Chapter 2

Isolation, characterization and identification of soil microbial isolates

2.1. Background study:

Soil microbes were isolated and characterized from their natural mixed wasteland populations to observe their role in environmental heavy metal removal and soil-plant interaction. While previous report informed that soil dwelling microbes can manage plant metal absorption by controlling bioavailability and mobility of metal [Stefanis et al. 2013], the underground ecosystem of a region is totally controlled by the soil microbes. And soil microbes play a crucial role in controlling soil quality by decomposing organic matter. Inspecting the character of the soil is important because the basic soil physiology is the relevant indicator of the soil microbial community diversity [Tripathi et al. 2013; Lauber et al. 2009]. As, the soil microbe produce different extracellular substances like enzymes to combat environmental and climatic stress occurred due to human disturbance, these microbes can be applied in biotechnological management of forestry, agriculture, biodegradation of pollutants and waste management. That's because now a day different biochemical and molecular means of bacterial characterization have evolved to classify and identify soil bacteria according to their taxonomic position beside the conventional morphological characterization.

The Miller Luria Bertani Agar [Atlas, 1993] media was chosen to isolate and maintain soil microbes because it provides adequate nutrient, help in rapid microbial growth and can carry easily differentiable large number of colonies. Primary characterization of the isolated strain was done by Gram staining [Bartholomew and Mittwer, 1952]. In Gram staining, when the bacteria are stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of Gram-positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan, and the lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink which closes the pores in the cell wall and prevents the stain from exiting the cell. Thus, the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan in Gram-positive bacteria and therefore appears blue or purple in colour. In case of Gram-negative bacteria, the cell wall also takes up the Crystal Violet-Iodine complex but due to the thin peptidoglycan layer and an outer thick lipid layer, the Crystal Violet-Iodine complex gets washed off. When they are exposed to alcohol, the decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Upon subsequent staining with safranin, they take up the stain and appear red in color.

The isolated microbes also characterized by endospore producing capability. Endospore is tough non-reproductive structure which helps the microbe to remain dormant in stressful condition. Endospore staining is a differential staining technique (the Schaeffer-Fulton method) [Pelczar, 2007; Oktari, 2017] which is used to distinguish between the vegetative cells and the endospores. When the vegetative cells of certain bacteria such as *Bacillus* sp. and *Clostridium* sp. are subjected to environmental stresses, they produce metabolically inactive or dormant endospores within different areas of the vegetative cells in order to survive. Since endospores resist staining, the primary stain malachite green is forced into the endospores (i.e., malachite green enter the spore wall) by heating. In this technique, heat acts as a mordant. The isolated microbes can also be characterized according to their extracellular chemical formation. Because, different extracellular lytic enzymes and organic acids formation designate the metabolic capabilities and nutritional means of microbes based on which they can be classified accordingly.

Gelatinase test: The gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase, a proteolytic enzyme that liquefies gelatin [Tille and Forbes, 2014]. The hydrolysis of gelatin indicates the presence of gelatinases.

Catalase test: Catalase is an enzyme that converts hydrogen peroxide into water and oxygen. The bacteria that produce this enzyme are usually aerobic, that is they require oxygen, or facultative anaerobes that can survive with or without oxygen [Reiner, 2010].

Amylase test: Amylases are a group of enzymes that hydrolyze starch [Simon, 2008], and are produced by some bacteria as well as other organisms.

Fluorescence test: Different components of aerosol like allegen-containing droplets can be determined by fluorescence test to detect bioaerosol forming bacteria [Dartnell et al. 2013]. Bioarezolic bacteria can transmitted through air and form different adverse effect on human health.

Methyl red test: In the methyl red test (MR test), the test bacteria is grown in a broth medium containing glucose. If the bacteria have the ability to utilize glucose with the production of a stable acid, the colour of methyl red changes from yellow to red when added into the broth culture [Stovall and Nichols, 1918].

Urease test: Urea is the product of amino acid decarboxylation. The hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the change in the color of phenol red from light orange at pH 6.8 to pink at pH 8.1 [Brink, 2013].

Diffusible pigmentation test: Some bacteria can be recognized by the kinds of pigment they produce [Krieg and Padgett, 2011].

Bacterial antibiotics resistance pattern had also been investigated as additional information required for bacterial identification [Ryan, 2020]. Four antibiotic resistance mechanism panels viz. bacterial cell wall degradation (Ampicillin), translation inhibition (Tetracycline), protein synthesis inhibition (Doxycycline) and DNA replication inhibition (Norfloxacin) generally been investigated. [Maugeri et al.2019]. Finally, the microbe should be identified through molecular means of identification because molecular investigation plays a critical role in bacterial identification. The sequence similarity in the ribosomal genes indicates taxonomic position of the microbes [Harada et al. 2001].

2.2. Methodology:

2.2.1. Sample collection from selected sewage sites:

Soil containing sludge (from 6 cm - 10 cm depth) samples were collected in triplicate from three selected sewage regions of south, east and north Kolkata, viz. Circular Canal (Tolly Nullah) [22.57^{o}N; 88.36^{o}E] East Kolkata Wetland [22.55^{o}N; 88.45^{o}E] and Kestopur Khal [22.59^{o}N; 88.43^{o}E] at post monsoon season with the help of sterile falcon tube and air dried at 35^{o}C for further investigation.

The effluent discharges were also collected for Physico-chemical tests. The collected samples were stored in sterile pouch at 4^{o}C and Physico-chemical characterization was started within two hours of collection.

Figure 2.2.1: Soil samples were collected at post monsoon season, from three heavy metal depository sewage reservoirs in the south, east, and north of Kolkata, namely, [A] Circular Canal (Tolly Nullah), [B] East Kolkata Wetland, and [C] Kestopur Khal

2.2.2. Physico-chemical profiling of soil:

After monitoring colour and texture, soil samples were dried. Dry soil was diluted five times in 0.01 (M) sterile $CaCl_2$ solutions and soil pH was checked in electronic pH meter (GeNei) [APHA 14th Ed, 1975] accordingly.

The Chemical Oxygen Demand (COD) of the soil samples was measured by the open reflux method [EPA, 1975; ASTM, 1995]. In open reflux method, mercuric sulfate, potassium dichromate, and concentrated sulfuric acid were added to 50% diluted sewage water, refluxed

for two hours, and diluted to 150 ml. After cooling, the sample was titrated against 0.25 (N) FAS (ferrous ammonium sulphate) using Ferroin as the indicator. The end point of the titration was indicated by the keen change of color from blue-green to wine red.

The quantity of Dissolved Oxygen (DO) in the collected sewage sludge was measured by the Azide modification method [Kroner et al. 1964; EPA, 1975; ASTM, 1976]. To conduct Azide modification methodology, 300 ml of the freshly collected sample was mixed thoroughly with an equal volume of manganese sulfate and alkaline iodide azide solution to obtain the precipitate. The settled precipitate was then dissolved in concentrated sulfuric acid and titrated against sodium thiosulphate using starch as an indicator.

In order to measure the soil dehydrogenase activity (DHA), 5 g soil was dissolved in 5 ml of 0.8% triphenyl tetrazolium chloride (TCC) and a few drops of 5% sodium sulfate was added to the mixture. Dissolved soil was centrifuged after one hour of dark shaking at 30°C, and the color intensity of the filtrate was measured by a spectrophotometer. The percentage of enzyme inhibition by the heavy metals was calculated from the standard curve prepared using a solution of tryphenyl formazan and ethyl alcohol [Weddle and Jenkins, 1971]. The dehydrogenase activity of the soil samples was measured by plotting the optical density (O.D.) values at 450 nm in the standard curve.

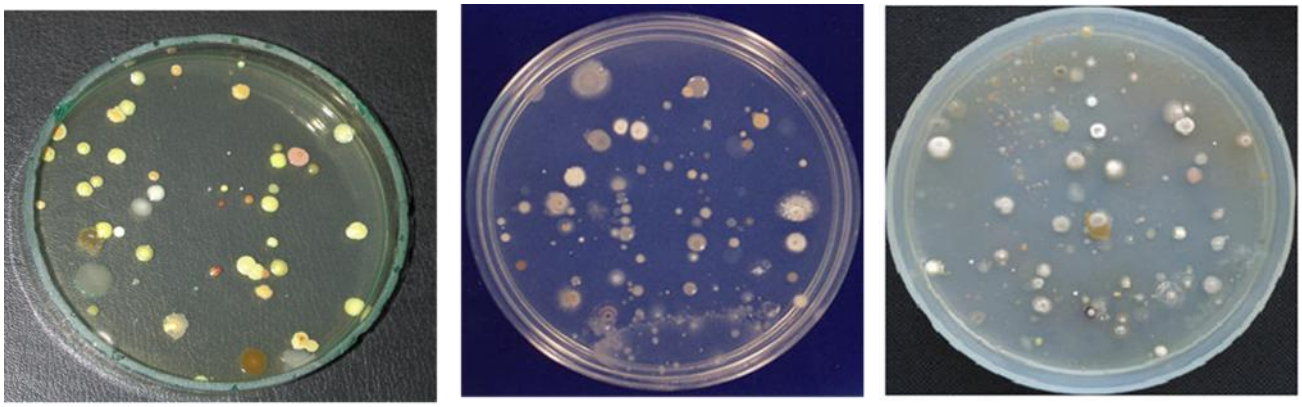
Figure 2.2.2: Pink colour developed in TCC and sodium sulphate containing soil filtrate after dark incubation due to dehydrogenase activity

The normalized values of four different sewage physico-chemical parameters were used to correlate them with microbial colony forming unit (CFU).

2.2.3. Isolation of soil bacteria:

One gram of soil samples collected from each sewage sites in triplicate, were dissolved in 10 ml of 0.85% NaCl containing sterile water and serially diluted. From each dilution (10^{-1} to 10^{-11}), 1 ml of sample was spread on Luria Burtani agar plates, followed by incubation at 37°C for 24 to 48 hours to obtain the number of colony forming units (CFU) [Brugger, 2012]

per gram of soil. The pure colonies, obtained from 10^{-7} times diluted soil suspension, were separated based on colony morphology and purified by repeated streaking. From the properly distinguished colonies, 54 microbial strains were differentiated by their colony morphology, streaked on master plate and also preserved in 80% glycerol stock for further experiments.



[A] [B] [C]

Figure 2.2.3: Bacterial isolates were obtained by spreading 10^{-7} times diluted soil samples collected from [A] East Kolkata Wetland, [B] Circular Canal and [C] Kestopur Khal

2.2.4. Morphological identification of the soil microbes:

The cellular morphology of the isolated microbes was determined by the conventional Gram staining procedure [Bartholomew and Mittwer, 1952] in which the bacteria were stained with crystal violet and safranin (counter stain) and observed under high power objective lens (Accu-scope). The endospore-forming capability of the microbial isolates was studied by the Schaeffer-Fulton method [Pelczar, 2007; Oktari, 2017] where Malachite green was used as the primary stain.

2.2.5. Biochemical characterization of the soil microbes:

The different bacteria produced extracellular substances which were monitored using different commercially available supplementary media and subjected to different biochemical tests, described hereafter:

Gelatinase test: Stab inoculates were prepared in nutrient gelatin tubes [MacFaddin, 1985] by puncturing agar columns. After freezing, the solidity of the medium was inspected.

Catalase test: 1% H_2O_2 was applied on the microbial colonies and the appearance of effervescence indicated the presence of the catalase enzyme.

Amylase test: 1% soluble starch supplemented nutrient agar [Wehr, 2004] plates were incubated at 28°C for 48 hours. A clear red zone around bacterial colonies after iodine application indicated starch hydrolysis.

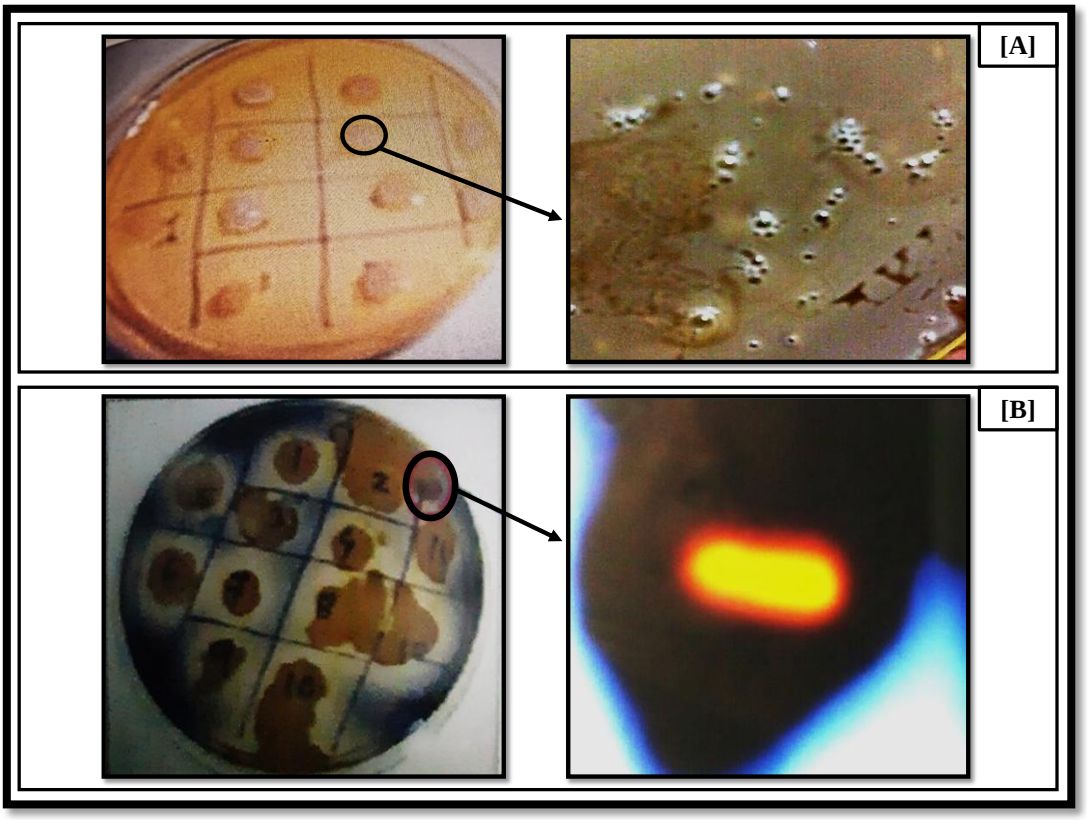


Figure 2.2.4: Extracellular enzymes formation by the bacterial isolates; [A]: Appearance of effervescence on the microbial colony after addition of 1% H_2O_2, indicated the presence of enzyme catalase [B]: After addition of 1% soluble starch, the clear red zone surrounding the bacterial colony had indicated the presence of enzyme amylase

Fluorescence test: King's B medium [King, 1954; FDA, 9th Ed. 1998] plates were incubated at 28°C. After 48 hours of incubation, the plates were placed in a UV chamber for analyzing the fluorescence characteristics of microbial isolates.

Methyl red Voges-Proskauer test: A few drops of methyl red indicator were applied onto inoculated MRVP broth [Voges and Proskauer, 1898; Clark and Lubs, 1915] tubes and the change of color was observed after 48 hours incubation at 35°C.

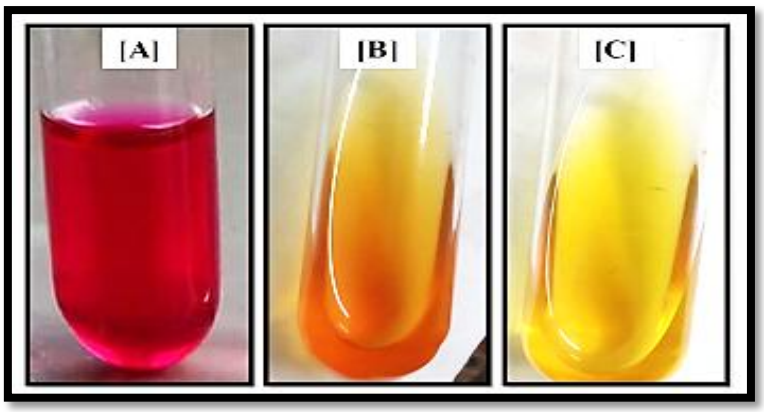


Figure 2.2.5: After application of methyl red to MR-VP (Methyl Red - Voges Proskauer) broth tubes, the change of colour denoted the pH of culture media and confirmed whether the bacterial isolates had taken part in mixed acid by fermentation or not. [A]: Development of the cherry red colour designated that the microbe went through acid fermentation and because of that the media became acidic (pH <4.5); [B] - [C]: orange to yellow colour development indicated that the pH of media raised towards neutrality (pH >6) and the respective microbe didn't taken part in mixed acid fermentation

Urease test: Phenol red supplemented urea agar medium [Christensen, 1946; Rustigian and Stuart, 1941] was inoculated and monitored the colour change was monitored after 48 hours of incubation.

Diffusible pigmentation test: In order to detect pigmentation, inoculated plates containing King's B medium [King, 1954; FDA, 9th Ed. 1998] were observed after first day and after six days of incubation.

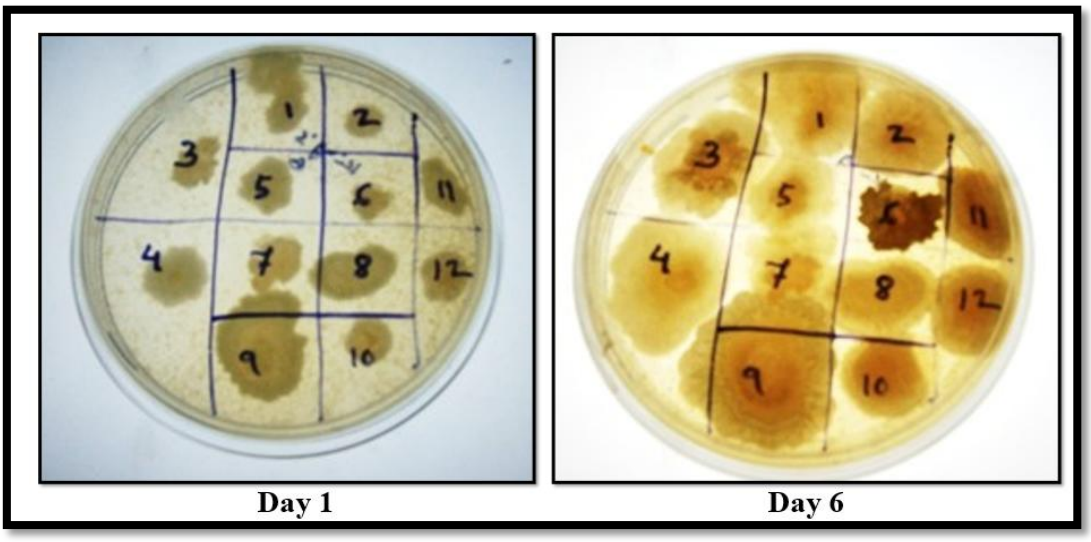


Figure 2.2.6: Diffusible pigment formation was detected amidst King's B media after first and sixth days of incubation

2.2.6. *Antibiotic screening*:

Four antibiotics viz. ampicillin (10 µg/ml), norfloxacin (10 µg/ml), doxycycline (30 µg/ml), tetracycline (30 µg/ml) were used to characterize microbial isolates. To measure bacterial resistance or sensitivity, antibiotic discs were applied to the Mueller Hinton Agar [Mueller and Hinton, 1941; Bauer et al. 1966; Ericsson and Sherris, 1971] supplemented with 10^{-2} times diluted mid log phase Mueller Hinton suspension culture [Mueller and Hinton, 1941; Jorgensen et al. 2015; Murray et al. 2003]. The zone of inhibition against different antibiotics was standardized by the commercially available National Committee for Clinical Laboratory Standard's (NCCLS) chart [NCCLS 5th Ed. 2000].

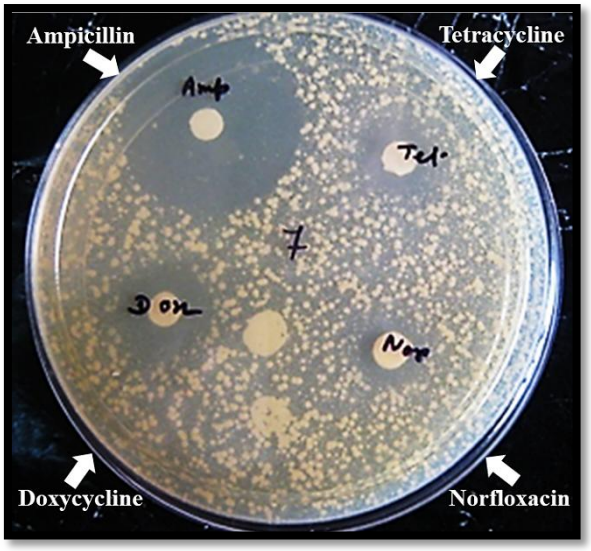


Figure 2.2.7: One of the isolated sewage bacteria manifested its resistance pattern against four applied antibiotic discs by forming clear zone of inhibition

2.2.7. Molecular characterization by 16S rDNA sequencing:

The bacterial genomic DNA was isolated with the microbial DNA isolation kit (GCC Biotech) according to the manufacturer's protocol, and the 16S rRNA region was amplified using BDT v3.1 cycle sequencing kit and 27F forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R reverse (5'-GGTTACCTTGTTACGACTT-3') primers. The purified PCR product was sequenced using ABI 3730Xl DNA analyzer executing Sanger sequencing method [Gupta et al. 2012].

The 16S rDNA sequences were annotated using Chromas Lite software (version 2.01), and their near relatives and taxonomic position were identified by the NCBI MEGABLAST algorithm [Altschul et al. 1990; Zhang et al. 2000]. Multiple sequence

alignments, phylogenetic analyses and molecular evolutionary studies of the 16S sequences were performed using the Clustal Omega [Maderia et al. 2019] and MEGA10 [Tamura et al. 2013; Kumar et al. 2018; Kimura, 1980]. The partial 16S rDNA sequences acquired herein were submitted to the NCBI GenBank database, and their accession numbers were subsequently obtained. The obtained microbial genera were compared with Bergey's manual of determinate bacteriology [Bergey and Holt, 2000] accordingly to fulfil the criteria of polyphasic microbial classification.

2.2.8. Standardize the optimal microbial growth condition:

The bacterial isolates were grown under different initial pH (6.0, 6.4, 6.8, 7.0, 7.2, and 7.4) and different thermal environments (27°C, 37°C, and 47°C) in LB broth media for 24 hours to standardize their suitable growth condition. The efficiency of bacterial growth was assessed by measuring the colour intensity and turbidity of different solutions were measured using visible spectrophotometer (Systronics 106) and UV-VIS spectrophotometer (Jasco V530) at 595 nm wavelength.

2.2.9. Statistical analysis:

Each experiment was repeated in triplicate. The data were standardized using one-way ANOVA and two tailed t test. Significant differences were compared using at $P \leq 0.05$ significance level (and $p < 0.01$ high significance level) and r value between +1 and -1 using Microsoft Excel and SPSS software.

2.2.10 Composition of used media:

2.2.10.1. Luria Burtani agar, Miller:

Ingredients	**g/liter**
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00
Agar	15.00
Final pH (at 25^{o}C)	7.5±0.2

2.2.10.2. Luria Burtani broth, Miller:

Ingredients	**g/liter**
Tryptone	10.00
Yeast extract	5.00

Sodium chloride	10.00
Final pH (at 25°C)	7.5±0.2

2.2.10.3. Nutrient gelatin:

Ingredients	**g/liter**
Peptone	5.00
Beef extract	3.00
Gelatin	120.00
Final pH (at 25°C)	6.8±0.2

2.2.10.4. Nutrient agar:

Ingredients	**g/liter**
Beef extract	3.00
Peptone	5.00
Sodium chloride	8.00
Agar	15.00
Final pH (at 25°C)	7.3±0.2

2.2.10.5. King's B medium:

Ingredients	**g/liter**
Proteose peptone	20.00
Dipotassium hydrogen phosphate	1.50
Magnesium sulphate	1.50
Agar	15.00
Final pH (at 25°C)	7.2±0.2

2.2.10.6. MRVP broth:

Ingredients	**g/liter**
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH (25°C)	6.9±0.2

2.2.10.7. Urea agar media:

Ingredients	g/liter
Dextrose	1.00
Peptic digest of animal tissue	1.00
Sodium chloride	5.00
Monopotassium phosphate	2.00
Urea	20.00
Phenol red	0.012
Agar	15.00
Final pH (25^{o}C)	6.8±0.2

2.2.10.8. Mueller Hinton broth:

Ingredients	g/liter
Beef infusion	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Final pH (25^{o}C)	7.3±0.1

2.2.10.9. Mueller Hinton agar:

Ingredients	g/liter
Beef infusion	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH (25^{o}C)	7.3±0.1

2.2.11. Composition of used buffer:

2.2.11.1. Tris acetate EDTA buffer (50X):

Ingredients	Amount/Liter
Tris acetate base	242.00 g
Glacial acetic acid	57.1 ml
0.5 (M) EDTA	100 ml
Final pH (25^{o}C)	8±0.3

2.3. Results:

2.3.1. Physico-chemical nature of soil:

Inspecting the character of the soil is important because the basic soil physiology is the relevant indicator of the soil microbial community diversity [Tripathi et al. 2013; Lauber et al. 2009]. The sewage soil of Kestopur Khal and Tolly Nullah were blackish, while that of East Kolkata Wetland was greyish in colour (Figure 2.3.1).

[A] [B] [C]

Figure 2.3.1: Soil samples collected from different sewage sites (A: East Kolkata Wetland; B: Circular Canal; C: Kestopur Khal)

Different soil physico-chemical parameters like soil pH, Dissolved Oxygen (DO), Chemical Oxygen Demand (COD), Dehydrogenase activity (DHA) were measured and compared (Figure 2.3.2).

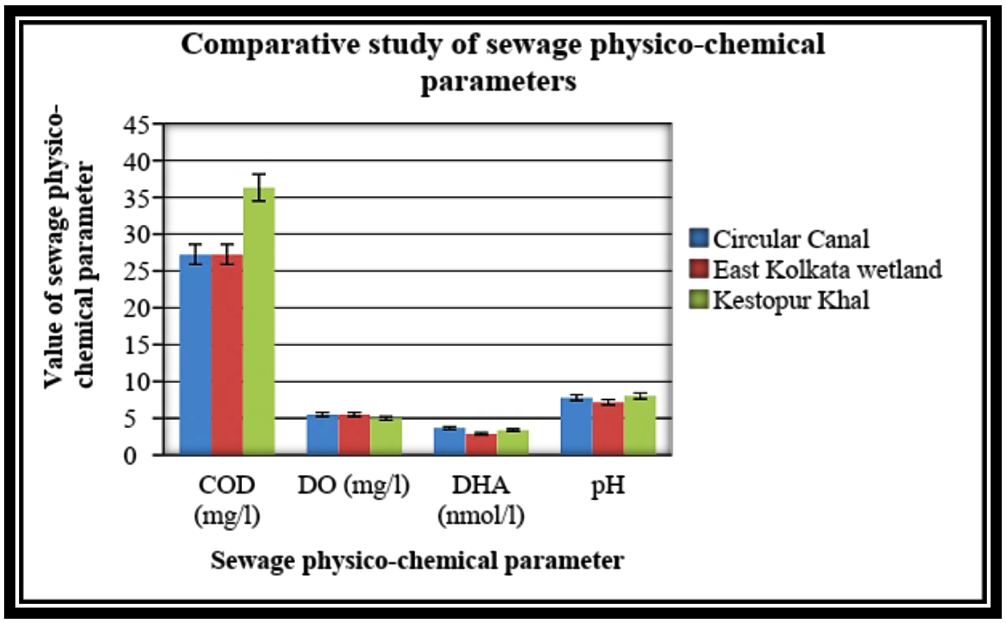


Figure 2.3.2: Comparative study of different soil physico-chemical parameters from different sewage sites

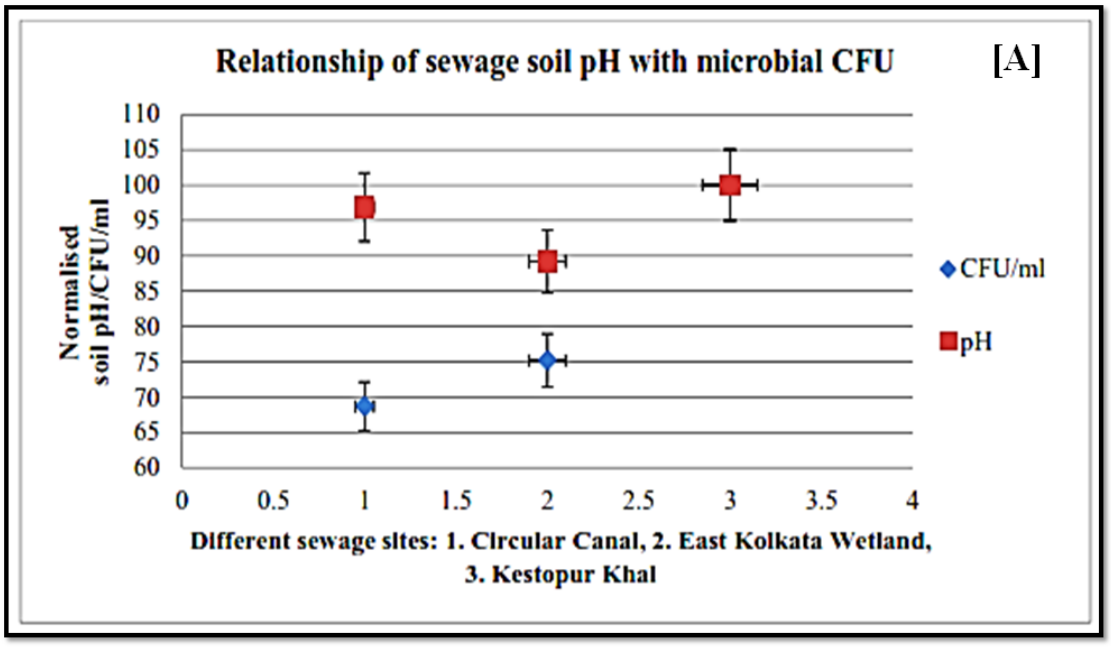
The pH of the soil samples collected from the different sewage regions were found to be neutral to slightly alkaline (7.8±0.05, 7.18±0.25, and 8.05±0.02 at Circular Canal, East Kolkata Wetland, and Kestopur Khal, respectively). Owing to the suitability of mineral

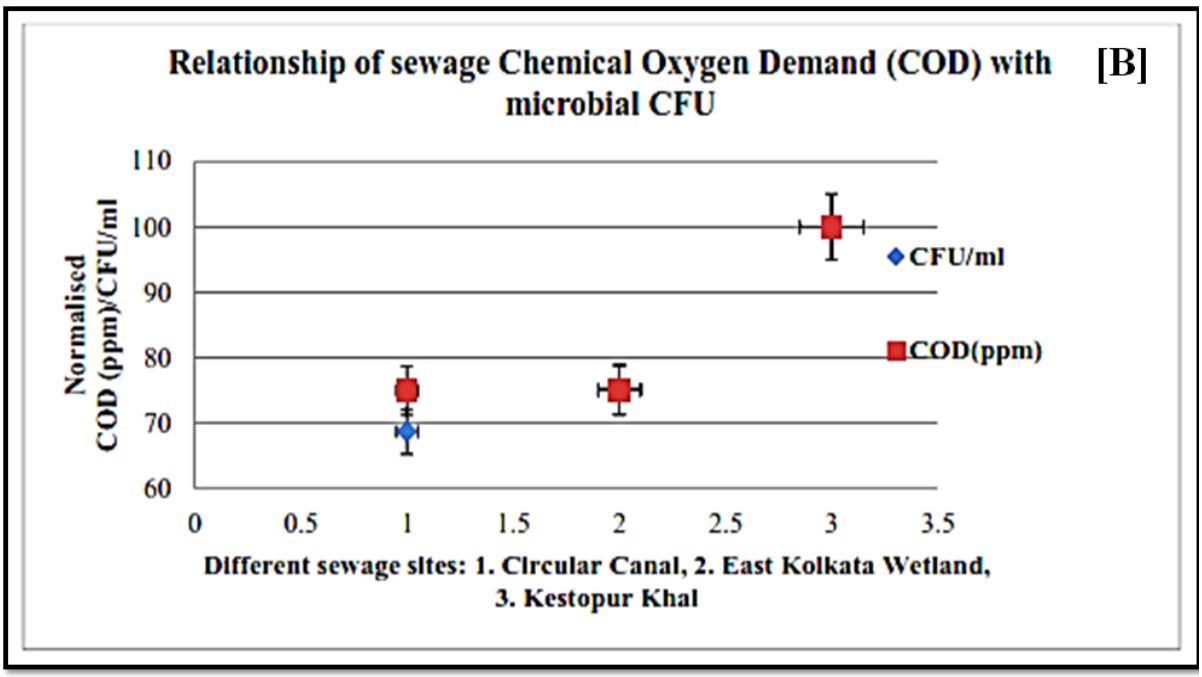
constituents and the availability of carbon source, generally, pH values ranging between 4.5 and 8.3 are optimum for microbial growth and our result showed the same trend (Figure 2.3.3 A).

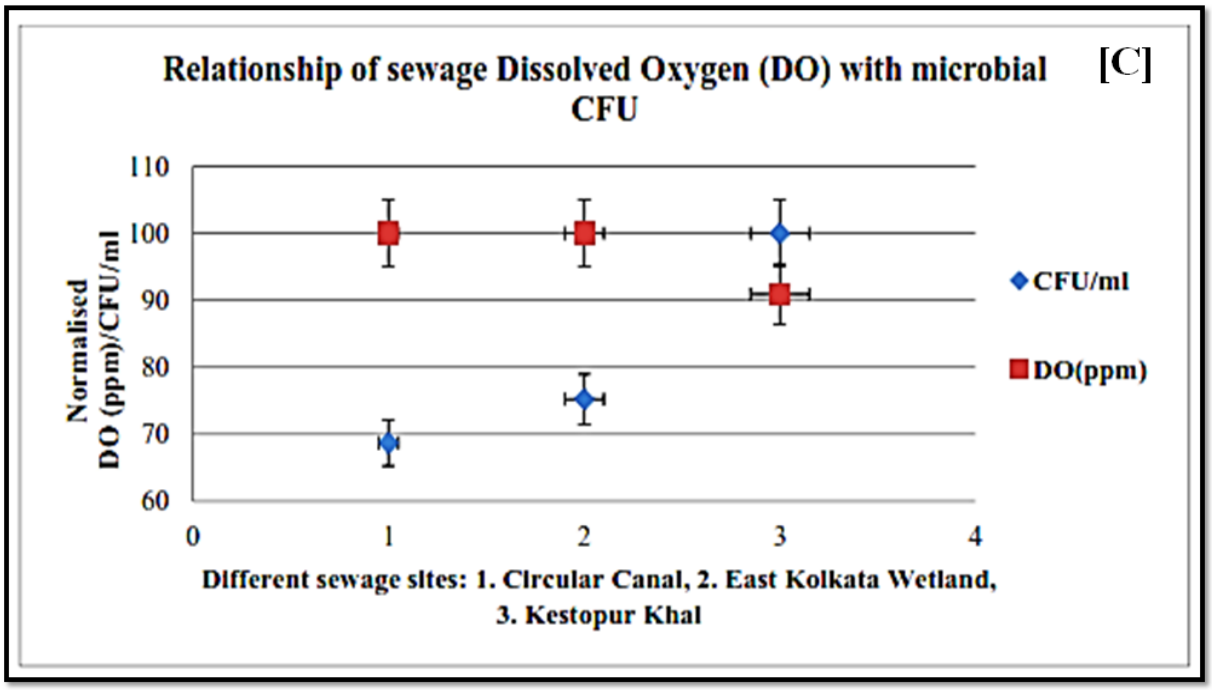
COD of sewage is the amount of oxygen consumed mainly by the sewage bacteria. The COD of the sewage effluent of Kestopur Khal was much greater (36.3±1.1 mg/l) than the effluents collected from the two other sewage regions (27.25±0.9 mg/l and 27.25±1.3 mg/l), indicating that the soil of Kestopur Khal is more polluted than those of the other two regions. The results revealed that the sewage sample of Kestopur Khal contained a greater amount of oxidizable organic material (Figure 2.3.3 B).

The concentration of DO in the sewage water of Circular Canal, East Kolkata Wetland and Kestopur Khal was 5.5±0.01 mg/l, 5.5±0.5 mg/l and 5±0.001 mg/l, respectively. Amount of DO reduced gradually with microbial growth because microbes utilized it to decompose organic matter (Figure 2.3.3 C).

The value of soil DHA was much greater at Circular Canal and Kestopur Khal (3.7±0.9 nmol/l and 3.4±1.0 nmol/l, respectively) than at East Kolkata Wetland (2.9±0.7 nmol/l). Lower soil DHA indicated that the three wastewater bodies herein were contaminated with heavy metals and amongst the three East Kolkata Wetland was highly polluted (Figure 2.3.3 D).




Relationship of sewage Chemical Oxygen Demand (COD) with microbial CFU
[B]
Normalised COD (ppm)/CFU/ml
110
100
90
80
70
60
0
0.5
1
1.5
2
2.5
3
3.5
CFU/ml
COD(ppm)
Different sewage sites: 1. Circular Canal, 2. East Kolkata Wetland, 3. Kestopur Khal


Relationship of sewage Dissolved Oxygen (DO) with microbial CFU
[C]
Normalised DO (ppm)/CFU/ml
110
100
90
80
70
60
0
1
2
3
4
CFU/ml
DO(ppm)
Different sewage sites: 1. Circular Canal, 2. East Kolkata Wetland, 3. Kestopur Khal

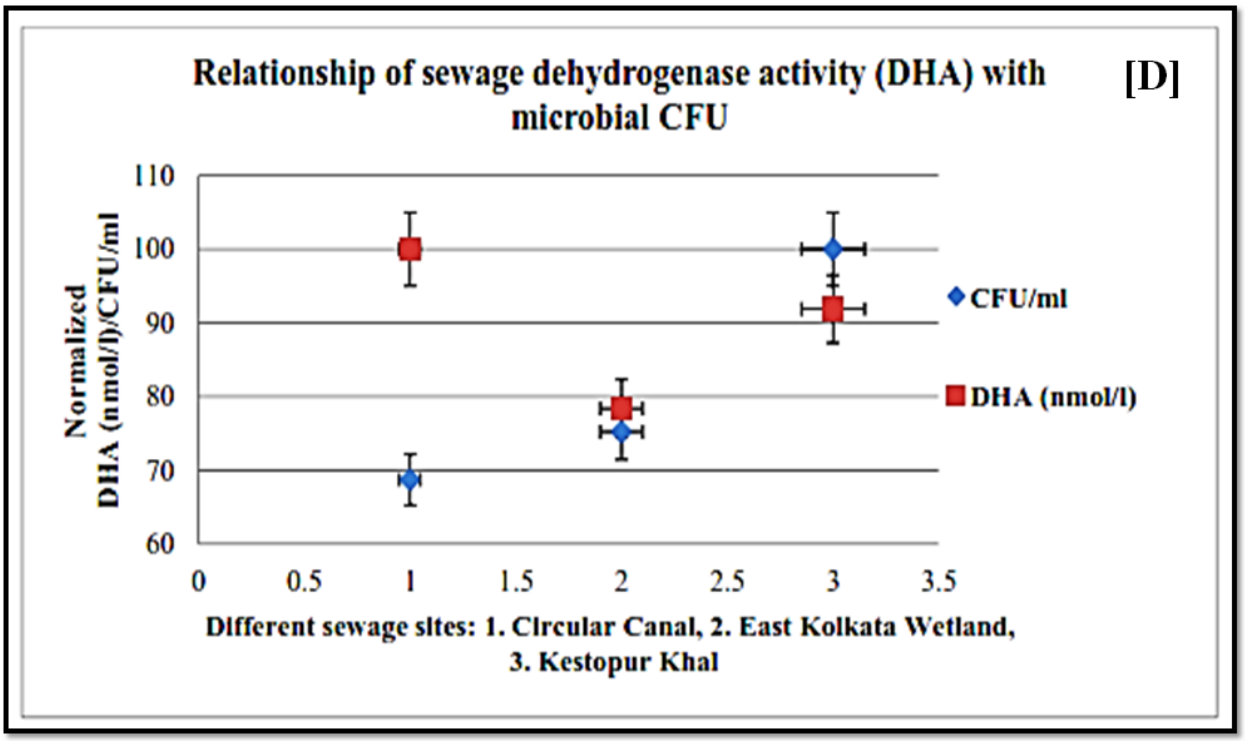


Figure 2.3.3 (A-D): Relationship of different soil physico-chemical characters (normalized) with bacterial Colony Forming Unit (CFU/ml) shown that some parameters (pH and COD) had related positively [A & B] and some others (DO and DHA) had negatively related [C & D].

The results presented in Table 2.3.1 indicate that at the sewage sites of Kolkata, bacterial CFU is positively correlated with pH (r = 0.57; p = 0.612) and COD (r = 0.98; p = 0.126) and negatively correlated with DO (r = -0.98; p = 0.126) and DHA (r = -0.05; p = 0.967).

Table 2.3.1: Relationship between Colony Forming Unit (CFU) and different colony forming parameters:

Soil Parameter	Correlation with CFU (r value)	Significance (p value)
Chemical Oxygen Demand (COD)	0.98	0.126
Dissolved Oxygen (DO)	-0.98	0.126
Soil pH	0.57	0.612
Soil Dehydrogenase (DHA) activity	-0.05	0.967

2.3.2. Morphological characters of the isolated soil microbes:

Forty-seven soil microbes isolated from different sewage regions, were chosen first by observing their colony morphology. The microbes were then differentiated in terms of colony characteristics, including colony form, elevation, surface, edge, colour (Figure 2.2.3).

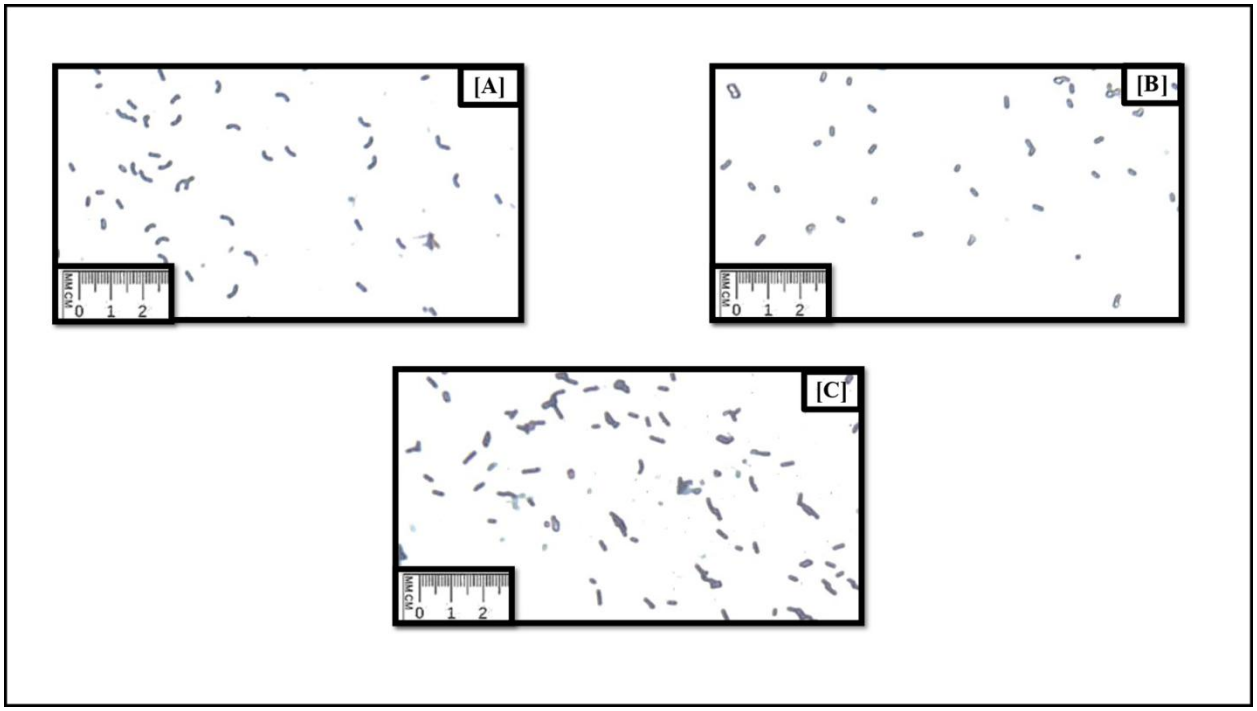


Figure 2.3.4: Gram-stained population [500X] of isolate 12C [A], 1E [B] and 26K [C] which later identified as strain *Microbacterium radiodurans* K12016 [A], *Bacillus xiamenensis* 1E0018 [B], and *Bacillus xiamenensis* 26K018 [C]

From the properly distinguished single colonies, microbial strains were selected on the basis of their colony morphology (Figure 2.3.4; Figure 2.3.5.). Most of them exhibited the presence of entire edges, smooth surfaces, flat elevation, circular to elongated form, and rapid growth (Table 2.3.2).

The cellular morphology of the isolated microbes was determined that most of the bacterial isolates were Gram positive rods and possessed endospore-forming ability (Table 2.3.2).

Table 2.3.2: Morphological characterization of isolated soil bacteria:

Strain No.	Colony Character		Cell Character		
	Color	Nature	Gram Nature	Shape	Endospore
1-C	Straw yellow	Rapid, entire, circular, smooth, flat	Gram +ve	Cocci	Present
2-C	Whish orange	Rapid, entire, circular, smooth, concave	Gram +ve	Rod	Present
3-C	Orange	Less rapid, entire, circular,	Gram +ve	Rod	Present

		rough, flat			
4-C	Yellow	Rapid, entire, circular, smooth, flat	Gram –ve	Cocci	Present
5-C	Whitish yellow	Less rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
6-C	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
7-C	Whitish yellow	Rapid, slightly wavy, circular, smooth, flat	Gram +ve	Rod	Present
8-C	Translucent	Rapid, entire, circular, smooth, concave	Gram –ve	Rod	Present
9-C	White	Rapid, entire, circular, smooth, flat	Gram +ve	Cocci	Present
10-C	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Long rod	Present
11C	Yellow	Rapid, entire, circular, smooth, flat	Gram +ve	Rod	Present
12-C (*Microbacterium radiodurans* K12016)	Grayish white	Rapid, wavy, circular, rough, flat	Gram + ve	Short Rod	Present
1-E (*Bacillus xiamenensis* strain 1E0018)	Straw Yellow	Rapid, entire, circular, smooth, concave	Gram +ve	Rod	Present
2-E	White	Less rapid, entire, circular, rough, flat	Gram +ve	Rod	Present
3-E	White	Rapid, entire, circular, smooth, flat	Gram +ve	Rod	Present
4-E	White	Rapid, entire, circular, smooth, concave	Gram +ve	Cocci	Present
5-E	Whitish Yellow	Rapid, entire, elongated, smooth, flat	Gram +ve	Cocci	Present
6-E	White	Rapid, entire, circular, smooth, flat	Gram -ve	Cocci	Present
7-E	Whitish	Rapid, entire, circular, smooth, concave	Gram +ve	Rod	Present
8-E	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Cocci	Present
9-E	White	Rapid, entire, elongated,	Gram +ve	Rod	Present

		smooth, flat			
10-E	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
11-E	White	Rapid, entire, circular, smooth, concave	Gram -ve	Rod	Present
12-E	White	Rapid, entire, circular, smooth, flat	Gram +ve	Rod	Present
13-E	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
14-E	White	Rapid, slightly wavy, circular, smooth, flat	Gram +ve	Cocci	Present
15-E	White	Rapid, entire, circular, smooth, concave	Gram +ve	Cocci	Present
1-K	White	Rapid, entire, circular, smooth, concave	Gram +ve	Cocci	Present
2-K	White	Rapid, entire, circular, smooth, convex	Gram +ve	Rod	Present
3-K	Translucent	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
4-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
5-K	White	Rapid, entire, circular, smooth, flat	Gram +ve	Rod	Present
6-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
7-K	White	Rapid, entire, circular, smooth, concave	Gram +ve	Rod	Present
8-K	White	Rapid, slightly wavy, circular, smooth, flat	Gram +ve	Rod	Present
9-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
10-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Cocci	Present
11-K	White	Rapid, entire, circular, smooth, concave	Gram +ve	Rod	Present
12-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Cocci	Present
13-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Cocci	Present

14-K	White	Rapid, slightly wavy, circular, smooth, flat	Gram +ve	Rod	Present
15-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Cocci	Present
16-K	White	Rapid, entire, circular, smooth, concave	Gram +ve	Rod	Present
17-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
18-K	White	Rapid, entire, elongated, smooth, flat	Gram +ve	Rod	Present
25-K	White	Rapid, slightly wavy, circular, smooth, flat	Gram +ve	Rod	Present
26-K (*Bacillus xiamenensis* strain 26K018)	Greyish White	Rapid, slightly wavy, circular, smooth, flat	Gram +ve	Rod	Present

The endospore forming (Figure 2.3.5) microbial isolates indicated that they had been evolved themselves to survive in hazardous metal polluted environment.

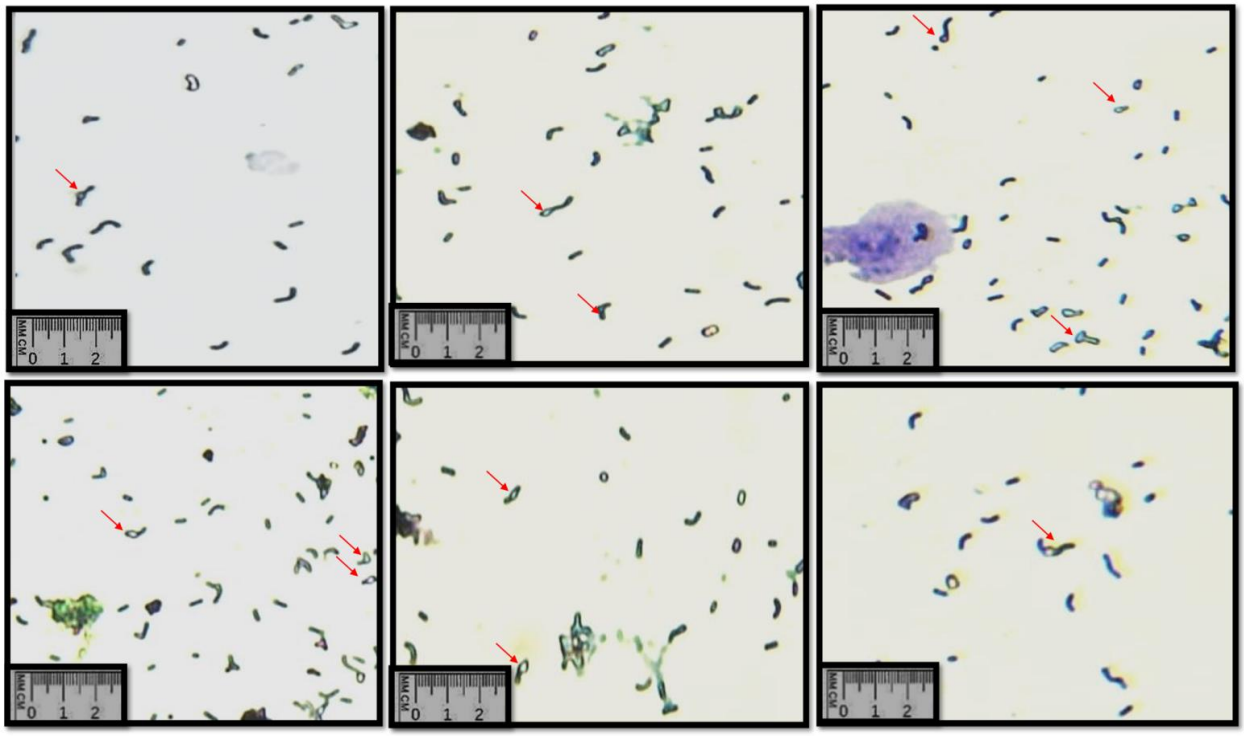


Figure 2.3.5: Malachite green strained bacterial population [500X] showed endospore formation

2.3.3. Biochemical characterization of isolated soil microbes:

The results of biochemical characterization revealed that the majority of the isolated microbial strains produced catalase and α-amylase enzymes of extracellular origin along with the carotenoid pigment, and had participated in the mixed acid fermentation test (Table 2.3.3).

Table 2.3.3 (A-C): Biochemical characters of the isolated soil bacteria ("+" denotes the enzyme producing property of the isolate and "–" denotes the absence of the enzyme producing property):

A. Bacteria Isolated from Circular Canal (**Microbacterium radiodurans* **K12016)**

Biochemical Tests	Bacterial strains											
	1-C	2-C	3-C	4-C	5-C	6-C	7-C	8-C	-9C	10-C	11-C	12-C*
1.Gelatinase test	-	+	+	-	-	-	+	-	-	-	+	-
2. Catalase test	+	+	-	+	++	+	+	+	-	+	+	+++
3. Amylase test	+	+	-	+	+	++	+	+	+	+	+++	+
4.Fluorescence test	-	-	-	-	-	-	-	-	-	-	-	-
5. Methyl red test	++	+++	+	+	+	+	+++	+	+++	+	+	+
7. Urease test	+	+	-	+	+	-	+	-	-	-	-	-
8. Diffusible pigmentation test												
After 1 days	-	+	-	-	+	+	-	-	-	-	-	-
After 6 days	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	+++

B. Bacteria isolated from East Kolkata Wetland (*_Bacillus xiamenensis_ strain 1E0018)

Biochemical tests	Bacterial strains											
	1-E*	2-E	3-E	4-E	5-E	6-E	7-E	8-E	9-E	10-E	11-E	12 -E
1.Gelatinase test	-	-	-	-	-	-	-	+	-	-	-	-
2.Catalase test	+++	-	-	-	++	-	-	+	-	++	-	+
3.Amylase test	+	+	+	+	+	+	+	+	-	+	+	+
4.Fluorescence test	-	-	-	-	-	-	-	-	-	-	-	-
5.Methyl red test	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++
7.Urease test	-	-	-	-	-	-	-	+	-	-	-	-
8.Diffusible pigmentation test												
After 1 days	-	-	-	-	-	-	-	-	-	-	-	-
After 6 days	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	+++

C. Bacteria isolated from Kestopur Canal (*_Bacillus xiamenensis_ strain 26K018)

Biochemical Tests	Bacterial strains											
	1-K	2-K	3-K	4-K	5-K	6-K	7-K	8-K	11-K	12-K	25-K	26-K*
1.Gelatinase Test	+	++	+	+	+	+	-	-	-	-	-	-
2. Catalase Test	-	-	-	-	++	+	-	-	+	-	-	+++
3. Amylase Test	+++	+++	+++	+++	+++	-	+++	+++	+++	+++	+	+++
4. Fluorescence Test	-	-	-	-	-	-	+	-	-	-	+	-
5. Methyl	++	-	-	++	-	-	++	-	-	-	-	++

red Test	+			+			+					
7. Urease Test	-	-	-	-	-	++ +	-	-	-	+	-	-
8. Diffusible pigmentation Test												
After 1 days	-	-	-	-	-	-	-	-	-	-	-	+
After 6 days	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	+++	+++	+++	+++

2.3.4. Antibiotic sensitivity index:

The antibiotic profiling data indicated that most of the isolated soil microbes were sensitive to ampicillin and resistant to Norfloxacin (Table 2.3.4).

Table 2.3.4: Microbial antibiotic sensitivity (µg/ml) index in Mueller Hinton Agar [The antibiotic concentration (in µg) was measured before applying it to disc]:

Bacterial Strain	Antibiotics			
	Ampicillin (10 µg/ml)	**Tetracycline (30 µg/ml)**	**Doxycycline (30 µg/ml)**	**Norfloxacin (10 µg/ml)**
1-C	Sensitive	Resistant	Intermediate	Resistant
2-C	Intermediate	Resistant	Resistant	Resistant
3-C	Intermediate	Resistant	Sensitive	Intermediate
4-C	Sensitive	Intermediate	Resistant	Resistant
5-C	Sensitive	Resistant	Intermediate	Resistant
6-C	Intermediate	Intermediate	Sensitive	Intermediate
7-C	Sensitive	Intermediate	Intermediate	Resistant
8-C	Intermediate	Sensitive	Intermediate	Sensitive
9-C	Sensitive	Intermediate	Sensitive	Intermediate
10-C	Sensitive	Resistant	Resistant	Resistant
11-C	Resistant	Resistant	Resistant	Intermediate
12-C (*Microbacterium radiodurans* K12016)	Sensitive	Resistant	Intermediate	Resistant
1E(*Bacillus xiamenensis* strain 1E0018)	Sensitive	Intermediate	Resistant	Intermediate
2-E	Intermediate	Sensitive	Intermediate	Resistant
3-E	Sensitive	Intermediate	Intermediate	Resistant
4-E	Intermediate	Resistant	Intermediate	Resistant
5-E	Resistant	Resistant	Resistant	Sensitive
6-E	Sensitive	Intermediate	Intermediate	Sensitive
7-E	Sensitive	Resistant	Resistant	Resistant
8-E	Sensitive	Resistant	Intermediate	Resistant
9-E	Intermediate	Resistant	Resistant	Resistant
10-E	Sensitive	Resistant	Intermediate	Sensitive

11-E	Resistant	Intermediate	Intermediate	Sensitive
12-E	Sensitive	Intermediate	Resistant	Resistant
13-E	Sensitive	Intermediate	Sensitive	Sensitive
14-E	Resistant	Resistant	Sensitive	Sensitive
15-E	Sensitive	Resistant	Sensitive	Resistant
1-K	Sensitive	Resistant	Intermediate	Sensitive
2-K	Sensitive	Resistant	Sensitive	Sensitive
3-K	Sensitive	Resistant	Intermediate	Resistant
4-K	Sensitive	Resistant	Sensitive	Intermediate
5-K	Intermediate	Intermediate	Intermediate	Sensitive
6-K	Sensitive	Resistant	Resistant	Resistant
7-K	Sensitive	Resistant	Resistant	Intermediate
8-K	Sensitive	Resistant	Resistant	Sensitive
9-K	Intermediate	Intermediate	Sensitive	Sensitive
10-K	Sensitive	Resistant	Sensitive	Sensitive
11-K	Sensitive	Intermediate	Sensitive	Intermediate
12-K	Sensitive	Intermediate	Intermediate	Resistant
13-K	Sensitive	Intermediate	Resistant	Intermediate
14-K	Sensitive	Resistant	Resistant	Resistant
15-K	Intermediate	Resistant	Sensitive	Sensitive
16-K	Sensitive	Resistant	Resistant	Resistant
17-K	Sensitive	Intermediate	Sensitive	Resistant
18-K	Intermediate	Resistant	Sensitive	Resistant
25-K	Intermediate	Resistant	Resistant	Intermediate
26-K (*Bacillus xiamenensis* strain 26K018)	Intermediate	Intermediate	Sensitive	Intermediate

2.3.5. Molecular characteristics of microbe:

Three microbial strains (one from each site) showed considerably Cr^{6+} resistance afterwards, were thoroughly studied to find their chromium removing capacity and identified by 16S rDNA sequencing.

The 16S rDNA of the strain 12C (isolated from the Circular Canal), 1E (isolated from East Kolkata Wetland), and 26K (isolated from Kestopur Khal), were sequenced, identified by sequence alignment and phylogenetic analyses with their near relatives (Figure 2.3.6, Figure 2.3.7) and their taxonomic positions were further identified by standard identification hierarchy. The 12C strain possessed 91.01% sequence identity with *Microbacterium radiodurans* N28_137 (Table 2.3.5). Zhang *et al.* (2010) previously reported one UV radiation resistant strain of *Microbacterium radiodurans* from soil. There are different chromium and other heavy metal removing *Microbacterium* species but according to our literature review no report found earlier regarding chromium resistance property of *Microbacterium radiodurans*. Since our finding indicated that *Microbacterium radiodurans* K12016 absorbed chromium from surroundings, it might play an important role in chromium

bioremediation. Both the 1E and 26K strains had 99% similarity with the MCCC 1A00008 strain of *Bacillus xiamenensis* (Table 2.3.6 & Table 2.3.7, Figure 2.3.7). Preliminary morphological, biochemical, physiological characterization and taxonomic key analysis also confirmed these reports. The newly annotated partial 16S rRNA sequences of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 was submitted to the NCBI database with the GenBank accession number MF600628, MK353157 and MK353500. Previous study by Gupta et al (2012) was also reported different species of *Bacillus* and *Exiguobacterium* (accession numbers JN392001-JN3920013) from Kestopur Khal. Our present study confirmed the presence of different species of *Bacillus* as the most predominant bacteria in the microbial community of different wastelands of Kolkata (Table 2.3.8, Figure 2.3.8) by aligning the acquired partial 16S rDNA sequences through NCBI MEGABLAST, Clustal Omega and MEGA10 algorithm program and comparing the obtained microbial genera with Bergey's manual of determinate bacteriology [Bergey and Holt, 2000].

Table 2.3.5: The sequences selected and retrieved from NCBI GenBank database based on maximum identity score with the *Microbacterium radiodurans* K12016 and aligned individually using NCBI MEGABLAST algorithm to find sequence similarity index:

Sequence	% Identity
Microbacterium radiodurans N28 137	91.01
Microbacterium radiodurans N28 139	88.71
Desulfovibrio vulgaris	74.05
Desulfovibrio vulgaris Hildenborough	73.04
Bacillus acidicola 105-2	72.9
Ochrobactrum tritici	78.52
Bacillus cereus 6+	72.33
Microbacterium arborescens S6	73.95
Microbacterium arborescens S3-91	86.52

Microbacterium arborescens S1-81	90.15
Microbacterium arborescens MFB-39 16S	90.34
Cellulosimicrobium cellulans IARI-BHI-13	90.13
Microbacterium paraoxydans 6U	90.55
Microbacterium sp. DP6-560-600	90.34
Microbacterium arborescens MS4	90.55
Microbacterium flavum E2 AS-19	90.13
Marine_bacterium AK6 009	90.34
Marine_bacterium AK6 007	90.34
Marine_bacterium AK6 006	90.34
Microbacterium oleivorans BAS69	90.72

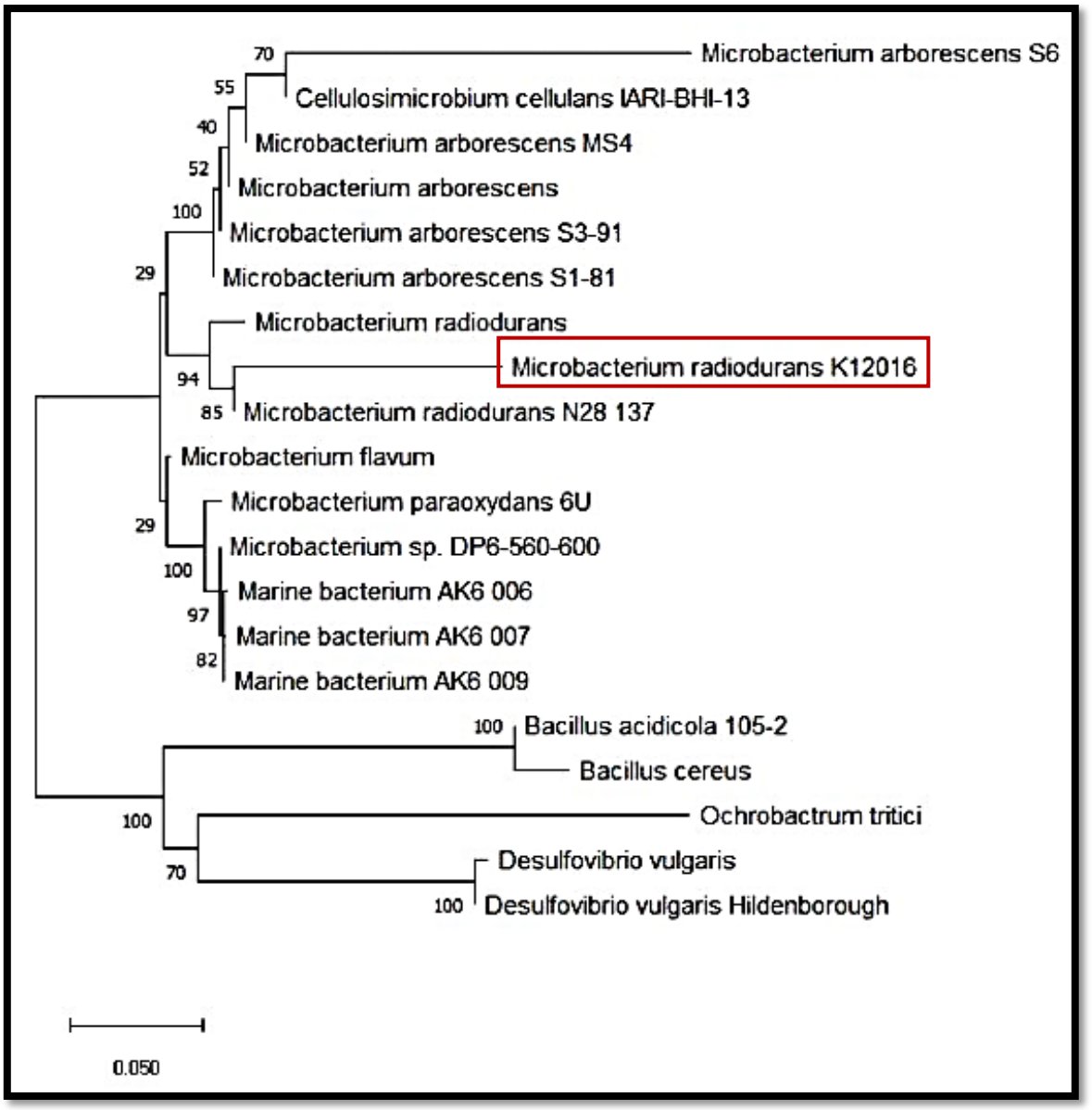


Figure 2.3.6: To identify the microbes isolated from sewage soil of Circular Canal, phylogenetic tree was generated using MEGA 10 software by applying the neighbor-joining method based on 16S rDNA sequences (using 600 bootstrap replicates) implying Kimura 2-parameter method (optimum branch length 0.93923707) and compared with Clustal Omega multiple sequence alignment algorithm. The analysis involved 20 different nucleotide sequences which shown maximum identity, higher query coverage and lower distance matrix (as per Kimura-2-Parameter model) with the newly obtained 16S rDNA sequence.

Table 2.3.6: The sequences selected from the NCBI genbank database based on maximum identity score with the *Bacillus xiamenensis* 1E0018 and aligned individually using NCBI MEGABLAST algorithms to find sequence similarity index:

Sequence	% Identity
Bacillus xiamenensis MCCC 1A00008	99.85
Bacillus xiamenensis 26K018	99.7
Bacillus cereus ATCC 14579	94.56
Bacillus mycoides DSM	94.45
Bacillus thuringiensis 24_1058	93.38
Exiguobacterium sp. 6b (2011)	91.31
Bacillus firmus KUCr1	95.67
Lysinibacillus sphaericus DSM	94.02
Bacillus oceanisediminis LNHL3	96

Table 2.3.7: The sequences selected from the NCBI genbank database based on maximum identity score with the *Bacillus xiamenensis* 26K018 and aligned individually using NCBI MEGABLAST algorithms to find sequence similarity index:

Sequence	% Identity
Bacillus xiamenensis MCCC 1A00008	99.48
Bacillus xiamenensis 1E0018	99.7
Bacillus cereus ATCC 14579	94.34
Bacillus mycoides DSM	94.16
Bacillus thuringiensis 24_1058	93.17
Exiguobacterium sp. 6b (2011)	89.82
Bacillus firmus KUCr1	95.58

Lysinibacillus sphaericus DSM	93.72
Bacillus oceanisediminis LNHL3	95.76

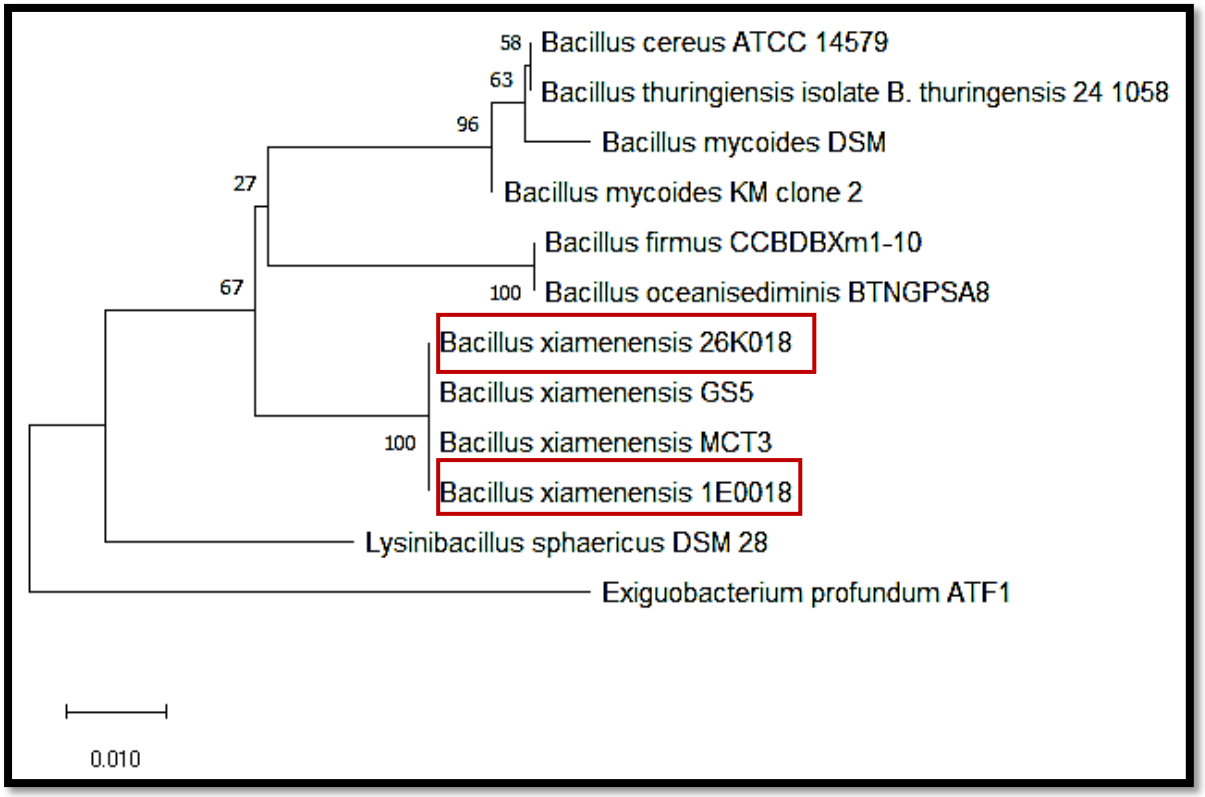


Figure 2.3.7: To identify the microbes isolated from sewage soil of East Kolkata Wetland and Kestopur Khal, phylogenetic tree was generated using MEGA 10 software by applying the neighbor-joining method based on 16S rDNA sequences (using 600 bootstrap replicates) implying Kimura 2-parameter method (optimum branch length 0.18200762) and compared with Clustal Omega multiple sequence alignment algorithm. The analysis involved 12 different nucleotide sequences which shown maximum identity, higher query coverage and lower distance matrix (as per Kimura-2-Parameter model) with the newly obtained 16S rDNA sequence.

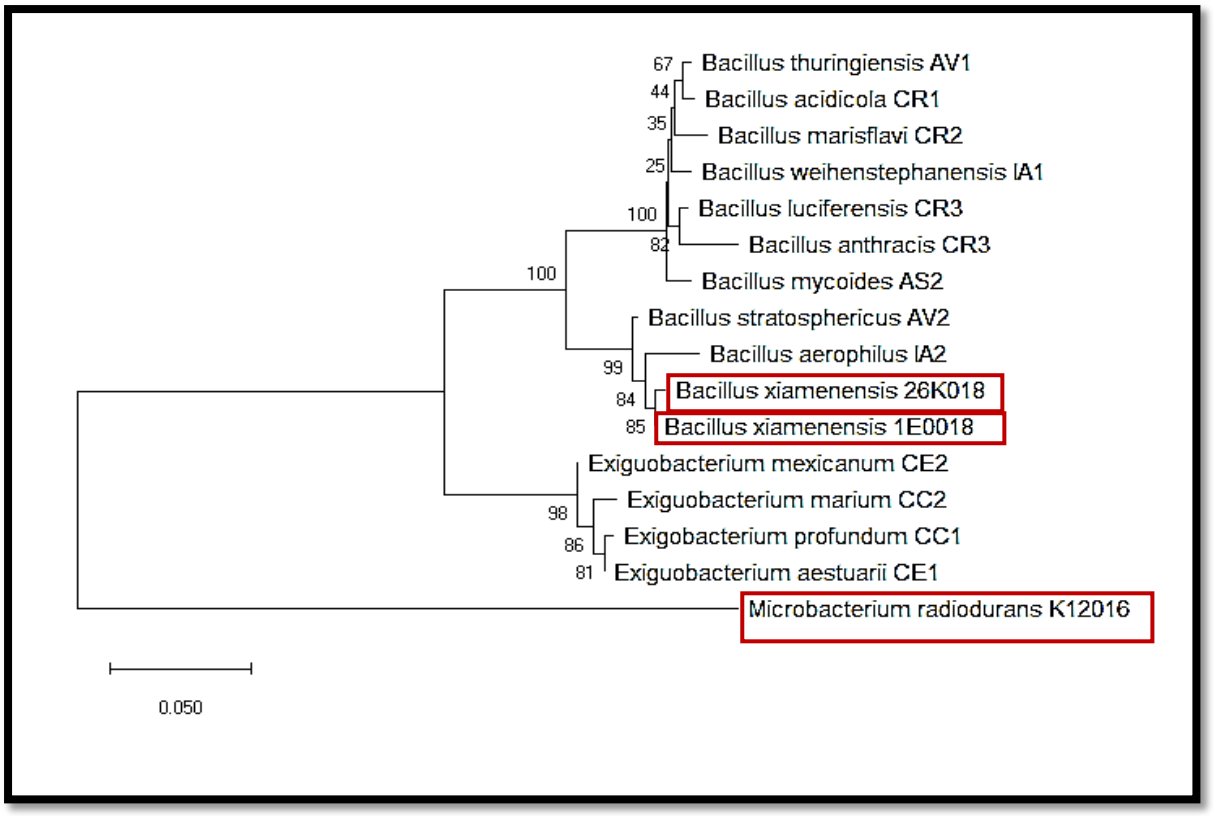


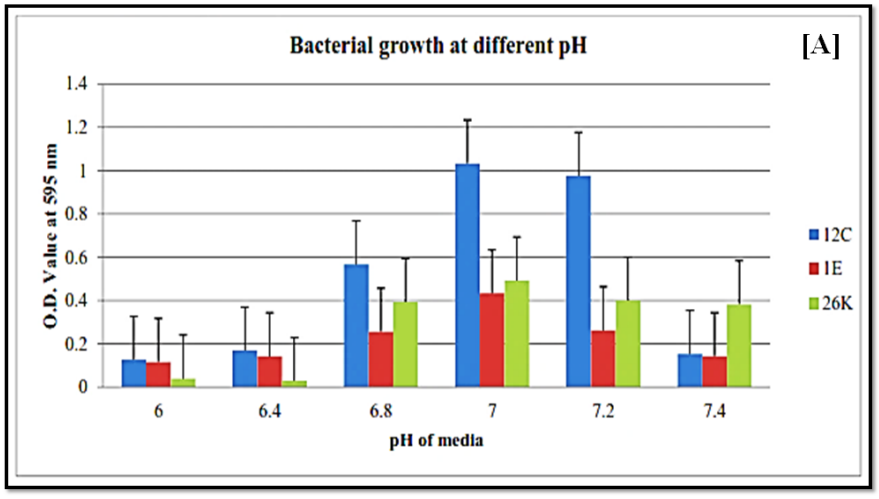
Figure 2.3.8: Phylogenetic tree constructed to observe the evolutionary relationship among the strains isolated from Circular canal, East Kolkata Wetland and Kestopur Khal in this present study with related strains isolated from sewage sites of Kolkata and reported in literature, using MEGA 10 software by applying the neighbor-joining method based on 16S rDNA sequences (using 600 bootstrap replicates) implying Kimura 2-parameter method (optimum branch length 0.64059896).

Table 2.3.8: The sequences of the isolated soil bacteria were subjected to 16S nucleotide BLAST analysis to find their closest neighbor; the microbial identity was decoded on the basis of the maximum percentage of similarity with their closest neighbors enlisted in the NCBI database:

Strain	Bacterial strain showing maximum homology	Identity (%)	Length (bp)	GenBank Accession number	Collected from
K12016	*Microbacterium radiodurans* strain GIMN 1.002	91.01	708	MF600628	Circular Canal
1E0018	*Bacillus xiamenensis* strain MCCC IA00008	99.85	1403	MK353500	East Kolkata Wetland
26K018	*Bacillus xiamenensis* strain MCCC IA00008	99.48	1418	MK353157	Kestopur Khal

2.3.6. Optimization of bacterial growth conditions:

Optimum growth condition for most of the microbes was 37°C temperature (figure 2.3.9 A) and neutral pH (pH 7) (Figure 2.3.9 B).



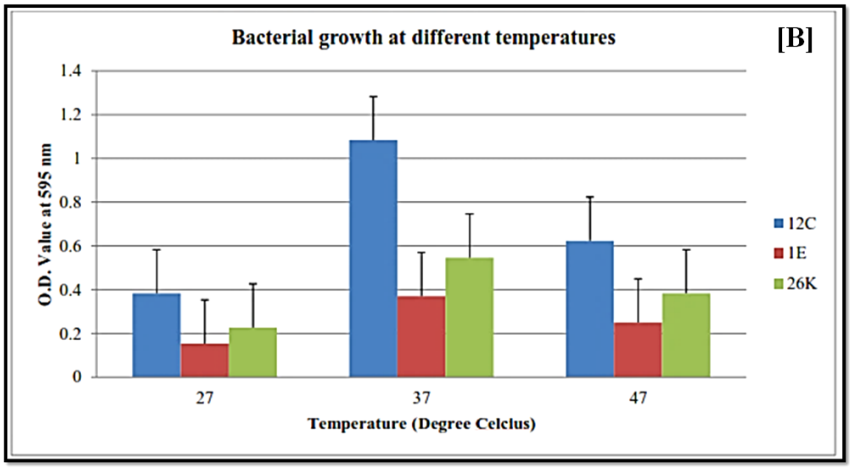


Figure 2.3.9 (A-B): Standardization the growth conditions of some selected microbes by optimizing the different important growth parameters like [A] pH, [B] temperature (12C: *Microbacterium radiodurans* K12016; 1E: *Bacillus xiamenensis* strain 1E0018; 26K: *Bacillus xiamenensis* strain 1E0018)

2.4. Discussion:

It can be stated after completion of polyphasic bacterial characterization, that the microbial populations of three different sewage regions were more or less similar in morphology (Figure 2.3.1). Most of the microbial populations from three previously mentioned sewage regions of Kolkata possessed gram-positive rod-shaped bacteria.

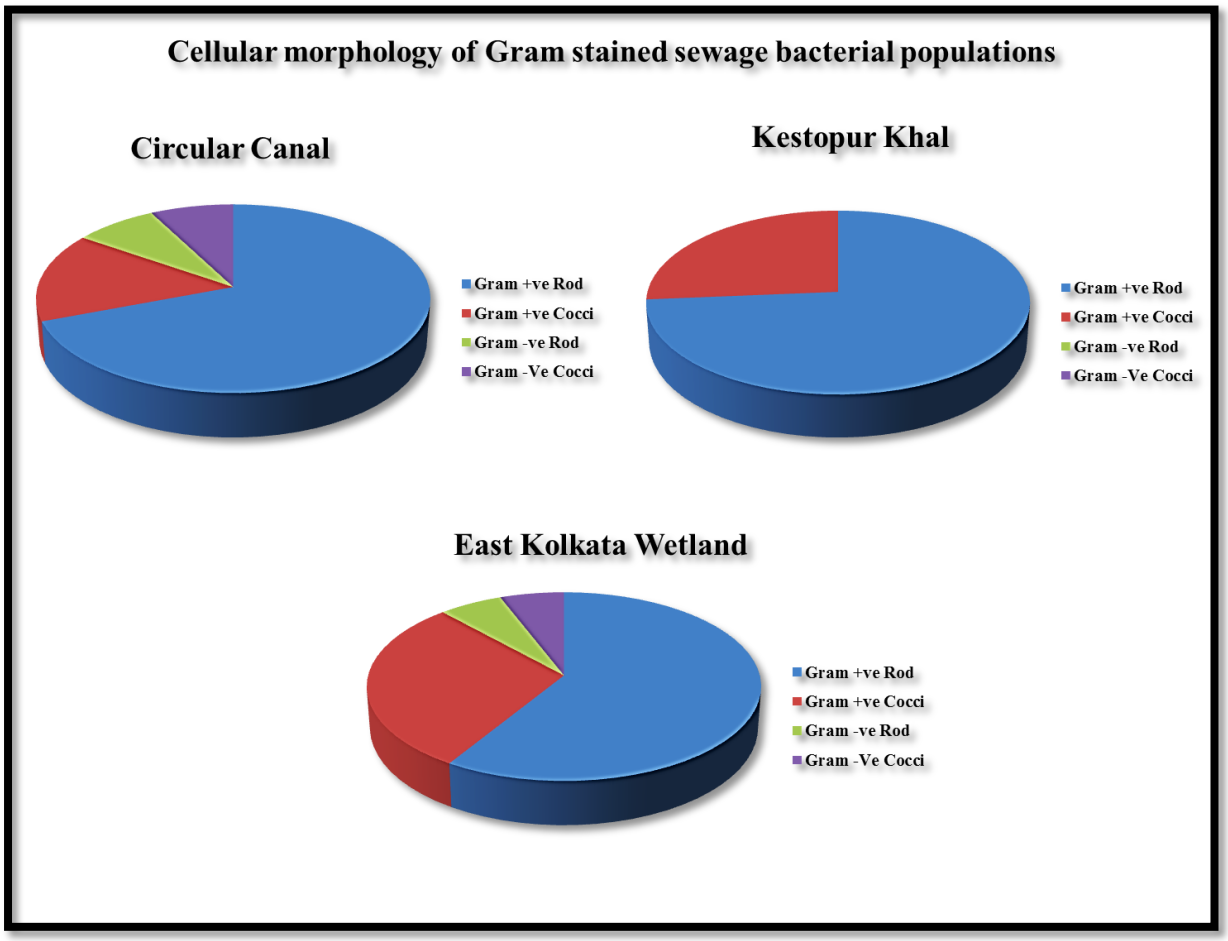


Figure 2.4.1: Comparative evaluation of morphological characters of microbes isolated from sewage regions of Kolkata

Temperature and pH requirement of all microbial isolates was alike (Figure 2.2.9) and they showed the same trend in metabolic and nutritional means (Figure 2.3.2).

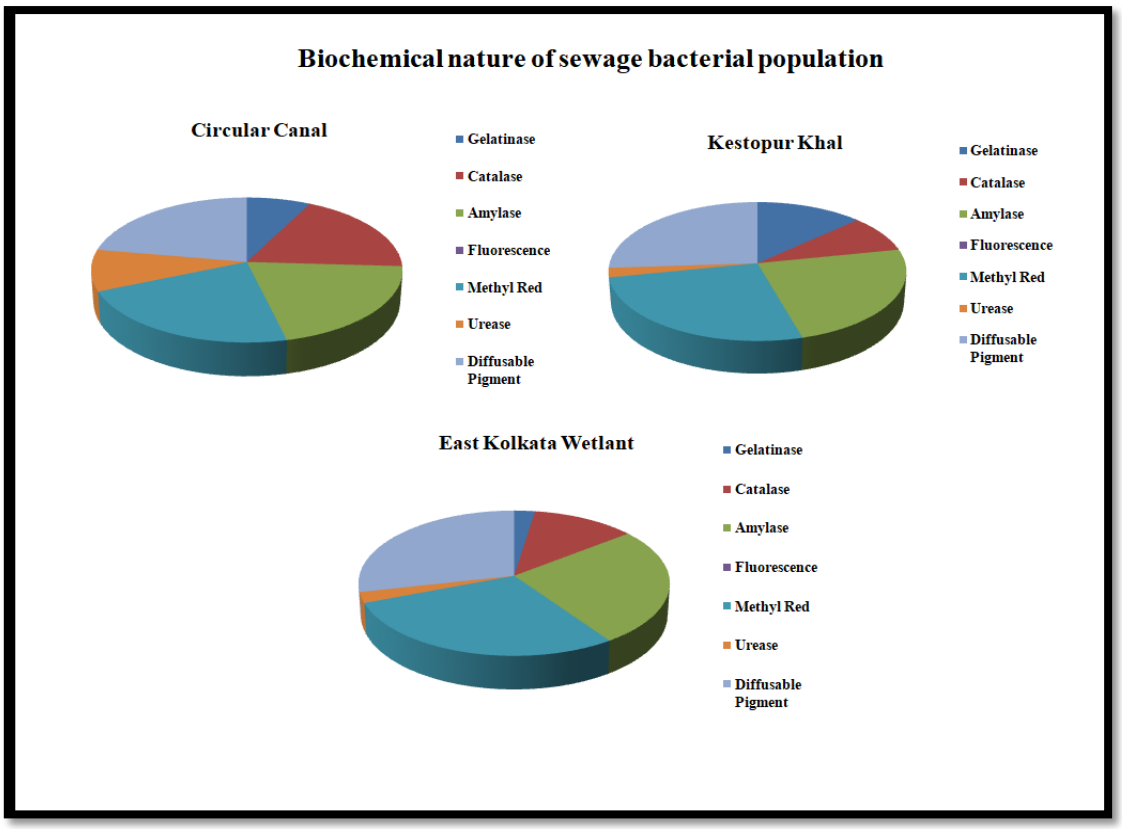


Figure 2.4.2: Comparative evaluation of biochemical parameters of microbes isolated from sewage regions of Kolkata

Different extracellular lytic enzymes (like amylase) formed by the microbes possessed a significant role in degradation of different soil macromolecules and their conversion to bioavailable simpler molecules. The organic acids formed by soil microbes help in mineral mobility. The endospore forming capacity of the microbial isolates designated that they can withstand toxic environment and can dormant for long time [Hobot, 2015]. So, it can be said from the findings that the morphologically similar sewage microbes could tolerate hazardous environment by implying different strategies.

The higher chemical oxygen demand (COD) and lower dissolved oxygen (DO) values indicated that, the three investigated wastewater bodies and its surrounding regions were severely polluted with oxidizable organic matter. Among the three, Kestopur Khal and its surrounding area had got tremendously contaminated.