CHAPTER 2
REVIEW OF LITERATURE

2.1. Lignocellulosic biomass

About half of the carbon fixed by plants by photosynthesis is incorporated into the structural polysaccharides and lignin, collectively named as ligno-cellulose that makes up plant cell walls and wood. Coughlan (1985) reported that annual production of lignocelluloses through photosynthesis, is 40 billion tons. Lignocellulosic biomass is a potential resource for production of biofuels because of its abundance and low cost. Agricultural residues are the easily available, unexploited, renewable and inexpensive source of lignocellulosic biomass. Lignocellulose consists of three major components:

i. **Cellulose**, the major constituent of all plant material and the most abundant organic molecule on earth, is a linear biopolymer of anhydroglucopyranose-molecules, connected by β-1, 4-glycosidic bonds. Cellulose or β-1-4-glucan is a linear polysaccharide polymer of glucose made of cellobiose units. Native crystalline insoluble cellulose consists of densely packed, hydrogen bonded anhydroglucose chains of 15 to 10,000 glucose units. The cellulose chains are packed by hydrogen bonds in so-called ‘elementary microfibrils’. These fibrils are attached to each other by hemicelluloses, amorphous polymers of different sugars as well as other polymers such as pectin, and covered by lignin. The microfibrils are often associated in the form of bundles or macrofibrils. Cellulose is resistant to both biological and chemical treatments because of its complex cross linkages which accumulatively makes its binding strong (Delmer and Amor 1995, Morohoshi 1991, Ha et al. 1998). Bioconversion of cellulosic biomass to develop novel bioprocesses and useful products is an important area of research in biotechnology.

ii. **Hemicellulose**, the second most abundant component of lignocellulosic biomass, is a heterogeneous polymer of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and uronic
acids of glucose and galactose. Hemicellulose is less complex, its concentration in lignocellulosic biomass is 25 to 35% and it is easily hydrolysable to fermentable sugars (Saha and Cotta 2007). The dominant sugars in hemicellulososes are mannose in softwoods and xylose in hardwoods and agriculture residues (Persson et al. 2006, Lavarack et al. 2002, Balan et al. 2009).

iii. **Lignin**, the third main heterogeneous polymer in lignocellulosic residues, generally contains three aromatic alcohols, serves as a sort of ‘glue’ giving the biomass fibers its structural strength. Lignin acts as a barrier for any solutions or enzymes by linking to both hemicellulososes and cellulose and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Lignin is the most recalcitrant component of lignocellulosic material to degrade (Zaldivar et al. 2001, Hamelinck et al. 2005).

### 2.2. Lignocellulose substrates used for ethanol production

In Brazil cane juice is the substrate for ethanol production whereas in USA starch crops are used (Sanchez 2009). But these substrates are also the edible substrates which will cause controversy for ethanol production. Thus a new biofuel research using lignocellulosic waste produced by different industries is used to produce up to 0.52 million gallons of ethanol per year (Hahn et al. 2006). China is the world’s largest sweet potato (*Ipomoea batatas*) producer (accounting for 85% of global production), with the output exceeded 100 million tons in 2005 (Lu et al. 2006). Zhang et al. (2011) reported sweet potato as an attractive feedstock for bioethanol production. Sipos et al. (2009) reported that sweet sorghum bagasse can be converted efficiently into fermentable sugars by SO$_2$ catalyzed steam pretreatment at 190°C for 10 min or 200°C for 5 min followed by enzymatic hydrolysis with a result of 89-92% glucan conversion. Hemp and ensiled hemp has been reported to produce ethanol with steam pretreatment (2% SO$_2$ catalyst, 210°C for 5 min) followed by simultaneous saccharification and fermentation at high solid loading (7.5% water insoluble solids [WIS]) with a result of 171-163 g ethanol/kg raw material (Sipos et al. 2010).
Lignocellulosic wastes are produced in large amounts by different industries including forestry, pulp and paper, agriculture and food, in addition to different wastes from municipal solid waste (MSW), and animal wastes (Sims 2003, Kim and Dale 2004, Kalogo et al. 2007, Champagne 2007, Wen et al. 2004). Products of agricultural activities like straw, stem, stalk, leaves, husk, shell, peel were treated as waste in many countries in the past, and present in some developing countries, which raises many environmental concerns (Palacios-Orueta et al. 2005). Howard et al. (2003) reported that lignocellulosic residues can be converted to valuable products such as biofuels, chemicals and animal feed. As banana peel is rich in carbohydrate, crude proteins and reducing sugars, thus can be used as a substrate for ethanol production. Moreover, accountability of banana peels increases as it is affordable and renewable low cost raw material (Bhatia and Paliwal 2010, Thakur et al. 2013). Similarly pineapple is the second harvest of importance after bananas, contributing to over 20% of the world production of tropical fruits (Coveca 2002). Thailand, Philippines, Brazil and China are the main pineapple producers in the world supplying nearly 50% of the total output. Other important producers include India, Nigeria, Kenya, Indonesia, Mexico and Costa Rica and these countries provide most of the remaining fruit available (50%). Isitu and Ibeh (2010) assayed the feasibility of obtaining ethanol from pineapple waste with the purpose of obtaining a valuable product from the residues of the juice and canning industries.

Large volume of bagasse is generated during sugarcane processing. Agricultural profitability and environmental protection issues are associated with disposal of bagasse. In recent years, potential efforts have been directed towards the effective utilization of cheap renewable agricultural resources, such as sugarcane bagasse as alternative substrate for ethanol production (Bhatia and Paliwal 2011). Rice is the one of the major crop grown worldwide with the productivity around 800 million metric tons per year that corresponds with the large production of rice straw leading to as a suitable lignocellulosic waste for ethanol production (Wati et al. 2007).
Feasibility of lignocellulosic material for ethanol production has been explored and accepted globally because of its availability. Production of ethanol from wheat straw, one of the most abundant agricultural wastes, has been extensively studied (Curreli et al. 1997, Curreli et al. 2002, Ballesteros et al. 2004, Talebnia et al. 2010). The average yield of wheat straw is 1.3–1.4 lb per lb of wheat grain (Montane et al. 1998). According to Ballesteros et al. (2006), under the 60% ground cover practice, about 354 millions of tons of wheat straw could be available globally and could produce 104 GL of bioethanol. Europe production would account for about 38% of this world bioethanol capacity. In Spain, grain industry generates important amounts of wheat straw, a part of which is used as bedding straw and the remainder is burned or left on the land to fertilize the soil. Bioconversion of this residue to fuel ethanol would provide an attractive possibility to boost the development of biofuels in a sustainable way (Hansen et al. 2013).

Cotton linters and cotton seed hulls generated from cotton industry; paper pulp and paper mill sludge generated from paper industry; saw dust generated from saw mill industry; rice husk and rice bran generated from rice milling industry are also the potential agroindustrial cellulosic residues that can be used for bioethanol production.

2.3. Overview of lignocellulosic fermentation

Schematic picture for the conversion of lignocellulosic biomass to ethanol, including the major steps can been seen in figure 1. Pretreatment of the lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow, and results in low product yield. Some pretreatment methods increase the pore size and reduce the crystallinity of cellulose (Dawson and Boopathy 2007). Pretreatment also makes cellulose more accessible to the cellulolytic enzymes, thereby reducing enzyme requirements and cost of ethanol production. In addition to enhancing the biodigestibility of the wastes for ethanol production, it also causes enrichment of the difficult biodegradable materials, leading to increased yield of ethanol from the wastes.
After pretreatment, the recalcitrant lignocellulosic biomass becomes susceptible to acid and/or enzymatic hydrolysis as the cellulosic microfibrils are exposed and/or accessible to hydrolyzing agents (Jacobsen and Wyman 2000). In the pretreatment process, small amounts of cellulose and most of hemicellulose is hydrolyzed to sugar monomers; mainly D-xylose and D-arabinose. The pretreated biomass is then subjected to filtration to separate liquids (hemicellulose hydrolysate) and solid (lignin and cellulose). After detoxification, the liquid is sent to a xylose (pentose) fermentation column for ethanol production. Second stage hydrolysis involves hydrolysis of solids. This process is mainly accomplished by enzymatic methods using cellulases. Mild acid hydrolysis using dilute sulfuric and hydrochloric acids is an alternative procedure (Zhang and Lynd 2004). The hydrolyzed sugars such as D-glucose, D-galactose, and D-mannose, can be readily fermented to ethanol using various strains of *Saccharomyces cerevisiae*. The pentoses (D-xylose and D-arabinose) from hemicellulose hydrolysis are not easily utilized by Saccharomyces strains; therefore, genetically modified strains of *Pichia stipitis*, *Zymomonas mobilis*, are used for their fermentation. *Candida shehatae* is capable of co-fermenting both pentoses and hexoses to ethanol and other value-added products at high yields (Betancur 2005, Senthilkumar and Gunasekaran 2005).

Mosier et al. (2005), Sun and Cheng (2007), Yang and Wyman (2008) proposed numerous pretreatment strategies to enhance the reactivity of cellulose leading to increase in the yield of fermentable sugars. It is required to produce
highly digestible solids that enhances sugar yields during enzyme hydrolysis, without degradation of sugars (mainly pentoses) including those derived from hemicelluloses. To reduce the cost by operating in reactors of moderate size and by minimizing heat and power requirements and also minimizing formation of inhibitors for fermentation process and recovery. Figure 2 depicts schematic of goals of pretreatment on lignocellulosic material followed by headings that will elaborate the major steps involved in the conversion of lignocellulosic biomass to ethanol and various post analysis.

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Figure 2- Schematic representation of goals of pretreatment on lignocellulosic material
(http://www.amb-express.com/content/2/1/65)

2.4. Substrate pretreatment

Physical pretreatments methods such as ball milling and grinding have been used for degradation of lignocelluloses with limited success. This method of pretreatment is not only cost effective but ecofriendly too. Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce
and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millet et al. 1976). The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics (Cadoche and Lopez 1989). Pyrolysis was used for pretreatment of lignocellulosic materials. Cellulose rapidly decomposes to produce gaseous products and residual char, when the materials are treated at temperatures greater than 300°C (Kilzer and Broido 1965, Shafizadeh and Bradbury 1979).

Vinctoru et al. (1999) showed the efficiency of ultrasound in the processing of vegetal materials and observed swelling of vegetal cells and fragmentation due to the cavitation effect, which increases the yield and reduces the extraction time. The effect of ultrasound on lignocellulosic biomass has been employed in order to improve the extractability of hemicelluloses (Ebringerova et al. 2002), cellulose (Pappas et al. 2002), lignin (Sun and Tomkinson 2002) or to get clean cellulosic fiber from used paper (Scott and Gerber 1995). Rolz (1986) found out that ultrasound has a beneficial effect on saccharification processes. He reported that sonication decrease cellulase requirements by \( \frac{1}{3} \) to \( \frac{1}{2} \) and increases ethanol production from mixed waste office paper by approximately 20% (Wood et al. 1997). Gama et al. (1997) noticed that the effect of ultrasound fragmentation of avicel (microcrystalline cellulose formed by acid treatment) is similar to that of the enzymes for short incubation intervals. The time needed for ultrasonic treatment could be reduced when increasing the irradiation power (Imai et al. 2004).

Bases, such as sodium, potassium, calcium, and ammonium hydroxide, are involved in alkaline pretreatment for the pretreatment of lignocellulosic biomass, causes the degradation of ester and glycosidic side chains resulting in structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose (Cheng et al. 2010, Ibrahim et al. 2011) and partial solvation of hemicelluloses (McIntosh and Vancov 2010, Sills and Gossett 2011). Sodium hydroxide has been shown to disrupt the lignin structure of the biomass, increasing the accessibility of enzymes to cellulose and hemicellulose (MacDonald et al. 1983, Soto et al. 1994, Zhao et al. 2008). Lignocellulosic
feedstocks that have been shown to benefit from this method of pretreatment are corn stover, switchgrass, bagasse, wheat, and rice straw.

Hamelinck et al. (2005) suggested mild treatments for longer durations the conditions for alkaline pretreatment which are usually less severe than other pretreatments can be performed at ambient conditions, but longer pretreatment times are required. The advantage of lime pretreatment is basically its cost effectiveness. Most commonly used alkali in the alkali pretreatment processes are sodium hydroxide and calcium hydroxide results in (i) the removal of all lignin and part of hemicellulose, and (ii) increased reactivity of cellulose in further hydrolysis steps, especially, enzymatic hydrolysis. Effective removal of lignin minimizes adsorption of enzyme onto lignin and thus allows for effective interactions with cellulose (Aswathy et al. 2010). Pretreatment with Ca(OH)$_2$ is preferable over NaOH because it is less expensive, more safer as compared to NaOH and it can be easily recovered (Mosier et al. 2005b).

Acid pretreatment breaks the rigid structure of the lignocellulosic material. The most commonly used acid is dilute sulphuric acid (H$_2$SO$_4$), which has been commercially used to pretreat a wide variety of biomass. Acid pretreatment (removal of hemicellulose) followed by alkali pretreatment (removal of lignin) has shown to yield relatively pure cellulose (Widgren et al. 2003, Taherzadeh and Karimi 2008). Strong acid allows complete breakdown of the components in the biomass to sugars, but also requires large volumes of concentrated sulfuric acid and can result in the production of furfural, an inhibitory byproduct (Goldstein and Easter 1992). Dilute acid allows reduced acid concentrations, but requires higher temperatures, and again gives furfural. They reported the advantage of acid pretreatment is that thereby enzymatic hydrolysis step can be ignored, as the acid itself hydrolyses the biomass to yield fermentable sugars (Zhu et al. 2009). A mixture of H$_2$SO$_4$ and acetic acid resulted in 90% saccharification (De Moraes-Racha et al. 2010). Hemicellulose and lignin are solubilized with minimal degradation, and the hemicellulose is converted to sugars with acid pretreatment. The major drawback to these acid processes is the cost of acid and the requirement to neutralize the acid after treatment.
Wet oxidation consists of drying and milling lignocellulosic biomass to obtain particles that are 2 mm in length, to which water is added at a ratio of 1 liter to 6 g biomass. (Martin et al. 2007, Martin et al. 2008, Banerjee et al. 2009, Ruffell et al. 2010, Szijarto et al. 2009, Martin and Thomsen 2007). During wet oxidation, lignin is decomposed to carbon dioxide, water and carboxylic acids. Biomass such as straw, reed and other cereal crop residues have a dense wax coating containing silica and protein which is removed by wet oxidation (Schmidt et al. 2002). Wet oxidation has been combined with other pretreatment methods to further increase the yield of sugars after enzymatic hydrolysis. Combining wet oxidation with alkaline pretreatment has been shown to reduce the formation of byproducts, thereby decreasing inhibition. Bjerre et al. (1996) used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170°C, 5–10 min), and achieved 85% conversion yield of cellulose to glucose. Wet oxidation combined with base addition readily oxidizes lignin from wheat straw, thus making the polysaccharides more susceptible to enzymatic hydrolysis. Furfural and hydroxymethylfurfural, known inhibitors of microbial growth when other pretreatment systems are applied, were not observed following the wet oxidation treatment (Azzam 1989, Martin et al. 2007).

McMillan (1994) reported that steam-explosion pretreatment was 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 material. Hydrothermal pretreatment method when subjected to high pressures and temperatures for a short duration of time rapidly depressurizes the system, disrupting the structure of cellulose microfibrils which increases the accessibility of the cellulose to the enzymes during hydrolysis. Studies have reported that steam explosion is typically initiated at a temperature of 120-260°C (corresponding pressure 0.69–4.83 MPa) for a few minutes to several seconds before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (Ballesteros et al. 2006, Chornet et al. 1988, Focher et al. 1991). The factors that affect steam explosion pretreatment
that have been reported by Duff and Murray (1996) are residence time, temperature, chip size and moisture content. Some studies indicate that lower temperature and longer residence time is more favorable (Wright 1998).

After the removal of hemicellulose from the substrate during the acid/base treatment, the leftover solid material is called cellulignin which is accessible for enzymatic saccharification. Critical factors such as lignocellulosic substrate, temperature, acid load, residence time, and substrate-to-liquid ratio play key roles in breaking down hemicellulose into its monomeric constituents. Parawira and Tekere (2011) reported that, the non-specificity of acidic treatment leads to the formation of complex sugars and compounds inhibitory to the microorganisms for ethanol production.

Degradation of hemicelluloses and cellulose during pretreatment leads to glucose is the most ubiquitous monomeric carbohydrate in contemporary bioethanol literature, owing to its well-studied conversion to ethanol via yeast fermentation. However, many other monomeric carbohydrates are present in biomass hydrolysates as a result of hemicellulose hydrolysis, which occurs during chemical pretreatment. In addition to monomeric sugars, many di- and oligosaccharides are formed during pretreatment (Dien et al. 2006, Gomez et al. 2008). Many of these additional carbohydrates (especially xylose) are substrates of various fermentation strategies including fermentation by alternative yeasts (Mosier et al. 2005, Nichols et al. 2008). Thus, techniques capable of quantifying the various carbohydrates present in a given biomass hydrolysate is paramount to realization of the full energy content of lignocellulosic materials.

The optimization criterion for pretreatment step is the fermentable sugar yields. It can be analyzed by High Performance Liquid Chromatography (HPLC). HPLC is a common approach for separation and analysis of non-volatile compounds. Since the first application towards carbohydrate analysis in biomass hydrolysates in the early 1980’s, the use of HPLC methods to analyze sugars has been growing rapidly (Ehrman 1996, Black and Fox 1996, Molnar-Perl 2000, Churms 1996, Davies and Hounsell 1996). Owing to the hydrophilic nature of sugars, traditional, reverse phase liquid chromatography (RPLC) is not a practical
Both refractive index (RI) and evaporative light scattering (ELS) detectors are attractive universal detectors for HPLC analysis of sugars. RI detectors respond to the change in refractive index of a solution in the presence of analyte, and ELS detectors measure light scattered by analyte molecules. The use of RI detection for the analysis of carbohydrates in biomass hydrolysates is fairly prevalent (Balan et al. 2009, Dadi et al. 2007, Gomez et al. 2002, Kaar et al. 1998, Mosier et al. 2005, Ehrman 1996).

Xylose constitute the major fraction and arabinose, mannose, galactose, and glucose in smaller fractions in addition to potential microbial inhibitors when the depolymerization of hemicellulose takes place by chemical process (Chandel et al. 2013). These inhibitors were divided into three major groups, i.e. i. organic acids (acetic, formic and levulinic acids), ii. furan derivatives [furfural and 5-hydroxymethylfurfural (5-HMF)], iii. phenolic compounds (Palmqvist et al. 1999). Overall cell physiology is affected by these inhibitors which often results in declined viability, ethanol yield and productivity (Chandel et al. 2007).

Rate of sugar uptake is affected due to toxic components that ultimately hinder the growth of microorganism with simultaneous decay in product formation. These inhibitors affect the cellular physiology by disturbing the function of biological membranes which affects microbial growth lingering towards extended incubation time with poor metabolite production. However, the yield may remain unaltered. The mechanism of inhibition of some compounds such as phenolics and plant cell wall derived extractives are yet to be known (Chandel et al. 2013).

Among sugar derived inhibitors, furfurals have been found to inactivate the cell replication that reduces the growth rate and the cell mass yield on ATP, volumetric growth rate and specific productivities (Taherzadeh et al. 1999, Palmqvist et al. 1999). Furfurals have been found toxic to Pichia stipitis under aerobic condition, whereas the growth of Saccharomyces cerevisiae was less affected under anaerobic condition by converting into furoic acid (Palmqvist et al. 1999).
Many researchers have reported the adaptation of microorganisms on high furfural concentration, a successful option to decrease the furfural effect on growth. It may be due to the synthesis of new enzymes or co-enzymes for furfural reduction (Boyer et al. 1992, Villa 1992). Furans (furfurals and 5-HMF) in association with acetic acid have been reported highly affective to the growth of *P. stipitis*, *Pachysolen tannophilus* and *Escherichia coli* (Martinez et al. 2000, Lohmeier-vogel et al. 1998).

The ethanologenic microorganisms have ability to degrade some of the inhibitors (Mussatto and Roberto 2004). Progress has been made to achieve higher levels of sugars by diminishing the overall impact of fermentative inhibitors which in-turn improves the fermentability of lignocellulosic hydrolysates (Alriksson et al. 2011, Sun and Liu 2011, Parawira and Tekere 2011). The ion exchange resins, active charcoal, enzymatic detoxification using laccase, alkali treatments and overliming with calcium hydroxide are among selective detoxification strategies which have been investigated in the past (Jurado et al. 2009). Other strategies include changes in fermentation methodologies and metabolic engineering (incorporation of ligninase or laccase genes) have been introduced to overcome from the cell wall degrading inhibitors (Larsson et al. 2001). Treatment with the soft-rot fungus *Trichoderma reesei* and other microorganisms to degrade inhibitors in a hemicellulase hydrolysate has also been proposed (Yu et al. 2011, Fonesca et al. 2011).

**2.5. Detoxification of hemicellulosic hydrolysates**

Due to highly acidic nature of hemicellulosic hydrolysates, the neutralisation of is unavoidable step before using the hydrolysate for fermentation. Alkali most preferably calcium hydroxide or sodium hydroxide are used for neutralization of hydrolysates (pH 6.0- 7.0). During the process, furfurals and phenolics may be removed by precipitation to some extent.

Since detoxification increases the cost of the process, it is important to either overcome the need for detoxification steps or develop cheap and efficient detoxification methods. Over-liming with a combination of high pH and temperature has for a long time been considered as a promising detoxification...
method for dilute sulfuric acid-pretreated hydrolysate of lignocellulosic biomass (Chandel et al. 2007, Martinez et al. 2000). This process has been demonstrated to help with the removal of volatile inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) from the hydrolysate additionally causing a sugar loss (~10%) by adsorption (Chandel et al. 2011). The dried calcium hydroxide is added in acidic hydrolysates converting into gypsum which can be used as plaster of Paris having many commercial values.

Activated charcoal treatment is an efficient and economical method of removing phenolic compounds, acetic acid, aromatic compounds, furfural, and HMF by adsorption without affecting levels of sugar in hydrolysate (Canilha et al. 2008). The effectiveness of activated charcoal treatment depends on different process variables such as pH, contact time, temperature and the ratio of activated charcoal taken versus the liquid hydrolysate volume (Prakasham et al. 2009).

### 2.6. Fourier transform infrared analysis

The enzymatic hydrolysis of cellulose (saccharification) is affected by several factors, viz., degree of polymerization, degree of crystallinity, structural composition and availability of surface area etc (Qi et al. 2009). Crystallinity of cellulose is one of the main factors influencing enzymatic hydrolysis (Kumar et al. 2009). Therefore, pretreatment is one of the key strategies for enhanced enzymatic saccharification of lignocellulosics. Monomeric sugars released after enzymatic saccharification of the free cellulose, can be converted into bioethanol. The texture of native and pretreated samples of *Lantana camara* was investigated by Kuila et al. (2011). The cellulose crystallinity value of untreated sample of *Lantana camara* was 19.57 % while that of pretreated sample was 25.21 %. For lignocellulosic biomass, crystallinity measures the relative amount of crystalline cellulose in the total solid. The crystallinity of the pretreated sample was increased due to removal of lignin and hemicellulose (both of which are amorphous).
Differences between samples with regard to the relative amounts of amorphous and crystalline cellulose have earlier been described through infrared peak ratios. At least four different peak pairs have been proposed (Hulleman et al. 1994, Wistara et al. 1999). Absorption in the infrared region results in changes in vibrational and rotational status of the molecules. The absorption frequency depends on the vibrational frequency of the molecules, whereas the absorption intensity depends on how effectively the infrared photon energy can be transferred to the molecule, and this depends on the change in the dipole moment that occurs as a result of molecular vibration. As a consequence, a molecule will absorb infrared light only if the absorption causes a change in the dipole moment. Thus, all compounds except for elemental diatomic gases such as N₂, H₂ and O₂, have infrared spectra and can be analysed by their characteristic infrared absorption. For quantification of several components absorbing in the mid infrared region (400-5000 cm⁻¹), either conventional dispersive infrared analysis or Fourier Transform Infrared (FTIR) spectroscopy can be used. Compared to dispersive IR analysis, FTIR analysis is faster and has a better signal to noise ratio (Brink 1992, Griffiths and Haseth 1986, Karlsson et al. 1996, Kassman et al. 1997, Amand et al. 1997, Merk et al. 1998).

FTIR analysis provides a versatile tool for qualitative as well as quantitative analysis. ATR-FTIR (Attenuated Total Reflectance- Fourier Transform Infrared) spectroscopy was used as an analytical tool to qualitatively determine the chemical changes in the surface of pretreated straw to complement and understand the microscopic investigations. The FTIR spectra of untreated, hydrothermally pretreated, delignified hydrothermally pretreated and steam-explosion straw samples were reported by Kristensen et al. (2008).

Crystallinity of the treated and untreated woods was studied by FTIR spectroscopy by some researchers (Colom et al. 2003, Carrillo et al. 2004). The 1427 and 898 cm⁻¹ absorption bands, which were assigned to the respective crystalline cellulose I and cellulose II, were used to study crystallinity changes. The absorbance ratio A₁₄₂₇/A₈₉₈ is known as crystallinity index (CI). FTIR
spectroscopy was carried out to investigate the changes in hemicellulose and cellulose structure during oxalic acid fiber expansion (OAFEX) treatment and enzymatic hydrolysis (Chandel et al. 2013b).

Mirahmadi et al. (2010) determined the overall crystallinity of untreated and pretreated substrate (spruce and birch) samples by Fourier Transform infrared (FTIR) spectrometer. The baselines of the spectra were adjusted and normalized with the software, and the absorption bands at 1,427 and 898 cm\(^{-1}\) were used to calculate the crystallinity.

2.7. Microbial production of cellulase and enzymatic hydrolysis of pretreated substrate

Enzymes are the most important bioproducts and are utilized in a large number of processes in the areas of industrial, environmental and food technology. Moreover, current developments in biotechnology are yielding new applications by enzymes (Pandey et al. 2004). Filamentous fungi are preferred for commercially important enzyme productions, because the level of enzymes produced by these cultures is higher than those obtained from Yeast and Bacteria (Bakri et al. 2003). *Aspergillus niger* and *Tricoderma viride* are most important and safe organisms for industrial use and good producers of cellulases (Berka et al. 1992, Comacho and Aguilar 2003). These had been in already use for many decades to produce extracellular enzymes (Schuster et al. 2002) with homologous and heterologous proteins due to high capacity of their protein secretion machinery (Iwashita 2002, Pandey et al. 2004). Filamentous fungi play a key role in SSF (here- “solid state fermentation”) because their hyphal development allows them to effectively colonize and penetrate the solid substrate (Pandey et al. 2000, Machida 2002). In its natural environment *A. niger* is involved in degradation of plant cell material by producing highly specialized enzymes like pectinases, cellulases, hemicellulases and xylanases (de Vries and Visser 2001, de Vries et al. 2002, de Vries 2003).
Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars (Kotchoni et al. 2003). Native cellulose is an unbranched homopolysaccharide consisting of D-glucose residues linked by β-1, 4-glucosidic bonds to form a linear polymeric chain. The smallest repetitive unit in cellulose is cellobiose, which consists of two glucose units. Cellulose is regarded as a valuable resource largely because it can be hydrolysed into soluble cellobiose and glucose sugars when β-bonds are broken.

Cellulolytic enzymes are formed by a large number of microorganisms. A wide variety of bacteria and fungi produce cellulolytic enzymes able to hydrolyze cellulose. However, relatively few fungi and bacteria produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively (Bhat and Bhat 1997, Mach and Zeilinger 2003). Fungi producing the necessary enzymes for the cell free degradation of crystalline cellulose generally belong to Ascomycetes and Deuteromycetes group or to the white rot Basidiomycetes (Ljungdahal and Eriksson 1985).

Cellulases are produced by wide variety of bacteria, fungi, actinomycetes, aerobes and anaerobes, mesophiles and thermophiles. However, fungi are the most studied organisms because of their higher enzyme yields and capacities to produce complete cellulase complex. The cellulase systems of the aerobic fungi *Trichoderma reesei*, *Trichoderma viride*, *Penicillium pinophilum*, *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), *Fusarium solani*, *Talaromyces emersonii*, *Trichoderma koningii* and *Rhizopus oryzae* are well characterized (Bhat and Bhat 1997, Murashima et al. 2002). Some thermophylic aerobic fungi *Chaetomium thermophile*, *Humincola insolens*, *Humicola grisea*, *Thermocola moidea*, *Myceliophthora thermophila*, *Talaromyces emersonii* and *Thermoascus aurantiacus* (Maheswari et al. 2002) and mesophilic anaerobic fungi (*Neocallimastix frantalis*, *Paromonas communis*, *Sphaeromonas communis*) (Bhat and Bhat 1997) also produce cellulases.

Cellulolytic enzymes are by necessity secreted into the medium or bound to the outer surface of cellulolytic microorganisms. The enzymology of cellulose
degradation is documented in several reviews (Bayer et al. 1994, Singh and Hayashi 1994, Leschine 1995, Gielkens et al. 1999, Kang et al. 1999, Lynd et al. 2003). Four classes of enzymes are involved in the biodegradation of cellulose (Klyosov 1990) viz.-

- **Endoglucanases** (Endo-1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) randomly hydrolyse internal 1,4-glucosidic linkages within the cellulose chain resulting in formation of gluco-oligosaccharides. Large numbers of reducing and non-reducing ends are created in the oligosaccharides.

- **Exoglucanases** (Exo-1,4-β-D-glucan-4-glucanohydrolase) cleave only external β-1,4-glucosidic bonds from nonreducing end of cellulose and also oligosaccharides and split off glucose units.

- **Celllobiohydrolases** (Exo-1,4-β-D-glucan-4-cellbiohydrolase EC 3.2.1.91) release cellbiose units from nonreducing end of cellulose. The distinction between exoglucanases and celllobiohydrolases is always not clear.

- **β-Glucosidases** (β-D-glucoside glucohydrolase EC 3.2.1.21) hydrolyse cellbiose and low molecular weight dextrins to glucose.

Cellulases are characterized by a multiplicity of enzyme components whose exact number varies from one organism to another. Two immunologically distinct celllobiohydrolases (CBH I and CBH II) have been detected in the extracellular medium of *Trichoderma* spp. in particular *reesei* (Fagerstam and Pettersson 1980, Enari and Niku-Paavola 1987, Kubicek and Pentilla 1998). These enzymes act in a synergistic or cooperative manner. The endoglucanase opens up linear cellulose molecules, producing reducing and non-reducing ends that, in turn, can be attacked by exoglucanase. Exoglucanases, in turn, act to remove molecular strand of cellulose and expose more internal sites for endoglucanase binding. Activities of both of these enzymes, in particular, CBH are inhibited by celllobiose. The cleavage of celllobiose to glucose by β-glucosidase greatly reduces this inhibition and allows continued cellulytic activity. This classical synergism between exoglucanase and endoglucanase has

One of the major obstacles for exploitation of cellulose is the cost of cellulase production. One very successful strategy has been to increase the productivity of cellulase production with use of high yielding cellulolytic organisms, locally available cheap raw material and optimizing culture conditions. Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues, particularly those originating from tropical regions. In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues such as sugarcane bagasse, cassava bagasse, wheat bran, rice bran, sugar beet pulp and apple pomace, etc.

Agro-industrial residues are generally considered the best substrates for solid state fermentation (SSF) processes (Pandey et al. 1999). Substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals as well as growth factors (Chahal 1985, Nandakumar et al. 1994). They have a capacity to absorb water and meet the vital requirement of water for microorganisms. The filamentous fungi are able to grow deep into the substrate particles. Thus, substrate also provides anchorage to microbial cells (Larroche et al. 1998). The oxygen requirement for growth and metabolism of the culture is derived largely from gaseous state and also to a lesser extent from that present in dissolved form in the water associated with the solids.

Solid substrate fermentation involves “the growth of microorganisms on moist solid substrates in the absence or near absence of free flowing water” (Ellaiah et al. 2004). As microorganisms on a solid substrate are growing under conditions similar to their natural habitat, they may be able to produce certain enzymes, metabolites, proteins and spores more efficiently than in submerged fermentation (Goes 1999).
Different solid substrates have been used for the production of cellulolytic enzymes in solid state fermentation. Muniswaran and Charyulu (1994), demonstrated the production of cellulolytic enzymes by SSF of *Trichoderma viride* NCIM 1051 on coconut coir pith. Comparison of production of cellulolytic enzymes by *Trichoderma harzianum* on use of different substrates viz., wheat bran, sugarcane bagasse, wheat straw either in single or in combination in a SSF system revealed a mixture of sugarcane bagasse and wheat bran at 80:20 (w/w) ratio suitable for higher yields (Roussos et al. 1991). The intake of available nutrients from wheat bran by fungi depends on their capacity to penetrate into the wheat bran particle (Chahal 1983, Lonsane et al. 1985). The nutrients present in the wheat bran at places, where mycelia could not penetrate, are thus not available to the culture during fermentation. Some of the important nutrients necessary for growth and product formation for microorganisms under study may also be present at sub optimal levels in wheat bran. Hence, the supplementation of wheat bran with other solid or water soluble nutrients were found to enhance the product formation in solid state fermentation (Kumar and Lonsane 1987). Wheat bran is a familiar as a complete medium for producing cellulases, amylases and xylanase in solid substrate fermentation (Smits et al. 1996).

### 2.8. Ethanologenic microorganisms

Historically, the most commonly used microbe has been yeast, among the yeasts, *Saccharomyces cerevisiae*, which can produce ethanol to give concentration as high as 18% of the fermentation broth, is the preferred one for most ethanol fermentation. This yeast can grow both on simple sugars, such as glucose, and on the disaccharide sucrose. Saccharomyces is also generally recognized as safe (GRAS) as a food additive for human consumption and is therefore ideal for producing alcoholic beverages. This yeast does not exhibit many of the limitations encountered with bacteria. However, *S. cerevisiae* is not able to ferment xylose. Therefore, metabolic engineering of xylose fermentation in *S. cerevisiae* is an attractive approach (Sonderegger and Sauer 2003).

*Zymomonas mobilis* is GRAS and has simple nutritional needs. It is so well suited for ethanol production that in the 1970s and 1980s, some researchers
reported it as superior to *S. cerevisiae*. Despite its advantages as an ethanologen, *Z. mobilis* is not well suited for all of the biomass resources conversion because it ferments only glucose, fructose, and sucrose. Moreover, for *Z. mobilis* on synthetic media containing either glucose, fructose or sucrose, the specific rates of sugar uptake and ethanol production are at a maximum when utilizing the glucose medium. In addition, *S. cerevisiae* is still preferred by the industry because of the yeast hardiness (Lin and Tanaka 2006).

Among the pentose fermenting organisms, *Pichia stipitis* has been shown to have most promise for industrial applications (Agbogbo et al. 2006). For example, the hemicellulosic hydrolysates of *Prosopis juliflora* (18.24 g sugar/L broth) when fermented with *P. stipitis* produced 7.13 g/L ethanol. Detoxified xylose rich hydrolysate of *L. camara* when fermented with *P. stipitis* 3498 at pH 5 and 30°C for 36 h resulted 0.33 g alcohol/g lignocellulose used (Kuhad et al. 2010). In yet another example, the detoxified water hyacinth hemicellulose acid hydrolysate (rich in pentose sugars) fermented with *P. stipitis* NCIM-3497 at pH 6.0 and 30°C resulted 0.425 g ethanol/g lignocellulose. *Pichia stipitis* is one of the best explored xylose-fermenting yeasts and has a substrate range including all the monomeric sugars present in lignocellulose (Jeffries and Shi 1999). Some *P. stipitis* strains produce low quantities of various cellulases and hemicellulases to breakdown wood into monomeric sugars although it cannot utilize polymeric cellulose as carbon source (Jeffries et al. 2007). Among the enzymes that are naturally produced are a β-glucosidase that allows the yeast to ferment cellobiose. Endoglucanases were successfully produced in *P. stipitis* (Piotek et al. 1998). As this yeast is capable of growth on cellobiose these recombinant strains should theoretically have the ability to hydrolyse amorphous cellulose. The xylanolytic ability of *P. stipitis* was enhanced by the co-expression of xylanase and xylosidase encoding genes (Den Haan and Van Zyl 2003). The resulting strains displayed improved biomass production on medium with birchwood glucuronoxylan as sole carbohydrate source. Even though mutant strains of *P. stipitis* with increased ethanol tolerance were recently isolated, *P. stipitis* remains a relatively poor fermentor (Watanabe et al. 2011). However, its ability to
consume acetic acid and reduce the furan ring in furfural and hydroxymethylfurfural (HMF) creates an opportunity for this yeast to clean up some of the toxins in cellulosic biomass conversion (Agbogbo and Coward-Kelly 2008).

Among yeasts, *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* resulted very interesting for their capacity to ferment xylose. Yeasts metabolize xylose by means of the xylose reductase (XR) that converts xylose to xylitol and xylitol dehydrogenase (XDH) that convert xylitol to xylulose. After phosphorylation, xylulose is metabolized through the pentose phosphate pathway (PPP) (Zaldivar et al. 2001). Among the wild type yeasts fermenting xylose, *Pichia stipitis* was considered the most promising (Agbogbo and Coward-Kelly 2008) because it has a XR capable to use as cofactor both NADPH and NADH. For this reason, under anaerobic conditions, xylose fermentation in *Pichia stipitis* is carried out by using NADH.

Good performances have been recently obtained with the thermotolerant strain *Kluyveromyces marxianus* 6556 that showed promising results in the Simultaneous Saccharification and Fermentation (SSF) of lignocellulosic agricultural wastes at 37°C (Zhang et al. 2010). In fact various strains of the *K. marxianus* species have the ability to grow at temperature around 40°C and ferment mixed sugars such as glucose, xylose, mannose and galactose (Fonseca et al. 2008). In this regard, Ballesteros et al. (2001) carried out several fed-batch SSF tests using *K. marxianus* at 42°C and obtaining ethanol yield of 76% for olive pulp. Rudolf et al. (2008) also demonstrated that undetoxified steam-pretreated bagasse could be successfully fermented to ethanol in a SSF process using both natural yeasts (*P. stipitis* CBS6054) that recombinant yeast (*S. cerevisiae* TMB3400). Interesting results were obtained using Simultaneous Saccharification and Fermentation (SSF) with other materials such as industrial wastes (Kadar et al. 2004), wheat straw, and sweet sorghum bagasse (Ballesteros et al. 2004). Vallet et al. 1996 also reported the use of *K. marxianus*. Strains of the yeast *K. marxianus* can grow at temperatures as high as 52°C and have a short generation time and high growth rate at elevated temperatures (Rajoka et al.
*K. marxianus* can convert a wide range of substrates, including xylose, to ethanol and successful SSF with a variety of feedstocks at elevated temperatures was demonstrated with *K. marxianus* (Fonseca et al. 2007, Fonseca et al. 2008). Thermotolerant celllobiohydrolase, endoglucanase and β-glucosidase encoding genes were expressed in combination in a strain of *K. marxianus* (Hong et al. 2007). The resulting strain was able to grow in synthetic media containing cellobiose or carboxymethylcellulose as sole carbon source but the hydrolysis of crystalline cellulose was not shown. Recently, a *K. marxianus* strain was engineered to display *T. reesei* endoglucanase II and *Aspergillus aculeatus* β-glucosidase on the cell surface (Yanase et al. 2010). This strain successfully converted 10 g/l of a cellulosic β-glucan to 4.24 g/l ethanol at 48°C within 12 h.

Sanchez et al. (1999) reported the use of *Pachysolen tannophilus*. In an attempt to develop a xylose-fermenting yeast for industrial ethanol production, UV light-induced mutants of *P. tannophilus* have been isolated, which can grow faster on xylose. Several other yeast strains for xylose utilization have been reported (Jeewon 1997).

Recently, Sharifia et al. (2008) have discussed that yeast in the form of the fungus *Mucor indicus* showed faster ethanol production with an average productivity of 0.90 g/l h from glucose, fructose and inverted sucrose, than the filamentous form with an average productivity of 0.33 g/l h.

### 2.9. Fermentation

The term fermentation is derived from the Latin “fervor”, to boil, thus to describe the appearance of the action of yeast on the extracts of fruit or malted grains. The boiling appearance is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugars present in the extract. “Fermentation”, however has different meanings to “Biochemists” and “Industrial Microbiologists”. In biochemical terms it relates to the generation of energy by the catabolism of organic compounds, whereas it’s meaning in Industrial Microbiology tends to be much broader.

The production of alcohol by the action of yeast on malt or fruit extracts has been carried out on a large scale for many years and was the first “industrial
process for the production of a microbial metabolite”. Thus, the industrial microbiologists have extended the term fermentation to describe any process for the production of a product by the mass culture of a microorganism. The giant “microbial libraries” in current vogue can be studied for microbes that convert cheaper carbohydrates into value-added products, which can serve as raw materials for the fermentation of hemicellulosic-derived sugars into valuable commercial commodities (Singh and Kumar 2007). The bioconversion process holds more promise of utilizing both hexose and pentose sugars from lignocellulosic materials.

In the fermentation process, the hydrolytic products including monomeric hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) will be fermented to valuable products such as ethanol. Among these hydrolytic products, glucose is normally the most abundant, followed by xylose or mannose and other lower concentration sugars.

The last two steps of bioconversion of pretreated lignocellulolytic residues to ethanol (hydrolysis and fermentation) can be performed separately (SHF) or simultaneously (SSF). In the separate hydrolysis and fermentation (SHF), the hydrolysate products will be fermented to ethanol in a separate process. The advantage of this method is that both processes can be optimized individually (e.g. optimal temperature is 45-50°C for hydrolysis, whereas it is 30°C for fermentation). However, its main drawback is the accumulation of enzyme-inhibiting end-products (cellobiose and glucose) during the hydrolysis. This makes the process inefficient, and the costly addition of β-glucosidase is needed to overcome end-product inhibition (Elumalia and Thangavelu 2010, Kont et al. 2013).

Further process integration can be achieved by a process known as consolidated bioprocessing (CBP) which aims to minimize all bioconversion steps into one step in a single reactor using one or more microorganisms. CBP operation featuring cellulase production, cellulose/hemicellulose hydrolysis and fermentation of 5- and 6- carbon sugars in one step have shown the potential to provide the lowest cost for biological conversion of cellulosic biomass to fuels,
when processes relying on hydrolysis by enzymes and/or microorganisms are used (Lynd et al. 2005).

The simultaneous saccharification and fermentation (SSF) process was first studied by Takagi et al. (1977) for cellulose conversion to ethanol. The SSF process was originally developed for lignocellulosic biomass by researchers at Gulf Oil Company in 1974 (Blotkamo et al. 1978). The SSF process eliminates expensive equipment and reduces the probability of contamination by unwanted organisms that are less ethanol tolerant than the microbes selected for fermentation (Szczodrak 1989).

SSF combines the enzymatic saccharification of polymeric cellulose to simple monomeric forms such as glucose and its eventual fermentation by yeast to ethanol in the same vessel (Ikwebe and Harvey 2011). In simultaneous saccharification and fermentation (SSF), however, the end-products will be directly converted to ethanol by the microorganism. Therefore, addition of high amounts of β-glucosidase is not necessary and this reduces the ethanol production costs (Stenberg et al. 2000). Rapid conversion of the glucose into ethanol by yeast results in faster rates, higher yields, and greater ethanol concentrations than possible for SHF. The presence of ethanol in the fermentation broth also makes the mixture less vulnerable to invasion by unwanted microorganisms (Sasikumar and Viruthagiri 2010). However, the main drawback of SSF is the need to compromise processing conditions such that temperature and pH are suboptimal for each individual step. The development of recombinant yeast strains with improved thermotolerance can enhance the performance of SSF (Galbe and Zacchi 2002). It is reported that the major inefficiencies of biochemical process for lignocellulosic bioethanol production were identified as the simultaneous saccharification and fermentation (SSF) process accounting for 27% of the lost energy by thermodynamic analysis (Sohel and Jack 2010). Alkasrawi et al. (2003) reported that addition of surfactants as an additive in SSF can significantly lower the operational cost of the process because it increases the conversion rate of cellulose to glucose. Addition of Tween-20, 2.5 g/l not only reduces the time required to attain maximum ethanol concentration, but also enhances enzyme
activity in the liquid fraction at the end of SSF, probably by preventing unproductive binding of the cellulases to lignin, which could facilitate enzyme recovery.

Over the years, various groups have worked on the SSF process to improve the choice of enzymes, fermentative microbes, biomass pretreatment, and process conditions. Extensive studies on SSF have since been conducted focusing on the production of ethanol from cellulosic substrates. Phillipidis et al. (1993) have studied the enzymatic hydrolysis of cellulose in an attempt to optimize SSF performance. Ghose et al. (1984) have increased ethanol productivity by employing a vacuum cycling in an SSF process using lignocellulosic substances. Zhu et al. (2006) evaluated the suitability of production of ethanol from the microwave-assisted alkali pretreated wheat straw, the simultaneous saccharification and fermentation (SSF) of the microwave assisted and conventional alkali pretreated wheat straw to ethanol. *Candida brassicae* is accepted as the yeast of choice as far as SSF is considered, although both *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* have been found to offer similar rates. Several other yeasts as well as the bacteria like *Z. mobilis* have been studied with cellulases from *T. reseii* mutants for SSF processes. Researchers have also examined several combinations of enzymes with *Z. mobilis*, *S. cerevisiae*, and other ethanol producer, but they have only considered substrate levels lower than necessary to prove economic viability. Wyman et al. (1986) evaluated the cellobiose-fermenting yeast *Brettanomyces clausenii* for the SSF of cellulose to ethanol.

Ballesteros et al. (2006) reported that best pretreatment conditions to obtain high conversion yield to ethanol (140 l/t) of cellulose-rich residue after steam-explosion were 180°C for 10 min in acid impregnated (0.9%) wheat straw. The sugars recovery yield in the filtrate was 300 g/kg wheat straw. Yu and Zhang (2004) investigated glucose production from acid hydrolyzed cellulose and fermentation of glucose to ethanol by *S. cerevisiae*. They obtained ethanol yield of 14.2 g/l of a hydrolysate medium containing 31.6 g/l glucose after 24 h of
fermentation. They obtained 15.1 g/l ethanol when urea was used as nitrogen source.

2.10. Immobilization of yeast and estimation of ethanol

Immobilization of cells is a method to increase the cell mass concentration in the fermenter to increase the process productivity and minimize the production costs, while offering advantages over free cell fermentation operations (Santos et al. 2008). The higher the yield, the smaller the fermenter required and therefore the capital cost is usually lower (Olsson and Hahn-Hagerdal 1996). There has been a surge in the attempts towards finding out a renewable and biodegradable carrier which essentially is not synthetic, easy to use, cheaper and available naturally in abundance (Ogbonna et al. 1997).

Immobilization of microbial cells and enzymes have showed certain technical and economical advantages over free cell system. Using immobilized enzymes not only leads to greater product purity, cleaner processes, and economic operational costs but also makes the use enzyme cost effective and recoverable (Meena and Raja 2004). The immobilized biocatalysts have been extensively investigated during last few decades. An immobilized cellobiase enzyme system has been used in the enzymatic hydrolysis of biomass for the generation of cellulosic ethanol (Das et al. 2011). Production of alcohol and biodiesel fuel from triglycerides using immobilized lipase has been carried out using porous kaolinite particle as a carrier (Iso et al. 2001).

The use of an immobilized yeast cell system for alcoholic fermentation is an attractive and rapidly expanding research area because of its additional technical and economical advantages compared with the free cell system. A reduction in the ethanol concentration in the immediate microenvironment of the organism due to the formation of a protective layer or specific adsorption of ethanol by the support may act to minimize end product inhibition. The most significant advantages of immobilized yeast cell systems are the ability to operate with high productivity at dilution rates exceeding the maximum specific growth rate, the increase of ethanol yield and cellular stability and the decrease of process
expenses due to the cell recovery and reutilization (Lin and Tanaka 2006). Other advantages of immobilized cell system over presently accepted batch or continuous fermentations with free-cells are: greater volumetric productivity as a result of higher cell density; tolerance to higher concentrations of substrate and products; lacking of inhibition; relative easiness of downstream processing etc. in different types of bioreactors, such as packed bed reactor, fluidized bed reactor, gaslift reactor and reactor with magnetic field (Ivanova et al. 1996, Sakai et al. 1994, Perez et al. 2007).

Perspective techniques for yeasts immobilization can be divided into four categories: attachment or adsorption to solid surfaces (wood chips, delignified brewer’s spent grains, DEAE cellulose, and porous glass), entrapment within a porous matrix (calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatine, chitosan, and polyacrilamide), mechanical retention behind a barrier (microporous membrane filters, and microcapsules) and self-aggregation of the cells by flocculation (Ivanova et al. 2011). In immobilization, yeast cells are entrapped in an artificial polyacrylamide gel, calcium alginate. When sodium alginate slurry mixed with yeast suspension is added to calcium chloride solution, calcium alginate is formed.

\[ \text{CaCl}_2 \cdot 2\text{H}_2\text{O} + \text{Sodium alginate} \rightarrow \text{Calcium alginate} \]

There are number of different methods to quantitate ethanol in samples. HPLC has been utilized to monitor the fermentation process This method has the advantage of being able to monitor not only the production of ethanol, but also the reaction substrates and byproducts (Hall and Reuter 2007). Fourier transform infrared spectroscopy (Sharma et al. 2009), gas chromatography (Wang et al. 2003), and Infrared (Lachenmeyer et al. 2010) technologies have also been used to detect and quantitate ethanol in samples. While FTIR requires a large investment in instrumentation, the use or less expensive IR technology has been demonstrated to be just as accurate (Lachenmeyer et al. 2010). Gerchman et al. (2012) developed a cheap and rapid approach for ethanol quantification in aqueous media during fermentation steps as part of the conversion of biomass to ethanol. The suggested method requires a sample of a small volume and consists of organic
extraction, followed by direct use of gas chromatography with a flame ionization detector (GC-FID). The proposed method was also tested for its ‘real-life’ applicability for ethanol quantification from fermentation process.

2.11. Distillation of ethanol

The recovery of water-free ethanol after the fermentation of cellulosic sugars determines the overall cost and economics of ethanol. It is a challenging task to obtain full recovery of non-hydrous ethanol from hydrous fermented broth. There has been much discussion regarding biomass pretreatment, saccharolytic processes and ethanol fermentation, but ethanol recovery from cellulosic fermented broth needs to be researched to make process cost effective. In general, lignocelluloses hydrolysate is a diluted sugar solution, which means less ethanol is produced after fermentation, making the distillation process energy-intensive and costly. The possible solutions to this problem include concentrating the cellulosic sugar syrup, increasing the amount of substrate during the saccharolytic process, or using the high throughput distillation or membrane-assisted approaches. A number of novel distillation methodologies such as per-evaporation, supercritical solvent extraction, mechanical vapor compression, membrane assisted or molecular sieve adsorption could be the best technologies for ethanol recovery from a fermented cellulosic sugar solution (Gulati et al. 1996, Widgren et al. 2008).

According to Keller and Bryan (2000), distillation is still a “formidable competitor” as a major separation method even though much research has been thrust on its alternatives. Hence, distillation, especially simple distillation, tends to be the first choice in industry for separating a liquid mixture; other methods, including complex distillation, e.g., azeotropic distillation, come into play only when simple distillation is deemed to be technically infeasible or economical inviable because of typically three large stainless steel distillation towers, stainless steel heat exchangers and price of stainless up 400% in last six years, high operating costs because 280 MMBTU/hr energy is consumed (100 MGPY ethanol). Mole sieve drying adds to energy costs and that’s why energy costs up
significantly with price of crude oil. Under certain circumstances, retrofitting of an existing process can be economically far more viable than constructing a new process, especially when the financial resources are limited and/or when short term needs are to be met under a tight time constraint. Developing economically viable fermentation processes requires efficient downstream processing: selective product removal and avoiding byproduct streams. “ESepis a modular, low energy process for the recovery of ethanol from fermentation broth with an estimated reduction of up to 60% in both capital and operating costs versus conventional distillation. Use of non-stainless steel components also results in a substantial reduction in construction time”. It is applicable to new ethanol plants (corn, sugar and cellulosic). It replaces whole distillation train and mole sieve dryer. With new plants it reduces overall energy consumption by >60% (ESep 2008). The utilization of pervaporation for the production of absolute (anhydrous) ethanol through its coupling with the previous distillation step has been reported. The modeling and optimization of the process using MINLP tools showed 12% savings in the production costs considering a 32% increase in membrane area and the reduction in both reflux ratio and ethanol concentration in the distillate of the column (Lelkes et al. 2000, Szitkai et al. 2002). Through pilot-plant studies, the integration of distillation process with the pervaporation has been achieved resulting in good indexes in terms of energy savings. These savings are due to the low operation costs of pervaporation and to the high yield of dehydrated ethanol, typical of pervaporation processes (Tsuyomoto et al. 1997). The comparison between azeotropic distillation using benzene and pervaporation system using multiple membrane modules showed that, at the same ethanol production rate and quality (99.8 wt.%), operation costs, including the membrane replacement every 2–4 years, are approximately 1/3–1/4 of those of azeotropic distillation.

2.12. Response surface methodology

In an experiment, some input $x$’s transform into an output that has one or more observable response variables $y$. Therefore, useful results and conclusions can be drawn by experiment. When treatments are from a continuous range of
values then the true relationship between \( y \) and \( x \)'s might not be known. The approximation of the response function \( y = f(x_1, x_2, \ldots, x_q) + \varepsilon \) is called Response Surface Methodology where \( \varepsilon \) represents the noise or error observed in the response \( y \). The surface represented by \( f(x_1, x_2) \) is called a response surface.

As an important subject in the statistical design of experiments, the Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery and Douglas 2005). The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods and their associated numerical noise. When treatments are from a continuous range of values, then a Response Surface Methodology is useful for developing, improving, and optimizing the response variable. The RSM is important in designing, formulating, developing, and analyzing new scientific studies and products. It is also efficient in the improvement of existing studies and products (Giovanni 1983).

Box and Wilson in 1951 suggested to use a first-degree polynomial model to approximate the response variable. They acknowledged that this model is only an approximation, not accurate, but such a model is easy to estimate and apply, even when little is known about the process (Wikipedia 2006). Moreover, Mead and Pike stated origin of RSM starts 1930s with use of Response Curves (Myers et al. 1989). According to research conducted (Myers et al. 1989), the orthogonal design was motivated by Box and Wilson (1951) in the case of the first-order model. For the second-order models, many subject-matter scientists and engineers have a working knowledge of the central composite designs (CCDs) and three-level designs by Box and Behnken (1960). Also, the same research states that another important contribution came from Hartley (1959), who made an effort to create a more economical or small composite design. There exist many papers in the literatures about the response surface models. In contrast, 3-level fractional design has limited works. Thus, 3-level fractional design is an open research subject. Fractional Factorial Experiment Design for Factor at 3-
Levels (Connor and Zelen 1959) is a helpful resource conducting this kind of design. Many three-level fractional factorial designs and more importantly their alias tables can be found in their study. According to (Myers et al. 1989), the important development of optimal design theory in the field of experimental design emerged following Word World II. Mead and Pike (1975) investigated the extent to which RSM had been used in applied research and gave examples from biological applications. Myers et al. (1989) summarized the developments in RSM that had occurred since the review of Hill and Hunter (1966), while a more recent summary of the current status of RSM and some indication of possible developments was given by Myers (1999). Venter et al. (1996) have discussed the advantages of using RSM for design optimization applications.

The classical method of studying one variable at a time can be effective in some cases but it is useful to consider the combined effects of all the factors involved. The Response Surface Methodology (RSM), based on statistical principles, can be employed as an interesting strategy to implement process conditions that drive to optimal ethanol production from pretreated substrates by performing a minimum number of experiments. Thus, RSM experimental design is an efficient approach to deal with a large number of variables and there are several reports on applications of RSM for the production of primary and secondary metabolites through microbial fermentation (Balusu et al. 2005, Jargalsaikhan and Saracoglu 2009). The influence of process variables such as incubation temperature, initial pH and fermentation time on ethanol production from pretreated sugarcane bagasse was studied using CCD experiments (Sasikumar and Viruthagiri 2010). The use of Design Expert Software for result analysis using CCD is reported by Ali et al. (2012).