Chapter 7

Summary

This chapter presents a summary of the findings from the present research study and the important conclusions inferred there form. Out of 112 benthic fungal strains screened and collected from the bottom soil of the Subarnarekha river. The benthic environment of any riverine system is generally endowed with a galaxy of life forms (microbes, plants and animals). Out of all these biodiversity components, benthic fungi represent a very significant life form with a lot of ecological roles. Considering the culture of so many fungal strains with a differential pattern of their distribution and occurrence in the different eco-region of a transboundary river, the Subarnarekha, India, The present research studies have touched upon several aspects of benthic fungal ecology with special emphasis to the heavy metals bio-accumulation, tolerability and bio-removal potential. The *Aspergillous penicillioieds* (F12) is a filamentous fungus found in saline water bodies in Talsari of the Subarnarekha river commonly used as a bioremediating agent. The bioactive form *Aspergillous penicillioides* are known to have exopolysaccharide (EPS) producing and bio remediating effect. Fungal EPS, an exo-polymer is mainly used in the food and pharmaceutical industries. Various bioabsorption techniques have been in use for the absorption of heavy metals from waste water for purification. Living mycelium, dead biomass and extracted EPS of the fungal strain were reported to absorb heavy metals at the level of around 90%. Bio absorption by EPS was suitable other than other methods and the bioremediation efficiency has been significantly affected by the temperature (^{o}C), extraction time (hrs) andpH.

In such a context, this project work was undertaken to optimize the growth conditions of *Aspergillous penicillioides* (F12) because of its higher bio absorption capability, and to develop a suitable bio remediating agent. The summary of the results of various experimental along with field studies that were conducted in order to achieve the objectives and the conclusion are being presented in the below into different sections.

The entire work was carried out in five phases. In the **phase–I**, physico-chemical and bacteriological parameters including percentage (%) of heavy metals of water and soil of three eco-contrasting sites of six seasons spanning a period of two years (July, 2012-June, 2014) of Subarnarekha river, India was carried out. In **phase –II**, recording of fungal diversity of three eco contrasting sites and selection of most efficient fungal strain *Aspergillous penicilloides* (F12) (MN210327) on the basis of their highest heavy metals (Pb II, Cd II, and Hg II) absorption capability were performed. In **phase III**, the determination of heavy metal-binding regions within the fungus by FTIR, SEM and EDEX analysis was performed. **In phase IV**, estimation of heavy metal absorption activities by fungal mycelium, dry biomass, and optimization of heavy metal Pb (II) absorption by an extracellular polymeric substance (EPS) using box – behnken design were worked out. Phase **V** was devoted to the estimation of emulsifying, flocculating and antibacterial activity of fungal EPS.

Phase –I: Water and soil samples were collected in air tight sterilized containers at July 2012 to June 2014 from three eco- contrasting sampling sites namely Muri (S-I), Sonakonia (S-II), and Talsari (S-III). First of all this study had attempted to record physico-chemical as well as the coliform bacteriological parameters of six consecutive seasons of Subarnarekha river water and fungal diversity of three eco- contrasting sites of Subarnarekha river, India. For physico-chemical analysis of water, the temperature was measured by a mercury centrifuge thermometer, pH was measured by field P^H meter. Alkalinity was determined by the titrimetric method. Total hardness, ca-hardness and Mg-hardness were determined by the titrimetric method. By the Winkler method, DO was measured. For the BOD measurement water sample was collected by BOD glass bottle, after 5 days of dark incubation BOD were measured. Chemical oxygen demand was measured by the titrimetric method. The total dissolved solid was measured by an analytical balance method. Total nitrate nitrogen was measured by Kjeldal method. After digestion of soil by $CuSO_4$, $H2SO_4$, and Potassium sulphate total Phosphate was measured by spectroscopic method. Chloride content was measured by silver nitrate titrimetric method. For the coliform test, the collected water sample was analyzed by CO_2 gas-producing fermentation method which was detected by inverted dirhams tube. Organic carbon of soil was estimated by the dry combustion method after the treatment of HCl. For heavy metal analysis of water and soil sample, all samples were estimated by Atomic absorption Spectroscopy (ICP–MSThermo scientific) after digestion of nitric acid and hydrochloric acid at 100^{o}C for 1hour.

Phase –II: The fungal diversities of three eco-contrasting sites were carried out by potato dextrose agar medium. One (1) gm of each soil sample from three collected sites was dissolved in sterilized distilled water, whose total volume was 1000 ml. 0.1 ml solution was spreaded over potato dextrose agar plate. After 3- 5days incubation at 28^{o}C, fungal colonies were collected. Thereafter fungal pure culture appeared on another PDA medium. Pure culture were maintained in the regular interval in slant at 4^{o}C. All isolated fungus were inoculated in 40 ppm heavy metals (Pb II, Cd II, and Hg II) containing PDA medium at 28^{0} C for 4-5 days incubation. After that heavy metal resistance fungi were isolated and screened for test of heavy metal tolerance index. According to their heavy metal tolerance activity, the most dominant fungal strain was selected and identified by ITS sequencing followed by phylogenetic tree construction.

Phase III: The heavy metal binding regions of fungus were determined by FTIR, SEM and EDEX analysis. The FTIR spectra were studied by thin palate preparation in the KBr matrix of dry biomass as well as dry EPS of *Aspergillous penicillioides* (F12). The FTIR spectru collected by Nexus TM 870 FT- IR (Perkin Elmer). For SEM analysis heavy metal treated, dehydrated fungal biomass and EPS were fixed into a graphite stub and kept in an E 5200 auto sputter coater under vacuum for 15 minutes. Then photographs were taken in the SEM monitor. **Phase IV:** Temperature was varied from 20^{o}C- 40^{o}C while other parameters remained constant i.e. metal concentration of 40 ppm, absorption time at 16 hours, inoculums dose of 0.2mg/ml A range of pH solutions were prepared 3.0 to 8.0. Other parameters like metal concentration at 40 ppm, incubation period of 8 hrs, inoculums dose of 0.2 mg/ml and temperature at 30°C were fixed. The incubation time was ranged from 1h to 7hrs in order to identify the optimum incubation time. Different concentration of dry biomass was used from 5-50 mg/ml in 50 ml of total volume. All experiments were conducted in triplicates. After determining the optimum bioabsorption capability, the experimental parameters were statistically optimized by box- behnken design (BBD) with three independent variables (pH, time and temperature) and one dependent variable (absorption %). 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.

Phase V: The emulsifying assay was measured according to the standard assay. EPS was dissolved in 5 ml of distilled water (1mg/ml) and mixed with 5 ml of hydrocarbon or oil such as xylene, toluene, octane, tetradecane, hexadecane, mineral light oil and mineral heavy oil (Sigma), crude oil and petrol. Then the samples were incubated at 37° C temperature for 24 hrs. As controls we used Tween 20, Tween 80 and Triton X-100. Emulsion index (E_{24}) was then determined by the following equation: E_{24}=he/hT×100, where the height of the emulsion layer ishe (mm) and the total height of the mixture is hT.

For the flocculating activity measurement a mixture of 100 ml kaolin clay suspension (5 g/l, pH 7.0), 0.2 ml of EPS in different concentrations of 1-15 mg/ml and 1ml of $CaCl_2$ solution (1 mg/l) were mixed, agitated and the absorbance was measured by spectrophotometer (UV-1601, Shimadzu) at 550 nm. A control experiment was carried out with distilled water and the flocculating activity was calculated by using the following equation: Flocculating activity (%) = A-B/A × 100, where A and B are the optical density values of control and test samples respectively.

Antimicrobial activity was performed by the well diffusion method against two gram negative bacteria such as *E.coli, Vibrio cholera* and two gram positive bacteria such as *Bacillus subtilis, Staphylococcus aureus*. The 0.2 ml of test bacterial solution and 50 µl of fungal metabolite were evenly spreaded in sterile luria–bertani (LB) broth agar. They were incubated in a culture incubator at 37°C for 24 hrs. After incubation the diameter of each inhibition zone was measured with a millimeter scale. The pertinent results obtained at various stages of investigation and inferences are drawn are briefly summarized below.

Physico-chemical parameters of water

- Temperature revealed a wide range of variation with a minimum temperature of 14.5°C (post-monsoon, 2012- 2013) and that of a maximum of 34.2°C (pre-monsoon, 2013) at S-I; from 18.5° C (post-monsoon 2012-13) to 33.7° C (premonsoon, 2013) at S-II and from 17.5°C (post-monsoon, 2012-13) to 31.8°C (pre-monsoon, 2014) at S-III respectively.
- The pH of water showed a fluctuation in between from 6.2 (post-monsoon, 2013-14) to 7.5 (pre-monsoon, 2014) at S-I; from 6.3 (post-monsoon, 2013-14) to 7.6 (pre-monsoon2013) at S-II and from 6.1 (monsoon, 2013) to 7.6 (pre-monsoon 2014) at S-III.
- The alkalinity of water displayed a variation from a minimum of 70.7 mg/l (post-monsoon 2012-13) to a maximum of 98.2 mg/l (monsoon, 2013) at S-I; from 63.7 mg/l (post-monsoon 2012-13) to 86.8 mg/l (monsoon, 2012) at S-II and from 65.8 mg/l (pre-monsoon, 2013) to 85.2 mg/l (post-monsoon, 2013-14) at S-III.
- Total hardness fluctuated from a lowest of 63.8 mg/l (post-monsoon 2013-14) to a highest of 105 mg/l (pre-monsoon 2014) at S-I; from 62.7 mg/l (monsoon, 2013) to 93.6 mg/l (post-monsoon, 2013-14) at S-II and from 56.2 mg/l (monsoon, 2013) to 96.5 mg/l (pre-monsoon, 2014) at S-III.
- Ca-hardness of river water ranged from a minimum of 47.1 mg/l (post-monsoon, 2013-14) to a maximum of 82.1 mg/l (pre-monsoon, 2013) at S-I; from 44.8 mg/l (post-monsoon, 2012-13) to 59.4 mg/l (monsoon, 2012) at S-II and from 48.6 mg/l (post-monsoon, 2013-14) to 77.9 mg/l (pre-monsoon, 2014) at S-III.

- The values of Mg-hardness of water showed a fluctuation in between from 2.32 mg/l (monsoon, 2013) to 8.98 mg/l (monsoon, 2012) at S-I; from 2.15 mg/l (monsoon, 2012)to 9.32 mg/l (post-monsoon, 2012-13) at S-II and from 1.04 mg/l (monsoon, 2013) to 6.39 mg/l (post-monsoon, 2013-14) at S-III.
- The values of total dissolved solid ranged from 96 mg/l (post-monsoon, 2012-13) to 173 mg/l (pre-monsoon 2014) at S-I; from 104 mg/l (post-monsoon, 2012-13) to 222 mg/l (pre-monsoon, 2014) at S-II and from 185 mg/l (post-monsoon, 2013-14) to 336 mg/l (pre-monsoon, 2014) at S-III.
- The TSS of water displayed a variation from a minimum of 49.2 mg/l (pre-monsoon, 2012-13) to a maximum of 88 mg/l (pre-monsoon, 2014) at S-I; from 68 mg/l (post-monsoon, 2012-13) to 173 mg/l (monsoon, 2012) at S-II and from 152 mg/l (post-monsoon, 2013-14) to 290 mg/l (monsoon, 2012) at S-III.
- Dissolved oxygen fluctuated from a lowest of 5.2 mg/l (monsoon, 2012) to a highest of 7.2 mg/l (pre-monsoon, 2013-14) at S-I; from 4.62 mg/l (post-monsoon, 2013-14) to 7.85 mg/l (monsoon, 2012) at S-II and from 5.5 mg/l (monsoon, 2012) to 6.9 mg/l (pre- monsoon, 2013) at S-III.
- The values of chemical oxygen demand displayed a variation from a minimum of 20.1 mg/l (monsoon, 2012) to a maximum of 40.6 mg/l (pre-monsoon, 2014) at S-I; from 26.8mg/l (post-monsoon, 2012-13) to 48.5 mg/l (monsoon, 2012) at S-II and from 25.2 mg/l (post-monsoon, 2012-13) to 54.3 mg/l (pre-monsoon,2013) at S-III.
- BOD of river water ranged from a lowest value of 0.6 mg/l (monsoon, 2013) to 2.04 mg/l (pre-monsoon, 2014) at S-I; from 1.04 mg/l (post-monsoon, 2012-13) to 1.9 mg/l (pre-monsoon, 2013) at S-II and from 1.01 mg/l (pre-monsoon, 2014) to 1.52 mg/l (post- monsoon, 2012-13) at S-III.
- The values of total Kjeldahl nitrogen displayed a variation from a minimum of 1.28 mg/l (monsoon, 2013) to a maximum of 1.98 mg/l (post-monsoon, 2012-13) at

S-I; from 0.78 mg/l (monsoon, 2013) to 1.68 mg/l (monsoon, 2012) at S-II and from 1 mg/l (monsoon, 2012) to 2.65 mg/l (pre-monsoon, 2013) at S-III.

- Total phosphate phosphorus of river water ranged from a lowest value of 0.05 mg/l (monsoon, 2012; pre-monsoon, 2014; monsoon 2013) to 0.09 mg/l (several seasons) at S-I; from 0.32 mg/l (pre-monsoon, 2014) to 0.71 mg/l (pre-monsoon, 2014) at S-II and from 0.12 mg/l (post-monsoon, 2012-13) to 0.64 mg/l (pre-monsoon, 2013) at S-III

- Available chloride content displayed a variation from a minimum of 17.6 mg/l (monsoon, 2012) to a maximum of 37.6 mg/l (pre-monsoon, 2014) at S-I; from 24.8 mg/l (monsoon, 2013) to 38.2 mg/l (monsoon, 2013) at S-II and from102 mg/l (pre-monsoon, 2013) to 148 mg/l (post-monsoon, 2012-13) at S-III.

- Total coliform bacteria exhibited a minimum of 540 MPN/100ml (monsoon, 2012) to a maximum of >2400 MPN/100ml (several seasons) at S-I; from 920 MPN/100ml (post- monsoon, 2012-13 and 2013-14) to >2400 MPN/100ml (several seasons) at S-II and from 900 MPN/100ml (post-monsoon, 2012-13 and post-monsoon 2013-14) to >2400 MPN/100ml (several seasons) at S-III.

- Faecal coliform bacteria displayed a variation from a minimum of 350 MPN/100ml (post-monsoon, 2012-13 and 2013-14) to a maximum of >2400 MPN/100ml (several seasons) at S-I; from 540 MPN/100ml (several seasons) to 1600 MPN/100ml (several seasons) at S-II and from 540 MPN/100ml (several seasons) to 1600 MPN/100ml (several seasons) at S-III.

Heavy metal analysis of water

. • Available chromium in water displayed the highest value of 1.01 mg/l (monsoon, 2013) that of the lowest value of bdl (monsoon, 2012) at S-I; the minimum value of 0.021 mg/l (monsoon, 2013) and that of a minimum of 0.20 mg/l (post-monsoon, 2013-

14) at S- II and highest of 0.05 mg/l (several season) and that of the lowest bdl (monsoon, 2012) at S-III respectively.

- The minimum and maximum values of lead contents in water were estimated to be as 0.25 mg/l (monsoon, 2012) to 0.55 mg/l (pre-monsoon 2013) at S-I, 0.43 mg/l (monsoon, 2013) to 1.52 mg/l (pre-monsoon, 2013) at S-II and 0.33 mg/l (monsoon, 2012) to 0.41 mg/l (pre-monsoon, 2013) at S-III respectively.

- Available copper concentration in water exhibited lowest value of 0.02 mg/l (monsoon, 2012) and the highest value of 0.09 mg/l (pre-monsoon, 2013) at S-I, from 0.06 mg/l (several seasons) to 0.09 mg/l (several seasons) at S-II and also minimum value of 0.05 mg/l (several seasons) to that of 0.06 mg/l (post-monsoon, 2013-14) at S-III.
- The values cadmium content fluctuated from bellow detection level (monsoon, 2012)to 0.03 mg/l (several seasons) at S-I; from 0.02 mg/l (several season) to 0.03 mg/l (pre- monsoon 2013 and pre-monsoon, 2014) at S-II and from bellow detection level (monsoon, 2013) to 0.03 mg/l (pre-monsoon, 2013- 2014) at S-III.
- The values mercury content fluctuated from bdl (monsoon, 2012-13) to 0.01 mg/l (several seasons) at S-I; from bdl mg/l (monsoon, 2013) to 0.03 mg/l (pre-monsoon, 2013 and pre-monsoon 2014) at S-II and from bdl (monsoon, 2012 and monsoon 2013) to 0.02 mg/l (pre-monsoon, 2014) at S-III.

From cluster analysis in pre-monsoon, it was observed that less than 5 % of the maximum distance (Dmax) was noticed for the two pairs of variables: alkalinity, chemical oxygen demand (COD), total hardness (TH) with biological oxygen demand (BOD), total phosphate phosphorous (TPP), total nitrogen (TN), pH, dissolved oxygen (DO), temperature, and total faecal coliform (TFC). In monsoon less than 5 % of the maximum distance (D_{max}) is noted for the two pairs of variables: alkalinity and TH, with COD, TFC, temperature, DO, pH, TP, TN and BOD. For these two pairs of the results, the highest positive values of correlation coefficients were noted. In post-monsoon about

20% of the maximum distance D_{max} is noticed for the two pairs of variables: TH with alkalinity, and all others except TDS

Composition of soil sediment

- From the upper to lower courses the percentage distribution of the sand size sediment is gradually decreases from Muri (S-I) with 97.2% to 83.1% in Talsari (S-III) during the monsoon period. Similarly, during post-monsoon sand size varies between 95.9% (S-I) to 91.7% (S-II) and 83.1% (S-I) but in the pre-monsoon period sand size varies between 92.9% (S-I), 90.6% (S-II) and 87.7% (S-III) respectively.

Heavy metal analysis of soil

- Available lead in soil was showed the highest value from 1.27 mg/l (monsoon, 2013) to the lowest value of 1.63 mg/l (post-monsoon, 2012-13) at S-I; from 0.51 mg/l (post-monsoon, 2012-13) to 1.82 mg/l (pre-monsoon, 2013) at S-II and from 1.26 mg/l (monsoon, 2012) to 1.63 mg/l (pre-monsoon, 2013) at S-III.
- The minimum and maximum values of the copper content in the soil varied from 0.09 mg/l (pre -monsoon, 2013) to 0.39 mg/l (post- monsoon, 2012-13) at S-I; from 0.11 mg/l (post-monsoon, 2012-13) to 0.51 mg/l (Pre-monsoon, 2014) at S-II and from 0.05 mg/l (pre-monsoon, 2014) to 0.50 mg/l (pre- monsoon, 2013) at S-III.
- The values cadmium content were varied from 0.02 (monsoon, 2012) to 0.03 mg/l (several seasons) at S-I; from 0.01 mg/l (monsoon, 2013) to 0.05 mg/l (several seasons) at S-II and from 0.04 mg/l (several seasons) to 0.05 mg/l (pre-monsoon, 2014) at S-III.
- The values of zinc content were fluctuated from 0.41 mg/l (pre-monsoon, 2013) to 0.73 mg/l (pre -monsoon, 2014) at S-I; from 0.31 mg/l (post -monsoon, 2012-13) to 0.60 mg/l (pre-monsoon, 2013) at S-II and from 0.48 mg/l (post -monsoon, 2013-14) to 0.59 mg/l (pre-monsoon, 2013) at S-III.
- The values cadmium content were varied from 0.02 (monsoon, 2012 and post monsoon

2012-13) to 0.03 mg/l (several seasons) at S-I; from 0.04 mg/l (several seasons) to 0.05 mg/l (several seasons) at S-II and from 0.04 mg/l (several seasons) to 0.05 mg/l (pre-monsoon, 2014) at S-III.

- Available chromium in soil was showed the lowest value from 0.02 mg/l (several seasons) to a highest value of 0.04 mg/l (pre-monsoon, 2014) at S-I; from 0.05 mg/l (several seasons) to 0.06 mg/l (premonsoon, 2013-14 and premonsoon, 2014) at S-II and from 0.04 mg/l (monsoon, 2012) to 0.05 mg/l (several seasons) at S-III.
- The values mercury content were fluctuated from 0.01 (monsoon, 2012) to 0.032 mg/l (post-monsoon 2013-14) at S-I; from 0.01 mg/l (monsoon, 2013) to 0.056 mg/l (premonsoon, 2014) at S-II and from 0.01 mg/l (monsoon, 2013) to 0.03 mg/l (pre-monsoon, 2013 and pre-monsoon 2014) at S-III.

Isolation of fungal diversity and metal tolerance fungi

- Total numbers of 112 fungi were isolated. Out of all those species, 28 (%) werefound at S-I, 29(%) was observed at S-II and 43(%) were recorded at S-III (Figure 5.6). In addition, the radar chart highlighting the distribution of fungus family revealed that the highest fungal diversity were found at S-III followed by S-II and least at S-I (Figure 5.9).
- Several species of soil-inhabiting fungi viz. *Aspergillus sp., Pythium sp., Rhizopus sp., Penicilium sp.,* and *Fusarium sp.* have been isolated from the collected soil samples. From which, *Aspergillus penicillioides* has exhibited maximum tolerant activities up to 1000 ppm of heavy metals of Cd (II), and Pb (II) but 200ppm Hg(II).
- *Penicillium sp*. and *Aspergilus sp*. have altered up to first level of tolerance of lead followed by *Pythium sp., Fusarium sp.* and *Rizopus sp*. but TI of *Peniciliumsp*. was higher than others with respect to Cd (II).
- It has also been observed that *Aspergillus penicillioides* secrete a huge amount of exopolysaccharide that supports in immobilization of heavy metals. Consequently, the

result demonstrates that both biomass and exopolysaccharides are responsible for heavy metal bioremediation.

Determination of heavy metal binding region

- The SEM study has shown that the characteristic of the genus *Aspergillus sp.* F12 (Deposition No.MN210327) is the spore-like behavior structure that produces extracellular polymeric substances.
- The EDEX study of the dry mass also exposed the accumulation of target metals in the surface of the fungal cell surface. The maximum metal accumulation was detected in the biomass of fungi when supplemented combined with an equal concentration of Pb (II) and Cd (II). But the low concentration of Hg (II).
- The appeared peak at 1036 cm^{-1} corresponded to the interaction of C-O-C symmetric stretching mode. Further, it is also recorded that the number 1036 cm^{-1} appears to be due to the vibration connections to (-CN) extending including carbon of free structure and the amino group of nitrogen. Interestingly when fungal cells were treated with Cd II metal, the FTIR spectrum gained from fungal mycelia biomass displayed the value shift from 1036 cm^{-1} peak to 1023 cm^{-1} confirming that Cd (II) ion inclines to affect the C-O-C bond. Concurrently 965 cm^{-1} for Pb (II) treated biomass relate to C-O stretching beating. The absorption strip at 965 cm^{-1} is assigned to C-O stretching to ribose sugar and phosphodiester bond of DNA. The frequency region at 1140 to1185 cm^{-1} is mainly responsible for cellular protein and carbohydrate.

Optimized process parameters for Cd (II) and Pb (II) absorption by fungal biomass andExo polysaccharide (EPS)

- The Maximum metal removal ability by both dry biomass and EPS against Pb (II) and Cd (II) were recorded at an optimum pH of 6.0. At 30^{o}C higher percentage of metal removal was observed by the EPS in comparison to dry biomass at only 2 hrs of the incubation period.

- Interestingly, 90% metal removal was observed in optimized condition at a concentration of 2 mg/ml of biomass and EPS.

Biological assays

- The flocculating activity peak of 9.4 occurred at an EPS concentration of 0.5 mg/l corresponding to the flocculating rate of 88.4 %. The flocculating activity changes using MgO suspension showed a similar trend. Whereas the highest flocculating activity reached 29.4 and the flocculating rate was 82.2 % with EPS concentration of 5 mg/l.
- The major differences in the emulsification indexes were determined based on different emulsions having different EPS concentrations from 0.15 to 0.3 %. However, no significant variances in the emulsification index were found when the EPS concentration extended from 0.3 to 1 %, although at higher concentrations emulsion activity was to some extent increased.
- The results reported that *Aspergillus sp.* showed antibacterial activity followed by *Pythium sp., Fusarium sp., Rizopous sp.*, and *Penicillium sp.* against four reference humanpathogenic Gram-positive bacteria such as *E.coli, Vibrio cholerae,* and Gram-negative bacteria *Bacillus subtilis,* and *Staphylococcus aureus.*