Chapter-2

Literature Review
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Biotechnology involves the technical exploitation of biological processes. It is receiving increasing attention in past decades because of its commercial potential in many fields. Wood is the major raw material for the forest based industries. One of nature's most important biological processes is the degradation of lignocellulosic materials such as wood and agricultural wastes to carbon dioxide, water, and humic substances through the natural detoxification processes. The virtue of biotechnology lies in its potential to supply more specific reactions, to provide less environmentally deleterious processes, to save energy, and to be used where non-biological chemistry is impractical.

There are, at present, about 515 units engaged in the manufacture of paper, paperboards and newsprint in India. The country is almost self-sufficient in manufacture of most varieties of paper and paperboards. At present about 60.8 per cent of the total production is based on non-wood raw material and 39.2 per cent on woody material. In India 6,128,000 tonne of paper and paper board were produced in 2006-07. During the production of one tonne of paper about 150\text{m}^3 of effluent is generated (www.indiastat.com).

Manufacturing of paper is an elaborate process involving mainly two steps: pulping and bleaching. The wood is chopped into small pieces mixed with sodium hydroxide and sodium sulphite (kraft pulping) or acids (sulphite pulping) and heated at very high temperatures (~200°C) and pressure for 1-3 hours. The lignin and hemicellulose present in it degrades and cellulose is left as pulp. This pulp is washed with water. The effluent generated at this stage is called black liquor as its dark brown in colour due to the presence of lignin, hemicellulose, their degradation products, resin acids, lignosulphonics and phenols. Further the pulp is bleached as it has traces of lignin and hemicellulose which impart it yellow tinge. For bleaching nascent chlorine, hydrogen peroxide and ozone are used. The effluent generated at this stage
has adsorbable organic halides, chlorophenols, dioxin and furans. The bleached pulp is white in colour and is used to make sheets of paper.

The whole process of making paper generates effluent that is extremely toxic in nature. Use of biological approaches like effluent treatment and biopulping can be used to reduce the toxicity of the effluent. It has been observed that fungi are the main degraders of lignocellulosic materials, particularly wood. The fungi, by means of the enzymes secreted from their hyphae, attacks and penetrates the wood very rapidly, upto 1mm/hr (Eriksson, 1990). Apart from fungi there are studies that show bacteria, Bacillus and Pseudomonas, are also capable of wood degradation (Bourbonnais and Paice, 1987). A mixed culture of algae was able to decolourize pulp and paper mill effluent (Lee et al., 1978). This review will primarily focus on the use of fungi in pulp and paper industry especially at pulping stage.

Process of making paper

The pulp and paper production is an elaborate process having high demand for water and electricity. The raw material can be wood, bagasse, wheat straw, rice straw and similar agricultural wastes having high fiber content. The steps involved in pulp and papermaking are (Figure 2.1.):

1. Raw material is cleaned and cut in small pieces.

2. Separation of cellulose fibers from lignin and hemicellulose is known as pulping. Pulping can be mechanical or chemical. In chemical pulping there are two basic methods, Kraft pulping (using sodium hydroxide and sodium sulphite) and Sulphite pulping (using sulphuric acid and bisulphite ions). Pulping contributes maximum to the pollution load.

3. For making low quality paper used for wrapping pulp is directly fed into paper making machines where it is turned into sheets, dried with steam, cut into the desired sizes and packed.

4. For making good quality writing paper, the pulp is bleached to remove residual lignin to make it white. For bleaching chlorine, hydrogen peroxide, oxygen, ozone and similar bleaching agents are used.

The effluent generated in digester house, i.e. pulping stage is dark brown in colour due to lignin, its degradation products, lignosulphonics, hemicelluloses, resin acid and
phenols (Chuphal et al., 2005). The effluent has very high chemical oxygen demand, biological oxygen demand, total solids and toxicity. The main problem at this stage is degradation of lignin. Lignin, a phenylpropanoid structural polymer of vascular plants, gives the plants rigidity and binds the wood cells together. It decreases water permeability across the cell walls of xylem tissue and makes the wood more resistant to attack by microorganisms (Sarkanen, Ludwig 1971). Though lignin is resistant to microbial attack, still it is degraded to humus, water, and carbon dioxide following the death of the plant tissues which indicates that in nature a number of fungi exist that are capable of lignin degradation. It is this property of fungi that is exploited by biotechnology to make the manufacturing process eco-friendly. The pulping process can be made environmental friendly in two ways: treatment of effluent generated at this stage and treatment of raw material, i.e. biopulping.

The effluent generated at the pulping stage in pulp and paper mill is highly coloured and toxic. Its alkaline in nature, has high COD, BOD and TSS. It contains lignosulphonics, resin acid, phenols, lignin and its breakdown products and hemicellulose (Pokhrel and Viraraghavan, 2004). These are complex organic compounds, when released in environment without treatment; reacts with a wide variety of other chemicals in presence of light and heat to form highly toxic and recalcitrant compounds (Kinae et al., 1981; Zacharewski et al., 1995). Thus it is obligatory to treat the effluent before disposal into the environment.

**Treatment of effluent generated at pulping stage**

Various physiochemical methods like sedimentation, flotation, screening, adsorption, coagulation, oxidation, ozonation, electrolysis, reverse osmosis, ultrafiltration, and nano-filtration technologies have been used for treatment of suspended solids, colloidal particles, floating matters, colors, and toxic compounds (Pokhrel and Viraraghavan, 2004). However, they have disadvantages like high cost and sludge generation. Sludge has to be landfill. This further increases the cost of treatment. Sometimes the sludge is burned to save cost of disposal. On burning, huge quantities of volatile organic toxic compounds are formed. These include dioxins, furfurals and other volatile organic compounds. Thus in general, these processes only change the state of pollutants from liquid to solid then to gas rather than treating them. On the other hand biological methods involve degradation of pollutants, solving the problem permanently.
The soil, dirt and barks are removed from the wood and chips are separated from the barks. Wastewater contains suspended soils, BOD, dirt, grit, fibers.

Black liquor is generated. Wastewater contains resins, fatty acids, lignosulphonics, colour, BOD, COD, AOX, VOCs (terpenes, alcohols, phenols, methanol).

The wastewater from the pulp contains high pH, BOD, COD and suspended solids. It is dark brown in colour.

The wastewater from bleaching contains dissolved lignin, colour, COD, AOX, inorganic chlorine compounds, organochlorine compounds, dioxins etc.

The wastewater generated from paper making contains waste, organic compounds, inorganic dyes, COD, acetone etc.

Figure 2.1. Steps involved in manufacturing of paper along with characteristics of effluent generated (Pokhrel and Viraraghavan, 2004)

Biological treatment can be divided into aerobic and anaerobic depending on the availability of oxygen. Aerobic treatment involves activated sludge treatment, aerated lagoons and aerobic biological reactors. Anaerobic filter, upflow sludge blanket (UASB), fluidized bed, anaerobic lagoon, and anaerobic contact reactors are anaerobic processes, that are commonly used to treat pulp and paper mill effluents. Among these treatments one thing is common, use of microbes (Pokhrel and Viraraghavan, 2004). A number of fungi, bacteria and algae have been reported to have effluent treatment capabilities.

Decolourization of effluent by bacteria

Few bacteria have been reported for treatment of pulp and paper mill effluent (Thakur et al., 2001; Chuphal et al., 2005; Thakur, 2004). Although many groups of bacteria are able to metabolize the monomeric constituents of the aromatic lignin polymer, their activity on polymeric lignin substrates is limited and low rates of conversion of radiolabelled lignin substrates to CO₂ were observed with most of the bacteria tested (Zimmermann, 1990). In pulp and paper industry, bacteria are
generally used for degrading chlorinated phenols (Chuphal et al., 2005). Bourbonnais and Paice (1987) tested \textit{Bacillus cereus} and two strains of \textit{Pseudomonas aeruginosa} for decolourization of bleach kraft effluent. Colour was primarily removed by adsorption with little depolymerization.

**Decolourization of effluent by algae**

It has been reported that some algae can decolourize diluted bleach kraft mill effluents (Lee et al., 1978). It was found that pure and mixed algal cultures removed up to 70% of colour within 2 months of incubation. All cultures exhibited a similar colour reduction pattern consisting of a phase with rapid and accelerating removal rate and a phase with declining rate. Colour removal was most effective during the first 15 to 20 d of incubation, and then gradually dropped off. Complete removal of colour did not occur. Colour removal by algae is caused by metabolic transformation of coloured molecules to noncoloured molecules with limited assimilation or degradation of molecular entities. Adsorption is not a major colour removal mechanism.

**Decolourization of effluent by fungi**

Published papers report the use of wide variety of fungi like \textit{Merulius aureus} syn. \textit{Phlebia} sp. and \textit{Fusarium sambucinum} Fuckel MTCC 3788 (Malaviya and Rathore, 2007), \textit{Trametes versicolor} (Pedroza et al. 2007), \textit{Paecilomyces} sp (Singh and Thakur, 2006; Chuphal et al. 2005), \textit{Coriolus versicolor} and \textit{Rhizo mucorpusillus} strain RM7 (Driessel and Christov et al. 2001) for decolourization of pulp and paper mill effluent. The decolourization of the pulp and paper mill effluent by fungi involves two main mechanisms; first use of various enzymes like lignin peroxidase, manganese peroxidaes, laccase, xylanase and second adsorption.

**Role of bioreactors in effluent treatment**

Many studies have reported high removals of organic pollutants of kraft mill wastewater by sequential batch reactor (SBR) treatment (Franta and Wilderer, 1997; Milet and Duff, 1998). Substantial removal of COD, TOC, BOD, lignin and resin acids of TMP wastewater using high rate compact reactors (HCRs) at a retention time of 1.5 hrs had been reported (Magnus et al., 2000a and b). Removal of COD in a moving bed biofilm reactor (MBBR) had been demonstrated (Jahren et al., 2002; Borch-Due et al., 1997). Berube and Hall (2000) showed that approximately 93%
removal of TOC could be achieved by a membrane bioreactor. These studies show the importance of bioreactors in treatment studies.

**Alternative technologies for decolourization**

Recently some studies have reported the use of integrative approach. An integrated or hybrid system is designed to take advantage of unique features of two or more processes. A combination of coagulation and wet oxidation removed 51% of COD, 83% of color and 75% of lignin (Verenich et al., 2001; Verenich and Kallas, 2001). A combination of ozone and biofilm reactor removed 80% COD (Helble et al., 1999). A combination of activated sludge and with ozonation (as tertiary treatment) removed 87–97% COD, and 97% BOD (Schmidt and Lange, 2000). However, all these techniques have high cost of treatment.

**Biopulping**

Apart from effluent treatment modification in process i.e. use of biopulping as a pretreatment before chemical and mechanical pulping is one economically viable alternative to make the manufacturing process cleaner and greener. Biopulping involves the biotreatment of lignocellulosic material by fungus having lignolytic enzyme system and the subsequent processing by mechanical or chemical pulping (Saad et al., 2008). In wood cellulose fibers are embedded in hemicellulose and lignin. Paper is made from cellulose thus hemicellulose and lignin is waste. These cellulose fibers need to be separated from hemicellulose and lignin to make paper. Pulping process involves separation of cellulose from lignin and hemicellulose. Also pulping is most polluting step (Pokhrel and Viraraghavan, 2004). Pulping technologies have undergone constant improvements due to market demands and new developments in research. The need for sustainable technologies has also brought biotechnology into the realm of pulp and papermaking. Enzymatic processes are being developed to increase pulp brightness, to reduce troublesome pith, to improve paper quality and to purify the effluent (Messner and Srebotnik, 1994).

Efforts have been made to improve pulp-producing process by using isolated enzymes. These efforts have limited success as lignin, which is the major problem, lacks the regular and ordered repeating units found in other natural polymers. It is an amorphous, polyphenolic complex polymer formed from dehydrogenative
polymerization of three phenylpropaniod monomers, coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol (Lin and Dence, 1992).

Microbiology of biopulping

The wood chips are inoculated with fungus, having preference for lignin as compared to cellulose, and left for few days to ferment. It is generally solid-state fermentation. During this duration, the lignin degrading or lignolytic fungus grows and solubilizes the lignin. They create cavities and tunnels in the wood chips and the cellulose fibers become loose (Daniel, 1994).

Selection of proper biopulping fungi is very important as different fungi behave differently with different wood types. The screening method includes chemical analysis along with electron microscopic method. Chemical analysis allows for quantification of residual lignin and wood sugars. However, a single fungus can selectively remove lignin (and hemicellulose) in one part of the wood block, and simultaneously remove all wood components in another part. Thus electron microscope studies are necessary before selecting a strain. The process of delignification depends on the fungal species and the type of wood. In white rot fungi delignification starts in wood cells, secondary cell wall are delignified easily, followed by middle lamellae, while cell corners and vessels are least delignified. Apart from that the process of delignification is not uniform i.e. an advanced stage of delignification can be found adjacent to apparently unaffected area. *Phanerochaete chrysosporium* is suitable for biopulping of hard wood and *Ceriporiopsis subvermispora* is used for both soft and hard wood. These two fungi are the most studied fungus for biopulping (Breen and Singleton, 1999)

Biochemistry of biopulping

The biochemical process of biopulping is still not fully understood. The three most important lignolytic enzymes, which are supposed to play a major role in biopulping, directly or indirectly, are lignin peroxidase, LiP (Figure 2.3.), manganese peroxidase, MnP (Figure 2.4.) and laccase (Figure 2.5.). It is unclear whether the solubilization of lignin from wood cell walls is caused by a direct interaction of lignolytic enzymes with lignin or whether it is caused by enzymatically generated low molecular mass agents (Messner and Srebotnik, 1994).
Process optimization for biopulping

The optimum condition for biopulping varies from fungus to fungus and with different substrate. Process parameters generally include the time for biopulping, temperature and moisture conditions, inoculum size etc. In the delignification of aspen wood by *Phlebia tremellosa* did not required agitation but needed forced aeration. It also needed sterilization of wood. However in some fungi, sterilization is not required. Biopulping is generally used as a pretreatment before mechanical or chemical pulping. It helps in reducing energy requirements during the mechanical pulping by approximately 25%. It also improves the quality of pulp. In chemical pulping it helps reducing the need for chemicals and the cooking time. Economic analysis of biopulping has shown that this process is economical (Messner and Srebotnik, 1994).

Enzymatic processes for lignin degradation

In pulp and paper industry, cellulose is used for paper production while lignin and hemicellulose end up in effluent. The fungi capable of degrading lignin and hemicellulose can be used for treatment of effluent. The degradation process involves use of number of enzymes collectively called ligninase. Ligninase is a generic name for a group of isozymes that catalyze the oxidative depolymerization of lignin. They include lignin peroxidase, manganese peroxidase and laccase. Another enzyme xylanase plays a crucial role in hemicellulose degradation. These enzymes are extracellular, are non-substrate specific and aerobic in nature. This is an essential requirement for lignin degradation as it is a randomly synthesized biopolymer that can not enter inside the cell and degradation involves the cleavage of carbon-carbon or ether bond, that link various sub-units, in oxidative environment (Breen and Singleton, 1999). The mechanism of action of these enzymes is as follows:

Xylanase

Xylan, a major constituent of hemicellulose, is composed of β- 1, 4-linked xylopyranosyl residues which can be substituted with arabinosyl and methylglucuronyl sidechains. Xylanases (endo-1, 4- β-D-xylan xylanohydrolase; E.C. 3.2.1.8) are a group of enzymes that hydrolyse xylan backbone into small oligomers (Kiddinamoorthy et al., 2008). The xylanolytic enzyme system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-
endoxyrananase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and p-coumaric acid) esterase (Figure 2.2.). The presence of such a multifunctional xylanolytic enzyme system is quite widespread among fungi, actinomycetes, and bacteria (Beg et al., 2001).

Figure 2.2. A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beg et al., 2001)

Lignin peroxidase (LiP)

Lignin peroxidase is a heme-containing glycoprotein which requires hydrogen peroxide as an oxidant. Fungi secrete several isoenzymes into their cultivation medium, although the enzymes may also be cell-wall bound (Lackner et al., 1991). LiP oxidizes non-phenolic lignin substructures by abstracting one electron and generating cation radicals which are then decomposed chemically (Figure 2.3.). Reactions of LiP using a variety of lignin model compounds and synthetic lignin have thoroughly been studied, catalytic mechanisms elucidated and its capability for C—C bond cleavage, ring opening and other reactions has been demonstrated (Eriksson et al., 1990; Higuchi, 1989). LiP is secreted during secondary metabolism as a response to nitrogen limitation. They are strong oxidizers capable of catalyzing the oxidation of phenols, aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons (Breen and Singleton, 1999).
Manganese peroxidase (MnP)

Manganese peroxidase is also a heme-containing glycoprotein which requires hydrogen peroxide as an oxidant. MnP oxidizes Mn(II) to Mn(III) which then oxidizes phenol rings to phenoxy radicals which lead to decomposition of compounds (Figure 2.4.). Evidence for the crucial role of MnP in lignin biodegradation are accumulating, e.g. in depolymerization of lignin (Wariishi et al., 1991) and chlorolignin (Lackner et al., 1991), in demethylation of lignin and delignification and bleaching of pulp (Paice et al., 1993), and in mediating initial steps in the degradation of high-molecular mass lignin (Perez and Jeffries, 1992).

Laccase (Lac)

Laccase (EC No. 1.10.3.2. (benzenediol: 0, oxidoreductase) is a true phenoloxidase with broad substrate specificity. It is a copper containing glycoproteins widely reported in fungi and plants. Most famous are rot fungi like Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Coriolus versicolor var. antarcticus, Pycnoporus sanguineus, Trametes elegans, Bjerkandera adusta, Pleurotus eryngii, Phlebia radiata, etc (Baldrian, 2006). It has also been reported in some plants like
Acer pseudoplantanus, Aesculus parviflora, Populus euramericana etc. In plants laccase participates in the radical-based mechanisms of lignin polymer formation (Sterjiades et al., 1992), whereas in fungi laccases probably have more roles including morphogenesis, fungal plant-pathogen/host interaction, stress defense and lignin degradation (Thurston, 1994). The presence of laccase has been reported in bacteria, however, such reports remain controversial (Diamantidis et al., 2000).

**Structural properties**

Current knowledge about the structure and physico-chemical properties of fungal laccase proteins is based on the study of purified laccases. Up to now, more than 100 laccases have been purified from fungi and been more or less characterized. Typical fungal laccase is a protein of approximately 60–70 kDa with acidic isoelectric point around pH 4.0. Several laccase isoenzymes have been detected in many fungal species. Most fungal laccases are monomeric proteins. Several laccases, however, exhibit a homodimeric structure, the enzyme being composed of two identical subunits with a molecular weight typical for monomeric laccases. Like most fungal extracellular enzymes, laccases are glycoproteins. The extent of glycosylation usually ranges between 10% and 25%, but laccases with saccharide content higher than 30% have been reported (Mayer and Staples, 2002).

**Mechanism of action**

The reactions catalysed by laccases proceed by the monoelectronic oxidation of a suitable substrate molecule (phenols and aromatic or aliphatic amines) to the corresponding reactive radical. The redox process takes place with the assistance of a cluster of four copper atoms that form the catalytic core of the enzyme (Figure 2.5.); they also confer the typical blue colour to these enzymes because of the intense electronic absorption of the Cu–Cu linkages (Piontek, 2002).

The overall outcome of the catalytic cycle is the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals (Claus, 2000; Solomon, 1996). These reactive intermediates can then produce dimers, oligomers and polymers. The physiological function of laccases, which can be extracellular or intracellular, is different in the various organisms but they all catalyse polymerization or depolymerization processes.
Differences between blue and yellow laccases

Yellow laccases were first reported in *P. tigrinus*. It was found that laccases produced by *P. tigrinus* in lignin-containing culture had different catalytic properties as compared to the laccases produced in non-lignin rich culture. The unusual properties of yellow laccase from *P. tigrinus* suggest its probable modification by the products of lignin degradation. The primary products of the enzymatic oxidation of lignin by blue laccases are reactive compounds of radical nature, phenols, and quinones. There is a probability of binding of such compounds to amino acid residues of the polypeptide chains. The binding of reactive compounds may cause reduction of copper atoms of types 1 and 2 in the active center of the enzyme. This was evident by the absence of the typical absorption maximum of Cu\(^{+}\) at 610 nm by yellow laccases. The molecular masses of blue and yellow laccases of *P. tigrinus* were found to be identical and their specific activities were of the same order of magnitude. Changes in the protein conformation make the yellow laccase less stable. The yellow laccase can oxidize non-phenolic substrates without exogenous addition of mediators (Leontievsky et al., 1997). It is assumed that the reactive compounds may be
reversibly substituted upon the enzyme saturation with substrate and these reactive compounds perform the function of electron-transfer mediator (Pozdnyakova et al., 2004).

**Functions of laccases**

Lignin is formed via the oxidative polymerization of monolignols within the plant cell wall matrix. Peroxidases, which are abundant in virtually all cell walls, have long been held to be the principal catalysts for this reaction. Recent evidence shows, however, that laccases secreted into the secondary walls of vascular tissues are equally capable of polymerizing monolignols in the presence of O₂. The role of laccases in lignification has often been debated. Laccase from *Acer pseudoplatanus* was able to polymerize monolignols, in the complete absence of peroxidase (Sterjiades et al., 1992). This shows that laccase was involved in the early stages of lignification, while peroxidases were involved later.

Laccase has been shown to be an important virulence factor in many diseases caused by fungi. Among other roles, laccase can protect the fungal pathogen from the toxic phytoalexins and tannins in the host environment (Pezet et al., 1992). For example, in the root pathogen, the aggressiveness of *Heterobasidion annosum* is related to the presence of laccase (Johansson et al., 1999). *Cryptococcus neoformans* is an encapsulated fungus that has emerged as a life-threatening infection in immunocompromised patients, especially those infected with human immunodeficiency virus. Laccase and its product melanin have been described as an important virulence factor of *C. neoformans* (Williamson, 1997). *Cryptococcus albidus* is also reported to produce laccase along with melanin production (Labrecque et al., 2005).

The use of laccases in bioremediation has also been reported. The toxicity of the isomers of 2,4,6-trichlorophenols was studied in liquid cultures of the white rot fungi, *Panus tigrinus* and *Coriolus versicolor*. In both cases the ligninolytic enzyme systems of both fungi were found to be responsible for the transformation of trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone. Laccase from the white-rot fungus, *Trametes hirsuta*, has been used to oxidize alkenes (Niku-Paavola and Viikari, 2000). The oxidation is the effect of a two-step process in which the enzyme first catalyzed the oxidation of primary substrate, a mediator added to the reaction, and then the oxidized mediator oxidizes the secondary substrate, the
alkene, to the corresponding ketone or aldehyde. Laccase purified from a strain of *Coriolopsis gallica* oxidized carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of 1-hydroxybenzotriazole and 2,2-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid as free radical mediators (Bressler et al., 2000). An isolate of the fungus, *Flavodon flavus*, was shown to be able to decolourize the effluent from a Kraft paper mill bleach plant. *F. flavus* decolourized several synthetic dyes like Azure B, Brilliant green, Congo red, Crystal violet, and Remazol Brilliant Blue R in low nitrogen medium (Raghukumar, 2000). Partial decolorization of two azo dyes (orange G and amaranth) and complete decolorization of two triphenylmethane dyes (bromophenol blue and malachite green) was achieved by cultures of *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase (Pointing and Vrijmoed, 2000). *Trametes hirsuta*, and a laccase purified from the fungus, was able to degrade triarylmethane, indigoid, azo, and atraquinonic dyes used in dyeing textiles (Abadulla et al., 2000) as well as 23 industrial dyes (Rodriguez et al., 1999).

Laccases are able to catalyze electron transfer reactions without additional cofactors, hence their use has been studied in biosensors to detect various phenolic compounds, oxygen or azides. Moreover, biosensors for detection of morphine and codeine (Bauer et al., 1999), catecholamines (Ferry and Leech, 2005), plant flavonoids (Jarosz-Wilkolazka et al., 2004) and also for electroimmunoassay (Kuznetsov et al., 2001) have been developed. An enzyme electrode based on the co-immobilisation of an osmium redox polymer and a laccase from *T. versicolor* on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, attaining nanomolar detection limits (Ferry and Leech, 2005). Laccase can also be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems (Chen et al., 2001; Calabrese et al., 2002).

The potential use of laccase for bleaching has been investigated and this has even led to the esoteric suggestion of using laccases in the presence of hydroxy stilbenes as hair dyes (bleaches) (Onuki et al., 2000; Pruche et al., 2000). To improve the production of fuel ethanol from renewable raw materials, laccase from the white rot fungus, *Trametes versicolor*, was expressed under control of the PGK1 promoter in *S. cerevisiae* to increase its resistance to phenolic inhibitors in lignocellulose
hydrolysates (Larsson et al., 2001). Laccase immobilized on a copper-chelate carrier that can be regenerated was used successfully to remove phenols from white grape must (Servili et al., 2000). Laccases are currently of interest in baking due to its ability to cross-link biopolymers. Laccase from the white-rot fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough (Selinheimo et al., 2006). The moonlighting property of the laccases can be attributed to its ability to produce a free radical from a suitable substrate. The ensuing secondary reactions are responsible for the versatility of laccases in producing so many varied products.

On the basis of ligninase enzymes produced, fungi are classified into five main groups (Tuor et al., 1995):

I. White-rot fungi expressing Lip, MnP and laccase. This group contains the best known white-rot fungi *Coriolus Lversicolor*, *Phanerochaete chrysosporium* and *Phlebia radiata*. *P. chrysosporium* is listed within this group since laccase production was reported (Ander et al., 1980; Eriksson et al., 1983). However, this fungus is generally considered not to produce laccase. All of them usually colonize deciduous trees, only *Phlebia radiata* occasionally degrades conifers.

II. White-rot fungi simultaneously produce both types of phenoloxidases MnP and laccase, but reportedly do not secrete detectable levels of lignin peroxidase. Nevertheless, these fungi are strong lignin degraders. *Dichomitus squalens* and the edible fungus *Lentinula edodes* belong to this group.

III. White-rot fungi with LiP and one of either phenoloxidases. Lactase is the predominant phenoloxidase produced, only in case of *Coriolus pruinosum* was MnP production reported. These fungi grow on hardwood. As an exception, only *Phlebia tremellosus* degrades coniferous wood.

IV. Four white-rot fungi secrete LiP without phenoloxidases. Again with one exception they are hardwood degraders.

V. The last group probably consists of fungi which are incompletely characterized. Notably *Fomes lignosus* and *Trametes cingulata* are white-rot degraders, but neither of the oxidative enzymes was detected.
Role of biosorption in effluent decolourization

There are two main processes acting during biological decolourization. One is enzymatic action and second adsorption. Biosorption is mainly a physio-chemical process involving a biological entity like live or dead biomass of fungi or bacteria (biosorbent) and some chemicals, metals or dyes (sorbate). The biosorption process involves a solid phase (sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, chemicals present in effluent, metal ions). Due to higher affinity of the sorbate for the sorbent species, the latter is attracted and bound by different mechanisms. The process continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbate affinity for the sorbent determines its distribution between the solid and liquid phases.

A wide variety of biological materials are used as biosorbents. For example the waste mycelia available from fermentation processes, olive mill solid residues (Pagnanelli, et al., 2002), activated sludge from sewage treatment plants (Hammaini et al., 2003), biosolids (Norton et al., 2003), live fungi and bacteria (Srivastava and Thakur, 2006; Srivastava and Thakur, 2007).

The mechanism of biosorption is complex, involving ion exchange, chelation, adsorption by physical forces, entrapment in inter and intrafibrilliar capillaries and spaces of the structural polysaccharide network as a result of the concentration gradient and diffusion through cell walls and membranes. There are several chemical groups that would attract and sequester the sorbate in biomass: acetamido groups of chitin, structural polysaccharides of fungi, amino and phosphate groups in nucleic acids, amido, amino, sulphhydryl and carboxyl groups in proteins, hydroxyls in polysaccharide and mainly carboxyls and sulphates in polysaccharides of marine algae that belong to the divisions Phaeophyta, Rhodophyta and Chlorophyta.

Mechanism of biosorption

The biosorption mechanisms are various and are not fully understood due to the complex nature and structure of microorganisms. They may be classified according to various criteria. According to the dependence on the cell's metabolism, biosorption mechanisms can be divided into:

1. Metabolism dependent and
2. Non-metabolism dependent

According to the location where the sorbate removed from solution is found, biosorption can be classified as

1. Extra cellular accumulation/ precipitation
2. Cell surface sorption/ precipitation and
3. Intracellular accumulation

Non-metabolism dependent biosorption mechanism

During non-metabolism dependent biosorption, sorbate uptake is by physico-chemical interaction between the sorbate and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cell’s metabolism. Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids have abundant sorbate binding groups such as carboxyl, sulphate, phosphate and amino groups (Figure 2.6.). This type of biosorption, i.e., non-metabolism dependent is relatively rapid and can be reversible (Kuyucak and Volesky, 1988).

**Physical adsorption**

Physical adsorption takes place with the help of van der Waals' forces. Kuyucak and Volesky (1988) hypothesized that uranium, cadmium, zinc, copper and cobalt biosorption by dead biomasses of algae, fungi and yeasts takes place through electrostatic interactions between the metal ions in solutions and cell walls of microbial cells. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chiarella vulgaris* (Aksu et al. 1992), and for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger* (Srivastava and Thakur, 2006b).

**Ion Exchange**

Cell wall of microorganisms contain polysaccharides and sorbates having charges which can be exchanged with the counter ions of the polysaccharides. For example, the alginates of marine algae occur as salts of K+, Na+, Ca²⁺, and Mg²⁺. These ions can exchange with counter ions such as CO³⁻, Cu²⁺, Cd²⁺ and Zn²⁺ resulting in the biosorptive uptake of heavy metals (Kuyucak and Volesky 1988). The biosorption of copper by fungi *Ganoderma lucidium* (Muraleedharan and
Venkobachr, 1990) and Aspergillus niger was also up taken by ion exchange mechanism.

**Chemical adsorption**

The sorbate removal from solution may also take place by complex formation on the cell surface after the interaction between the metal and the active groups. Aksu et al. (1992) hypothesized that biosorption of copper by C. vulgaris and Z. ramigera takes place through both adsorption and formation of coordination bonds between metals and amino and carboxyl groups of cell wall polysaccharides. Complexation was found to be the only mechanism responsible for calcium, magnesium, cadmium, zinc, copper and mercury accumulation by Pseudomonas syringae. Microorganisms may also produce organic acids (e.g., citric, oxalic, gluonic, fumaric, lactic and malic acids), which may chelate toxic metals resulting in the formation of metallo-organic molecules. These organic acids help in the solubilisation of metal compounds and their leaching from their surfaces. Metals may be biosorbed or complexed by carboxyl groups found in microbial polysaccharides and other polymers (Figure 2.6.).

**Extra cellular accumulation/ precipitation**

In the case of precipitation, the sorbate uptake may take place both in the solution and on the cell surface (Ercole, et al. 1994). Further, it may be dependent on the cell's metabolism if, in the presence of toxic compounds and metals, the microorganism produces compounds that favour the precipitation process. Precipitation may not be dependent on the cells' metabolism, if it occurs after a chemical interaction between the metal and cell surface (Figure 2.6.).

**Metabolism dependent biosorption mechanisms**

Transport of the sorbate across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of biosorption may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal. Chemicals, i.e. heavy metals and dyes transport across microbial cell membranes, may be mediated by the same mechanism used to convey metabolically important ions such as potassium, magnesium and sodium (Figure 2.6.). Basically biosorption by living organisms comprises of two steps. In first case a metabolism independent binding where the sorbate is bound to the cell walls. In other case
metabolism dependent intracellular uptake is carried out where sorbate is transported across the cell membrane (Gadd et al., 1988).

Factors affecting biosorption

The investigation of the efficacy of the sorption uptake by the microbial biomass is essential for the industrial application of biosorption, as it gives information about the equilibrium of the process which is necessary for the design of the equipment. The sorbate uptake is usually measured by the parameter 'q_e' which indicates the milligrams of sorbate accumulated per gram of biosorbent material and 'q_m' is reported as a function of sorbate accumulated, sorbent material used and operating conditions.

The following factors affect the biosorption process:

1. Temperature seems not to influence the biosorption performances in the range of 20-35°C (Aksu et al., 1992).

2. pH seems to be the most important parameter in the biosorptive process: it affects the solution chemistry of the metals, the activity of the functional groups in the biomass (Galun et al., 1987).

3. Biomass concentration in solution seems to influence the specific uptake: for lower values of biomass concentrations there is an increase in the specific uptake. Interference in between the binding sites due to increased biomass was suggested as a possible reason (Gadd et al., 1988).

Effect of Pre-treatment of sorbent on biosorption process

The affinity of sorbate to the biomass can be manipulated by pre-treating the biomass with alkali, acid, detergents and heat. Effect of these treatments is highly variable for different microorganism and different sorbates as the biosorption process is very complex involving a number of processes. It is difficult to predict the effect of pretreatment without experimentation.

Effect of heat pretreatment

The biosorption capacity of autoclaved *Mucor rouxii* decreased as compared to the live fungus, attributed to the loss of intracellular uptake (Yan and Viraraghavan, 2000). Whistler and Daniel (1985) reported that the heat treatment could cause a loss of amino-functional groups on the fungal surface through the non-enzymatic
browning reaction. However, in case of *Pencillium* biomass pretreatment at 100°C for 5 minutes increased the biosorption of lead, cadmium, nickel and zinc and the increase was attributed to the exposure of latent binding sites after pre-treatment (Galun et al., 1987).

![Figure 2.6. Cartoon representation of different mechanisms of biosorption. Microbial cell is shown in light blue colour and effluent, metal or dye (adsorbate) are shown in black colour circles. (1) Precipitation, on and outside surface, (2) physical adsorption, electrostatic force, (3) active diffusion in cell, (4) ion-exchange (5) chemical adsorption by bond formation (6) complexation, production of organic acids to form complex with adsorbate, (7) deposition on surface and (8) entrapment in the surface structures of cell](image)

**Effect of alkali pre-treatment**

In the case of alkali pre-treatment, biosorption capacity of *Mucor rouxii* biomass was significantly enhanced in comparison with autoclaving (Yan and Viraraghavan, 2000). Alkali treatment of *Pencillium digitatum* also showed
enhancement of cadmium, nickel and zinc biosorption (Galun et al., 1987). Removal of surface impurities, rupture of cell-membrane and exposure of available binding sites for metal biosorption after pre-treatment may be the reason for the increased biosorption. Alkali treatment of biomass may destroy autolytic enzymes that cause putrefaction of biomass and remove lipids and proteins that mask reactive sites (McGahren et al., 1984; Brierly et al., 1985; Muraleedharan and Venkobachar, 1990). The cell wall of *Mucor rouxii* was ruptured by NaOH treatment. Besides, the pre-treatment could release polymers such as polysaccharides that have a high affinity towards certain metal ions (Mittelman and Geesey, 1985).

**Effect of acid pretreatment**

Acid pretreatment of *Mucor rouxii* significantly decreased the biosorption of heavy metals (Yan and Viraraghavan, 2000), which is in agreement with the case of *A. niger* (Kapoor and Viraraghvan, 1998). Bux and Kasan (1994) suggested that higher the biomass electronegativity, greater will be the attraction and adsorption of heavy metal cations. Thus the remaining H+ ions on the acidic pretreated *M. rouxii* biomass may change the biomass electronegativity, resulting in a reduction in biosorption capacity. However, Huang and Huang (1996) reported that acid pretreatment can strongly enhance the adsorption capacity of *Aspergillus oryzae* mycelia. In case of *A. oryzae*, live biomass after acid pre-treatment was directly used in biosorption of heavy metals instead of being autoclaved and dried. The difference in results after a specific pretreatment may be attributed to the different strains of fungi used and whether the biomass was live or dead when it is used in biosorption of metal ions.

**Biosorption equilibrium models**

The equilibrium of the biosorption process is often described by fitting the experimental points with models (Gadd, et al. 1988) usually used for the representation of isotherm adsorption equilibrium. They include models like Freundlich, Langmuir, Elovich, Temkin, Fowler–Guggenheim, Kiselev, Hill–de Boer. The two widely accepted and linearised equilibrium adsorption isotherm models for single solute system are Freundlich and Langmuir.
Optimization methods for process parameters

The bioprocess optimization through statistical design, especially for culture conditions, is a common practice in biotechnology. These statistical methods, as compared to the common ‘one-factor-at-a-time’ method, proved to be useful tools. The classical method of discussing one variable at a time while holding others constant at unspecified levels is usually inefficient in many cases. It has some limitations for complete optimization and cannot provide information about the interaction of different effective factors. Statistical experimental design techniques are very useful tools for this purpose, as they can provide statistical models that assist in understanding the interaction of different variables and predict the maximized product formation. The use of statistically designed experiments can allow the rapid and economical determination of the optimal culture conditions with fewer experiments and minimal resources (Wang et al., 2005). Commonly used statistical methods are response surface methodology, Plackett-Burman design and Taguchi approach.

Response surface methodology (RSM)

The methodology requires the identification of those factors that are significant predictors of the response variable (quality characteristics) to be optimized. The initial experimentation identifies the various levels to be used for each factor considered to be important in predicting the response variable. Data are analyzed using multiple regression analysis. This yields an estimate of the unknown model parameters, thereby, providing a predicted response function. Tests are performed to check model adequacy and to determine whether certain terms can be eliminated from the model.

Generally, the experimentation begins with a first order model. After the model parameters are estimated, model lack of fit is determined. If the first order model is adequate, the fitted model is used to locate the area where more desirable response values can be found. This is known as the Method of Steepest Ascent (Descent). The predicted model is used to move in the direction of maximum increase (decrease). The movement is incremented and the Method of LaGrange Multipliers is used to identify the new design center. Experiments are conducted to identify the maximum response. This is followed by another experiment using the factor settings at this location as the design center. The procedures detailed above are repeated. If a
first-order model is inadequate, the design is augmented to fit a full second order model. Once the design is fitted to the data, the predicted model is checked for adequacy. If model adequacy is assured, the surface is mapped and optimum factor settings are identified (Osborne and Armacost, 1996).

**Plankett-Burman design (PBD) and uniform design (UD)**

Plankett-Burman design (PBD) and uniform design (UD) are efficient and effective approaches for systematic investigation of the target factors. PBD is an effective screening design which considerably diminishes the number of experiment, and gives as much as possible information for the evaluation of the target factors. Only the most effective factors with positive significance can be selected out for further optimization, others with less significance or high negative effect on response value may be omitted in further experiment. UD can be used to optimize target parameters within the designed scopes. The number of the trials is equal to the maximum number of the designed levels of the target factors, therefore, UD method presents the attractive advantage that it needs much fewer trials when compared with the other statistical designs of experiments such as response surface design. A combination of PDB followed by UB gives a powerful tool for optimizing processes (Chen et al., 2006).

**Taguchi approach**

Treatment of effluent or production of enzymes in batch reactor is static design where the optimum levels for each factor are determined to achieve the desired levels of output. The factors that are not feasible to control and thus lead to variation in output are called noise. The purpose of optimization is to give robust design that is capable of giving desired output even in presence of noise (Figure 2.7.). This is achieved by identifying the influence of individual factors, establishing the relationship between variables and operational conditions and finally establishes the performance at the optimum levels obtained with a few well-defined experimental sets (Joseph and Piganatiells, 1988; Roy, 2001).

There are three signals to noise ratios for optimization of static problems. They are (A) Smaller the better, i.e. lesser is the output better is the process (B) Larger the better, more is the output better is the process (C) Normal the best, this is used when the target value is pre-defined; closer the output to the defined value more
robust is the system. They are also called quality characteristics. These quality characteristics are in terms of output.

![Diagram](image)

**Figure 2.7.** Flow diagram showing variations in output (a) before optimization (b) after optimization

Taguchi approach involves two important features, use of orthogonal arrays for designing the experiments and Analysis of Variance (ANOVA) for generation and significance of results. Orthogonal arrays are well defined tables that are used to reduce the number of experiments to be conducted. This is a fractional factorial approach. The benefit of using fractional factorial approach over full factorial approach is the drastic reduction in the number of experiments. Full factorial approach will require 1024 experiments to be conducted for optimizing a process with five parameters at four levels while in fractional factorial using M-16 orthogonal array the number of experiments reduces to sixteen (Adewuyi and Oyenekan, 2007).

After designing, the analysis of experimental data was performed using ANOVA which is defined as collection of many statistical techniques used to test statistical significance between means, which in turn is determined by analyzing the variances between them. Use of ANOVA in Taguchi approach facilitates the evaluation of parameters involved in the process that are statistically significant. This approach not only helps in considerable saving in time and cost but also leads to a more fully developed process by providing systematic, simple and efficient methodology for the optimization of the near optimum design parameters with only a few well defined experimental sets (Adewuyi and Oyenekan, 2007; StatSoft, 2007; Phadke and Dehnad, 1988). The whole process of optimization by Taguchi approach
can be divided into four steps: design of experiment, DOE (I), experimentation (II), analysis of data (III) and validation of results (IV).

Figure 2.8. Four steps involved in optimization of process parameters using Taguchi approach (modified from Prakasham et al., 2005)

The designing of experiments and calculations are extremely complex hence there is a need of computer based software for performing optimization studies. Qualitek-4 is one such software developed by Nuteck Inc., U.S (Roy, 2001). It is a window based software that has inbuilt functions to perform various steps. The experiments are designed using software and experiments are conducted. The results are feed in the programme and then analysis is done. Software provides optimized conditions which are further validated by performing experiments (Figure 2.8.).

**Contribution of instrumentation techniques in lignin degradation**

To study the degradation of lignin by microbes, a number of instruments are used. They include electron microscopy (EM), infrared spectroscopy (FT-IR) and gas chromatography and mass spectrophotometer (GC-MS).
Electron microscopy (EM)

Electron microscopy (EM) (scanning (SEM), scanning-transmission (STEM) and transmission (TEM)) and ancillary techniques (e.g. X-ray microanalysis, electron diffraction) are now routine procedures which have been successfully applied to an array of problems in lignocellulose biotechnological research. These problems range from conventional studies on morphological aspects of wood cell wall ultrastructure (Fengel, and Wegener; 1984), biodegradation (Blanchette et al., 1990) and biopulping (Sachs et al., 1989, 1990), to enzyme interactions with pulp fibers (Mora et al., 1986) and more recently pitch problems in paper mills (Blanchette et al., 1992). Conventional SEM and TEM have been used essentially to confirm the ability of various microbes to modify and degrade wood cell walls and to visualize these events in time and in space. One of the greatest advantages of EM is its ability to show aspects of wood degradation and decay at higher magnification which proves the ability of microbes to degrade lignocellulose. Indeed, in several cases EM has provided the only conclusive evidence for true wood degradation as opposed to decay of lignin model compounds. With the further use of ancillary cytochemical, immunocytochemical and X-ray techniques it has also been possible to visualize spatial distribution of enzymes, their substrates and substrate removal during lignocellulose degradation, thus combining morphological features with biochemical aspects (Daniel, 1994).

**Scanning and transmission electron microscopy**

Conventional SEM was generally the choice for early morphological studies on wood degradation. This presumably reflects the relative ease by which specimens can be prepared for SEM in comparison to the relatively longer and more labor-intensive procedures for TEM. SEM gives the picture of surface view while TEM gives the idea of the changes taking place inside the wood. Thus TEM is much more informative as compared to SEM however, its not always possible to prepare sample. Sample preparation for TEM requires cutting very thin sections of the wood which is quite hard (Figure 2.9.). By using electron microscopy, particularly conventional TEM it has now been possible to confirm wood degradation by members from the major groups of microbes colonizing wood including bacteria, actinomycetes, ascomycetes, higher ascomycetes and basidiomycetes; the latter including both white
(i.e. simultaneous and preferential lignin degraders) and brown rot fungi (Daniel, 1994).

Figure 2.9. Scanning electron micrographs of delignified wood from ‘palo blanco’ (A) Delignified wood showing cells that are detached from each other. Individual fibers and vessel elements can be seen. Bar, 100μm. (B) Cells from an area of decay with only secondary wall layers evident. The middle lamella between cells has been completely removed. Bar, 10μm. (C) Nonselective white rot causing an attack of all cell wall layers. Cell walls are eroded, and many voids can be seen in the wood where cells have been removed. Bar, 100μm (Agosin et al., 1990).

SEM/TEM-EDXA

EDXA stands for energy dispersive X-ray analysis. Using this technique it is possible to analyze the elements present in the sample. It had been used to confirm the presence of elevated levels of manganese in black spots or flecks in wood degraded by various white rot fungi, including Heterobasidion annosum, Fomes fomentarius and T. versicolor (Blanchette, 1984). Although the existence of such flecks were reported as early as 1878 by Hartig, and previously used as a diagnostic macroscopic feature of certain types of wood decay (Hartig, 1978); it is only by the use of EDXA that elevated levels of Mn in flecks could be confirmed. The presence of calcium oxalate associated with P. radiate (wild-type) hyphae and attacked wood cell walls. At present, the role of inorganic elements during wood degradation is unknown; however, the frequent presence of MnO₂, calcium oxalate and indeed P suggests a possible role in degradation processes (Figure 2.10.). Mn forms an important component of Mn(II) peroxidases while pyrophosphate has also been reported as an important chelator of various enzymes systems (Popp et al., 1990; Fernandes et al., 2005).
Fourier-transformation infrared spectroscopy (FT-IR)

FTIR is a useful technique for studying wood decay chemistry, since minimal sample preparation is required and very small quantities of wood can be analysed (a few milligrams) as compared to conventional gravimetric techniques where several grams are required. FTIR had been used to characterize the chemistry of wood (Fauix, 1992; Owen and Thomas, 1989; Pandey, 1999) and determine lignin content in pulp, paper and wood (Schultz et al., 1985; Berben et al., 1987; Rodrigues et al., 1998). It has also been used for analysing chemical changes that occur in wood during weathering, decay and chemical treatments (Table 2.1. and 2.2.). FT-IR is generally used to give qualitative changes in wood chemistry and it need correction using other analysis before using for quantitative analysis (Pandey and Pitman, 2003). Apart from studying degradation of wood, FT-IR has also been used as a tool for quality control in the preparation of alkaline solutions of bagasse lignin (Käuper and Ferri, 2004).

Gas-chromatography mass spectrophotometry

Analytical techniques such as Py-GC/MS are useful for the chemical characterization of lignin-containing materials, providing data on the relative amounts of different types of lignin units (Calvo et al., 1995a,b). Curie-point pyrolysis-gas chromatography mass spectrometry (Py-GC-MS) is a rapid microanalytical method for the structural analysis of lignin polymers on a molecular level. The technique requires minimum sample preparation and preserves side-chain information of the phenylpropane structural units. Py-GC-MS has been applied to pulp mill effluents, chlorolignins in xylan (Erik et al., 1993). The main disadvantage of GC relies in the
Table 2.1. Summary of IR bands shown by cellulose (Pandey, 1999).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Band Position</th>
<th>Absorbance</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3348</td>
<td>0.78</td>
<td>O-H stretch (hydrogen-bonded)</td>
</tr>
<tr>
<td>2</td>
<td>2902</td>
<td>0.27</td>
<td>C-H stretching</td>
</tr>
<tr>
<td>3</td>
<td>1640</td>
<td>0.21</td>
<td>Adsorbed O-H, conjugated C=O</td>
</tr>
<tr>
<td>4</td>
<td>1430</td>
<td>0.37</td>
<td>C-H deformation (asymmetric)</td>
</tr>
<tr>
<td>5</td>
<td>1372</td>
<td>0.43</td>
<td>C-H deformation (symmetric)</td>
</tr>
<tr>
<td>6</td>
<td>1336</td>
<td>0.39</td>
<td>O-H in-plane deformation</td>
</tr>
<tr>
<td>7</td>
<td>1318</td>
<td>0.41</td>
<td>CH_2 wagging</td>
</tr>
<tr>
<td>8</td>
<td>1201</td>
<td>0.35</td>
<td>O-H deformation</td>
</tr>
<tr>
<td>9</td>
<td>1163</td>
<td>0.67</td>
<td>C-O-C asymmetric vibration</td>
</tr>
<tr>
<td>10</td>
<td>1112</td>
<td>0.80</td>
<td>Glucose ring stretch (asymmetric)</td>
</tr>
<tr>
<td>11</td>
<td>1059</td>
<td>1.00</td>
<td>C-O stretch</td>
</tr>
<tr>
<td>12</td>
<td>1033</td>
<td>0.90</td>
<td>C-O stretch</td>
</tr>
<tr>
<td>13</td>
<td>897</td>
<td>0.12</td>
<td>Glucose ring stretch, C=H deformation</td>
</tr>
</tbody>
</table>

Table 2.2. Summary of IR bands shown by softwood and hardwood lignin (Pandey, 1999)

<table>
<thead>
<tr>
<th></th>
<th>Softwood</th>
<th>Hardwood</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. No.</td>
<td>Band Position</td>
<td>Absorbance</td>
</tr>
<tr>
<td>1</td>
<td>3414</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>2935</td>
<td>0.58</td>
</tr>
<tr>
<td>3</td>
<td>2842</td>
<td>0.45</td>
</tr>
<tr>
<td>4</td>
<td>1714</td>
<td>0.35</td>
</tr>
<tr>
<td>5</td>
<td>1606</td>
<td>0.76</td>
</tr>
<tr>
<td>6</td>
<td>1502</td>
<td>0.99</td>
</tr>
<tr>
<td>7</td>
<td>1462</td>
<td>0.93</td>
</tr>
<tr>
<td>8</td>
<td>1425</td>
<td>0.75</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>1268</td>
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<tr>
<td>11</td>
<td>1214</td>
<td>0.97</td>
</tr>
<tr>
<td>12</td>
<td>1140</td>
<td>0.79</td>
</tr>
<tr>
<td>13</td>
<td>1086</td>
<td>0.79</td>
</tr>
<tr>
<td>14</td>
<td>1030</td>
<td>0.85</td>
</tr>
<tr>
<td>15</td>
<td>866</td>
<td>0.365</td>
</tr>
</tbody>
</table>

fact that derivatization is necessary and the life of some derivatives is reduced to 12–24 h (Latorre et al., 2003).

GC-MS studies helps in understanding the process of degradation. If samples of different durations are analyzed then it is possible to study the process of degradation as the intermediates formed in between can be detected. Detection of intermediates or end products of degradation gives the conclusive evidence of lignin degradation. Recently, instead of using pyrolysis GC-MS, in which sample is suddenly heated at very high temperature to make it volatile, silylation is done. Samples are treated with silylating agents [BSTFA (N, O-bis (trimethylsilyl)]
trifluoroacetamide) and TMCS (trimethylchlorosilane)] and trimethyl silyl derivatives are analyzed (Raj et al., 2006).

**Denaturing gradient gel electrophoresis (DGGE)**

Bioremediation, the use of microbes to degrade environmental contaminants is receiving increased attention as an effective biotechnology to clean up polluted environments as it offers several advantages over the traditional chemical and physical treatments for diluted and widely dispersed contaminants. The establishment of methods to monitor microbes and their genes in the natural environment is desirable because it is necessary to understand the dynamics of microbes that degrade pollutants in order to carry out bioaugmentation efficiently and safely (Tani et al., 2002). To achieve this goal denaturing gradient gel electrophoresis can be used.

In denaturing gradient gel electrophoresis (DGGE), DNA fragments of the same length but with different base-pair sequences can be separated. Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is decreased, compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE (Muyzer et al., 1993). By comparing the pattern of bands or amplifying the separated bands and then sequencing them, it is possible to track the presence or distribution of microbes of interest.

Most of the studies done have not included evaluation of toxicity along with effluent treatment, an important goal for any treatment process. The studies in general do not take into consideration the optimization of the process. Moreover, the mechanism behind the treatment process, whether decolourization of effluent or biopulping, have not been studied properly. Such studies have heavy dependence on instrumentations. For performing the treatment processes efficiently and safely it is
important to keep a tract of the microbial strains during the whole process. This can be achieved by techniques like DGGE. Therefore, in the present study novel fungal strains are identified from indigenous sources having catabolic and non-catabolic (biosorption) efficiency for removal of colour through the optimization of process parameters. The experiments were designed to evaluate the metabolites formed during decolourization, and fate and effect of microorganism were measured. Removal of toxic nature was effluent was also performed for safe disposal in to the environment.