CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulosic biomass

In term "Lignocellulosic biomass" is used when referring to higher plants, softwood or hardwood. The main components of the lignocelluloses are cellulose, hemicelluloses and lignin. Lignocellulosic biomass includes materials such as agricultural residues (*e.g.* corn stover and rice bran), forestry residues (*e.g.* mill wastes and sawdust), portions of municipal solid waste (*e.g.* waste paper), and woody can also be used (Eriksson *et al.*, 1990). The wood cells are composed of different layers, which differ from one another with respect to their structure and chemical composition. Basically, cellulose forms a skeleton which is surrounded by other substances functioning as matrix (hemicelluloses) and encrusting (lignin) materials (Fig. 2.1). Cellulose, hemicelluloses and lignin are closely associated and covalent cross-linkages occur between lignin and polysaccharides (lignin-carbohydrate complexes, LCC). The side-groups arabinose, galactose and 4-O-methylglucuronic acid are most frequently perceived as connecting links to lignin (Fengel and Wagerner, 1983).

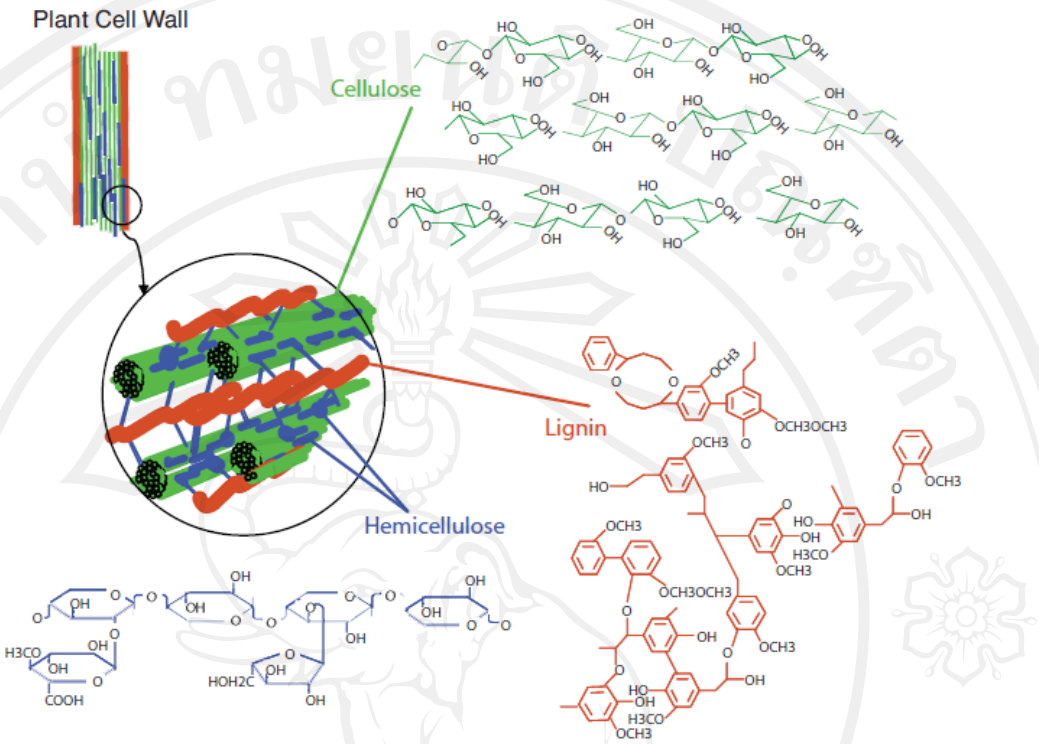

Fig. 2.1 The structure of lignocellulosic biomass, mainly cellulose, hemicelluloses and lignin are presented (Fengel and Wagerner, 1983)

2.1.1 Cellulose

Cellulose is the main constituent of plant cell walls comprising about 50% of wood. Cellulose is the β-1,4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose) and bound by β-1,4- glycosidic linkages. The chemical formula of cellulose is $(C_6H_{10}O_5)_n$, the structure of one chain of the polymer, and the monosaccharide (glucose) are presented in Fig. 2.2.

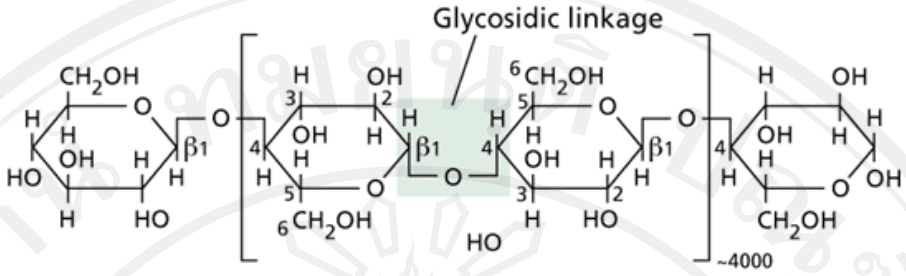

Fig. 2.2 Schematic illustration of the cellulose chain with glycosidic linkage (Taiz and Zeiger, 2010).

The average degree of polymerization (DP) of plant cellulose varies 7,000 and 15,000 glucose unit, depending on the source (Fengel and Wagerner, 1983). The nature of bond between the glucose molecules allows the cellulose polymer is the long straight chains. The latter arrangement of the molecule, together with the fact that the hydroxide is evenly distributed on both sides of the monomers, allows for the formation of hydrogen bonds between the molecules of cellulose. The hydrogen bonds in turn result in the formation of a compound that is contained of several parallel chains attached to each other (Faulon *et al*., 1994). Cellulose is found in both the crystalline and non-crystalline structure. The coalescence of several polymer chains leads to the formation of microfibrils, which in turn are united to form fibres. In this way cellulose can obtain a crystalline structure in the plant cell wall. Cellulose is insoluble in water, where it swells. Moreover, it is also insoluble in dilute acid solutions at low temperature. The solubility of the polymer is strongly related to the degree of hydrolysis achieved. The factors that affect the hydrolysis rate of cellulose also affect its solubility that takes place, however, with the molecule being a different form than the native one.

2.1.2 Hemicelluloses

Hemicelluloses are generally classified according to the main sugar residue in the backbone, including, xylans, mannans, galactans and glucans. The dominant sugars in hemicelluloses are mannose in softwoods and xylose in hardwoods and agriculture residues (Lavarack *et al*., 2002; Emmel *et al*., 2003). As shown in Fig. 2.3, the molecule of a xylan involves 1->4 linkages of xylopyranosyl units with α-(4-O)-methyl-D-glucuronopyranosyl units attached to anhydroxylose units. The result is a branched polymer chain that is mainly composed of five carbon sugar monomers, xylose, and to lesser extent six carbon sugar monomers such as glucose. Important aspects of structure and composition of hemicelluloses are the lack of crystalline connected to the polymer chain.

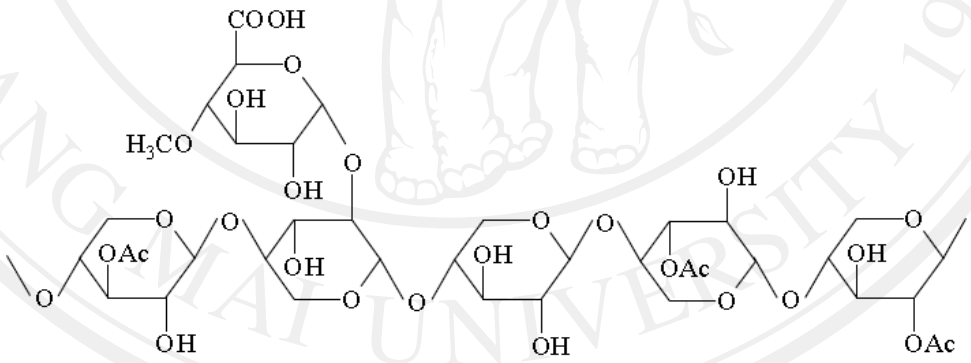

Fig. 2.3 Structure of xylans, partial structure from hardwood chain (Bastawde, 1992).

Hemicellulose is often reported to be chemically associated with cross-linked to other polysaccharides, proteins or lignin. Hemicellulose is insoluble in water at low temperature, however, its hydrolysis starts at a temperature lower than cellulose. It is more soluble than that of cellulose, and they can be isolated from wood by extraction.

The presence of acid highly improves the solubility of hemicellulose in water. The degree of polymerization of hemicellulose does not exceed the 200 units whereas the minimum limit can be around 150 monomers.

2.1.3 Lignin

Lignin is aromatic, 3-dimensional and amorphous. It is synthesized from phenyl propanoid precursors by polymerization in higher plants. It comprises 20-30% of woody plant cells and by forming a matrix surrounding the cellulose and hemicellulose, it provides strength and protection, for example, against biodegradation. Lignin is highly resistant to biodegradation and only higher fungi are capable of degrading the polymer via an oxidative process (Hans and Klaus, 1996). The hydroxyl and methoxyl groups in lignin precursors and oligomers may interact with cellulose microfibrils despite the fact that lignin is hydrophobic in character (Houtman and Atalla, 1995).

The lignin precursors ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol consist of an aromatic ring and a 3-carbon side chains (Brown, 1985). A typical finding for the lignin polymer is that there is no single repeating bond between the subunits but a random distribution of at least 10 different types of bond, the most common being the β-aryl ether (β-O-4) bond (Argyropoulos and Menachem, 1997). As showed in Fig. 2.4, illustrates only the major linkages; the dominant linkages are the, β-O-4 type (50%), seen between units 1 and 2, 2 and 3, 4 and 5, 6 and 7, 7 and 8, and 13 and 14, and β-1 type, seen between units 9 and 10 (Kirk, 1987). Due to its complex, hydrophopic, cross-linked aromatic polymer, lignin is more difficult to breakdown than cellulose and hemicelluloses. The molecular weight of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell (Eriksson

et al., 1990). Therefore, the biological degradation of macromolecular lignin must appear through the activity of extracellular enzymes. Moreover, the chemical structure of native lignin is essentially changed under high temperature and acidic conditions, such as the conditions during steam pretreatment (Tanahashi *et al*., 1983).

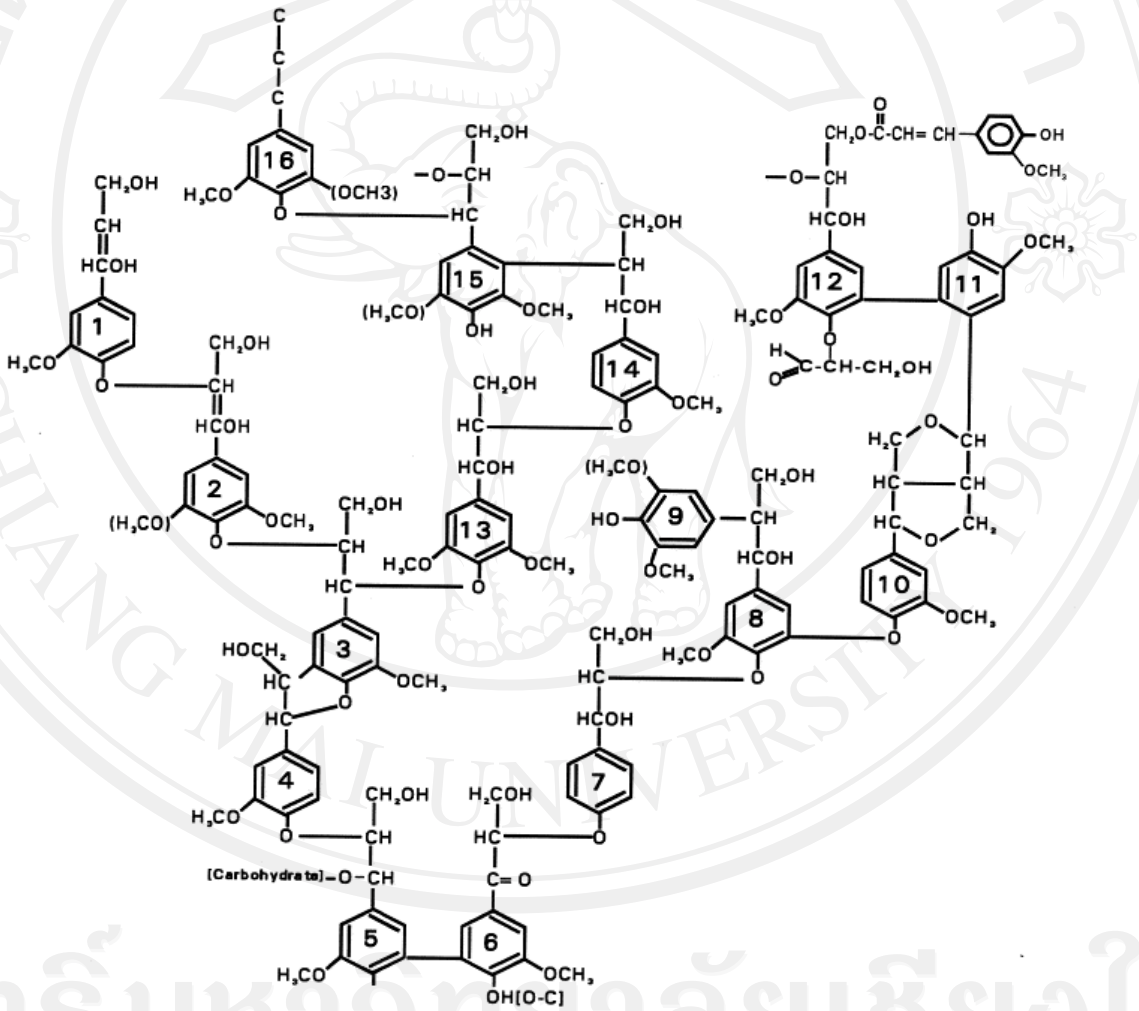

Fig. 2.4 Schematic structural formula of lignin (Kirk, 1987)

The composition of lignocelluloses highly depends on its source, derived from hardwood, softwood, or agriculture residues. As shown in Table 2.1, summarizes the composition of lignocelluloses encountered in the most common sources of biomass.

Table 2.1 cellulose, hemicelluloses and lignin content in several sources (% dry mass)

Lignocellulosic materials	cellulose	hemicellulose	lignin	references
Sugarcane bagasse	30-43	22-26	10-22	Gawande and Kamat, 1999; Dawson and Boopathy, 2008
Coffee husk	19-26	24-45	18-30	Bekalo and Reinhardt, 2010
Rice husk	25-30	18-21	26-31	Gerardi *et al*., 1998; Ludueňa *et al*., 2011
Orange peel	13.61	6.10	2.10	Ververis *et al*., 2007
Wheat straw	31-39	22-24	16-25	Kabel, 2007; Thomsen *et al*., 2008
Corn stover	39-42	22-28	18-22	Kim *et al*., 2002; Zhu *et al*., 2009
Hardwoods stems	40-55	24-40	18-25	Sun and Cheng, 2002
Softwoods stems	45-50	25-35	25-35	Sun and Cheng, 2002

2.2 Bio-ethanol production from lignocellulosic biomass

The most common renewable fuel today is ethanol derived from corn grain (starch) and sugar cane (sucrose). It is expected that there will be limits to the supply of these raw materials in the near future (Kevin *et al*., 2006). Lignocellulosic biomass has long been known as a potential low cost source of mixed sugars for fermentation to fuel ethanol and it seen as an alternative raw material for future supplies for ethanol production. Lignocellulosic biomass resulting complex composition, cellulose, hemicellulose, and lignin which present in wood represents a major problem for the development of biomass conversion process. In general, ethanol production from lignocellulosic biomass grouped as:

(1) crop residues (sugarcane bagasse, corn stover, wheat straw, rice straw, rice hull and pulp *etc*.);

(2) hardwood (aspen, poplar);

(3) softwood (pine, spruce);

(4) cellulose wastes (newsprint, waste paper, recycled paper) and

(5) municipal solid wastes (Oscar and Carlos, 2008).

Therefore, the biological process of ethanol fuel production utilizing lignocelluloses as substrate requires:

(1) delignification or pretreatment to liberate cellulose and hemicelluloses from their complex with lignin,

(2) depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and

(3) fermentation of mixed hexose and pentose sugars to produce ethanol (Lin and Tanaka, 2006).

Ethanol production from lignocellulosic biomass, major step showed in Fig. 2.5

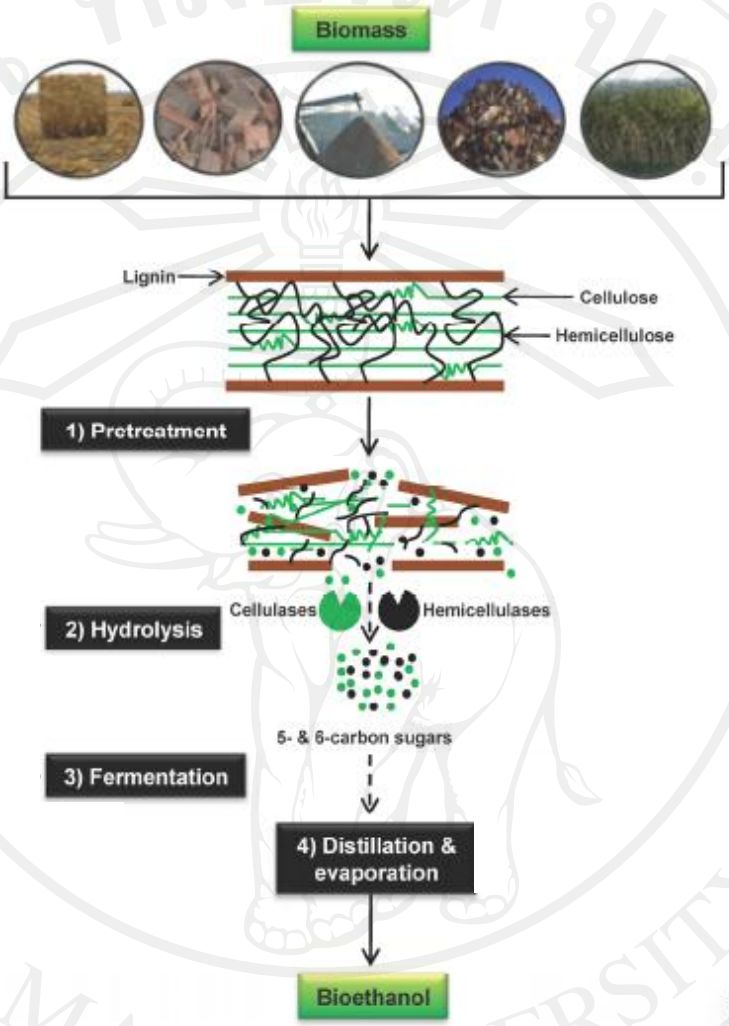

Fig. 2.5 Schematic picture for the conversion of lignocellulosic biomass to ethanol, including the major steps, pretreatment, hydrolysis, fermentation and distillation (Dashtban *et al*., 2009).

2.2.1 Delignification (pretreatment)

Pretreatment is an importance process step for the biochemical conversion of lignocellulosic biomass into bioethanol. It is required to alter the structure of strongly cellulosic biomass to break the complex lignocellulose (Fig 2.6) and make cellulose

more accessible to the enzyme to convert the polysaccharide polymers into fermentation sugars (Mosier *et al*., 2005).

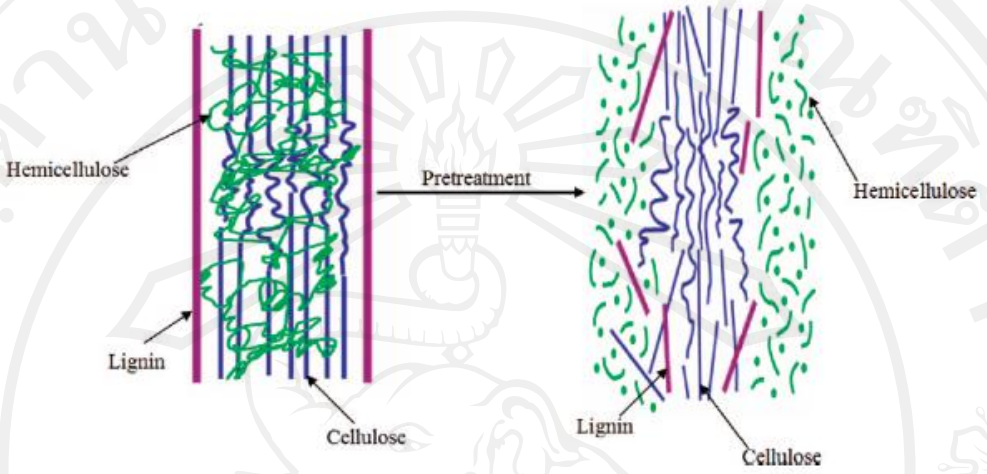

Fig. 2.6 Schematic of the role of pretreatment in the conversion of lignocellulosic biomass to fuel (Kumar *et al*., 2009).

The goals of the pretreatment are:

(1) to remove and separate hemicelluloses from cellulose,

(2) to disrupt and remove the lignin sheath,

(3) to decrease the crystallinity of cellulose

(4) to increase the accessible surface area of cellulose, and

(5) to increase the pore size of cellulose to facilitate the penetration of hydrolysis agents (Gong *et al*., 1999).

Pretreatment has been viewed as one of the most expensive processing steps within the conversion of biomass to fermentation sugar (Galbe and Zacchi, 2007). However, it has great potential for improvements in efficiency and lowering of costs through further research and development. Normally, agricultural residues are more

easily treated than hardwood materials. The most difficult substrate for treatment is softwood materials because they have a relatively high lignin content (Chahal, 1991).

Pretreatment method can be roughly divided into different categories: mechanical and physical, physico-chemical, chemical pretreatment, and biological.

2.2.1.1 Mechanical and physical pretreatment

Machanically based pretreatment methodologies are aimed at reducing the size of lignocellulosic biomass to facilitate subsequent treatment (Mtui, 2009). Mechanical pretreatment increase the digestibility of cellulose and hemicelluloses in the biomass. The use of mechanical chopping (De Sousa *et al*., 2004), ball milling (Mais *et al*., 2002), hammer milling (Mani *et al*., 2004), roll milling (Tassinari and Macy, 1997) and vibratory ball milling (Yue *et a*l., 2012) have proved success as a low cost pretreatment strategy.

Physical pretreatment does not use chemical agents. The most common pretreatment method for lignocellulosic biomass is uncatalyzed steam-explosion, also named autohydrolysis, in which only steam water is used. Uncatalyed stream-explosion is typically conducted at a temperature of 160-270^{o}C for several seconds to a few minutes (Chandra *et al*., 2007). Extensive research work has been done on this method (Verga *et al*., 2004; Chandra *et al*., 2007; Hu *et al*., 2008). In this method, particles of biomass are rapidly heated by high-pressure saturated steam for a period time to promote the hemicelluloses hydrolysis. One of the most successful physical pretreatment is irradiation, cause significant breakdown of structure of lignocellulosic biomass. High energy radiation methods, including γ-ray (Aziz and Mohsen, 2002; Yang *et al*., 2008), ultrasound (Kim *et al*., 2000; Imai *et al*., 2004), UV (Kang and Kim, 2012), microwave heating (Zhu *et al*., 2005), and electron beam (Sung and Shin,

2011). The action mode behind the high energy radiation could be one or more changes of features of lignocellulosic biomass, including increase of specific surface area, decrease of the degrees of polymerization and crystallinity of cellulose, hydrolysis of hemicelluloses and partial depolymerization of lignin. However, the high radiation treatments are usually slow, energy-intensive, and prohibitive expensive (Zheng *et al*., 2009).

2.2.1.2 Physico-chemical pretreatment

Combined chemical and physical treatment systems are importance in dissolving hemicelluloses and alteration of lignin structure, providing an improved accessibility of the cellulose for hydrolytic enzymes (Hendriks and Zeeman, 2009). The common physico-chemical pretreatment that use for lignocellulosic biomass are steam explosion with addition of chemical, Ammonia fiber explosion (AFEX) and CO_2 explosion.

Steam pretreatment at elevated temperature, e.g. 160-230^{o}C, for period about 10 min and the chemical such as SO_2 and H_2SO_4 have been studied (Galbe and Zacchi, 2002; Zimbardi *et al*., 2007 and Cara *et al*., 2008). While, the objective of adding this chemical is to improve recovering both cellulose and hemicelluloses fractions.

In the AFEX process, biomass is treated with liquid ammonia at high temperature and pressure for a few seconds and then the pressure is rapidly reduced (Teymouri *et al*., 2005). It reduces lignin content and removes some hemicelluloses while decrystallising cellulose. However, the cost of ammonia and especially of ammonia recovery drives the cost of the pretreatment.

CO_2 explosion is similar to steam and ammonia fibre explosion, high pressure CO_2 is injected into the batch reactor and then liberated by an explosive

decompression. It belived that CO_2 reacts to carbonic acid (carbon dioxide in water), thereby improving the hydrolysis rate. Studies indicate that combined capital and operating costs of the carbonic acid system are slightly high than a sulfuric acid-based system and highly sensitive to reactor pressure and solids concentration (Jayawardhana and Van, 2004).

2.2.1.3 Chemical pretreatment

The most common chemical pretreatment methods used for cellulosic feedstocks are dilute acid, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide or other chemicals to make the biomass more digestible by the enzyme. The acidic pretreatments will hydrolyze the hemicelluloses fraction while leaving the cellulose and lignin intact in the residual solid (Lloyd and Wyman, 2005). Sulfuric acid (H_2SO_4) is the most applied acid, while other acids such as hydrochloric acid (HCl) and nitric acid (HNO_3) were also reports (Xiao and Clarkson, 1997; Taherzadeh and Karimi, 2007). However, acidic pretreatments result in high concentrations of furfurals in the liquid phase (Eggeman and Elander, 2005). Furfural had a great negative effect on specific growth rate. Moreover, furfural and catechol showed a synergistic effect on toxicity with respect to ethanol yield and biomass yield (José *et al*, 2006).

The alkaline pretreatment refer to the application of alkaline solution to remove lignin and a part of the hemicelluloses, and efficiently increase the accessibility of enzyme to the cellulose (Taherzadeh and Karimi, 2008). The alkaline pretreatment was shown to be more effective on agricultural residues than on wood materials (Xu *et al*., 2007). The alkaline solutions such as NaOH, $Ca(OH)_2$, Na_2CO_3 and H_2O_2 were reported (Gomes *et al*., 2007; Silverstein *et al*., 2007; Zhao *et al*., 2007). However, the

alkaline methods may result in high concentrations of ferulate and acetate in the hydrolysate. These compounds present in the sugar stream and have deleterious effects on the fermentative microorganism (Eggeman and Elander, 2005).

Pretreatment chemistry also affects the non-sugar composition of the hydrolysate; for example, acidic pretreatments may result in high concentrations of furfurals, hydroxyl-methylfurfural, formic acid etc. in the liquid phase. The alkaline pretreatment may result in high concentration of ferulate and acetate in the hydrolysate. These compounds will be present in the sugar stream and may have deleterious effects on that fermentative microorganism (Eggeman and Elander 2005).

2.2.1.4 Biological pretreatment

Most pretreatment technologies require expensive instruments or equipment that have high energy requirement, depending on the process. Biological pretreatment refer to wood degrading microorganisms, including brown-, white-, soft-rot fungi, and bacteria to modify the chemical composition and/ or structure of the lignocellulosic biomass so that the modified biomass is more amenable to enzyme digestion (Taherzadeh and Karimi, 2008; Kumar *et al*., 2009). Recently this environmentally friendly approach has received enzymatic saccharification of lignocelluloses in ethanol production process (Alvira *et al*., 2010).

The advantages of biological delignification over previous methods are mild reaction conditions, higher product yields and fewer side reactions, and less energy demand and less reactor resistance to pressure and corrosion (Lee, 1997). Fungi have distinct degradation characteristics on lignocellulosic biomass. In general, brown- and soft-rot mainly attack cellulose while inparting minor modifications to lignin component (Schurz, 1978). White-rot fungi present more effectively and more

specifically degrade lignin than brown- and soft- rot fungi. Lignin degradation by white-rot fungi is the most effective for biological pretreatment of lignocellulosic biomass, occurs through the action of lignin-degrading enzyme (ligninolytic enzyme) such as peroxidases and laccases (Zheng *et al*., 2009).

2.2.1.4.1 Biological pretreatment by ligninolytic enzyme

White-rot fungi (Basidiomycetes) produce various isoforms of extracellular ligninolytic enzymes, such as laccases (Lac) and different peroxidases including lignin peroxidase (Lip) and manganese peroxidase (MnP), which are involved in the degradation of lignin and their natural lignocellulosic materials (Nagai *et al*, 2002). Some white-rot fungi produce all the three lignin modifying enzymes while others produce only or two of them. Lignin modifying enzymes are produces by these fungi during their secondary metabolism since lignin oxidation provide no net energy to the fungus (Eggert *et al*., 1996).

Lignin peroxidase (Lip); EC. 1.11.1.14 or ligninase (Tien and Kirk, 1983) is a glycoprotein and has been characterized as a peroxidase (Harvey *et al*, 1985). The enzyme catalyses degradation of lignin and low-molecular-mass lignin model compounds by one-electron oxidations (Schoemaker *et al*, 1985). The substrates are 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol and H_2O_2, whereas its 3 products are 3,4-dimethoxybenzaldehyde, 1-(3,4-dimethoxyphenyl)ethane-1,2-diol, and H_2O. Manganese peroxidase (MnP); EC.1.11.1.13 is considered to be an important factor in lignin biodegradation (Blanchette, 1984). This enzyme is an enzyme that catalyzes the 3 substrates, Mn(II), H^+, and H_2O_2, whereas its two products are Mn (III) and H_2O.

Several fungi show better selectivity for lignin removal. In the most widely studied ligninolytic fungus, *Phanerochaete chrysosporium*, Lip and MnP appear to be

the enzyme leading to polymer fragmentation (Asit *et al*., 1991), able to degrade all of major polymers in wood (Glenn and Gold, 1983). The other fungi, *Trametes versicolor* (*Coriolus versicolor*) was studied of the enzymatic activities show the high production of laccase activity on synthetic growth media (Manzanares et al., 1995) and on solid substrate, rice bran (Chawachart *et al*.,2004). Moreover, some fungi *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996), and *Stereum ostrea* (Buddolla *et al*., 2008) were reported as ligninolytic enzyme producing fungi. The major enzyme that initiates ring-cleavage of lignin is laccase, which catalysed the addition of second hydroxyl group to phenolic compounds.

Laccase (Lac); EC. 1.10.3.2, is an important enzyme in fungal ligninolytic systems involved in lignin degradation (Gianfreda *et al*., 1999). This enzyme, belonging to a family of multicopper-containing enzymes, catalyzes the oxidation of a variety of phenolic compounds, polyamines, aryl diamines, and lignin, with concomitant reduction of O_2 to water (Thurston, 1994; Solomon *et al*., 1996). Lac act on phenol and similar molecules, performing a one-electron oxidations, which remain poorly defined. Most Lac were reported and studied from fungal organism. Activity can also be monitored with an oxygen sensor as the oxidation of the substrate is paired with the reduction of oxygen to water. The application of laccase was used in pulp and paper industry, pulp bleaching (Bajpai, 1999). In additions, beverages industries such as fruit juices, wine and beer also used this enzyme as phenol derivatives remover (Minnussi *et al*., 2002).

In ligninolytic enzyme system, Lac is thought to contribute to degradation of lignin, a polymer incorporated into plant cell walls in response to pathogen attack. Since pectin can be esterified to phenolic compounds like ferulic acid and covalently

linked with lignin, Lac may also be required for mycelia penetration though plant cell wall (Nicole *et al*., 1992). The function of fungal hyphae in wood and the hypothetical relationship between enzymes and radicals in the process of lignocelluose transformation are shown in Fig. 2.7. Enzymes are secreted from the fungal hyphae close to the plant cell wall where they cooperate with each other and with mediating factors (work as enzyme messengers in lignocellulose degradation) such as veratryl alcohol, oxalate, malate, fumarate and 3-hydroxy anthranilic acid (Leonowicz *et al*., 2011). Lac oxidized lignin-derived radicals to quinines which serve as the oxygen source for mediating factor (Marzullo *et al*., 1995). The latter veratryl alcohol oxidase produces H_2O_2 and prevents the polymerizing activity of Lac, with formed H_2O_2 serving as co-substrates for the Lip and MnP.

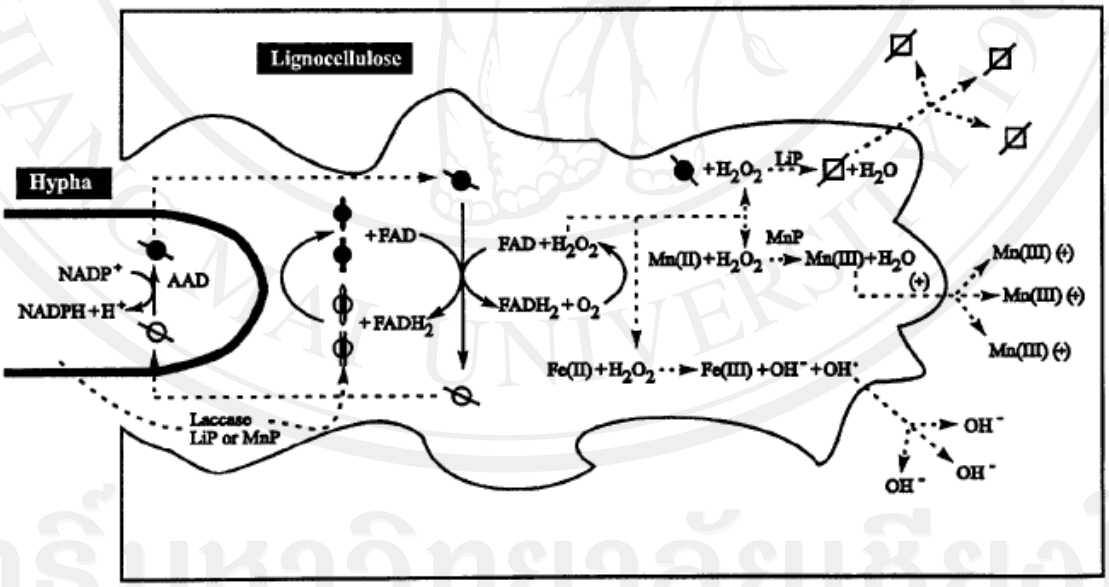

Fig. 2.7 The interaction of fungal hyphal enzymes and veratryl alcohol oxidase with mediators and mediating radicals during transformation of lignocellulose (Ander and Marzullo, 1997)

As shown in Fig. 2.7, from left to right: AAF (aryl alcohol dehydrogenase) with NADP as prosthetic group; ( )aryl alcohol; ( )aryl aldehyde; LiP, lignin peroxidase; MnP, manganese dependent peroxidase;(, ,) lignin derived radicals or quinones and their reduced forms, VAO, veratryl alcohol oxidase with FAD in prosthetic group; LiP with Fe; MnP with Mn; metal chelating agents, e.g., oxalic acid (Ander and Marzullo, 1997).

2.2.2 Hydrolysis

After pretreatment, cellulose and hemicellulose are hydrolyzed to soluble monomeric sugars (hexoses and pentoses) (Fig. 2.8) using cellulases and hemicellulases, respectively The major fermentatable sugars in biomass bydrolysate are glucose and xylose with significantly less amounts of arabinose, galactose and mannose (Gray *et al*., 2006).

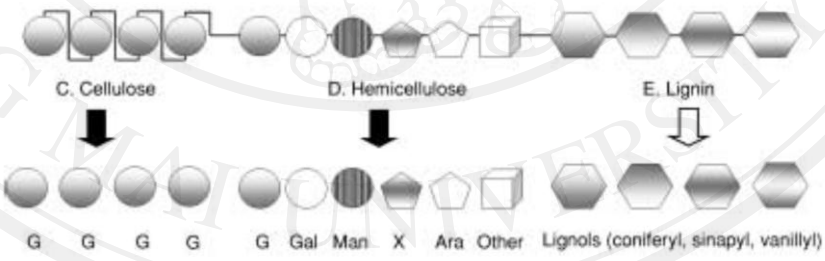

Fig. 2.8 Sources of sugars for ethanol production from lignocellulose (Zaldivar *et al*., 2001).

Arrows represent hydrolysis (only monomers generated from hydrolysis, represented by *dark arrows*, can be fermented).*G* Glucose, *Gal* galactose, *F* fructose, *Man* mannose, *X* xylose, *Ara* arabinose, *Other* L-rhamnose, L-fucose, uronic acids

2.2.2.1 Enzymatic hydrolysis

Hydrolysis or depolymerization of lignocellulosic biomass by enzymes is an effective method. Cellulase (E.C. 3.2.1.4), the most commonly used enzymes for depolymerization of cellulose to glucose, consist of three major classes: endoglucanases, excoglucanases and β-glucosidases. Whereas, xylanase (E.C. 3.2.1.8) hydrolyze hemicelluloses to xylose, arabinose, mannose, galactose etc. (Fig. 2.9). Biomass processing by enzymatic or microbial hydrolysis commonly involves four biologically mediated transformations (Lynd *et al*., 2005):

(1) production of saccharolytic enzyme (cellulase and xylanase)

(2) hydrolysis of carbohydrate components present in pretreated biomass to sugars

(3) fermentation of hexose sugar (glucose, mannose, and galactose) and

(4) fermentation of pentose sugar (xylose and arabinose)

Enzymatic hydrolysis yield sugar less than 20% of theoretical quantity are obtained without pretreatment, on the other hand, sugars more than 90% of theoretical quantity are obtained with pretreatment (Kumar *et al*., 2009). However, enzymatic hydrolysis is a promising alternative that generally provides hydrolysates with a lower inhibitory impact on the fermentation step, but it is more costly than chemical hydrolysis (Galbe and Zacchi, 2002). The use of less expensive enzyme promote several advantages, for example the quite high efficiency, low by-product formation, less expensive materials for construction due to mild process conditions, and low process energy requirement.

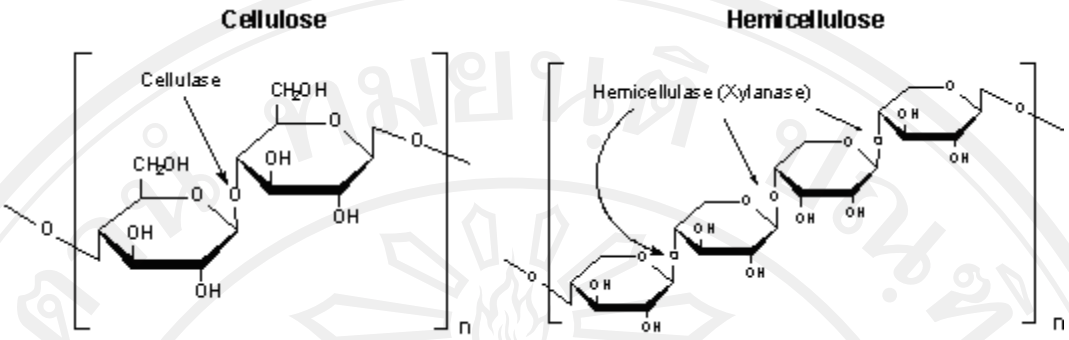

Fig. 2.9 Cellulose and hemicelluloses hydrolysis by cellulase and hemicellulase (Slavin *et al*., 1981).

Microbial cellulases have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture (Kuhad *et al*., 2011). Many studies of cellulase were successfully produced on lignocellulosic biomass under solid state fermentation, for example Milala *et al*. (2005) were used corn straw, rice husk and maize straw as substrates for cellulase production under solid state fermentation and found the optimal cellulase secretion by *Aspergillus niger* was shown at 5% concentration of substrates and pH 3.0.

About xylanase, these enzymes are important in the bioconversion of hemicelluloase into their consitituent sugars (Gawande and Kamat, 1999). Their potential applications include pulp bleaching, bread making, clarifying fruit juices and wines, improving the nutritive value of animal feed and ethanol production (Gilbert and Hazlewood, 1993; Tebka *et al*., 2006; Royer and Nakas, 1989; Wong *et al*., 1996). Many studies on xylanase production have used solid state fermentation by using agricultural residues as solid substrate. For example, Sanghvi *et al*. (2010)

produced thermostable xylanase by *T. harzianum* and found that wheat straw produced the highest yields (146 IU/ml) over the various different substrates.

2.2.3 Ethanol fermentation

When cellulose and hemicellulose are hydrolyzed, a mixture of monosaccharide was fermented. Fermentation involves microorganisms which consume sugar as a food source. Ethanol fermentation from hexose sugar (glucose) results in 4 major products, additional yeast cells (cell division), ethanol, carbon dioxide, and heat. One molecule of glucose will yield 2 molecules of ethanol and 2 molecules of carbon dioxide;

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Carbon dioxide}}{2\ CO_2} + \underset{\text{Ethanol}}{2\ C_2H_5OH}$$

Production of ethanol from sugar derived from starch and sucrose has been commercially dominated by the yeast *Sacchromycetes cerevisiae* (Yu *et al*., 2009; Hawkins and Doran-Peterson, 2011). However, *S. cerevisiae* could not used pentose sugar (primarily xylose), derived from lignocellulosic biomass for ethanol fermentation. A number of naturally occurring xylose-fermenting yeast species, including *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus*, have been found to be highly efficient xylose-fermenting strains that can be used in ethanol production (Eken-Saraçoğlu and Arslan, 2000; Sun and Tao, 2010; Joshi *et al*., 2011). The conversion of pentose sugars in fungi are explained, D-xylose is first reduced to xylitol by D-xylose reductase (aldo reductase) and subsequently oxidized to D-xylulose by xylitol dehydrogenase to form D-xylose-5 phosphate. More scientists used bioengineering methodology to enhance the conversion efficiency in the ethanol

production, the organisms used should be able to ferment all monosaccharides in the medium (Taniguchi *et al*. 1997; Zaldivar *et al*., 2001).

2.3 Solid State Cultivation (SSC)

SSC or solid state fermentation is defined as the growth of microorganisms in absence or near absence of free water with inert substrate or natural substrate as solid support. SSC processes generally employ a natural raw material as carbon and nitrogen source (Pandey *et al*., 1999). Substrates from agricultural or industrial residues (lignocellulosic biomass such as, sugarcane bagasse, coffee husk, rice husk, orange residues, among other) or inert materials (such as resins of ionic exchange, acreolite of polyurethane foam) can be used (Aguilar *et al*., 2008). However, solid substrates must contain enough moisture. Water is essential for the microbial growth and in SSF and it is present in thin layers and in occasions absorbed inside the substrates (Mudgett, 1986). Among microorganisms, filamentous fungi are the best studied for SSC due to their hyphal growth, which have capability to not only grow on the surface of the substrate particles but also penetrate though them. However, at industrial level, most enzyme production using submerged fermentation (SmF) or liquid surface fermentation (SLF) techniques (Aguilar *et al*., 2002). In the western world, the SSC has been fewer studied than the SmF and SLF. Even though, there are many advantages of SSC over submerged fermentation for examples; less space requirements, lower costs, the abundance of agricultural residue as a substrate for the production of enzymes and the low technology fermentation process are suitable for the production process particularly in developing countries (Sathish *et al*., 2008).

2.3.1 The advantages and disadvantages of the SSC in comparison to the SmF (Aguilar *et al*., 2001).

Advantages

- The culture media are simple. Some substrates can be used directly as a solid media or enriched with nutrients and better oxygen circulation compared with SmF.
- The product of interest is concentrated, that which facilitates its purification and higher yields in a shorter time period over SmF.
- The used inoculum is the natural flora of the substrates, spores or cells. Wild type strains of microorganisms perform better in SSF than do genetically modified onces, reducing energy and cost requirements.
- The low humidity content and the great inoculums used in a SSC reduce vastly the possibility of a microbial contamination.
- The quantity of residue generated is smaller than the SmF.
- Less effort in downsteam processing.
- The enzymes are low sensitive to catabolic repression or induction

Disadvantages

- The used microorganisms are limited those that grow in reduced levels of humidity
- The determination of parameters such as humidity, pH, free oxygen and dioxide of carbon, constitute a problem due to the lack of monitoring devices
- The scale up of SSC processes has been little studied and it presented several problems.
- Higher impurity product, increasing recovery product costs.

SSC are traditional fermentation such as Japanese “Koji”, Indonesian “tempeh” and French “blue cheese”. Moreover, SSC processes have shown to be particularly suitable for the production of enzyme by filamentous fungi (Rodríguez and Sanromán, 2005).

2.4 Statistical analysis

2.5.1 Analysis of variance (ANOVA)

ANOVA is a set of statistical methods used mainly to compare the means of two or more samples. Estimates of variance are the key intermediate statistics calculated, hence the reference to variance in the title ANOVA. One factor analysis of variance (Snedecor and Cochran, 1989) is a special case of ANOVA, for one factor of interest, and a generalization of the two-sample *t*-test. The two-sample *t*-test is used to decide whether two groups (levels) of a factor have the same mean. One-way ANOVA is a simple special case of the linear model. The one-way ANOVA form of the model is

$$y_{ij} = \alpha_j + \varepsilon_{ij}$$

Where y_{ij} is a matrix of observations in which each column represents a different group. $\alpha_{.j}$ is a matrix whose columns are the group means. (The "dot *j*" notation means that α applies to all rows of column *j*. That is, the value α_{ij} is the same for all *i*.) and ε_{ij} is a matrix of random disturbances.

The model assumes that the columns of *y* are a constant plus a random disturbance. You want to know if the constants are all the same. For example, data collected on, say, five instruments have one factor (instruments) at five levels. The ANOVA tests whether instruments have a significant effect on the results.

2.5.2 Experimental design

2.5.2.1 Central composite design (CCD)

A Box-Wilson Central Composite Design, commonly called a central composite design; CCD, contains an imbedded factorial or fractional factorial design with center points that is augmented with a group of star points' that allow estimation of curvature. If the distance from the center of the design space to a factorial point is ± 1 unit for each factor, the distance from the center of the design space to a star point is $\pm\alpha$ with $|\alpha| > 1$. The precise value of α depends on certain properties desired for the design and on the number of factors involved. In addition, the number of center point runs the design is to contain also depends on certain properties required for the design. A CCD always contains twice as many star points as there factors in the design. The star points represent new extreme values (low and high) for each factor in the design. Some of CCD is rotatable design and the value of α is chosen to maintain rotatability, the values of α depends on the number of experimental runs in the factorial portion of the central composite design:

$$\alpha = [\text{number of factorial runs}]^{1/4}$$

If the factorial is a full factorial, then

$$\alpha = [2^k]^{1/4}$$

Some typical values of α as a function of the number of factors are showed in table 2.2.

Table 2.2 Determining α for rotatability of CCD experiment

Number of factors	Factorial portion	Scaled value for α relative to ±1
2	2^2	$2^{2/4} = 1.414$
3	2^3	$2^{3/4} = 1.682$
4	2^4	$2^{4/4} = 2.000$
5	2^{5-1}	$2^{4/4} = 2.000$
5	2^5	$2^{5/4} = 2.378$
6	2^{6-1}	$2^{5/4} = 2.378$
6	2^6	$2^{6/4} = 2.828$

The second-order model equation following CCD experiment are as follows;

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

Where, Y is the variable response and β is the regression coefficients given by the model and X_i and X_j are the independent factors of the experiment (Akay, 2007).

2.5.2.2 Mixture design experiment

Mixture design experiment, determination of the best model for modeling the mixture system is significant in both understanding and interpreting the system. A mixture experiment involves mixing various proportions of two or more components to make different compositions of an end product (Akey, 2007). The mixture experiments are commonly encountered in industrial product formulation including food processing, chemical formulation, and pharmaceutical drugs (Moros *et al*., 2005; Martinello *et al*., 2006; Zorba and Kurt, 2006), but there is not any report on the application of the mixture design method on solid substrate for enzymes production

(Techapun *et al*., 2002). One type of mixture is simplex-centroid designs. In the *q*-component simplex-centroid design, the number of distinct points is $2^q - 1$, where *q* is components consists of points defined by the following coordinate settings. The *q*-component simplex-centroid design, the number of distinct points is 2^q - 1. These points correspond to *q* permutations of (1, 0, 0, ..., 0) or *q* single component blends, the (*q*/2) permutations of (.5, .5, 0, ..., 0) or all binary mixtures, the (*q*/3) permutations of (1/3, 1/3, 1/3, 0, ..., 0), ..., and so on, with finally the overall centroid point (1/*q*, 1/*q*, ..., 1/*q*) or *q*-nary mixture. In general, the mixture models or equation (with the asterisks removed from the parameters) are linear, quadratic, cubic and special cubic. The design points in the simplex-centroid design will support the polynomial are as follow;

$$Y = \Sigma\beta_i X_i + \Sigma\beta_{ij} X_{ij}$$

Where, *Y* is the variable response and β is the regression coefficients given by the model and X_i and X_j are the independent factors of the experiment (Akay, 2007).