PRODUCTION OF BIOETHANOL FROM LIGNOCELLULOSIC BIOMASS

1.ABSTRACT

One of the most urgent needs of our time is the development of new and cleaner energy sources in order to reduce the use of polluting and unrenewable fossil fuels responsible for the “greenhouse effect”, the main cause of the current global warming trend. The purpose of this review is to expose the high potential of second generation bioethanol as a more environmentally friendly substitute for traditional fossil-derived fuels. The work will focus on the production process, explaining the technologies which are currently available, analyzing the critical issues and exposing the most promising future developments. Particular attention will be paid to the pretreatment processes -necessitated by the high recalcitrance of lignocellulosic feedstocks- also taking into account the collateral production of inhibitory compounds detrimental to the following enzymatic hydrolysis and fermentation steps.

2.INTRODUCTION

Ethanol for use as biofuel is mainly produced by fermentation from yeasts or bacteria which metabolize sugars in oxygen-lean conditions converting them to ethanol and carbon dioxide.[1] Bioethanol can be used in many ways: it can be added to petrol in different percentages -until a maximum of 30%- without having to modify the engine; it can be used as a pure fuel for dedicated engines; it can be used in gasoline as an octane enhancer and in bioethanol-biodiesel blends as an additive capable of reducing pollutant emissions and improving air quality, thanks to a lower release of un-burnt or partly oxidized compounds.[2]

Bioethanol can be produced using different feedstocks. First generation bioethanol is produced using sugar-based plants -the major world producers Brasil and the USA mainly exploit corn and sugarcane. Since these are food plants, the recent aim is to replace them with non-food plants or wastes in order to make the use of biofuel not competitive for the exploitation of the environment. Second generation bioethanol is

produced from lignocellulosic raw materials, whereas third generation bioethanol is made from algae but it is still in early stage of investigation.[1]

A lot of researches are being made to implement the production of bioethanol from lignocellulosic biomass: the primary obstacle is the absence of a low-cost technology for overcoming the recalcitrance of these materials, due to the rigid and compact structure of plant cell wall. Several factors affect the accessibility of biomass cellulose, including biomass structure-relevant factors (pore size and volume, particle size, and specific surface area), chemical composition (lignin, hemicelluloses, and acetyl group), and cellulose structure-relevant factors (cellulose crystallinity and degree of polymerization).[3]

3.LIGNOCELLULOSIC BIOMASS

3.1.Lignocellulose Composition

Lignocellulose is the principal component of the plant cell wall and it is mainly composed of cellulose, hemicellulose, lignin, extractives and inorganic materials, the composition of each varying depending on the origin of the lignocellulosic material.[4]

Cellulose is a linear syndiotactic homopolymer composed of D-anhydroglucopyranose units linked together by β 1-4-glycosidic bonds; taking the dimer cellobiose as the basic unit, cellulose can be considered as an isotactic polymer of cellobiose. In native cellulose the degree of polymerization can be as high as 15000, and the individual molecules form microfibrils stabilized by hydrogen bonds, thus giving cellulose a crystalline structure and making it difficult to hydrolize. There are also some amorphous regions which alternate with crystalline regions.[5][6] Cellulose fibrils are attached to each other by hemicellulose and other polymers such as pectin and are covered by lignin.[7]

Hemicellulose is a branched heteropolysaccharide, has a low degree of polymerization and is easy to hydrolize. It is mainly composed of pentoses -like xylose and arabinose, predominant in hardwoods and annual plants- and hexoses -like mannose, glucose, galactose, predominant in softwoods.[7]

Lignin is a heterogeneous polycrystalline reticulated polymer which belongs to the polyphenol compounds, a kind of polymers consisting of phenylpropane structural units linked through carbon–carbon and ether linkages: it adds compressive strength and stiffness to the cell wall and confers resistance against microbial attack.[8]

Wood extractives are a heterogeneous group of compounds that can be extracted with polar or non-polar solvents: they consist of terpenes, fats, waxes, phenolics, and their content and composition vary among species, location and season.

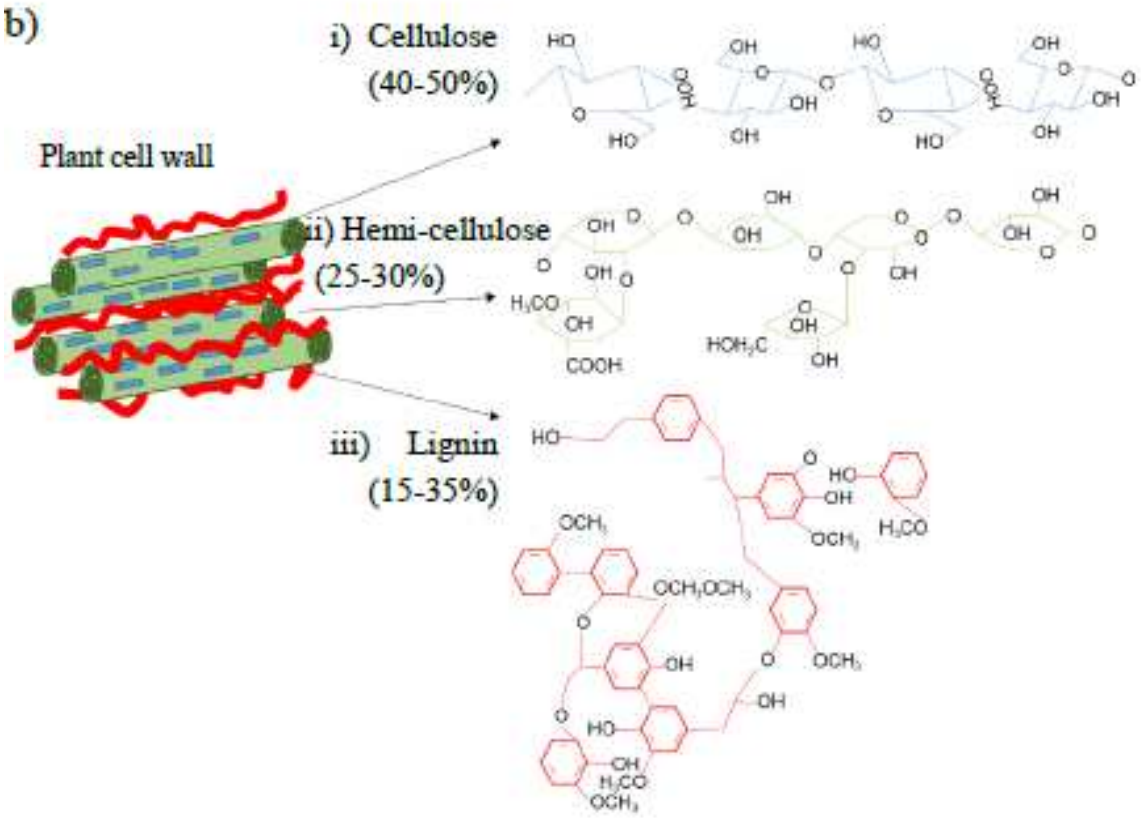

Figure 3.1. Main Components of Lignocellulosic Biomass [9]

3.2. Different sources of lignocellulosic biomass [1][10]

Available biomass can be broadly classified into virgin biomass, energy crops and waste biomass. Virgin biomass includes all naturally occurring plants such as trees, bushes and grass. Energy crops are crops with high yield of lignocellulosic biomass produced to serve as a raw material for biofuel production. Waste biomass includes municipal waste and low value byproducts of various industrial sectors, agriculture and forestry.

The organic fraction of municipal solid waste is an inexpensive source of biomass. Agroindustrial biomass residues are a byproduct of agriculture or its related industry, such as rice husks and sugarcane bagasse: they are produced decentralized and have low density and, due to the high transportation cost, it would be expensive to apply

them as the main fuel in power stations. Forestry residues include biomass, not harvested or removed from sorting regions in commercial hardwood and softwood production, through forest management operations. Forest waste includes wood chips, sawdust and bark. The expensive extraction costs and required transportations make forest fuels suitable for energy production at a district level in decentralized small plants.

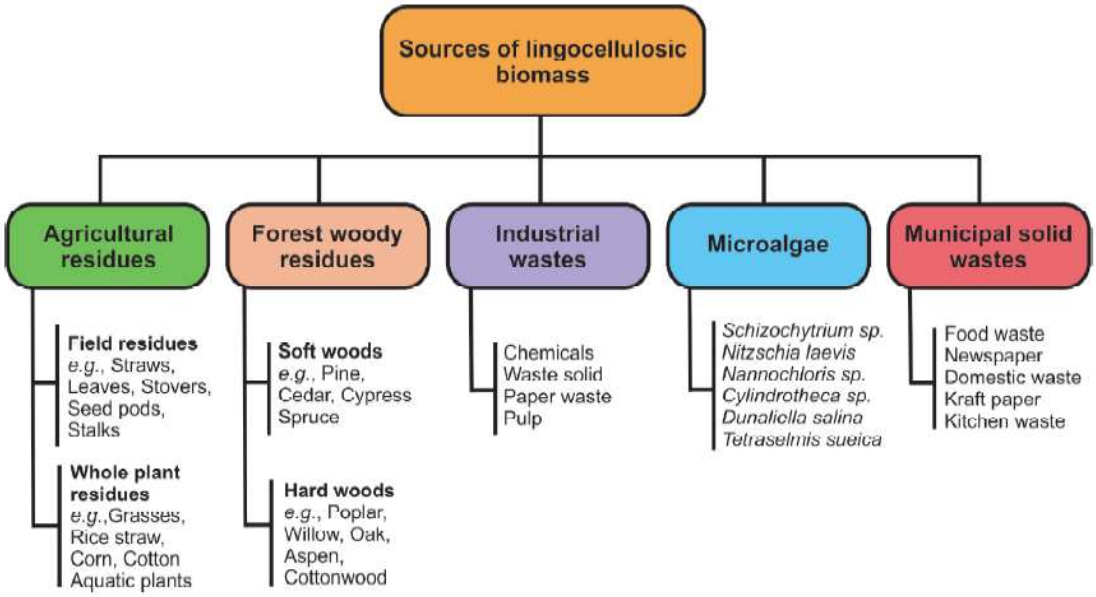

Figure 3.2.[10] Different sources of lignocellulosic biomass

4.PROCESSING OF BIOMASS TO ETHANOL

Independently on the type of lignocellulosic biomass requested, the production of bioethanol consists of four main stages:

1-**Pretreatment** of the lignocellulosic biomass, necessary for destroying its structure thus reducing its recalcitrance

2-**Enzymatic hydrolysis** through which carbohydrates are converted into simple sugars;

3-**Fermentation**, which is the conversion of sugars into ethanol and carbon dioxide;

4-Recovery of ethanol through **distillation**.

The general scheme is summarized in the following scheme. [10]

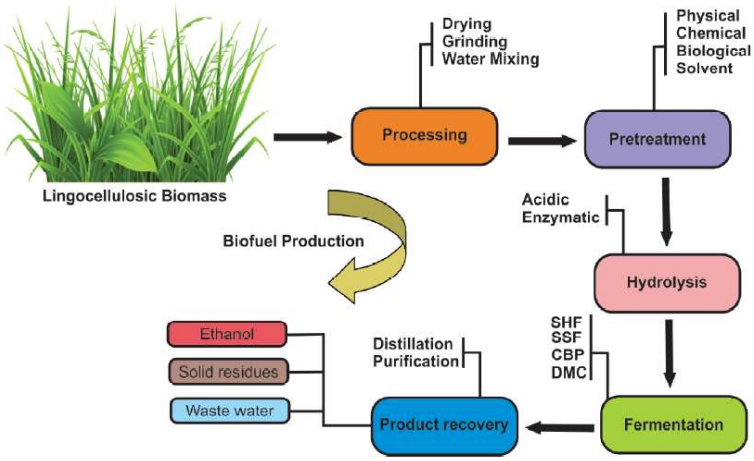

Figure 4.1.[10] General scheme for the production of second generation bioethanol from lignocellulosic biomass.

4.1.Pretreatment

The main aim of every pretreatment method is that of changing the chemical composition, macrostructure and microstructure of the lignocellulosic raw material, thus maximizing the enzymatic digestibility of the pretreated material.[10]

The aim of an effective pretreatment of lignocellulosic mass should be focused on:

- increasing the accessible surface area and decrystallizing cellulose;
- obtaining partial depolymerization of cellulose and hemicellulose;
- minimizing the formation of compounds showing inhibitory activity towards hydrolytic enzymes and fermenting microorganisms ;
- solubilizing hemicellulose and/or lignin;
- recoverying lignin for the convertion into valuable coproducts;
- minimizing the loss of sugars, also preserving the pentose fractions derived from hemicellulose degradation
- minimizing capital and operating costs;
- avoiding the need for the reduction of biomass particles size.[4]

Pretreatment technologies are usually classified into physical, chemical, physiochemical and biological.

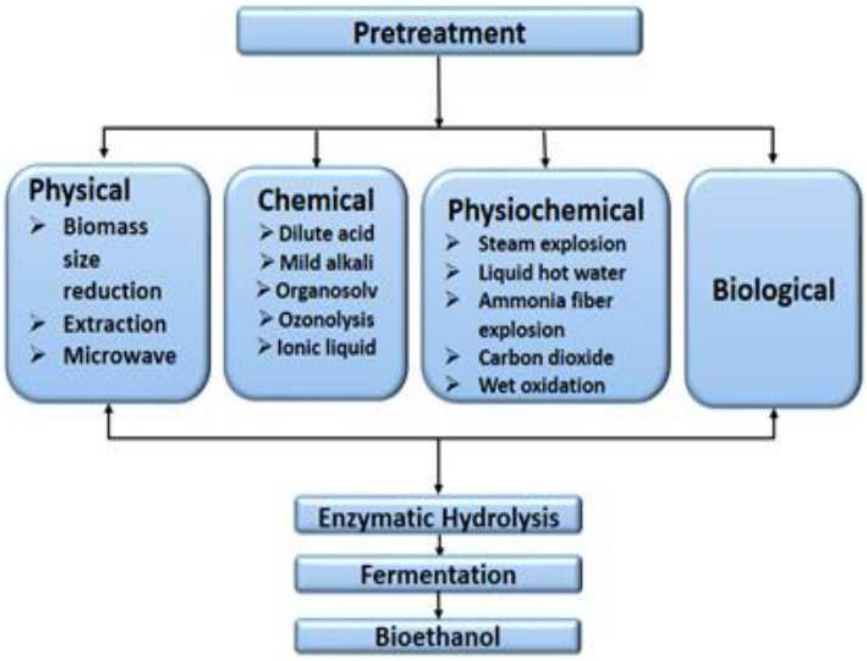

Figure 4.2.[8] Different types of pretreatment

4.1.1.Physical pretreatments[8]

Physical pretreatments are methods that lead to the mechanical size reduction of lignocellulosic biomass in order to enhance enzymatic digestibility. These methods involve operations such as chipping, shredding, grinding, coarse size reduction and milling thus decreasing the cellulose crystallinity and the degree of polymerization as well as increasing the specific surface area. The energy demand of the pretreatment depends on agricultural biomass features and final particle size requested. Examples of physical pretreatments include extrusion and microwaves.

4.1.2.Biological pretreatment[8]

Biological pretreatment involves the use of microorganisms possessing enzymes to degrade cell wall. This kind of pretreatment uses mild conditions, does not lead to the production of unwanted byproducts or inhibitory compounds, does not request the addiction of any chemicals nor does require great energy supply and is cheap. On the other hand, the disadvantages are the low hydrolysis rate and the fact that most degrading microorganisms do not degrade only lignin but also cellulose and hemicellulose, so that extensive research is required before this method can be applied on an industrial scale.

4.1.3.Chemical pretreatments

Chemical pretreatments are amongst the most widely used and comprise a lot of different methods requesting the addiction of chemicals in order to achieve cell wall degradation.

- ALKALINE PRETREATMENT [7]

  This kind of pretreatment involves the use of bases -the most studied are sodium hydroxide and lime. Alkaline pretreatment leads to the degradation of ester and glycosidic side chains, thus resulting in structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose and partial solvation of hemicellulose. The advantages of this method are the efficient removal of lignin -which improves the accessibility of cellulose to hydrolytic enzymes-, low inhibitors formation and less severe conditions than requested for other pretreatments. The process involves the soaking of the biomass in alkaline solutions, mixing it at a target temperature -that determines the amount of time requested to achieve an effective pretreatment-, then a neutralizing step designed to remove lignin and inhibitors like salts, carboxylic acids, phenolic acids, acetic acid, furfural, aldehydes and other compounds. Despite several positive aspects, this pretreatment also implies some not negligible disadvantages such as the high cost of alkaline catalysts and the structural modification of lignin.

- ACID PRETREATMENT [7]

  Acid pretreatment is one of the most promising methods with respect to industrial implementation and involves the use of concentrated or diluted acids to break the rigid structure of lignocellulosic materials –the most commonly used acid is dilute sulphuric acid. The process consists of the addition of concentrated or diluted acids to the biomass, followed by constant mixing at temperatures between 130℃ and 210℃ for a time that depends on the conditions. The advantages of this method are a high glucose yield, the solubilization of hemicellulose and lignin with minimal degradation, the conversion of hemicellulose to sugars. The disadvantages are the extensive production of fermentation inhibitors, the need for subsequent extensive washing and detoxification steps to remove the acid and inhibitors before the enzymatic hydrolysis and fermentation steps and the need for specific reactors due to the corrosive nature and toxicity of most acids.

- GREEN SOLVENTS [7]

  One of the methods that gained increasing importance in the last years is processing lignocellulosic biomasses with ionic liquids (IL) and other solvents. Ionic liquids are salts, usually consisting of a small anion and a large organic cation. The advantage of IL is the vast range of cations and anions amongst which choosing according to the type of lignocellulosic feedstock. The features that ionic liquids must have in order to be suitable for lignocellulosic biomass treatment are: good dissolution capacity, low melting point, low vapor pressure (in order to be recoverable), low viscosity, low toxicity and high stability. This kind of pretreatment can be performed at ambient pressure and requests temperatures of 90℃-130℃ for amounts of time that vary from one to 24 hours. After dissolution, the biomass is reprecipitated by adding water and washed several times to completely eliminate the ionic liquid that would otherwise irreversibly disactivate callulases in the following enzymatic hydrolysis step. The action of ionic liquids in lignocellulosic biomasses degradation can be ascribed to the capability of the anion to form hydrogen bonds with cellulose, thus breaking its crystalline structure and making it more amorphous and accessible to enzymatic hydrolysis. Another advantage of using this kind of pretreatment is the possibility to tune the chemistry of the solvent in order to dissolve also the other components of the lignocellulosic biomass, lignin and hemicelluloses. The main drawbacks are the high cost of the solvents and the need for their recovery. Recent researches are investigating new methods to synthesize ILs at lower costs and on a larger scale, as well as the possibility of microorganisms to ferment sugars in the presence of these solvents –factors that would be fundamental for further applications on an industrial scale.

- OZONOLYSIS [4]

  In this kind of chemical pretreatment the oxidizing agent is ozone O_3, which degrades lignin by attacking the aromatic rings structure leaving the hemicellulose and cellulose fractions unaffected. Ozonolysis is usually performed passing the ozone gas through the reaction vessel at room temperature and pressure and does not lead to the production of toxic byproducts. The major drawback of the process is the requirement of large amounts of ozone, which is very expensive.

4.1.4.Physicochemical pretreatments

- STEAM-EXPLOSION

  Steam explosion pretreatment combines hydro-thermal and sudden pressure change in pretreating the lignocellulosic biomass. At first, the biomass is exposed to high temperature and pressure for a time ranging from a few seconds to some minutes; then, sudden depressurization occurs causing the degradation of the material structure, making cellulose more susceptible to enzymatic attack and also causing lignin modification and hemicellulose solubilization. From the physical point of view, the effect of this treatment is that of separating and shortening fibres, whereas from the chemical point of view, the effect of high temperature is the auto-hydroysis of the acetyl groups of hemicellulose, forming acetic acid which creates an acidic environment.[11] A promising finding is the fact that large particle size have been able to yield maximum sugar concentrations, so that further mechanical fragmentation of the mass and consequent costs are not requested. The main drawbacks of the process are the partial degradation of hemicellulose and the formation of toxic components that can damage enzymatic hydrolysis and fermentation processes.[4]

  Some experiments have been conducted in order to optimize the yield of the process. It has been demonstrated that a two step process increases the downstream ethanol yield by increasing the accessibility to cellulose by reducing the hemicellulose fraction. Despite this positive achievement, a two step process involves increased costs for process equipments and additional use of energy during the second steam explosion. Another way that can be used in order to improve hemicellulose hydrolysis during pretreatment and cellulose hydrolysis in the following step is the use of dilute acid catalysts, which reduce retention time and the temperature needed for the steam explosion process; other advantages are the complete removal of hemicellulose and improved hydrolysis of cellulose. Despite the positive aspects, this acid catalyzed treatment also has some disadvantages, which are the acid catalyst costs, the need for acid-resistant equipments and the increased formation of degradation products that must be neutralized and separated from the system. [7]

- LIQUID HOT WATER [4]

  This method closely resembles the steam-explosion process: it involves the usage of high temperature water at high pressures, in order to obtain destruction and separation of the lignocellulosic matrix. Temperature range between 170 and 230℃ and pressure

higher than 5 MPa are commonly used in order to maintain water liquid at high temperature. The pretreatment removes hemicellulose from the matrix thus making cellulose more susceptible to enzymatic hydrolysis. After pretreatment, the slurry can be filtered, thus obtaining two fractions: a solid cellulose-enriched fraction and a liquid fraction rich in hemicellulose-derived sugars, mostly in form of oligomers.

The major advantages of this pretreatment are: no requirement for chemicals and corrosion-resistant materials; no need for size reduction of the biomass; higher pentoses recovery and lower inhibitors production (compared to steam explosion). However, this method is still far from application at industrial level because of high water and energy requirements.

- WET OXIDATION [4]

  Wet oxidation involves the treatment of lignocellulosic biomass with water and air/oxygen as an oxidant agent at temperatures higher than 120℃ for about 30 minutes. The parameters that mainly affect wet oxidation are: temperature, reaction time and oxygen pressure. This kind of pretreatment leads to the formation of acids as a consequence of hydrolytic processes and oxidative reactions. All three main fractions of lignocellulosic biomass are affected by the pretreatment: lignin undergoes cleavage and oxidation; hemicellulose in cleaved to low molecular weight sugars that become soluble in water, whereas cellulose is partially degraded thus becoming more susceptible to the attack of hydrolytic enzymes. The addition of alkaline agents such as sodium carbonate has been proved to result in a better solubilization of the hemicellulose fraction and also helps avoiding the formation of byproducts. The disadvantages of this method are the request for high temperature and pressure conditions and the formation of strongly oxidizing species, conditions that lead to high costs of maintenance.

- AMMONIA FIBER EXPLOSION [4]

  In this pretreatment lignocellulosic materials are treated with liquid anhydrous ammonia under high pressure and moderate temperature (90-100℃), and are then rapidly depressurized. This results in a rapid expansion of the liquid ammonia, which causes swelling and physical destruction of biomass fibers and partial decrystallization of cellulose. Parameters that determine the degree of destruction of the biomass structure are temperature, residence time and ammonia loading. This method shows lots of advantages –lower moisture content, lower formation of sugar degradation products,

complete recovery of solid material, the ability of ammonia to lessen lignin's effect on enzymatic hydrolysis- but also some important disadvantages, which are the high cost of ammonia and the need for its recovery.[7]

- AMMONIA RECYCLE PERCOLATION [7]

  In this process aqueous ammonia (5-15% wt) passes through a packed bed reactor containing the biomass material at a rate of 5 mL/min. The main advantages of this physicochemical pretreatment are the ability to remove a majority of lignin and solubilize more than a half of hemicellulose, still maintaining high cellulose content. In this way it is possible to obtain a solid material containing short-chain cellulosic material with high glucan amounts. Another positive aspect of this method is the limited production of inhibitors, even if high energy costs –higher temperatures and longer times are requested in comparison to AFEX- and high liquid loadings are problems that need to be solved before an application of this procedure on an industrial scale is possible.

- SUPERCRITICAL FLUID PRETREATMENT

  A supercritical fluid is a material which can be either liquid or gas, used in a state above the critical pressure and temperature where gases and liquids can coexist. It has very particular features: it possesses liquid-like density and solvating properties and exhibits gas-like transport properties of diffusivity and viscosity. Their properties are very dependent on temperature and pressure conditions and can be tuned with little changes of them.[7] Supercritical carbon dioxide has been successfully tested in lignocellulosic mass pretereatment, showing interesting advantages such as little production of inhibitory compounds and efficient removal of lignin at mild conditions thus increasing the accessible surface for hydrolytic enzymes. The main drawback of this method is the high pressure requirement which makes the process not large scale economically viable. [11]

4.2.Inhibitors

With bioethanol production from lignocellulosic biomass, chemical inhibition is a more severe problem than encountered in first generation raw materials due to the pretreatment and hydrolysis processes necessary to reduce the recalcitrance of second generation feedstocks.

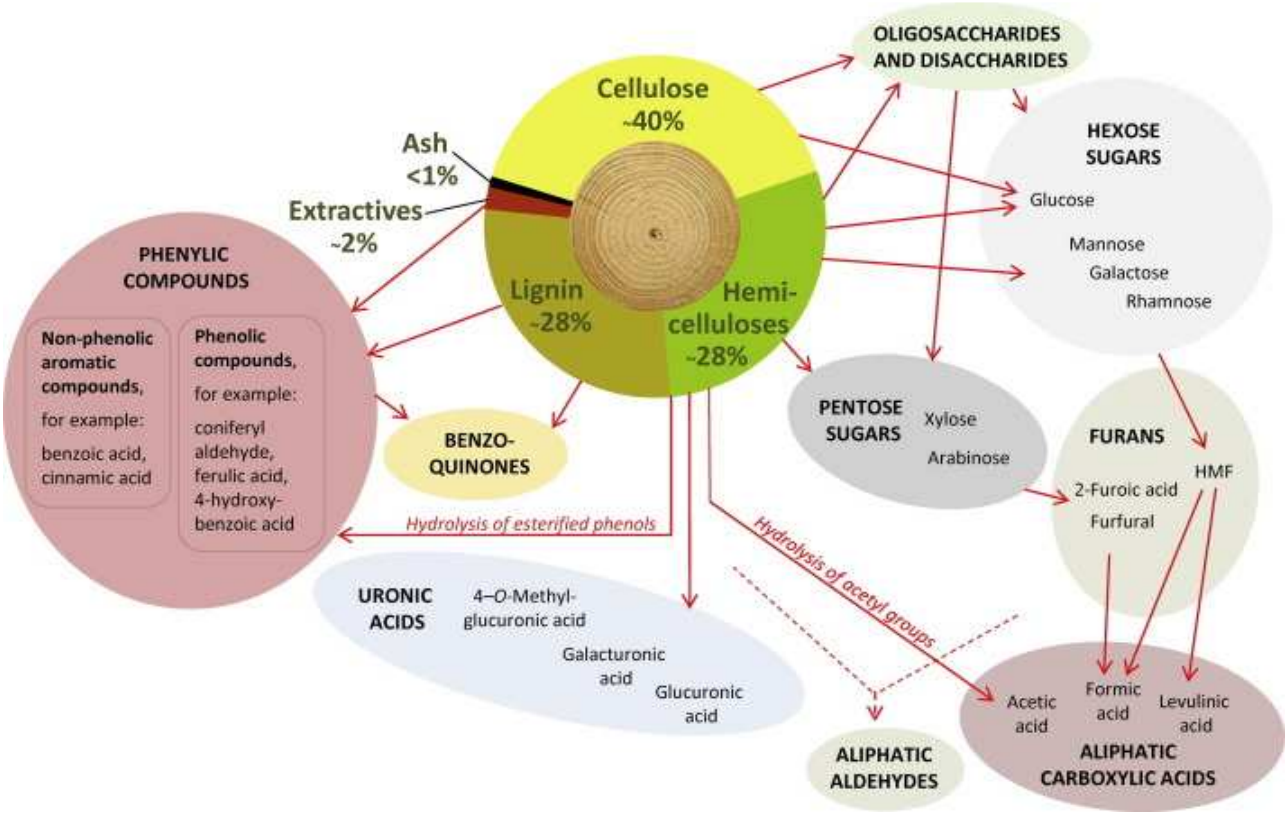

Figure 4.3.[6] Different types of inhibitors and their origin

4.2.1.Inhibition of microorganisms

By-products of pretreatments can be divided into groups on basis of chemical functionality, origin and effect on the fermenting microorganisms.

- SHORT CHAIN ALIPHATIC ACIDS

  Short-chain aliphatic acids found in lignocellulose hydrolysates mainly include formic acid, acetic acid and levulinic acid. Acetic acid mainly derives from the hydrolysis of acetyl groups of hemicellulose, while formic acid and levulinic acid arise as acid-catalyzed thermochemical degradation products from polysaccharides.[12] Due to the low acetyl content, softwood hydrolysates have relatively low concentrations of acetic acid, whereas hardwood and agricultural residues with high acetyl content result in high concentrations of acetic acid.[6]

  Acid toxicity on microbial growth depends on both exposed concentration and acid chain length and structure -the last two factors both influencing the ability to establish hydrophobic interactions with cell membranes; for this reason, long chain acids are more toxic to microbial growth than short chain ones and straight chain acids are more

toxic than branched ones.[13] The toxic effect on *S.Cerevisiae* is attributed to the undissociated form and increases in the order: acetic acid < levulinic acid < formic acid.[12]

- PHENOLIC COMPOUNDS

  These compounds, including acids (ferulic acid, vanillic acid, 4-hydroxybenzoic acid and syringic acid), alcohols (guaiacol, catecol and vanillyl alcohol) and aldehydes (vanillin, syringic aldehyde and 4-hydroxylbenzaldehyde) originate from lignin or from hydrolysis of esterified phenols during biomass pretreatment processes by acid-based, alkaline, hydrothermal and oxidative methods. [13]. They are present in lower concentrations than aliphatic carboxylic acids and exhibit a much stronger inhibitory effect.[6] The toxicity of phenolic compounds can be attributed to their ability to penetrate cellular membranes and damage them. Phenolic compounds of lower molecular weight are the most dangerous, due to their ability to penetrate membranes more easily than higher molecular weight compounds. Another negative effect of phenolic compounds is their action as reactive oxygen species, which can cause severe DNA damages and eventually lead to cell death.[13]

- FURAN ALDEHYDES

  Production of furan aldehydes furfural and HMF results in a decreased yield in sugars because they are produced from the degradation of pentoses and hexoses respectively.[12] The amount of furan aldehydes in the hydrolysate depends on both the lignocellulosic feedstock and the pretreatment adopted.[13] These products show lower inhibitory activity compared to aromatic aldehydes, but their concentration can be in some cases relatively high.[6]

- INORGANIC COMPOUNDS

  Inorganic ions that are present in lignocellulose hydrolysates originate from the lignocellulosic feedstocks, from chemicals added during pretreatment, conditioning and hydrolysis, and possibly from process equipment. The addition of salts results in a higher osmotic pressure, which might have inhibitory effects.[12]

Other inhibitors include quinines, benzoquinones and small aliphatic aldehydes. [6]

4.2.2.Inhibition of cellulolytic enzymes [6]

There are many products that can inhibit the catalytic action of cellulolytic enzymes: the inhibitory effect is due to the non-productive binding of enzymes to different components of the solid fraction, such as lignin and residual hemicellulose. Other compounds exhibiting inhibitory activity are carbohydrates and aromatic substances in the pretreatment liquid, which are monosaccharides such as glucose, disaccharides such as cellobiose, oligosaccharides and phenolics. Several experiments show that the inhibitory action of aromatic substances is strictly related to the hydrophobicity of these compounds, due to the hydrophobic interactions that occur between inhibitors and the active site on the enzyme; evidence of this is the fact that the treatment with sulfite or dithionite, which increases the polarity of the compounds, results in a loss in inhibitory activity towards cellulolytic enzymes, whereas the treatment with sodium borohydride is only capable of reducing microbial inhibition.

4.2.3.Strategies to counteract inhibition problems

There are different strategies that are being investigated in order to solve problems related to the presence of inhibitors.

- FEEDSTOCK SELECTION AND ENGINEERING

  One possibility is that of choosing feedstocks with low recalcitrance and that can thus be pretreated under mild conditions.[12] As the study of Jeon et al. demonstrates, of the various raw materials evaluated, the highest ethanol yields and productivities were achieved with herbaceous raw materials (e.g. sugarcane bagasse and wheat straw), while fermentation of woody hydrolysates resulted in relatively low ethanol concentrations.[14]

  Another approach to reduce recalcitrance and thereby inhibitors release is feedstock engineering targeting components such as lignin, hemicellulose and pectin. By choosing or engineering plants characterized by low acetyl content, it is possible to minimize the production of acetic acid derived from the hydrolysis of these groups. [6]

- DETOXIFICATION/CONDITIONING

  One of the most useful strategies in order to contrast the formation of inhibitors is detoxification or conditioning of lignocellulosic hydrolysates. One possibility is the use of additives such as alkali, reducing agents and polymers; other ways of detoxification

include heating and vaporization, liquid-liquid extraction and solid-liquid extraction.[6] The treatment with calcium hydroxide (overliming) is one of the most efficient and economical methods. Another promising option for detoxification is the use of reducing agents, such as sulfur oxyanions or sulphydryl agents: the action of these reactants is due to the sulfonation of inhibitors, such as phenolic compounds, thus making them hydrophilic and unreactive.[12]

- MICROBIAL DETOXIFICATION

  There have been several attempts to treat lignocellulosic hydrolysates using biological methods: several microorganisms including yeasts, fungi and bacteria naturally have the ability to detoxify inhibitory compounds and might thus be used to pre-detoxify lignocellulosic hydrolizate before fermentation with *S.cerevisiae* for ethanol production.[15] Microbial treatment can be used to improve both fermentability and enzymatic hydrolysis of cellulose. In order to make this solution economically available, time requested to perform the treatment as well as the microbial request of sugars (thus resulting in a decreased final yield in ethanol) must be taken into account.[6]

- ENZYMATIC DETOXIFICATION

  Another possible approach to detoxify lignocellulosic hydrolysates is enzymatic detoxification. This method differs from microbial detoxification because single enzymes instead of living microorganisms are used. The advantages of this method include the possibility to use higher temperatures in order to obtain efficient detoxification and the high catalytic efficiency of pure enzymes, which makes the process faster. The main drawbacks of this method are the long time of incubation requested for detoxification and the expensive production costs of enzymes. [15]

- CULTURING SCHEMES [16]

  Also the design of the overall process plays an important role in order to minimize the production of species that show inhibitory activity.

  Four process configurations for ethanol production are possible as shown in **Figure 4.4.**

  -In **Separate Hydrolysis and Saccharification (SHF)** the enzyme production, hydrolysis, hexoses and pentoses fermentation are carried out in separated reactors. This allows every process to occur in the optimal conditions but, on the other hand, has the disadvantage that glucose and cellobiose produced during hydrolysis –which exhibit

inhibitory activity towards cellulases- are not removed, thus causing a loss in efficiency of hydrolytic enzymes.

-In **Simultaneous Saccharification and Fermentation (SSF)**, both hydrolysis and hexoses fermentation occur in the same reactor. This has the advantage of continuously removing sugars which show inhibitory activity towards hydrolytic enzymes. The disadvantage is that cellulases and fermenting microorganisms usually have different optimal work pH and temperature, so that it is necessary to work at conditions compatible for both enzymes and microorganisms.

-In **Simultaneous Saccharification and Co-Fermentation (SSCF)** glucose and xylose are co-fermented in the same reactor. Strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis* are genetically engineered to co-ferment both glucose and xylose.

-In **Consolidated Bio Processing (CBP)**, one single microorganism is capable of both producing the hydrolytic enzymes and fermenting sugars. This is a very promising process, but such microorganisms are still in the early stages of development.

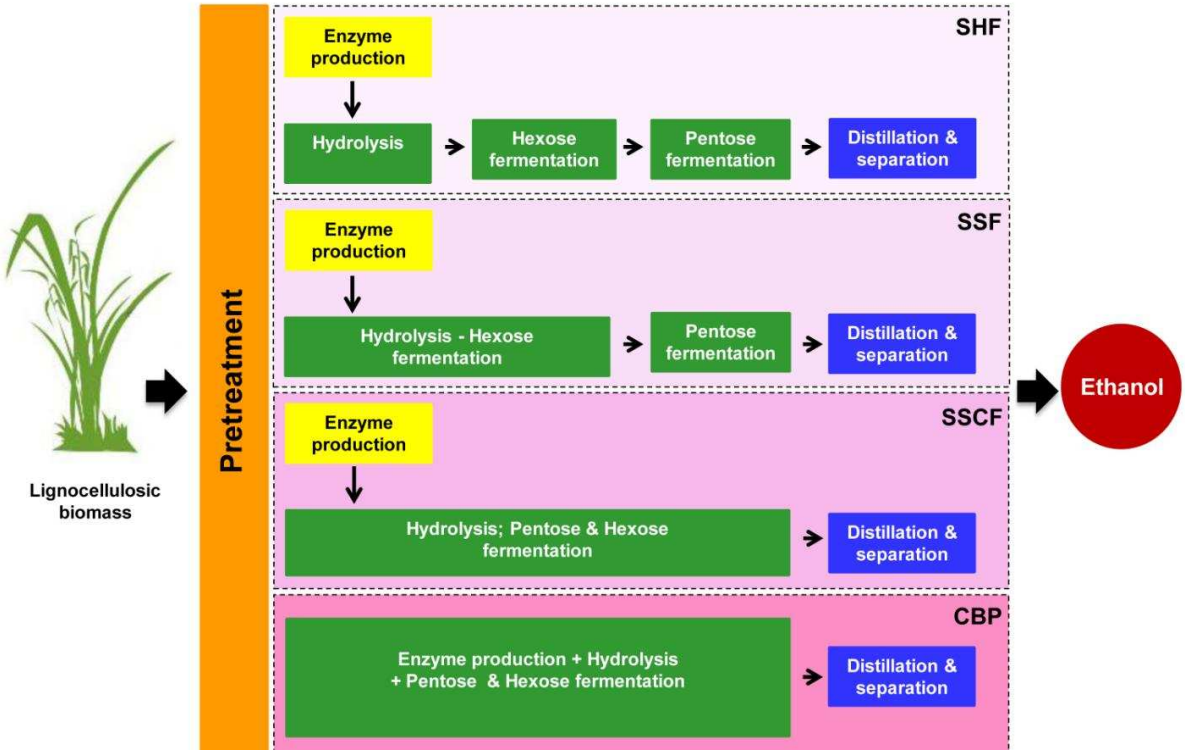

Figure 4.4.[16] Possible process configurations for bioethanol production

- SELECTION OF MICROORGANISMs [17]

Saccharomyces cerevisiae is the most used microorganism at industrial level. However, in order to be used in second generation bioethanol production, it still has to overcome a variety of stresses present during the process that damage cell metabolism and

consequently reduce ethanol yield and fermentation rate. There are some less studied but not less interesting yeasts –known as non conventional yeast species- which present better tolerance to some of these stresses and could potentially be used as model organisms to study the molecular basis of these tolerances in order to further develop *S.cerevisiae*.

-High osmotic pressure: there is a yeast species, *Z.rouxii*, which is able to grow in salt concentrations of 3 M NaCl and sugar concentrations up to 90% due to its particular plasma membrane sugar transporters.

-High temperature: tolerance to high temperature is an essential requirement for bioethanol production, especially in SSF, where hydrolytic enzymes work at an optimal temperature of 55℃, a temperature which is quite different from the optimal fermentation temperature of S.cerevisiae, which ranges from 25 to 37℃; *K.marxianus* and *O.polymorpha* are able to ferment xylose at 45℃.

-Presence of inhibitors: acetic acid and furan derivatives inhibit efficient ethanol fermentation by *S.cerevisiae*: however, *Z. bailii* and *P.kudriavzevii* are the most tolerant yeast species respectively towards acetic acid and furan derivatives.

-Ethanol tolerance: as far as this limiting factor is concerned, *S.cerevisiae* seems to be the most ethanol tolerant yeast.

- EVOLUTIONARY ENGINEERING [18]

  Resistance to inhibitors can also be obtained through adaptative evolution due to the fact that cell tolerance to lignocellulosic inhibitors is strictly correlated and dependent on the environment that the cell population has experienced before exposure. The level of cellular resistance is determined by both stress-specific and general mechanisms. As far as stress-specific mechanisms are concerned, pre-cultivation in lignocellulosic hydrolysate containing furfural and HMF leads to induced expression of genes coding for specific NADPH-dependent oxidoreductases that reduce the aldehydes into less inhibitory furfuryl alcohols. Similarly, tolerance to vanillin is obtained through reduction to the less toxic vanillyl alcohol. Also tolerance to acetic alcohol is increased by pre-cultivation in the presence of acetic acid.

  Addictionally, as far as the general adaptative mechanisms are concerned, it has been discovered that cells in stationary phase (SP) are characterized by increased cell robustness towards different non-related stresses -heat shock, osmotic stress, freeze-thaw stress, weak acid stress- due to the activation of multiple cellular regulatory events upon nutrient starvation, including the environmental stress response (ESR). This

suggests that increased tolerance to lignocellulosic conditions can be reached without pre-exposure to inhibitors, simply allowing cells to reach SP by carbon starvation before the fermentation step.

- GENETIC AND METABOLIC ENGINEERING

  Using genetic engineering, recombinant microorganisms exhibiting improved resistance to lignocellulosic hydrolysates have been developed.

4.3. Enzymatic hydrolysis [9]

Cellulases are enzymes synthetized by a large variety of microorganisms including both fungi and bacteria. These enzymes are responsible for the hydrolysis of the β-1,4 glycosidic bonds in cellulose and are members of the glycoside hydrolase family of enzymes. Although cellulases cleave one specific type of bond, the complex intermolecular bonding pattern of cellulose generates a crystalline structure that requires multiple enzymes for degradation. From a structural point of view fungal cellulases are simpler compared to bacteria cellulase systems, cellulosomes. In particular, fungal cellulases typically have two separated domains: a catalytic domain (CD) and a cellulose binding module (CBM), joined by a short polylinker region to the catalytic domain.[19]

The cellulase system consists of three main groups of enzymes:

-endoglucanases (endo-1,4-β-glucanases);

-exoglucanases (exo-1,4-β-glucanases);

-β-glucosidases (1,4-β-glucosidases).[19]

These three key enzymes have similar catalytic domains which cleave β-glycosidic bonds between glucose molecules through acid-catalysed hydrolysis (the mechanism is shown in **Figure 4.5.**), but they differ in their binding substrates and substrate interacting domains that act to tether them to their binding substrate.

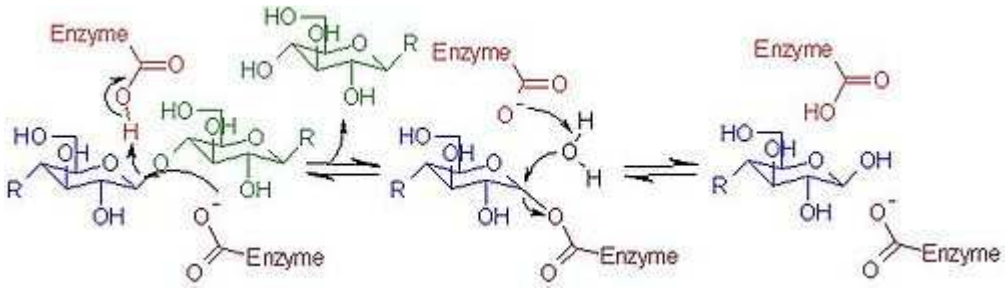

Figure 4.5. Mechanism of action of cellulolytic enzymes

-Endoglucanases (EGs) cleave the more amorphous regions of cellulose and are capable of slicing β-1,4- glycosidic bonds from the internal structure thanks to a cleft shaped active site. They facilitate the hydrolysis of cellulose by rapid depolymerization, producing more chain ends for processive enzymes to act upon.[20]

-Exoglucanases, also named cellobiohydrolases (CBHs), are progressive enzymes that act on both reducing and non-reducing ends of crystalline cellulose chains in a continuous manner, thus releasing cello-oligosaccharides and cellobiose.[9]

-The last step in cellulose degradation is β-glucosidase (BGLs) degradation of cellobiose and other longer cello-oligomers produced by EGs and CBHs to glucose.[20]

Enzymatic hydrolysis of cellulose consists of three main steps: absorption of cellulases to the surface of cellulose, hydrolysis of cellulose, desorption of cellulases.[9]

In practice, the hydrolysis of cellulose is influenced by external factors including cellulose structure and complex enzyme-substrate interactions.[21] Hydrolysis rate progressively declines with conversion -leading to decreased yeld, longer reaction time and higher enzyme usage- and this has often been attributed to declining reactivity of the remaining substrate as the degradation proceeds. Many hypotheses have been presented to explain this observation, including thermal instability of cellulases, hydrolysis products inhibition, cellulase inactivation, enzyme slowing down/stopping, substrate transformation into a less digestible form, and/or the heterogeneous structure of the substrate.[22]

There are various ways in which cellulose effectiveness can be enhanced, such as the supplementation with BGLs during hydrolysis and removal of sugars during hydrolysis by ultra-filtration or SSF in order to reduce inhibition of cellulose degradation process.[9]

An important phenomenon in cellulose degradation is synergism, by which a mixture of enzymes exhibit higher specific activity compared to the sum of the individual enzymatic activities.[23] There are four different types of synergy that have been described with regards to cellulolytic enzymes: [9]

- Endo-exo synergy through the simultaneous action of EGs and a CBHs.
  Endoglucanases attack the bulk cellulose, creating new chain ends that can be further degraded by exoglucanases. On the other hand, exoglucanases create more substrate for endoglucanases by destroying the crystalline structure of cellulose and by exposing previously inaccessible areas;
- Exo-exo synergy between two CBHs acting on the reducing and non reducing chain ends of cellulose;
- EGs-BGLs synergy, which remove cellobiose that inhibits the first two enzymes;

- Intramolecular synergy between CBMs and catalytic domains.

  It has been demonstrated that the synergistic cooperation not only substantially enhances the hydrolysis rate, but also dramatically reduces the required amount of cellulases needed to achieve reasonable cellulose hydrolysis yield.

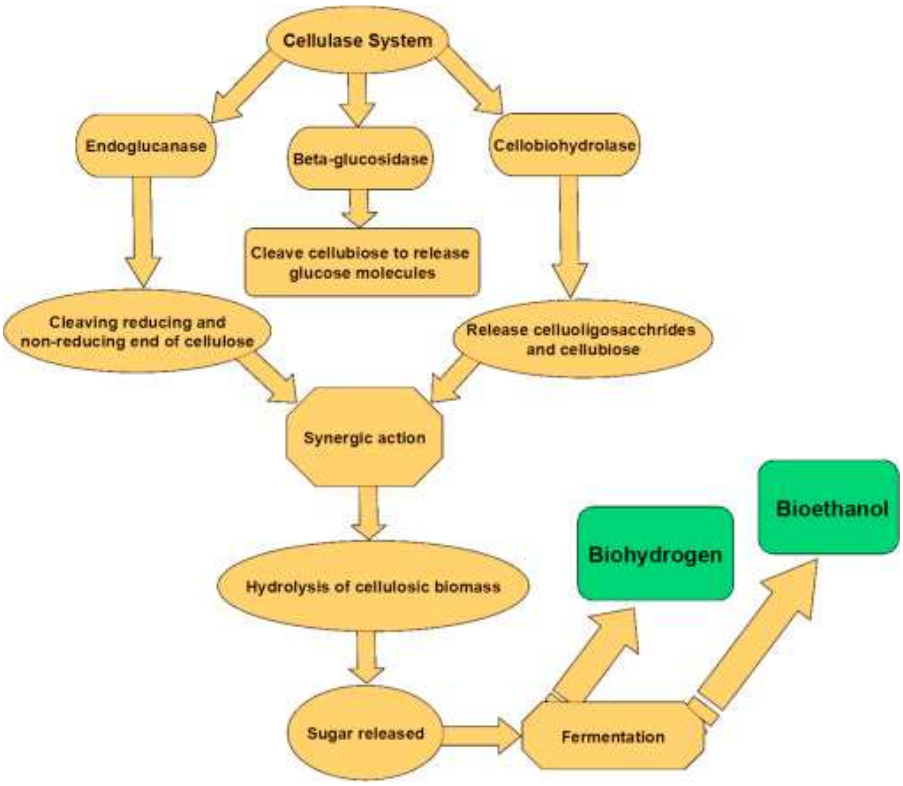

Figure 4.6.[20] Different actions of cellulolytic enzymes

4.4.Fermentation

Fermentation is the process by which microorganisms such as bacteria, fungi or yeasts, convert hexoses and pentoses into ethanol. The general reactions are as follow: [1]

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

$3\ C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5\ CO_2$

One of the main obstacles to second generation production of bioethanol on an industrial scale is the lack of microorganisms able to efficiently ferment hexoses and pentoses. Ideally, fermenting microorganisms should fulfill the following conditions: potential for broad substrate utilization, high ethanol yield, high toleration to concentrated ethanol and heat, resistance to inhibitory products derived from the pretreatment processes. Natural microorganisms are not able to carry out the fermenting process fulfilling these requirements.[10] Moreover, most employed

microorganisms –*S.Cerevisiae* and *Z.Mobilis*- are not capable of fermenting pentose sugars, whereas species which are able to are usually not efficient. This is the reason why lots of researches are focusing on the development of genetically modified microorganisms.[11]

4.5.Distillation

The ethanol solution resulting from fermentation process needs to be further processed in order to obtain anhydrous ethanol, which can contain a maximum of 0.5 % of water. The main technique used is distillation, based on the difference in boiling point of the components of the solution.

5.CONCLUSIONS

Second generation bioethanol is a promising environmentally friendly alternative to fossil derived fuels, based on the exploitation of non-food lignocellulosic biomass. Still, the high recalcitrance of the raw materials is an obstacle to extensive production of this new energy source, due to the necessity of expensive pretreatment processes. Other factors that make second generation bioethanol still far from large scale application are the need for great amounts of cellulose degrading enzymes and the lack of microorganisms capable of efficient conversion of both hexoses and pentoses to ethanol through fermentation. For these reasons, recent researches are focusing on the development of efficient pretreatment methods, capable of degrading lignocellulosic raw materials in an energy-efficient way, limiting the production of inhibitory compounds and the use of strong chemicals. Other researches are investigating different process configurations, such as Simultaneous Saccharification and Co-Fermentation (SSCF) and Consolidated Bio Processing (CBP) in order to minimize the requested steps and increase process yields as well as production costs.

6.REFERENCES

[1] Kang, Q., Appels L.; Tan, T.; Dewil, R. “Bioethanol from Lignocellulosic Biomas: Currrent Findings Determine Research Priorities” *The Scientific World Journal*, **2014**, 2014, Article ID 298153

[2] De Bari, I.; Dininno, G.; Braccio, G. “Bioetanolo da residui della lavorazione del mais: process design e analisi del ciclo di vita” ENEA, **2009**

[3] Zhao, X.; Zhang, L.; Liu, D. “Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocelluloses” *Biofpr Journal*, **2012**, 6 (4), 465-482

[4] Devendra, P.M.; Ankit, S.; Sangeeta, N. “An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol” *3 Biotech*, **2015**, 5, numero 5, 597-609

[5] Mota, T.R.; de Oliveira, D.M.; Marchiosi, R.; Ferrarese-Filho, O.; dos Santos, W.D.; “Plant Cell Wall Composition and Enzymatic Deconstruction” *AIMS Bioengineering*, **2018**, 5 (1), 63-77

[6] Jönsson, L.J.; Martìn, C.; “Pretreatment of Lignocellulose: Formation of Inhibitory By-Products and Strategies for Minimiziang their Effects” *Bioresource Technology*, **2015**, 1999 (2016), 103-112

[7] Brodeur, G.; Yau, E.; Badal, K.; Collier, J.; Ramachandran, K.B.; Ramakrishnan, S. “Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review” *Enzyme Research*, **2011**, vol.2011, Article ID 787532, 17 pages

[8] Tayyab, M.; Noman, A.; Islam, W.; Waheed, S.; Arafat, Y.; Ali, F.; Zaynab, M.; Lin, S.; Zhang, H.; Lin, W. “Bioethanol Production from Lignocellulosic Biomass by Environment-Friendly Pretreatment Methods: A Review” *Applied Ecology and Environmental Research*, **2018**, 16(1), 225-249

[9] Davison, S.A. “Heterologous Expression of Cellulase Genes in Natural *Saccharomyces Cerevisiae* Strains” Graduation Thesis for the degree of Masters of Science in the Faculty of Microbiology.-Stellenbosch University, **2016**

[10] Fatma, S.; Hameed, a.; Noman, M.; Ahmed, T.; Shahid, M.; Tariq, M.; Sohail, I.; Tabassum, R. “Lignocellulosic Biomass: A Sustainable Bioenergy Source for the Future” *Protein & Peptide Letters,* **2018**, 25, 1-16

[11] Aditiya, H.B.; Mahlia, T.M.I.; Chong, W.T.; Hadi Nur; Sebayang A.H. “Second Generation Bioethanol Production: A Critical Review” *Renewable and Sustainable Energy Reviews*, **2016**, 66, 631-653

[12] Jönsson, L.J.; Björn, A.; Nilvebrant, N.O. “Bioconversion of Lignocellulose: Inhibitors and Detoxification” *Biotechnology for Biofuels,* **2013**, 6; 16

[13] Wang, S.; Sun, X.; Yuan, Q. “Strategies for Enhancing Microbial Tolerance to Inhibitors for Biofuel Production: A Review” *Bioresource Technology,* **2018** (article in press)

[14] Jeon, Y.J.; Xun, Z.; Rogers, P.L. " Comparative Evaluations of Lignocellulosic raw Materials for Second Generation Bioethanol Production" *Letters in Applied Microbiology*, **2010**, 51, 518-524

[15] Parawira, W.; Tekere, M. "Biotechnological Strategies to Overcome Inhibitors in Lignocellulose Hydrolysates for Ethanol Production: A Review" *Critical Reviews in Biotechnology,* **2011**, 31(1), 20-31

[16] Devarapalli, M.; Atiyeh, H.K. "A Review of Conversion Processes for Bioethanol Production with a Focus on Syngas Fermentation" *Biofuel research Journal,* **2015**, 7, 268-280

[17] Radecka, D.; Mukherjee, V.; Mateo, R.Q.; Stojiljkovic, M.; Foulquié-Moreno, M.R.; Thevelein, J.M. "Looking Beyond *Saccharomyces*: the Potential of Non-Conventional Yeasts Species for Desirable Traits in Bioethanol Fermentation" *FEMS Yeast Research*, **2015**, 15

[18] Narayanan, V.; Schelin, J.; Gorowa-Grauslund, M.; van Niel, E.W.J.; Carlquist, M. "Increased Ligniocellulosic Inhibitor Tolerance of *Saccharomyces Cerevisiae* Cell Polpulations in early Stationary Phase" *Biotechnology for Biofuels*, **2017**, 10:114

[19] Kuhad, R.C.; Gupta, R.; Singh, A. "Microbial Cellulases and Their Industrial Applications" *Enzyme Research*, **2011,** vol.2011, Articlw ID 280696, 10 pages

[20] Srivastava, N.; Srivastava, M.; Mishra, P.K.; Gupta, V.K.; Molina, G.; Rodriguez-Couto, S.; Manikanta, A.; Ramtekea, P.W. "Applications of Fungal Cellulases in Biofuel Production: Advances and Limitations" *Renewable and Susteinable Energy Reviews*, **2017** (Article in Press)

[21]Yang, B.; Dai, Z.; Ding, S.Y.; Wyman, C.E. "Enzymatic Hydrolysis of Cellulosic Biomass" *Biofuels*, **2011**, 2(4), 421-450

[22] Yang, B.; Dai, Z.; Ding, S.Y.; Wyman, C.E. "Changes in the Enzymatic Hydrolysis Rate of Avicel Cellulose With Conversion" *Wiley InterScience*, **2006**, DOI: 10.1002/bit.20942

[23] Kostylev, M.; Wilson, D. "Synergistic Interactions in Cellulose Hydrolysis" *Biofuels*, **2012**, 3(1), 61-70