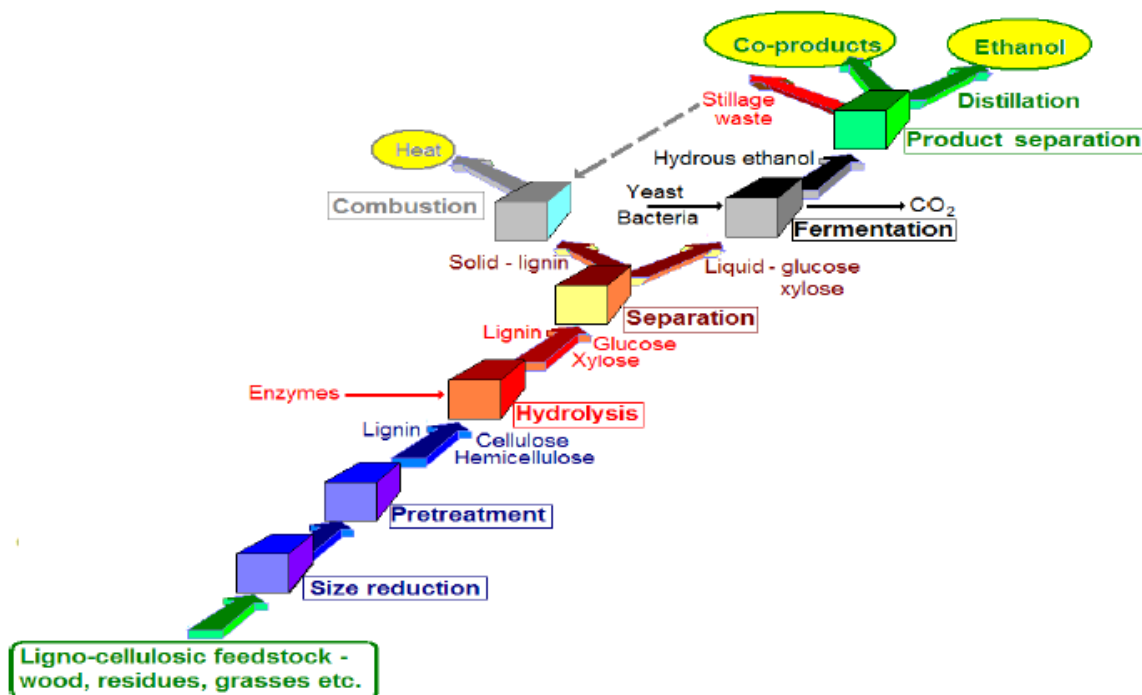

Fig. 1.6 - Ethanol production from lignocellulosic biomass via the bio-chemical route.

1.3.2.1 – Pretreatment

To make possible the use of lignocellulosic materials as feedstocks for the production of ethanol and other chemicals following the biochemical platform, it is necessary to separate their main components. For this separation, a pretreatment stage is essential, which aims at fundamentally disorganizing the lignocellulosic

complex. The pretreatment can be realized through physical, physical-chemical, chemical or biological processes, and can be either associated or followed by hydrolysis procedures of the polysaccharides (hemicellulose and cellulose) in their respective monomeric units (pentoses and hexoses).

Designed to optimise the biomass feedstock for further processing, the aim of a pretreatment process is to expose cellulose and hemicellulose for subsequent enzymatic hydrolysis, and is one of the most critical process steps. The key performance goals for pretreatment technologies and processes are to:

- ✓ maximise the yields of both hexose and pentose sugars in downstream processing;

- ✓ facilitate the recovery of lignin for later combustion;

- ✓ minimise the production of chemicals that inhibit downstream enzymatic processing such as furfural and hydroxymethyl furfurals;

- ✓ be flexible enough to cope with varying ligno-cellulosic feedstocks;

- ✓ avoid expensive biomass comminution (size reduction by grinding, milling, pulverising etc.);

- ✓ utilise low cost chemicals and minimise waste streams;

- ✓ have low energy requirements and low capital costs.

Due to the strong bonds of the lignocellulosic structure, pretreatment processes are generally severe and represent a significant cost element of the whole pathway. So optimization of this process is important. The aim is to open up the cellular structure of the plant material so that the cellulose is exposed to the enzymes that can then start hydrolyzing the polysaccharide polymers into sugars.

The more appropriate visual demonstration may be shown as follow, i.e. after pretreatment, the hemicellulose, some soluble lignin, and some cellulose in amorphous regions are extracted or removed, leaving the lignocellulosic matrix with somewhat alteration.
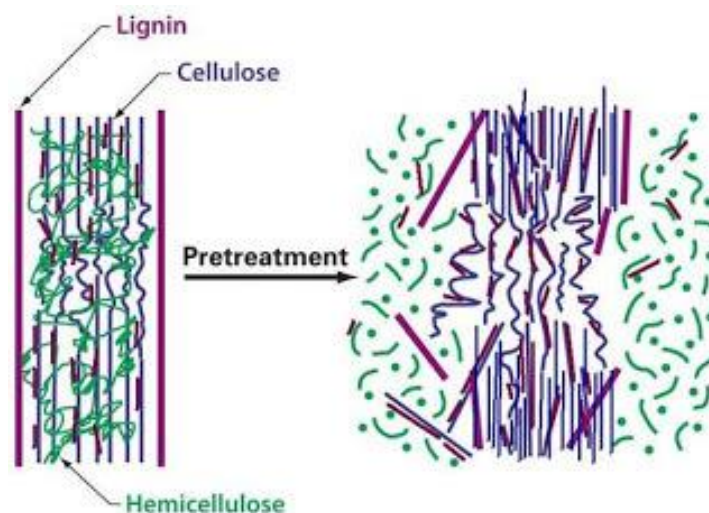
Fig. 1.7 – Effects of pretreatment.

The key for the pretreatment is to open channels/pores due to the removal of these chemical components and somewhat alteration or dislocation of the cell wall structure[23]. As a result, it allow enzymes or chemicals more easily to transport into cell structure and function. No doubt, a relatively severe treatment causes more damage to the cell wall structure. The question is how much more degradation occurs for removed hemicellulose and cellulose (monomer sugars).

In sugar and starch based processes, pretreatment can be as simple as pressing sugarcane or sugar beet to extract sucrose, or shucking corn cobs in order to separate the starch-rich kernels for further processing. When dealing with lignocellulosic feedstocks however the pretreatment step uses techniques analogous to traditional mechanical pulp processing where the lignin is either softened to release individual cellulosic fibers and/or removed to create a low-lignin pulp. Traditional mechanical pulping isolates fibers through a physical refining process which leaves much of the intact lignin present in the resulting pulp.

The pretreatments can be classified as biological, physical, chemical or a combination.

- Biological pretreatment typically utilise wood-degrading fungi to modify the chemical composition of the feedstock. In essence, soft and brown rot fungi primarily degrade the hemicellulose while white-rot fungi more actively attack the lignin. A major disadvantage of biological/fungal treatments is the typical residence time of 10-14 days, which reduces the commercial potential of these approaches. For these reasons, biological pretreatments are considered to be less attractive commercially.

- Physical pretreatment involves mechanical breakdown of the biomass feedstock by hammer- or ball-milling into smaller particles to increase the rate of subsequent enzymatic hydrolysis. Physical treatments improve hydrolysis yields by disrupting cellulose crystallinity and increasing the specific surface area of the feedstock rendering it more accessible to attack by cellulases (Mais et al. 2002). Physical pretreatment is relatively insensitive to the differing physical and chemical characteristics of the biomass but the processes do not result in lignin removal. Hence the ability of cellulases to access the cellulose that remains is limited and inhibited (Berlin et al., 2006). Furthermore, the high energy inputs required mean that physical pretreatment has yet to be proven economically viable at the commercial scale.

- Chemical pretreatment pulping processes in commercial use today involve the removal of lignin to produce pulp for various paper products. Although these processes could be considered as potential pretreatment methods they are optimized to maintain the fiber/strength integrity of the pulp, rather than to increase accessibility to the cellulose. The relatively high value of pulp can justify the high capital and operating costs of chemical pulping, whereas lower-value biofuels must seek cheaper pretreatment options. Acid and alkali elements as used in pretreatment that have been assessed to

date have had the primary goal of enhancing enzyme accessibility to the cellulose by solubilizing the hemicellulose and lignin, and to a lesser degree decreasing the degree of polymerization and crystallinity of the cellulosic component. Pretreatments that reduce cellulose crystallinity include mild swelling agents such as NaOH, hydrazine and anhydrous ammonia, and extreme swelling agents such as sulphuric or hydrochloric acid (Wood & Saddler, 1988).

- <u>Combination pretreatment</u> using physio-chemical processes have received considerable attention in recent years. AFEX, *organosolv* pulping and steam pretreatments are attractive because of their ability to generate readily hydrolysable substrates from the lignocellulosic biomass. Treatments that reduce the lignin content of the substrate include organosolv pulping, where the pulping liquor used could be one of a range of organic solvents including ethanol, glycerol, or ethylene glycol.

Several aspects that affect the viability of the overall process include the handling and preparation of the feedstock prior to the pretreatment step, the need to minimise processing costs, and the need to maximise the value of co-products derived from the hemicellulose and lignin streams. For example, if a process has a requirement for very fine, uniform feedstock with a maximum particle size of less than 10 mm, this will have a significant impact on the overall economic viability of the overall process because of the energy requirements to produce this fine material (Wooley et al. 1999).

Various pretreatments have been shown to be better suited for specific feedstocks. For example, alkaline-based pretreatment methods such as lime, can effectively reduce the lignin content of agricultural residues but are less satisfactory for processing recalcitrant substrates such as softwoods (Chandra et al., 2007). Acid-based pretreatment processes have been shown to be effective on a range of lignocellulosic substrates, but are relatively expensive and not suitable for all feedstocks (Mosier et al., 2005).

Pretreatment Type	Advantages	Disadvantages
Dilute Acid	The hemicellulose fraction is hydrolyzed into pentose sugars and the downstream hydrolysis of the cellulose is improved.	Requires expensive reactor components and downstream acid neutralization which increase capital costs. High temperatures can degrade sugars, resulting in lower yields and elements toxic to fermentation organisms. Can be addressed with two temperature process, or use counter-current reactor.
Concentrated Acid	Allows lower temperature. Reactions that reduce degradation of sugars are reduced.	Expensive. Requires acid to be recovered and re-used to become more competitive.

Steam Explosion	Well known route (for fiberboard), works well for hardwoods. Hemicellulose partially hydrolyzed. Adding dilute acid can raise pentose sugar yields.	Requires after-wash to remove degraded components, leading to loss of solubilized sugars. Some inhibitory by-products possible.
Liquid Hot Water	May require little feedstock reduction. Dissolves hemicellulose and makes cellulose fraction receptive to hydrolysis.	As with steam explosion, some degraded products formed that are toxic to fermentation organisms. Still at development stage.
AFEX	Similar to steam explosion, except using ammonia, but hemicellulose fraction is not significantly solubilized to sugars. Lignin-hemicellulose bonds are ruptured and some hydrolysis of the hemicellulose. Greatly reduced production of degraded products that are toxic to downstream fermentation microorganisms. Ultimate sugar yields could be high.	Significant capital costs due to need to capture and recycle the ammonia.

Tab. 1.2 – Main characteristics of the various types of pretreatments.

The comprehension of the mechanisms involved in the pretreatment processes is still not completely elucidated and the project of those processes is normally made on empirical basis. It can be affirmed that exist different levels of importance of the involved mechanism in function of the different pretreatment processes. In the lignocellulosic materials found in the nature, the cellulose is intimately associated with the hemicellulose and with other structural macromolecules, being the microfibrils rich in carbohydrates involved with a kind of "glue" constituted of lignin (Lynd, 1996). Clearly, the primary function of thepretreatment is to open up the multi-component matrix, in the way of turning cellulose more accessible to the biocatalysts, as described previously.

In this way, several factors assume outstanding importance, according to the following summary:

- Time vs Temperature: in a first moment, it is possible to believe that, as higher the temperature is, as greater is the hydrolysis efficiency. Nevertheless, higher temperatures can lead to the degradation of

free sugars to furfurals (furfural from pentoses and hydroxymethyl furfural from hexoses), reducing the efficiency of the pretreatment and increasing the toxicity of the hydrolysate. On the other hand, even with lower temperatures, the same thing can take place when the time of exhibition is very long. In a general way, higher temperatures implicate in shorter times and vice versa (Mc Millan, 1994; Mosier et al., 2005). Additionally, it should be noticed that the greater the extension of the prehydrolysis step, the greater the cost is with heat to maintain the temperature and the slower the overall process.

- Size of the particles: generally, the pretreatment efficiency when using processes with diluted acid is increased in systems with particles of smaller size. The size of particles affects the available surface area for further actuation of the hydrolysis agent, as well as the cellulose crystallinity and its degree of polymerization. On the contrary, when steam-explosion is used, especially with cane bagasse, it is more convenient to work with particles of bigger size (Lynd, 1996).

- Structure of the cellulose molecule: the crystallinity and the polymeration degree of the cellulose molecules are closely associated to its reactivity. Greater efficiencies are reached with a lower degree of polymerization and crystallinity indexes (Mc Millan, 1994; Mosier et al., 2005).

- Acidity of the medium: the proton concentration has an important role for the chemical reactions involved in the pretreatment stage. Even though the technology does not involve the addition of acid to the process, with the use of high temperatures the medium acidity is favoured by the releasing of endogenous organic acids, principally acetic acid, as well as by the protonation resulting from the water dissociation (Lynd, 1996; Mosier et al., 2005).

- Humidity: The role of the water in the pretreatment processes should not be underestimated. The presence of water decreases the temperature of lignin degradation, facilitating its removal from the fibers. Moreover, the glycosidic linkages in the cellulose as well as in the hemicellulose are cleaved by hydrolysis, and in case of the hemicelulose - the most susceptible fraction to the hydrolysis - partial desacetylation and depolymerization take place. Similarly, the lignin also suffers from partial depolymerization. The cleavage of the glycosidic bonds can be incremented with the increase of the water deprotonation constant in high temperatures, what causes a drop in the medium pH. For example, at 220 °C, the medium pH reach 5.6, while at room temperature the value is equal to 7.0. Due to this effect, it is believed that, in high temperatures, the role of the water is more relevant than the role performed by the free organic acids (Lynd, 1996)

Finally, it should not be neglected that the efficiency of the pretreatment processes is a result of the synergism between temperature, time and medium acidity. The combination of this factors defines the parameter "degree of severity", which is intrinsically associated with the toxicity and fermentability of the

hydrolyzate. Generally, there exists an optimum degree of severity, under which the hydrolysis efficiency will be lower, and above which there will be degradation of sugars and formation of other derivative inhibitors from lignin.

In this work, the liquid hot water pretreatment will be fine-tuned to work with our lignocellulosic biomass feedstock.

The liquid hot water (LHW) treatments are also called hot compressed water treatments, hydrothermolysis, aqueous or steam/aqueous fractionation, uncatalyzed solvolysis, and aquasolv. LHW is based on the use of pressure to keep water in the liquid state at elevated temperatures (160–240 °C). This process changes the biomass native structure by the removal of its hemicellulose content alongside transformations of the lignin structure, which make the cellulose more accessible to the further enzymatic hydrolysis step. Differently from steam-explosion treatment, LHW does not use rapid decompression and does not employ catalysts or chemicals. Nevertheless, as with the acid treatment, LHW depolymerizes hemicelluloses to the liquid fraction. In this case, sugars are removed mostly as oligosaccharides, and the formation of the inhibitors furfural and 5-hydroxymethyfurfural (HMF) is at a slightly lower level, depending on the process conditions. To avoid the formation of inhibitors, the pH should be kept at between 4 and 7 during the pretreatment, because at this pH, hemicellulosic sugars are retained in oligomeric form, and monomer for- mation is minimized. The removal of hemicellulose also results in the formation of acetic acid in the liquid fraction.

Fig 1.8 – Various sizes of LCW batch reactors.

LHW pretreatment, whose most important parameters are the biomass moisture content, the operation temperature, and the residence time, is usually done in a pressure tank reactor where two streams can be obtained after filtration of the biomass slurry: a solid, cellulose-enriched fraction and a liquid fraction rich in hemicellulose-derived sugars. The solid phase is therefore constituted by cellulose and lignin along with residual hemicellulose. There are three types of rector design for LHW pretreatment.

For co-current reactors, the biomass liquid slurry passes through heat exchangers where it is heated to the appropriate temperature (140–180 °C) and kept for 10–15 minutes as the slurry passes through an insulated plug-flow snake-coil, followed by the slurry-cooling concomitant to heat recovery via the countercurrent

heat exchange with the incoming slurry. Flow-through technologies pass hot water at 180–220 °C and approximately 350–400 psig. The resulting pretreated biomass has enhanced digestibility and a significant portion of the lignin is also removed. In counter-current pretreatment, the biomass slurry is passed in one direction while water is passed in another in a jacketed pretreatment reactor. Temperatures, back pressures, and residence times are similar. In the flow-through pretreatment reactor, water or acid is passed over a stationary bed, and removes some of the biomass components including lignin. Although LHW can result in the partial depolymerization and solubilization of lignin, the re-condensation of lignin-derived, soluble compounds is also observed. Flow-through systems have been reported to be more efficient in terms of hemicellulose and lignin removal in comparison to batch systems for some types of biomass via the addition of external acid during the flow-through process.

Several works have reported about the optimal LHW pretreatment conditions in terms of temperature and residence time. For the pretreatment of corn stover the best conditions were reported to be 190 °C and 15 min, resulting in a 90% of cellulose conversion after enzymatic hydrolysis, while for wheat straw the optimum treatment temperature was found to be 188 °C during 40 min, which resulted in 79.8% of cellulose conversion and releasing of 43.6% of hemicellulose derived sugars to the liquid fraction. Nonetheless, when response variables were analyzed separately, the best conditions for the recovery of hemicellulose-derived sugars from wheat straw, at up to 71.2%, were found at 184 °C during 24 min, whereas the optimal conditions for a cellulose conversion of 90.6% were found to be 214 °C during 2.7 min.

Before the hydrothermal, the biomass can undergo a mechanichal treatment that prepares his structure; Mechanical pretreatments of biomass aim primarily to increase the surface area by reducing the feedstock particle size, combined with defibrilization or reduction in the crystallinity degree. This approach facilitates the accessibility of enzymes to the substrate, increasing saccharification rates and yields. The most studied biomass mechanical pretreatment for biomass is the milling process, mainly the ball-milling and disk-milling pretreatments. Another mechanical treatment to be considered is extrusion, even though this process involves additional thermal and/or chemical pretreatments.
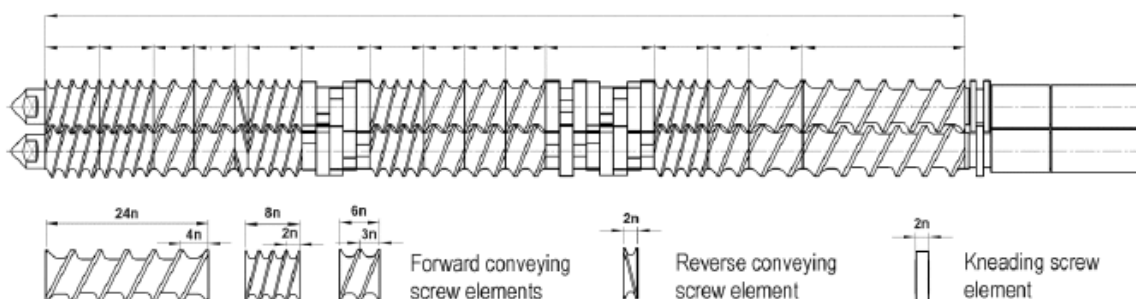

Fig 1.9 – Twin-screw extruder

The ball milling process uses mechanical shear stress and impaction to produce powdered material. This process can be done in the wet or dry state. A combination of chemicals, such as acids, bases and organic

solvents, can also be applied depending on the main treatment purpose. In general, the process uses a rotary drum and balls of different sizes made from different materials (tungsten, ceramic or stainless steel). The effect of ball milling on the biomass particle size, structure and crystalline degree depends on the rotation speed, operation time and ball size. Ball milling treatment can be considered a kind of ultra-fine grinding and fibrous materials can present between 10-20 µm in terms of particle size. For biomass pretreatment after the material is fed into the rotary drum equipped with balls, drum rotation around a horizontal axis causes a reduction on the material particle size. The ball milling process can drastically alter the complex heterogeneous network structure of wood cell walls, and with a long pretreatment time, cellulose crystallinity can be significantly reduced, which increases the ratio of amorphous cellulose, thus improving the saccharification yields.

Wet disk milling is a recently introduced biomass pretreatment process able to produce milled biomass with low levels of inhibitors; it is considered to be feasible for industrial implementation. This technique has been shown to increase the degree of biomass fibrillation and the nanospace between the micro fibrils, thus promoting the accessibility of the cellulolytic enzyme pool to cellulose. The disk mill is a type of crusher that can be used to grind, cut, shear, fiberize, pulverize, granulate or blend. In general, the suspended material is fed between op- posing disks or plates that can be grooved, serrated or spiked. The force applied in the material will depend on the type of disks, the distance between the disks and its rotation speed.

Screw extruders were originally designed to extrude polymers and were also developed for food and feed processing. An extruder can provide many functions, such as cooking, forming, kneading, degassing, dehydration, expansion, homogenization, mixing, sterilization, shaping, densification and shearing. These functions can be performed in the same process, depending on the size of the extruder and the screw design. Since the 1990s, there has been an increase in the number of studies that use extrusion for biomass processing, such as for the extraction of compounds, densification and biomass pretreatment for enzymatic saccharification. For lignocellulosic biomass pretreatment, extrusion processing can provide a unique continuous reactor working at higher throughput and solid levels. The extrusion equipment provides temperature control and efficient pulverization by applying a high shearing force. This process also allows the advantageous simultaneous combination of thermo-mechanical and chemical pretreatment.

The extruder consists of a barrel with a rotating screw (or screws) that squeezes and conveys the material continuously from the input to the output. The barrel is normally segmented, which allows temperature control (heating or cooling) along its length and feeding ports for additive injection.

1.3.2.2 – Enzymatic Hydrolysis

Cellulose, a bioorganic linear polymer and the most abundant renewable resource, is composed of D-glucose monomer units joined by β-1,4-glycosidic bonds. Native cellulose is built from several thousands (~10,000)

of β-anhydroglucose residues to form a long linear chain molecule and that explains why its molecular weight is above 1.5 million[29].

Cellulose dissolution involves disengaging the inter-chain hydrogen bonding between layers of cellulose chains thereby making the hydroxyl (OH) on each of the glucose units available for bonding with the component of the dissolving solvent. The dissolution is preceded by swelling of the cellulose chain thereby facilitating accessibility of the degradative agent in breaking apart the inter-chain hydrogen bonding within the crystalline structure. The dissolved cellulose can be further converted to lower molecular compounds such as the oligomers and fermentable sugars. The reaction of degradating cellulose into glucose can be summarized in:
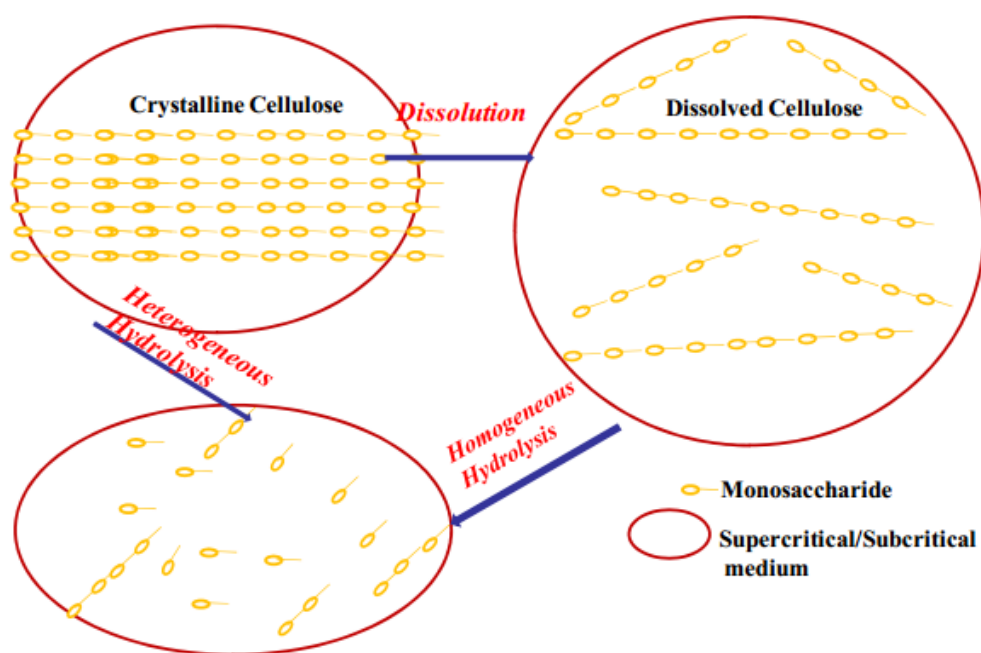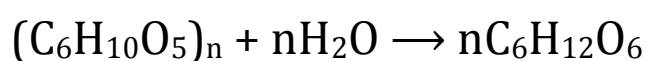
$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6$$

Fig. 1.10 - Schematic diagram of crystalline cellulose dissolution and hydrolysis[29].

The option for the enzymatic hydrolysis of the cellulose comes from the absence of severe conditions, typically of the chemical hydrolysis. This technological strategy differs from the conception of old processes in which the chemical hydrolysis of cellulose and hemicellulose (polysaccharides with different susceptibilities to the hydrolytic attack) was taking in one step. These processes generated hydrolyzates with high toxicity, which hindered the metabolism of the microorganisms agents of the fermentative processes. Nevertheless, the cellulose chemical hydrolysis has been left behind and substituted by the enzymatic hydrolysis[28].

Enzymatic hydrolysis of starch, as used in 1G ethanol production, requires a single family of amylases. By comparison, the challenge is much more significant for ligno-cellulose since the effective hydrolysis of the interconnected matrix of cellulose, hemicellulose and lignin requires a number of cellulases, those most commonly used being produced by wood-rot fungi such as *Trichoderma*, *Penicillum*, and *Aspergillus*.

Commercial cellulase preparations are typically deficient in *β-glucosidase* so require supplementing in order to relieve product inhibition caused by accumulation of cellobiose and other soluble sugars (Tengborg et al. 2001). This adds to the already substantial cost for enzymes. Although the cost of commercial cellulase preparations has been reduced by up to 20-fold in recent years, enzyme costs are still an obstacle to full-scale process commercialization.

1.3.2.3 – Fermentation to Ethanol

The ethanol production technologies of sugary (sugar cane juice) and starchy (corn) feedstocks are commercially established and carried out by the yeast *Saccharomyces cerevisiae*. However, as described before, the hydrolyzates of the polysaccharides of the lignocellulosic materials possess a mixture of hexoses (mainly glucose) and pentoses (mainly xylose), being the naturally-occuring strains of *Saccharomyces cerevisiae* unable to metabolizing xylose.

The fermentation of glucose occurs primarily when the glucose concentration is high or when oxygen is not available. The cells attain a maximum specific growth rate of about 0.45 hr^{-1} with a low biomass yield of 0,15 g dry mass per gram glucose consumed and a high respiratory quotient (the ratio of CO_2 production rate to O_2 consumption rate), resulting in a low energy yield of only about 2 ATP molecules per mole of glucose metabolized[28]. The stoichiometry of this reaction is:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \varepsilon$$

The conversion of glucose to ethanol during fermentation of the enzymatic hydrolysate is not difficult provided there is an absence of inhibitory substances such as furfural, hydroxyl-methyl-furfural, or natural wood derived inhibitors such as resin acids (Weil et al., 2002). For more than 20 years, research activities have been directed towards the development of improved micro-organisms for the fermentation of the pentose sugars. For cost effective processing, such organisms must be able to co-ferment both glucose and xylose streams together. Although C5 pentoses are generally more difficult to ferment, new yeast strains are being developed that can effectively use these sugars. Currently, there are no known natural organisms that have the ability to convert both these C6 and C5 sugars at high yields.

Even though certain naturally-occurring yeasts, as for example: *Pichia stipitis*, *Pichia segobiensis*, *Candida tenius*, (Toivola et al., 1984) are capable to ferment xylose to ethanol, the production rates are more reduced, compared to those of the alcoholic fermentation of glucose.

Aiming at the integration of these two processes (xylose and glucose fermentations), researches have basically been developed with two approaches. In the first, it is searched for the construction of a recombinant with additional hability to ferment xylose, inserting genes which codify for xylose transport and metabolim and in the second, it is aimed at increasing the ethanol yield through the Genetic Engineering in microrganisms which already possess the hability to ferment hexoses and pentoses.

1.3.2.4 – Resume of Technological Challenges in 2G Ethanol Production

The lignocellulosic materials, especially the residues of the agroindustry, have been object of intensive researches all over the world because they are renewable feedstocks of carbon and energy available in great quantities. The integral and rational utilization of these abundant feedstocks can revolutionize a series of industrial segments, such as the liquid fuels, the food/fodder and the chemical supplies, bringing immeasurable benefits for countries with great territorial extensions and with high productivity of biomass. In spite of the great potential of this residual biomass of lignocellulosic composition (50-70% carbohydrates) for the production of fuels and chemicals, the majority of it is burnt in sugar mills and alcohol distilleries for energy generation, and a smaller fraction is used for animal feeding, yet there still have surpluses (Zanin et al., 2000).

The effective utilization of the lignocellulosic materials in biological/fermentative processes faces us two principal challenges: the crystalline structure of the cellulose, highly resistant to the hydrolysis and the lignin-cellulose association, which forms a physical barrier which hinders the enzymatic access to the cellulose fibers. Additionally, the cellulose acid hydrolysis presents the inconvenient of requiring the use of high temperatures and pressures, leading to the destruction of part of the carbohydrates (sugars) and the generation of toxic substances, derived from lignin partial degradation (Jacobsen & Wyman, 2000). On the other hand, the enzymatic saccharification requires the use of physical (grinding, heating, and irradiation) or chemical (sulphuric acid, phosporic acid, alkalis) pretreatments, to reach viable yields.

To make possible that the ethanol production technology from lignocellulosic biomass can be implemented industrially, the following aspects should be focused[28]:

1. Development of pretreatment technologies which should be efficient and do not generate toxic substances that can hinder the alcoholic fermentation, neither should require onerous high pressure equipments;

2. Combination of cellulose enzymatic conversion with alcoholic fermentation to maintain low levels of sugars, resulting in improvements of the enzymatic conversion rates due to the

minimization of enzymes inhibition by their final hydrolysis products (cellobiose and glucose);

3. Construction of "optimum" microorganisms, through Molecular Biology, for an efficient fermentation of pentoses and hexoses;

4. Development of cellulase production processes by submerged and solid state fermentations (with natural-occurring or recombinant microorganisms), as well as to develop a deep knowledge about their structures and properties in order to formulate an enzymatic preparation (product engineering) for an efficient hydrolysis of cellulose;

5. The industrial production of cellulases should be in plant as for reducing the inherent costs with enzymes in the process;

6. Incorporation of reduced temperatures for ethanol separation to allow the enzyme recycling without thermal denaturation;

7. Realization of a detailed study of process integration (mass and energy), including all the streams, be they of the process or utilities, in order to favour the input/output ratio of energy;

8. Realization of a detailed technical-economic evaluation of the process viability for the utilization of agricultural and agroindustrial residues, including the logistic issues.