CHAPTER – 2

REVIEW OF LITERATURE
2.1 Lignocellulose

Lignocellulose refers to plant dry matter (biomass), so called lignocellulosic biomass. It is the most abundantly and low cost available raw material on the Earth for the production of bio-fuels, mainly bio-ethanol. In general, ethanol production from lignocellulosic feed stocks is grouped as:

1. Crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp)
2. Hardwood (aspen, poplar)
3. Softwood (pine, spruce)
4. Cellulose wastes (newsprint, waste paper, and recycled paper)
5. Municipal solid wastes (MSW) (Taherzadeh and Karimi, 2007)

In structure of Lignocellulosic biomass there are three parts, cellulose, hemicellulose and lignin. (Fig 2.2) Cellulose is a polysaccharide consisting of D-glucose, and it forms the backbone structure of lignocelluloses; hemicellulose is composed of a matrix of different polysaccharides, such as xylan, arabinoxylan and xyloglucan; in addition, lignin is a complex aromatic polymer, functioning as the supportive structure of lignocellulose.

Lignocellulose is well suited for energy applications because of its large-scale availability, low cost, and environmentally benign production. (Demirbas, 2009)
Figure 2.1: Lignocellulose structure (Potters et al., 2010)

However in comparison to sugar- or starch-containing crops, producing monomer sugars from cellulose and hemicellulose at high yields is far more difficult. Therefore, although the cost of lignocellulosic biomass is far lower than that of sugar and starch crops, the cost of obtaining sugars from such materials for bioethanol production has historically been far too high to be a focus for industrial interest. For this reason, it is vital to solve the problems involved in the conversion of lignocellulose to ethanol. (Galbe and Zacchi, 2002)

2.2 Different sources for bioethanol production

There are several agricultural products that are rich in carbohydrates due to high starch content, such as corn, wheat, potato and cassava, or due to high sugar content, such as sugar beet, sugar cane, fruit or palm juice (Naik et al., 2010). These types of biomass can
be converted into ethanol via fermentation (of the sugars) or after hydrolysis of the substrate into fermentable sugars (Walter and Ensinas, 2010).

The first generation of bio fuels was from classical sources like vegetable oil (soybean, sunflower, and palm) or starch (potatoes, cassava, wheat) that was grown in farms all over the world. More recently, sources of woody material (the second generation of lignocellulose producers) have been considered, and currently microalgae and their oil products are the more promising third generation. Each of these types of bio fuel producers has its own advantage, obstacles, availability and readiness to be implemented on a large scale, depending on its yield or sustainability. However, they all constitute a marked improvement over the use of fossil fuels as sustainable resources. (Naik., et al. 2010).

### 2.3 Water hyacinth as a biomass for bioethanol production

Features of an ideal crop for biofuels production are:

- Naturally grown vegetation, preferably perennials.
- High cellulose with low lignin content per unit volume of dry matter.
- Easily degradable.
- Should not compete with arable crop plants for space, light and nutrients.
- Resists pests, insects and disease.
- Not prone to genetic pollution by cross breeding with cultivated food crops.

(Bhattacharya and Kumar, 2010)

Water hyacinth has low lignin content of 10% and contains high amounts of cellulose (20%) and hemicellulose (33%). Lignin content in water hyacinth is low therefore cellulose and hemicellulose can be easily converted to fermentable sugars which results in enormous amount of usable biomass for the biofuels industry (El-Shinnawi et al., 1989). Water hyacinth is able to grow in any habitat and require little maintenance but it prefer to grow in warm climate. Further, it can be used to remove heavy metals from contaminated
waters, for the production of biogas and the byproducts can be used as organic manure or for producing bioethanol by further decomposition of fermentable saccharides (Nigam 2002).

In addition, aquatic plants such as water hyacinth do not compete with land resources used in arable food crop cultivation and thus are an incentive factor when it comes to the production of biofuels (Mishima et al., 2008). Water hyacinth can be fairly compared with other lignocellulosic materials like agricultural waste used for the production of bioethanol (Mishima et al., 2008), thus it can be considered as a potential new source of biomass for biofuels production and employment generating industry.

2.4 Water hyacinth as a biomass for xylitol production

At present, xylitol production by chemical methods is expensive, especially the purification process. Therefore, biotechnological production of xylitol from fermented hemicellulosic hydrolysate using microorganisms such as fungi, bacteria and yeast has become more attractive since the downstream process is expected to be cheaper (Pessoa et al., 1997). A large amount of xylitol is obtained using Candida yeasts like C. mogii, C. tropicalis and C. guilliermondii. (Granstrom et al., 2001). In water hyacinth, hemicellulose content is high (Table 1.5) and it is suitable source for xylose and xylitol production by biotechnological methods.
Table 2.1: Chemical composition of water hyacinth (average reported by Chanakya et al., 1993, Patel V et al., 1993, Abraham and Kurup 1996, Kumar A et al, 2009 and Ahn et al., 2012)

<table>
<thead>
<tr>
<th>Main composition</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>24.6</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>32.39</td>
</tr>
<tr>
<td>Lignin</td>
<td>10.36</td>
</tr>
<tr>
<td>Other compounds</td>
<td>33</td>
</tr>
</tbody>
</table>

2.5 Pretreatment and hydrolysis of lignocelluloses.

Due to the rigid structure of lignocellulosic biomass, very few microorganisms can use the biomass directly for growth and production. Therefore, prior to feeding the biomass into fermentors, a pretreatment-hydrolysis step is carried out to break down the structure of lignocellulosic biomass and hydrolyze the exposed polysaccharides into monomers. (Mosier et al., 2005)

But this degradation process is complicated, energy-consuming and not completely developed (Sims et al., 2010, Scordia et al., 2011, Sanchez and Cardona, 2008)

![Lignocellulose and pretreatment](image)

**Figure 2.2: Lignocellulose and pretreatment (Zha and Punt, 2013)**
There are at least two hydrolysis methods which are still of interest to many researchers for breaking down lignocelluloses to fermentable sugars in the development of ethanol production. The most commonly applied methods can be classified into two groups: chemical hydrolysis and enzymatic hydrolysis. In addition, there are some other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocelluloses may be hydrolyzed by gamma-ray or electron-beam irradiation, or microwave irradiation. However, these processes are far from being commercially applied (Taherzadeh 1999).

2.5.1 Chemical hydrolysis

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and release sugar monomers from cellulose and hemicellulose polymers. Chemical hydrolysis involves the use of dilute or concentrate acids, sulfuric acid is the most investigated acid (Taherzadeh and Karimi, 2007), although other acids such as HCl has also been used. There are two types of acid hydrolysis processes commonly used: Dilute and concentrated acid hydrolysis.

The dilute acid process is conducted under high temperature and pressure that has short reaction time. The concentrated acid process is conducted under mild temperatures, but at high concentration of sulfuric acid and a minimum pressure. Reaction times are typically much longer than that of dilute acid process (Tsoutsos and Bethanis, 2011)
2.5.2 Pretreatment of lignocellulosic materials

The aim of the pretreatment is to make the cellulose or hemicellulose more accessible to enzymatic attack due to weakening of the protecting lignin matrix or due to alteration of the pores in the material. There are a wide range of different pretreatment methods. Steam explosion and dilute-acid pretreatment are among the most common (Sun and Cheng, 2002)

Typical goals of pretreatment include

- Production of highly digestible solids that enhances sugar yields during enzyme hydrolysis.
- Avoiding the degradation of sugars (mainly pentoses) including those derived from hemicellulose.
- Minimizing the formation of inhibitors for subsequent fermentation steps.
- Recovery of lignin for conversion into valuable coproduces.
- To be cost effective by operating in reactors of moderate size and by minimizing heat and power requirements. (Brodeur Get al., 2011)
Pretreatment has been recognized as one of the most expensive processing steps in bioconversion of lignocellulose to fermentable sugars and several recent review articles provide a general overview of the field (Alvira et al., 2009, Carvalheiro et al., 2008; Hendriks and Zeeman, 2008, Taherzadeh and Karimi, 2008).

In an ideal case, the pretreatment employed leads to a limited formation of degradation products that inhibit enzymatic hydrolysis and fermentation and is also cost effective. However, these are actually the primary challenges of current pretreatment technologies.

2.5.2.1 Physical pretreatments

Physical pretreatments can be classified into two categories, mechanical and non-mechanical pretreatments (irradiation, high pressure steaming and pyrolysis).

A common purpose of both categories is to converse lignocellulosic materials into fine particles which are substantially susceptible to saccharification. The smaller particles have a large surface-to-volume ratio thus making cellulose or hemicellulose more accessible to hydrolysis. Mechanical pretreatment is usually carried out before following the processing step. (Harmsen et al., 2010)

2.5.2.1.1 Milling

Reduction of particle size is often needed to make material handling easier and to increase surface/volume ratio. Reduction of particle size can be done by chipping, milling or grinding. The desired particle size is dependent on these subsequent steps. (Yadav et al., 2011).
2.5.2.1.2 Ultrasonic pretreatment

The method of ultrasonic for lignocellulosic biomass is investigated at laboratory scale. It is a well known technique for treatment of sludge from waste water treatment plants. The issue of lignocellulose pretreatment is not addressed in the experiments. The experimental results showed that when a suspension of cellulose is provided with energy by irradiation, the reaction rate of the following enzymatic hydrolysis is increased by approximately 200% (Imai et al., 2004)

2.5.2.2 Chemical and combined pretreatment

This group belongs to the pretreatments that are only initiated by chemical reactions for disruption of the lignocellulose structure.

2.5.2.2.1 Liquid hot water

Liquid hot water (LHW) processes are biomass pretreatments with water at high temperature and pressure. Other terms are hydrothermolysis, hydrothermal pretreatment, aqueous fractionation, solvolysis or aquasolv (Mosier et al., 2005).

2.5.2.2.2 Weak acid hydrolysis

Dilute acid treatment is one of the most effective pretreatment methods for lignocellulosic biomass. In general there are two types of weak acid treatment:

1. High temperature and continuous flow process for low-solids loading (T> 160 °C, 5-10 wt% substrate concentration).

2. Low temperature and batch process for high-solids loading (T≤160 °C, 10-40% substrate concentration) (Chen et al., 2007).
In addition to inorganic acids, organic acids (e.g. maleic acid, fumaric acid) can be used for dilute acid pretreatment (Kootstra et al., 2009).

2.5.2.2.3 **Strong acid hydrolysis**

Concentrated strong acids such as $\text{H}_2\text{SO}_4$ and $\text{HCl}$ have been widely used for treating lignocellulosic materials because they are powerful agents for lignocellulose hydrolysis (Sun Y and Cheng, 2002), and no enzymes are needed subsequently for acid hydrolysis. The advantage of concentrated acid hydrolysis is the flexibility in terms of feedstock choice, high monomeric sugar yield as well as mild temperature conditions that are needed. (Yadav et al., 2011).

2.5.2.2.4 **Alkaline pretreatment**

The main effect of alkaline pretreatment is the removal of lignin from the biomass, thus improving the reactivity of the residual polysaccharides (Chang and Holtzapple, 2000).

2.5.2.2.5 **Calcium or sodium hydroxide**

Typically lime (calcium hydroxide) or sodium hydroxide is used to form salts that may be integrated in the biomass and need to be removed or recycled (Gonzalez et al., 1986). Process conditions are comparatively mild but reaction times can be long. These mild conditions prevent condensation of lignin, resulting in high lignin solubility, especially for biomass with low lignin content such as softwood and grasses. Due to the mild conditions, degradation of sugars to furfural, HMF and organic acids is limited (Chang and Holtzapple, 2000).
2.5.2.2.6 Ammonia

Reaction of biomass with aqueous ammonia at high temperatures reduces lignin content and removes some hemicellulose while de-crystallizing cellulose. (Kim et al., 2008).

2.5.2.2.7 Organosolv pretreatment

Organosolv processes use an organic solvent or mixtures of organic solvents with water for removal of lignin before enzymatic hydrolysis of the cellulose fraction. In addition to lignin removal, hemicellulose hydrolysis leads to improved enzymatic digestibility of the cellulose fraction. Frequent solvents for the process include ethanol, methanol, acetone, ethylene glycol and inorganic or organic acids (Sun and Cheng, 2002).

2.5.2.2.8 Oxidative delignification

Pretreatment of lignocellulose can also be achieved by treatment with an oxidizing agent such as hydrogen peroxide, ozone, oxygen or air. (Bujanovic et al., 2010).

2.5.2.2.9 Hydrogen peroxide

An oxidative compound commonly used is hydrogen peroxide ($\text{H}_2\text{O}_2$). Dissolution of about 50% of lignin and most of the hemicellulose has been achieved in a solution of 2% $\text{H}_2\text{O}_2$ at 30 °C. The yield of $\text{H}_2\text{O}_2$ treatment followed by enzymatic hydrolysis followed can be as high as 95%. (Efanov and Averin, 2004).

2.5.2.2.10 Ozonolysis

Ozone treatment can be used to disrupt the structure of many different lignocellulosic materials, such as wheat straw, bagasse, pine, peanut, cotton straw and poplar sawdust
Ozone treatment focuses on lignin degradation by attacking of aromatic rings structures, while hemicellulose and cellulose are hardly decomposed (Sun and Cheng, 2002).

2.5.2.2.11 Wet oxidation

Wet oxidation can be used to fractionate lignocellulosic material by solubilizing hemicellulose and removing lignin (Martín et al., 2007). There are two reactions that occur during this process. One is a low temperature hydrolysis reaction and the other is a high temperature oxidation reaction. During wet oxidation, lignin is decomposed to carbon dioxide, water, and carboxylic acids (Banerjee et al., 2009).

2.5.2.2.12 Steam explosion

Steam-Explosion pretreatment is one of the most commonly used pretreatment options, as it uses both chemical and physical techniques in order to break the structure of the lignocellulosic materials. This hydrothermal pretreatment method subjects the material to high pressures and temperatures for a short duration of time after which it rapidly depressurizes the system, disrupting the structure of the fibrils. The disruption of the fibrils increases the accessibility of the cellulose or hemicellulose to the enzymes during hydrolysis. Particle size is a major causative factor on the effectiveness of the process, and it has been seen that relatively large particle sizes have been able to yield maximum sugar concentrations. This is a promising finding, as decreasing the particle sizes of the material requires further mechanical processing of the raw material driving up the production costs (Ballesteros et al., 2002).
2.5.2.2.13 Ammonia fiber explosion (AFEX)

In the AFEX process, lignocellulose is treated with liquid ammonia at high temperature and pressure (Teymouri et al., 2005). After a few seconds, pressure is quickly reduced. A typical AFEX process is carried out with 1-2 kg ammonia/kg dry biomass at 90 °C during 30 min. It reduces the lignin content and removes some hemicellulose while de-crystallizing cellulose.

The AFEX processes are used for different feed stocks (Mosier et al., 2005, Kumar et al., 2009).

2.5.2.2.14 Ammonia Recycle Percolation (ARP).

In this process, aqueous ammonia of concentration between 5–15% is sent through a packed bed reactor containing the biomass feed stock at a rate of about 5 mL/min. Moderately high temperatures (140 °C to 210 °C) and longer reactions times are seen in comparison to the AFEX process, creating higher energy costs (Kim et al., 2006). Ammonia recycle percolation (ARP) has been paired with the AFEX pretreatment process by many researchers but it can have some different characteristics that needs to be taken into consideration when looking at different pretreatment options (Brodeur G et al., 2011).

2.5.2.2.15 CO₂ explosion

This method is similar to steam and ammonia fiber explosion; high pressure CO₂ is injected into the batch reactor and then liberated by an explosive decompression. It is believed that CO₂ reacts to carbonic acid (carbon dioxide in water), thereby improving the hydrolysis rate. Yields of CO₂ explosion are less than those obtained with steam or
ammonia explosion but they are higher than those that reached enzymatic hydrolysis without pretreatment (Sun and Cheng, 2002).

2.5.2.2.16 Mechanical/alkaline pretreatment

Combined mechanical/alkaline pretreatment consists of a continuous mechanical pretreatment (e.g. milling, extrusion, refining) of lignocellulosic biomass with the aid of an alkali. The resulting fractions consist of a soluble fraction (containing lignin, hemicellulose and inorganic components) and a cellulose-enriched solid fraction (Andersen, 2007).

2.5.2.2.17 Biological pretreatment

Biological pretreatment, as normally clear, involves the use of microorganisms (mainly fungi) to degrade lignin and hemicellulose but leave the cellulose intact (Kumar and Wyman 2009, Shi et al., 2008). Lignin degradation occurs through the action of lignin degrading enzymes secreted by the microorganisms. Even though biological pretreatment involve mild conditions and are of low cost, the disadvantages include low rates of hydrolysis and long pretreatment times (Sun and Cheng, 2002). Current efforts in biological pretreatments are combining this technology with other pretreatments and are developing novel microorganisms for rapid hydrolysis (Sanchez, 2009).

2.6 Enzymatic hydrolysis of lignocellulose materials

After initial biomass processing by milling, the production of fermentable sugars is usually approached in two steps:

1. A pretreatment process in which the cellulose polymers are made accessible for further conversion. In this step hydrolysis of hemicellulose may occur (depending on the process
conditions) as well as separation of the lignin fraction (for production of chemicals, combined heat and power production or other purposes);

2. Enzymatic cellulose hydrolysis (Harmsen et al., 2010).

The advantages of enzymatic hydrolysis are high yields due to the highly specific conversion and enzymatic hydrolysis is performed at moderate temperatures. Furthermore, the by-product formation is low. The disadvantages of enzymatic hydrolysis are the slow reaction rate and the high enzyme cost (Brodeur et al., 2011).

Factors effecting enzymatic degradation of Lignocellulose can be divided in two groups:

1. Enzyme related factors.
2. Substrate related factors.

2.6.1 Enzyme related factors

Several factors associated with the nature of the enzyme system have been suggested to be significant during the hydrolysis process. These include:

- Enzyme concentration
- Adsorption of Enzyme
- Synergism reaction
- End-product inhibition
- Mechanical deactivation (fluid shear stress or gas-liquid interface),
- Thermal inactivation
- Irreversible (non-productive) binding to lignin. (Andersen N, 2007)

2.6.2 Substrate related factors

The rate of enzymatic hydrolysis of Lignocellulose is affected by the structural features of lignocelluloses used as substrates. main substrate related factors are ;

- Crystalline nature of cellulose,
Degree of polymerization (DP), i.e. molecular weight of cellulose,
Available/accessible surface area,
Structural organization, i.e. macro-structure (fiber) and microstructure (elementary micro fibril) and particle size, and,
Presence of associated materials such as hemicellulose and lignin (Andersen, 2007).

2.6.3 Types of enzyme hydrolysis and ethanol production from lignocelluloses

In separate hydrolysis and fermentation (SHF), cellulose is hydrolyzed enzymatically into glucose and/or hemicellulose to pentose at first, and then sugars are fermented into ethanol by microorganism (Fig 2.5). Its primary benefit is its ability to perform each step at its optimum temperature range: 45-50°C for the enzymatic hydrolysis and around 30°C for the fermentation (Philippidis, 1996). The co-fermentation scheme involve the presence of a co-culture capability of converting the mixed sugars into ethanol. It is known that when the glucose level in the feedstock is much higher than xylose, co-fermentation could be a more efficient approach since the cost of separate processes would be high (Wyman et al., 2005, De Bari et al., 2004).

![Figure 2.4: Separate hydrolysis and fermentation (Chandel et al., 2007)](image-url)
Simultaneous saccharification and fermentation (SSF) involves the enzymatic hydrolysis of cellulose and hemicellulose to sugars, and the conversion of fermentable sugars to ethanol in the same vessel (Fig 2.6). The Simultaneous saccharification and fermentation technique provides the possibility to overcome the main difficulty of enzymatic hydrolysis i.e., decreasing the enzyme loading and therefore the production cost, making application of SSF for conversion of lignocellulosic to ethanol a more cost-effective process. The main problem of this technique is the difference among the optimum temperatures used for the enzymatic hydrolysis and the optimum temperatures used in the ethanol fermentation (Wyman et al., 2005, Kadar et al., 2004).

2.7 Fermentation inhibitors

During biomass hydrolysis by different pretreatment methods, inhibitor agents were generated. Most of these components are toxic to microorganisms showing negative effects on the subsequent fermentation process. This is a well-known problem associated with the acid hydrolysis process yet to be resolved (Millati et al., 2011).

Figure 2.5: Simultaneous saccharification and fermentation (Chandel et al., 2007)
Some of inhibitor elongate lag- reduce growth rate, some lower product yield, while others stop growth completely, these inhibitory compounds are mostly sugar and lignin degradation products, which can be different in each hydrolysate (Zha and Punt, 2013).

The crude sugar solution from the acid process contains various degradation products (Fig 2.6). The major types of inhibitors are discussed below and summarized in Figure 2-7. The inhibitory effect of these compounds is higher when they are present together due to a synergistic effect (Mussatto and Roberto, 2004).

![Figure 2.6: Composition of wood and compounds generated during acid hydrolysis (Ibraheem and Ndimba, 2013)](image)

### 2.7.1 Avoiding inhibition problems

The level of toxicity depends partially on fermentation variables including cell physiological conditions, dissolved oxygen concentration, pH of the medium etc. In addition, the fermenting organisms to some extent may be resistant to inhibitors or may
gradually get adapted to their presence. One of the major challenges faced in commercial production of lignocellulosic bioethanol is the inhibitory compounds generated during the thermo-chemical step of biomass.

Different ways for reduce negative effects of inhibitors:

- Special design of the fermentation process (Lin and Tanaka, 2006).
  - Batch mode fermentation
  - Fed-batch fermentation
  - Continuous fermentation
- Selection of highly resistant microorganisms, strain (Lin and Tanaka, 2006).
- Strain adaptation
- Genetic engineering for strain improvement (Larsson et al., 2001).

2.7.2 Detoxification

To reduce the toxicity of biomass, detoxification methods have been developed which remove the inhibitors present in hydrolysates. The effects of detoxification were improved fermentability and increased product yield (Alriksson et al., 2011).

2.7.2.1 Detoxification methods

Detoxification can be physical, chemical, and biological (Larsson et al. 1999a).

2.7.2.1.1 Chemical detoxification methods:

- Neutralization (Yu and Zhang, 2003).
- Alkaline detoxification (over liming) (Martin et al., 2007).
- Detoxification with sulfite.
- Ionic exchange detoxification: (Palmqvist and Hahn-Ha, 2000).
- Detoxification with wood-ash (Miyafuji et al., 2003a,b).
2.7.2.1.2 Physical detoxification methods:

- Evaporation (Converti A et al., 2000)
- Supercritical fluid extraction (Persson et al. 2002a,b)
- Adsorption by resins Lee et al. (1999)
- Adsorption by activated charcoal (Mussatto et al., 2004)

2.7.2.1.3 Biological detoxification method

There are two main types of biological detoxification method:

Enzymatic detoxification (Martin et al., 2002) and microbial detoxification i.e. treatment with *Trichoderma reesei* (Larsson et al., 1999b), using native or recombinant microorganism (Lopez et al., 2004), adaptation of microorganism (Silva and Roberto, 2001) and high amount inoculum (Taherzadeh et al., 1999).

2.8 Biotechnological production of ethanol

Fermentation is the breakdown of carbohydrates to ethanol, carbon-di-oxide and water using micro organisms. The raw materials used in the manufacture of ethanol via fermentation are classified under three types of agricultural raw materials:

1. Sugar
2. Starches
3. Cellulose materials (Osunkoya and Okwudinka, 2011)

Processing of lignocelluloses to ethanol consists of four major division operations: pretreatment, hydrolysis, fermentation, and product separation/purification.

Ethanol fermentation can be carried out by three main strategies: simultaneous saccharification and co-fermentation (SSCF), simultaneous saccharification and fermentation (SSF) and/or separate hydrolysis and fermentation (SHF) (Peterson, 2006).

Ethanol is recovered from the medium by distillation or distillation combined with
adsorption. The residual lignin, un-reacted cellulose and hemi cellulose, ash, enzyme, microorganism and other components end up in the bottom of the distillation column (Lin and Tanaka 2006). These materials may be concentrated and used as fuel to power the process or converted to various co-products (Mosier et al., 2005).

Figure 2.7: Different raw materials for biotechnological ethanol production

2.9 Fermentation

The anaerobic conversion of sugar to carbon dioxide and alcohol by yeast is known as Fermentation. Since fruits ferment naturally, fermentation precedes human history. Fermentation has been used by humans for the production of food and beverages since the Neolithic age. Fermentation is a process by which the living cell is able to obtain energy through the breakdown of glucose and other simple sugar molecules without requiring oxygen. (Pearson 2006).

2.9.1 Microorganism

The sugar syrup obtained after lignocellulosic hydrolysis is used for ethanol fermentation. The ability to ferment pentoses along with hexoses is not widespread among microorganisms S. cerevisiae is capable of converting only hexose sugars to ethanol. The
most promising yeasts that have the ability to use both C5 and C6 sugars are *Pichia stipitis*, *Candida shehatae* and *Pachysolan tannophilus* (Table 2.2)

**Figure 2.8:** Different types of fermentation products (Pearson 2006)

### 2.9.2 Why Thermotolerant yeast?

Yeast in general, grows at relatively temperatures in comparison with bacteria with upper temperature limits for thermotolerant and thermophilic yeasts being 42 °C and 45 °C respectively (Koedritha et al., 2008). Some species are more thermo tolerant than others; the maximum temperature for growth and fermentation depends on many factors. The optimum temperature can be defined as the temperature at which growth or fermentation rates or the cellular yield is the highest (Koedritha P et al., 2008)
Thermo tolerant yeast could be more suitable for ethanol production at industrial level. At higher temperature, energy savings can be achieved through a reduction in cooling costs. Considering this approach, solid state fermentation system for ethanol production is carried out using thermo tolerant *S. cerevisae* strain (VS3) (Sree et al. 1999, Zhang et al 2012).

**Table 2.2: Different types of microorganism capable fermenting xylose**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Ability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 <em>P. tannophilus</em></td>
<td>Ferment xylose, glucose and glycerol</td>
<td>Zhao L et al., (2010)</td>
</tr>
<tr>
<td>2 <em>Mucor species</em></td>
<td>Ferment pentoses</td>
<td>Ueng and Gong, (1982)</td>
</tr>
<tr>
<td>3 <em>C. shehatae</em></td>
<td>Has both active and positive transport system for xylose uptake: produces moderate amount of xylitol and does not grow anaerobically: requires biotin and thiamine</td>
<td>Jeffries and Shi, (1999)</td>
</tr>
<tr>
<td>4 <em>P. stiptis</em></td>
<td>Ferment all sugars found in wood, some strains ferment xylan</td>
<td>Mujgan and Nurdan, (2006)</td>
</tr>
<tr>
<td>5 <em>C. boidinii</em></td>
<td>Produces large amount of xylitol: oxidizes methanol</td>
<td>Winkelhausen et al., 1996</td>
</tr>
<tr>
<td>6 <em>F. oxysporum</em></td>
<td>Ferments different carbon sources</td>
<td>Singh and Kumar, (1991)</td>
</tr>
</tbody>
</table>

**2.9.3 Types of fermentation process:**

Fermentation can be performed as a batch, fed batch or continuous process. The choice of most suitable process will depend upon the kinetic properties of microorganisms and type of lignocellulosic hydrolysate in addition to process economics aspects.

**2.9.3.1 Batch fermentation processes**

At present, almost all of the ethanol fermentation industry uses the batch mode. In batch fermentation, the microorganism works in high substrate concentration initially and a high
product concentration at the end (Olsson and Han-Hagerdal, 1996). The batch process is a multi-vessel process, which allows flexible operation and easy control. In batch fermentation productivity is low with an intensive labour. Batch fermentation is not a suitable method for lignocellulosic hydrolyzates, since a high concentration of the inhibitors at the beginning of fermentation deactivates the yeasts and decreases productivity (Gupta 2006, Sanchez et al., 2004 and Beatriz et al, 2005).

2.9.3.2 Fed batch fermentation process:

In fed batch fermentation, the microorganism works at low substrate concentration with an increasing ethanol concentration during the course of fermentation process. Fed-batch technique is a promising method for the fermentation of dilute-acid hydrolyzates (Nilsson et al. 2001; 2002, Taherzadeh et al., 2007).

2.9.3.3 Continuous fermentation process:

The major drawback of continuous fermentation is that inhibitors present in the medium will limit the specific growth rate of the cells. This will result in wash-out of the bioreactor, unless a very low dilution rate is applied resulting in low productivity. Furthermore, at a very low dilution rate, the conversion rate of the inhibitors can be expected to decrease due to the decreased specific growth rate of the biomass. Thus, wash-out may occur even at very low dilution rate. Cell retention by "immobilization," encapsulation," "filtration," and "cell recirculation" by using e.g. centrifuges or flocculating organisms are solutions to overcome the wash-out problem in continuous cultivation of dilute-acid hydrolyzates. This mode of fermentation can be performed in different bioreactors (single or series of stirred tank reactors, plug flow reactors). This mode of
fermentation often gives higher productivity than batch fermentation but at low dilution rates which offers the highest productivities. (Karhumaa et al., 2006, Hahn-Hagerdal et al., 2007; Zhang et al., 2010).

2.10 Xylitol production from lignocellulose materials

Xylitol can be found naturally in various fruits and vegetables such as strawberries, raspberries, yellow plum, lettuce and cauliflower (Prakasham et al., 2009). Several technologies are available for xylitol production. Xylitol can be produced by the extraction from natural sources, chemical conversion of xylose or biotechnological processes. It is produced mainly by chemical processes. (Prakasham et al., 2009).

The chemical process has some drawbacks such as high cost of purification processes. To avoid aggressive stages of the chemical processes, biotechnological processes were studied for xylitol production. This alternative production is bioconversion of D-xylose to xylitol by microorganisms. To reduce costs and environmental problems renewable biomass from agro-industrial waste can be used as source of D-xylose (Domínguez et al., 2012).

2.10.1 Extraction

Xylitol can be extracted from natural sources (fruits and vegetable) by solid-liquid extraction, but its small proportion in the raw materials (less than 900 mg/100g) is a major economic problem (Hyvonen et al., 1982). It would be very uneconomical to extract pure xylitol from natural sources due to their own high cost and relatively low concentrations of xylitol.
2.10.2 Chemical process

On a large scale, xylitol is currently produced by the chemical conversion of xylose as shown in figure 2.9. The potential sources of xylose are hard woods or soft woods containing xylan such as birch wood, sugar cane bagasse, straw and corn cobs. This process is the hydrogenation of the five-carbon sugar D-xylose in the presence of nickel as catalyst at elevated temperature and pressure (Prakasham et al., 2009).

![Chemical production of xylitol](image)

**Figure 2.9: Chemical production of xylitol (Hyvonen et al., 1982)**

2.10.3 Biotechnological processes

The chemical process has some drawbacks such as high cost of purification processes. To avoid aggressive stages of the chemical processes, biotechnological processes were studied for xylitol production. They are based on the utilization of microorganisms (bacteria, fungi and yeast), enzymes or both (Parajo et al., 1998a). To reduce costs and environmental problems renewable biomass from agro-industrial waste can be used as source of D-xylose. Biotechnological production of xylitol was extensively studied as an alternative in order to clarify the metabolic pathways involved in microbial growth in the presence of non-conventional compounds (Dmytruk et al., 2008).

Biotechnological methods for xylitol production are becoming attractive and are currently being developed.
2.11 Factors influence xylitol production in yeast

The biotechnological production of xylitol using yeasts is controlled by a series of factors such as: substrate concentration, carbon source, inoculums etc. (Ghindea et al., 2010).

Figure 2.10: Production of xylitol from lignocellulose materials (Chen et al., 2010)

Figure 2.11: Xylose Metabolism in yeasts (Parajo et al., 1998)
2.11.1 Temperature

Xylitol is produced by most yeast in the range of 24 - 45°C, and depends on the microorganism. The optimal temperature range is between 28 - 30°C. Xylitol production was uninterrupted in temperature range between 35 - 40°C for Candida sp. (Cao et al., 1994) and at a temperature range between 28 - 37°C for D. hansenii (Dominguez et al., 1997, Sampaio et al. 2006).

2.11.2 pH

The optimum initial pH cultivation for xylitol production depends on the yeast used (Table 2.3). Converti A et al., 2003 reported that with increase in pH, the optimum xylose transport across the cell membrane is limited due to this limitation at sun-optimum pH values xylitol yield and productivities decrease.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Initial pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida sp.</td>
<td>6</td>
<td>Cao NJ et al. (1994)</td>
</tr>
<tr>
<td>C. shehatae</td>
<td>4.5</td>
<td>Kastner et al. (1996)</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>4</td>
<td>Yahashi Y et al. (1996)</td>
</tr>
<tr>
<td>C guilliermondii</td>
<td>6</td>
<td>Nolleau et al. (1995)</td>
</tr>
<tr>
<td>Debaryomyces hansenii</td>
<td>5.5</td>
<td>Dominguez et al. (1996)</td>
</tr>
<tr>
<td>P. tannophilus</td>
<td>8</td>
<td>Debus et al. (1983)</td>
</tr>
</tbody>
</table>

2.11.3 Aeration

The dissolved oxygen concentration is an important parameter in xylitol production by yeast. The oxygen transfer rates (OTR) effects production of xylitol. Among Pichia stipitis, Pachysolen tannophilus, C. shehatae and C. parapsilosi. The C. parapsilosis culture gave high xylitol yield and higher rates of production at a KLa (oxygen transfer
An oxygen transfer rate (OTR) of 2.2 mmol/l.h produced a maximum xylitol yield of 0.66 g/g in *C. guilliermondii*, whereas oxygen transfer rate (OTR) of 0.4 mmol/l.h produced a maximum xylitol yield of 0.75 g/g at pH 4.75 in *C. parapsilosis* (Nolleau et al., 1995).

### Table 2.4: Effect of aeration on xylitol production by yeast (Source: Saha and Bothast, 1997)

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Aeration</th>
<th>Xylose (g/l)</th>
<th>Xylitol yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. parapsilosis</em></td>
<td>Microaerobiosis</td>
<td>100</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Semiaerobiosis</td>
<td>100</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>Aerobiosis</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td><em>C. guilliermondii</em></td>
<td>700 ml/min</td>
<td>100</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Microaerobiosis</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Semiaerobiosis</td>
<td>100</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Aerobiosis</td>
<td>100</td>
<td>0.56</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>100 ml/min</td>
<td>100</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>400 ml/min</td>
<td>100</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>500 ml/min</td>
<td>100</td>
<td>0.45</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>0.15 vvm</td>
<td>10</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>0.30 vvm</td>
<td>10</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>0.60 vvm</td>
<td>10</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>1.00 vvm</td>
<td>10</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>1.50 vvm</td>
<td>10</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>2.00 vvm</td>
<td>10</td>
<td>0.04</td>
</tr>
</tbody>
</table>

### 2.11.4 Xylitol inhibition

Accumulation of xylitol can effect on microorganism as inhibitor. Xylitol consumption has been observed after complete utilization of xylose in yeasts (Parajo et al., 1998). Accumulation of 50 g/l xylitol caused inhibition in fermentation by *C. shehatae*. Da Silva
and Afschar (1994) also observed inhibition at 200 g/l xylitol during *C. tropicalis* fermentation.

### 2.11.5 Carbone source

It is important to understand the effect of hexose and other pentose sugars in xylose utilization and xylitol production as hydrolysates contain sugar mixtures of varied compositions. D-galactose, D-cellobiose and L-arabinose do not affect D-xylose utilization, while D-mannose and D-glucose affect xylose utilization (Winkelhausen and Kuzmanova 1998, Lucas et al., 1986). Glucose when added as a supplement for fermentation by *C. tropicalis* growing on xylose in 3-L batch fermentation increases xylitol production (104.5 g/l) as much as 1.3 times (Saha and Bothast, 1997). *C. boidinii* in batch fermentation containing a mixture of glucose and xylose showed a faster growth compared to xylose alone in the medium. Maximum xylitol production of 59.3 g/l was obtained with a mixture of glucose and xylose in culture medium compared to 41g/l obtained with only xylose as carbon source in culture medium.

### 2.11.6 Nitrogen source

The type and concentration of the nitrogen source in the medium influences the xylitol production and bioconversion of xylose by the microorganisms. Organic nitrogen supplement like yeast extract have been shown to increase xylitol production compared to nitrogen salts (Saha and Bothast, 1997 and Horitsu et al., 1992). Results of analyzing eight ammonium salts and four organic nitrogen sources used for xylitol production with *P. albertensis* showed that ammonium acetate was most effective nitrogen source all the
among ammonium salts and organic nitrogen sources as the most suitable for xylitol formation (Saha and Bothast, 1997, Kim and Moon, 2003).

### 2.11.7 Inoculum size and age

The age of inoculum has played an important role in metabolic activity and viability of cells (du Preez, 1994). A 24 h old inoculum of *C. shehatae* produced 20 g/l (Sreenath et al., 1986) after 22 h with an yield of 0.24 g/g, whereas a 72 h old inoculum produced only 9 g/l xylitol after 65 h with a yield of 0.13 g/g. Productivity and cell growth were desirable when inoculum age was 15 h - 24 h for *C. guilliermondii* (Pfeifer et al., 1996).

### 2.11.8 Initial Substrate concentration

Growth rate will decrease with increase in initial xylose concentration, unless the aeration rate was increased. Da Silva and Afschar, (1994) observed inhibition in growth due to the high initial substrate concentration. Optimum initial xylose concentration is necessary for growth and xylitol production. Initial xylose concentration in the range of 20-50 g/l produced the highest specific growth in *C. guilliermondii* (Meyrial et al., 1991). The best initial xylose concentration reported for xylitol production was 60 g/l for *P. tannophilus*, 200 g/l (Gong et al., 1981) and 100 g/l (Da Silva and Afschar, 1994) for *C. tropicalis* and 200 g/l for *C. guilliermondii* (Meyrial et al., 1991).

### 2.11.9 Cell density

Another effective factor in bioconversion of xylose to xylitol by microorganisms is cell density. The effect of cell density on xylitol production has been studied in different yeasts. In *C. boidinii* high cell density has been shown to increase the xylitol yield and specific productivity of xylitol (Saha and Bothast, 1997, Winkelhausen and Kuzmanova...
In *Debaryomyces hansenii*, increase in the initial cell density from 0.3 g/l to 3 g/l increased xylitol productivity from 0.68 g/l/h to 2.25 g/l/h (Dominguez et al., 1997, Cao et al., 1994) reported an increase in xylitol production when the initial cell concentration was increased from 3.8 to 14 g/l in *Candida sp.*

2.11.10 Vitamin supplementation

Vitamins in the medium as supplement have been shown to increase productivity and enhance growth in yeasts. Lee et al., (1988) observed a productivity increase from 0.002 g/l/h to 0.009 g/l/h, while a 0.25 μg/l biotin supplementation increased the productivity to 0.044 g/l/h. They also observed biotin supplementation increased xylitol production in *Pachysolen tannophilus*.

2.11.12 Fermentation strategy

Xylitol production in batch processes has high initial substrate concentration at the beginning of the process, but low substrate concentration and high product concentration and at the end of the process. The high xylose and xylitol in the media of batch process can inhibit xylitol production and reduce productivity (Winkelhausen and Kuzmanova, 1998). Continuous culture processes have shown better productivity and yield in many microorganisms. To achieve high production rates in continuous culture system low dilution rate is essential. But the low dilution rate makes the process imperial due to the increase in residence time.

In fed-batch techniques, a constant substrate concentration can be maintained during the fermentation to achieve higher productivity (Horistsu et al., 1992).