CHAPTER 3

MATERIALS AND METHODS

The methods followed for the isolation and identification of yeast from dairy effluent for the removal of Cu (II) and Pb (II) were discussed in detail this section. In the present study, Cu (II) and Pb (II) were removed by different temperature, initial metal concentrations, biomass concentration were investigated in a batch mode using free and immobilized yeast species. The best species was used for continuous experiments in a packed column reactor. The influence of initial bed height, flow rate and concentrations were investigated and modeled using BDST, Thomas and Yoon-Nelson models. The experimental methods followed are explained elaborately and discussed in detail.

3.1 CHARACTERIZATION AND IDENTIFICATION OF YEAST ISOLATES

The yeasts were isolated from the effluent collected from a dairy industry situated in Chennai, India. The samples were plated on YPD and incubated at 30°C for 72 h, the colonies were plated on YPD medium supplemented with 30mg/mL Erythromycin and incubated at same conditions. Thus the colonies were plated on YPD medium supplemented with 6g/L tartaric acid and incubated. The grown colonies were plated in 30mg/mL Chloramphenicol and incubated at 30°C for 72h. The isolated colonies are grown and preserved on YPD tubes at 4°C.
3.2 PHYLOGENETIC TREE ANALYSIS

Nucleotide sequence was initially analyzed at NCBI server using BLAST tool and the corresponding sequences were downloaded. The yeast nucleotide sequences were aligned using the CLUSTALX program. The Phylogenetic tree was constructed by the neighbor-joining method using MEGA - 4.1 (Beta) software.

3.3 MEDIA OPTIMIZATION

Various media are selected for the studies with reference to the literature are shown in the Table 3.1. Growth yield was characterized by performing a viable cell count. Samples of 1000 µL were withdrawn aseptically for analysis of viable cell numbers at the exponential phase of the organism (13-24 h). Colony-forming-units (CFU) were measured by serial plating technique on yeast-malt extract agar. A volume of 100 µL from the dilutions was plated onto Malt Extract Agar and incubated for 48 h at 37°C. After the incubation time, number of colonies in each plate was counted. The optimum concentration of the media components that showed maximum cell growth was studied. The concentration of the components was varied and the cell growth was measured by counting the viable number of cells as described before.
<table>
<thead>
<tr>
<th>S. No</th>
<th>Medium</th>
<th>Components</th>
<th>Concentration (mg/L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sabouraud (Liu et al 2004)</td>
<td>Dextrose, Peptone</td>
<td>4, 1</td>
<td>5.6</td>
</tr>
<tr>
<td>2</td>
<td>YD (Kushi et al 2000)</td>
<td>Dextrose, Yeast extract</td>
<td>2, 1</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>YP (Martins et al 2002)</td>
<td>Yeast extract, Peptone</td>
<td>1, 2</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>YPL (Becerra et al 1998)</td>
<td>Lactose, Peptone, Yeast extract</td>
<td>4, 1, 1</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Maltose (Becerra et al 1998)</td>
<td>Maltose, Peptone, Yeast extract</td>
<td>4.2, 1, 1</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>YM (Duvnjak et al 1981)</td>
<td>Dextrose, Yeast extract, Malt extract, Peptone</td>
<td>1, 0.3, 0.3, 0.5</td>
<td>5.8</td>
</tr>
<tr>
<td>7</td>
<td>Mineral (Kitamoto et al 1998)</td>
<td>Lactose, Yeast extract, Dipotassium hydrogen phosphate, Ammonium dihydrogen phosphate, Diammonium hydrogen phosphate, Magnesium sulphate</td>
<td>3, 1, 0.2, 0.1, 0.1, 0.01</td>
<td>5.5</td>
</tr>
</tbody>
</table>
3.4 MINIMAL INHIBITORY CONCENTRATION (MIC)

The minimal inhibitory concentration (MIC) was determined by inoculating overnight grown culture of yeast isolate into freshly prepared agar plates containing different concentrations of copper and lead (50 to 600 mg/L) at pH 7.0 and at 37°C. The incubation period maintained was 48 h. The minimal concentration of metal, inhibiting the growth completely was taken as MIC.

3.5 GROWTH CURVE STUDY

Growth curve of the yeast was determined in the presence and absence of the metal. Inoculum concentration of 1% (v/v) was used for the study. The cells were grown at 30ºC in the growth medium broth with metal and without metal. Samples were collected at various time intervals and the optic density of the culture was determined at 600 nm using spectrophotometer.

3.6 BIOMASS AND METAL REMOVAL STUDIES

At the exponential phase of the yeast, 10mL of sample was collected and centrifuged at 6000 rpm for 10 min. The supernatant was discarded and the biomass was collected. The biomass was diluted with 10mL of distilled water. The sample was then serially diluted and the OD was calculated at 600nm. The samples were taken in pre-weighed centrifuge tubes. The samples were centrifuged and the supernatant was discarded. The tubes are again weighed to calculate the dry weight of the biomass. A graph was plotted between dry weight and OD. The values of optical density measured were correlated with the concentrations of cells, in terms of dry weight of cells per liter of suspension (g/L) and calibration curve is plotted (Anagnostopoulos et al 2010b).
The percentage removal of the metal ions (Copper and Lead ions) by the biomass at the corresponding equilibrium conditions was calculated using the equation shown as follows

\[
\% \text{ Removal} = \frac{c_i - c_f}{c_i} \times 100
\]

where,  
- \( c_i \) - Initial concentration of the metal, mg/L  
- \( c_f \) - Final concentration of the metal, mg/L  

3.6.1 Batch Study with Free Cells  
Batch adsorption experiments were carried out by shaking the flasks at 150 rpm for a period of contact time using a rotary shaker. The effects of various parameters on the growth and metal removal capacity of the yeast were studied. Samples were collected at regular intervals (2 h). The samples were centrifuged and the concentration of metals in the supernatant solution was measured using AAS and the biomass concentration was measured using Spectrophotometer.

3.6.2 Effect of pH on metal removal  
The effect of pH on the metal removal capacity of the yeast was investigated using solution of 20 mg/L of metal solution. The growth medium was prepared and its pH was adjusted for a pH range of 3 to 7. The medium was then inoculated with 1 % (v/v) of inoculum and incubated at room temperature for 48 h. The samples were centrifuged and the concentration of metals in the supernatant solution was measured using AAS. Experiments could not be performed at higher pH values due to hydrolysis and precipitation of the metal ions.
3.6.3 **Effect of Temperature on metal removal**

Growth medium with a solution of 20 mg/L of metal solution was prepared and its pH was adjusted to optimum value. The medium was then inoculated and incubated at various temperatures 15 to 45°C for 48h. The samples were centrifuged and the concentration of metal in the supernatant solution was measured for heavy metal content.

3.6.4 **Effect of Metal Concentration on metal removal**

Growth medium with a solution of 20-500 mg/L of metal solution was prepared and incubated at optimum operating conditions. The samples were centrifuged and the concentration of metals in the supernatant solution was measured for heavy metal content. The growth kinetics of the organism was also determined and the Monod curve was plotted.

3.6.5 **The Estimation of Total Protein of the Yeast**

Growth medium with a solution of 20-500 mg/L of metal solution was prepared and batch study was conducted. At the stationary phase of the organism, 10mL was withdrawn and centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and the biomass was dried. Dried samples of washed cells were incubated in 5mL of 1N NaOH at 90°C for 10 min. to solubilize cellular protein. The cellular protein was estimated using Lowry method (Hassen et al 1998).

3.7 **IMMOBILIZATION STUDIES**

3.7.1 **Immobilization of the Yeast**

Alginate solution of 3% by weight was used for the cell immobilization. The entrapment of cells was performed according to the method reported in the literature (Johnsen & Flink 1986). Alginate was
dissolved in boiling water and autoclaved at 121°C for 15 min. Cells were harvested during the mid-logarithmic growth phase by centrifugation (6000 rpm, 10 min), resuspended in 15 mL of saline and added into 100 mL of sterilized alginate solution. This alginate/cell mixture was extruded drop by drop into a cold, sterile 3% CaCl$_2$ solution through different size nozzle pipette. Gel beads obtained were hardened by resuspending into a fresh 0.01M CaCl$_2$ solution for 24 h with gentle agitation. Finally, these beads were washed with distilled water to remove excess calcium ions and free cells. Various size alginate beads without *Kluyveromyces* Sp cells were also prepared to carry out control experiments.

### 3.7.2 Mechanical Strength of Beads

Bead mechanical resistance assessment was performed according to the method described by Wang et al (1989). 50 beads were placed in a shaking flask containing 62.5 mL of 0.85% sodium chloride with ten glass beads and incubated at 37°C under shaking at 185 rpm for 6 h. After that the flask contents were filtered by a sieve and gel beads were observed (Reyes et al 2008). Mechanical resistance is expressed based on the fracture frequency which is given as

$$Fracture frequency \% = \frac{N}{N_t} \times 100$$  \hspace{1cm} (3.2)

where, $N$ is the number of fractured beads and $N_t$ is the total number of gel beads.

### 3.7.3 Determination of Bead Density and Bead Size

Bead density was measured by displacement methods. Beads were dried with filter paper before measuring the mass. The total mass of 50 beads was measured and the beads were placed in a measured volume of water in a
graduated cylinder. The increase in volume after the bead addition was measured and the density was calculated from the mass and volume bead diameter was calculated. Bead size was found using Vernier Caliper.

3.7.4 Viable Cell Count

The bead was submerged in 1mL of saturated phosphate buffer solution until the alginate cell suspension was completely dissolved. The viability of the cell was studied by serial dilution and spread plate method.

3.8 BATCH STUDY WITH IMMOBILIZED CELLS

The batch experiments using immobilized cells were carried out in 100 mL of solution consisting of heavy metals at desired concentration, lactose (3.0 g/L) and predetermined quantities of immobilized beads. The effect of pH (3-7) and temperature on metal removal (15-45ºC) was investigated. Further experiments were carried out at optimum conditions in an orbital shaker at 150 rpm. The influence of initial metal concentration (20-500 mg/L) was investigated with the various bead diameters such as 0.33, 0.52 and 0.74 cm. The cell free beads were used to carry out control experiments. In all experiments, the initial pH was adjusted to 5.0 by adding 0.1N H₂SO₄/0.1N NaOH. Samples were analysed for heavy metal content.

3.9 PACKED BED REACTOR (PBR)

The schematic diagram of the column used in the present study is shown in Figure 3.1. The column was constructed using Perspex. The length and diameter of the column was 35 cm and 2 cm respectively. It was packed with immobilized beads of diameter 0.33 cm. The total volume of packed bed reactor was 110 cm³ and the bed volume was 66 cm³. The experimental setup
of up flow Packed Bed Column is shown in Figure 3.2. The reactor was operated in an up flow mode and the flow rates (5, 10 and 15 mL/min) were regulated using peristaltic pump. To minimize the effects of air bubble at the inlet and outlet regions of a packed column, a supporter mesh was provided inside the column. Immobilized beads without the addition of biomass were used as control. Effluent was taken out through an outlet port provided at the top of the reactor. The reactor system was operated at room temperature. The influence bead size (0.33, 0.52 and 0.74 cm) on removal of heavy metal was also investigated. The feed metal concentration was varied in the range of 100-500 mg/L for 5, 10 and 15 mL/min metal solution flow rate. Samples collected are analyzed for heavy metal concentration.

Figure 3.1 Schematic diagram of Packed Bed Column
5. Glass wool 6. Immobilized beads 7. Effluent tank
3.10 CHARACTERIZATION OF THE YEAST

3.10.1 SEM Analysis

The surface morphologies of the live cells and immobilized yeast cells before and after metal adsorption were examined with a SEM of Hitachi make. The ACC Voltage and Magnification of the instrument are 0.3+030KV
and 5X+0300000X, respectively. The resolution power of it ranges from 3.0-10nm (3KV HV mode). The Coating unit is an ion sputter coated with gold target.

3.10.2 SEM-EDX Analysis

Elemental analysis of product obtained after heavy metal removal was done with scanning electron microscopy coupled with energy dispersive X-ray analysis (EDX) spectrum. The elemental composition of the soil before and after adsorption was also analyzed using SEM-EDX analysis (Hitachi-3400N).

3.10.3 FTIR Study

FTIR spectra of the live cells and immobilized cells of the yeast before and after adsorption of heavy metals were taken. The Fourier transform infrared spectroscopy (FTIR) measurement was carried out in Bruker Tensor 27 spectrometer.

3.10.4 XRD Study

The Yeast sp. and product obtained after metal removal were properly dried in a hot air oven and powered. Then, samples were subjected to XRD analysis. The analysis was carried out with a Bruker AXS D4 Endeavor Diffractometer operating with radiation source filtered with a graphitic monochromator (λ = 1.5406). The fine powder was pressed into a sample holder and the XRD scan was done from 2θ = 10° to 80° using a step size of 0.02° and a count time of 6.
3.11 MODELING FOR MICROBIAL GROWTH STUDY

3.11.1 Monod Model

Growth medium with a solution of 20-500mg/L of metal solution was prepared and experiments are conducted using the optimum conditions. The growth kinetics of the organism was determined and the Monod curve was plotted. The relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics. In this case we assumed metal concentration (Substrate), that as growth rate limiting for the microorganisms. At higher substrate concentrations, substrate inhibition occurs and hence, Monod model might not hold well under such conditions. To overcome this problem, Andrews and Yano model are normally used to represent microbial growth (Shuler & Kargi 2005).

Kinetic models have been applied to the experimental growth data to estimate the biokinetic constants are shown in Table 3.2.

### Table 3.2 Kinetic models used in this study

<table>
<thead>
<tr>
<th>Models</th>
<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monod model (Kratochvil &amp; Volesky1998)</td>
<td>( \mu_g = \frac{\mu_{\text{max}}[S]}{K_s + [S]} ) (3.3)</td>
</tr>
<tr>
<td>Yano model (Kratochvil &amp; Volesky1998)</td>
<td>( \mu_g = \frac{\mu_{\text{max}}[S]}{[K_s + [S] + ([S^2]/K_i)}\right}{1 + (1/K)} ) (3.4)</td>
</tr>
<tr>
<td>Andrews model (Shuler &amp; Kargi 2005)</td>
<td>( \mu = \frac{\mu_{\text{max}}[S]}{K_s + [S] + [S^2]/K_i} ) (3.5)</td>
</tr>
</tbody>
</table>

where, \( \mu \) - Specific growth rate, \( t^{-1} \), \( \mu_{\text{max}} \) - Maximum specific growth rate, \( S \) - Substrate, mg/L, \( K_s \) - Saturation constant or half-velocity constant, mg/L, \( K_i \) - substrate inhibitory concentration (mg/L)
3.12 MODELING OF BATCH DATA- ADSORPTION ISOTHERM STUDY

Equilibrium data, commonly known as adsorption isotherms, are basic requirements for the design of adsorption system. In adsorption isotherm study, adsorption isothermal equations usually utilized are Langmuir, Freundlich and Redlich-Peterson model isotherms for the liquid-solid system.

The simplest proposed model for characterizing adsorption is the Langmuir isotherm. This model is an adequate approximation for uniform surfaces (Langmuir 1918). The Langmuir isotherm is developed by assuming that a fixed number of adsorption sites are available, and that the adsorption is reversible.

The Langmuir isotherm is expressed as below:

$$q_e = \frac{q_{\text{max}} b C_e}{1 + b C_e}$$  \hfill (3.6)

The Freundlich isotherm is an improvement over the Langmuir isotherm by including physical adsorption of species. This equation is a special case for heterogeneous surface energies. (Freundlich 1906). The Freundlich equation is an empirical equation that is very useful as it accurately describes much adsorption data.

The Freundlich isotherm is expressed as:

$$q_e = k C_e^{1/n}$$  \hfill (3.7)

The Redlich Peterson isotherm model is expressed as (Redlich & Peterson 1959):
\[ Q_e = \frac{K_r C_e}{1 + a_r C_e^b} \]  \hspace{1cm} (3.8)

where \( Q_e \) represents the equilibrium adsorption capacity (mg/g), \( C_e \) is the equilibrium metal concentration (mg/L), \( b \) is the Langmuir isotherm constant (L/g), \( Q_{\text{max}} \) is the maximum metal sorption (mg/g), \( K_r \) represents the capacity of adsorption (L/g) and \( \beta \) represents the Redlich–Peterson constants. The model parameters were estimated using Microsoft Excel and MATLAB.

3.13 KINETIC STUDY

Pre-equilibrium kinetic profiles were characterized in order to determine the rate limiting steps involved in the process of removal of metal by the immobilized beads. In order to examine the controlling mechanism of the biosorption process such as chemical reaction and kinetic models are used to test the experimental data. The large number and different chemical groups on the cell wall constitute the yeast cell (e.g. \(-\text{COOH}, -\text{NH}_2, -\text{NH}, -\text{SH}, -\text{OH}\)) imply that there are many types of yeast–metal ion interactions. The kinetic models including the pseudo-first order and pseudo-second order equations can be used in this case assuming that measured concentrations are equal to cell surface concentrations.

The pseudo first order equation

The pseudo-first order reaction equation for solid–liquid sorption system was proposed by Lagergren (Lagergren 1898) which is expressed as follows:

\[ Q_t = Q_s \left(1 - \exp(-k_1 t)\right) \]  \hspace{1cm} (3.9)

where, \( k_1 \) - pseudo first order rate constant (min\(^{-1}\))
The pseudo-second order model is based on the amount of sorbate which was sorbed onto the sorbent proposed by (Ho & McKay 2000). The second order equation can be derived from chemical equations and generally express as follows:

\[ Q_t = \frac{K_2 Q_e^2 t}{1 + K_2 Q_e t} \]  

(3.10)

where, \( K_2 \) is the rate constant of second-order rate constant (g/(mg /min)).

### 3.14 COLUMN MODELING

The dynamic behavior of the column can be predicted by various simple mathematical models. In this study Thomas and Yoon-nelson models were used to predict the performance of the column in adsorption studies. The flow rate, bed heights and metal concentrations on heavy metal ions removal in column operation was investigated.

#### 3.14.1 Thomas Model

Successful design of the column sorption process requires prediction of the concentration-time profile or breakthrough curve for the effluent (Yan & Viraraghavan 2003). Various mathematical models can be used to describe fixed bed adsorption. Among these the Thomas model is simple and widely used by several investigators (Yan & Viraraghavan 2003, Aksu & Gonen 2004). The linearized form of the Thomas model is expressed as follows:

\[ \ln \left( \frac{C_0}{C} - 1 \right) = \frac{k_{TH} Q_M}{F} - \frac{k_{TH} C_0}{F} v \]  

(3.11)
where $k_{Th}$ is the Thomas model constant (L/mg h), $Q_0$ is the maximum solid phase concentration of solute (mg/g) and $V$ is the throughput volume (L). The model constants $k_{Th}$ and $Q_0$ can be determined from a plot of $\ln \left( \frac{C_0 - C}{C} - 1 \right)$ against $t$ at a given flow rate.

### 3.14.2 Yoon-Nelson model

Yoon–Nelson model (Yoon–Nelson 1984) developed a model based on the assumption that a rate of decrease in the probability of the adsorption of adsorbate molecule is proportional to the probability of the adsorbate adsorption and the adsorbate breakthrough on the adsorbent. The linearized model equation is given below:

$$\ln \left( \frac{C_t}{C_0 - C_t} \right) = k_{YN} t - \tau k_{YN}$$  \hspace{1cm} (3.12)

where $k_{YN}$ (min$^{-1}$) is the rate velocity constant, $\tau$ (min) is the time required for 50% adsorbate break through. From a linear plot of $\ln \left( \frac{C_t}{C_0 - C_t} \right)$ against sampling time (t), values of $k_{YN}$ and $\tau$ were determined from the intercept and slope of the plot $\ln \left( \frac{C_t}{C_0 - C_t} \right)$ versus t.

### 3.14.3 BDST model

The BDST model is based on physically measuring the capacity of the bed at different breakthrough values. The BDST model works well and provided useful modeling equation for the changes of system parameters (Ko et al 2000). BDST column model is the simplest model which gave the useful information about the relationship between bed height ($Z$) and service time ($t$) of a column.

A modified form of the equation is given below (Goel et al 2005):
\[ t = \frac{N_0 Z}{C_0 v} - \frac{1}{K_a C_0} \ln \left( \frac{C_0}{C_b} - 1 \right) \]  

(3.13)

where \( C_b \) is the break through metal ion concentration (mg/L); \( N_0 \) the sorption capacity of bed per unit volume; \( v \) the linear velocity (cm/h) and \( K_a \) the rate constant (mg/h).

\[ Z = \frac{v}{k_a N_0} \ln \left( \frac{C_0}{C_b} - 1 \right) \]  

(3.14)

A simplified form of the BDST model is:

\[ t = aZ + b \]  

where,

\[ a = \frac{N_0}{C_0 F} \]  

(3.15)

\[ b = -\frac{1}{K_a C_0} \ln \left( \frac{C_0}{C_t} - 1 \right) \]  

(3.16)

3.15 ERROR ANALYSIS

The least sum of the squares (SS), of the difference between the three experimental data obtained from the experiments (by calculation), could be computed. If all three data from the experiments are similar, SS will be a small number; if they are different, SS will be a large number. In order to point out the actual data, the average between three values is used in the graphs and their deviation is given in the form of standard error (error bar).

\[ \text{Standard error (SE)} = \frac{\text{Standard deviation of the three experimental data}}{\text{Square root of the total number of experiments}} \]  

(3.17)

\[ SE = \frac{\text{STDEV(range of values)}}{\text{SQRT(number)}} \]  

(3.18)
3.16 METAL DESORPTION AND BIOSORBENT REUTILIZATION

Bioadsorbed heavy metals ions were desorbed from the saturated immobilized beads by different molar (0.5, 0.75, 1.0 and 1.5M) solution of HCl and EDTA in a batch recirculation mode. The solution was then filtered through Whatmann filter paper 42 and the filtrate was used to determine the metal released. The total desorbed metal was established by comparing the metal released to the amount of metal previously adsorbed to the biosorbent. All experiments were run in triplicates.

The metal stripped biosorbent was rinsed with 50 mL of double distilled water for 15 min for two times. The acid treated and base treated biomass was activated by treating them with 0.1 M H₂SO₄ and 0.1N NaOH respectively for 30 min at 150 rpm. The resulting biomass was then reloaded to packed bed reactor for metal solutions removal and the desorption treatment was repeated.

3.17 KITS, CHEMICALS, MATERIALS AND SOFTWARE

The potential yeast species for the removal of heavy metal was isolated from Dairy industrial sludge, Chennai, India. The nutrient broth and nutrient agar were purchased from (Himedia, India), the 18SrRNA sequence of the related organisms and Phylogenetic tree was constructed using BLAST, CLUSTALX, & MEGA 4.1 (beta). All the chemicals used in this study were analytical grade and were purchased from S.D Fine Chemicals Ltd (Mumbai, India), SRL Pvt. Ltd, (Mumbai, India), and Sigma Aldrich (USA). The heavy metal content in aqueous solution was measured in a Perkin-Elmer atomic absorption spectrophotometer model 2380. The model parameters were estimated using Sigma plot and MATLAB.