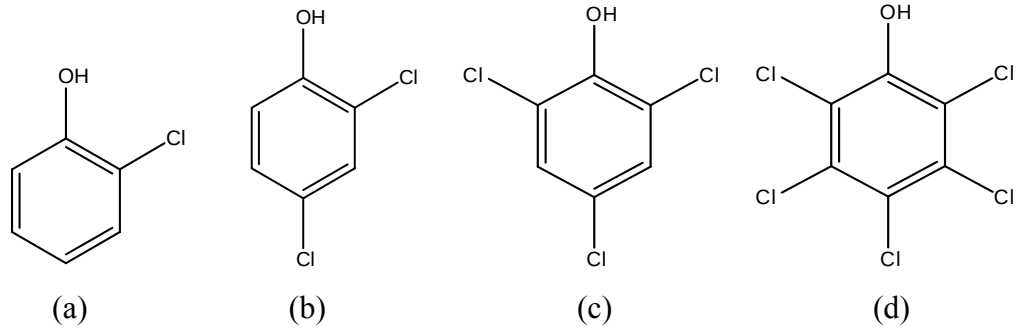
The prolific industrialization and anthropogenic activities owing to the blooming and booming economies all over the world has led to diminishing forests, extinction of species, release of toxic compounds in the environment and a collapse of fragile ecosystems (Worm *et al.,* 2006). Among the toxic compounds released, many are new to the environment and hence cannot be degraded as such by indigenous flora and fauna (Kuperman and Carreiro, 1997; Singh, 2003). These compounds being foreign to the biological world have been aptly described as Xenobiotic compounds (*Lt. Xenos = Foreign; Bios = living*). As the organisms are not exposed to these compounds naturally, they do not have a metabolic pathway for degrading them. Thus, these compounds pose a serious threat to the environment by bioaccumulation, biomagnification, rendering carcinogenic effects etc. (Bhattacharyya and Banerjee, 2007).

Chlorophenols are one of the most prominent xenobiotic compounds that pose a serious threat to the ecology and economy of a region. These compounds are extremely cytotoxic. They have genotoxic effects as well resulting in carcinogenicity (International Agency for Research on Cancer (IARC) Carcinogen List, 2003; Pesticide Action Network (PAN) Pesticide database, 2003).

Chlorophenols are widely used in paper, herbicide and pesticide industries. They are also used as wood preservatives (Ribeiro *et al.,* 2002). As chlorophenols are among the most widely used industrial compounds, an evaluation of toxicity of these compounds is of great importance both in natural environment and bioremediation technologies (Jin and Bhattacharya, 1997). They have been listed as priority pollutants by the U.S. Environmental Protection Agency (U.S. EPA). Although toxicity of chlorophenols may show variation depending on both the number and the location of chlorine atoms, it may be generalized that the toxicity of chlorophenols increases with increase in the number of chlorine atom in aerobic environment.

Copyright IIT Kharagpur



(a) (b) (c) (d)

Fig. 1.1: Structure of some major chlorophenols (a) 2-chlorophenol (b) 2,4-di chlorophenol (c) 2,4,6-trichlorophenol (d) pentachlorophenol

Ozkaya *et al.,* (2004) observed that although 25 mg L^{-1} 2,4-DCP caused around 50 % inhibition (IC_{50}), 300 mg L^{-1} of 4-chlorophenol caused the same degree of inhibition. Unlike to aerobic environment, under anaerobic conditions, the rate of reductive dechlorination increases with increasing the number of chlorine atoms (Armenante *et al.,* 1999). This highlights the potential of higher chlorophenols in rendering toxicity to the environment.

1.1 2,4-dichlorophenol: A potent xenobiotic in its class

Widely used as a soil fumigant as well as in many other industrial applications, 2,4-dichlorophenol (2,4-DCP) poses serious threat to the environment due to its toxicity to living cells, obnoxious odour and suspected mutagenic effects. Several studies have been carried out in different models to assess the toxic effects of 2,4-DCP. The studies in human models largely revealed their detrimental effects on the human erythrocytes. A study carried out by Bukowska (2003) revealed the effects of *in vitro* exposure of human erythrocytes to different concentrations of 2,4-DCP and its precursor 2,4-dichlorophenoxyacetic acid (2,4-D). The activity of superoxide dismutase, glutathione peroxidase and reduced glutathione level (GSH) were considered. It was revealed that the activity of erythrocyte superoxide dismutase (SOD) decreased with increasing dose of 2,4-D and 2,4-DCP, while glutathione peroxidase activity increased. 2,4-D (500 ppm) decreased the level of reduced glutathione in erythrocytes by 18 % and 2,4-DCP (250 ppm) by 32 %, respectively, in

Copyright IIT Kharagpur

comparison with the controls. These results lead to the conclusion that *in vitro* administration of herbicide-2,4-D and its metabolite 2,4-DCP causes a decrease in the level of reduced glutathione in erythrocytes and significant changes in antioxidant enzyme activities. Comparison of the toxicity of 2,4-D and 2,4-DCP revealed that the most prominent changes occurred in human erythrocytes incubated with 2,4-DCP. Another important study was carried out by Bukowska *et al.,* (2000). They studied the effect of phenolic compounds namely, phenol, 2,4-DCP, 2,4-dimethylphenol (2,4-DMP) and catechol on human erythrocytes. The level of oxidation of the fluorescent label 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, a marker for studying oxidative stress at cellular levels by phenolic compounds in erythrocytes as well as the carbonyl group content and haemoglobin denaturation were monitored. It was noted that 2,4-DCP, 2,4-DMP and catechol induced an increase in the concentration and time-dependent oxidation of the marker. To sum up, the kind of the substituent in a phenolic ring determines the molecular mechanism of action of the individual compound and the capacity of reactive oxygen species generation and thus damages the specified structures in human erythrocytes.

Studies have also been carried out on several other organisms to find out the effect of 2,4-DCP. Tessier *et al.,* (2000) studied the effects of 2,4-DCP exposure on the net-spinning behavior of *Hydropsyche slossonae* by examining the anomalies after 0, 5, 10, 15, and 20 exposure days to gradient concentration of 2,4-DCP. The net-spinning behavior was significantly affected when larvae were exposed to 1.0, 10, 25, and 50 $\mu g\ L^{-1}$, as expressed by the occurrence of two distinct abnormalities. The first one was a distortion of the midline meshes, where the normal diamond-shape structure is disrupted and the meshes are separated by extra strands (called "midline" anomaly). The second aberration observed was called "chaotic" net, where the nets are highly irregular without any real structure or well-defined areas.

Evidences from the studies carried out on cell, laboratory animal, wildlife and human revealed that exposure to endocrine disrupting pesticides can promote hormone dependant cancers (McDuffie, 2001). Case control studies investigating the

Copyright IIT Kharagpur

relationship between women's body burdens of organochloride pesticides and breast cancer risk have found that women with higher burdens are more likely to develop the disease. Organochloride pesticides such as 2,4-DCP have been shown to alter tumour suppressor genes *in vitro*, promoting the growth of breast, ovarian and prostate cancer tumour cells (Payne, 2001; Frigo *et al.,* 2004; Lewis *et al.,* 2005; Rattenborg, 2002; Tessier, 2001).

1.2 Degradation of 2,4- DCP

The widespread persistence, acute toxicity and suspected mutagenic effects of the 2,4-DCP has prompted researchers all over the globe to find solution to this widely rampant problem. All the attempts for removal of chlorophenols can be divided into the following major heads:

a. Photocatalytic methods (Nissen *et al.,* 2009; González *et al.,* 2010)
b. Electrochemical methods (Song-hu and Xiao-hua, 2005; Wang *et al.,* 2010)
c. Ozonation methods (Contreras *et al.,* 2003; Tsai *et al.,* 2004; Xiao *et al.,* 2008).
d. Other physicochemical methods (He *et al.,* 2002; Yasman *et al.,* 2006)
e. Biological methods

Owing to the several limitations of the physicochemical methods, such as huge start up expenses, requirement of skilled personnel on ground, hazardous operational factors such as toxic intermediates, biological solutions to the problem are sought after.

Many investigators have reported biodegradation of toxic compounds in the presence of non-toxic easily biodegradable organic compounds (Loh and Wang, 1998, Fakhruddin and Quilty, 2005, Uysal and Türkman, 2005). Several researchers have investigated biodegradation of 2,4-DCP, using different bacterial species and fungi. *Micrococcus* sp., *Chrysosporium* sp. and *Mucor* sp. have been found capable of degrading 2,4-DCP (Gallizia *et al.,* 2003, Vroumsia *et al.,* 2005). Immobilized cells of *Achromobacter* sp. in an air-lift bioreactor have also been used for the degradation of 2,4-DCP (Xiangchun *et al.,* 2003, Xiangchun *et al.,* 2004). The

removal of 2,4-DCP in a conventional activated sludge by bioaugmentation has also been reported (Chen *et al.,* 2006).

Despite their capacity to degrade chlorophenols, their usage for the degradation is being limited due to their lack of resistance to chlorophenols at a higher concentration. Chlorophenols at high concentrations usually inhibit cell growth. These toxic effects can be alleviated to some extent by subsequent adaptation of microorganisms to chlorophenols and by addition of conventional growth substrates in the medium (Van der Meer *et al.,* 1992; Andretta *et al.,* 2004). Despite acclimatization of the microorganisms capable of degrading chlorophenols, most of them are unable to thrive in 2,4-DCP contaminated effluents where the concentration levels varies from 0.15 μg mL^{-1} to 100–200 μg mL^{-1} (Valo *et al.,* 1990). As living cells are susceptible to such chlorophenols, the degradation of such compounds can alternatively take place by using some biocatalysts, i.e., enzymes, that catalyse the cleavage of such molecules resulting in mineralization.

1.3 Alternative strategies

Alternatives to circumvent the problems associated with the present biological processes of toxicity to the live cells had to be employed. The use of enzymes for bioremediation instead of whole cells could be an approach that could solve the problem. In this process, the exposure of live cells to 2,4-DCP would be evaded thereby solving the problem of cytotoxicity. Several reports were available on the use of laccase for bioremediation purposes such as degradation of industrial dyes such as Remazol brilliant blue R (Soares *et al.,* 2001), diazo dyes (Soares *et al.,* 2002), reactive blue textile dye 220 (Niebisch *et al.,* 2010) etc. Reports were also available on the use of laccase for oxidation of polycyclic aromatic hydrocarbons (PAHs) (Dodor *et al.,* 2004). However, systematic attempts for bioremediation of 2,4-DCP using laccase were lacking. Most of the attempts revolved around the use of whole cells capable of producing laccase rather than employing cell free laccase for 2,4-DCP degradation. Hence, a solution to the problem at hand may be provided

by a detailed study on the feasibility of laccase mediated degradation of 2,4-DCP, along with the kinetic parameters and degradation intermediates.

1.4 Role of laccases in bioremediation

Laccases (EC 1.10.3.2), *p*-diphenol:dioxygen oxidoreductase, are part of a larger group of copper containing metallozymes which includes among others ascorbic acid oxidase and ceruloplasmin. Laccase was first described by Yoshida in 1883. It was characterized as a metal containing oxidase by Bertrand (1985). This makes it one of the oldest enzymes ever described. Laccases can be roughly divided into two major groups which show clear differences, i.e. those from higher plants and those from fungi (Harvey and Walker, 1999; Mayer and Harel, 1979; Solomon *et al.,* 1996).

Fig. 1.2: Fruiting bodies of *Phanerochaete chrysoporium*

Fig. 1.3: Fruiting bodies of *Trametes versicolor*

Fig. 1.4: Fruiting bodies of *Coprinus cinereus*

Fig. 1.5: Fruiting bodies of *Bjerkandera adusta*

Several fungi, mainly from the basidiomycetes group, also known as white rot fungi, are the most well known producers of laccase.

Laccase like enzymes have also been reported in bacteria (Claus and Filip, 1997; Givaudan *et al.,* 1993) and insects (Diamantidis *et al.,* 2001; Hopkins and Kramer, 1992; Kramer *et al.,* 2001). However, many researchers do not consider the laccases present in insects as laccase. For instance, Sidjanski *et al.*, (1997) reported that the enzyme present in the hemolymph of *Anopheles* is probably not a laccase.

The reviews by Messerschmidt (1993) and Solomon *et al.,*. (1996) provide excellent summaries of the enzymology and electron transfer mechanism of the laccases, and a book edited by Messerschmidt (1997) contains a series of articles dealing with different aspects of laccase kinetics and mechanism of action and the possible roles of the enzymes. The relationship between laccases and other multi-copper enzymes is stressed.

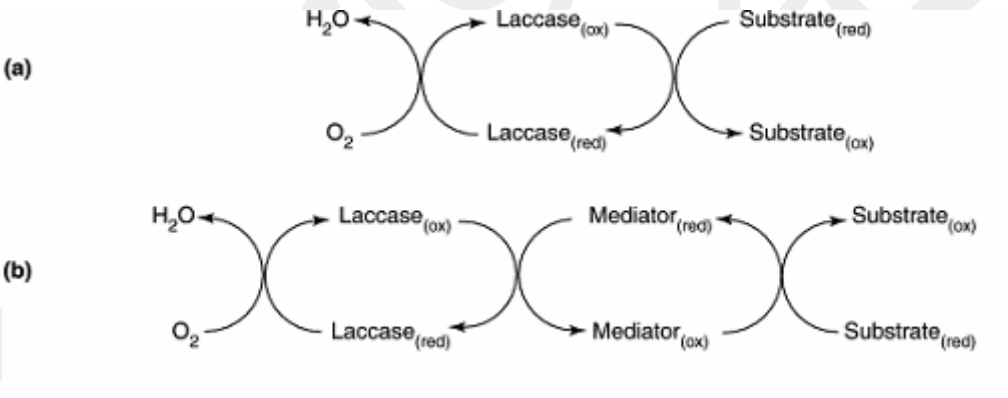


Fig. 1.6: Mechanism of laccase action (a) in the absence of the mediators (b) in the presence of the mediators

Spectroscopic techniques have provided further insights into the molecular mechanisms of copper proteins (Randall *et al.,* 2001; Solomon *et al.,* 1998). Laccase from *Coprinus cinereus,* expressed in *Aspergillus oryzea* has been crystallized and its three-dimensional structure determined (Ducros *et al.,* 2001). Laccase contains four copper atoms termed T1 (where the reduction of the substrate takes place) and a

trinuclear copper cluster T2/T3 where molecular oxygen binds and is reduced to water. As one electron oxidation is coupled like four electron reduction of oxygen, the reaction mechanism cannot be entirely straightforward. Laccases can be thought to operate as battery storing electrons from individual oxidation reactions in order to reduce molecular oxygen. Hence, the oxidation of four reducing substrate molecules is necessary for the complete reduction of molecular oxygen to water. In general terms, substrate oxidation by laccase is a one-electron reaction generating a free radical. The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation or otherwise a non-enzymatic reaction such as hydration, disproportionation or polymerisation. The bonds of the natural substrate, lignin, that are cleaved by laccase include Cα- oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage.

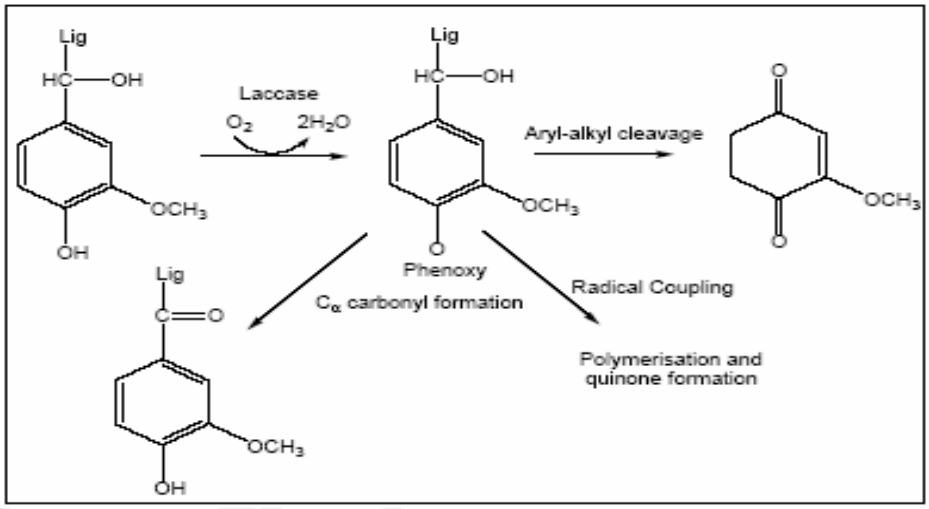


Fig. 1.7: Mode of laccase action in phenolic substrates

1.5 Optimization of laccase production

For implementation of a particular process in field condition it is necessary for the process to be economically feasible. The main constraint in the feasibility of an enzymatic process in the field conditions is the cost of the enzyme itself. An increase in the titer value and/or reduction in its production costs would tilt the process economics in favor of the enzymatic processes. There can be two main approaches for increasing the laccase production or increasing its titer value. One of the approaches

is the media manipulation by changing its composition, addition of limiting factors, inducing stress by making the media deficient in certain nutrients etc. The second approach can be the manipulation of the organism itself by the use of the tools of molecular biology.

1.5.1 Media manipulation for increasing the laccase yield

Several workers carried out optimization studies of laccase production from wild as well as genetically manipulated strains to maximize the production of laccase. The main alteration that was carried out by different investigators involved the effect of pH, temperature, additional carbon sources, cofactors etc. Wang *et al.,* (2006) investigated the effects of the carbon and nitrogen sources, initial pH and incubation temperature on laccase production by the endophytic fungus *Monotospora* sp. It was found that maltose and ammonium tartrate were the most suitable carbon and nitrogen sources for laccase production. Hou *et al.,* (2004) found that using cellobiose and peptone as carbon and nitrogen source, a higher activity of laccase was obtained from *Pleurotus ostreatus*. 2,2′-Azino-bis-(3-ethylbenzothialozin-6-sulfonic acid) (ABTS) was shown to be the best inducer of laccase production. Cu^{2+} also had a positive effect on laccase production. A similar observation was made by Revenkar and Lele (2006) who optimized the fermentation medium using a combination of one factor at a time and orthogonal array methods. Optimized medium containing 2 % starch was found to give enhanced laccase activity. The enhancement of laccase production by incorporation of copper in the medium was also reported. The addition of aromatic compounds such as 2,5-xylidine, lignin, and veratryl alcohol is known to increase and induce laccase activity (Xavier *et al.,* 2001). Many of these compounds resemble lignin molecules or other phenolic chemicals (Marbach *et al.,* 1984; Farnet *et al.,* 1999). Veratryl alcohol is an aromatic compound known to play an important role in the synthesis and degradation of lignin. The addition of veratryl alcohol to cultivation media of many white-rot fungi has resulted in an increase in laccase production (Barbosa *et al.,* 1996). Some of these compounds affect the metabolism or growth rate while others, such as ethanol, indirectly trigger laccase production (Lee *et al.,* 1999). Eggert *et al.,* (1995) found that the addition of 2,5-xylidine as inducer had the

most pronounced effect on laccase production. The addition of 10 µM 2,5-xylidine after 24 h of cultivation gave the highest induction of laccase activity and increased laccase activity nine-fold. At higher concentrations the 2,5-xylidine had a reduced effect, probably due to toxicity. The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals (Farace *et al.,* 2002). These can bind to the recognition sites when present in the substrate and induce laccase production. White-rot fungi were very diverse in their responses to inducers tested for laccase. The addition of certain inducers can increase the concentration of a specific laccase or induce the production of new isoforms of the enzyme (Robene-Soustrade and Lung-Escarmant, 1997). Some inducers interact variably with different fungal strains. Lee *et al*., (1999) investigated the inducing effect of alcohols on the laccase production by *Trametes versicolor*. The enhanced laccase activity was comparable to those obtained using 2,5-xylidine or veratryl alcohol. Meza *et al.,* (2007) studied laccase production by the strain *Pycnoporus cinnabarinus* ss3 solid-state culture on sugar-cane bagasse using chemical compounds as inducers (ethanol, methanol, veratryl alcohol and ferulic acid) and found that laccase productions were about 5 to 8.5 fold higher than non-induced cultures.

1.5.2 Use of molecular biology for the improvement of laccase yield

Reducing the costs of laccase production by optimizing the fermentation medium is the basic research for industrial applications. Another approach is the overproduction of laccase in a suitable host. However, ligninolytic enzymes are difficult to overexpress heterologously in an active form (Jönsson *et al.,* 1997) contrary to other oxidoreductases such as glucose oxidase which are industrially produced by recombinant strains of filamentous fungi. Fungal laccases are glycosylated enzymes whose sugar moieties are involved in the stabilisation process against proteolysis (Yoshitake *et al.,* 1993). Consequently, it is necessary to express these enzymes in eukaryotic microorganisms able to perform such post-translational modifications. Several fungal laccase genes have been cloned and heterologously expressed in the filamentous fungi *Aspergillus niger* (Record *et al.,* 2002; Larrondo *et al.,* 2003),

Aspergillus oryzae (Yaver *et al.,* 1996; Ducros *et al.,* 1997 and Hoshida *et al.,* 2005) and *Trichoderma reesei* (Kiiskinen and Saloheimo, 2004 and Bailey *et al.,* 2007). The latter gave high laccase production levels.

Yeasts are suitable as hosts for heterologous protein production because they combine a high capacity for growth, the easy manipulation of unicellular organisms and the post translational modification capacity of eukaryotic organisms. Laccase genes have been heterologously expressed in the yeasts *Saccharomyces cerevisiae* (Kojima *et al.,* 1990; Hoshida *et al.,* 2001; Larsson *et al.,* 2001; Bulter *et al.,* 2003; Kiiskinen and Saloheimo, 2004; Necochea *et al.,* 2005; Piscitelli *et al.,* 2005), *Pichia pastoris* (Jönsson *et al.,* 1997; Otterbein *et al.,* 2000; Hoshida *et al.,* 2001; Brown *et al.,* 2002; O'Callaghan *et al.,* 2002; Soden *et al.,* 2002; Liu *et al.,* 2003; Colao *et al.,* 2006), *Pichia methanolica* (Guo *et al.,* 2005; Guo *et al.,* 2006), *Kluyveromyces lactis* (Piscitelli *et al.,* 2005) and *Yarrowia lipolytica* (Jolivalt *et al.,* 2005 ; Madzak *et al.,* 2005). The latter led to very promising results opening the way to the use of directed mutagenesis or *in vitro* evolution for improvement of laccase for industrial applications. Also, recently Hong *et al.,* (2007) expressed a new laccase gene isolated from a novel laccase-producing fungus *Trametes* sp. 420 in *P. pastoris* obtaining a high laccase yield (8.3×10^4 U L^{-1}).

1.6 Types of fermentation used in laccase production

The types of fermentation processes used for enzyme production can be divided into two major classes:

a. Solid State Fermentation (SSF)
b. Submerged Fermentation (SmF)

Among these two types of fermentation, all the fermentation processes used in ancient times were based on the principles of solid-state fermentation technology. However, it was lost in oblivion in western countries after 1940 due to emergence of submerged fermentation technology. Perhaps SSF was neglected because development of wonder drug, penicillin took place in submerged fermentation (SmF),

which was having enormous importance at that time. Research related to SSF always continued, though in isolated pockets. During 1950–1960, steroid transformation was reported using fungal culture and reports on mycotoxin production employing SSF appeared during 1960–1970, which enabled SSF to attain another milestone and it continued with the reports on production of protein enriched cattle feed by SSF utilizing agro-industrial residues, thus offering a unique process development for value addition of these low cost residues which are also considered as pollutant to some extent. There has been a continuous extension of SSF arena, for the development of bioprocesses, such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products, such as biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids, biopesticides, including mycopesticides and bioherbicides, biosurfactants, biofuels, aroma compounds, etc. (Pandey, 2003).

1.6.1 Solid-state fermentation

Solid-state fermentation has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water. However, the substrate must possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients so as to allow the development of the microorganisms. The potential of SSF lies in bringing the cultivated microorganism in close vicinity of substrate and achieving the highest substrate concentration for the fermentation. SSF resembles the natural habitat of microorganism and is, therefore, preferred choice for microorganisms to grow and produce useful value added products. Fact, that SSF is well adapted to the metabolism of fungi; characteristic of the microorganism employed in this technique is an important feature of this process. At laboratory scale, many of the papers have been published and still publishing over study of effects of various factors on fungal metabolism. SSF reproduces the natural microbiological processes like composting and ensiling. On one hand by utilizing the

Copyright IIT Kharagpur

low cost agricultural residues SSF adds on to economic feasibility of the process and on other hand it solves the problem of its disposal which otherwise causes pollution.

For affecting the solid state fermentation, recently a number of attempts have been taken for devising effective bioreactors that would give better process controls as well as higher yields. There are several parameters (aeration, pH, humidity, agitation, temperature) relevant for the selection of the suitable bioreactor for each particular fermentation process (Rodriguez-Couto and Sanroman, 2005).

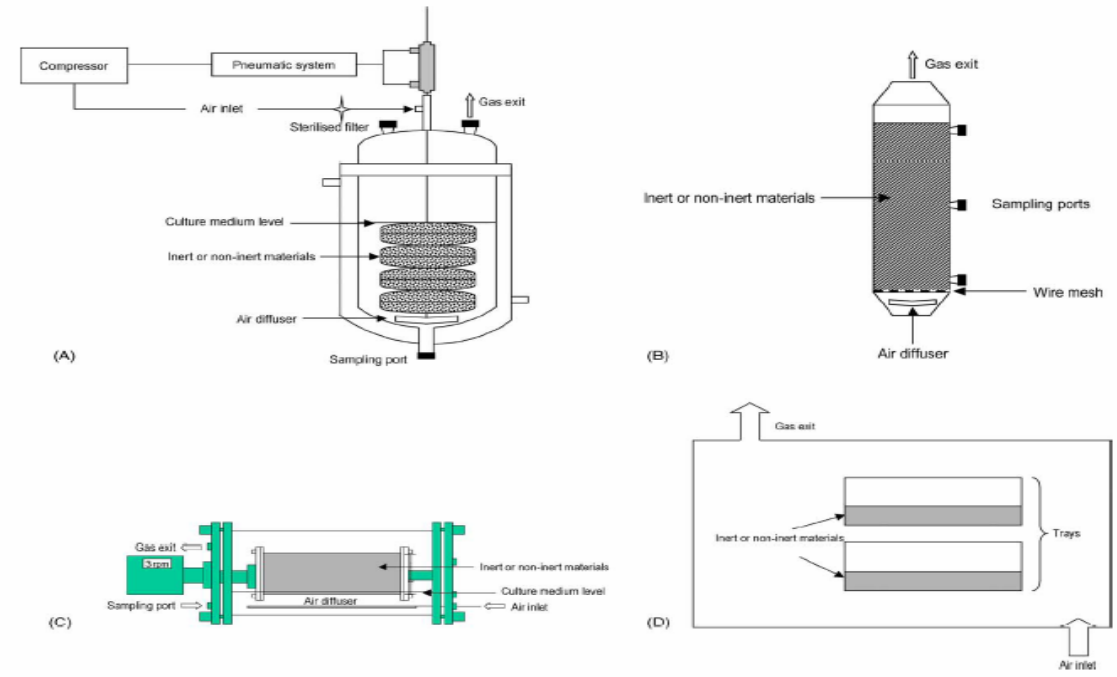


Fig. 1.8: Different types of bioreactors used for carrying out SSF (A) Immersion (humidified air; mechanical agitation) (B) Packed bed (humidified air; static) (C) Rotating drum (humidified air; mechanical agitation) (D) Tray (passive aeration; static) (Rodriguez-Couto and Sanroman, 2005)

Though different types of reactors have been used in SSF, it remains a fact that most of the instrumentation developments in fermentors are concerned with SmF. Tray reactors are still the most popular reactor systems used in SSF processes.

1.6.1.1 Important aspects of SSF

There are various important factors that produce immense impact on success of a particular technology hence, needed to be considered for the development of any bioprocesses and so is the SSF. It includes selection of microorganism and substrate, optimum process parameters and also purification of the end product, which has been a challenge for this technology. Fungi and yeast were termed as suitable microorganisms for SSF according to the theoretical concept of water activity, whereas bacteria have been considered unsuitable. Still, availability of several research articles (Chinn *et al.,* 2007; Gupta *et al.,* 2008; Mukherjee *et al.,* 2008; Singhania *et al.,* 2009) proves that bacterial cultures can also be well manipulated and managed for SSF process even for scarcely produced tannase enzyme. *Bacillus thuringenesis* production was standardized by SSF on wheat bran to obtain maximum toxin and was found to be cost effective (Devi *et al.,* 2005).

The establishment of the relationships between the physiology of the microorganisms and the physico-chemical factors is the aim for the development of proper models. These factors include temperature, pH, aeration, water activity and moisture, bed properties, nature of solid substrate employed, etc. Among several critical factors moisture and nature of solid substrate employed are the most important factors affecting SSF processes. Selection of moisture depends on microorganism employed and also on the nature of substrate. Fungi needs lower moisture, 40–60 % moisture could be sufficient but selection of substrate depends upon several factors mainly related with cost and availability and thus may involve the screening of several agro-industrial residues.

SSF is defined as any fermentation process occurring in absence or near absence of free liquid, employing an inert substrate (synthetic materials) or a natural substrate (organic materials) as a solid support (Pandey *et al.,* 1999a). In recent years, SSF has received more and more interest from researchers, since several studies have shown superior product yields and simpler downstream processing than SmF (Robinson and Nigam, 2003). SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi (Moo-Young *et al.,* 1983; Pandey *et al.,*

Copyright Kharagpur

1999a), since they reproduce the conditions under which these fungi grow in nature (Pandey *et al.,* 1999b). Moreover, Téllez-Jurado *et al.,* (2006) have shown recently that the expression of a heterologous laccase in *A. niger* substantially improved using the SSF technique.

The use of natural solid substrates, especially lignocellulosic agricultural residues, as growth substrates for fungi has been studied for laccase production in recent years (Rodriguez-Couto and Sanromán, 2005). Furthermore, such residues contain lignin or/and cellulose and hemicellulose, which act as inducers of laccase. Moreover, most of them are rich in sugars, which due to their organic nature are easily metabolised by the microorganisms. This makes the whole process much more economical. Nevertheless, the use of lignocellulosic materials presents several problems such as support degradation and support accretion, causing mass and oxygen restrictions

1.6.1.2 Challenges and advantages of SSF

Today's environment is rapidly changing, where we can experience constant technological advancement backed by innovation playing major catalyst in this race. SSF appears to possess several biotechnological advantages, though at present mostly on a laboratory scale, such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of micro-organisms specialized for the water-insoluble substrates or mixed cultivation of various fungi, and last but not least, lower demand on sterility due to the low water activity used in SSF (Hölker *et al.,* 2004). González *et al.,* (2003) have attempted to develop a general approach for the comparison of productivity of enzymes employing SSF and SmF and have tried to explain the reason for higher production in SSF. Higher biomass, high enzyme production and lower protein breakdown, contributes to the better production in SSF.

1.6.2 Submerged fermentation

Submerged fementation (SmF) can be considered as a violation to their natural habitat, especially of fungi (Pandey, 2003). Not even marine microorganism prefer swimming in free water, since more than 98 % of isolates from marine environment

have been obtained from the underwater surfaces of solid substrates, found in marine habitats. Submerged fermentation (SmF) involves the growth of microorganisms in a liquid medium rich in nutrients and with a high oxygen concentration (aerobic conditions). The industrial production of enzymes is mainly performed by SmF.

Growth patterns in submerged cultures usually result into uncontrolled growth of mycelium. The extension of the fungal biomass has profound effects on mass transfer, metabolic rate and product secretion. Fungal mycelia can wrap around impellers, cause blockages and spread into sampling and nutrient feed lines as well as increase broth viscosity, which results in mass and oxygen transfer limitations. These drawbacks limit the time of operation in bioreactors.

Several strategies have been proposed by different workers to overcome this problem of lack of control over fungal growth in bioreactors. Lema *et al.,* (2001) developed a pulsed system that allows controlling pellet growth, making possible a long-term operation. Recently, this system has been applied to decolouration of a synthetic dye by the white-rot fungus *Trametes versicolor* (Blánquez *et al.,* 2004; Blánquez *et al.,* 2006; Romero *et al.,* 2006; Blánquez *et al.,* 2007) making possible to operate the bioreactor in a continuous mode for prolonged times with high efficiency.

Fig. 1.9: A Continuous Stirred Tank Reactor

Fungal laccases are extracellular enzymes that are secreted into the medium by the mycelia of filamentous fungi (Bollag and Leonowicz 1984; Agematu *et al.,* 1993b). The highest amounts of laccases are produced by white-rot fungi (Leonowicz *et al.,* 1997), which are the only organisms able to mineralize all components of lignin to carbon dioxide and water. One of the factors limiting the large-scale application of fungal laccases is the lack of an efficient production system at bioreactor scale. The different morphological growth forms of the filamentous fungi have a significant effect on the rheology of the fermentation broth and, thus, in the performance of the bioreactor. The effects of broth rheology on mass, momentum and heat transfer within a bioreactor have been well studied (Charles, 1985; Moo-Young *et al.,* 1987; Funahashi *et al.,* 1988). Thus, different morphological forms result in different types of broth rheology. Cultures with filamentous growth usually exhibit a high apparent viscosity and non-newtonian rheology. At moderate to high biomass levels, these broths display shear thinning or pseudoplasticity (Harvey and McNeil, 1994). These effects can have a number of undesirable results such as poor mass transfer, which would decrease the overall productivity. In addition, the control of hyphal extension is of great importance to operate the bioreactor in a continuous mode. In the last years different cultivation techniques and strategies have been used to produce laccase at bioreactor scale by wild-type strains of filamentous fungi (Table 1.1). Laccase production has been carried out successfully owing to the manifold advantages that the solid substrate fermentation has over submerged fermentation (SmF).

Table 1.1: Different types of reactors used in different fermentation conditions for laccase production

Fungus	Type of reactor	Type of cultivation	Inducer	Max. laccase activity ($U\ L^{-1}$)	Reference
Pycnoporus cinnabarinus	10-L packed-bed	SmF, immobilised on nylon cubes	10 mM VA	280	Schliephake *et al.,* (2000)
Trametes pubescens	20-L STR (150 rpm)	SmF, free cells	2 mM Cu^{+2}	61,900	Galhaup and Haltrich (2001)
Neurospora crassa	Capillary membrane	SmF, immobilised on membrane supports	1 µM cyclohexi mide	10,000	Luke and Burton (2001)
Phanerochaete flavido-alba	Bioflo III (975 mL, 70 rpm)	SmF, free cells	OMW	72	Blánquez *et al.,* (2002)
T. pubescens	20-L STR (100 rpm)	SmF, free cells	2 mM Cu^{+2}	333,000	Galhaup *et al.,* (2002)
T. pubescens	20-L STR (100 rpm)	SmF, free cells fed-batch	2 mM Cu^{+2}	740,000	Galhaup *et al.,* (2002)
Trametes multicolor	STR	SmF	–	–	Hess *et al.,* (2002)
Coriolus hirsutus	10-L jar fementor (160 rpm)	SmF, free cells, semi-continuous	0.25 g L^{-1} Cu^{+2}	83,830	Koroleva *et al.,* (2002)
Pycnoporus sanguineus	2-L Biostat C	SmF	16 mM VA	460	Van der Merwe (2002)
P. sanguineus	15-L Biostat C (8 L; 250 rpm)	SmF, free cells	16 mM VA	8131	Van der Merwe (2002)

Continued in the next page

Copyright IIT Kharagpur

Fungus	Type of reactor	Type of cultivation	Inducer	Max. laccase activity ($U\ L^{-1}$)	Reference
Pleurotus ostreatus	Benchtop fermenter (3 L; 200 rpm)	SmF, free cells	OMW	65	Aggelis *et al.*, (2003)
Panus tigrinus	3-L STR (2 L; 250 rpm)	SmF, free cells	OMW	4600	Fenice *et al.*, (2003)
P. tigrinus	3-L ALR (2.5 L)	SmF, free cells	OMW	4300	Fenice *et al.*, (2003)
P. tigrinus	20-L RDR	SSF (maize stalks)	OMW	1309	Fenice *et al.*, (2003)
Trametes versicolor	Biostat Q (4 reactors of 330 mL each)	SmF (pellets)	–	–	Font *et al.*, (2003)
Trametes versicolor	0.5 L fluidised-bed	SmF (pellets)	–	1187	Font *et al.*, (2003)
T. versicolor	0.5 L pulsed-bed	SmF (pellets)	–	16,000	Font *et al.*, (2003)
Irpex lacteus	Packed-bed (27 mL)	SmF (immobilised on PUF)	–	–	Kasinath *et al.*, (2003)
I. lacteus	Packed-bed (27 mL)	SmF (immobilised on PW)	–	–	Kasinath *et al.*, (2003)
T. versicolor	ALR (2 L)	SmF free cells	Tween 80	1670	Rancaño *et al.*, (2003)
T. versicolor	Immersion (2.5 L)	SSF (nylon sponge)	Tween 80	229	Rodriguez-Couto *et al.*, (2003)
T. versicolor	Immersion (2.5 L)	SSF (barley bran)	Tween 80	600	Rodriguez-Couto *et al.*, (2003)

Continued in the next page

Copyright IIT Kharagpur

Fungus	Type of reactor	Type of cultivation	Inducer	Max. laccase activity ($U\ L^{-1}$)	Reference
T. versicolor	Expanded-bed (300 mL)	SSF (nylon sponge)	Tween 80	126	Rodriguez-Couto *et al.*, (2003)
T. versicolor	Expanded-bed (300 mL)	SSF (barley bran)	Tween 80	600	Rodriguez-Couto *et al.*, (2003)
T. versicolor	Tray (1 L)	SSF (nylon sponge)	Tween 80	343	Rodriguez-Couto *et al.*, (2003)
T. versicolor	Tray (1 L)	SSF (barley bran)	Tween 80	3500	Rodriguez-Couto *et al.*, (2003)
T. versicolor	2-L STR (1.5 L)	SmF free cells	–	5.3	Sedarati *et al.*, (2003)
T. versicolor	2-L STR (1.5 L)	SmF (immobilised on nylon mesh)	–	5.3	Sedarati *et al.*, (2003)
T. versicolor	Fluidised (1.5 L)	SmF (pellets)	–	1685	Blánquez *et al.*, (2004)
P. ostreatus	Solid-substrate	Trickle-film processing (sugarcane bagasse)	Sugarcane bagasse	3500	Lenz and Hölker (2004)
Bjerkandera adusta	STR (5 L)	SmF (immobilised on plastic net)	–	4	Mohorčič *et al.*, (2004)
Trametes hirsuta	1-L fixed-bed	SmF (immobilised on stainless steel sponges)	Cu^{+2}	2206	Rodriguez-Couto *et al.*, (2004a)
T. hirsute	0.5-L immersion	SSF	Cu^{+2}	4892	Rodriguez-Couto *et al.*, (2004b)
P. cinabarinus ss3	STR (12 L), 150 rpm	SmF	–	2800	Sigoillot *et al.*, (2004)

Continued in the next page

Copyright IT Kharagpur

Fungus	Type of reactor	Type of cultivation	Inducer	Max. laccase activity ($U\ L^{-1}$)	Reference
T. versicolor	Fluidised-bed with air pulses (10 L)	SmF pellets	–	2700	Blánquez (2005)
T. hirsute	ALR (2 L)	SmF (immobilised in alginate beads)	4 Mm veratryl alcohol	1043	Domínguez *et al.*, (2005)
P. ostreatus	Packed-bed (280 mL)	SmF (immobilised on PUF)	Cu^{+2}	1403	Prasad *et al.*, (2005)
T. pubescens	ALLR (3.5 L)	Pellets	Phenolic effluent	11,800	Ryan *et al.*, (2005)
T. versicolor	Fluidised-bed with air pulses (1.5 mL)	Pellets	–	1160	Blánquez *et al.*, (2006)
T. versicolor	RITA® System	SSF (palm oil fibre)	–	138.6	Böhmer *et al.*, (2006)
T. versicolor	RITA® System	SSF (pine wood chips)	–	54	Böhmer *et al.*, (2006)
Phanerochaete chrysosporium NCIM 1197	Bench scale bioreactor (800 mL)	Batch fermentation	30 mM Cu^{+2}	30.2	Gnanamani *et al.*, (2006)
P. cinnabarinus ss3	Vapour phase bioreactor (300 mL; 18 L)	SSF (sugarcane bagasse)	Ethanol vapour	10,000	Meza *et al.*, (2006)
P. ostreatus	ALLR (5 L)	SmF pellets	OMW	1200	Olivieri *et al.*, (2006)
Funalia trogii	2-L STR	Immobilised on Na-alginate beads	–	1000	Park *et al.*, (2006)
T. hirsute	Tray (0.2 L)	SSF (nylon songe)	–	6898	Rodriguez-Couto *et al.*, (2006a)

Continued in the next page

Copyright ITKharagpur

Fungus	Type of reactor	Type of cultivation	Inducer	Max. laccase activity ($U\ L^{-1}$)	Reference
T. hirsuta	Tray (0.2 L)	SSF (grape seeds)	–	18,715	Rodriguez-Couto *et al.*, (2006a)
T. hirsuta	Immersion (0.5 L)	SSF (grape seeds)	–	12,877	Rodriguez-Couto *et al.*, (2006a)
T. hirsuta	ALLR (6 L)	SmF free cells	Cu^{+2}, glycerol	19,400	Rodriguez-Couto *et al.*, (2006b)
T. versicolor	Pulsed fluidised-bed (0.5 L)	Pellets	–	> 1500	Romero *et al.*, (2006)
T. versicolor	STR (1 L)	SmF	30 µM xyldine	11,403	Tavares *et al.*, (2006)
T. versicolor	Fluidised-bed with air pulses (1.5 mL)	Pellets	–	2123	Blánquez *et al.*, (2007)
T. hirsuta	250-mL fluidised-bed (200 mL)	SSF (orange peels)	5 mM Cu^{+2}	3000	Rosales *et al.*, (2007)
T. hirsuta	1.8-L tray (200 mL)	SSF (orange peels)	5 mM Cu^{+2}	12,000	Rosales *et al.*, (2007)

From Table 1.1 it is evident that there is a huge difference in the titer values of the enzymes obtained depending upon the strain used, culture conditions, inducers etc. Hence, studies are needed to further increase the titer values so as to tilt the process economics in favor of laccase mediated degradation of 2,4-DCP.

Copyright Universitat Rovira i Virgili

1.7 Characterization of laccases

Laccases (EC 1.10.3.2) exhibit a great diversity in their characteristic properties as well as in the substrates they degrade. Primarily secreted by white rot fungi to degrade lignin to access carbon source such as cellulose and hemicellulose in lignocellulosics, they have found widespread application in the degradation of recalcitrant compounds such as chlorophenols (Fahr *et al.,* 1999, Grey *et al.,* 1998, Ricotta *et al.,* 1996, Roy-Arcand and Archibald, 1991, Bhattacharya and Banerjee, 2008), PAHs (Majcherczyk *et al.,* 1998), lignin-related structures (Bourbonnais and Paice, 1996, Boyle *et al.,* 1992), organophosphorous compounds (Amitai *et al.,* 1998), nonphenolic lignin model compounds (Kawai *et al.,* 1989; Majcherczyk *et al.,* 1999), phenols (Bollag *et al.,* 1988; Xu, 1996) and aromatic dyes (Chivukula and Renganathan, 1995; Abadulla *et al.,* 2000). This group of N-glycosylated extracellular blue oxidases with molecular masses of 60–390 kDa (Reinhammar, 1984; Call and Mücke, 1997), contain four copper atoms in the active site (as Cu^{2+} in the resting enzyme) that are distributed among different binding sites, and are classified into three types with differential specific characteristic properties (Messerschmidt, 1997; McGuirl and Dooley, 1999). An important feature is a covalently-linked carbohydrate moiety (10–45 % of total molecular mass), which may contribute to the high stability of the enzyme. The sugar composition has been analyzed in several examples, such as *Podospora ansenna,*and *Botrytis cinerea* , *Trametes hirsuta*, *Trametes ochracea, Cerrena maxima* and *Coriolopsis fulvocinerea* and *Melanocarpus albomyces* .

Laccases catalyze the oxidation of a variety of aromatic compounds with the concomitant reduction of oxygen to water. Moreover, laccases not only oxidize phenolic and methoxyphenolic acids, but also decarboxylate them and attack their methoxy groups resulting in demethylation.

Many laccase producing fungi secrete isoforms of the same enzyme. These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme (Archibald *et al.,* 1997). The number of isozymes present differs

Copyright IIT Kharagpur

between species and also within species depending on whether they are induced or non-induced (Assavanig *et al.,* 1992). They can differ markedly in their stability, optimal pH and temperature and affinity for different substrates (Assavanig *et al.,* 1992; Heinzkill, 1998). Furthermore, these different isozymes can modulate different roles in the physiology of different species or in the same species under different conditions (Assavanig *et al.,* 1992). *Cerrena unicolor* secreted two laccase isoforms with different characteristics during the growth in a synthetic low-nutrient nitrogen/glucose medium. Various laccase encoding gene sequences have been reported from a range of ligninolytic fungi; these sequences encode for proteins between 515 and 619 amino acid residues and close phylogenetic proximity between them is indicated by sequence comparisons.

Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidized varies from one laccase to another. These enzymes catalyse the one-electron oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols, aromatic amines and ascorbate with the concomitant four-electron reduction of oxygen to water. Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups found in lignin. Kinetic data of laccases from different sources were reported. K*m* values are similar for the cosubstrate dissolved oxygen (about 5-10 M), but V*max* varies with the source of laccase (50–300 M s^{-1}). The turnover is heterogeneous over a broad range depending on the source of enzyme and substrate/type of reaction. The kinetic constants differ in their dependence on pH. K_m is pH-independent for both substrate and co-substrate, while K_{cat} is pH-dependent. The wide substrate specificity of laccase is an important reason that contributes to its popularity for a wide range of biotechnological applications.

However, the feasibility of using an enzyme in an industrial process will center on several important criteria including: (1) low costs of production; (2) inexpensive co-factors substrates and/or additives, if required (3) good stability when stored under

Copyright IIT Kharagpur

moderate conditions (4) ability to exert its catalytic activity at high reaction rates under desired reaction conditions and (5) broad substrate specificity. The laccases under investigation were found to have the potential to meet these criteria as they were produced from a hyperactive strain of *Pleurotus sp.,* exhibiting high rates of catalysis and used oxygen as an inexpensive and readily available oxidant while oxidizing aromatic substrates like 2,4 DCP and other PAHs. It has been reported that various fungal laccases are relatively stable when stored at or near neutral pH and below room temperature (Bonomo *et al.,* 2001 and Xu *et al.,* 1996) and can exert their catalytic activities over relatively wide ranges of pH and temperature (Call and Mucke, 1997 and Xu, 1997). Despite the basic reaction mechanism remaining the same, the laccases are actually a family of enzymes showing diversity in their molecular mass, isoelectric points and amino acid sequences. Many workers have revealed the presence of two or more laccases in a single organism.

1.8 Immobilization Studies

The use of laccase for biodegradation of 2,4-DCP has two major constrains. The first one is the cost of the enzyme and the second is the additional cost incurred for the secondary treatment to remove the laccase present in water. Immobilization provides a solution to both these problems.

Immobilization can be defined as the fixation of the biocatalysts (e.g. enzymes, microorganisms and organelles) to insoluble solid supports. The most important advantage of immobilization is that it makes continuous bioreactor operations possible and this is especially useful in the production of fine chemicals and bio-treatment of industrial and agricultural wastes (Mosbach *et al.,* 1976; Yoshida *et al.,* 1979; Arica and Bayramoglu, 2004; Arica *et al.,* 1999, 2004). To make an enzymatic process economically viable and/or to operate a system in a continuous mode, it is essential to immobilize the enzyme. Several attempts have been made by the workers all around the globe to immobilize laccase.

Copyright IIT Kharagpur

The attempts of immobilization can be divided into:

a. Entrapment

b. Adsorption

c. Covalent bondings

Dye decolorization using laccase immobilized on imidazol-modified silica gel (Peralta-Zamora *et al.,* 2003) or silanized alumina particles (Zille *et al.,* 2003) occurred mainly by adsorption, and to a lesser extent, by enzymatic decolorization. More recently, Champagne and Ramsay (2007) demonstrated that laccase immobilized on controlled porosity carrier (CPC) glass beads using APTES-glutaraldehyde decolorized a single anthraquinone dye, Reactive blue 19, mainly by enzymatic degradation. However, decolorization of other dyes was not evaluated. Champagne and Ramsay (2010) reported the decolorization and detoxification of textile dyes by fungal laccase immobilized on porous glass beads and subsequently their role in anthraquinone (reactive blue 19 and dispersed blue 3), indigoid (acid blue 74) dyes and azo dyes. Rekuć *et al.,* (2009) immobilized extracellular laccase produced by the wood-rotting fungus *Cerrena unicolor* covalently on the mesostructured siliceous cellular foams (MCFs) functionalized with various organosilanes with amine and glycidyl groups. The experiments indicated that laccase bound via glutaraldehyde to MCFs modified using 2-aminoethyl-3-aminopropyltrimethoxysilane remained very active. Silva *et al.,* (2007) used polyamide matrices, such as membranes, gels and non-wovens, as supports for laccase immobilization. Rekuć *et al.,* (2007) immobilized extracellular laccase produced by *Cerrena unicolor* by adsorption and covalent bonds on the cellulose-based carrier Granocel. Immobilization was optimized by changing the anchor groups and the methods of activation/immobilization. Yang *et al.,* (2006), immobilized laccase on water-soluble chitosan and chitosan microsphere by various methods, and their properties were compared with transitional metal (Fe^{3+})-immobilized laccase in chelation.

Copyright IIT Kharagpur

1.9 Statistical optimization approaches

Recent advances in computational techniques have enabled experimental workers to go in for optimization using computational modeling techniques for maximization of the processes as well as their deployment under field conditions (Sinha and Minsker, 2007). Till date several workers have tried to optimize the process parameters using one factor at a time approach. However, the one factor at a time approach suffers from several disadvantages as the inability to study the interaction between different parameters as well as its shortcoming in predicting the response in the presence of more than one variable (Montgomery and Runger, 2002). As a result several workers employed different optimization tools such as Response surface methodology (RSM), EVOP etc. to optimize the process. Several workers have used one factor at a time approach followed by statistical techniques such as Orthogonal array method to optimize laccase production (Revenkar and Lele, 2006). During the last few decades there has been a growing interest in solving optimization problems using evolutionary and hereditary principles; these systems maintain a potential population of candidate solutions to the problem at hand. In recent years, the subsurface simulation model has been combined with techniques of optimization to address important problems of contaminated site management. Modeling of site remediation was useful for dynamic evaluation/prediction and/or real-time control of the remediation systems. Campagnolo and Akgerman (1994) proposed a model for simulating soil vapor extraction systems that was used for biodegradation of petroleum-contaminated soils while Christodoulatos and Mohiuddin (1996) suggested generalized models for prediction of pentachlorophenol adsorption by natural soils.

Bhattacharya and Banerjee (2008) studied the interaction of different parameters in the biodegradation of 2,4-DCP using RSM and got a regression coefficient of ~89 %. In order to improve the efficacy of an enzymatic process, response surface models can be coupled with modern optimization techniques such as Genetic algorithm (GA) to attain the optimal conditions for enzymatic treatment (Liu *et al.,* 2008). The predicted optimum degradation condition by GA is validated with an experimental measurement.

Copyright IIT Kharagpur

Many global optimization methods have been proposed, such as GA (Holland, 1975 and Mitchell, 1996) and swarm intelligence (SI) which differ from other traditional search techniques in that they search among a population of points and use probabilistic rather than deterministic transition rules. The RS model and GA developed and utilized in this study present several advantages over other methods in the literature. The RS model is a higher order and more sophisticated polynomial model with greater accuracy (Myers and Montgomery, 2002). The GA eliminates the difficulty of user defined parameters of the existing RS models.

1.10 Identification of the research problem

From literature review, it can be ascertained that the target compound, 2,4-DCP, was extremely cytotoxic and a suspected carcinogen. Several attempts for the removal of 2,4-DCP have been taken which includes both physicochemical methods as well as biological methods. Physicochemical methods involved huge start up costs and presence of skilled personnel which act as detrimental factors for the processes to be widely used in field conditions. On the other hand, biotechnological methods were time consuming as well as ineffective above a certain concentration due to the cytotoxicity of 2,4-DCP. Hence, a feasible solution would be the development of a biotechnological method which could be used at higher concentrations of 2,4-DCP and would have a high rate of biodegradation. A cell free enzyme mediated process, by employing the lignolytic enzyme laccase (which uses molecular oxygen unlike other lignolytic enzymes which need hydrogen peroxide), for the biodegradation of 2,4-DCP can be a potential solution. 2,4-DCP having structural homology with lignin, the natural substrate of laccase, can be effectively degraded by it. Optimization of the biodegradation conditions using statistical and computational tools will outline the most favorable conditions for the process to be carried out. The process economics can be improved by optimization of the fermentation conditions of laccase thereby reducing the cost of the enzyme. Purification and characterization of the enzyme is crucial for gaining insights into the process as well as to understand different conditions affecting the efficacy of the enzyme. In order to further economize the process as well as to remove the residual enzyme from the treated water (which

Copyright IIT Kharagpur

would again incur the cost of downstream processing), immobilization can be carried out along with the optimization of the parameters of immobilization. The efficacy of the immobilized enzyme must be compared with the free enzyme to ensure the feasibility.

1.11 Objectives of the present work

After going through the literature to solve the problem at hand, the following objectives were fixed for investigation:

1. Production optimization of laccase from a locally isolated strain of *Pleurotus* sp.
2. Purification and characterization of laccase from the strain of *Pleurotus* sp.
3. Optimization of laccase mediated degradation of 2,4-DCP and its kinetic studies
4. Identification of the intermediates and propose a degradation mechanism
5. Immobilization of laccase and its kinetic studies

Copyright IIT Kharagpur