PART – I
CHAPTER – I

GENERAL INTRODUCTION

1. THE COMPOSITION OF WOOD

The characteristics of wood, a major raw material for the production of pulp and paper, differ in various types of plant, for instance, conifers (gymnosperms) produce softwoods whereas angiosperms produce hardwoods. Softwoods are mainly composed of three types of cells, tracheids, the axial cells and ray parenchyma cells. Hardwoods are mainly made of fibers, vessels, and axial and ray parenchyma cells. The three major components of wood cell walls are cellulose, hemicellulose, and lignin (Table-1). Long molecules of cellulose provide the skeleton of the walls. Hemicelluloses and other carbohydrates provide the matrix of cell wall, whereas lignin, a heterogenous hydrophobic phenolic polymer, encrusts the other wall components to waterproof and strengthens the wall.

Table 1: Chemical composition of softwoods and hardwoods (Aitken, 1988)

<table>
<thead>
<tr>
<th>Components</th>
<th>Type</th>
<th>Softwoods</th>
<th>Hardwoods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td></td>
<td>42-44%</td>
<td>43-47%</td>
</tr>
<tr>
<td>Lignin</td>
<td></td>
<td>25-30%</td>
<td>17-26%</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>Hexosans</td>
<td>± 15%</td>
<td>5-8%</td>
</tr>
<tr>
<td></td>
<td>Pentosans</td>
<td>10-15%</td>
<td>15-35%</td>
</tr>
<tr>
<td>Waxes, resins, fats</td>
<td></td>
<td>1-10%</td>
<td>0.5-2%</td>
</tr>
<tr>
<td>Mineral substances</td>
<td></td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

In a transverse plane, the parietal structure of wood cells is made of a primary and a secondary wall. The secondary wall consists of two or three layers, designated $S_1$, $S_2$, $S_3$ (Figure 1). Outside the primary wall, the middle lamella connects adjacent
cells. The various cell wall layers differ in chemical composition (Mellerowicz, 2001).

Studies on the distribution of lignin by UV microscopy show that the middle lamella and the primary wall of secondary thickened cells are rich in lignin (70-80% lignin, which is 15-20% of the total lignin in the cell wall). The secondary wall is composed of only 20-25% lignin but contains 80% of the total lignin because they constitute the largest part of the total cell wall (Fergus et al., 1969).

The cells of certain parts such as pericycle, phloem, xylem of the plants undergo heavy thickening of their walls. The thickening materials of the cells are secreted by the protoplasm. These materials are deposited in the cell walls in such a manner that the cell wall becomes stratified in appearance. The cells which ultimately develop into vessels, tracheids and fibres show the thickening of the cell wall in various ways. The thickening takes place due to the deposition of the hard substance,
lignin, on the inner surface of the cell wall. Usually lignin of the secondary wall is not laid down in uniform thickness but it may form the special patterns such as annular, spiral, scalariform, reticulate and pitted (Figure 2).

Figure 2: Deposition of lignin in the cell walls

In annular or ring-like thickenings [A] the deposition of lignin takes places in the form of rings on the inner surface of the cell wall. These lignified rings are placed
one above the other like coins leaving sufficient space in between each other. The gaps of the walls remain unthickened.

In spiral thickening [B], the deposition of thickening material lignin takes place in the form of complete spiral bands. The number of such bands may be one or more than one.

In scalariform or ladder-like thickenings [C], the lignin is deposited in the form of the transverse rods of the ladder. In reticulate or net-like thickening [D], lignin is deposited in the form of a net or reticulum. In pitted thickenings [E], of the cell wall, the whole inner wall is more or less uniformly thickened, leaving here and there small unthickened areas, the pits.

Natural biopolymers formed in nature during the growth cycles of living organisms are classified as plant, animal and microbial based depending on their origin. Lignin is a plant based biopolymer. The biopolymers are classified into eight classes (Table 2) based on their occurrence. Lignin does not occur in prokaryotes but does occur in Eukaryotes (in plants only).
Table 2: Classes of biopolymers and their occurrence

<table>
<thead>
<tr>
<th>Class</th>
<th>Synthesised in</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Prokaryotes</td>
<td>Eukaryotes</td>
<td></td>
</tr>
<tr>
<td>1 Nucleic acids</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>2 Proteins, polypeptides</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>3 Polysaccharides</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>4 Polyhydroxyalkanoates</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5 Polyphosphates</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>6 Polyisoprenoids</td>
<td>No</td>
<td>Plants, some fungi</td>
<td></td>
</tr>
<tr>
<td>7 Lignin</td>
<td>No</td>
<td>Only plants</td>
<td></td>
</tr>
<tr>
<td>8 Polythioester</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

Source: Everslo et al., 2001

Lignin the most abundant renewable aromatic compound on earth is second only to cellulose in its contribution to living terrestrial biomass (Crawford, 1981). A naturally produced organic recalcitrant, lignin is a polymer of units linked together in a complex and irregular pattern which varies from species to species, tissue to tissue, and cell to cell. Vascular plants use lignin to line their conductive tissues as a barrier to water loss; thus lignin was responsible in the spread of these plants throughout the terrestrial landscape. Plants have subsequently harnessed lignin to bind cells together, rigidify their lamellate cell walls into micro composite structures of remarkable strength and provide a physical barrier against invading microorganisms. The stable, irregular cross-links and hydrophobicity make lignin an ideal material to limit water loss and stifle pathogen invasion. The heterogeneity of the polymer and the high proportion of covalent bonds linking different monomers are responsible for the difficulty in determining the amount, structure, or monomeric composition of lignin in plants.
Comprising 20-30% of the total dry weight of wood, lignin is the principal barrier for the production of pulp and paper. Historically, efforts to manufacture paper more economically have provided the principal impetus for studies on lignin structure, and biosynthesis. Consequently, the accumulated information about lignin produced in arborescent plants tends to show our perception of what lignin is and does. Thus, Freudenberg (Freudenberg, 1968) defined lignin as the heteropolymer resulting from the dehydrogenation of a mixture of three p-hydroxycinnamyl alcohols—coumaryl, coniferyl and sinapyl alcohols—best exemplified by spruce milled—wood lignin. This definition was drawn specifically to delineate a perceived difference between the polymer isolated from trees and other higher order vascular plants, and the aromatic polymers that can be isolated from various bryophytes and pteridophytes.

However, studies of lignin mutants in both herbaceous and woody plants have more recently provided us with a greater appreciation of the true diversity of function to which plants have adopted this polymer. Consequently Freudenberg’s definition of what constitutes lignin seems increasingly restrictive. Ralph (Ralph et al., 1999) and co-workers observed that plants can incorporate a wide variety of phenolic precursors into lignin as anticipated form Freudenberg’s definition. It can also be speculated that lignin composition and structure could be changed even more drastically by drawing on our expanding knowledge of developmental processes that parallel lignification in other organisms, e.g., sclerotization of insect cuticle (Andersen et al., 1996) and melanization of fungal rhizomorphs (Butler et al, 1998).
2. HISTORICAL OUTLINE

The most abundant source of the benzene nucleus on this planet, lignin, probably began to appear on Earth some 350 million years ago during the late Silurian age when plants first began to invade the land. Such invasion required the additional rigidity, conferred by lignification, of plant organs that were to be borne above the soil surface and subjected to the mechanical stresses of wind and rain. Lignin is also found in other tissues such as roots and in husks, and shells where it has a protective role.

Against the current debate concerning a chemical definition for lignin (Lewis, 1999), it would be appropriate to delve into the origin and definition of the term, lignin. The dispassionate collection and evaluation of knowledge indicates that the foundation stone for the study of lignin was laid way back in the beginning of the nineteenth century. Prior to 1811 wood was considered to be a uniform chemical compound. In 1811, Gay-Lussac and Thenard found that the organic components of wood consist only of carbon, hydrogen and oxygen and that no other elements were present. Around the same time Prout carried out an analysis of wood fibres which he called "fibres ligneus". At a time when it was thought that wood is a chemical entity, about 1815, Raspail expressed the opinion that wood may consist of several components and that the cell wall is built of gum and lime. In 1819 Braconnot and later in 1822 Authenrieth and Bayerhammer showed that wood is not a uniform chemical compound.

Derived from the Latin for wood (lignum), the first use of the word, lignin in the English language is ascribed to Imison, who was describing what was then
considered an elemental and non divisible material remaining after wood fibers were first boiled in water and then in alcohol. In 1834, Runge discovered that wood gives a green colour reaction with phenol and hydrochloric acid, and a yellow colour with aniline hydrochloride. It was Anselme Payen who in 1838, succeeded in isolating, a uniform compound which he called “cellulose”. He treated wood alternatively with nitric acid and caustic soda and obtained cellulose fibers which he considered to be the basic substance of the cell membrane. By this treatment he also succeeded in removing a substance different from cellulose, which he called “matiere incrustante” (incrusting material). This material had a higher carbon and hydrogen content than cellulose. Although he isolated a series of products which he called “lignose”, “lignon”, “lignin” and “lignireose”, these products were a mixture of carbohydrates with incrusting material and did not have a definite composition.

In the following years Payen arrived at the conclusion that the cellulose fiber in wood must be surrounded by the incrusting material based on which he postulated the incrustation theory in which he postulated that cellulose is the fiber-forming material in wood and other cell membranes and it is embedded in and impregnated with varying amounts of incrusting material. In 1851, Claussen tried to remove the noncellulosic materials by treating wood alternatively with sulphurous acid and alkali. This process led, fifteen years later, to the development of the manufacture of wood cellulose by the sulphate cooking process. In 1853, Watt and Burgen and in 1854, Coupier and Melhier applied hot alkali to remove the incrusting material in order to isolate cellulose from wood destroying the noncellulosic components. Schulze removed the incrusting material which he called “lignin”. The term “lignin” however had been introduced into the scientific literature by de Condolle in 1833. Schulze failed in his attempt to isolate lignin but in agreement with Payen, concluded that in wood and other cell membranes, the cellulose is the fiber-forming material and that
the fiber is surrounded by various amounts of lignin. Fremy and Terrell made the first attempt to isolate incrustants from various cell membranes for analytical proposes. Erdmann after a series of findings concluded that lignin is chemically combined with cellulose or other carbohydrates of the cell wall. He found numerous supporters of his theory that lignin is at least partially aromatic in nature.

The first analytical data of the presence of methoxyl group in lignin was provided by Benedict and Bamberger discovered that lignified plants gave methyl iodide when heated with hydroiodic acid and phosphorous. Because cellulose did not give this product, they concluded that it must have originated from the lignin and that lignin must contain methoxyl groups. Almost simultaneously Tollens and Lindsey in Germany and Klason in Sweden began the first investigation on lignosulphonic acid. Klason produced the first of his classical publications on lignin. He found that coniferyl alcohol, under the conditions of the sulphite pulping process gave a sulphonic acid which possessed properties almost identical with those of lignosulphonic acid. He was the first to suggest that a phenyl propane derivative of the coniferyl type might be the basic parent substance of lignin. He also proposed the first method for the direct determination of lignin in lignified substances and was the first to isolate lignin for analytical purposes with 72% sulphuric acid, a process which forms the basis for the present standard lignin determination. It was Klason who discovered the isolation of lignin by means of alcohols in the presence of small amounts of mineral acids. Since modern lignin chemistry has tended more and more to probe Klason’s original concept that lignin is built up from phenylpropane building stones is correct, he may justly be called “the father of lignin chemistry”.

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After world war I a tremendous amount of lignin research work has been carried out, particularly in Europe. It was during this time that Brauns isolated native spruce lignin (Brauns, 1952). This brief historical review shows that the problem of lignin is an old one.

Although many investigations have attempted to define lignin in terms of its chemical structure, none have succeeded in proposing an adequate definition. After Klason in 1908 and Willslatter and Zechmeister in 1913 isolated lignin by subjecting wood to hydrolysis with strong mineral acid, Kalb in 1932 defined lignin as a complex compound or cell membrane constituent consisting of carbon, hydrogen, and oxygen with relatively high carbon and methoxyl content, unhydrolyzable, readily oxidized, soluble in hot alkali or bisulphite, easily condensable with phenols, and giving a series of colour reactions. This, of course, is not a definition at all, but a listing of physical and chemical properties associated with known lignin materials.

As the chemistry of lignin evolved, Brauns in 1952 updated Kalb’s description and defined lignin as that incrusting material of the plant which is built up mainly if, not entirely, of phenyl propane building stones, which carries the major part of the methoxyl content of the wood, which is unhydrolyzable with acids, readily oxidized and soluble in hot alkali and bisulphite, and which readily condenses with phenols and thio compounds. In 1960, on the basis of intern studies, Brauns broadened his definition to include products of aromatic aldehydes upon the oxidation of lignin with alkaline nitrobenzene and the production of “Hibbert’s monomers” (Vanillin - α - ethoxy propiovanillone, and vinilloyl methyl ketone) upon its subjection to
ethanolysis. Efforts to refine the chemical definition of lignin have resulted only in still greater complexity.

Over the years the term lignin has come to have different meanings to different persons (Irwin). To a sulphite pulp producer lignin represents a sulphonated wood component that must be washed from his pulp and be withheld from an adjoining stream or waterway. A fisherman thinks of lignin as a polluting agent. A kraft, or soda pulp manufacturer thinks of lignin as a product that can be precipitated from his black liquour with acid but that which is usually employed as the source of heat for running his mill. Lignin to a bleach plant operator is the material he must remove from the pulp to increase its brightness. An analytical chemist regards lignin as that material with 72% sulphuric acid followed by dilution and boiling or that material which gives ultraviolet absorption maximum at 280m\( \mu \). To a botanist lignin is a metabolite of the growing plant or a structural element contributing strength to the mature plant and may be located by means of specific colour reactions such as that with phloroglucinol and hydrochloric acid. The microbiologists and soil chemists consider lignin a residue of decay, formed by the enzymatic dehydrogenation of specific phenyl propane monomers. Lignin to the organic chemist is a complex polymer that challenges his fundamental interest in chemical structure, a biochemical constituent of plants to be degraded to identifiable fragments in the search for its ultimate structure; while some regard lignin only as a probable raw material for the production of commercially useful organic chemicals. Thus it is apparent that different kinds of scientists have varying concepts of lignin contributing to the complexity of the meaning of the term lignin (Kirk).
3. CHEMICAL CONSTITUTION OF LIGNIN

Dehydrogenative polymerization of three different cinnamyl alcohols, p-coumaryl, coniferyl, and sinapyl alcohol which differ in the degree of methoxylation at the C₃ and C₅ positions of the aromatic ring (Figure 3) produce the lignin polymer in plants.

When incorporated into lignin, these alcohols are called the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the polymer, respectively. In addition to the three main monolignols, lignin contains traces of units from incomplete monolignol biosynthesis and incorporates various other phenylpropanoid units, such as hydroxycinnamyl aldehydes, acetates, p-coumarates, p-hydroxybenzoates, and tyramine ferulate (Sederoff et al., 1999; Boeijan, 2003). A variety of chemical linkages, including ether and carbon-carbon bonds, connect the units in lignin (Boerjan 2003, Higuchi 1990, Ralph et al, 1998). The complexity and heterogeneity of the polymer depend on the relative proportions of the three principal monolignol units as well as the different types of interunit linkages (Campbell et al., 1996, Monties, 1998). For example, lignin from gymnosperms consists mainly of G units and low levels of H units, whereas lignin from angiosperms is predominantly made up...
of both G and S units along with traces of H units. Lignin from grasses incorporates G and S units at comparable levels and more H units than dicots. Softwoods are less susceptible to Kraft lignification than hardwoods. Lignin rich in G units has relatively more carbon-carbon bonds than lignin rich in S units because the aromatic C₅ position of G units is free to make linkages. Wood lignins essentially made of G units (Softwoods) are therefore less susceptible to Kraft delignification than lignins made of G and S units (hard woods) (Chiang et al., 1990, Ona 1996, Lapierre et al., 1999).

The structure of lignin makes it the recalcitrant material that it is. Lignin is formed in vascular plant cell walls by the oxidative coupling of several related phenyl propanoid precursors: coumaryl, coniferyl, and sinapyl alcohols. Peroxidases or laccases in enzymes in the plant cell wall oxidize these monomers by one electron, yielding transient resonance stabilized phenoxy radicals which then polymerize in a variety of configurations. The possible ways that the precursors can couple can be portrayed on paper simply by drawing the conventional resonance forms of the phenoxy radicals, and then by linking the most important of these in various pairwise combinations. This subject has been extensively reviewed (Higuchi 1990, Adler 1977).

Lignin consists primarily of the inter-monomer Linkages shown in Figure 4. The arylglycerol-β-aryl ether structure is quantitatively the most important of these, constituting over 50% of the polymer.
Figure 4: Structure of lignin macro molecule

Numerous types of covalent linkages bind lignin with hemicelluloses in the cell wall, the most important being the ether bonds between the benzylic carbon of lignin and the carbohydrate moiety, ester bonds between the benzylic carbon of lignin and uronic acid residues, and lignin – glycosidic bonds.
4. ROLE OF LIGNIN IN THE BIOSPHERE

When vascular plants die or drop litter lignified organic carbon is incorporated into the top layer of the soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the earth’s carbon cycle. Were this not so, all carbon would eventually be irreversibly sequestered as lignocellulose. Lignin biodegradation has diverse effects on soil quality.

A quantitatively important sink for fixed carbon in the biosphere, lignin is mineralized via the carbon cycle. This natural plastic is not catabolized “Sensu strictu” but is biologically eroded in the presence of air and is biologically inert in the absence of molecular oxygen. The majority of biomass on earth is in the form of lignocellulose produced via vascular plant photosynthesis. The relation of lignocellulose to the carbon cycle is as shown in Figure 5.
Figure 5: Relation of lignocellulose to the carbon cycle (Boerjan, 2003)
The absence of anaerobic lignin biodegradation has profound environmental complication. The gradual accumulation of lignin and lignin-derived materials over extended periods of time forms the basis for coat and peat deposits. If nature withdraws its helping hand—if the carbon sinks stop absorbing some of our excess carbon dioxide—we could be facing drastic changes even before 2050, a disaster too swift to avoid. But if the carbon sinks hold out or even grow, we might have extra decades in which to wear the global economy from carbon-emitting energy sources. Some scientists and engineers believe that by understanding natural carbon sinks, we may be able to enhance them or even create our own places to safely jail this threat to global climate (Tim Appenzeller, 2004).

The microbial degradation of litter results in the formation of humus, and ligninolysis probably facilitates this process by promoting the release of aromatic humus precursors from the litter. These precursors include incompletely degraded lignin, flavanoids, terpenes, lignans, condensed tannins, and uberins (Hudson, 1986). Undegraded lignocellulose for example in the form of straw has a deleterious effect on soil fertility because decomposing lignocelluloses supports high populations of micro organisms that may produce phototoxic metabolites. High microbial populations in undecomposed litter also compete with crop plants for soil nitrogen and other nutrients (Lynch and Harper, 1985). By breaking down the most refractory component of litter, lignolysis thus contributes to the removal of conditions that inhibit crop productivity (Hammel, 1997). The more highly lignified litter is, the less digestible it is, and the more its decomposition depends on the unique organisms that can degrade lignocellulose.
The industrial utilization of ligninaceous materials results in the large scale production of aromatic chemical wastes (Figure 6) in particular in the paper and pulp industry. Environmental pollution caused by accumulation and toxicity of aromatic chemical wastes are of special importance in anaerobic environments because here they are more refractory or biochemically inert. The lack of biodegradability, growing water and land surface litter problem and pollution of incinerated synthetic plastics has raised worldwide concern on use of synthetic plastics. The need of the hour, therefore is a worldwide research to develop new biodegradable alternatives to synthetic plastics. Biodegradable polymers or bioplastics are important and interesting areas that are being worked out as alternatives for synthetic plastics. There are a new generation of materials able to significantly reduce the environmental impact in terms of energy consumption and greenhouse effect. ISO 472-1988, a standard authority defines a biodegradable plastic as, a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastics and application in a period of time that determines its classification. The change in chemical structure results from the action of naturally occurring micro organisms. Biodegradation is a process by which bacteria, fungi, yeasts and their enzymes consume a substance as food source so that its original form disappears (Chandra and Rustgi, 1998).
Figure 6: Polymer waste management options (SCOTT, 2000)
Wood is an industrial raw material and a renewable energy resource. During the manufacture of high quality paper, lignin is chemically separated from the polysaccharide components of wood during pulping and bleaching reactions. Lignin extraction consumes large quantities of chemicals and energy leading to a poor environmental image for the industry (Higuchi, 1985; Marie Baucher et al., 2003). Paper mills use chlorine to rinse lignin out of the wood pulp they need to make paper. Once the lignin is washed away and the pulp is ready to be made into paper, chlorine is used again to make paper white. When wood pulp or recycled paper is bleached the reactions that take place between the chlorine, lignin and cellulose produce the most toxic substances ever created. The most dangerous of these include a family of seventy five different chemicals known as dioxins, the most carcinogenic chemicals known to science, not readily degraded.

While we talk about the advantages that have been derived from notable research in chemistry, it will be a job half done if we do not focus on the hazards created by the advances in chemistry during the past few decades. Dealing with the hazardous and toxic substances along with the adverse impact of over exploitation of research by virtue of the scientific developments would be the challenges for the future. Not denying the fact that notable research in chemistry has been responsible for several hazardous substances, it is to be noted that the existing knowledge to use them can be enough to get rid of these substances besides finding newer and green routes to manufacture alternatives.

With the exception of synthetic polymers, most economically important products, such as paper, cordage (cords and rope) and textiles are derived from plant
fibers. Fibers are elongate cells with tapering ends and very thick, heavily lignified cell walls. The plant cell is protected by a cell wall that has a structure analogous to reinforced concrete. The cellulose fibrils play the role of steel reinforcing rods, while concrete is represented by lignin. Lignin determines the rigidity, strength and resistance of a plant structure.

When wood fiber is processed to make paper or composite products, lignin must be removed using polluting chemicals and a great deal of energy. The digestibility of animal feed is influenced by lignin content, the greater the lignin content, the poorer the food source. Genetic engineering is now being used to fundamentally modify the lignin of forest trees and animal feed. The costs of preparing fiber and improved digestibility of fodder and forage can be greatly reduced by reducing lignin content of fiber and forage. However, the advantages of reduced lignin are offset by the disadvantage of plants with reduced lignin, which are more readily attacked by predators such as insects, fungi and bacteria. Increasing lignin content has been promoted as a defense against pests.

The importance of lignin in disease resistance has been known for well over twenty years. The process of lignification is crucial in reducing predation by spruce beetles, and lignin in the roots of the date palm plays a key role in defence against the fungus fusarium. A guaiacyl rich lignin is produced as “defense” lignin when eucalyptus is wounded by a predator. Lignin content of larch species determines the level of heart wood brown - rot decay. Genetic modification of plants to enhance lignin production is covered in United States patent No. 5,728,570 (Cummins, 2005). However, Arabidopsis plants modified in the metabolic pathway leading to lignin formation produced abnormal lignin that was associated with severe fungal attacks.
Genetic modifications for reduced lignin level results in reduced fitness including increased winter mortality and decreased biomass.

Genetically modified low lignin trees are called "super" trees with little consideration of past resistance and genetic stability. The forage and fodder with reduced lignin and lignin with improved composition are more desirable food sources for grazing animals. Lignin modification of trees and forage crops has been a focus of research in genetic engineering. But lignin provides both fundamental structural features and resistance to animal and microbial pests. Lignin enhancement that leads to greater forage or tree pulp quality also leads to susceptibility to diseases, while lignin enhancement that leads to greater disease resistance makes forage less digestible and tree pulp more expensive to process.
5. BIOSYNTHESIS OF LIGNIN

Lignin monomers are produced intracellularly and exported to the cell wall and subsequently polymerized. The monomers are products of the phenylpropanoid pathway starting from phenylalanine (Figure 7). The lignin biosynthetic pathway has been the subject of many recent reviews, but is still being continually revised and updated to incorporate the results of new research (Marie et al., 2003).

A central question in lignin biosynthesis is how guaiacyl intermediates are hydroxylated and methylated to the syringyl monolignol in angiosperms. To address this question, Keishi et al., (1999) cloned cDNAs encoding a cytochrome P450 monooxygenase and a caffeate O- methyl transferase from sweetgum xylem.
Figure 7: Phenylpropanoid and monolignol biosynthesis pathways

(Source: Marie Baucher et al., 2003)
The hydroxylation and methylation reactions that ultimately determine the monomeric composition of lignin (because the three monomers differ only in their degree of methoxylation) occur at the level of the cinnamic acids (Higuchi, 1985). The final steps in the biosynthesis of lignin are the oxidation of the cinnamyl alcohols to the corresponding radicals in the cell wall and their subsequent polymerization. A significant extent of research during the past decade has proposed that peroxidases, laccases, and other phenol oxidases may be involved in the radical formation process. Due to the complexity of these enzymes that exist in plants cells, determining the role of individual enzymes in the lignification process is difficult. Conclusion is yet to be reached as to whether monomer coupling is a random or a highly orchestrated process.

A persistent challenge is the understanding of how similar enzymes in different plants can give rise to the striking natural heterogeneity that exists in lignin. The content and composition of lignin differs not only among plant taxa, but also between different cell types of a single tissue and even within a single cell wall. Lignin is also influenced by environmental stress. Heterogeneity of lignin could be a product of the spatio-temporal and conditional expression of the genes involved in the lignin pathway and of the differences in the substrate specificity and kinetics of the enzymes they encode. These processes may also be regulated by pathway intermediates because the concentration of certain intermediates has been shown to effect enzyme activity and gene expression. Studying lignin-biosynthetic-pathway mutants and transgenics provides insights into plant responses to perturbations of the lignification system, and enhances our understanding of normal lignification. When enzymes late in the pathway are down regulated, significant changes in the
composition and structure of lignin may result. Ralph et al., (2001) have shown the importance of NMR spectroscopy as a powerful diagnostic tool for elucidating structures in the difficult lignin polymer, hinting at the chemical and biochemical changes that have occurred during the biosynthesis of lignins.

The exact composition of *in situ* lignin and its biosynthesis pathway has been and is still a challenge to chemists and biochemists to this day.

Echoing the words of Joe Cummins (2005), the economic consequences of effective lignin modification could be tremendous, but producing forests and rangelands highly susceptible to insects, fungi and bacteria would lead to economic and environmental disaster. The low lignin trait is comparable to a loss in immune functions similar to AIDS in mammals.

Lignin is perhaps the most unusual of all natural products in that it is optically inactive even with the presence of asymmetric carbon atoms in its molecule, which is in striking contrast to other naturally occurring chiral biopolymers, such as nucleic acids, protein and polysaccharides.
CHAPTER – II

REVIEW OF THE LITERATURE

1. ISOLATION AND CHARACTERISATION OF LIGNIN

As early as 1893, Klason thought he had isolated an unchanged lignin from spruce ground wood. By alternate extractions with boiling water and hot alcohol he dissolved 12% of the wood and isolated from the alcohol solution 2% based on wood a light brown powder containing 59.97% C and 5.66% H. Thirteen years later he repeated the experiment but dried at 130°C and C: 63.9% and H: 5.94% were calculated. In 1939, Brauns made an attempt to isolate native lignin from spruce and later from western Hemlock.

Bondi and Meyer (1948) isolated lignin in a state of comparative purity from a number of young plants such as wild barley Bersem clover, Fahli clover, Peanut hay and Cyprus vetch representing the Leguminoeae and four plants from the Gramineae family using dilute alkali. The OCH$_3$ groups were determined by methylation of lignin by two different methods, with dimethyl sulphite and with diazomethane. The OCH$_3$ content of lignin from Leguminocae is trebled on methylation with diazomethane, whilst the content of Gramineae lignin is only approximately doubled. They concluded that OCH$_3$ groups in lignin from Gramineae which have a higher OCH$_3$ content before methylation, correspond to unsubstituted hydroxyl groups in the lignin from Leguminocae. Comparison of the results of methylation with dimethyl sulphate and diazomethane therefore shows that in all plant lignin there are many more phenolic and enolic hydroxyl groups than aliphatic ones. Their solubilities have
been studied in different solvents. Molecular weights were obtained by the depression of freezing point method using β - naphthol.

Bondi and Meyer (1948) used the following process for the degradation of lignin (1) oxidation with nitrobenzene in alkaline solution (2) fusion with potassium hydroxide (3) hydrolysis with acid ethanol under pressure. Surprisingly they found that syringaldehyde is totally absent from the plant lignin investigated by them. Syringaldehyde is a characteristic component of the aldehyde fraction of hardwood lignin. The Graminea lignin gave a larger quantity of aldehydes on oxidation than lignin from Leguminoeae. Though the total percentage of aldehyde obtained varies greatly, the ratio of vanillin to p-hydroxybenzaldehyde remains constant throughout and is approximately 2:1 without being influenced either by the botanical family or the degree of woodiness of the plant from which the lignin is derived. The plant lignin contain in contrast to wood lignin, nitrogen as a characteristic component. They classified lignin by their increasing N and decreasing OCH₃ content as softwood lignin, Gramineae Lignin and Leguminoeae lignin. George and Nord in 1952 isolated both the native and enzymatically liberated lignin from white Scots Pine, Oak, Birch, Maple, Bagasse and Kiri. The lignin isolated were subjected to oxidation and the degradation products quantitatively determined. The results of the oxidation process and degradation are recorded and discussed with respect to the nature of the products formed from representative softwoods and hardwoods.

Pepper in 1954 made a study on the conditions affecting the isolation of a lignin fraction by the procedure involving a moderate temperature catalytic hydrogenation of pre-extracted aspen wood meal. The effect of change in the initial
hydrogen pressure, the catalyst, the time and maximum temperature of reaction, and the nature and acidity of the suspending medium were studied. The weight of the residual pulp, the effectiveness of lignin removal, the weight and methoxyl content of the resulting chloroform-soluble fraction containing the lignin and the yields of vanillin and syringaldehyde obtained by the alkaline nitrobenzene oxidation of this same fraction were the factors serving as the basis for comparison. Data are represented and interpreted to indicate the existence of a lignin-carbohydrate complex which is cleaved under the conditions of catalytic hydrogenation. It is indicated that the use of copper chromium oxide may be preferred over Raney nickel as a catalyst for this isolation procedure. Continuing their work on lignin Pepper et al., experimented with lignin fraction from spruce and aspen wood meals using a low temperature (90-95°C) acidolysis involving a dioxane-water (9:1, V/V) solvent medium containing the equivalent of 0.2N hydrochloric acid. The effect of extraction time on the yield of precipitated lignin and ether-soluble material, and on the methoxyl content of these products, was investigated. For both species prolonged extraction times led to greater yield of lignin with lower methoxyl content. These results indicate the rearrangements that occur intramolecularly in isolated lignin. They emphasize too the requirement of short times of extraction in order to obtain a representative lignin fraction. The application of this procedure of lignin isolation to both spruce and aspen woods gives good yields of a readily obtained product, which on preliminary examination, appears to be only moderately changed from protolignin. In comparison, it appears that aspen wood lignin is isolated rather more easily than that from spruce wood. They have proof to prove the dinuclear characters of the hardwood lignin as compared to the greater homogeneity of the softwood lignin,
suggesting the greater ease of cleavage, and subsequent dissolution of fragments with syringyl type nuclei.

Sarkanen and Ludwig (1971) have brought out an excellent publication relating to the occurrence, formation, structure and reactions of lignin. Cotin in 1973 carried out experiments on a range of samples of grass and one clover by a method of lignin determination which utilizes the solubility of lignin in HCl-activated trimethylene glycol at 121°C. The method is simple, fast and reproducible, and relates well to in vitro organic matter digestibility. Trimethylene glycol is relatively non-toxic and non-corrosive. Determination of lignin is indirect, but gravimetric, so that standards are not required.

With the object of exploring the isolation of plant lignin from different sources and their overall characterization by elemental analysis and proton nuclear magnetic resonance spectroscopy, Iradj in 1976 isolated pulp lignin with TGA. TGA has been shown to react readily with lignin's benzyl alcohol and benzyl ether groups if hydrogen chloride or boron trifluoride etherate are present. The reaction has been noted for its reproducibility, high yields, and mild conditions. Since the extent of thioglycolization depends upon the concentration of reactive benzyl functions, this reaction had been applied only to protoligins (lignin in wood), but not to pulp lignin. These have lost most of their reactive sites during pulping.

As part of a study of the charring rate of wood, Robert (1987) determined the lignin heating values and the chemical composition of samples from four hardwoods and four softwoods. The four softwoods were Engelmann spruce, western red cedar,
southern pine and redwood. The four hardwoods were hard maple, yellow-poplar, red
oak, and basswood. The higher heating value of wood was correlated with lignin and
extractive contents.

Two fairly comprehensive guides to the use of spot tests for the detection of lignin are:

1. TAPPI Official Test Method 401, “Fiber Analysis of Paper and Paperboard”.
2. Paper Conservation Catalog, part 10, “Spot Tests”.

The Paper Conservation Catalog is available from the American Institute for conservation (202/232 – 6636); it gives sources of supply for each stain. It refers frequently to relevant TAPPI standards and has a 41 item bibliography, which overlaps partly with the TAPPI 401 bibliography.

A succinct review of 45 methods for chemical analysis is given in TAPPI, 1987. This is followed by reviews of 12 studies on preparative lignin, of 25 on characterization of lignin, and of 13 on chemical modification and degradation. In the section on pulps the accuracy of lab tests for lignin content is discussed; “Accurate analytical determination of the lignin content of pulps has constituted a particular problem... The kappa number lignin content relationship differs with pulping method and species; therefore it must be established for each combination of the factors”.

Galen in 1987 methylated and acetylated the solid lignin preparations from common woods red oak and lodgepole pine in order to examine the relationships between the $^{13}$C NMR chemical shift and molecular structure in solid lignin samples.
Oskar et al., (1987) subjected wood and milled wood lignin from beech, spruce, bamboo, and teak wood to two types of pyrolysis: (1) Direct pyrolysis – gas chromatography-mass spectrometry (Py-GC-MS) and off-line Py-GC with flame ionization detection (FID). It was demonstrated that off-line Py-GC-FID is a suitable method for lignin classification of composed lignocellulosic materials without isolation of lignin. Quantitative pyrolysis results were used to calculate H.G.S ratios of lignin and the results were compared with data obtained from nitrobenzene oxidation and Fourier transform infrared analysis.

Martin et al., in 1991 isolated two milled wood lignin (MWLs) from tobacco leaves and midrib material according to the Bjorkman procedure. Prior to MWL isolation, the material was extracted with cyclohexane / ethanol and hot water to remove extractives which could interfere with this isolation. FT-IR spectroscopy was used for quality control and classification of the isolates. Tobacco lignin contains approximately 10% 4-hydroxyphenylpropane, 78 - 82% guaiacylpropane and 10-13% syringylpropane moieties.

Richard and Gosta (1992) subjected a released suspension culture lignin from Norway spruce to a detailed two-dimensional NMR study. It was found that the most informative technique was homonuclear Hartmann–Hahn spectroscopy and $^{13}$C-decoupled $^1$H-detected multiple quantum $^1$H-$^{13}$C-correlation (HMQC) spectroscopy. While correlations from the side chains of most of the commonly proposed lignin structural units were observed, no resonances assignable to the non-cyclic benzylarylether or diarylpropane-1, 3-diol substructures which have previously been claimed to account for up to 20% of the inter unit linkages in lignin could be
observed, suggesting that the relative importance of these units to the structure of lignin is somewhat questionable. From the ROESY data, and by consideration of molecular modeling results, it was concluded that in the predominant β-aryl ether inter unit linkage most of the side chains were in the gauche conformation, with some in an anti conformation.

Erik et al., (1993) have characterized a set of lignin polymers using temperature-resolved in-source pyrolysis-mass spectrometry (Py-MS) and complementary curie- point pyrolysis-gas chromatography - mass spectrometry (Py-GC/MS). Combined results showed that enzyme- treated cottonwood-milled wood lignin is a relatively homogeneous polymer with a large fraction of preserved alkyl-aryl ether linkages. Technical lignin isolated by the steam-explosion, organiosolv and Kraft pulping processes, were found to be modified depending on, the severity of the isolation procedure employed, and contained large amounts of β1- linked stilbene and β-β-linked resinol types of structure. Bagasse lignin shows the most condensed polymer structure with a large proportion of ether bonded p-coumaric acid and ferulic acid.

James and Walter (1994) used solid-state $^{13}$C NMR to investigate changes in the composition of residue from red clover and peanut hulls obtained by four commonly used gravimetric methods for lignin in forages. The samples had also previously been analyzed by infrared and pyrolytic mass spectrometric procedures. The solid-state $^{13}$C NMR spectra enable the conformation of a structural interpretation of why the different gravimetric procedures are not chemically equivalent. The three instrumental techniques together enabled a more accurate evaluation of the chemical
composition of lignin obtained by each of the four different gravimetric procedures as well as a comparison of the advantages and limitations of each of the instrumental methods for forage analysis.

Alessandro and Argyropoulos (1995) thoroughly examined the use of 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane as a phosphitylation reagent in quantitative $^{31}$P NMR analysis of the hydroxyl groups in lignin. Quantitative analysis of six standard lignin gave results comparable to those obtained by other methods of analysis. Excellent resolution of the various phenolic hydroxyl environments including those present in condensed moieties was observed. However this was at the expense of resolution in the aliphatic hydroxyl region, where no distinction between primary, secondary and the erythro and threo forms of the secondary hydroxyls of the $\beta$-0-4 bonds can be made.

Mark et al., (1996) have reported a rapid isolation and structural characterization of alkali-soluble lignin during alkaline treatment and atmospheric refining of wheat straw. The chemical compositions of two alkali-soluble wheat straw fractions isolated by two steps of precipitation from two different alkali-refining treatment regimes are reported. The physicochemical properties of the two lignin samples were characterized by using gel permeation chromatography (GPC), ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy and alkaline nitrobenzene oxidation. No significant difference of the phenolic monomers composition was found in the case of the two lignin samples. The isolated lignin contain rather low amounts of polysaccharide sugars (0.7%) and have low molecular weights (MW, 1906-1937). The results also showed that two lignin
fractions (alkali lignin), contained roughly equal amounts of guaiacyl (G) and syringyl (S) units with relatively fewer p-hydroxyphenyl (H) units and appeared to be very closely associated to phenolic acids and glucuronic acid or 4-O- methylglucuronic acid.

By combining mild alkaline hydrolysis with quantitative $^{13}$P NMR Claudia and Dimitris in 1997 have been able to arrive at a protocol for determining the various ester linkages and their relative contributions to the overall structure of wheat straw lignin. Additional information on the identity and location of these bonds was sought by the application of GC / MS and two-dimensional $^{13}$C-$^1$H heterocorrelation NMR experiments. Milled straw lignin was found to contain about 12 ester units per 100 phenylpropane units. Approximately 77% of the carboxyl fraction of these ester bonds was found to be composed of p-coumaric acid while the rest was other aromatic acids bound to lignin via intra-and / or intermolecular ester bonds.

Fachuang and John (1997) introduced a new method for selective and efficient cleavage of arylglycerol/β-aryl (β-0-4) ether linkages in lignin. The acronym “DFRC” relates to the reactions involved. Derivatization followed by Relative Cleavage Derivatization (DFRC), accompanied by cell wall solubilization, is accomplished with acetyl bromide in acetic acid; reductive cleavage of resulting β-bromoethers utilizes zinc in an acetic medium. Following acetylation, degradation, monomers (4-acetoxy cinnamyl acetates) are quantified by GC, providing data analogous to those from analytical thioacidolysis.

Isolation of lignin from waste black liquor produced from Kraft pulping of baggage (kraft lignin) and soda pulping of rice straw (soda lignin) were carried out by
Precipitated kraft lignin was subjected to different treatments such as acid hydrolysis, oxidation with H$_2$O$_2$ and thermal treatment at different temperature (120-150°C). Infrared spectra of these treated lignins were used to follow the effect of treatments on the different functional groups of lignin. Hydrolysis of lignin with acid shows an increase of phenolic hydroxyl groups due to not only degradation of β-0-4 ether linkages, but also to hydrolysis of methoxyl groups. Hydrolysis also causes depolymerization of lignin. Thermal treatment of lignin causes a decrease of OH functional group and an increase in C=O group especially at elevated temperature. Quinonoid structures can be observed on treatment of lignin with high concentrations of hydrogen peroxide.

Fachuang Lu and John (1998a) have identified the monomers released from derivatization followed by reductive cleavage (DFRC degradation) of lignin by mass spectra and/or comparison of their GC retention times with authentic compounds. The primary monomers from several isolated lignins representing softwoods, hardwoods, grasses, and dicots have been quantified by GC sources. The minor monomeric components have been rationalized on the basis of DFRC mechanisms. Products from cinnamyl alcohol, end groups, aldehydes, arylglycerols, and α-carbonyl units have been identified. From isolated lignin, the DFRC method produces its diagnostic primary monomers, the hydroxy peracetates, with yields comparable to the production of monomers from other ether-cleaving analytical methods.

The structural units in lignin are linked to each other randomly by several kinds of ether and carbon-carbon bonds. The most frequent interunit linkages are arylglycerol β-aryl (β-0-4) ethers. Others include β-5, β-1, β-β, 5-5, and 5-O-4
linkages that are more resistant toward degradation. Because of the dominance of β-arylethers in lignin, their cleavage has been studied extensively. It has long been one of the most important research targets to find mild selective and efficient methods for cleaving β-O-4 ethers. Such a cleavage is a key either for an efficient degradation of polymeric lignin during chemical pulping or for analysis of various linkages present in lignin. Fachuang and John (1988b) have developed the DFRC method, a simple and powerful method which selectively and efficiently cleaves α-ether and β-ether linkages and allows quantitative analysis of lignin structural units involved in uncondensed structure.

Two key reactions in the DFRC method have been examined by NMR. Both acetyl bromide derivatization of lignin and Zn metal reductive elimination of the β-bromo derivatives from lignin were highly selective and essentially quantitative. Treatment with AcBr in acetic acid efficiently converted β-aryl ether substrates of lignins into β-bromo ethers while γ-hydroxyl and phenol groups were acetylated, the following Zn reductive step cleaved brominated β-uryl ethers forming the expected cinnamyl acetates. In view of the high selectivity of AcBr reactions with lignocellulose materials, AcBr derivatization of lignin can be used for NMR characterization of whole lignin.

Jumpeng et al., (1998) directly isolated sixteen lignin dimers by gel permeation and reverse phase TLC and HPLC from pine sapwood following large scale application of the new derivatization followed by reductive cleavage (DFRC) procedure. Their structures were elucidated by GC/MS and NMR. These dimers
included representatives from all of the common inter unit linkages in softwood lignin (β-1, β-β, 5-5, and 5-O-4).

Ana et al., (1998) isolated dioxane and milled wood lignin from the core and bark of kenaf *Hibiscus Cannabinus*, variety Salvador. These lignin were characterized by $^{13}$C and $^1$H, NMR, FTIR, and UV spectroscopies, permanganate oxidation, alkaline hydrolysis followed by GC and GC-MS analysis of the released products and by functional group analysis. The permanganate oxidation and alkaline hydrolysis were also applied to “in situ” lignin. Isolated and in situ lignin showed significant differences in composition and structure. Strong structural differences were observed between bark and core lignin, suggesting their different biosynthetic routes. Permanganate (oxidation showed that both core and bark “in situ” lignin are HGS-type lignin with HGS proportions of 15:66:19 and 12:5:32, respectively. The β-O-4 type linkages are the main inter unit linkages and are more abundant in bark than in core lignin. The core lignin is more “condensed” and shows higher contents of β-β plus β-5 linkages than those in the bark lignin.

A semi quantitative analytical method has been developed by Dimitris and Liming (1998) for the determination of the total quinonoid content (o- benzoquinones and p-benzoquinones) of soluble lignin. The method is based on detailed measurements and observations made with model o-and p-quinones, which in dry organic solvents were shown to from adducts with trimethyl phosphate in quantitative yield.
Taking advantage of the large dispersion of $^{13}$C chemical shifts to resolve individual H chemical shifts, Erja and Gosta (1998) studied the structure of an acetylated $^{13}$C-enriched poplar wood lignin preparation using three dimensional HMQC-HOHAHA NMR spectroscopy. The whole spin system of a $^1$H-$^{13}$C correlation observed in an HMQC spectrum, even for minor components and unknown structural units, can be traced out. It is shown here that both trans- and cis-isomers of 6,7-dihydrodibenzo for example 1,4-dioxocin a recently discovered prominent linkage in softwood lignin occur in hardwood lignin too. Moreover, the poplar lignin preparation contains small amounts of non-cyclic $\alpha$-aryl ether linkages. Signals from a side chain of unknown structure are tentatively assigned to a Spiro-cyclohexadienone.

Lignin from sugarcane bagasse and eucalyptus, isolated respectively from soda and kraft liquor by precipitation with HCl or H$_2$SO$_4$, have been investigated together with samples of lignin isolated after organosolv pulping by Cotrim et al., (1999). Sugarcane bagasse lignin precipitated by HCl underwent oxidation by O$_2$ in aqueous NaOH. The reaction products, the oxidized lignin, reprecipitated with HCl, and the soluble products, isolated by evaporation, formed two other groups of samples. Fourier transform infra-red (FTIR) spectra of 49 of these lignin samples in KBr disks were recorded and analysed by principal component analysis (PCA) and statistical isolinear multicomponent analysis (SIMCA).

Ana et al., (2000) isolated milled wood lignin and dioxane lignin from different morphological regions (nodes and internodes) of *Arundo donax* reed and subjected them to a comprehensive structural characterization by $^{13}$C, $^1$H NMR,
FTIR, and UV spectroscopies and functional analysis. The permanganate and nitrobenzene oxidation methods were also applied to the in situ lignin. Both node and internode lignin are HGS-type lignin, with a significant amount of H units (including p-coumaric acid type structure). The S/G ratio (1.13-1.32), the weight average molecular weight (2040-2450), the methoxyl group content (0.23-0.27) and the aliphatic hydroxyl content (1.00-1.09) are not very different in the lignin from nodes and internodes. However, some structural differences between node and inter node lignin were observed.

Romualdo and Ronold (2001) investigated lignin extracted with acidic dioxane as a possible standard for quantitatively determining lignin content in plant samples using the spectrophotometric method employing acetyl bromide. Acidic dioxane lignin was analyzed for carbohydrate, total protein, nitrobenzene oxidation products, and UV spectral characteristics.

Dmitry et al., (2001) have presented the results of a comprehensive study on the chemical structure of lignin from plantation Eucalyptus globules Labill. Lignin has been isolated by a modified mild acidolysis method and thoroughly characterized by functional group analysis, by a series of degradation techniques and $^1$H and $^{13}$C. NMR spectroscopy. Plantation Eucalyptus globules lignin was found to be of the S/G type with an extremely high proportion of syringyl (S) units (82-86%) and a minor proportion of p-hydroxyphenyl propane (H) units (roughly 2-3 mol %). Unknown C-6 substituted and 4-O-5' type syringyl substructures represent about 65% of lignin condensed structures.
Run-Cang Sun et al., (2001) have described the first comprehensive study on the lignin from barley straw. They concluded that barley straw lignin are typical grass lignin and comprise substantial amounts of guaiacyl and syringyl units and significant quantities of p-hydroxyphenyl units. The lignin had weight average molecular weights between 1750 and 2190. Their results underscore the structural differences between alkali and alkaline peroxide soluble lignin from barley straw.

Tsutomu et al., (2002) analysed the structures of milled wood lignin, cellulolytic enzyme lignin, and residual lignin from a loblolly pine using a modified derivatization followed by reductive cleavage (DFRC) method developed to allow the quantitative determination of three different structural monomeric products originating in lignin, phenolic β-O-4, α-O-4, and etherified β-O-4 structures. A distinct advantage of the DFRC method for lignin structure analysis is its relatively simple protocol and applicability to all types of woody tissues.

Yang et al., (2002) subjected a series of sweet gum Kraft pulps with initial kappa numbers ranging from 29 to 13.6 to oxygen delignification employing 1.00 and 4.00% charges of caustic. The O-delignified pulps were characterized according to lignin content, pulp yield, and viscosity. In addition, they isolated residual lignin samples from the Kraft brown stock and the post-oxygen-delignified Kraft pulps, along with the corresponding O-effluents. The structural changes of this lignin during oxygen delignification were investigated using UV spectroscopy and advanced NMR techniques. Differential UV spectra indicated that lignin isolated from oxygen-delignified Kraft pulp and its effluent were significantly reduced at \( \lambda_{\text{max}} \) 260, 280 and 370 nm in comparison to the brownstock lignin. These changes in absorbance are
attributed to the degradation of phenolic and stilbene structures during oxygen delignification. $^{13}$P NMR lignin data revealed that the primary sites of oxidation during an oxidation stage were syringyl and guaiacyl phenoxy groups.

Sun et al. (2002) extracted seven acid insoluble lignin preparations from barley straw with alkaline hydrogen peroxide in order to study how the delignification and degradation of the lignin is influenced by aqueous 1.5% $\text{H}_{2}\text{O}_{2}$ extractant to straw ratios. The results showed that treatment of dewaxed barley straw with 1.5% $\text{H}_{2}\text{O}_{2}$ at 450°C for 14 h (pH 12.0) under the extractant to straw ratios of 10:1, 13:1, 15:1, 18:1, 20:1, 25:1 and 30:1 resulted in dissolution of 65.8%, 68.4%, 69.0%, 69.7%, 71.6% and 72.3% of the original lignin respectively. The degraded seven lignin samples were analyzed with respect to their chemically linked polysaccharides, molecular weights and structural changes.

Morrison et al. (2003) have subjected the lignin obtained from isolated flax fibers to chemical and spectroscopic analyses. Chemical analysis using 4 N NaOH at 170°C to degrade the sample has been used to characterize the lignin associated with plant fibers. The modified procedure of Mikhail et al. for the isolation of enzymatic residual lignin gave preparations with rather high yields and with appreciably lower protein contamination. The HMQC NMR technique has been shown to be a very useful method in the structural characterization of lignin preparations isolated after kraft pulping, providing important information on the pulping mechanisms. Elucidation of the role of condensation reactions shows that the increase in the degree of lignin condensation after the pulping resulted from accumulation of the native condensed lignin moieties rather than the formation of new alkyl-aryl structures.
Heikkinen et al. (2004) have utilized thermogravimetric analysis to investigate pyrolosis of individual waste components and waste mixtures. Based on the measured weight loss characteristics, single waste components are divided into three classes, low stability organic, lignocellulosic and plastic materials. In order to calculate the composition of an unknown waste mixture, it is assumed that the thermal degradation curve of a mixture is obtained as a sum of the contributions of the corresponding single components. Lignin starts decomposing at lower temperatures than cellulose. It decomposes over a wide temperature range having a long tailing section.

Gosselink et al. (2004) have characterized sulphur-free lignin coming from a novel alkaline pulping process called Nova Fiber and evaluated them for their potential applications. Compared to kraft lignin, Nova Fiber lignin differ in amount of functional groups, molecular weight and acidity. The high molecular Nova Fiber lignin is totally different from the other lignin due to a high ash content, as a result of the different precipitation procedure used.

Tatiana et al. (2004) devoted their work to studies of the radical scavenging properties of lignin, which are recognized as efficient antioxidants of natural origin. Radical scavenging efficiency of a series of lignin isolated from deciduous and coniferous wood species and 10 lignin related monomeric compounds were examined against 1,1-diphenyl-2-picrylhydrazyl (DPPH· ) radical in homogeneous conditions using ESR and spectrophotometry methods. Some structure-activity relationships are proposed, pointing out the importance of the non-etherified OH phenolic groups, ortho-methoxy groups, hydroxyl groups and the double bond between the outermost carbon atoms in the side chain for increasing scavenger activity. Analysis of rate
constants for the lignin-DPPH interaction revealed the contribution of polymer molecular weight and π-polyconjugation systems. The π-conjugation systems of lignin operate as catalysts / activators of the interaction with DPPH.

Sarwar et al. (2005) isolated lignin from cotton stalks, jute stick and dhaincha by acidolytic dioxane and characterized them by alkaline nitrobenzene oxidation, elemental analysis, methoxyl analysis and molecular weight analysis and UV, IR, ¹H NMR spectroscopy. The C₉ formulae for cotton stalks, jute stick and dhaincha (Sesbania aculeate) lignin were C₉H₉.36O₄.5(OCH₃)₁.23, O₉H₉.02O₄.57(OCH₃)₁.35 and C₉H₈.88O₄.65(OCH₃)₁.5 respectively. All three lignins were of the guaiacyl-syringyl type. Cotton stalks lignin, contained more P-hydroxy phenyl units than dhaincha and jute stick lignin as observed by alkali nitrobenzene oxidation products.

Small differences in the isolation techniques of lignin can result in significant changes in its molecular structure and configuration. Aarthi Gidh et al. (2006a) have evaluated the characteristics of lignin by light scattering (evaluated at 18 different angles in a plane), Atomic force microscopy (AFM) and Near Infrared spectroscopy (NIR). Marques et al. (2006) separated extracted Douglas-fir bark (Pseudotsuga menziesii) and submitted it to suberin depolymerization by transesterification with sodium methoxide in methanol. From the saponified cork fraction a milled cork lignin was isolated using the Bjorkaman procedure with a yield of 0.75%. The isolated Douglas-fir milled cork lignin was characterized by elemental analysis and OMe determination, FTIR spectroscopy and analytical pyrolysis (Py-GC/FID). It has been shown that the lignin polymer isolated by them consists of approximately 97% guaiacyl, 2% p-hydroxyphenyl and 1% syringyl units.
Aarthi et al. (2006b) developed a method using high-performance size exclusion liquid chromatography (HPSEC) with multiangle laser light scattering (MALLS), quasi-elastic scattering (QELS) interferometric refractometry (RI) and UV detection to characterize and monitor lignin.
2. BIODEGRADATION of lignin

Kirk et al., (1975) developed a definitive assay for microbiological and biochemical research on the biodegradation of lignin using radioactive synthetic lignin specifically labeled in the side chains aromatic rings, or in the methoxyl groups. The $[^{14}\text{C}]$ lignin were prepared by oxidative polymerization with peroxidases and H$_2$O$_2$ of specifically labeled coniferyl alcohol. The synthetic polymers were shown by spectroscopic and chemical methods to contain the same intermonomer linkages found in natural lignin, obtained form Engelmann spruce wood. Incubation of the $[^{14}\text{C}]$ lignin with known lignin degrading fungi and with a forest soil resulted in $^{14}\text{CO}_2$ evolution. Synthetic $^{14}\text{C}$ labeled lignin prepared as described here will permit investigation of such hitherto unresolved problems as (a) the role of various bacteria and fungi in lignin biodegradation, (b) the cultural and nutritional parameters affecting biodegradation, (c) the turnover of lignin in nature, including the conversion of lignin humic substances, and (d) the biochemistry and molecular biology of microbial decomposition.

A great deal of noteworthy contributions towards lignin biodegradation have been made by Kirk et al., (1980) and Crawford (1981).

The conversion of lignin to any form that is chemically different from lignin, can be measured either by the disappearance of lignin or by the appearance of products. Determination of residual lignin gives the more definitive measure of total lignin degradation. Reid (1983) included both acid-insoluble, and acid-soluble lignin in aspen wood for the chemical determination of residual lignin. Conversion of $[^{14}\text{C}]$ lignin to $^{14}\text{CO}_2$ and water soluble form can be expected to slightly overestimate bulk lignin degradation if all lignin carbon is being converted to the products measured.
However, recovery of $^{14}$C seriously underestimated lignin degradation in the presence of peptone and yeast extract. Significant amount of lignin carbon were converted into some form other than CO$_2$ or water-soluble materials.

Major advances in our understanding of the biochemical and enzymological mechanisms of lignin biodegradation have been made in the past two decades. Research has principally involved two ligninolysis microorganisms, the white rot fungus _Phanerochaete chrysosporium_ and the actinomycete _Streptomyces viridosporus_. Ronald and Don (1984) proposed the possibility that lignin degradation might be non-enzymatic and mediated by extracellular reduced oxygen species such as hydrogen peroxide (H$_2$O$_2$), super oxide (O$_2^-$), hydroxyl radical (.OH) or singlet oxygen and has been investigated with both microorganisms. Evidence has been presented to show that _S. Viridosporus_ produces a lignolytic enzyme complex involved in demethylation of lignin’s aromatic rings, in the oxidation of lignin side chains and cleavage of β-ether linkages within the polymer. The combined activities of these enzymes generate water soluble polynemic modified lignin fragments, which are then slowly degraded further by _S. Viridosporus_. The first isolations of lignolytic enzymes has changed the course of basic research on lignin biodegradation.

Thomas and Donald (1984) studied the ability of 12 _Cyathus_ species to degrade $^{14}$C-labeled lignin in Kenaf. The sum of $^{14}$C released into solution plus $^{14}$C released into the gas phase over a 32-day fermentation period was used to determine average daily rates of lignin biodegradation. The apparent ability of fungi to metabolize low-molecular-weight lignin break down products correlated well with their overall delignification rates. _Synthus stercoreus_ metabolized degradation products
of lignin from wheat straw, better than those from kenaf lignin based on the amount of low-molecular weight products left in solution. *Cyathus* species having unique substrate preferences may differ in their ability to attack the substructures of native lignin and lignocellulose complexes.

Significant quantities of lignin-containing plant materials eventually enter anaerobic zones and are believed to be rate-limiting components in methane production processes. Benner et al., (1984) reported that lignin component of intact softwoods and hardwoods is partially degraded to gaseous end products under anaerobic conditions. Colberg and Young (1985) reported the results of a study in which lignin-derived substrates separated into three-component molecular size fractions. Each fraction is then used as the sole source of carbon for the degradation studies by strictly anaerobic enrichment cultures. Anaerobic environments such as those found in sediments, soils and digesters have the capacity for the mineralization of lignin-derived materials. Anaerobic populations of bacteria could serve as members of a microbial food chain which utilize the oligolignin released by for example, fungi, which are considered the primary lignolytic organisms. Understanding the cycling of lignin carbon in the environment necessitates an examination of its little understood anaerobic fate.

Due to Lignin's challenging structural complexity, its potential as the renewable resource of aromatic chemicals, and the fact that its degradation is required for subsequent enzymatic hydrolysis of wood's cellulose and hemicellulose, a great deal of work has been performed on lignin biodegradation (Higuchi, 1985). Kersten et al., (1985) and Schoemaker et al., (1985) have shown that Ligninase catalyses a one
electron oxidation of lignin in the presence of H₂O₂ and thus mechanistically resembles a peroxidase. Jonathan et al., (1986) have reported that horseradish peroxidase and milk lactoperoxidase, while inactive toward lignin in water, can vigorously depolymerize both synthetic and natural lignin in organic media.

Plant peroxidases play a major role in lignin formation and wound healing and are believed to be involved in auxin catabolism and defense to pathogen attack. Anionic peroxidases isoenzymes catalyze the formation of the lignin polymer and form rigid cross-links between lignin, cellulose, and extension in the secondary plant cell wall.

Hartman and Kent (1987) examined the relationship between the molecular size of lignin in several preparations and extent of degradation (mineralization) by Xanthomonas sp. strain 99. They interpreted their results to indicate that this species degrades the low molecular weight portions of lignin but probably is unable to depolymerize the high molecular weight backbone lignin polymer. Their results suggest further that the bacterial cells are able to take up lignin molecules of up to molecular weight 600 to 1000 and then to metabolize them to CO₂.

Hans and David in 1989 developed a method for the isolation of lignin from wheat straw by ball milling and enzyme treatment. The objective of their study was to develop a method for isolation of relatively unmodified lignin from forage to use in more extensive chemical characterization work. They concluded from all of the spectral and chemical information that it would be acceptable to use 50% (v/v) dioxane – water to extract a lignin material that would be just as representative of
polymeric lignin as extraction with standard method (96% dioxane) from wheat strew and similar materials. Thus, advantage can be taken of the increased yield that this procedure provides for grasses and other herbaceous tissues that have relatively low lignin content when compared to wood. Further research is necessary to verify this conclusion in less mature forage material and to pinpoint the major source of the phenolic acids in these materials.

Alice et al., (1994) evaluated the commercial button mushroom *Agaricus bisporus*, grown under standard composting, for its ability to produce lignin-degrading peroxidase, which have been shown to have an integral role in lignin degradation by wood-rotting fungi. Characterization of the enzyme was done with a crude compost extract. The correlation between the activities of manganese peroxidase and laccase and the degradation of lignin in *A. bisporus* suggests significant roles for these two enzymes in lignin degradation by this fungus.

Coir, fiber of coconut used for making ropes results in the accumulation of huge quantities of lignin waste Uma et al., (1994) showed that enrichment technique yielded a lignin-degrading bacterium characterized as *Pseudomonas* sp. KU03. This organism was able to degrade acid dioxane and fiber lignins which are the true representatives of native lignin. The direct polyphenol oxidase and laccase enzyme assays and the indirect ligninase assay with α-keto-γ-methyl thiol butyric acid and the concomitant release of phenols and sugars proved the organism's ability to degrade lignin.
The differential biodegradation of phenolic and nonphenolic (C-4-etherified) lignin units in wheat straw treated with the white rot fungi *Pleurotus eryngii* and *Phanerochaete chrysosporium* was investigated by Susana et al., (1994) under solid-state fermentation conditions. Two analytical techniques applied to permethylated straw were used for this purpose, namely, alkaline CuO degradation and analytical pyrolysis both followed by gas chromatography, mass spectrometry for product identification.

Medvedeva et al., (1995) studied the pathways of lignin biodegradation by *Trametes villosus* and *Phanerochaete sanguinea* fungi. By use of chromatography and spectroscopy methods it was shown that in vivo the biotransformation of aspen lignin and lignin-modeling-compounds is effected due to distraction-polymerization reactions. Polymerization involves model compounds and lignin fragments having a free phenolic OH. The reaction proceeds at the expense of both the phenolic OH and aromatic ring hydrogen atoms mainly in the position 5 to form C-C and C-O-C bonds.

Becker et al., (1995) have evaluated the ability of *Bjerkandera adusta* grown on lignosulphonates to produce lignin-degrading peroxidases, which have been shown to play an important role in lignin degradation by white-rot fungi.

In 1995, Juraj et al., working on white-rot fungi showed that as *Trametes cingulata* grows vegetatively in culture solution, the microorganisms first polymerizes the dissolved lignin components, and then in subsequent phases, completely degrades the polymerized substrate. They have concluded that *Trametes cingulata* is quite capable of degrading lignin very effectively without resort to any peroxidase activity.
In extracellular solutions from white-rot fungal cultures, lignin is both polymerized and depolymerized. The enzymes which bring about lignin depolymerization namely ligninperoxidase, manganese-dependent peroxidase and laccase act as single-electron oxidants. Therefore, either directly or indirectly, they may produce from particular lignin monomer residues, phenoxy radicals that will undergo bimolecular coupling, in the absence of other competing reactions. Working on this aspect Vugzar et al., (1998) have explored the connection between lignin polymerization and depolymerization \textit{in vivo}. Their results suggests that as the white-rot fungi \textit{Tramates cingulata} grows vegetatively, it polymerizes the dissolved lignin components and after cessation of primary growth, it completely degrades the high molecular weight polymerized substrate. The overall effect of \textit{T. cingulata} upon the kraft lignin substrate may therefore, be governed by the consecutive release of distinct polymerizing and depolymerizing enzymes.

Lignin peroxidase is employed by lignolytic fungi to degrade the recalcitrant biopolymer lignin, a cell wall constituent of woody plants. Due to its enlarged substrate range in the presence of specific mediators and due to its high redox potential this enzyme has the potentiality for the application in various industrial processes. Klaus Piontek (2002) has summarized the major outcome of these investigations and described the underlying structural factors that govern substrate interaction and electron transfer in lignin peroxidase.

The common mushroom \textit{Agaricus bisporus} is a non-white rot saprophytic fungus that can degrade lignin to free and utilize holo cellulose embedded in fermented straw as present in compost. Rimko et al. (2003) have discussed within the scope of the \textit{A. bisporus} mushroom yield and lignin degradation by white rot fungi during growth on lignocellulose-containing materials.
3. MUSA SPECIES

With the aim of determining the nutritive value of banana stalk (*Musa Cavendishi*) as a feed for sheep, Viswanathan et al., (1989) found that the stalk of banana plants (*Musa Cavendishi*) contain (in percent dry matter) crude protein, 7.2; ether extract, 1.8; crude fiber, 31.5; total ash, 21.4; neutral detergent fiber (NDF) 67.2. The fiber components were (in percent) acid detergent fiber (ADF), 45.3 hemicellulose, 21.9; cellulose, 35.9; Lignin, 9.4.

Sarathathevy Suntharalingam et al., (1993) working on physical and biochemical properties of green banana flour have reported the banana flour prepared from two cooking banana varieties, namely ‘Alukehel’ and ‘Monthan’ for their physical and biochemical characteristics. The lignin composition was found to be 1% of the dry matter.

Fekadu and Ledin separated weighed and analysed the different plant parts including the leaf lamina, leaf midribs, pseudostem sheath, pseudostem core and corm of three varieties of *enset*, Agade, Badedat and Lemat. The pseudostem sheath of Badedat had a lower content of lignin compared to the other two varieties and the average value in percent of lignin of the total dry matter is 1.8. The dry matter content was 15.8% in the pseudostem.

In their report on anaerobic digestion of banana stem waste, Kaalia et al. (2000) have stated that waste banana stem has a high organic content (83%); with 15-20% (w/w) lignin and cellulose which gives it a sheath-like texture.
A very interesting publication on the recycling of banana pseudostem waste for economical production of quality banana by Phirke et al. (2001) reports as to how an agro-waste is converted into an eco-friendly agro-input for sustainable productivity. Banana pseudostem biomass, traditionally incinerated and wasted, has been conserved and recycled by solid state fermentation into plant growth stimulating soil conditioner.

Cordeiro et al. (2004) have determined the chemical composition and pulping potentialities of banana pseudostems growing in Madeira Island. First, the raw material was both studied as a whole and as the outer bark part, which is richer in cellulose fibers. Before starting the cooking of banana wastes, the main components of the two types of material were quantified, and showed that the polysaccharide content was high enough (about 60-70) to justify the pulping investigations. The lignin content was about 12%.
CHAPTER - III

OBJECTIVES AND SCOPE OF THE PRESENT WORK

India has 16 agro-climatic zones, and 45,000 different plant species. It is the second largest producer of bananas in the world. Karnataka is one of the leading producer of banana in India. Banana trees fruit only once in their life time. After harvesting, the banana stem is considered as waste material. About 1000 banana plants are estimated to yield 20 to 25 tonnes of pseudostem. If this banana pseudostem biomass traditionally incinerated and wasted, could be conserved and used as plant stimulating soil conditioner, for carpet backing and padding, for automobiles, roofing felt, fire logs, cardboard, composite board in place of fiber glass, food wrappers, fast food containers, wall paper, manufacturing papers, and so on, the loss of our forest trees could be reduced by half. All these applications are dependent on the quality and quantity of lignin in the pseudostem. Hence our interest in isolating and characterizing lignin in the pseudostem of the locally available banana plant variety Yellakki Bale.

Lignin acts as a copolymer in adhesive manufacturing. Increasing lignin content has been predicted as a defense against pests. The importance of lignin in disease resistance has been known for well over twenty years. In liquid formulation, lignin acts as complexing agent keeping micronutrients in an available form under specific pH conditions, which would normally cause insolubalization. Lignin dietary fiber controls cholesterol levels and prevents gallstones. Lignin is used as a plant nutrient and soil agglomerator and is known for biodegrability, low cost and low toxicity. Lignin is non-toxic on its own, extremely versatile in performance, qualities that have made it extremely important in many applications. We have just set out to
isolate and characterize lignin from the banana plant pseudostem which we hope to use in the near future to carry out experiments to study its medicinal properties.

An extensive literature review has indicated that information on the detailed chemical and structural analysis of lignin isolated from various banana plants is quite sparse and disperse. To the best of our knowledge, no comprehensive work on the detailed characterization of the lignin component isolated from the Musa species (AB) Yellakki Bale has been published. So far a serious attempt has been made in this research to isolate and chemically characterize lignin from the pseudostem of the locally available banana plant Yellakki Bale.
The banana plant differs considerably from the majority of horticultural plants. It is described as a monocotyledonous, herbaceous, evergreen perennial. It is herbaceous because after fruit harvest the aerial parts die down to the ground and there are no woody components. It is perennial because new suckers grow up from the base of the mother plant to replace aerial parts which have died.

The true stem of the banana plant is partly or wholly underground and is known technically as a tuberous rhizome. The banana does not have extended horizontal growth like most rhizomes but, never the less, suckers grow successively outwards and there is a small amount of horizontal growth before it turns upwards. Although the mature pseudostem is quite sturdy, it is very fleshy, comprising about 95% water. The pseudostem can be as short as 2m or as tall as 7m. Pseudostem circumference near ground level can be 1 m on a vigorous ratoon plant, but somewhat less on a mother plant in the first cycle (Robinson, 1996).

The generic name Musa is derived from the Arabic word *Mouz*. The earliest scientific classification of banana were made by Karl Linnaeus in 1783. He gave the name *Musa sapientum* to all dessert bananas which are sweet when ripe and which are eaten fresh. The name *Musa paradisiaca* was given to the plantain group which are cooked and consumed when still starchy. However it is now known that these two apparent species are not species at all but both refer to closely related interspecific triploid hybrids of the AAB group. They are general names and cannot be used to differentiate between bananas and plantains. Subsequently, taxonomists gave species names to the many diverse forms of edible bananas that were found, for example,
Musa nava for Dwarf Cavendish, Musa rubra for Red Dacca, Musa corniculata for Horn plantain and many more.

This endless addition of coining of new names was put an end to by Simmonds and shepherd in 1955 who suggested that the Linnaeus classification was based on their hybrid progenies and not on the original parental species. The original progenitors of present day bananas was concluded to be Musa acuminata and Musa balbisiama. It has now been confirmed that two other species Musa textiles and Musa schizocarpa are also involved (Carreel, 1995).

Banana and plantain belong to the family Musaceae comprising of two genera Ensete Horn and Musa L. Genus Ensete has 8-9 species with its distribution in South and South East Asia and also in Africa, while genus Musa. L. has four sections namely Eumusa, Australimusa, Callimusa and Rhodochlayms.

Genus Musa has contributed greatly to the present day edible bananas. Section Eumusa contributes Musa acuminata and Musa balbisiana the major progenitors of present day edible bananas. Musa acuminata (AA) has 9 sub-species recognised (Horey et al., 1997) and have their distribution in South East Asia and pacific islands. In their areas of origin and diversification triploids (AAA) evolved due to spontaneous mutations expressing sterility and parthenocarpy. But selected by man and domestication over a course of time they were taken to South Asia and part of South East Asia where Musa balbisiana has its home of origin. Both being cross compatible and fertile, led to natural hybrids, thus resulting in hybrids of various combinations receiving A genome from Musa acuminata and B genome from Musa balbisiana. These bispecific hybrids inherited many biotic and abiotic stress resistant genes from Musa balbisiana. Owing to their many desirable traits most of the bispecific hybrids and hardy, parthenocarpic, acuminata diploids drifted slowly far and wide and became the backyard cultivars of South and South-East Asia.
Figure 8 gives the classification of *Musa* accessions at National Research Centre for Banana (NRCB) field genebank.

**Figure 8**: Classification of *Musa* accessions at NRCB field genebank

Source: Uma et al., 2002
For easy search, the clones are dealt as genome, subgroup and cultivars. The classification followed is represented in Figure 8.

All banana taxonomists seem to agree that no single scientific name can be given to all the edible bananas. To avoid confusion, therefore, it is internationally accepted that all banana cultivars should be referred to by the genus Musa followed by a code devoting the genome group and ploidy level followed by the popular name of the cultivar. Some examples are:

- Musa AAA (Cavendish subgroup) ‘Grand Nain’
- Musa AAB (Plantain subgroup) ‘Horn’
- Musa BBB Saba
- Musa AB Ney Poovan

Table 3 shows the names and synonyms of Bananas and Plantains of Karnataka belonging to the Genome/Subgroup AB

<table>
<thead>
<tr>
<th>Genome / Subgroup</th>
<th>Name</th>
<th>Synonyms</th>
</tr>
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<tbody>
<tr>
<td>AB Kunnan</td>
<td>Kunnan</td>
<td>Chitti Bale, Firige Bale</td>
</tr>
<tr>
<td></td>
<td>Poovilla Chundan</td>
<td>Randu Bale, Kaththu Bale, Mambilla Bale</td>
</tr>
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Yellakki Bale belongs to the Genome/Subgroup AB Ney Poovan. This variety of Banana is a major fruit crop growing in and around Mysore, India.