CHAPTER 4

RESULTS AND DISCUSSION
The combined industrial effluent was treated with *Phanerochaete chrysosporium* containing solution for the reduction of hazardous organic pollutants as well as the remediation of metals by biotechnological approach. Shake flask as well as bioreactor level experiments were conducted to determine the growth of the fungus. The results obtained are illustrated in the form of Figures 4-1 to 4-28, Tables 4-1 to 4-6 and Appendices I - VIII. The results obtained from experiments reveal that the fungus, *Phanerochaete chrysosporium* can be employed for the bioremediation of liquid effluents. This could be due to its capability to secrete characteristic degradative enzymes, produced in idiophase of the fungal growth cycle under nitrogen limiting conditions [Barr and Aust, 1994].

4-1 Subculturing and biomass determination

The subculturing and maintenance of cultures were carried out at regular intervals on malt extract medium slants. The mycelium appeared as white mat on solid medium after incubation [Figure 4-1 [a] & [b]]. The agitated cultures in mineral salt medium appeared as whitish puffballs [Figure 4-1 [c]]. The growth profile of the organism was determined by measuring wet and dry weights as shown in Figure 4-2. The biomass concentration increased substantially with time of incubation. After the initial lag phase for first eight hours, the growth increased with consumption of nutrients in the medium. A rapid logarithmic growth phase upto 56 hours of incubation, followed by a decline phase was observed. Maximum fungal growth was between 56-64 h of incubation in mineral salt medium. The above findings were further confirmed by the relative enhancement in the dry weight of the fungus.
Figure 4-1: The appearance of fully grown white rot fungus, *Phyrsosporium* on the media.

The growth profile of the organism is shown in the Figure 4-2, with increasing wet and dry weights, upon incubation. The higher growth can be attributed to the utilization of media components by diffusion through cellular membranes. The lag phase up to 16 h and the real start of the log phase at 24 h was convenient to measure the rate of reaction. Glucose, being the principal carbon source diffuses easily through the porous fungal cell wall and gets assimilated by triggering various biochemical reactions inside the cellular compartments. Other nutrients are also assimilated by diffusion from bulk phase of the medium. The effect of temperature and time may be the key factors for the optimum growth of the organism. *Phanerochaete chrysosporium* has a broad range of temperature for growth and metabolism (known to grow in mesophilic as well as in lower thermophilic range), however the optimum growth was at 39 °C (not shown as a figure). Fungal spores initially start forming at the tapering end of the slant and proceed further down leaving brown color on the medium, which is a specific character of the white rot fungi [Kent et al., 1976].
It was observed in Figure 4 -3 that there was a linear decrease in substrate concentration (i.e. Glucose- in MSM) from 10 gL⁻¹ to 5.51 gL⁻¹ after 72 h of incubation. This observation was also supported by a concomitant rise in the biomass concentration. Another significant parameter, the specific growth rate of the fungus was determined and found to be in the range of 0.089 - 0.102 h⁻¹ as mentioned in Table 4 -1. The maximum specific growth rate was found to be 0.10188 h⁻¹ as determined from the Lineveawer – Burk plot [Figure 4-4].

There was a linear decrease in the concentration of the substrate, which was reduced more than half to its initial concentration at the end of 72 h. It was also demonstrated that there was a slow reduction in the substrate concentration during the lag phase of the growth cycle and an increased nutrient uptake during accelerated log phase [Figure 4-2]. In general for fungi, the specific growth rate is reported to vary between 0.01-0.61 h⁻¹ [Andersson et al., 2000], the specific growth rate of P.chrysosporium falls in the normal range. Having established the growth rate of the organism in MSM, the growth rate studies were carried out in the effluent and it was found to be low. It could be that the available population in the medium might have utilized the pollutants (as source of food) for its growth and release of characteristic secondary metabolites. The findings were in agreement to a report stating a slow and moderate specific growth rate of 0.02-0.07 h⁻¹ by Thermus sp. favoring BTEX degradation, while a high growth rate, as 0.16 h⁻¹, was inhibitory to degradation[Brigita et al., 1999]. For clean up activity like bioremediation, the stationary phase of P.chrysosporium is of significance, as it produces lignolytic enzyme systems in this stage under nitrogen deficient conditions. And secrete into the external environment, where they act upon the complex organic molecules and resulting in reduction of toxicity.

4-1-1 Modeling

Mathematical modeling of growth kinetics was also attempted to determine the diffusion of nutrients into the fungal cell system. Glucose, the principal carbon source acts as a limiting substrate in the growth phase. The growth and multiplication of the microbe can be visualized as a chemical reaction where the nutrients are reactants and the cell biomass as the desired product. The uptake of nutrients is a process of diffusion through the cell walls.

The equations were derived by considering the single cell and then extending the same to a multi-cellular level. Few realistic assumptions were made in the process of obtaining the equations.
Assumptions:

The cell possesses spherical geometry.

Resistance to mass transfer is only across the Nernst diffusion layer.

The conditions of agitation provided cause only laminar flow of the fluid medium across the cell wall.

The diffusion process is such that substrate is diffusing through stagnant component i.e. cell/biomass.

The single cell under consideration is surrounded on all sides by the medium containing the nutrients.

The following parameters are defined to completely describe the system depicted in Figure 4 -5.

Considering Monod's growth kinetics as mentioned below:

\[
\frac{dX}{dt} = \mu X
\]

\[
\mu = \frac{\mu_{max} S}{S + K_s}
\]

A shell mass balance over a shell of thickness 'dr' at a distance 'r' from the center of the cell is written as :-

rate of input of S + rate of product formation
= rate of output of S + rate of disappearance + rate of accumulation of S

The rate of accumulation of S throughout the film and at the cell interface is zero. The component S is taken into the cell to produce biomass; hence its output is zero.

The flux N is given by

\[
N = -D \left( \frac{C}{C - C_s} \right) \frac{dC}{dr}
\]

The rate of input of S is

\[
= N 4\pi r^2
\]

Product formation rate = \( r_x^* V \)
Disappearance rate = \( r_s \cdot V \)

The shell mass balance equation becomes

\[ N4 \pi r^4 = Vr_s(l-Y_{s,s}) \]

Boundary conditions

At \( r = r_1 \), \( C_s = C_s^* \)

At \( r = r_2 \), \( C_s = C_m \)

Assuming the film thickness to be twice of the cell radius

\[ 3 \; r_1 = r_2 \]

Integrating the differential equation over the given boundary conditions

For low \( Re \)

\[ Sh = 2 \]

\[ r_s = \frac{D_{s,f}r^2}{V(l-Y_{s,s})} \cdot \ln \frac{C_s - C_m}{C_s^*} \]

\[ D = k_f \]

The rate equation may be written as

\[ r_s = \frac{2.3 l_s^2}{V(l-Y_{s,s})} \cdot k_f (C_m - C_s^*) \]

Then the effective diffusivity is defined as

\[ D_{eff} = \frac{D_s}{r} \]

The rate of substrate consumption becomes

\[ r_s = \frac{2 \pi R^2 k_{eff}}{V(l-Y_{s,s})} (C_s - C_s^*) \]

Which is of the form

\[ r_s = K(C_{s,0} - C_s^*) \]
4-1-2 Reaction Mechanism

Breakdown of carbohydrate molecules like glucose to CO$_2$ and H$_2$O, where the corresponding derivation of energy and reproduction of cells can be considered as a series of chemical reactions. The pathways native to the microbes like Glycolysis, Entner-Duodorooff pathway and the TCA cycle are representative of the exact response of the microbial system to its surroundings. In order to understand the manner in which the organism takes up the nutrients and exhibits growth, a reaction mechanism analogous to chemical reactions has been postulated. Since the emphasis is on the modeling of the biosystem, it becomes imperative to focus attention on the deviation of the actual mechanism from the predicted one.

In the presence of few cells

$$S \rightarrow X + P$$

The reaction mechanism may be postulated as

$$S \leftrightarrow SC^* (k_1, k_2)$$

$$SC^* + S \rightarrow S_1^* + SC^* (k_3)$$

$$S_1^* + SC^* \rightarrow X + P \text{ (slowest step) } (k_4)$$

where $S_1^*$ and $SC^*$ are intermediates.

By the pseudo steady state hypothesis, the rate equation is

$$r_s = \frac{k_2k_sC_s^2}{k_5 + 2k_5C_s}$$

An expression relating the substrate concentration and rate of consumption was obtained as follows.

$$r_s = a + bs + cs^2$$

$a$, $b$, $c$ are constants with values

$$a = 80.684, \ b = -43.24, \ c = 9.6242$$
Assuming that the microbial cell is a spherical body and the diffusion of nutrients is carried out by selectively porous cellular membranes. The standard Monod's microbial growth parameters and Nernst diffusion concept with related equations enabled in development of an acceptable mathematical model for fungal growth kinetics. In reality, cells do not exist individually, but as clusters. They take up the shape of spherical beads because of maximal surface area and minimum surface tension. This bead is not a tightly packed structure but a porous one. The pores are not all straight or have uniform cross section. Because of the non-uniformity within the bead, the porosity and tortuosity factor of the pores need to be considered (as mentioned in section 4-2) [Padmini et al., 2003]. Considering the pseudo-steady state hypothesis, the rate equation for substrate consumption was derived, which states that the concentration of the substrate was found to fall with time of incubation [Thobanoglous, 1990]. This decline in substrate concentration is because of the consumption of nutrients from the medium. The variation of substrate concentration with time is depicted in Figure 4-3.

4-1-3 Determination of Effectiveness factor

The system in consideration for determination of specific growth rate and diffusion of nutrients, is known to have a non-uniform pore structure and therefore effective mass transfer parameters were used to describe the diffusion in the system. There are two resistances that control the metabolic reaction - diffusion and chemical reaction. This results in the variation of the substrate concentration with the pellet radius. This variation can be described by the following differential equation:

\[
\frac{d^2C}{dr^2} + \frac{2}{r} \frac{dC}{dr} + (\frac{d}{r}) = 0
\]

To know which of the resistance controls the uptake of the substrate, an effectiveness factor is considered.

The effectiveness factor is defined as

\[
\eta = \frac{\text{actual reaction rate}}{\text{ideal reaction rate}}
\]

The ideal reaction rate is that which is obtained when diffusion does not limit the process of reaction. In the rate equation obtained from the experimental data, the ideal reaction is that which occurs when all the cells are exposed to the same concentration. In reality however, the
cells are not all exposed to the bulk concentration. In fact inside the spherical pellet, some cells are not all exposed to the substrate.

There exists internal mass transfer resistance to the diffusion of the substrate within the spherical pellet.

The boundary conditions are

\[ \begin{align*}
  & \text{At } r = R, C_s = C_s^\infty \\
  & \text{At } r = r, C_s = C_s
\end{align*} \]

The above differential equation when solved for the experimental and ideal rate equation leads to concentration profiles as shown Figure 4-6. The system was found to possess large diffusional resistance as compared to reaction resistance.

In a constantly moving system (bioreator), it is difficult to know the extent of availability of nutrients for the cellular system to carry out metabolic activities. Considering the mass and oxygen transfer phenomena it can be stated that the effectiveness factor plays a significant role. For its determination, the specific growth rate and its mathematical modeling were taken into account [Christopher et al., 2000].

The difference in rate equations obtained from theoretical approach and from the experiments has to be considered.

These can be attributed to the following facts.

- In the absence of substrate, the rate of its consumption must be zero. But the rate obtained from experimental analysis indicates a constant value for zero substrate concentration. In the absence of favorable conditions for growth, the cell wall is utilized resulting in endogenous metabolism. This explains the presence of a constant in the rate equation.

- The unsteady state transient behavior of the reaction rate in a batch bioreactor signifies three different rates over the time integral - in the complete absence of substrate, substrate at low concentration and the substrate at high concentration. When the substrate is at low concentration, the reaction follows second order kinetics. On the other hand when the substrate concentration
is high, the reaction follows first order kinetics. Thus the rate equation obtained from experiments accounts for all the three possible transient phases in the growth.

Also, the observed deviation highlights the difference between the behavior of a living system and that of a non-living reaction system i.e. biochemical reaction vs. chemical reaction. In a shake flask or in an aerobic reactor, both the biological system and the nutrient moieties are in a constant motion. It forms a difficult situation for the substrate molecules to be available for utilization, by the microbial cell. It is well illustrated in Figure 4 -6, in agreement to this factor, where there is a marked difference in the radius of the pellet to the concentration of the substrate under ideal and real conditions during microbial growth phenomenon. Under ideal conditions all cells have to be supplied with equal concentrations of the substrate at a given point of time. But in reality there exists a mass transfer resistance within the cells of the aggregate and there is considerable decrease in the concentration of substrate consumed.

4 -2 Adaptation studies [Effect of dilution]

The selected sample is a combined industrial effluent sample, a complex mixture of effluents of more than 30 industries in a typical industrial estate. When the fungus, *Phanerochaete chrysosporium*, was inoculated and incubated under optimal growth conditions, in the undiluted combined industrial effluents sample, no growth was observed. Attempts were made to grow the fungus by diluting the combined industrial effluent sample by five and ten fold with water. The results obtained upon incubation at optimal conditions reveal that ten-fold diluted sample have shown good growth of the fungus when compared to the traces of it in five fold diluted sample. This was confirmed by the reduction in the metal concentrations present in the ten fold effluent after incubation.

The flask and bioreactor level studies revealed that the *Phanerochaete chrysosporium* could not survive in the undiluted combined industrial effluent sample. This can be attributed to the inhibitory concentrations of the effluent sample for the growth of the fungus. Analysis shows that the toxic organic and inorganic moieties present in the combined effluent sample were found in varied concentrations.
In order to make the environment conducive for the growth and multiplication of the fungus, dilution of the sample was carried out [Figure 4-7]. Initially, the sample was diluted five-fold and there was no growth of the fungus observed. Then the sample was diluted ten-fold, which showed fairly good growth. Further experiments and analyses were carried out with the ten-fold diluted sample. Many compounds were present in the CIE, and the Figure 4-8 defines percentage reduction profile of various metals detected upon incubation with the ten-fold diluted sample.

Figure 4-7: The conical flasks showing the undiluted, five and ten fold diluted effluent sample.

4.3 Effect of pH

In the studies conducted it was observed that there is a decline in pH of the medium. As the time of incubation increased, the pH of the medium decreased and the reduction from 4.5 to 2.73 after seven days of incubation are shown in the Figure 4-9.

The overall pH of the medium affects the activity of cellular enzymes and therefore the microbial growth rate. Different organisms have different pH optima for growth and metabolism, likewise Pchrysosporium has an optimum of 4.5 and all experiments were conducted at that pH. Naturally, white rot fungi have the capability to adjust the external pH to their optimum growth [Barr and Aust, 1994]. The Figure 4-9 shows a linear reduction in the pH to 2.73 with time of incubation in the medium, with the fungus. The drop in pH could be a reflection of the degradation and also the production of large intermediary pools of organic compounds with acidic nature [Yateem et al., 1998].
Solubility of metal ions and ionization state of the functional groups (i.e. carboxylate, phosphate and amino groups) in the fungal cell wall [Gadd and White, 1988; Gadd 1986] is also affected by pH. The groups responsible for metal binding are carboxyl groups, which have pKa values between 3.0 and 4.0 [Shumate and Standberg et al., 1985]. The optimal pH for growth may be different from that for product formation. pH also plays a significant role in the adsorption of the metals over the surface of the fungus, as discussed in section 4.4.

4.4: Preliminary Tests

The selected sample was a combine industrial effluent sample as mentioned elsewhere. The untreated effluent sample was characterized and found to contain:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solids</td>
<td>45.56 gL⁻¹</td>
</tr>
<tr>
<td>Total Dissolved Solids</td>
<td>43.68 gL⁻¹</td>
</tr>
<tr>
<td>Total Suspended Solids</td>
<td>1.88 gL⁻¹</td>
</tr>
<tr>
<td>Dissolved Oxygen</td>
<td>0.5 mgL⁻¹</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 ± 0.2</td>
</tr>
</tbody>
</table>

The combined industrial effluent was dark brown in appearance, as it is a mixture of effluents of varied nature of pollutants of many industries. It forms mandatory for the individual industries' owners to check for the absence of Cyanide traces and also to adjust the pH to neutrality prior to sending their respective effluent samples to the CETP. All these parameters make the sample non-conducive for growth and multiplication of biological systems. Hence, dilution of the sample was carried out in order to lower the strength of the sample and allow the microbial population to flourish resulting in reduction in level of toxic pollutants. The microbial consortium adapted for biological treatment in activated sludge treatment pond were found to grow well at pH 7.0 ± 0.2. The selected fungus *Phytophthora* found to show 4.5 as optimum growth pH. Therefore, the samples were adjusted to pH 4.5 before treatment for the bioremediation of organic and inorganic contaminants.
4.5 COD Removal

The combined industrial effluent sample before treatment (at the plant) was checked for the organic load and COD was around 11,000 mgL⁻¹. When treated with *Phanerochaete chrysosporium* by applying seven days old biomass in mycelium form (6000 mgL⁻¹) and the spores (4800 mgL⁻¹), there was reduction in COD observed. Comparatively the samples incubated with the spore suspension showed higher reduction in COD than the fully-grown biomass (Figure 4-10).

The combined industrial effluent sample was checked for the organic load after the routine treatment methodology of the CETP. The COD was in the range of 3800 mgL⁻¹. The white rot fungus, *Phanerochaete chrysosporium* showed reduction in COD around 1700 mgL⁻¹ and 900 mgL⁻¹ (Figure 4-11) respectively, for seven days old biomass and the spores. Comparatively the samples incubated with the spore suspension showed higher reduction in COD than the fully-grown biomass (Figure 4-11).

The shake flask and FBR studies exhibited similar trends in reduction of COD in CIE. The Figure 4-10 distinctly demonstrates that there was reduction in the COD of the untreated samples (i.e. influent of CETP), when treated with *Phanerochaete chrysosporium* for seven days. The reduction in COD when biomass was employed was around 48% when compared to 57% reduction with spores. Similarly, Figure 4-11 depicts the reduction in COD in the samples treated with *Phanerochaete chrysosporium* in treated effluents (i.e. effluent of CETP) (i.e. 55% reduction in COD with biomass and 75% with that of spores). This may be because of utilization of available nutrients by spores where germination, propagation, growth and multiplication of spores is faster and higher when compared to the biomass.

These findings were well in agreement with earlier reported literature. The mixed culture was very well adopted to the two stage immobilized fluidized bed bioreactor system and was able to bring down the chemical oxygen demand (COD) to approximately 96% [Bhagvanth Rao et al., 2003]. Around 60% reduction in COD was found in the influent sample and 75% reduction in COD of the effluent sample with the selected fungus. About 50% reduction in COD and BOD with *P. chrysosporium* observed in experiments aimed at reduction in colour of pulp and paper mill effluents [Dev et al., 1992]. 56% COD reduction was observed in removal in synthetic and
industrial wastewaters by *R. erythropolis* UPV-1 [Hidalgo et al., 2002]. Complete COD removal in 2,4,6 TCP degradation by consortium of microorganism was reported [Kharoune et al., 2002, Kenneth and Diemer, 1999]. The average rate of COD biodegradation over a period of digestion was 0.74 gL\(^{-1}\)h\(^{-1}\) at 45°C, whereas at 65°C, it was twice (approx. 1.57 gL\(^{-1}\)h\(^{-1}\)). Approximately 100% COD reduction was achieved in batch cultures after 80 h using mesophilic and thermophilic conditions—a two-stage technology [Kosseva et al., 2001]. Another report confirmed that COD fell by 86% after six days treatment of whey by a protozoan ciliate, *Tetrahymena pyriformis*, in a fermentor at 28 °C [Bonnet et al., 1999].

4-6 Organic Components present in CIE

The composition of CIE is very complex in its contents. The GC-MS analysis of the sample after liquid—liquid extraction and silica gel clean up procedures, showed hundreds of contaminants with varied concentrations. Some of the major contaminants were identified from the GC-MS database and were found to be as mentioned in Table 4-2. When checked for the difference in their concentrations after treatment with the fungus, considerable reduction observed. The results could not be presented with their exact degradation values as it was difficult to calibrate and compare with original standard molecules and moreover it was out of the scope of this study [Figure 4-12].

The GC-MS analysis of the sample showed many contaminants with varied concentrations. The percentage reduction of some of the major contaminants in the effluent sample is reported in Table 4-2, which was identified from the GC-MS database. It was a significant observation where the major contaminants have shown reduction in respective concentrations after the treatment with the *Rhysosporium* as shown in Figure 4-13.
4-7 Application of dead and living fungus for metal adsorption studies

The fungus in its living and dead form was applied for adsorption of eight metals. Biosorption of metal ions increased as the initial concentration of metal ions increased in the medium. Biosorption equilibrium was established in about 1 h in cultures incubated for ten days and the concentration of adsorbed metal ions did not change further with time. The equilibrium was well described by Langmuir, Freundlich and Reidlich – Peterson isotherms. The Table 4-3 describes the percentage reduction of metals by Phanerochaete chrysosporium. The percentage reduction level was observed to be different for different metals where it was higher for Zn and Mn and lowest for Pb. The reduction in metal concentrations found to be more in ten days incubated samples than that of five day incubated culture.
Biosorption is the process by which metals are sorbed and/or complexed to either living or dead biomass [Volesky and Holan, 1995]. Extending the biosorption process by living and dead biomass as a biosorbent for remediation of toxic metals from the undiluted, five fold and ten fold diluted aqueous combined industrial effluents. Chromium, Zinc and Manganese were reduced to a considerable extent. The other metals were also reduced but to a lesser extent when compared to the mentioned metals (Table 4–4). Comparatively, the dead fungal biomass was proved to be effective in adsorbing the metal ions in solutions. The living fungus tends to absorb the metal ions into the intracellular compartments where they are utilized for metabolic activities as cofactors and co-enzymes. The dead biomass provided the highest biosorption capacity, while live fungus exhibited the lowest for all the metal ions analyzed. Similar observations have been reported for other fungi (in living conditions) and can be devoted to a variety of resistance mechanisms[Brady et al., 1994]. These mechanisms include extracellular complexation with metal binding proteins such as metallothionein and phytochelatins and efficient pumping out if metal ions enter the cell[Gadd, 1986]. Several explanations for biosorption of heavy metal ions occurred on the cell surface or within the cell wall matrix[Huang et al., 1990]. The fungal cell walls have a negative charge due to the arrangement of the carboxyl and phosphate groups of the cell wall components, which are primarily responsible for metal binding[Brady et al., 1994]. The biosorption on the fungal cell surface is a result of the complexation reactions between heavy metal ions and the charged constituents of the cell wall components[Huang et al., 1990].

This difference in the adsorption and reduction in metals by the fungus after treatment can be attributed to co-ion effect on the biosorbent by metal ions in the solution. Several researchers have reported metal biosorption using heat inactivated or living cells, since bioadsorptive capacity of the inactivated cells might be greater, equivalent or less than that of living cell[Gadd, 1996, Gadd and White, 1988, Kapoor et al., 1999, Osteen and Bibler, 1991, Sing and Yu, 1998]. However, the use of heat inactivated or dead biomass in industrial application may offer some advantages over living cells, such as less sensitivity to heavy metal ion concentrations and adverse operating conditions[Kapoor et al., 1999, Volesky and Holan, 1995]. A rapid release of sorbed metal was observed and about 40% of the metal removal might be by intracellular accumulation and the rest of the metal biosorption was by extracellular and cell surface removal (Senthilkumaar et al. 2000).
In biosorption processes, several parameters determine the biosorption rate, including structural properties of biosorbent (e.g., protein and carbohydrate composition and surface charge density, topography and surface area). The sorptive capacity of dead cells was relatively high indicating the occurrence of both surface biosorption and bioaccumulation mediated by enzymes which may be active in complexing and binding the metal and also transporting and eventually depositing the metals into the vacuoles [Chang and Hong, 1994]. The biosorption of metals onto the biosorbent are due to electrostatic attractions between the positively charged metal ions and the negatively charged groups of cell wall constituents of the fungal mycelia.

In the first stage of biosorption process, a rapid equilibrium is generally established between metal ions adsorbed on cell wall and metal ions remaining in solution [Pagnanelli et al., 2001]. Wastewaters usually contain not one but many ions making the description of metal adsorption difficult. When several ions are present in solution, interference and competition phenomena for adsorption sites can occur and lead to more complex mathematical formulations. Several isotherms have been proposed to describe equilibrium and competitive adsorption for such a system. Two-component models can be characterized only by the single metal system parameters or by additional correction factors [Pagnanelli et al., 2001].

The decrease in metal uptake with time could be attributed to the change in cell wall composition or the chitin content of the cell wall. The role of chitin in the uptake and sequestering of metallic species has been well established [Volesky, 1987]. The probable mild negative charge on the cell surface of hyphae of the fungus adsorbs positively charged metal ions. There occurs a cell-mediated transformation resulting in the variation in oxidation state of the metal ions. This phenomenon ultimately results in reduced toxic nature of the metals, which can be released into the natural streams.

The results shown in the Figure 4 illustrate that an average 80% reduction in copper and 65% reduction in case of nickel was observed with non-living biomass. The percentage reduction level in copper and nickel were only 49% and 53% after incubation with living cells. The dead fungal biomass has exhibited greater and faster biosorption/reduction levels when compared to living biomass at minimum time of contact.
The pH of the system plays a significant role on the metabolic activities of the fungus. The reduction of chromium present in the combined industrial effluent sample was checked at various pH values [Figure 4 -15]. It was observed that there was a substantial increase in reduction in chromium in samples incubated at pH 3.0 to 6.0. Maximum reduction in chromium (80%) was at pH 6.0.

Earlier there were doubts whether the microbial reduction of Cr(VI) to Cr(III) be competitive to the chemical treatment methodologies, but it was proved to be effective manifold, especially for the in situ treatment[Derek and John, 1997]. Further trials were made to bring toxic hexavalent chromium in the effluents into non-toxic trivalent form by biological systems. The total chromium present in the effluent sample was analyzed for reduction levels. The percentage reduction of chromium in the effluent sample was found to be high at pH 6.0 as shown in the Figure 4 -15.

The feasibility of metal ions adsorbing onto the biomass decreases, with the increase in proton concentrations reveals the pH dependant nature of biosorption.

The maximum chromium biosorption occurred at pH 6 and the interaction of Cr(VI) with the fungal cell wall components of the mycelium took place at that pH. This is due to the physico-chemical state of Cr ions in the aqueous medium and the total charge produced on the surface of both live and dead Pchrysosporium mycelia at pH values studied. There was an increase in Cr(VI) biosorption capacity with increasing pH from 3 to 5, which reached a plateau between pH 5.0 and 6.0. At acidic pH (pH=3), protonation of the cell wall component adversely affected the biosorption capacity of the fungal biomass, but its effect becomes minor with increasing pH in the medium. As in the case of a pH higher than 6.0 for biosorption of Cr ions, at pH 3-6.0 hydroxo species of the metal ions may begin to form in the system and which are not bound to the functional surface ligands[Sripathi et al., 2002]. This could have caused a drop in the adsorption capacity of the biosorbent for biosorption of heavy metals by using different kinds of microbial biomass. For example, the biosorption of Cd(II), Pb(II) and Cu(II) on inactivated Pchrysosporium was pH dependant and maximum biosorption was obtained at pH 6.0[Neod et al., 1999]. The optimum pH for Zn(II) uptake for Zoogloea ramigera cells was 4.0[Park et al., 1999]. Kapoor et al. in 1999 reported that biosorption of heavy metals on A.niger was selected to pH 3.5.
A number of recent studies have indicated that organic contaminants such as aromatic compounds are suitable electron donors for Cr(VI) reduction. This suggests that microorganisms may be able to simultaneously remediate organic and chromium contaminants [Derek and John, 1997].

4-9 Comparison of Standard Biosorption Isotherms

The Langmuir and Freundlich isotherms exhibit large deviations from the experimental data as can be seen from Figure 4-16. When the equilibrium data is linearized as shown in Figure 4-17, the exact fit was found to be far from a straight line because of the previously discussed factors. The curve obtained from the proposed model equation coincides with many points of the curve generated from the experimental data. This reinforces the conformance of the experimental data to the proposed model equation.

4-9-1 Modeling of metal adsorption

Langmuir and Freundlich isotherms have played a major role in giving a better understanding of the adsorption process. Their application in the chemical process industry has been more than satisfactory. However, the same cannot be said with respect to biosystems because of the incertitude involved in the intrinsic process mechanism. The attempt made to arrive at a generalized model equation for the biosorption of metals by dead fungal biomass has yielded an equation, which is mainly characterized by its generality. Also, the conformance of equilibrium data to this equation is seen by the relatively lower values of 'sse' (Table 4-4 & 4-5). The model was found to accommodate the deviations of the standard isotherms (Figure 4-16). The reliability of this equation is reinforced by the closeness of the experimental and calculated values of Qmax.

The various attempts made to study biosorption have concentrated only on the Langmuir and Freundlich isotherms for quantification. Certain deviations were observed in most of the cases. The linearization of the isotherms did not give an exact representation of the equilibrium data (Figure 4-17). The isotherms also did not yield a good fit for high values of concentration.
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4.9.1 Modeling of metal adsorption

Langmuir and Freundlich isotherms have played a major role in giving a better understanding of the adsorption process. Their application in the chemical process industry has been more than satisfactory. However, the same cannot be said with respect to biosystems because of the incertitude involved in the intrinsic process mechanism. The attempt made to arrive at a generalized model equation for the biosorption of metals by dead fungal biomass has yielded an equation, which is mainly characterized by its generality. Also, the conformance of equilibrium data to this equation is seen by the relatively lower values of 'sse' (Table 4-4 & 4-5). The model was found to accommodate the deviations of the standard isotherms (Figure 4-16). The reliability of this equation is reinforced by the closeness of the experimental and calculated values of Qmax.

The various attempts made to study biosorption have concentrated only on the Langmuir and Freundlich isotherms for quantification. Certain deviations were observed in most of the cases. The linearization of the isotherms did not give an exact representation of the equilibrium data (Figure 4-17). The isotherms also did not yield a good fit for high values of concentration.
Limitations of the standard isotherms:

The Langmuir model is given by the following equation

\[
\text{Lineweaver - Burk Plot}
\]

\[
Q = \frac{aC^*}{b + C^*} \quad => \quad \frac{1}{Q} = \frac{1}{a} + \frac{b}{a} \cdot \frac{1}{C^*}
\]  

(1)

The Freundlich isotherm is given by

\[
\text{Eadie - Hofstee Plot}
\]

\[
Q = \frac{aC^{ab}}{b + C^*} \quad => \quad \frac{Q}{a} = 1 - \frac{b}{a} \left( \frac{Q}{C^*} \right)
\]  

(2)

For each one of the metal-fungal biomass systems, referred to above, it was seen that neither the Langmuir nor the Freundlich models did give the reasonable representation of the biosorption equilibrium. The Freundlich model did not account for the saturation of the sorbent with the sorbate, whereas, the saturation of the biosorbent with the metal in contact was a commonly observed phenomenon. Langmuir isotherm, in some cases predicts the sorption equilibrium closer to the one observed experimentally and in most cases, the Langmuir isotherm is linearized into either the Lineweaver-Burk plot or the Eadie-Hofstee plot. Although the Lineweaver-Burk plot relates \(1/Q\) and \(1/C^*\), it relies heavily on the accuracy of measurements at lower concentrations of metal in solution. Eadie-Hofstee plot exhibits a more evenly spaced data distribution but it involves \(Q\) in both the coordinates. Linearization of the isotherm fits better in the Langmuir plot because it gives a good fit at lower concentration of metals. However, at higher metal concentration, the data points deviate to a large extent from the linear relationship. All these deviations discourage the use of the Langmuir and Freundlich isotherms in the linearized form to quantify the biosorption process. Therefore, non-linear regression analysis is the safest way to estimate the Langmuir and Freundlich parameters. The magnitude of error due to approximation increases as the scale of operation increases. This may be attributed to the fact that biosorption of metals is affected by factors other than the metal concentration in solution and the pH. These factors may possibly affect the ultra-structure of the surface- existence of different types of binding sites on the biomass surface; non-uniform distribution of the sorption sites throughout the surface.
Development of model equation:

Obtaining a relation that fits the experimental data with a lower variance as compared to the Langmuir and Freundlich isotherms is best done by the quantification of biosorption process. Hence, a suitable model is proposed. The data used to develop the present model was collected from literature for the biosorption of metals from the solutions by live/dead fungal biomass [Puranik, 1995, Puranik et al., 1995, Sameer and Zdravko, 1995, Tobin et al., 1984, Gadd and White, 1988] and [Puranik and Paknikar, 1997]. The model was developed following observations.

- The specific metal uptake reaches saturation at higher values of metal concentration in solution.
- At low values of $C^*$, the value of $Q$ increases steeply.

Also, the model equation was developed on the basis of following requirements.

- In the absence of physiological reactions, it is assumed that only reversible physical adsorption takes place on the biomass surface. This assumption is validated by the fact that desorption for metal recovery occurs almost as easily as adsorption. Therefore, the model must provide a good fit for metal sorption onto fungal biomass irrespective of the metal fungi system.
- The sum of squares of errors 'sse' which represents adequacy of the model must be lower than either or both the Langmuir / Freundlich models.

The equilibrium data being hyperbolic in nature, polynomial, logarithmic and power law models were first attempted. The model equation is required to fit both the steep increase and saturation behavior of the metal uptake for low and high metal concentration in solution respectively. The model equation must mathematically satisfy certain constraints as the value of $C^*$ goes on increasing as the system attains saturation. Mathematically, the limit of $Q$ as $C^*$ tends to very large values should be a constant.
The following model was proposed on the basis of the aforementioned observations

\[ Q = \frac{aC^*}{b + C^*} + dC^{**} \quad (3) \]

The model equation is essentially the algebraic summation of the Langmuir and Freundlich isotherms. The values of the constants \(a, b, d,\) and \(e\) are different from those of the individual models. For each of the metal-fungi systems studied by many investigators are tabulated in Table 4-5. The values of the sum of squares of the errors are evaluated for each model. This gives a measure of how well the data fits the model equation and vice-versa. The proposed model was found to give lower variance than the Langmuir or Freundlich model in most of the cases. In the case of Cu biosorption by \(R.\) arhizus, the sum of squares of errors was found to be lower by two orders of magnitude as compared to that of Langmuir and Freundlich equations. For Cu biosorption by \(A.\) carbonarius and Cd biosorption by \(S.\) pimprina the sum of squares of errors was close to half of that of the standard isotherms. This indicated the better fit for model equation when compared to the Langmuir and Freundlich isotherms. This directs towards an overall model that can be applied irrespective of the metal-fungi system.

Large deviations from this model equation were observed in cases where the biomass concentration was very high. The equilibrium data in this case did not even conform to the Langmuir and Freundlich isotherms. The equilibrium curve was closer to a straight line and saturation was not observed. This behavior can be explained by the lack of sufficient metal ions in solution to saturate the sorbent surface. The sorbent was exposed to the same solution concentrations as in the cases of lower biomass concentration. The low concentration of metal solutions compared to biomass present for sorption could be a reason for non-conformance of the system to any isotherm. It may be possible that if more metal ions were present in solution, saturation would have occurred.

When either of the terms in the proposed model equation was ignored the results were far from the experimental data. This explains the importance of both the terms in the equation. In some cases the values of \(d\) are negative. Therefore when the first term is ignored extremely erroneous results are obtained for the values of \(Q\). It is therefore not possible to give the values
of \( Q_{\text{max}} \) in terms of the constants alone. This is possible in the Langmuir equation with 'a' denoting the value of \( Q_{\text{max}} \). In case of the model equation, the value of \( Q_{\text{max}} \) depends on 'a', 'b', 'c', 'd', and 'e'. Therefore to give a direct value for the maximum specific uptake might not be possible. However, the evaluation of the value of \( Q_{\text{max}} \) from the model equation gives a value very close to that of the value obtained from experiments. The higher value of \( Q_{\text{max}} \) signifies more uptake of the metal by the living/dead biomass from the solution (Table 4 -4).

Data analysis:

\[
Q = \left[ \left( C_{0} - C \right) V \right] / M
\]

Mathematical model for Biosorption of metals:

Assuming the biosorbent particles to be spherical in shape, two aspects of diffusion come into play: external diffusion and internal pore diffusion. The operation of the system is in unsteady state throughout. The concentration of the metal in solution varies until equilibrium is attained.

External diffusion-

The rate of change of metal concentration can be related to the diffusional flux by

\[
\nu \frac{dC}{dt} = n \frac{4}{3} \pi r^3 N
\]

The change in the metal concentration in the sorbent can be related to the metal concentration on the sorbent. A simple material balance gives

\[
n \frac{4}{3} \pi r^3 N = n \frac{4}{3} \pi r^3 \left( \frac{dC}{dt} + (1 - \varepsilon) \frac{dq}{dt} \right)
\]

The system of differential equations can be solved simultaneously given the initial conditions.

At \( t = 0 \), \( C_A = C_{A0} \)

\( C_K = 0 \)

The expression for \( Q_K \) as a function of \( C_K \) is found from the experimental results. This expression is then inserted into equation II in order to obtain the variation of the concentration in the solution and on the biosorbent with time.
The variation of concentrations with time in both these systems are given by Figures 4-18 and 4-19. The simulation studies were carried out for nickel and copper concentrations. The data obtained was first used to fit the isotherm relationship (Figures 4-20 & 4-21). For a 10 fold diluted sample of the effluent in contact with 0.1 g of biosorbent, the proposed isotherm model gave lower variance than the Langmuir and Freundlich models.

\[ Q_e = \frac{aC_0}{b + C_e} \]

The parameter values for Nickel and copper are

<table>
<thead>
<tr>
<th>Metal</th>
<th>'a'</th>
<th>'b'</th>
<th>'d'</th>
<th>'e'</th>
<th>'C_{ao} (mgL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>-7.1655</td>
<td>1.633 X 10^7</td>
<td>4.22411</td>
<td>0.86041</td>
<td>0.59</td>
</tr>
<tr>
<td>Nickel</td>
<td>1.2528</td>
<td>25.2058</td>
<td>49.2794</td>
<td>0.916762</td>
<td>4.24</td>
</tr>
</tbody>
</table>

The values used for simulation are

'k' = 1 X 10^{-3} ms^{-1}

D = 1 x 10^{-9} m^2s^{-1}

\[ r = 88 \mu m \]

which are obtained from appropriate correlations.

The concentration of the metal in solution falls rapidly in the beginning and later the drop in concentration is relatively lower. The percentage reduction on the concentration level of copper is 80.42% at the end of 2.5 hours and 69.3% for nickel. The variation of concentrations with time in both these systems are given by Figure 4-18 and Figure 4-19.

4-10 Biodegradation of Hydrocarbons

The Figure 4-22 describes the reduction of the aromatic hydrocarbons when treated with the Phanerochaete chrysosporium. The extent of biodegradation in Benzene, Toluene and Xylene was in the range of 65-80%. There was very low biodegradation in the case of Phenol, around 35%.
The experiments conducted for biodegradation of hydrocarbons showed that except Phenol the rest of the hydrocarbons found degraded to 65-80% when incubated for 7 days. Phenol degradation was at a slow rate and only 35% reduction was observed (Figure 4-22). Xylene reduction is more over benzene and toluene degradation owing to weakly linked ortho- or para-position of methyl groups to benzene. The available literature shows that BTEX compounds were biodegraded under sulfate-reducing conditions (toluene was biodegraded under methanogenic conditions). After 5 days of incubation, impressive degradation was found where, Benzene was reduced by 18%, toluene by 41%, ethylbenzene by 99%, o-xylene and p-xylene by 67% [Andrzej and Ronald, 1995]. Biomass formation (2.5g cell dry weight L^-1) and BTEX degradation (Specific Activity of 2 -10 nmol degraded BTEX mg/cell dw/min) were higher in fed-batch culture than in batch culture. The obtained results are in agreement with previous reports and the extent of biodegradation can be improved by optimizing other physico-chemical parameters.

4-11 Biodegradation of Polynuclear aromatic hydrocarbons

The organic compounds present in the CIE were degraded to a marked extent when treated with the *Phanerochaete chrysosporium*. The reduction levels of some of the components in the synthetic effluent are depicted in the Table 4-6 and Figure 4-23 & 4-24. The percentage reduction of Fluorene was 47.43% and Chrysene has lower reduction level(39.16%).

Many PAHs and their epoxides are highly toxic, mutagenic and/or carcinogenic to microorganisms as well as to higher systems including humans[Sudip et al., 2002] [Appendix VI]. Reductive dechlorination, hydrogenolytic denitrations, and reduction of nitro groups decrease the redox potential of the xenobiotic compounds to such an extent that the metabolites generated are now susceptible to oxidative treatment process. Increased reactivity of the reduced products with molecular oxygen can facilitate a subsequent oxidative treatment process[Parales et al., 2002, Brown et al., 1995]. Considerable reduction was observed with the PAHs stock incubated with the *Phyrsosporium*. Under aerobic conditions, the native soil flora mineralized 20% of [14C]phenanthrene, but the addition of *Phyrsosporium* enhanced mineralization to 38% in 21 days of incubation [Andrzej and Ronald, 1995].
Table 4 - 6 shows the reduction of the synthetic effluent mixture containing Acenaphthylene, Fluorene, Anthracene, Pyrene, Chrysene, was observed after 20 days treatment. Highest reduction reported for Fluorene and the lowest for that of Chrysene. The total concentration of PAHs was reduced to 55% in the samples treated with 100g kg⁻¹ Phryosporium, a significant reduction of naphthalene (62%), phenanthrene (92%), fluoranthe, pyrene, benzo[a]anthracene(70%) and chrysene (70%) was noticed[Yateem et al., 1998, Canet et al., 2001, Lea et al.,1996, Cantuti et al., 1965]. Around 80% of acenaphthene and fluorene were degraded in systems by Phryosporium without surfactants. Naphthalene and phenanthrene on degradation in a partitioning bioreactor with Sphingomonas aromaticivorans were 15g and 300g achieved in 21 h for both the organisms, resulting in a volumetric PAH degradation rate of 238 mgL⁻¹h⁻¹[Andrew and Tine, 2002, Lea et al., 1996]. Significant degradation of pyrene, a tetracyclic compound, was observed after 10 days when most of the fluorene and the phenanthrene had been exhausted from the mixture. This was related to low water solubility as well as its ring structure[Seung and Jong, 1999].

In separate experiments the Benzo[a]pyrene and Ben[e]acephenanthrylene were found to be degraded upto 50% within 20 days of incubation when compared to other PAH molecules by the Phryosporium with reference to standard chromatograms. (Figure 4-25, 4-26, 4-27) [Appendix VII]. This is in agreement with the report submitted by Sudarat et al. in 2000, that Benzo[a]pyrene, to an extent of 25% was mineralized to CO₂ by the cocultures of bacteria and fungus (Stenotrophomonas maltophilia and Penicillium janthinellum) over 49 days, accompanied by accumulation and disappearance of intermediates. Michael and Xiu in 1995 reported, around 62.8% reduction of Benzo[a]pyrene in soil composting systems amended with Phryosporium. These observations were agreeable to that of the earlier reported work[Andrzej et al., 1998, Liao and Dyi-Hwa, 1996]. Of the 16 PAH compounds found on the US EPA's priority pollutant list, it has been found that the higher molecular weight compounds (i.e., those containing four or more fused benzene rings) are resistant to biological transformation and persist in contaminated soil environments[Michael and Xiu, 1995].

From the results obtained it can be stated that the degradation efficiency of the microbe is inversely proportional to the complexity of the organic contaminant in the system. Lower the number of rings, higher will be the degradation efficiency, where the lignolytic enzymes directly take part in the reduction process.
Fluidized Bed Bioreactor Studies

The fluidized bed bioreactor studies were carried out for effective degradation of COD, organic pollutants and metals etc. The outcome of the bioreactor studies suggest that, there was a substantial increase in the biomass concentration with concomitant decrease in substrate concentrations in the CIE. Reduced concentrations of organic compounds when analyzed with GC-MS and also reduction in the COD after treatment of the sample have confirmed these results.

As shown in the schematic diagram (Figure 3–1), the turbulent field on the fungal cells that were suspended in the medium imposes shear stress. The fluid motion was induced by the difference in density and there was an overall directionality of liquid flow, which generated a homogeneous field of shear [Erin and Argyrios, 2001]. The fluidized bed bioreactor system requires high recirculation to maintain uniform conditions in the reactor [Michael and Fikret, 2002].

The interwoven fibers of the nylon sponge offer good area for the attachment of the fungal hyphae. They also permit proper diffusion of nutrients and oxygen into the culture. There will be severe problems of clogging of bed due to the clinging of the cubes together with the fungal hyphae forming agglomerates, thereby hindering the rate of mass and oxygen transfer. It is necessary to modify the reactor in order to avoid such problems. [Rodriguez et al., 2002]. The possibility to control and regulate hyphal extension and pellet size is of great importance for its potential application in continuous operation, but the low- shear stress existing in an airlift reactor makes impossible to control the mycelial growth. An attempt was made to avoid the agglomeration of the nylon cubes as well as the hyphal extension of the fungus [Alberto et al., 2001].

The attempt made in treating the CIE led to logical conclusions that this organism can reduce COD, adsorb toxic metals and can metabolize single and polynuclear aromatic hydrocarbons. Therefore, successful attempt to reduce pollution of complex effluent of typical industrial estate prompted us to come to the conclusions mentioned in the following section.
Figure 4-28: The laboratory fluidized bed bioreactor employed for reactor level bioremediation and biosorption studies.