CHAPTER 3

Materials and methods

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3.1 Collection of biological waste

Rice husk was used for the preparation of ferrous ion coated rice husk (FeRH) and used for removal of heavy metal ions. Rice husk was taken from a local industry situated in Varanasi, India. *Citrus limetta* peels (CLPs) were collected in airtight plastic box from a fruit shop situated in the University campus. CLPs were used for synthesis of chitosan coated MnO_2 particles (CMNPs) and chitosan coated *Citrus limetta* peels (Ch-CLPs).

3.2 Preparation of stock solutions

3.2.1 Chemicals and reagents

Chemicals and reagents used in the present study were of analytical grade with 98% purity. Potassium dichromate ($K_2Cr_2O_7$), Potassium permanganate ($KMnO_4$), Sodium bicarbonate ($NaHCO_3$) and ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) were procured from HiMedia Laboratories Pvt. Ltd. make, India. The stock solution of Cr (VI), Cd (II) and Pb (II) of 1000 mg/L strength was prepared by using Potassium Dichromate ($K_2Cr_2O_7$), Cadmium Nitrate Tetrahydrate and Lead (II) Nitrate, respectively. 0.1 M $KMnO_4$ was prepared by dissolved 3.16 g in 200 mL double distilled water. 0.1 M $FeSO_4.7H_2O$ was prepared by dissolving 5.56 g of $FeSO_4.7H_2O$ in 200 mL double distilled water. 1% sodium bicarbonate solution was prepared by dissolving 5 g of sodium bicarbonate in 500 mL water. 0.01N NaOH and 0.01N HCl were used for maintaining the pH.

3.2.2 Preparation of biosorbent

3.2.2.1 RH and FeRH

The rice husk was crushed to make powder in a ball mill. The crushed rice husk was sieved through 45 - 52 mesh sieve (BSS). After size reduction, 20 g rice husk was soaked in 200 mL (0.1M) of $KMnO_4$ solution for 30 minutes and agitated at 150 rpm to allow complete mixing. Thereafter, soaked RH was washed thrice with deionized water and filtered by

Whatman filter paper No.1. The filtered rice husk was mixed with 200 mL (0.1M) $FeSO_4.7H_2O$ solution and incubated at 30°C, 150 rpm for 10 hours. After incubation, the mixture was filtered again and the residual was washed thrice with 1% sodium bicarbonate solution. Iron treated rice husk was then soaked in sodium bicarbonate solution for 5 hours. Thereafter, iron doped material was washed with double distilled water. After air drying, it was dried in a hot air oven for two hours at 105°C.

3.2.2.2 Synthesis of CMNPs

Citrus limetta peel extract was prepared by mixing 5g of *Citrus limetta* peel powder in 100 ml deionised water and boiled for 10 minutes. After boiling, the mixture was centrifuged at 5000 rpm for 15 minutes. Curcumin solution was prepared by adding 3g turmeric powder in 100 ml ethanol and the solution was boiled for 5 minutes. The boiled mixture was centrifuged at 5000 rpm for 12 minutes, supernatant was collected and used as curcumin extract. 100 ml of 0.10 mM manganese acetate was mixed with 100 ml *Citrus limetta* peel extract. 20 ml curcumin extract was added as a stabiliser for MnO_2 nanoparticles. pH 8 was maintained in the mixture by adding 0.10 M NaOH and HCl. The mixture was continuously stirred for 120 minutes at 37°C and subsequently centrifuged at 5000 rpm. The MnO_2 nanoparticles were collected as precipitate and washed thrice by ethanol and deionised water.

0.10% solution of chitosan was coated on MnO_2 nanoparticles to enhance the magnitude of surface functional groups. 0.1 % of chitosan solution was prepared by adding 0.1 g chitosan powder in 100 ml of 1% (w/v) acetic acid. 5 g of MnO_2 nanoparticles were mixed with 25 ml of 0.10 % chitosan. The mixture was continuously stirred at 2000 rpm for 2 hours at 35°C. Thereafter, CMNPs were centrifuged at 5000 rpm and washed thrice with ethanol and deionized water. Finally, the pellet of CMNPs was dried overnight at 50°C in an oven.

3.2.2.3 Preparation of ChCLP

CLPs were collected in airtight plastic box from a fruit shop situated in the University campus. The procured peels were washed thrice with the deionized water. Thereafter, CLP was sundried (39 ± 2 ºC) for 4 days and then dried at 50ºC for 24 hours in a hot air oven, Equitron Medica Private Limited, Make India. The CLPs were crushed to make powder in a ball mill. The powder was sieved through 45 - 52 mesh sieve (BSS). After size reduction, chitosan was coated on the surface of CLP. 0.5 g of chitosan was added in 500 ml of 1% (w/v) acetic acid and 0.1 % chitosan solution was prepared. 10 g of CLP was added with 60 ml of 0.1 % chitosan solution and incubated at 180 rpm for 120 minutes at 37°C. After incubation, ChCLP was filtered and dried overnight at 50 ºC.

3.3 Characterization of synthesized biosorbents

Surface morphology of biosorbents samples determined by Scanning electron microscope (SEM) (ZEISS MA15/18 make, Germany and Nova Nano SEM 450, FEI Company make, USA). The elemental composition of samples were determined by energy dispersive X-ray analyser (EDAX Inc. make, USA). The functional moieties on the surface were determined by fourier transform infrared spectrophotometer (FTIR) (NICOLET spectrophotometer (iS5) make, USA). The sample were mixed with the photometric grade potassium bromide (KBr) in ratio of 1:3. The FTIR spectra was analysed between 400 - 4000 cm^{-1}. Proximate analysis was conducted according to ASTM Standard D5142 (ASTM, 2009). C, H, N, O and S contents were determined using CHNSO organic elemental analyzer (PerkinElmer make, USA). The X-ray photoelectric spectroscopy (XPS) (Thermo Fisher Scientific make, USA) analysis realised the reduction of chromium and its oxidation states on the biosorbent surface. Experimental procedure mentioned in Mondal and Roy [Mondal and Roy, 2018] was adapted to measure the point of zero charge (pH_{pzc}) on biosorbents. Brunauer–Emmett–Teller (BET) surface area analyser (Microtrac BEL Corporation, Bellsorp Max II & Belcat-II make, Japan) was used to assess the surface area and pore volume of biosorbents. Size of CMNPs was measured at room

temperature in double distilled water and physiological pH using Zetasizer Ver. 7.12, Malvern Instruments Ltd, make United Kingdom. HR-TEM analysis was also used to confirm size, shape and crystallinity of the CMNPs. The sample for HR-TEM was prepared on copper grid. 1 mg CMNPs was added to 10 ml absolute alcohol and the solution was sonicated for proper mixing and particle separation. After sonication, copper grid for TEM analysis was prepared. The surface morphology of adsorbent in three dimension (3D) and surface roughness was analyzed by atomic force microscopy (AFM), NT-MDT Service and Logistics Limited make, Ireland.

3.4 Dimensionless numbers and Artificial neural network (ANN)

The adsorption of metal ions from the bulk to the solid-liquid interface is controlled by bulk diffusion, surface (film diffusion), intra-particle diffusion and rearrangement [Imaga and Abia, 2015]. The rate determination step can be one of the above. Rearrangement of ions is relatively rapid and is not often considered as a rate-controlling step. Dimensionless numbers address the type of diffusion during adsorption. The details of dimensionless numbers are given as follows

3.4.1 Derivation of dimensionless number

Fick's second law of diffusion controls the spherically symmetrical diffusion of ligands (Equation 1).

$$\frac{\partial C}{\partial t} = \frac{D}{r^2}\frac{\partial}{\partial r}\left(s^2\frac{\partial C}{\partial r}\right) \quad (1)$$

where, D is the diffusion coefficient, C is the bulk concentration and r is the spherical radial coordinate.

In addition, the movement of molecules from the subsurface to the surface has been defined. The mass balance at the interface for the i^{th} ligand molecule is

$$\frac{dS_i}{dt} = \emptyset_a^i - \emptyset_d^i \tag{2}$$

where, S_i = i^{th} surface concentration of molecule at ϕ_a and ϕ_d.

ϕ_a. = adsorption flux

ϕ_d = desorption flux

In several physical models, adsorption flux depends on ligand's surface (S) and subsurface (Cs) concentrations, while desorption flux depends only on S [Biswas et al., 2005]. Control Equations have been changed to develop mathematical solutions (Equation 3):

$$C^* = \frac{C}{C_0} \qquad \varsigma = \frac{r}{r_s}, \qquad S^* = \frac{S}{S_\infty}, \qquad \tau = \frac{tD}{k^{2'}}, \tag{3}$$

where, C^* is dimensionless concentration, C is bulk concentration, C_0 is initial bulk concentration, ς is dimensionless distance, r is spherical radial coordinate, r_s is drop radius, S^* is dimensionless surface concentration, S_∞ is maximum surface concentration, τ is dimensionless time and k is ratio of the surface concentration at equilibrium to bulk concentration.

Replacement of Equation 3 by 1 gives Equation 4:

$$\frac{\partial C^*}{\partial t} = \varphi^2 \left[\frac{\partial^2 C^*}{\partial \varsigma^2} + \frac{2}{\varsigma}\frac{C^*}{\partial \varsigma}\right] \tag{4}$$

where, $\varphi = \frac{h}{r_s}$ and, 'h' is the adsorption thickness.

$$\frac{dS^*}{d\tau} = N_k \left[\frac{C_s^*}{S^*}(1 - S^*) - \lambda \frac{S^*}{C_s^*}\left(\frac{1}{1 - S^*}\right)\right] \tag{5}$$

The dimensionless numbers φ, λ and N_k are shown in Equation 6

$$\varphi = \frac{h}{r_s} \quad , \quad \lambda = \frac{h^2}{K} \quad , \quad N_k = \frac{K_a}{DC_0} \tag{6}$$

where, K_a is adsorption rate constant (mol m^{-1} s^{-1}) and,

$$K = \frac{K_a}{K_d}$$

The lower values of φ and λ indicate that the ligand covers the entire adsorbent surface with least surface tension [Biswas et al., 2005].

According to Biswas et al., 2005, molecular dynamics is

i. diffusion-controlled if values of N_k is $10^1 \leq 10^4$

ii. mixed diffusion and transfer controlled if N_k is $10^{-3} \leq 10^1$

iii. transfer controlled for values if N_k is $10^{-4} \leq 10^{-3}$

3.4.2 ANN

ANN is a network capable of predicting an output pattern when relevant input data is given [Yildiz, 2018]. Levenberg-Marquardt algorithm (LMA) is usually used in engineering disciplines as a training algorithm. Generally, this algorithm is preferred due to its ease of use and high training competence. The pH, dosage, temperature, contact time and agitation rate were used as input constraints.

3.5 Adsorption study

3.5.1 Adsorption of Cr (VI) using RH and FeRH

0.25 g of FeRH and RH were separately added to 50 mL Cr (VI) solutions having 50 mg/L in a 250 mL conical flask. The effect of the initial metal ion concentration (50 - 250 mg/L), biosorbent dose (0.50 – 2.5 g/L), pH (2 - 10), temperature (20 - 50°C), contact time (60 - 360 min) and agitation speed (120 - 280 rpm) were evaluated. The mixture was equilibrated for 4 hours at 150 rpm using orbital shaker (REMI CIS-18 PLUS make, India). After equilibration, the mixture was filtered and Cr (VI) in filtrate was determined.

3.5.2 Adsorption of Cr (VI), Cd (II) and Pb (II) using CMNPs

Biosorption of Cr (VI), Cd (II) and Pb (II) was performed in the 250 mL conical flask with 100 mg/L metal ions in 50 mL working solution. The pH was maintained at 2 for Cr (VI), and 6 for Cd (II) and Pb (II). The effect of the initial metal ion concentration (50 - 200 mg L^{-1}), CMNP dose (0.5 – 2.5 g L^{-1}), pH (2 - 10), temperature (20 - 50°C), contact time (60 - 300

min) and agitation speed (120 - 240 rpm) were evaluated. pH was adjusted by adding 0.1 M of HCl and NaOH. The samples were withdrawn from batch reactor at intermittent time interval and centrifuged at 5000 rpm for 15 minutes. Ternary metal ion complex was also studied under similar experimental conditions.

3.5.3 Adsorption of Cr (VI), Cd (II) and Pb (II) using ChCLP

The effect of parameters like pH (1-10), temperature (20-50°C), initial metal ions concentration (50-200 mg/L), agitation rate 120-240 rpm, time interval between 60-240 minutes and ChCLP dose (1 -10 g/L) were studied in terms of percentage removal of Cr (VI), Cd (II) and Pb (II) in the single and ternary metal ion system. 50 ml working solution of desired strength was added to 0.5 g amount of adsorbent at 150 rpm agitation rate and 40°C temperature in a conical flask of 250 ml capacity. The solution pH was regulated by adding 0.1M HCl and NaOH.

3.5.4 Determination percentage removal and adsorption capacity

The percentage removal of heavy metal ions and uptake capacity (q_e) of FeRH, CMNPs and ChCLP were calculated by Equations 7 and 8.

$$\text{Removal (\%)} = \frac{C_i - C_e}{C_0} \times 100 \qquad (7)$$

$$q_e = \frac{(C_i - C_e) \times V}{W} \qquad (8)$$

where, C_i and C_e are the initial and equilibrium concentration of heavy metals (mg/L), V is the volume of metal ion solution (L) and W is the weight of biosorbent (g). All experiments were carried out thrice. All the parameters were kept constant unless stated. The concentration of Cr (VI), Cd (II) and Pb (II) in the water was measured by 1, 5-Diphenylcarbazide (DPC) method

(only for Cr (VI)) and the Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES), Perkin Elmer, Optima 7000 DV instrument make, USA.

3.6.0 Isotherms, Kinetics, Thermodynamics and Mechanistic study

3.6.1 Isotherm models

Langmuir, Freundlich, Temkin, Dubinin-Radushkevich (D-R), Halsey, Harkin-Jura (H-J), Jovanovic and Redlich-Peterson (R-P) isotherms are studied to determine the goodness of fit of the data.

Langmuir isotherm

Langmuir isotherm assumes the monolayer coverage and homogenous adsorption of metal ions. The mathematical expression is shown in Equation 9.

$$\frac{C_e}{q_e} = \frac{1}{Q^0 b} + \frac{C_e}{Q^0} \qquad (9)$$

where, Q^0(mg/g) is maximum uptake capacity and b (L/mg) is isotherm constant which can be calculated from the slope and intercept of the plot between C_e/q_e *vs* C_e.

Freundlich isotherm

Freundlich isotherm assumes that adsorption of heavy metal ions occurs on heterogeneous surface. The linear form of Freundlich isotherm is given in Equation 10.

$$\log q_e = \log k_f + \frac{1}{n}\log C_e \qquad (10)$$

where, k_f (mg/g) and n are the isotherm constants and calculated from intercept and slope of the plot between $log\ C_e$ *vs*. $log\ q_e$.

Temkin isotherm

Temkin isotherm is related to the heat of adsorption of all molecules in the layer that decreases linearly rather than logarithmically with the increase in uptake capacity. The Temkin isotherm is shown in Equation 11.

$$q_e = \frac{RT}{b_T}\ln A_T + \frac{RT}{b_T}\ln C_e \qquad (11)$$

where, R is the universal gas constant (8.341 J mol^{-1} K^{-1}), T is temperature (K), b_T and A_T are Temkin isotherm constant, A_T is related to heat adsorption (L/g) and b_T is related to the maximum binding energy (kJ/mol). A_T and b_T were calculated from the intercept and slope of between q_e *vs* ln C_e plots.

Dubinin-Radushkevich (D-R) isotherm

D-R isotherm was studied to determine the nature of binding between metal ions and adsorbent. The linear form of D-R isotherm is shown in Equation 12.

$$\ln q_e = \ln Q_{D\text{-}R} - \beta \varepsilon^2 \quad (12)$$

where, $Q_{D\text{-}R}$ (mol/g) and β (mol2 kJ-2) are the D-R constants, calculated from the intercept and slope of the plot between ln qe and ε^2. Here, ε is polanyi potential and is calculated from Equation 13.

$$\varepsilon^2 = RT \ln\left(1 + \frac{1}{C_e}\right) \quad (13)$$

where, R is universal gas constant (8.341 J mol^{-1} K^{-1}) and T is the temperature (K). The free energy of adsorption is related to D-R isotherm constant. The free energy denotes the adsorption energy of one mole of adsorbate. It can be calculated from Equation 14.

$$E = \frac{1}{\sqrt{-2\beta}} \quad (14)$$

where, E (kJ/mol) is the free energy which denotes whether the adsorption system is physical or chemical.

Halsey isotherm

Halsey isotherm defines multilayer adsorption at a relatively larger distance from the adsorbent surface. Halsey isotherm is shown in Equation 15.

$$q_e = \frac{1}{n_H} I_n K_H - \frac{1}{n_H} \ln C_{qe} \quad (15)$$

where, K_H and n_H are the Halsey isotherm constants. K_H and n_H can be obtained from slope and intercept of the plot between lnq_e and lnC_e.

Harkin-Jura (H-J) isotherm

H-J isotherm (Equation 16) represents the possibility of multilayer adsorption on the adsorbent surface.

$$\frac{1}{q_e^2} = \frac{B}{A} - \left(\frac{1}{A}\right) logC_e \quad (16)$$

where, B and A are the model constants. B and A can be calculated from the slope and intercept of plot between $\frac{1}{q_e^2}$ versus $log\ C_e$.

Jovanovic isotherm

Jovanovic isotherm assumes mechanical contact between adsorbate and adsorbent. The model equation is shown in Equation 17.

$$\ln q_e = \ln q_{max} - \mathrm{k_j C_e} \quad (17)$$

where, q_e is the adsorption capacity at equilibrium (mg/g) and q_{max} is the maximum adsorption capacity (mg/g) of adsorbent that can be calculated from the plot between ln q_e versus C_e.

Redlich-Peterson (R-P) isotherm

R-P isotherm (Equation 18) is a combination of Langmuir and Freundlich isotherm that does not follow monolayer adsorption.

$$\ln \frac{C_e}{q_e} = \beta \ln C_e - \ln A \quad (18)$$

where, A (Lg^{-1}) and B (Lmg^{-1}) are the R-P isotherm constant and β is model exponent (0 to 1). C_e and q_e are the metal concentration in solution at equilibrium (mg/L) and adsorption capacity at equilibrium (mg/g), respectively.

3.6.2 Thermodynamic

The change in Gibbs free energy (ΔG^o), enthalpy (ΔH^o) and entropy (ΔS^o) were studied to explore the nature and thermodynamic feasibility of adsorption. Thermodynamic parameters were calculated by using Equations 19, 20 and 21.

$$\Delta G^o = -RT \ln k_c \quad (19)$$

$$k_c = \frac{C_{Ae}}{C_e} \quad (20)$$

$$\ln k_c = \frac{\Delta S^o}{R} - \frac{\Delta H^o}{RT} \quad (21)$$

where, C_{ae} (mg/L) is the equilibrium concentration, C_e (mg/L) denotes equilibrium metal ion concentration in the bulk solution, T is the reaction temperature (K) and R is the universal constant (8.314 J mol^{-1} K^{-1}). The value of ΔS^o and ΔH^o were determined using intercept and slope of the plot between ln k_c versus 1/*T*.

3.6.3 Kinetics

Study of adsorption kinetics is helpful in understanding the mechanism of adsorption of metal ions onto the surface of adsorbent. It is highly dependent on physical and chemical nature of adsorbent and useful in design and scale-up of the wastewater treatment systems [Torrik et al., 2019]. In the present study, pseudo-first order (PFO), pseudo-second order (PSO) and Elovich kinetic model was tested to predict the adsorption kinetics of heavy metal ions on the surface of biosorbent.

PFO kinetics

The PFO model considers the reversible attachment of ligand with adsorbent surface. The linear expression of PFO is shown in Equation 22.

$$log(q_e - q_t) = \log (q_e) - \frac{k_s}{2.303} t \quad (22)$$

where, k_s is the equilibrium rate constant and calculated from the slope $log(q_e - q_t)$ vs time (t). The q_t and q_e are the adsorption capacities (mg/g) at time *t* and equilibrium, respectively.

PSO kinetics

It assumes surface sorption as the rate-limiting step that incorporates chemisorption in the adsorption. The mathematical expression of PSO model is shown in Equation 23.

$$\frac{t}{q_t} = \frac{1}{k'_2 q_e} + \frac{1}{q_e}\mathrm{t} \qquad (23)$$

where, k'_2 is the equilibrium constant

$$h = k'_2 q_e^2 \qquad (24)$$

Here, k'_2 and h are constants that can be calculated from the plot between t/q_t *vs* t.

Elovich model

This model assumes that solid surfaces of adsorbent is energetically heterogeneous. This model also explains the rate of chemisorption on the energetically heterogeneous adsorbent surface. Elovich's kinetic model is shown in Equation 25.

$$q_t = \frac{1}{\beta}\ln(\alpha\beta) + \frac{1}{\beta}\ln t \qquad (25)$$

where, α (μg/g/min) is the initial adsorption rate and β (g/μg) indicates the extent of surface coverage and activation energy for chemisorption. The value of α and β can be calculated from the slope and intercept of the plot between q_t and ln (t).

3.6.4 Mechanistic study

The transfer of metal ions to and from the surface of adsorbent is a complex phenomenon. Liquid -Film diffusion (LFD), intraparticle diffusion (IPD) and rearrangement are involved in adsorption across liquid phase. The rearrangement of ions on the surface of adsorbent is considered as a rapid process and therefore, it is not a rate limiting step [Chaudhry et al., 2016].

IPD

During adsorption, there is a possibility of diffusion of metal ions into the pores or intraparticle space of adsorbent which may be a rate-controlling step. The mathematical expression of IPD is shown in Equation 26.

$$q_t = k_{id} t^{0.5} + C \quad (26)$$

where, k_{id} is the intraparticle diffusion rate constant (μg/g/min$^{1/2}$) which can be calculated from the slope of q_t versus $t^{0.5}$and C is intercept of plot.

LFD

This model deals with the interaction of solid-liquid interface with metal ions during adsorption. If LFD is rate controlling step, then the rate depends on the thickness of the liquid film around adsorbent. Equation 27 depicts LFD model

$$\ln (1 - F) = -k_{fd} + C \quad (27)$$

where, k_{fd} is film diffusion rate constant and calculated from the slope of plot between ln (1 – F) vs. t. F is the fractional attainment of the equilibrium (Equation 28).

$$F = \frac{q_t}{q_e} \quad (28)$$

Bangham's model

Possibilities of pore diffusion are explored through Bangham's model (Equation 29).

$$Log\ log\left(\frac{C_o}{C_o - q_t M}\right) = log\left(\frac{K_j M}{2.303V}\right) + \alpha \log t \quad (29)$$

where, C_o is the initial metal concentration in the bulk phase (mg/L), V is the volume solution (L), M is the mass of the adsorbent (g/L), q_t is the amount of heavy metal ion adsorbed on the adsorbent at time t (min), α and K_j are Bangham's constants and can be calculated from the slope and intercept of plot between $\mathrm{Log\ log}(C_o/C_o - q_t M)$ versus log (t).

The film diffusion coefficient (D_f) and the particle diffusion coefficient (D_p) were calculated to reassure the rate limiting step. The D_f and D_p were calculated from the Equation 30 and 31 [Mishra et al., 2012].

$$D_f = 0.23\left[\frac{R_p \varepsilon}{t^{1/2}}\right]\left[\frac{q_e}{C_0}\right] \quad (30)$$

$$D_p = 0.03\left[\frac{R_p^2}{t^{1/2}}\right] \quad (31)$$

where, R_p is the radius of the adsorbent particle (0.023 cm), ε is the thickness of the liquid film = 10^{-3} cm, q_e is the metal uptake capacity (mg/g), C_0 is the initial metal ions concentration (mg/L) and $t^{1/2}$ is related to the time for adsorption of heavy metals on the surface of biosorbent.

If adsorption is film diffusion limited, then D_f exists between 10^{-6} to 10^{-8} cm^2 s^{-1} [Mishra et al., 2012]. On the other hand, if particle diffusion is rate controlling step then D_p lies between 10^{-11} to 10^{-13} cm^2 s^{-1} [Mishra et al., 2012].

3.7.0 Desorption of heavy metal ions: regeneration of biosorbent

Desorption of the heavy metal ions from the biosorbent was studied by solvent elution method. Desorption experiment was performed using 0.10 M HNO_3 as stripping agent. 0.10 g of metal ions loaded dried biosorbent was mixed with the 50 ml 0.10 M HNO_3 and incubated for 1 hour at room temperature. After incubation, the solution was filtered and metal ions concentration was measured. The % desorption was calculated by Equation 32

$$\%\ \text{Desorption} = \frac{100[C_D V_D]}{q_{em}} \quad (32)$$

where, C_D is metal ion concentration (mg/L) in the desorbed solution, V_D is desorbed solution volume in Litre (L), m is mass (g) of biosorbent and q_e (mg/g) is biosorption capacity. The five cycle of adsorption-desorption was performed to estimate reusability of the biosorbent. At the end of each cycle, biosorbent were washed with double distilled water and dried in oven at 105 ºC.

3.8 Continuous column study

The continuous flow operation experiments for removal of Cr (VI), Cd (II) and Pb (II) were carried out in a cylindrical glass column (2 cm internal diameter and 60 cm height). The adsorbent was filled in the column up to desired bed height (45 cm). The solution of Cr (VI), Cd (II) and Pb (II) of 100 mg/L concentration was pumped into the column by peristaltic pump at various flow rates 10, 20 and 30 ml/min. The treated sample was collected from the exit of the column at different intervals of time until the equilibrium was achieved and remaining concentrations of Cr (VI), Cd (II) and Pb (II) was determined.

3.8.1 Parameters and design of packed bed column

The 25 g of adsorbent was added in the column up to bed height 40 cm.

- The internal diameter of the column: 2 cm.
- Height of the column: 60 cm.
- Bed height: 45 cm.
- Adsorbent weight: 15 g.
- Initial metal ion concentration: 100 mg/L.
- Effect of flow rate: 10, 20 and 30 ml/min
- Temperature: 25 – 30 ℃.
- pH of the heavy metal solution: 6

The continuous column unit is shown in Figure 3.1

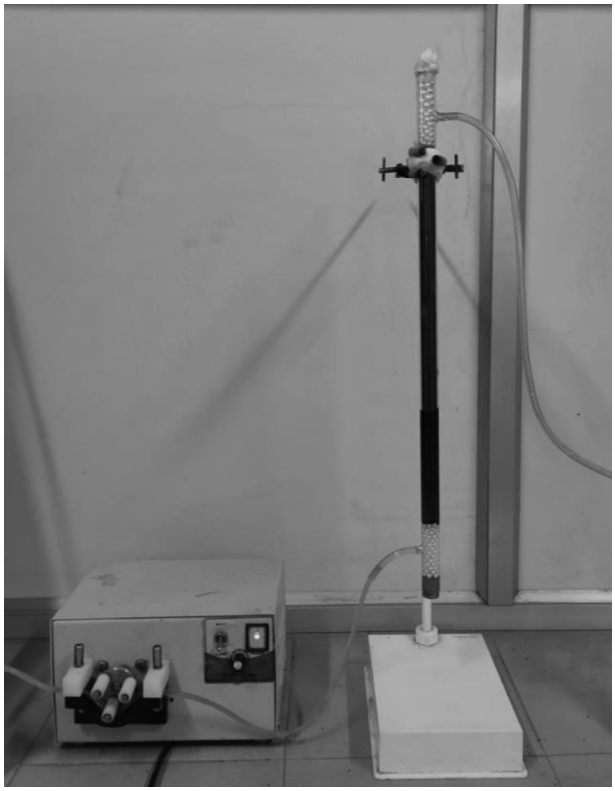

Figure 3.1 Continuous fix bed column

3.9.0 Isolation of new bacterial strain from the site contaminated with coal mine effluents: characterization and heavy metal removal

3.9.1 Collection and physicochemical characterization of wastewater

Wastewater was collected from the Baliya Nala (a drain), Singrauli, Madhya Pradesh, India. This drain is an open source of wastewater, and several coal mining units are situated in Singrauli discharge their effluents after treatment into this drain. The wastewater was collected in May, 2019. The atmospheric temperature was approximately 39.33 ºC. The wastewater was collected in the sterile amber coated screw capped sample bottles, kept between ice bags and finally stored at 4ºC in dark till further use.

The physico-chemical parameters such as pH, temperature and suspended solids, biological oxygen demand (BOD), phosphate, nitrate and ammonia concentration in wastewater were analyzed as per the American Public Health Association (APHA) guidelines.

3.9.2 Isolation of heavy metal tolerant bacteria

The heavy metal tolerant bacterial species was isolated from wastewater by the pour plating method. 1 ml of wastewater was mixed with normal saline and serially diluted up to 10^{-10}. The desired volume from each dilution was poured on Luria-Bertani (LB) agar plates containing Cr (VI), Cd (II) and Pb (II). Further, LB agar plates were incubated for 24 hours at 37ºC. After 24 hours of incubation, three morphologically distinct colonies out of all those were carefully chosen. These isolated colonies were grown in the LB broth containing Cr (VI), Cd (II) and Pb (II) in the range of 50 to 200 mg/l and one of the highly heavy metal resistant colonies was selected.

3.9.3 Genomic DNA isolation

The genomic DNA of bacterial strain was isolated by using the method described in Minas et al., 2011 and Manta et al., 2020. Bacterial culture was picked up and homogenized in 1 ml of extraction buffer. The fixed ratio (25:24:1) of phenol, chloroform and isoamyl alcohol was added in the supernatant and mixed properly. The mixture was centrifuged at 14,000 rpm for 15 minutes. After centrifugation, aqueous and organic phase appeared in the centrifuge tube. Aqueous phase was transformed in a new centrifuge tube and an equal volume of chloroform-isoamly alcohol (24:1) was added and centrifuged at 14,000 rpm for 10 minutes. The supernatant was collected and 0.1 volume of 3M sodium acetate and 0.7 volume isopropanol was added in the supernatant and incubated at room temperature for 15 minutes. After incubation, mixture was centrifuged at 4 ºC for 15 minutes at 14,000 rpm. Supernatant was discarded and pellet was washed with chilled 70% ethanol and dried in air. The DNA in the form of pellet was dissolved in nuclease free Milli-Q water or TE buffer (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). The RNA contamination was removed by using 5 µl RNase A (10 mg/ml).

3.9.4 16S rRNA gene sequencing and molecular identification

The 16S rRNA gene of the bacterial isolate was amplified for molecular identification through polymerase chain reaction (PCR). 66.4 ng of extracted bacterial DNA was used in the PCR reaction and 10 pM of each primer. Forward primer (63F) 5'-GGATGAGCCCGCGGCCTA-3' and reverse primer (1387R) 5'-CGGTGTGTACAAGGCCCGG-3' were used for 16S rRNA gene amplification. The amplified PCR product was analysed through agarose gel electrophoresis at 70 V for 2 hours.

The amplified 16S rRNA gene product was purified from gel and sequenced with the same primer, which was used for PCR reaction. The obtained nucleotide sequence was analyzed via Basic Local Alignment Search Tool (BLAST), NCBI. BLAST sequence search was based on the maximum sequence similarity with other bacterial species. The cut off value (E value) for the blast result was 0.0. The phylogenetic tree was constructed for the bacterial isolate with the reference sequence available in the GenBank, NCBI, USA. ClustarW alignment tool was used for the nucleotide sequence alignment. The phylogenetic tree was created in the MEGA X software by using the Maximum Likelihood method and Tamura-Nei model. The 16S rRNA gene sequence of the bacterial isolate was submitted to the GenBank, NCBI and accession number was obtained.

3.9.5 Effect of various parameters on the microbial growth

The bacterial cells were grown in 50 ml LB broth in 250 ml flask in the control. The effect of temperature on bacterial growth was analyzed at temperatures ranging from 25 to 50ºC for 24 hours. The effect of pH on bacterial growth was observed at pH 4-10 at 37 ºC for 24 hours. The bacterial growth in the culture medium was estimated by measuring optical density at 600 nm by using UV-Visible spectrophotometer. The microbial viability was also estimated in terms of colony forming units (CFUs/ ml). The bacterial culture was grown (for

CFU calculation) in control (0 mg/L heavy metal), 100 mg/L and 200 mg/L heavy metal concentration and spread on the LB agar plate. The bacterial colonies grown were counted and CFUs/ml was calculated [Lima et al., 2012].

3.9.6 Analysis of antioxidants activity in heavy metal exposed bacterial isolate

The activity of antioxidant enzymes in heavy metal exposed bacterial isolate and in control was observed. Bacterial isolate was grown in the 500 ml conical flask containing 200 ml LB broth spiked with 100 mg/L heavy metals and incubated in a rotatory shaker-cum-incubator at 37ºC for 48 hours. The bacterial cells pellet was obtained by using centrifugation at 10,000 rpm for 10 minutes. The pellet was gently dissolved in phosphate buffer through sonication. The sonicated bacterial pellet was used for the analysis of antioxidants. The activity of glutathione S-transferase (GST) was evaluated by using the protocol given in Habig et al., 1974. The activities of the superoxide dismutase (SOD) and catalase were measured by protocol described in BeersJr and Sizer, 1952 and Ewing and Janero, 1995, respectively. Peroxidase (POX) activity was analyzed by the protocol of Reuveni et al., 1992.

3.9.7 Analysis of heavy metal removal

The bacterial cells was inoculated in the LB broth containing Cr (VI), Cd (II) and Pb (II) of single and ternary metal ion system in the range of 50 to 200 mg/L and incubated at 37ºC for 1 to 5 days. The sampling was done at 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} day and bacterial cells were separated by centrifugation at 10,000 rpm for 10 minutes. The residual heavy metal concentration in the supernatant was measured by ICP-OES. The heavy metals removal from aqueous medium was estimated by using equation 7.

3.9.8 SEM and EDX analysis of bacterial isolate

Surface morphology and elemental composition of the bacterial isolate was analysed through SEM-EDX (ZEISS EVO, Carl Zeiss Microscopy make, Germany). The standard

protocol was used for the preparation of the sample [Juma et al. 2020]. The images were taken at 20 keV.

3.10.0 Bioremediation of toxic metal ions from coal washery effluent (CWE) by *Pleurotus florida*

3.10.1 Collection and characterization of CWE

Wastewater sample was collected from the effluent treatment plant of Northern Coalfields Limited (NCL), Kakri Project, Sonbhadra district, Uttar Pradesh, India. The CWE was collected in the month of December, 2018. The temperature of the effluent was around 20°C. The color of the CWE was black and samples were collected in the dark amber coated bottles. The wastewater sample was stored as per guidelines of APHA, USA.

Physiochemical characterization and heavy metal analysis of CWE was carried out as per APHA standard methods. pH, electrical conductivity and turbidity were measured by digital pH-cum-conductivity meter and nephelometer (Thermo Scientific make, Singapore). Total solids, dissolved solids and suspended solids were analyzed by hot air oven (Equitron Medica Private Limited make, India) and furnace (Narang Scientific Work Pvt. Limited make, India) at various temperatures. Biological oxygen demand (BOD_5) and dissolved oxygen were also determined using standard APHA method. Phosphate, ammonia and nitrate concentration was determined using standard methods of APHA, USA. The concentration of heavy metal was determined by ICP-OES, Perkin Elmer, Optima 7000 DV instrument make, USA.

3.10.2 Collection and preparation of the substrate for mushroom cultivation

Paddy straw waste was purchased from the local market, near Sheer gate of Banaras Hindu University (BHU), Varanasi, India. The paddy straw was chopped into two to three inches size. The chopped paddy straw was soaked into hot water and left for one hour for sterilization. After sterilization, the excess water was removed from paddy straw.

3.10.3 Experimental design for bioremediation of CWE

3.10.3.1 Spawn preparation

Spawns were prepared in the glass bottles. The wheat grains were boiled for 35 minutes in the glass beaker of 2 L capacity. After boiling the excess water was discarded through filtration. Thereafter, 2% of calcium carbonate and 0.5% of calcium sulphate powder (on the dry weight basis) were mixed with the boiled wheat grains. The wheat grains were transferred to the glass bottles and autoclaved for 15 min at 121°C. The autoclaved grains were allowed to cool at room temperature and were inoculated with the active mycelium of *Pleurotus florida* grown on from potato dextrose agar (PDA) plate inside the laminar flow unit. Inoculated wheat grains were incubated at 27 ± 2 ºC in dark place for 15 days until the proper mycelium growth covered the grains. These mycelial covered grains were called as spawn and were used as inoculum for bioremediation.

3.10.3.2 Bioremediation of CWE

The range of process parameters studied in present work was as follows: concentration of CWE: 0% to 100% (intermittent interval of 25% each) and growth period: 20 days to 40 days (intermittent interval of 5 days each). Other than these, other parameters such as temperature (27 ± 2ºC), pH of CWE (7.5 ± 0.2) and dosage of spawn (30 mg g^{-1}) were kept constant throughout the study.

The CWE was filtered using Whatman filter paper No. 42 and also autoclaved to remove the microbial and other solid particle contamination. The various concentrations (100% CWE, 75% CWE, 50% CWE, 25% CWE and 0% CWE) of CWE were prepared in double distilled water. The 0% CWE contained 100% double distilled water and was taken as control. All dilutions were prepared in triplet for further experiments. Sterilized paddy straw was dipped into the sterile CWE and double distilled water (control) in the separate beakers for 24 hours.

After CWE treatment, the excess water was removed from the paddy straw and the straw was packed in polyethylene bags. Each polyethylene bag contained 500 grams of paddy

straw. 30 grams of spawn was added to every bag. Finally, the inoculated bags were stored at optimized conditions (27 ± 2ºC) in the sterile box made up of perspex sheet (2.5 m × 1.5 m × 0.5 m; thickness of sheet is 10 mm) and was incubated at optimum growth conditions (temperature (27 ± 2 °C), pH of CWE (7.5 ± 0.2) and dosage of spawn (30 mg g^{-1}). The lid of the box was movable (sliding) and to maintain the moisture content during the growth of *P. florida* over paddy straw the lid was removed every alternate day and the double distilled water was sprayed on the surface of polyethylene bags. After spraying the lid was again closed. The box was kept in dark in air-conditioned atmosphere.

The growth of *P. florida* was observed till the end of 40th day and samples of fruits bodies were collected at 20th, 25th, 30th, 35th and 40th day from every dilution of CWE including control. The paddy straw substrate samples were also collected at zero days and after bioremediation. No growth was recorded in the beginning of 41th day. However, degradation of the cell mass was observed from 40th day onwards. The samples of paddy straw and fruit body were collected till the end of 40th day and samples were stored at -20°C.

3.10.4 Analysis of metallothionein concentration in *P. florida* fruit body grown in CWE containing substrate

Concentration of metallothionein in the fruit bodies was analyzed using standard method of Griffith, 1980. 600 mg of mushroom fruit bodies were homogenized in sulphosalicylic acid. The homogenized samples were pellet down at 12,000 × g for 20 minutes at 4°C and supernatants were recovered. Two separate solutions were prepared (solution A and B), the compositions of solution A was 100 mM sodium monohydrogen phosphate heptahydrate, 15 mM Ethylenediaminetetraacetic acid (EDTA), 1.8 mM 5, 5-dithiobis (2-nitrobenzoic acid) and 0.04 % bovine serum albumin and solution B of 50 mM imidazole, 1 mM EDTA, 0.2 % (w/v). The pH of both solutions was adjusted to 7.2. The metallothionein

fraction in the supernatant was determined by 800 µl solution A, 640 µl of solution B and 800 µl supernatant. The concentration of metallothionein in solution was determined at 412 nm.

3.10.5 Analysis of antioxidant enzymatic system in *P. florida* grown in CWE containing substrate

Antioxidant enzymes such as GSH, SOD, lipid peroxidase and catalase were analyzed using standard methods described by Moron et al. 1979; Das et al. 2000; Ohkawa et al. 1979; and Aebi 1984. The fruit bodies of mushroom were homogenized in chilled 0.1M phosphate saline buffer (PBS) using mortar pistil. The homogenized samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant was again centrifuged at 10,000 rpm for 10 minutes and supernatant obtained was stored at -80°C.

GSH was estimated using the method described by Moron et al. 1979. The GSH was allowed to react with 5, 5-dithiobios nitro benzoic acid which generates yellow colored compound. The absorbance was measured at 412 nm. The SOD activity was measured using the standard method of Das et al. 2000. The Lipid peroxidase was measured by the method described by Ohkawa et al. 1979. The catalase activity was estimated using the method suggested by Aebi 1984. All antioxidant enzymes were measured in the *P. florida* fruit bodies cultivated in CWE containing medium and also in control. The analysis was performed in mushroom samples collected from 0%, 25%, 50%, 75% and 100% CWE containing medium from 20^{th} to 40^{th} days.

3.10.6 Growth modelling of *P. florida* in CWE

Autoclaved potato dextrose agar (PDA) media containing CWE in various concentrations (0%, 25%, 50% and 100% of CWE) was dispensed into petri plates of size 9 cm. Approximately, 35-40 ml of sterile PDA was dispensed in each plate. The spores of *P. florida* were inoculated in the centre of plates. The lids of plate were closed and sealed with parafilm tapes and the lids were stroked with radii which crossed the inoculum site (ten radii

were marked). To prevent desiccation of plates, routinely plates were packed in polyethylene bags and incubated at 28°C in dark in a BOD incubator (REMI Laboratory Instruments make, India). The mycelia growth of *P. florida* was marked under compound microscope (10 x) with a marker at every 1 to 2 days. At the completion of growth, the marks were measured from the point of inoculation with a ruler having a precision of 1 mm. The peripheral growth (P_l; in mm) was measured according to Trinci, 1974.

Growth kinetics calculation of *P. florida*: growth fitness functions shown in Equation 33 and 34 has been used in the present work to elucidate the growth kinetics of *P. florida* [Prosser, 1995].

$$\beta = \beta_{0.} \exp^{\alpha.t} \quad (33)$$

$$\beta = \beta_0 + K_r.t \quad (34)$$

where, α and K_r are growth rates expressed as growth rate (d^{-1}) and growth per day (mm d^{-1}), respectively. β and β_0 represent the growth at time t and time t = 0. For growth kinetics integration, the real values of t (at β) were substituted in linear functions rather than intercepts (t at β_0). The weight of these growth fitness functions was determined by comparing the experimental value of β (at time t) with the theoretical value which was calculated by fitting of data approximately closure to the value of β.

3.10.7 FTIR and SEM analysis of *P. florida* fruit body

The FTIR spectra of both mushroom samples *P. florida* exposed CWE and *P. florida* grown in the distilled water were analyzed. The samples were prepared using standard method suggested by of Xu et al., 2012 and Das and Guha, 2009. The sample of mushroom fruit bodies was mixed with the photometric grade KBr in ratio of 1:3. The spectra were analyzed between the range of 400-4000 cm^{-1} (wave number). The FTIR spectra were analyzed using JASCO-6300 FTIR instrument make, Japan.

SEM (ZEISS EVO, Carl Zeiss Microscopy make, Germany) was performed to study the surface morphology of *P. florida* mushroom. The gold layer was coated on the sample. The gold coated sample was placed in the SEM equipment chamber and images were taken at voltage of 20 keV. EDX was used for the elemental analysis and this technique worked along with SEM analysis. The energy of electron beam was kept in the range between 10-20 keV.

3.10.8 Sample preparation for heavy metal analysis

Samples of paddy straw and fruit body were dried at 120°C in hot air oven. Dried samples were powdered and one gram of each sample was digested with diacid solution of nitric acid (HNO_3) and perchloric acid ($HClO_4$). The digestion process was also performed on hot plate until complete solubility of sample was attained. After the acid digestion, the samples were diluted with doubled distilled water and heavy metal analysis was performed by ICP-OES.

The percentage of heavy metal removal from paddy straw substrate was calculated using equation (32).

3.10.9 Statistical data analysis

All experiments were carried out in triplicate (n=3) and the average values of the experimental data were used to plot the graphs. The experimental errors (± standard deviation) were also calculated and are shown as error bars in graphs.

Model errors were calculated by using error functions like sum of square error (SSE) and chi square (χ^2) are used (Equation 35 and 36)

$$SSE = \sum_{i=1}^{n} (qe.calc - qe.\exp)^2 \quad (35)$$

$$\chi^2 = \sum_{i=1}^{n} \left(\frac{qe.calc - qe.\exp}{qe.\exp}\right)^2 \quad (36)$$

where, qe. *calc* and *qe. exp* represent the calculated and experimental uptake capacities, respectively.