

Exploring rhizosphere bacteria of *Eichhornia crassipes* for metal tolerance and biological activity

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Abstract - With urbanization and rapid industrial progress, water bodies around the world are getting highly polluted with metals. Bacteria have evolved several mechanisms to adapt to heavy metal contaminants thus representing a potential source for bioremediation process. The present study deals with isolation, identification and characterization of metal tolerant bacteria (MTB) from the rhizosphere of *Eichhornia crassipes* (water hyacinth) collected from the backwaters of Cochin (South India, 9°58' N, 76°17' E). Depending on the metals they tolerated (Pb, Cu, Fe, Zn, & Co), the metal tolerant bacteria (MTB) were labeled as Pb-1 to Pb-3, Cu-1 to Cu-4, Fe-1 to Fe-4, Zn-1 to Zn-4, and Co-1 to Co-2. No cultures tolerated and grew in the presence of Hg and Cd. Cultures were identified on the basis of 16S rRNA gene sequence analysis. Morphological and compositional studies were conducted using computer-assisted Scanning Electron Microscopy equipped with Energy Dispersive Spectroscopy (SEM/EDS). The maximum tolerable concentration (MTC) which is the highest concentration having minimum effect on the growth of the isolates were determined and found to be <2mM for CuSO₄, <3mM for ZnSO₄, <3.5mM for CoCl₂, <12mM for PbNO₃, and 25mM for FeCl₃. They were further screened for antibiotic sensitivity and biological activity according to Kirby-Bauer disc diffusion method. The MTB under metal stress condition showed significant biological activity against clinical pathogens, fouling bacteria and selective plant and fish pathogens indicating their biotechnological application in medicine, agriculture and aquaculture.

Keywords Rhizosphere; Metal tolerant bacteria; *Eichhornia crassipes*; biological activity

I. INTRODUCTION

Backwaters of Cochin are subjected to increasing human interference, laced with chemical industries, domestic sewage, coconut husk retting yards, port activities to mention a few. These industries eventually empty their wastes into the surrounding waters. Water hyacinth, *Eichhornia crassipes*, is a free-floating invasive perennial aquatic plant growing in all types of freshwater, often choking waterways with aerial leaves and thick heavily branched fibrous root system. Although they perish at higher salinity (especially during the pre-monsoons) they continue to grow when the salinity levels falls during the monsoons and post-monsoon seasons. Their thick mat which covers the entire surface of water bodies often causes oxygen depletion and fish kill. They have received considerable attention due to their ability to grow and thrive in highly polluted and stagnant waters, proving them to be highly resistant to toxicants and adverse water conditions [1-6]. The rhizosphere being the zone surrounding the roots of the *Eichhornia* plant, in which complex relations occur between

the root and the soil microorganisms, harbor bacteria possessing increased metal removal efficiency [7]. Although it is known to be the world's worst aquatic weed, there are areas where this plant and its associated microorganisms may find application in biotechnology that are useful to mankind [8]. The main aim of this study was to isolate, identify and characterize rhizosphere bacteria able to tolerate and grow in the presence of metals. An attempt has also been made to screen bacterial metabolites produced on exposure to metals by MTB for various antibiotic activity (including drug resistant pathogens). SEM/EDS analysis was used to provide elemental information of the sediment samples as well as of the bacterial cells on their exposure to metals.

Historically, several metals have been used as drugs and diagnostic agents to treat/diagnose several diseases and conditions [9-13]. For example, organo arsenic/bismuth compounds have been used for the treatment of syphilis and other infectious diseases, including colitis, gastritis, diarrhea etc [14-18]. Gold preparation like auranofin was used for oral antiarthritic purposes [19] and diagnostic agents for MRI (Gd, Mn, Fe) etc. Platinum compound cisplatin (cis-[Pt(NH₃)₂Cl₂]), carboplatin and oxaloplatin are among the most widely used cancer therapeutic agents [20,21]. Gold drugs myocrisin and auranofin are used as therapeutic agent and for the treatment of rheumatoid arthritis [22]. Myocardial tissue selectively takes up ^{99m}Tc which is used to image the heart. Radionucleolide ¹⁸⁶Re/¹⁸⁸Re also has therapeutic potential. Hence, biological activity of the metabolite formed by the metal tolerant bacteria when grown in the presence of metals was assayed and their probable use as metal-based antimicrobial agents in medicine, agriculture and aquaculture is summarized.

II. MATERIALS AND METHODS

A. Sample collection and isolation of metal tolerant bacteria

Ten young and healthy, freshly collected plant of *E. crassipes*, with adherent soil was collected from Cochin backwaters (9°58' N, 76°17' E) in sterile polyethylene bags using sterile spatula and transported to the laboratory in an icebox. On reaching the laboratory, the roots of the sample were rinsed in sterile distilled water (SDW) and observed under the microscope (Olympus BX43). Known weight of the soil (1g) associated with *E. crassipes* roots was diluted 10 to 10,000 fold in sterile distilled water and spread plated on Nutrient Agar (NA) plates (composition: peptone 0.5g, yeast extract 0.1g, KH₂PO₄ 0.01g, Agar 2.5g dissolved in 100ml deionized water) containing 50ppm of metal salts. Stock

solutions of metal salts (1000mg/L) were prepared in deionized water. The metal salts employed were $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, PbNO_3 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, HgCl_2 and CdCl_2 which were prepared separately and added (50ppm) to the medium after autoclaving and before bacterial cultivation. The respective metal tolerant bacteria (MTB) which grew on the plates after 24-72h were picked on the basis of colony morphology. Cultures in the presence of cobalt took 6-7 days to appear on the plates. Pure cultures were obtained using four way streaks on respective metal-containing nutrient agar plates. The cultures obtained from lead treated plates were called Pb-tolerant bacteria (PbTB) and designated as Pb-1, Pb-2 & Pb-3, on copper plates as CuTB and labeled as Cu-1, Cu-2, Cu-3 & Cu-4, Zn-tolerant bacteria (ZnTB) as Zn-1, Zn-2, Zn-3 & Zn-4, Fe-tolerant bacteria (FeTB) as Fe-1, Fe-2, Fe-3 & Fe-4 and Co tolerant bacteria (CoTB) as Co-1 & Co-2. No cultures grew on Cd and Hg-containing NA plates.

B. Study of colony, cell morphology and elemental composition

Isolated colonies of purified bacterial strain grown on solidified NA plates were observed and data was recorded regarding the form (circular, filamentous and irregular), elevation (flat, convex, and umbonate), margin (entire, undulate, erose and filamentous) and optical feature (opaque, translucent, and transparent). Colonies were visualized by Gram's staining using standard techniques and cell morphology was observed on an OLYMPUS BX Microscope and Scanning Electron Microscope (SEM, JOEL Model, 5800LV). Energy dispersive spectroscopic (EDS) analysis using Oxford ISIS 300 EDS instrument provided elemental information. For SEM studies the cultures were grown in 500ml flasks, each containing nutrient broth to which 1mM metal salt solution was added separately. Bacterial cultures were incubated at 27°C overnight (except for cobalt which took 5-7 days to grow). At the end of incubation period, cultures were transferred into centrifuge tubes and centrifuged for 3 minutes at 2500rpm to separate the cell pellets from the supernatant. The clear supernatant was discarded using disposable pipette and to this was added 0.1 M phosphate buffer, centrifuged and the process was repeated thrice. The bacterial cell surface was dehydrated by resuspending them in a series of ethanol solution (10, 30, 60, 75 and 100% ethanol). Cells were allowed to remain in 100% ethanol for 10 minutes in 3 intervals. The cells were finally dried at 37°C for 30 minutes and loaded onto stubs using double sided stickers. The samples on stubs were sputter coated with a thin layer of gold to minimizing charging during observation of bacteria using SEM.

Energy Dispersive Spectroscopic (EDS) technique utilizes high-energy spark created across gold plated sample of the material to be analyzed. The spark creates an emission of radiation from the excited sample surface with wavelengths characteristics of the elemental composition. The spectrum of radiation is separated into distinct element lines and the intensity of each line can be measured. Sample analysis was carried out in triplicates.

C. Molecular identification of metal tolerant bacteria (MTB)

For molecular identification of MTB, 16S rRNA gene sequence method was used [23]. DNA extraction was performed using HiPura Bacterial and Yeast Genomic DNA MiniPrep Purification Spit Kit – BB505-50PR15. The DNA extracted from the bacterial cultures were analyzed by gel electrophoresis using 0.8% agarose and running the gel using 1X TAE buffer and visualized in Ingenius Syngene Bio Imagine GelDoc. PCR amplification was performed in a total volume of 50µl using qTaq PCR master mix and 10 µl of the universal primer 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1501R (5' TGAACAGTTGACCATGCTGG 3'). PCR was carried out for 30 cycles of 95°C for 1 minute, 52°C for 40 seconds and 72°C for 2 minutes following initial denaturation at 95°C for 2 minutes. Final extension was carried out at 72°C for 7 minutes. The PCR amplification product was purified using AxyPrep™ PCR cleanup kit. Quantification of nucleic acid was done using Thermo Scientific Nanodrop 2000R. Sequencing of the PCR product was done using 3130XL Genetic analyzer (Applied Biosystems). The sequences obtained were used to identify the bacterial cultures. For this purpose their nucleotide similarity was compared to standard sequences BLAST (Basic Logic Alignment Search Tool) available at <http://www.ncbi.nlm.nih.gov/blast>. The phylogenetic and molecular evolutionary analyses were conducted using Mega version 5 [24]. A phylogenetic tree was constructed using neighbor-joining method and bootstrapped 1000 times to obtain the most probable tree. The nucleotide sequence was deposited in the GenBank database.

D. Screening level of metal tolerance in bacteria

All the 17 strains were checked for metal tolerance as described by [25]. Maximum tolerant concentration (MTC) is the highest concentration of metal which has minimum effect on the growth of the strain. To determine MTC of metal salts such as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, PbNO_3 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1M stock solution of each metal salt was prepared and appropriate dilutions (0.5mM, 1.5mM, 2.5mM, 3.5mM, 5mM, 10mM 15mM, 20mM and 25mM) were made. Bacterial isolates were streaked from NA plates and grown in nutrient broth medium containing different concentrations of metal salts and incubated at 37°C for 24-48h. Flasks in duplicate were incubated on a shaker (120rpm) and the growth of bacteria was tested through optical density measurement at 600nm using a UV-VIS Spectrophotometer (UV-2401 PC-S, Shimadzu instrument).

E. Test for antibiotic resistance of bacterial isolates

The bacterial isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method [26]. For this test, 10µg/disc of nine antibiotics (from Himedia) were selected. These included nystatin, streptomycin, nalidixic acid, actidione, rifampicin, trimethoprim, penicillin, neomycin, ampicillin and ketoconazole. The selected bacterial culture broth was spread (after 16 hrs growing condition) on Muller-Hinton agar plates and antibiotic discs were placed aseptically. All the plates were incubated at 37°C for 24 hrs. The zone of growth inhibition formed around each disc was measured in

millimeter using a vernier calliper and on the basis of presence/absence of growth inhibition zone classified as sensitive or resistant respectively. Control plates were incubated without antibiotic discs and all the experiments were carried out in triplicate.

F. Extraction of fermentation medium and screening of extract for activity

The metal tolerant bacterial isolates were grown in the laboratory in the presence and absence (control) of metal salts. At the end of incubation period (24 h for Cu, Pb, Fe & Zn and 144 h for Co), the cell free fermentation medium (after centrifugation) was dried in a lyophilizer at low temperature. To the dry mass thus obtained was added methanol (10 ml) and sonicated. This process was repeated 3-4 times to obtain the methanol soluble fraction (Me-fraction). The methanol fractions referred to as organic fraction was subjected to biological screening to understand their ability to inhibit the growth of fouling bacteria, clinical pathogen (bacteria and fungi), plant and fish pathogens (fungi). Clinical bacteria also included 2 drug resistant bacteria viz *Acinetobacter* sp., and Methicillin Resistant *Staphylococcus aureus*.

The 10 fouling bacteria used for screening were isolated from various substrata collected from the coastal waters of Goa, while the clinical pathogens were generously provided by Goa Medical College and assay was carried out at the Dept of microbiology, GMC). The fish and plant pathogens were provided by CAS (Centre of Advanced Studies in Marine Sciences, Dept of Parasitology, Tamil Nadu) and ICAR (Indian Council of Agricultural Research, Goa) respectively. All the pathogens used for screening are listed in Table 3. The assay was carried out by the disc diffusion method as described earlier [27,28].

III. RESULTS

Microscopic examination of the roots of *E. crassipes* showed bacterial cells embedded in aggregates (mucigel) around the roots (Fig. 1a).

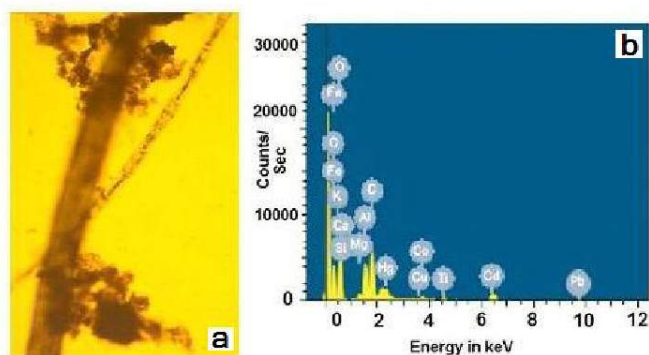


Figure 1 (a) Micrograph of *Eichhornia* root (10X) (b) EDS spectrum of sediment

Chemical microanalysis for elemental composition of rhizosphere soil particles from the *Eichhornia crassipes*-dominant region indicated the presence of Fe, Hg, Al, Mg, Ti, Cd, Zn, Co, Cu, and Pb (Fig. 1b). This was measured using energy and intensity distribution of x-ray signals generated by a focused electron beam on the specimen (EDS) analyzed on the scanning electron microscope (SEM).

The morphological details of the rhizosphere bacteria which could tolerate metals in the form of their salt solutions ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, PbNO_3 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, HgCl_2 and CdCl_2) are detailed in Table 1.

The bacterial cultures were identified by molecular methods and the PCR amplicons of bacterial isolates of the 16S rDNA genes is shown in Fig. 2A. A phylogenetic tree was constructed as shown in Fig. 2B and the identified bacteria had above 99% (99.56 to 99.93%) similarities with standard strains and the nucleotide sequences were deposited in the GenBank with accession numbers as shown in Table 2.

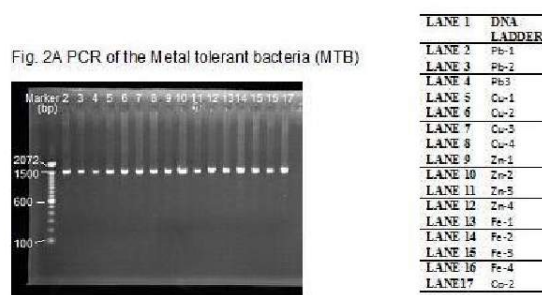


Figure 2A. PCR of the Metal Tolerant Bacteria (MTB)

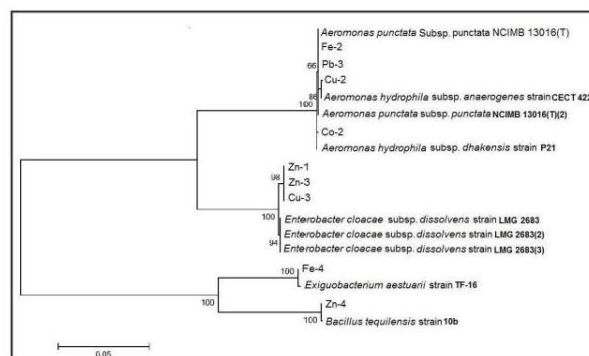


Figure 2B. Phylogenetic tree of the metal tolerant bacteria

Maximum tolerant concentration (MTC) estimated by recording the optical density (OD) of the fermentation media when grown in different concentrations of metal salts is represented in Fig. 3.

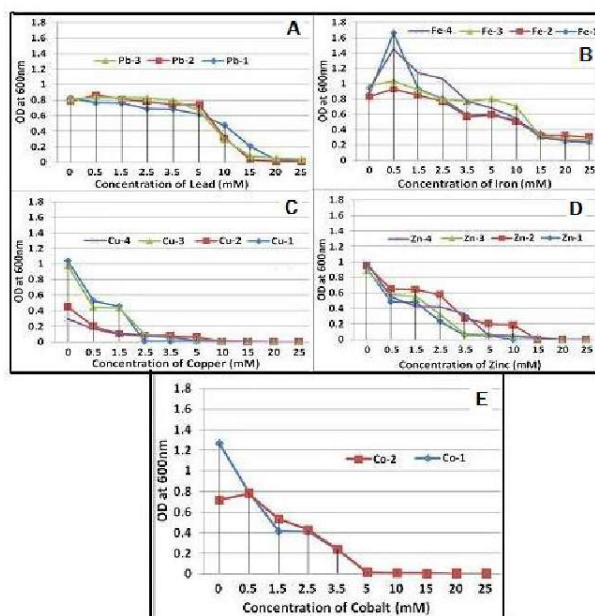


Figure 3. Maximum tolerant concentration (MTC) of various metal tolerant bacteria (MTB)

Among all the metal salts, copper was least tolerated (<2mM concentration) by bacteria followed by zinc (<3mM), cobalt (<3.5mM), lead (<12mM), and iron (<25mM) in the increasing order of tolerance. In most cases (salts of Pb, Cu, Zn & Co), good growth was evident in metal-free medium which gradually decreased with increase in metal salt concentration. However, in the case of iron tolerant bacteria (Fe-1 to Fe-4) better growth was evident when cultures were grown in the presence of FeCl_3 as compared to the control (when grown in its absence) as can be seen in Fig.3. All the iron tolerant cultures showed very marginal decrease in growth with increase in FeCl_3 concentration upto 25mM. In the case of Pb-tolerant bacteria also, there was no remarkable decrease in growth with increase in PbNO_3 concentration upto 5mM. However, with further increase in concentration to 10mM, there was a drop in the growth profile which showed almost no growth beyond a concentration of 20mM. In the case of salts of Cu, Zn and Co, the growth pattern was similar (Fig. 3) showing gradual decrease in cell number with increase in the respective metal salt concentration with no growth evident from 2.5mM for CuSO_4 , 5mM for CoCl_2 and 15mM for ZnSO_4 respectively. No cultures were able to grow in the presence of mercury and cadmium. SEM/EDS served well in determining that except for Cu, all other metals (Pb, Fe, Zn & Co) accumulated on the bacterial cell surface (Fig.4a-e).

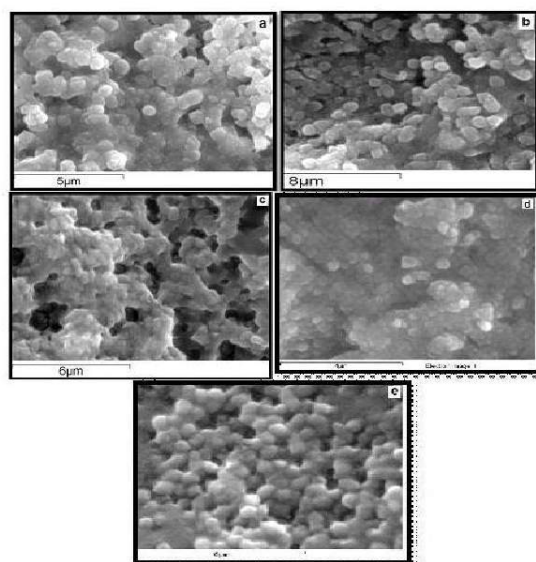


Figure 4a-e. SEM analysis of lead tolerant bacteria (a), copper tolerant bacteria (b) iron tolerant bacteria (c) zinc tolerant bacteria (d) and cobalt tolerant bacteria (e).

EDS analysis showed metal peaks at 20 kv representing the presence of Pb, Fe, Zn and Co (4A-E). Further, lysis of the cells, centrifugation and repeated examination using SEM/EDS indicated the metals to be of intracellular origin.

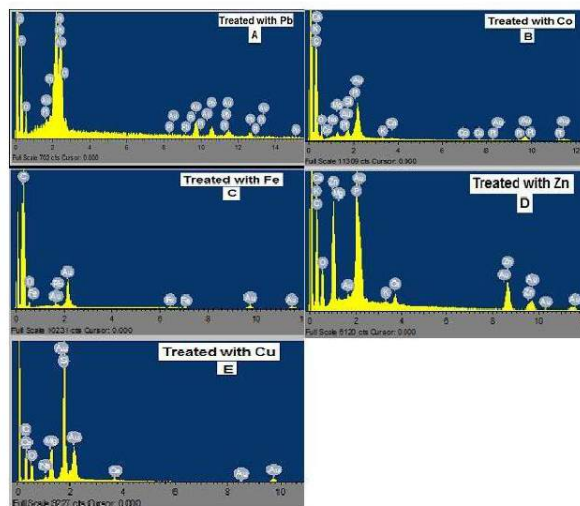


Figure 4A-E. EDS figures showing presence of Pb (A) Co (B) Fe (C) Zn (D) and undetectable Cu (E) on the cells surface.

Metal tolerant bacteria showed antibiotic resistance. The antibiotics employed were 10µg/disc of nystatin, streptomycin, nalidixic acid, actidione, rifampicin, trimethoprim, penicillin, neomycin, ampicillin and ketoconazole. Among the 3 Lead-tolerant bacteria all the three strains were sensitive to streptomycin while Pb-1 and Pb-2 were also sensitive to trimethoprim and Pb-3 was sensitive to rifampicin (Fig. 5).

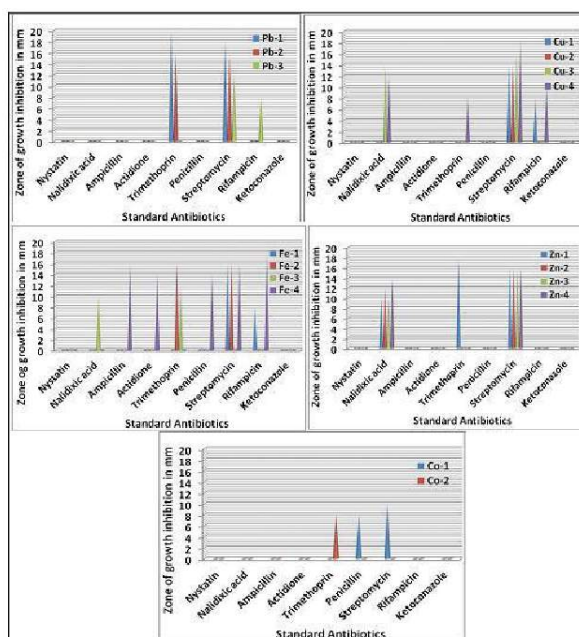


Figure 5. Antibiotic susceptibility of various metal tolerant bacteria (MTB).

In the case of copper-tolerant bacteria once again all the four isolates were sensitive to streptomycin and in addition, bacterial culture Cu-4 was also sensitive to nalidixic acid, trimethoprim and rifampicin while Cu-3 was sensitive to nalidixic acid and Cu-1 to rifampicin. All the remaining antibiotics under study had no effect on the growth of copper-tolerant bacteria. In the case of Iron tolerant bacteria a wide variation existed in the sensitivity to antibiotics. Fe-3 was the only bacterium which was resistant even to streptomycin but was sensitive to nalidixic acid and trimethoprim. The other three FeTB (Fe-1, Fe-2 & Fe-4) were sensitive to streptomycin and in addition Fe-4 was also sensitive to ampicillin, actidione, penicillin and rifampicin. Fe-2 was not only sensitive to streptomycin but also to trimethoprim and resistant to all other antibiotics used in the study. The 4 zinc tolerant bacteria were sensitive to nalidixic acid and streptomycin, in addition Zn-1 also showed sensitivity to trimethoprim. The cultures were resistant to all the other antibiotics used in the study. Finally, cobalt-tolerant bacteria (Co-1 and Co-2) were resistant to all the antibiotics except Co-1 which was sensitive to penicillin and streptomycin and Co-2 was sensitive to trimethoprim.

All bacterial isolates except Fe-3 & Co-1 were sensitive to streptomycin, 7 isolates were sensitive to trimethoprim and nalidixic acid each and 5 isolates were sensitive to rifampicin. However only 2 isolates were sensitive to penicillin (Fe-4 & Co-1), 1 isolate was sensitive to ampicillin (Fe-4) and actidione (Fe-4). None of the metal tolerant isolates had any effect on their growth in the presence of antibiotics nystatin and ketoconazole.

Screening of organic extracts of the metal tolerant bacterial isolates grown in the laboratory in the absence of metals (control) did not show any activity against test pathogens (negative results not shown). Screening of organic extracts of metal tolerant bacteria (MTB), when grown in the presence of

metal salts showed variable activity. Hence, it can be stated that the activity of the organic extracts could mainly be due to the metal-organic complex present in the fermentation medium or due to secondary metabolites produced by bacterial cultures when grown under metal stress conditions.

Fig.6 shows the antibiotic activity of the methanolic extract of the MTB against clinical pathogens.

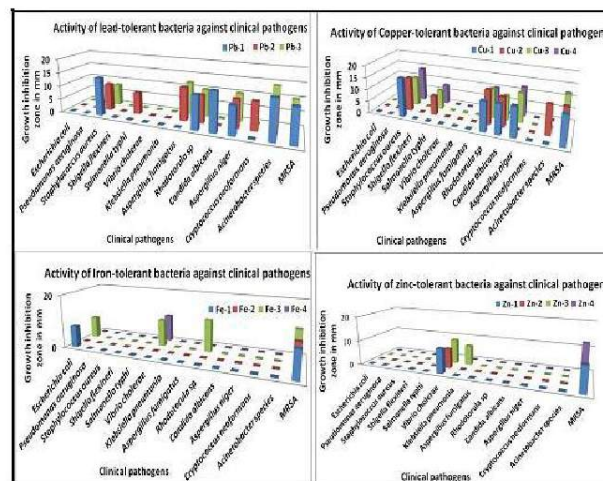


Figure 6. Antibiotic activity of metal tolerant bacteria (MTB) against clinical pathogens.

All the 3 Pb-tolerant cultures (Pb-1, Pb-2 & Pb-3) showed activity against *Staphylococcus aureus*, *Rhodotorula* sp. and *Aspergillus niger*. Pb-1 alone was active against fungal pathogen *Candida albicans* and along with Pb-3 showed activity against drug resistant bacteria *Acinetobacter* sp. and MRSA. Both Pb-2 and Pb-3 were also active against *Aspergillus fumigatus* and against *Cryptococcus neoformans* (Pb-2). The rest of the pathogens remained unaffected by the extracts of lead-tolerant bacteria.

When tested for antibiotic activity against clinical pathogens, organic extract of Cu-1, Cu-2, Cu-3 & Cu-4 showed mild to moderate activity against *Staphylococcus aureus*, *Salmonella typhi* (except Cu-1), pathogenic fungi *Rhodotorula* sp. *Candida albicans* (except Cu-4), *Aspergillus niger* (except Cu-2), drug resistant bacteria *Acinetobacter* sp. (except Cu-1, Cu-3 & Cu-4) and Methicillin resistant *Staphylococcus aureus* (except Cu-4). The organic extracts of all CuTB did not have any effect on the growth of pathogens *E. coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Vibrio cholerae*, *Klebsiella pneumoniae*, *Aspergillus fumigatus* and *Cryptococcus neoformans*.

The organic extract of the iron-tolerant-bacteria (Fe-1 to Fe-4) showed mild activity against pathogenic bacteria *E. coli* (Fe-1, Fe-3), *Vibrio cholerae* (Fe-3, Fe-4), pathogenic fungus *Rhodotorula* sp. (Fe-3) and significant activity against MRSA (Fe-1, Fe-2, Fe-3). Organic extract of the 4 zinc-tolerant bacteria did not show significant activity against most of the clinical pathogens except mild activity against *Vibrio cholerae* (Zn-1, Zn-2 & Zn-3), *Klebsiella pneumoniae* (Zn-3) and significant activity against MRSA (Zn-1 & Zn-4). Organic

extract of CoCl_2 treated cultures did not exhibit activity against any of the clinical pathogens tested.

Screening activity against fouling bacteria showed that neither copper nor lead tolerant bacteria showed any activity against fouling bacteria (Fig. 7), while ZnTB and FeTB showed variable activity against fouling bacteria used in the study.

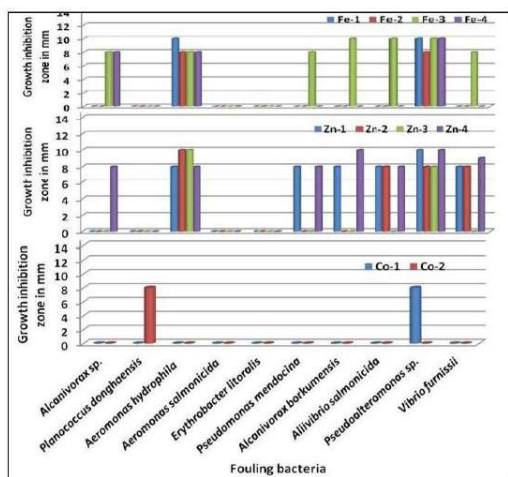


Figure 7. Antibiotic activity of metal tolerant bacteria (MTB) against fouling bacteria.

The organic extract of all the four FeTB showed activity against *Aeromonas hydrophila* and against *Pseudoalteromonas* sp. So also was the case with the four Zn-tolerant bacterial isolates (Zn-1, Zn-2, Zn-3 & Zn-4). The organic extract of Fe-3 showed a broad spectrum antibiotic activity against 7 of the fouling bacteria tested while Fe-4 showed antifouling activity only against *Alcanivorax* sp. Similarly, Zn-1 and Zn-4 also showed antifouling activity against 6 and 7 of the fouling bacteria tested respectively. Cobalt-tolerant bacterial isolates (Co-1 and Co-2) showed activity against *Pseudoalteromonas* sp. (Co-1) and *Planococcus donghaensis* (Co-2) respectively. None of the other isolates showed any antimicrofouling activity against fouling bacteria.

Fig. 8, shows activity of methanolic extract of metal tolerant bacteria against plant and fish fungal pathogens.

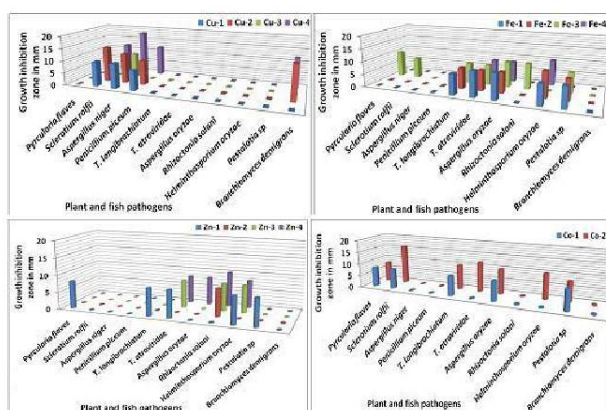


Figure 8. Antibiotic activity of metal tolerant bacteria (MTB) against plant and fish pathogens.

The organic extract of lead (Pb-1 & Pb-2) did not show any activity. However, Cu-1, Cu-2, Cu-3 and Cu-4 showed significantly strong activity against several plant pathogens (*Sclerotium rolfsii*, *Aspergillus niger* and *Penicillium piceum*) and against fish pathogen (*Branchiomyces demigrans*). All the other fungal pathogens tested showed resistance to the organic extracts of copper tolerant bacteria. Iron-tolerant-bacteria (Fe-3) showed mild to moderate activity against plant pathogens *Pyricularia flaves* and *Sclerotium rolfsii*. Mild activity against *T. longibrachiatum* (Fe-1, Fe-2, Fe-3), *T. atroviridae* (Fe-1, Fe-2, Fe-3 & Fe-4), *Aspergillus oryzae* (Fe-1, Fe-2, Fe-3 & Fe-4), *Rhizoctonia solani* (Fe-3), *Helminthosporium oryzae* (Fe-1, Fe-2 & Fe-4), and *Pestalotia* sp. (Fe-1, Fe-2 & Fe-3) was observed (Fig. 8). No activity was evident against fish pathogens by FeTB. When the organic extracts of zinc-tolerant bacteria were examined, mild activity was observed against several plant pathogens. No activity was evident against fish pathogens (Fig. 8). Cobalt-tolerant-bacteria (Co-1) showed activity against 5 plant pathogens while Co-2 showed activity against 7 plant pathogens. However, no activity was evident against fish pathogenic fungi.

IV. DISCUSSION

The narrow zone of soil around the plant root defined as the rhizosphere is a hot spot for microbial activity. Examination of the roots of *E. crassipes* showed (Fig. 1a) a kind of a symbiotic relationship between bacteria and the roots and these rhizospheric bacteria provide a crucial link between the plant and soil [29] and produce an immobilizing effect on the contaminants in the soil. They facilitate uptake of essential elements [30,31] and increase metal removal efficiency [7] and hence these bacteria may directly or indirectly influence metal uptake by *E. crassipes*. Metal bioremediation using metal tolerant bacteria is a widely discussed topic [32], however, very limited reports are available on the detailed antibiotic activity of metal tolerant bacteria against a wide range of pathogens for biotechnological application.

The rhizosphere soil when subjected to Energy dispersive spectroscopy (EDS) which is linked to Scanning Electron Microscopy (SEM) provided elemental analysis of the field of view (soil sample) indicating the presence of Fe, Hg, Al, Mg, Ti, Cd, Zn, Co, Cu, and Pb. (Fig. 1b). SEM/EDS is hence, considered a popular tool for the determination of trace metals in geological and environmental samples [33]. Sampling environment is known to play a significant role in determining the nature of microorganisms [34]. Hence, in the present study, the backwaters of Cochin dominant in water hyacinth, *Eichhornia crassipes* was sampled to obtain bacterial strains from the rhizosphere, possessing ability to grow in elevated metal concentrations (Pb, Cu, Fe, Zn and Co). Although, a number of metal contaminants existed in the study area the highly anoxic condition prevailing (during the growth of *E. crassipes*) created reducing condition with acidic pH below 7.0. At lower pH values the metals dissociate into its ions and become available to the organisms. At higher pH values the metals form metal carbonates and hydroxide complexes which control metal solubility. The anthropogenic sources contributed largely to the various metal contaminants in the said area. Among the metals studied, lead arose mainly due to the use of leaded gasoline (which accounted for approximately 90% of all

anthropogenic lead emission), manufacture of lead-containing products like lead based paint, lead solder in food cans, lead-arsenate pesticides etc. Since compounds of lead do not degrade, their concentration remains relatively high in the marine environment and the sink for lead is the soil/sediment. At pH above 6, lead exists as carbonates, or they exist as colloidal particles of lead oxide and lead hydroxide which limit the amount of soluble lead. Zinc is one of the most mobile metals because it is present as soluble compounds in an aquatic environment at neutral as well as acidic pH [35]. The main source of copper in the environment is from paints, mining activity, algicides, chromate copper arsenate, copper pipes etc. Under anaerobic condition, when sulfur content increases copper mostly forms CuS. Cu²⁺ sorbs strongly to mineral surfaces over a wide range of pH values [36]. The cupric ion Cu²⁺ is the most toxic species of copper [37]. Iron (Fe) is an essential micronutrient for marine organisms and it is well established that low Fe availability controls phytoplankton productivity [38]. However, at high concentration (>1ppm), it becomes toxic to the marine environment and microbes have known to well adapt to its high concentration. Co mostly exists in cationic form in seawater and can exist in oxidation states +II and +III in solution. Its mobility increases in acid soils, while it is significantly immobilized in organic soils. For all metals in addition to pH, its solubility also depends on the partial pressure of carbon dioxide and temperature [39,40].

In all, 17 bacterial cultures whose colony characteristics are detailed in Table 1 was isolated and among these 9 were identified using 16S rDNA methods. Most of the cultures belonged to genus *Aeromonas* followed by *Enterobacteriaceae* sp. and showed above 99% similarities with standard strains. Earlier report on *Enterobacteriaceae* isolated from rhizosphere of *E. crassipes* was reported by Zhang and group [41]. The purpose behind such studies was to explore the possibility of isolating metal tolerant bacteria for various biotechnological applications.

Maximum tolerant concentrations (MTC) of the 17 bacterial isolates were investigated using broth medium amended with different concentration of metal salts (CuSO₄.5H₂O, PbNO₃, FeCl₃.6H₂O, ZnSO₄.7H₂O, CoCl₂.6H₂O, HgCl₂ and CdCl₂). The ability of bacterial isolates to tolerate different metal salts was different. For example copper was least tolerated with maximum tolerant concentration for CuTB being <2mM. This was followed by zinc (<3mM), cobalt (<3.5mM), lead (<12mM), and iron >25mM) in the increasing order of tolerance (Fig.3). The ability of microorganisms to tolerate such high concentrations could be due to the variety of mechanisms specific to the metals and the organisms [42-47]. It was also observed that bacterial isolates (Fe-1 to Fe-4) grew better in the presence of Iron than when grown in the absence of Iron (control) and also FeTB could tolerate significantly higher concentration of Iron used as FeCl₃. This indicated that bacteria can control their iron requirements in response to iron availability and hence intracellular levels of free iron do not reach toxic levels [48]. The metals tolerated may either be incorporated inside the living biomass or biosorbed on the cell surface by different mechanisms [49]. SEM/EDS analyses of the bacterial cultures exposed to metal in the present study showed that except in the case of copper all other metals (Pb,

Fe, Zn and Co) accumulated on the bacterial cell surface (Fig 4a-e and 4A-E). In the case of CuTB, no copper was evident on the cell surface, careful lysis of the bacterial cells, centrifugation and repeated SEM/EDS analysis of the supernatant showed the metals (except copper) to occur intracellularly. This is in agreement with Burgos and group who also demonstrated that Cu is sequestered in the extracellular polymeric substances [50]. Hence, the method using SEM/EDS was found to be very suitable for the direct observation of heavy metal effects on bacterial cells. The unmatched combination of accuracy, high speed (maximum 2-3 minutes), precision (femtomolar level), stability (including biological samples) and reliability has made it an indispensable tool for such kind of studies [50].

When assayed for antibiotic sensitivity of the metal tolerant strains to 9 of the commonly used antibiotics, several strains showed resistance (Fig.5). Several metal tolerant strains were reported resistant towards one or more antibiotics indicating possible close association between metal tolerance and antibiotic resistance. This close association could be due to the fact that genes that code antibiotic resistance traits and that for metal resistance is stated to be carried on the same plasmid [51,52].

Furthermore, since multi-drug resistance in organisms are posing to be a huge threat in treatment procedure, the organic extract of the cell free fermentation media when grown in the presence and absence of metals was screened for biological activity against a list of pathogens. The test pathogens included clinical human pathogens (both bacteria and fungi) including multi drug resistant cultures (Fig. 6) fouling bacteria (Fig. 7), fungal-plant and fish pathogens (Fig. 8). Control flasks in which MTB was grown in the absence of metal salts did not show activity against any of the test pathogens unlike when grown in the presence of metals. This clearly indicated that the activity of the MTB is due to one of the following reasons. The compounds/products produced by the MTB under metal stress conditions or due to the metal-organic complex formed by bacteria when exposed to metal salts. As far as biotechnological application of these MTB are concerned, Zinc (ZnTB) and Iron tolerant bacteria (FeTB) and to a small extent Cobalt tolerant bacteria (CoTB) showed specific activity against fouling bacteria when compared to other MTB (lead, copper) (Fig. 7). Hence, this may provide an interest in probing innovative antimicrofouling technologies using MTB on improved coatings having application as antifouling agents. It may be expected that copper-tolerant bacteria show activity and resist the growth of fouling bacteria as it is a known fact that copper is generally toxic to bacteria and is many a time used as a positive control in antifouling studies [53-55]. However, this was not the case in the present study with CuTB not inhibiting the growth of fouling bacteria used in the study.

When tested against clinical pathogens (bacteria and fungi) the methanolic extracts of copper and lead tolerant bacteria showed better activity as compared to Fe and Zn extracts (which showed activity against fewer pathogens). On the other hand, Cobalt tolerant bacteria (CoTB) did not show any activity against clinical pathogens. When screened against plant and fish fungal pathogens, PbTB did not show any inhibitory effects. Better activity but against fewer pathogens

were exhibited by the Cu tolerant bacteria (Cu-1 to Cu-4). It was interesting to note that all the four CuTB showed activity against *Aspergillus niger* which is one of the most commonly occurring fungus, growing on almost all kinds of substrates. In addition, Cu-2 and Cu-4 were the only two isolates which showed activity against fish pathogen *Branchiomyces demigrans* which is responsible for causing fish disease, branchiomycosis, or gill rot in fish. Significant broad spectrum antifungal activity was also evident by Fe, Zn and Co tolerant bacteria. This remarkable difference in the activity of the MTB to different group of pathogens has led us to further study the nature of the metal-organic complex or the secondary metabolite produced when exposed to metal stress in greater details. Work is in progress to understand the chemical nature of all the MTB showing specific activity for application in medicine, agriculture and aquaculture.

TABLE II. MOLECULAR IDENTIFICATION WITH DEPOSITION NOS OF METAL TOLERANT BACTERIA (MTB)

Culture code	GeneSeq Acc. no.	Microbial culture Dep. No.	Bacterial strain	Similarity (%)
Pb-1	-	NIOBCC-Pb1TB	-	-
Pb-2	-	NIOBCC-Pb2TB	-	-
Pb-3	KJ847714	NIOBCC-Pb3TB	<i>Aeromonas punctata</i> subsp. <i>Punctata</i> NCIMB 3016(T)	99.93
Cu-1	-	NIOBCC-Cu1TB	-	-
Cu-2	KJ847715	NIOBCC-Cu2TB	<i>Aeromonas hydrophila</i> subsp. <i>anaerogenes</i> CECT 21(T)	99.93
Cu-3	KJ847716	NIOBCC-Cu3TB	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> LMG 2683(T)	99.56
Cu-4	-	NIOBCC-Cu4TB	-	-
Zn-1	KJ847719	NIOBCC-Zn1TB	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> LMG 2683(T)	99.64
Zn-2	-	NIOBCC-Zn2TB	-	-
Zn-3	KJ847720	NIOBCC-Zn3TB	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> LMG 2683(T)	99.64
Zn-4	KJ847721	NIOBCC-Zn4TB	<i>Bacillus tequilensis</i> 10b(T)	99.93
Fe-1	-	NIOBCC-Fe1TB	-	-
Fe-2	KJ847717	NIOBCC-Fe2TB	<i>Aeromonas punctata</i> subsp. <i>punctata</i> NCIMB 13016(T)	99.93
Fe-3	-	NIOBCC-Fe3TB	-	-
Fe-4	KJ847718	NIOBCC-Fe4TB	<i>Exiguobacterium aestuarii</i> TF-16(T)	99.85
Co-1	-	NIOBCC-Co1TB	-	-
Co-2	KJ847722	NIOBCC-Co2TB	<i>Aeromonas dhakensis</i> P21(T)	99.93

TABLE III. LIST OF PATHOGENS USED IN THE STUDY FOR SCREENING

Sr.No.	Nature of Pathogen	Name of Pathogen	Disease caused	Source
1	Bacterial pathogens	<i>Escherichia coli</i>	Gastrointestinal infection	Goa Medical College
2		<i>Pseudomonas aeruginosa</i>	Urinary tract infection (UTI)	
3		<i>Staphylococcus aureus</i>	Skin infection	
4		<i>Shigella flexineri</i>	Gastrointestinal infection	
5		<i>Salmonella typhi</i>	Typhoid	
6		<i>Vibrio cholerae</i>	Cholera	
7		<i>K. pneumonia</i>	Pneumonia/UTI	
8	Fungal Pathogens	<i>Aspergillus fumigatus</i>	Skin infection	
9		<i>Rhodotorula sp.</i>	Skin infection	
10		<i>Candida albicans</i>	Candidiasis	
11		<i>Aspergillus niger</i>	Skin infection	
12		<i>Cryptococcus neoformans</i>	Skin infection	
13	Multi-drug resistant bacteria	<i>Acinetobacter sp.</i>	UTI	
14		MRSA	Skin infection	
15	Fouling bacteria	<i>Alcanivorax sp.</i>	Microfouling	In-house NIO
16		<i>Planococcus donghaensis</i>	Microfouling	
17		<i>Aeromonas hydrophila</i> ATCC 7966	Microfouling	
18		<i>Aeromonas salmonicida</i>	Microfouling	
19		<i>Erythrobacter litoralis</i>	Microfouling	
20		<i>Pseudomonas mendocina</i>	Microfouling	
21		<i>Alcanivorax borkumensis</i>	Microfouling	
22		<i>Aliivibrio salmonicida</i>	Microfouling	
23		<i>Pseudoalteromonas sp.</i>	Microfouling	
24		<i>Vibrio furnissii</i>	Microfouling	
25	Plant pathogens	<i>Pyricularia flaves</i> sp.	Infects <i>O. sativa</i>	
26		<i>Sclerotium rolfsii</i>	Infects <i>S.uberosum</i>	
27		<i>Aspergillus niger gr</i>	Infects <i>P.guajava</i>	
28		<i>Penicillium piceum</i>	Infects <i>M. zapota</i>	
29		<i>Trichoderma longibrachiatum</i>	Infects <i>Mangifera indica</i>	
30		<i>Trichoderma atroviridae</i>	Infects <i>A.occidentale</i>	
31		<i>Aspergillus oryzae</i>	Infects <i>Triticum aestivum</i>	
32		<i>Rhizoctonia solani</i>	Infects <i>Amaranthus sp.</i>	
33		<i>Helminthosporium oryzae</i>	Infects <i>Oryza sativa</i>	
34		<i>Pestalotia sp</i>	Infects <i>C. nucifera</i>	
35	Fish Pathogens	<i>Branchiomyces demigrans</i>	Branchiomycosis (gill rot)	

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TABLE I Morphological characteristics of metal tolerant bacteria (MTB)

Isolates	Colour	Size	Shape	Elevation	Margin	Opaque	Consistency	Gram nature	Molecular identification
Pb-1	Brown	2mm	Circular	Raised	Entire	Opaque	Butyrous	positive	-
Pb-2	Cream	2mm	Circular	Raised	Entire	Opaque	Butyrous	negative	-
Pb3	Light brown	pinpoint	Circular	Raised	Entire	Semi translucent	Butyrous	negative	<i>Aeromonas punctata</i>
Cu-1	Off-white	pinpoint	Circular	Raised	Entire	Opaque	Butyrous	positive	-
Cu-2	Off-white	pinpoint	Circular	Raised	Entire	Opaque	Butyrous	negative	<i>Aeromonas hydrophila</i>
Cu-3	Off-white	2mm	Circular	Raised	Entire	Opaque	Butyrous	negative	<i>Enterobacter cloacae</i>
Cu-4	Off-white	1mm	Circular	Raised	Entire	Opaque	Butyrous	positive	-
Zn-1	Off-white	pinpoint	Circular	Raised	Entire	Opaque	Butyrous	negative	<i>Enterobacter cloacae</i>
Zn-2	Off-white	pinpoint	Circular	Raised	Entire	Opaque	Butyrous	positive	-
Zn-3	Off-white	1mm	Circular	Raised	Entire	Opaque	Butyrous	negative	<i>Enterobacter cloacae</i>
Zn-4	Off-white	1mm	Circular	Raised	Entire	Opaque	Butyrous	positive	<i>Bacillus tequilensis</i>
Fe-1	Orange	1mm	Circular	Raised	Entire	Opaque	Butyrous	negative	-
Fe-2	Orange	1mm	Circular	Raised	Entire	Opaque	Butyrous	negative	<i>Aeromonas punctata</i>
Fe-3	Orange	pinpoint	Circular	Raised	Entire	Opaque	Butyrous	negative	-
Fe-4	Orange	2mm	Circular	Raised	Entire	Opaque	Butyrous	positive	<i>Exiguobacterium aestuarii</i>
Co-1	Brown	1mm	Circular	Raised	Entire	Opaque	Butyrous	negative	-
Co-2	Brown	pinpoint	Circular	Raised	Entire	Opaque	Butyrous	negative	<i>Aeromonas dhakensis</i>