Chapter 4

4. Materials and Methods

4.1. Selection of study sites

The Subarnarekha River originates at Piska/Nagri village (23°18'N 85°11'E), 15 km away from Ranchi, Jharkhand and travels a long distance of 395 km through three important states of India viz. Jharkhand, West Bengal and Odisha before meeting Bay of Bengal near Talsari (21°33'1"N 87°23'31"E). The upstream of river bank harbours some mineral deposits and along with the river basin a few numbers of industries have been established. Several numbers of heavy metals like Fe, Cu, U, Cr, Al, Pb, Cd, and Hg etc. are discharged in river basin through industrial waste materials such as limestone, building stone, asbestos and dolomite (Wolkersdorfer and Bowell, 2005 and Giri et al., 2013). Along the Subarnarekha river bank major industrial areas such as Ranchi-Hatia industrial hub, Alumina processing industries at Muri, Jaduguda-Ghatshila, Industrial and mining area, Steel Authority of India (SAIL), Hindustan copper limited, Tata steel, TELCO, Uranium Corporation of India, Usha Martin industries and various number of medium and small industries are developed (Chakraborty et al., 2013). The water quality of river are very much influenced by the industrial effluents as well as sewage and agricultural runoff which are released into the riverian flow (Pakhira, 2019).

Subarnarekha river from an ecological point of view is one of the most important rivers in India that traverse within three different states viz, Jharkhand, Odisha and West Bengal. However, river passes from Dhalbhum district the state of Jharkhand, India through the south-west point of the Midnapore (West) district of West Bengal before entering into Odisha (Gupta, 2018). The river receives discharge as drainage water from industrial and agricultural areas which include heavy metals, solid & liquid waste, pesticide etc. Most of the stretchers of river up to the end of Midnapore (west) district of the state of West Bengal are fresh down while the end part passing through Odisha experience saline influence. The water in the river is mainly fresh water, but

increases salinity towards the downstream (Talsari). In order to assess the anthropogenic influences on the water quality three sampling sites were selected along the environmental flow of the river.

1. Study Site-1, Muri (S-I) (23^{o} 22'12" N, 85^{o} 51'36"E) at Purulia district, West Bengal, India is situated at upstream in close area of the very nearby discharge of industrial waste and also partly exposed to drainage of copper mines at Ghatshila.

2. The study Site -II, Sonakonia (S-II) (21^{o}86'25" N, 87^{o}25'51"E) at Midnapore (west) district, West Bengal, India experience considerable dilution at the middle stream of the river and is nearly free from either pollution or fresh water impact by agriculture and sand lifting.

3. The Site -III, Talsari (S-III) (21^{o}86'25" N, 87^{o}25'51"E) at the junction of Midnapore (East) district, West Bengal and Odisha positioned at the confluence of the Bay of Bengal with Subarnarekha estuary is also under risk of heavy metals discharge mostly out of ecotourism.

4.2. Collection of water samples

Field observation data in regular interval mode were accumulated by cluster wise during the period of July 2012 to June 2014 from three different eco-contrasting sites of the Subarnarekha river (Figure 4.1). Water samples were collected through ground truth verification by pilot survey and application of Global Positing System (GPS). For more potential assessment, the Subarnarekha riverian study site was divided into three (3) environmentally dissimilar major zones/cluster which are ecological differing sites namely Muri, Sonakonia, and Talsari. These are selected on the basis of water availability, source of pollution and fungal diversity.

The water samples of the river were collected in airtight sterilized containers from July, 2012-

June, 2014 from various sampling positions situated at the upstream (S-I), middle stretch (S-II), and downstream (S-III) on a regular basis of the different season (pre monsoon, monsoon, post-monsoon) as per standard sampling methods (Bhatt and Srinivasan, 2019; Premlata, 2009; Gupta 1qq11q1et al., 2009 and Patil, 2012). Collected water samples were reserved at airtight glass container in the refrigerator at 4 °C for further analysis.

Figure 4.1: Collection of soil samples from different sampling sites

4.3. Estimation and the recording of water quality parameter

The water quality of the river is monitored through different parameters viz. physico-chemical and biological including heavy metal contents of soil and water.

4.3.1. Physicochemical analysis of river water

During the period of investigation (July, 2012-June, 2014), water samples were collected monthly from different selected study sites in plastic containers. For dissolved oxygen (DO), however, water samples were collected in oxygen fixing bottles and fixed on the spot. At the time of sampling, soil and water temperature were recorded by the field thermometer. All physicochemical parameters of water were determined immediately

upon arrival at the laboratory with the collected samples to prevent the change of chemical composition of water and soil. All analyses were done in triplicate and taken the average value. The techniques useful for the determination of several physicochemical factors of water, during the period of study, are as follows.

- **Water temperature:** Water temperature was recorded with the help of a mercury centigrade thermometer. This was done by vertically dipping the thermometer into water.
- **pH:** pH of water samples were determined with the help of a portable field pH meter (Hanna).
- **Alkalinity:** Alkalinity, pH and hardness affects the toxicity of many substances in the water. Alkalinity or pH stabilizer composed by bicarbonate and carbonate which determined by HCl titration in presence of indicators methyl orange and phenolphthalein (Chichirov, 2019, July).
- **Total Hardness [**mg/l as calcium carbonate ($CaCO_3$**)]**: Total hardness was measured by the titrimetric method using EDTA solution in presence of Eriochrome Black – T as an indicator.
- **Ca-Hardness** [mg/l as calcium carbonate ($CaCO_3$)]: Ca-hardness was estimated by a titrimetric method using ethylene diamine tetraacetic acid (EDTA) solution in presence of Eriochrome Black – T as an indicator.
- **Mg-Hardness** [mg/l as calcium carbonate ($CaCO_3$)]: Mg-hardness was calculated by total hardness - Ca-hardness × 0.244.
- **Biochemical oxygen demand (BOD):** BOD is the measurement of dissolved oxygen (DO) required for the biochemical decomposition of organic compounds and the oxidation of certain organic materials (e.g., iron, sulphites) for domestic and industrial wastes (Pakhira, 2019), which specified by mg/l.

Typically the test for BOD is conducted over a five days dark incubation the period at 37°C. According to WHO, the BOD of drinking water is <6 mg/ l (Razif and Persada, 2015).

- **Chemical oxygen demand (COD)**: COD is the amount of dissolved oxygen required to cause chemical oxidation of the organic and inorganic material in water, which indicated by mg/l. For the determination of water health, BOD and COD are used as key indicators (Kim et al., 2017).
- **Dissolved oxygen** (**DO):** Dissolved oxygen was measured by using a modified Winkler's method (APHA, 1985).
- **Total dissolved solid (TDS):** TDS means dissolved solid (metal, salt, minerals and ion) in surface and groundwater which can hamper the function of the central nervous system of humans and also suffer from dizziness, paralysis. Few amounts of organic chemicals can change the odors, tastes and colors of aquatic plants (Crittenden, 2012). The method of measuring TDS is to be evaporated water samples and weigh the remains with a precision analytical balance.
- **Nitrate-Nitrogen (NO3-N):** During agricultural activities, nitrate concentration was increased in surface and groundwater (Savci, 2012). The amount of Nitrate-Nitrogen prescribe by, BIS, WHO and ICMR are 45 mg/l, 45 mg/l, 20 mg/l respectively (Gupta, 2017).

According to Johan Kjeldahl total nitrogen estimation was done by the following method. Soil samples in solution (10 g /100 ml) were taken in Kjeldahl flask then digested with concentrated sulphuric acid containing copper sulphate and potassium sulphate (50 ml; 6.7 ml of conc. H_2SO_4, 6.7 g K_2SO_4, and 0.365 g $CuSO_4$) (Sáez-Plaza et al., 2013). The sample was heated until the volume is significantly reduced (to about 25 to 50 ml) and copious white fumes were

appeared when turbid or colored samples will be converted into a transparent and pale green. After digestion, the solution was cooled and diluted with water and mixed well. 50 ml of sodium hydroxide-thiosulfate reagent was added to form an alkaline layer at the flask bottom. The flask was then connected to a steamed out distillation apparatus and mixed well. The liberated ammonia was collected in 50 ml of indicating boric acid as an absorbent solution and finally the ammonia was quantified by titrating with 0.02N H_2SO_4.

In the titrimetric method, Total kjeldahl nitrogen (TKN) calculation was done in mg/l and finally, the data were represented as total nitrogen in percentage (%).

$$\text{TKN, mg/l} = \frac{(A \times B)\, N \times F \times 1000}{S}$$

A = milliliters of standard 0.020 N H_2SO_4 solution used in titrating sample.

B = milliliters of standard 0.020 N H_2SO_4, the solution used in titrating blank.N = normality of sulfuric acid solution.

F = millgram equivalent weigh to nitrogen

(14 mg)S = milliliters of sample digested.

- **Phosphate (PO_4^{3-})**: Microbial growth depends upon phosphate of sewage and industrial organic pollutant. River water receives phosphate by mainly several ways (1) from industrial discharge and (2) runoff from the agricultural field. It was measured by spectrophotometrically followed by digestion with 4 ml H_2SO_4; 10 drops $CuSO_4$ solution; 6 gm of potassium sulphate for water and soil.
- Chloride content (mg/l): It was measured by Silver Nitrate (0.02 N) Titrametric Method.

4.3.2. Biological parameters of river water

- **Coliform - Total coliform and fecal coliform (MPN/100ml)**: The most probable number of coliforms in a water sample can be estimated using the

multiple tube fermentation techniques (Trivedy and Goel, 1984). This technique involves inoculating the water sample at several dilutions in a suitable liquid medium. All experimental tubes were placed in an incubator at 37^0C at 48 hrs. After the incubation period, they were examined for gas production by coliform organisms. The density of bacteria was calculated on the basis of positive and negative combinations of tubes using a standard MPN chart (Paria and Chakraborty, 2019).

4.4. Collection of soil sample

Soil samples were collected from10-12 cm depth of four separated position of three selected sites of Subarnarekha river, India. Then collected soil samples are mixed together site wise.

4.5. Analysis of physico-chemical parameters of soil

4.5.1. Analysis of soil texture

Soil plays a vital role in water percolation rate, P^H, alkalinity and able to control of productivity and quality of aquatic environment .Texture of soil depends upon environmental variable (Curcio, 2013). The texture of soil is strongly influenced by the composition of soil i.e. sand, silt, clay and mineral with organic matters. The textural composition of soil by mechanical analysis is followed by international pipette method (Sarkar, 2018). According to this method soil sample were washed with boiled distilled water to remove soluble salt following by treatment with 30% H_2O_2 and 2N HCl to separate organic matter and carbonate respectively. Then each sediment sample was wet sieved by 63 mm sieve (230ASTM). The 63 mm size fraction were dried, weighted and sand % were calculated. Very fine particle (<63mm) size were analyzed by pipette analysis method. About 10 gm of dry soil dissolved in 1000 ml distilled water to produce uniform concentration of dilute suspension. The slurry was shacked up to 2 hours. Then 10 ml and 20 ml suspension were separate from 10 cm and 290 cm depth respectively on a time schedule based on stock's law (Oguma, 2016).

4.5.2. Analysis of soil pH

50 gm of freshly mixed soil was collected (for each site) and it put in a 150 ml beaker and mixed with 50 ml sterilized distilled water. The soil and sterilized water thoroughly stirred with a vortex machine for 30 minutes. By digital pH meter, pH of the suspension was measured (Sharma, 2016).

4.5.3. Determination of soil moisture

The moisture content of the soil was determined by following the methods. In this method, 50g soil samples were taken in a clean150 ml beaker (Lyons, 2019). Before pouring of soil sample, the weight of the empty beaker was taken. The soil sample was kept in a hot air oven at 110^0C ± 3^0C for 24 hrs till constant weight. Soil moisture was calculated by the difference between the initial and final weight of soil.

4.5.4. Estimation of soil organic carbon by dry combustion method

The dry combustion method was employed for total organic carbon estimation (Page, 2014). Leco CN-2000 dry combustion analyzer was operated on a dry combustion principle, with infrared detection for carbon. In brief, weighed soil samples were treated with dilute HCl (HCl: H_2O in the ratio of 1:1) to remove carbonate carbon. The treated samples were dried on a hot plate.

4.6. Heavy metals analysis of water and soil

The content of six different heavy metals – Lead (Pb), Copper (Cu), Zinc (Zn), Chromium (Cr) Mercury (Hg) and Cadmium (Cd) in the water at different study sites were determined by the atomic absorption spectroscopy (APHA, 2005). Water samples were decomposed on the basis of acidification in the open system. At first 100 ml of water was mixed with 30 ml aqua regia but 1

gm soil dissolve in some amount of water that makeup up to 100 ml then mixed with 30 ml aqua regia (Skwarzec, 2009). The mixer was placed in a digester for about 1 hourat 100^0C temperature. The nitric acid (HNO_3) and hydrochloric acid (HCl) were continuously added to the distillation device due to prevention dry out. Along with test samples, one standard and one blank sample were ready to perform the total experiment. After cooled solutions were transferred to polyethylene weighing bottles which diluted with 10 ml of double-distilled water and filtered. Filtrates were injected into the atomic absorption spectrophotometer for the determination of heavy metals concentration (Paria et al., 2018).

4.7. Analysis of bacterial load of water

Bacterial contamination of water was measured by three methods simultaneously such as standard plate count (SPC), most probable number (MPN) method and membrane filtration Technique (MFT). All types of tests were accomplished within 24 hrs of sample collection (Paria and Chakraborty, 2019). The most probable number of total coliform per 100 ml indicate riverian water is contaminated by enteric disease-causing bacteria. According to WHO, the permissible limit of drinking water is zero (0) coliforms/100 ml (Hörman and Hänninen, 2006). The MPN study of water sample was revealed in Figure 4.2. The presence of >10 coliforms/dl in water is designated as polluted or unhealthy for drinking purposes. High MPN values in all the samples clearly indicate that the water is highly contaminated with coliform bacteria. The total coliform test has resulted in the growth of coliform bacteria at a temperature of 37^0C. On a global scale, water contamination by coliform is a major cause of morbidity and mortality, especially in children. As already it has been indicated coliform that is acquired directly or indirectly from a human or animal carrier. Risk from drinking water, therefore, only follows from fecal contamination of the supply (Shing and giri, 2018).

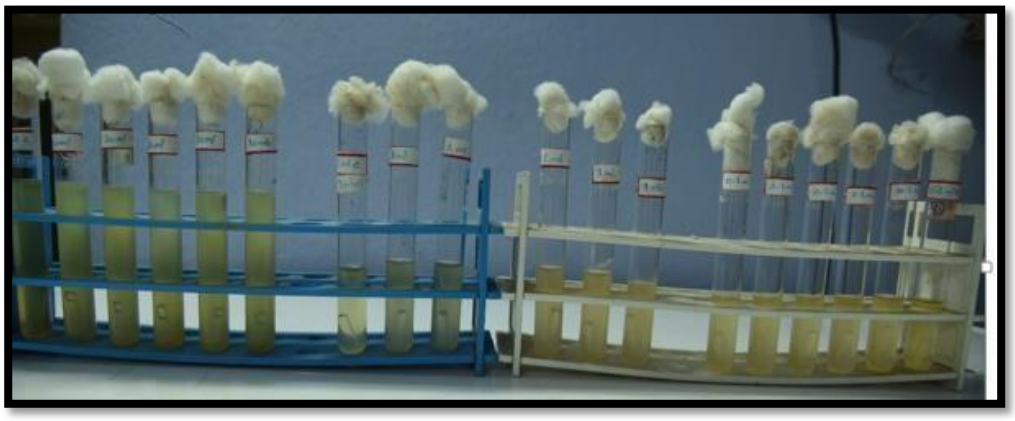

Figure: 4.2 MPN index analysis by test tubes method

4.7.1. Standard plate count (SPC) method

The total count of bacteria was determined by using nutrient agar plate. 200 ml of nutrient broth agar were prepared in a conical flask by mixing of nutrient broth and 2% agar. After sterilization by autoclave, hot nutrient agar poured into 90 mm diameter containing petri plates.

Figure 4.3 Isolation of fungal strain from soil sample in laminar air flow hood in university laboratory

Serially diluted water sample was spreaded over on to solidifying nutrient agar plate (Figure

4.3). Then all plates were incubated for 24 h at incubation temperature of 37 C. Bacterial colonies were appearing those counted by colony forming unit per milliliter (cfu/ml) of water.

4.7.2. Membrane filter technique

The membrane filters afford a fast and convenient sampling from water. Acetate cellulose type 0.45 μm membrane filter was used for the judgement of viable bacteria through membrane filtration technique (De Luca et al., 2013). Bacteria are taken on the surface of the membrane that is sited on a suitable selective medium in a sterile container and incubated at 37 °C temperature. After incubation, colony were counted and expressed as number of colonies per 100 ml sample by following formula:

No. of colonies per 100 ml = [(No. of colonies)/ (volume filtered)] × 100

4.7.3. Most probable number method

It is a usual report of the results of the multiple fermentation test tube for coliforms as a most probable number (MPN) index. This is a table of the amount of coliform bacteria that is more possibly than any other number, would give the results revealed by the test. It is not an amount of the definite number of indicator bacteria existing in the sample.

The MPN method was used to determine the presence of gas producing lactose fermenters and most probable number of total coliform and fecal coliforms present in 100 ml of river water. The standard MPN method (15 multiple tube dilution technique) was used for the detection of total coli forms by inoculation of samples at 37 ± 1^0C for 48 h into tubes of lactose broth (LB). For surface water analysis, 10 ml volume of double strength and single strength LB broth poured into 5 test tubes and 10 test tubes respectively. One inverted dirham's tube was placed in each lactose broth (LB) containing each test tube. After autoclaving of broth containing five test tubes filled by 10ml (in double strength LB broth), 1 ml, and 0.1 ml (in single strength LB

broth) water sample respectively. All test tubes were shake gently to mix the medium with the sample. Test tubes were placed the rack in an incubator for 48 hours at 37 ± 2^0C. After proper incubation, tubes with show turbidity or gas production and color changed indicate the production of acid, are regarded as positive. Last of all compare the pattern of positive results with a most probable number standard table.

4.8. Preparation of microbial culture medium for bacteria and fungi

a. For Fungi

24 gm of Potato dextrose media were dissolved in 1000 ml of distilled water for broth (liquid) culture preparation and for preparation of solid media 2% agar was mixed before sterilization. Alternatively 15 gm of Malt extract was dissolved in 1000 ml of distilled water for preparation of Malt agar medium. (Rauf and Javaid, 2013).

a. For Bacteria

13gm nutrient agar was dissolved in 1000 ml of water for preparation of nutrient agar media. The nutrient agar media was used for bacterial growth as well as assay of antibacterial activity of fungal metabolites (Rani et al., 2017).

4.9. Methods of isolation of Fungi

1 gm of freshly collected soil was dissolved in sterilized distilled water and made up to total volume of 100 ml. 1 ml stock sample was mixed in 9 ml of sterilized distilled water making 10^{-3} dilution. 100 µl 10^{-3} diluted sample was spread on to sterilized potato dextrose agar medium containing plate. After proper (28 °C temperature for 3-5 days) incubation fungal colony was appeared.

4.10. Development of pure culture of fungi

According to Paria et al., in 2018 fungal colony was inoculated in separate PDA medium

containing sterilized petri plate. Those petri plates were incubated at 28^0C for 7 days (Paria et al., 2018). After incubation individual fungal mycelium was stored at 4^0C by slant preparation during further experiment. As the pure cultures contain only one specific type of organism, these are very much suitable for study of their properties.

4.11. Pure Culture maintenance

During maintenance of purity sub culturing was done in regular interval. The metal tolerance fungal strain was used for further studies. Biochemical and morphological features of isolated fungal strain was given in Table 1.

Table 1: Morphological and biochemical characteristics of the isolated fungal species

Serial Number	Parameters
1	Colony shape
2.	Colony color
3	Mycelia shape
4	Spore color
5	Heavy metal tolerance activity
6.	Cross metal resistance assay

4.12. Selection of fungal isolates to be used in the experimental set up for studying toleranceto heavy metals against Pb, Cd and Hg

The fungi were cultured and isolated with a serial dilution of up to 10^{-5} at Potato Dextrose Agar (PDA) media (Hi-media) by standard spread plate technique in three replicates. Then the petri plates were placed in an incubator at 28^0C up to 7 days of incubation time. Cultured fungi were inoculated in seven different conical flasks containing 50 ml of potato dextrose broth (PDB) media. Then conical flasks were placed in a shaker incubator at 100 rpm but each 16 hours interval of 8 hours was stopped. Heavy metals tolerant ability by the fungal isolates were further

screened against each metal such as Pb (II), Hg (II) and Cd (II)) in different concentration having a range of 50, 100, 200 and 400 ppm on the PDA plate. The growth of fungal isolates was checked after 72 h of incubation. After incubation one fungal strain was selected on the basis of the highest Pb (II), Cd (II) and Hg (II) tolerance ability among all of the isolates which is designated as F12.

4.13. Microscopic identification of fungi

a. By light microscope

According to the morphological properties (microscopic shape and color of conidia) fungi were identified by light microscopic observation after staining the fungus by cotton blue (Hay and Ashbee, 2016). Although all fungi were identified by a light microscope. The highest heavy metal tolerant samples were visualized by electron microscopic studies according to standard protocol (Paria et al., 2018). In this respect, different species of *Aspergillus sp.* have been reported as efficient heavy metals reducers step by step (El Hameed, 2015).

b. By Phase contrast microscope

Cells were grown in potato dextrose broth (PDB) media at 28±2ºC. Few mycelia of the fungal cultures from the mid-exponential phase were spotted onto 0.1% (w/v) poly L-lysine coated slide and viewed by phase-contrast microscopy (Olympus 1x51). Images were captured by Image ProTM discovery software (Olympus).

4.14. Determination of heavy metal resistant fungal diversity in different study sites

Fungal strains were isolated from potato dextrose agar (PDA) plate by serial dilution methodin order to avoid over-lapping colonies. The soil samples (1g) were suspended in 100 ml of sterilized water whose total volume was 100 ml. The mixture was shaken at 200 rpm for 30min at room temperature. After that 0.1 ml of a sample of dissimilar dilutions were spread on petri-plates (diameter 90 mm) having 20 ml of potato dextrose agar (PDA) media. The solution is

prepared with Lead and cadmium compound with a concentration of 500 µg/ml of Pb, Cdand Hg were added to the medium after autoclaving at 15 pounds/inch^2 for 15 min at 121^{0}C. The plates were incubated at 28^{0}C and monitored up to 10 days, each developed colonies weresub-cultured and isolated into fresh PDA media containing petri-plate. Purified isolates werekept on a slant at 4^{0}C and re-cultured every 4 weeks (Ahmed, 2018).

4.15. Selection of metal tolerance fungi according to their maximum tolerance ability

5 types of fungus were inoculated into 200 ppm to 1000 ppm heavy metal containing Petri plates. Total experiments were achieved in separate heavy metal containing petri plate for each fungi. According to their tolerance ability F12 fungus was selected as the best fungal strain.

4.16. Selection of efficient strain for heavy metal tolerance

Pure culture of selected isolate (F12) was cultivated and maintained in PDA media and periodically checked for purity. The morphological and biochemical characterization of the isolate was made from a pure culture of F12 strain after 48 h growth in potato dextrose broth (PDB) at 28^{0}C in an incubator. The morphology of the selected strain was studied in PDB medium. The spore-producing ability was also checked at an interval of different incubation time and also by transferring the cells at the stationary phase of growth into PDB containing basal medium.

4.17. Identification of fungi by genome sequencing

a. DNA isolation from fungi

Prior to genomic DNA isolation, F12 fungal strain was inoculated into 20 ml of potato dextrose broth (PDB) and incubated for 3 days at 30 °C. After incubation, 2 ml of culture was taken in eppendrof tube and centrifuged at 10000× g for 15 mins to collect pellets. Then pellet was

added to 200 µl of TE buffer of pH 8.0, and after centrifugation (10000× g for 10 mins), TE buffer was removed and 300 µl of extraction solution (200 mM Tris HCl at pH 8.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added. After the addition of 150 µl sodium acetate (3M, pH 5.2) the mixture was grounded in an eppendorf. Thereafter mixture was homogenized by a homogenizer during obtained of uniform cell suspension. In order to avoid RNA contamination, 20 µl of RNase A was added, mixed well by brief vortexing and incubated at room temperature for 5 minutes. Treatment of Proteinase K also was finished by the addition of 20 µl Proteinase K solution. Then 500 µl of phenol- chloroform solution was added and centrifuged at 10000× g for 10 min. After that, the aqueous phase was transferred to another test tube and an equimolar amount of isopropanol was added to it. After 30 min of incubation at 4ºC, the supernatant was centrifuged (10000× g for 15 min) and the resulting DNA was washed with 300 µl of 70% ethanol. Again after centrifuging (10000× g for 15 min) and of ethanol removal, DNA was dried at 37 °C and dissolved in 50 µl of TE buffer. DNA thusly prepared was used for the PCR (Paria et al., 2018)

b. PCR and sequencing

Genomic DNA was isolated from strain F12, amplification of 5.8S ITS region by PCR, conserved primers were used (Paria et al., 2018). Using these primers (20 pmol each) and 200 ng genomic DNA in a 50µl reaction buffer containing 2mM dNTP, 1.5 mM magnesium chloride and 5 units Taq DNA polymerase (Bioline, USA), PCR was performed in a thermocycler (ABI, USA). The PCR conditions were an initial denaturation for 1 min and 30 sec, followed by 35 cycles of denaturing at 94^0C for 40 sec, annealing at 53^0C for 30 s and extension at 72^0C for 40 min and a final extension step for 7 min at 72^0C. PCR products were analyzed by 1.0% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV-trans illuminator. After amplification, the PCR product was eluted from gel, purified by QIA quick gel extraction kit (QIAGEN), sequenced via big dye terminator kit (ABI) and

similar primers (used for PCR) in an automated DNA sequencer (ABI model 3100, Hitachi).

4.18. Study of growth rate of *Aspergillus penicillioides* (F12) against two heavy metals The fungal strain was isolated on potato dextrose agar (PDA) media with serial dilution method to avoid over-lapping colonies. Lead (Pb II) and cadmium (Cd II) with concentration of 500 µg/ml were added to PDA medium after autoclaving at 15 psi for 15 mins at 121 °C. The organism was also tested for optimum pH and optimum the temperature in 100 ml nephalometric flasks containing medium, inoculated with 2% (v/v) culture and kept in shaker incubator at 28 °C. Growth of the organism was determined in terms of OD (Optical Density) value by spectrophotometer (Hitachi 166) at 600 nm, against a calibration curve.

4.19. Heavy metal tolerance activity of F12 fungus in liquid culture

Heavy metal uptake studies were performed in a batch process. Both dry and living mycelium were observed and evaluated separately for heavy metal removal efficiency. Adsorbents were taken in 100 ml Erlenmeyer flask containing heavy metal solution (40 ppm). Flasks were placed on a rotary shaker (160 rpm) at 28 ± 2^{0}C (Figure 4.4). After desired incubation condition, supernatant has been analyzed for each heavy metal concentration. The concentration of unabsorbed heavy metal in the medium was determined spectrophotometrically (Cheng, 2014). Dry and wet fungal mycelium were taken in a 100 ml Erlenmeyer flask containing 50 ml of heavy metals solution having a concentration of 20 ppm. It was then placed in a mechanical shaker at 160 rpm. The shaking was continued and the amount of metal remained in the liquid phase was determined at an interval of 2 h.

Assessment of the effect of heavy metals on the growth of the fungal isolates individually was measured by observing the radial colony extension against the control (without metal). The diameter of inoculums was subtracted from radial growth diameter (in cm) (Kim, 2017). The mean of perpendicular diameter measurements was recorded for each plate on the day 7^{th}.

Metal Tolerance Index (T_i) was calculated as the ratio of the extended radius of the treated colony and untreated colony.

$$Ti=Dt/Du$$

Where D_t is the circular extension (cm) of treated colony and D_u is the circular extension (cm) of untreated colony. The tolerance index (Ti), is an sign of the organism response to metal stress was calculated from the growth of strain showing to the metals divided by the growth in the control plate (Le et al., 2006).

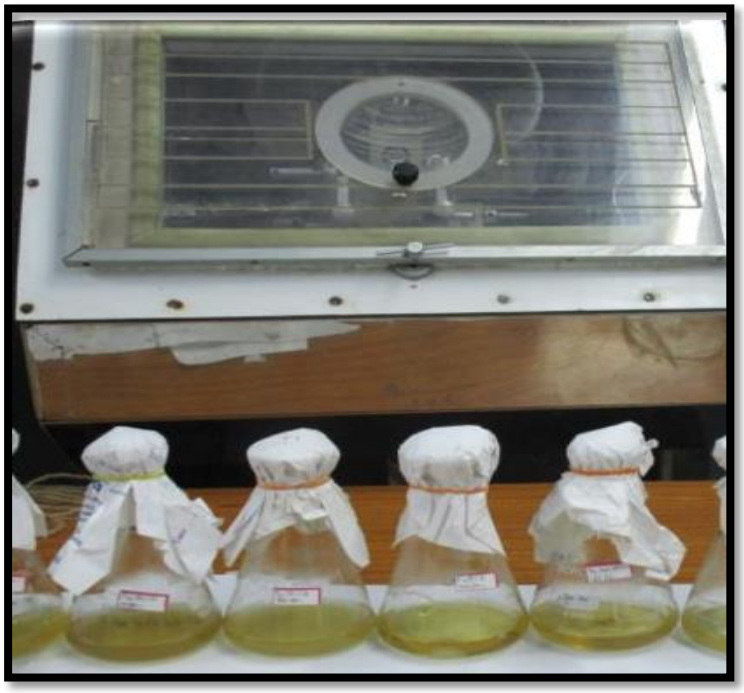

Figure 4.4: Experimental setup for studying heavy metal tolerance activities of F12 fungus in liquid culture

4.20. Determination of MICs (Minimum Inhibitory Concentrations)

By dilution method, a minimum inhibitory concentration (MICs) of the selected isolates to Pb II, Cd II and Hg II were determined. In the beginning metal ions were added separately to PDA medium at different concentrations. Agar plugs from young fungal colonies were inoculated

against control plates which were incubated at 28^0C for at least 7 days. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of metal that inhibit visible growth of the isolate (Ezzouhri et al., 2009).

4.21. Uptake of heavy metals (Cd II, Pb II and Hg II) by fungal isolates from liquid media

The highest tolerant fungal isolates, F12 were evaluated in potato dextrose broth (PDB) supplemented with 50 ppm of two heavy metals (Cd II and PbII). These flasks were inoculated with 1 ml of freshly prepared spore suspension of F12 fungal isolate and incubated at 160 rpm at 28^0C for 96 hrs. Un- inoculated flasks containing PDB of 50 ppm concentration of different heavy metals were used as control. The supernatant was collected after centrifugation at 10,000 rpm for 5 min. The harvested fungal biomass was rinsed with double distilled water 3–4 times and dried in hot air oven at 80^0C for 12 hrs. The dried fungal biomass was weighed and heavy metal concentration was estimated by digestion with nitric acid and hydro chloric acid (3:1 ratio). The digested fungal biomass was centrifuged and filtrate was collected. By atomic absorption spectrophotometer (AAS), heavy metals concentration was estimated by Greenberg et al., 1985.

4.22. Production and separation of extracellular polymeric substances (EPS) from *Aspergillus penicillioides* (F12)

In submerged culture, EPS production was performed by Papinutti, (Papinutti, 2010). Ten conical flasks containing potato dextrose broth (PDB) with fungal inoculum were incubated at 28 ± 2^0C on an orbital shaker at 160 rpm for 7 days (shaking were stopped for 8 hours for each day) of incubation time. After 7 days of incubation fungal mycelia were separated from the extracellular fluid by filter paper. The filtrate broth were then mixed with a double volume of ethanol and incubated at 4^0C for 24 hours (Figure 4.5). The precipitate substances were recovered by centrifugation at 5000 g for 15 min at 4^0C, and after that, the final product was

freeze dried to produce fine powder and stored in a desiccators (Kumar et al 2014). Yields of EPS were estimated by the phenol sulphuric acid assay (Notararigo et al., 2013) against freshly prepared nitrogen free basal glucose medium as control.

Figure 4.5: Production and collection of EPS from liquid culture broth

4.23. Optimization of parameters for metal removal study

4.23.1. Fungal Growth optimization with heavy metals

Different fungal strains were isolated on potato dextrose agar (PDA) media with serial dilution method to avoid over-lapping colonies. Lead (Pb II) and cadmium (Cd II) with concentration of 500 µg/ml but Hg concentration 200 µg/ml were added to PDA medium after autoclaving at 15 psi for 15 mins at 121^0C. The soil samples (1gm) were suspended in sterilized water and volume was made up to 100 ml by distilled water. Then at room temperature the mixture was shaken at

200 rpm for 30 min. Dilution factor of all samples were up to 10^{-5}. After that 0.1 ml of different dilution sample were spreaded on petri- plates (diameter10 cm) containing 25 ml of potato dextrose agar (PDA) media (Figure 4.6).

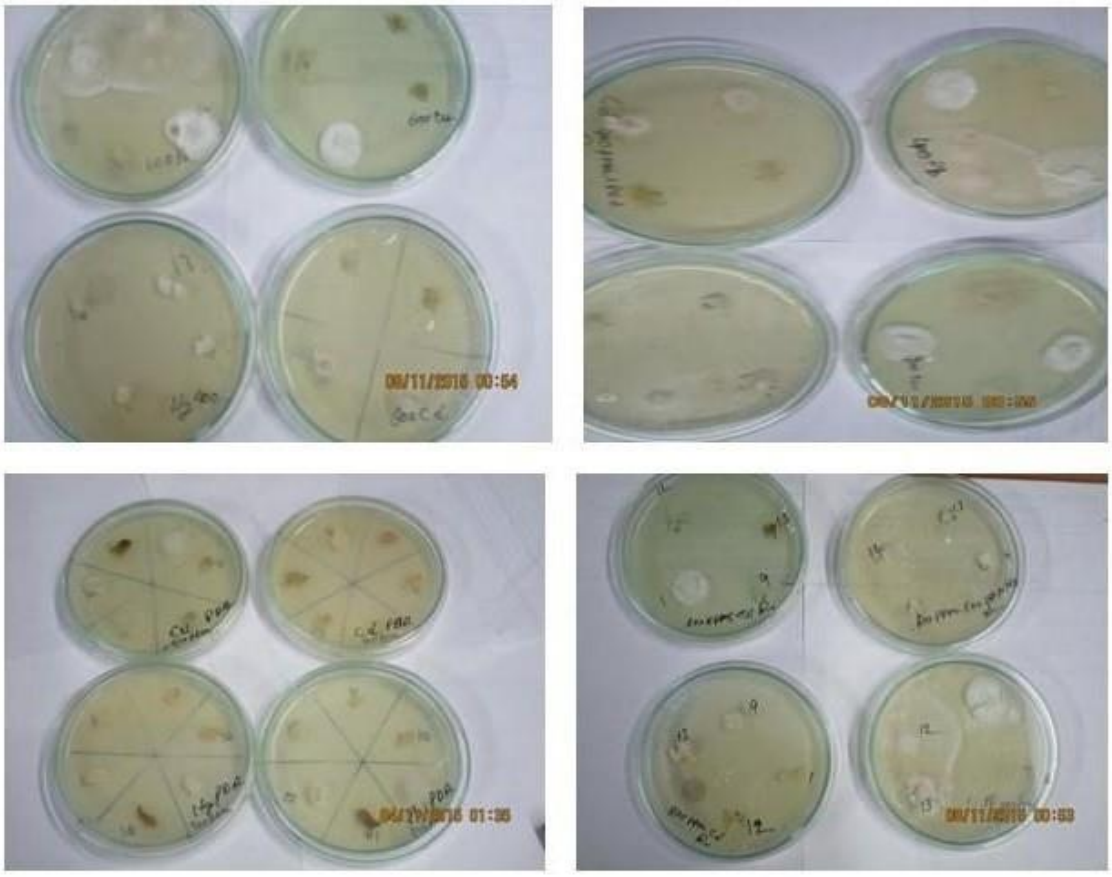

Figure 4.6: Fungal growth culture with different concentrations of heavy metals in agar plates

Plates were incubated at 28 °C temperature up to 10 days and each developed colonies was isolated into fresh PDA due to sub-cultured. Pure cultured stored on a slant at 4^0C and re-cultured every 4 weeks (Kathuria, 2018).

4.23.2. Parameters optimization (for simultaneously heavy metal Pb II and Cd II removal by dry biomass and EPS)

A range of pH solutions was prepared from 3.0 to 8.0. Other parameters like metal concentrationat

40 ppm, incubation period of 8 hrs, inoculums dose of 0.2 mg/ml and temperature at 30^0C were fixed. The temperature is another factor which influences on the bio-sorption of heavy metals by dry biomass was recorded which were found to vary from 20^0C- 40^0C while other parameters remained constant i.e. the metal concentration of 40 ppm, absorption time at 16 hours, inoculums dose of 0.2 mg/ml and optimized pH at 6. The contact time between dry biomass and the metal solution was observed to influences on the bio-sorption process. Sufficient contact time should be provided to the bio sorbent (dry biomass or EPS) and therefore, studied with different contact times ranging from 1h to 7hrs in order to identify the optimum incubation time when other parameters remained fixed. Biomass being the most important parameter in metal uptake kinetics, different concentration of dry biomass was used from 5-50 mg/ml in 50 ml of total volume. Other conditions like temperature 30 °C, metal concentration 40 ppm, incubation time 3 hrs and pH 6.0 were used as optimized earlier. All experiments were conducted in triplicates (Kalyoncu et al., 2010).

4.23.3. Optimization of heavy metal Pb (II) absorption by EPS using box–behnken design

After determining the optimum bioabsorption capability, the parameter was statistically optimized by Box-Behnken Design (BBM) with three independent variables (pH, time and temperature) and one dependent variable (% of absorption). A total of thirteen experiments (4 experiments repeated) were conducted at a low and high levels of the variables. The statistical software Design Expert 7.0 has been used for determining the optimum levels of absorption pH, time (hours), temperature (°C) of three responses. The response variables selected was % of absorption. These three factors namely pH (A), time (B) and temperature (C) were coded into three levels. The independent variables were added in the design with three levels with an equal difference (pH 4 to 11, time in the range of 0.25 hours to 8 h, temperature 25 °C to 40 °C).

In order to apply in response surface methodology (RSM), the real value of independent variables must be transformed into the coded variables. The coded and uncoded independent

variables were used in the RSM design that is shown in Table 5.8. In this study, the three independent variables were used and Y_i was set as response variables (pH, time and temperature) with the equation:

$Y_i = \beta o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2$ Equation 1

Where βo is the intercept; β_1, β_2 and β_3 the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients; β_{12}, β_{13} and β_{23} are the interaction coefficient. The model parameters were analyzed by multiple linear regression using Design Expert 7.0. The coefficient of determination (R^2), the Residual Standard Deviation (RSD) and the lack of fit was tested for the evaluation of the goodness of fit and presented in the ANOVA. The optimum conditions, that maximize the absorption %, were also determined by the software program from the fitted model. The obtained fitted quadratic a polynomial equation is showing the relationship between the response and independent variables was used to develop the three-dimensional surface response and contour plots (Liu et al., 2013) using Design Expert 7.0 (Stat-Ease Inc., Minneapolis, MN, USA). The significance level was based on a confidence level of 95.0.

4.24. Heavy metal absorption by biomass and EPS determined by SEM and EDEX Metal treated wetted fungus from the agitated flask was dehydrated by multiple washing with progressively concentrated acetone (20%, 30%, 50%, 70%, and 100%) for 20 min each. After fixed into a graphite stub samples kept in an E5200 Auto sputter coater (UK) under vacuum upto 15 minutes. At 20 kV accelerating voltage photographs were taken. For the EDAX study, fungi as well as fungal EPS were taken after absorption in optimum condition and dried at 40^{o}C in the oven. After that fungus, as well as EPS, was placed onto graphite stub for X-ray dispersion analysis (Muthu et al., 2018).

4.25. Determination of heavy metal binding region of fungi by fourier transforms infrare d spectroscopy (FTIR) method

The infra-red spectrum was studied of thin pellets of fungus mycelium and fungal exo-polysaccharide mix with dust KBr matrix. A Nexus TM 870 FT-IR (Perkin Elmer) spectrophotometer fitted out through a deuterated triglycine sulphate thermoelectric cool (DTGS-TEC) detector was used to collect the data over a range of 200-4000 cm^{-1} (Paria, et al., 2018).

4.26. Fungal metabolites extraction

Crude extracts of endophytic fungi were prepared as described by Wang et al., 2006 with slight modifications. Endophytic cultures were filtered to separate the culture broth and mycelia using filter paper (Liang et al., 2012). All filtrates were then added to 95% ethanol with fully stirring and left overnight (Figure 4.7). Further, the filtrate was concentrated in a rotary vacuum to remove organic solvents and was dried by freeze-drying. Then the sterile distilled water was added with powder extract to make a concentration of 10 mg/ml and sterilized through a 0.22 µm Millipore for antimicrobial activity assay.

Figure 4.7: Production of Fungal metabolites from *Aspergillus penicillioides* (F12)

4.27. Estimation of emulsifying activity of EPS

The emulsifying assay was measured according to the standard assay (Farvin, et al., 2014). EPS was dissolved in 5 ml of distilled water (1mg/ml) and mixed with 5 ml of hydrocarbon or oil then stirred in a vortex for 5 min. Then samples were stored at 37^{o}C temperature for 24 h. The hydrophobic substances tested were xylene, toluene, octane, tetradecane, hexadecane, mineral light oil and mineral heavy oil (Sigma), crude oil and petrol (Aizenberg et al., 2015). As controls we used the following chemical surfactants from Sigma: Tween 20, Tween 80 and Triton X-100. Emulsion index (E_{24}) was then determined after 24 hrs, which depends on assay E_{24}=he/hT×100, where the height of the emulsion layer is 'he' (mm) and the total height of the mixture is 'hT'. All experiments were performed in triplicate.

4.28. Estimation of flocculating activity of EPS

The flocculating activity of the EPS was produced by *Aspergillus penicillioides* fungus was carried out using the selective method (Kumar et al., 2019). A mixture of 100 ml kaolin clay suspension (5 g/l, pH 7.0), 0.2 ml of EPS in different concentrations (1 to 15 mg) and 1ml of $CaCl_2$ solution (1mg/l) were mixed, agitated and the absorbance was measured by spectrophotometer (UV-1601, Shimadzu) at 550 nm. A control experiment was carried out by replacing the sample with distilled water and the flocculating activity was calculated by using the following equation:

Flocculating activity (%) = A-B/A × 100, Where A and B is the optical density values of control and test samples respectively.

4.29. Determination of the antibacterial activity of the fungal extract (F-12)

Antimicrobial activity of the secondary metabolites from isolated fungi was carried out by the well diffusive method (Nwakanma et al., 2016) against two Gram-negative bacteria such as *E.coli, Vibrio cholera*, and two Gram-positive bacteria such as *Bacillus subtilis, Staphylococcus aureus*. 0.2 ml of test bacterial solution was evenly spread in sterile luria–

bertani (LB) broth agar. Then the fungal metabolite (50 µl) was poured on the inoculated agar plates, but one of them contained no extract. They were incubated at 37 °C for 24 h in aculture incubator. After incubation, the diameter of each inhibition zone was measured with a millimeter scale. All experimental assessments were conducted in triplicate.

4.30. Statistical Analysis

4.30.1. Correlation coefficient and cluster analysis

This test is utilized to calculate the linear correlation between two continuous variables. To analyze the statistical significance of the influence of different heavy metals and physicochemical parameters on the abundance of the fungal population of the study sites SPSS (Statistical Package for Social Science) version 20.0 has been used.

Cluster analysis is a method that a constituent in a similar cluster (called a cluster) are more similar (in some sense or another) to each other than to those in supplementary groups (clusters). It is the main tusk of exploratory data means and a common technique for statistical data analysis, used in ecological fields.

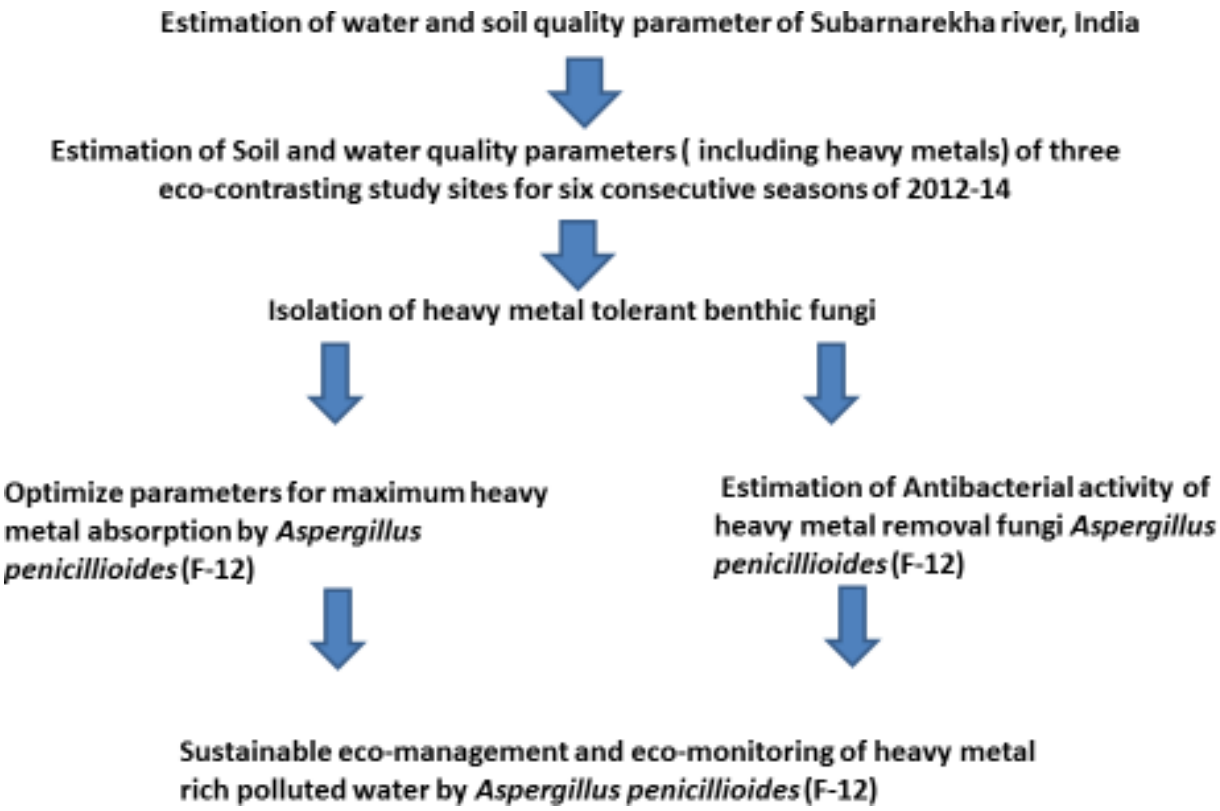

Figure 4.8: Schematic presentation of the entire study, undertaken emphasizing for eco-monitor of the selected eco-system