Chapter 4

Inspect the role of rhizospheric soil microbe in decreasing plant chromium accumulation and plant growth promotion

4.1 Background study:

The plants grow in heavy metal contaminated surroundings, absorb heavy metals from both surface-soil and underground soil micro-climate [Patra et al. 2004]. The absorbed heavy metals accomplished serious plant metabolic disorders by producing different substances like reactive oxygen species (ROS) or cytotoxic methylglyoxal (MG) which disturbed the ionic homeostasis within plant tissue and cease plant growth and metabolism [Hossain et al. 2012; Sytar et al. 2013]. A small amount of the non-biodegradable heavy metal can enter the food chain through bioaccumulation within plant tissue, get magnified across each and every trophic level and impactfully cause fatal consequences on human beings [Gadd, 2010]. Some previous studies revealed that the concentrations of heavy metals in dry fruit and other edible plant parts exceeded the values approved by WHO and FAO [Neiem et al. 2009; Oueirolo et al. 2000; Husain et al. 1995]. The entrance of heavy metal within the food chain depends on the composition of flora and fauna of regional micro-climate because the plant-alliance soil microbes play important role in soil metal mobility, plant metal uptake and biogeochemical cycling of soil heavy metal. Some heavy metal resisting soil rhizospheric microbes play a potent role in the survival and growth of the metal sensitive plant by alleviating the effect of heavy metal and providing nutrients to the plants [Benizri and Kidd 2018].

The plant growth-promoting rhizospheric bacteria (PGPR) promote plant growth by fabricating extracellular plant growth stimulating substances like Indole Acetic Acid (IAA), Indole Butyric Acid (IBA), siderophore, phosphate degrading enzymes in the root-adjacent soil. From this root-adjacent soil, the plants draw nutrients and water directly. Many previous workers have reported the role of PGPR bacteria in plant growth promotion. Heavy metal stress tolerating PGPR strains of Bacillus, Streptomyces, Pseudomonas, Methylobacterium have been reported for their potent role in the improvement of plant growth by decreasing the deleterious effects of heavy metal [Sessitsch et al. 2013]. Arsenic and lead resistant Bacillus and cadmium resistant Ochrobactrum was reported by Pandey et al (2013) which induced rice plant growth.

The Indole compounds synthesized by a few soil-dwelling microbes, play important role in root and shoot development for its centrifugal movement and also help in plant cell division and elongation in very low concentrations. Previously, Susilowati et al. (2002) reported different species of Bacillus, Pseudomonas which induced rice growth. Spaepen et al. (2007) had also reported many Indole producing microbes. The metal resistant sewage bacteria

produce siderophore as well phytohormones to help in the survival of the plants grown in heavy metal contaminated soil. Siderophore declines the deleterious effects of soil heavy metal and simultaneously provide growth stimulators especially iron to plant [Rajkumar et al. 2010]. Siderophore formation was checked in iron supplemented chrome azurol S (CAS) agar media taking hexadecyltrimethylammonium bromide (HDTMA) as an indicator [Schwyn and Neilands, 1987; Louden et al. 2011]. Mishra et al. (2017) stated that microbe produces some additive substances which serve as a more potent agent in the protection of plants grown in heavy metal contaminated soil. The different anionic functional group-containing extracellular polysaccharide substances like glycoprotein or lipoprotein secreted by bacteria to help in the removal of soil heavy metal pollutants [Ayangbenro and Babalola, 2017]. Some stress-tolerant microbe form biofilm with different exopolysaccharides and that convert the toxic heavy metal to the non-toxic one and protect themselves from surrounding stress [Gupta and Diwan, 2017]. The Gram-positive bacteria cells, which do not get surrounded by the outer membrane, also form exopolysaccharides. The Gram-positive bacteria produced exopolysaccharide has transported to the extracellular space directly [Whitfield et al. 2015]. The polysaccharide modification in Gram-positive bacteria has taken place within cytoplasm as like Gram-negative bacteria [Little et al. 2014a; Little et al. 2014b; Rozeboom et al. 2014].

In several ways, the plant and microbes help each other and have maintained a symbiotic association. Many of the bacterial genes have co-evolved with plant in a conserved and stable manner and has considered as selection unit [Rosenberg and Zilber-Rosenberg, 2016]. Because of the dependency of the plant with their rhizospheric microbe, recently different long-term and short-term plant-microbial volt has been created which have tried to help out the plant to grow in consortia with their rhizospheric microbes and form microbiome just as in their natural habitat [Gopal and Gupta, 2019].

4.2. Methodology:

4.2.1. Soil and plant Sample collection from selected sites:

Some herbs (*Eichhornia crassipes*, *Amaranthus viridis, Coccinia cordifolia, Ipomea aquatica, Alternanthera sp., Colocasia esculenta*) and their rhizosphere soil samples were collected in triplicate from three selected sewage regions of south, east and north Kolkata, viz. Circular Canal (Tolly Nullah), East Kolkata Wetland and Kestopur Khal. Most of the collected herbs were edible. The soil bacteria were isolated from this heavy metal containing rhizospheric soil. The herbs were identified by Bengal Plants (Vol. I & II).
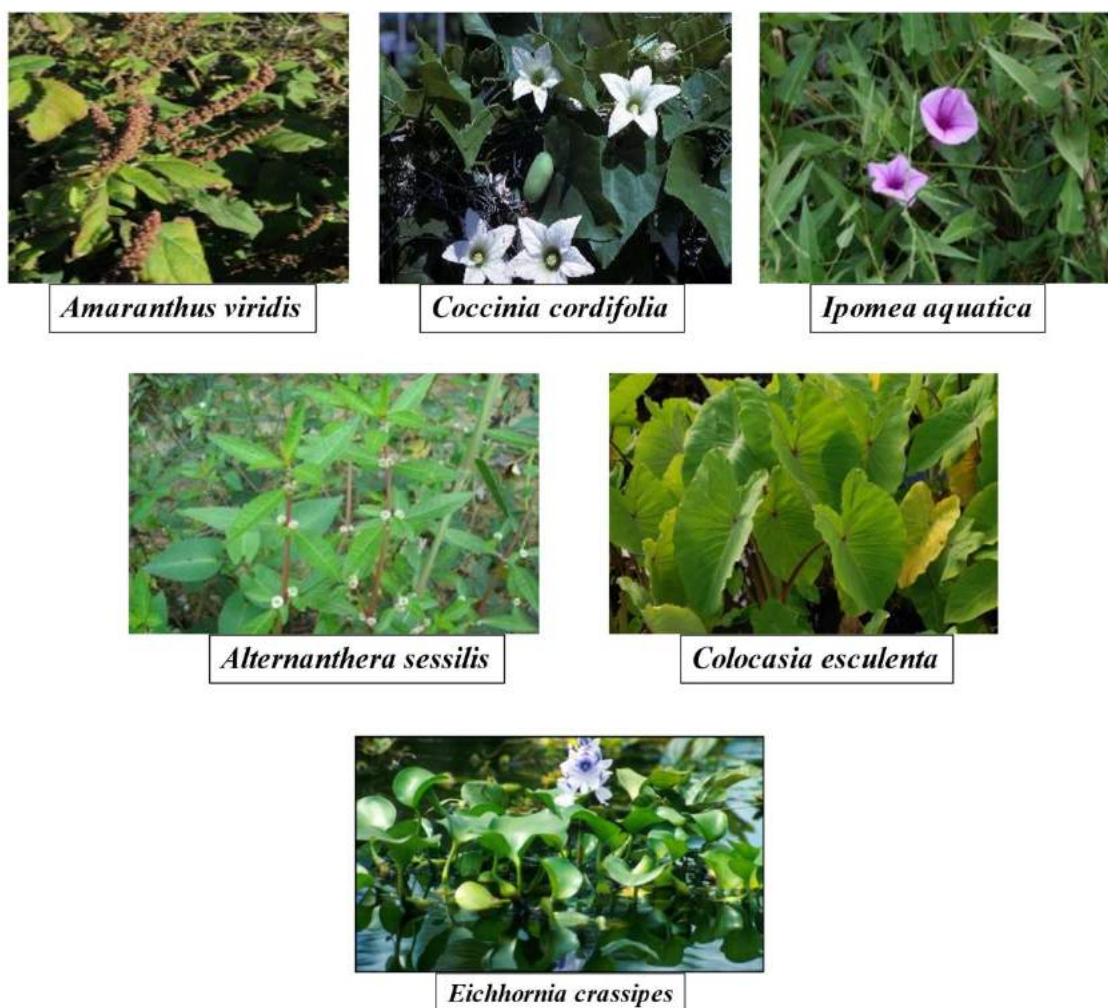
Figure 4.2.1: Some edible plants collected from sewage bank to measure chromium content

Figure 4.2.2(A): The dense population of native *Eichhornia crassipes*, which can significantly be induced biomagnification [Tiwari and Verma, 2007], was found in the vicinity of the fishing area of East Kolkata Wetland. Figure 4.2.2(B) The same trend published in the newsletter East Kolkata Wetland (2010) [Figure 4.2.2(B): Reprinted from East Kolkata Wetlands: a jointly published newsletter of East Kolkata Wetlands Management Authority and Wetlands International - South Asia (2010). 1:7]

Figure 4.2.3: Mixed vegetation at the bank of Kestopur Khal revealed the presence of *Colocasia esculanta* and *Cocos nucifera*, parts of which have consumed by human beings
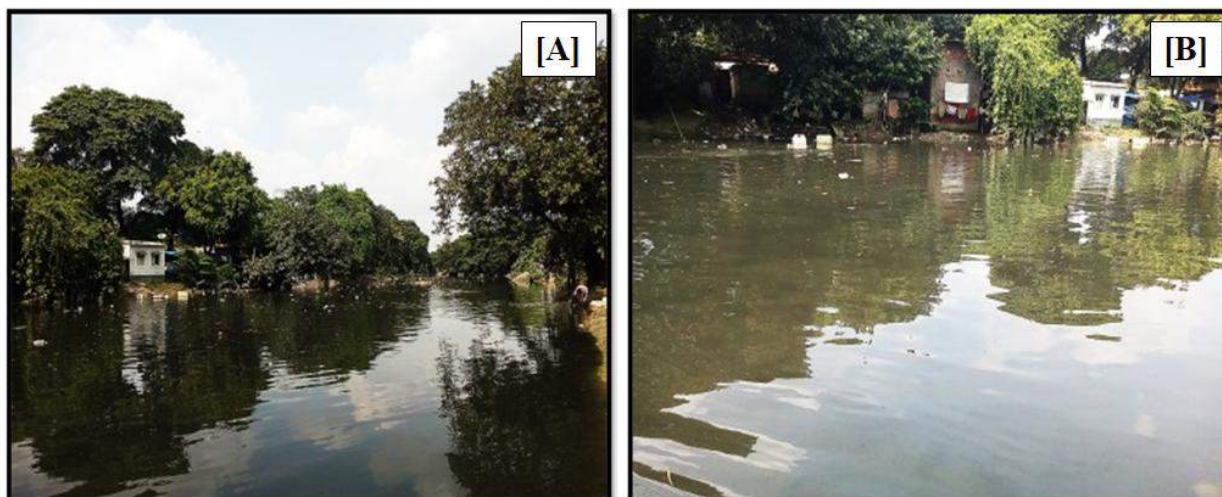
Figure 4.2.4(A-B): At the bank of Circular canal, [A] the rich vegetation and [B] household activities had noticed at a time

4.2.2. Grown plants in experimental condition:

To inspect the Bio-concentration factor of plants grown in the presence of the pure and mixed culture of isolated soil bacteria, and compare it with plants collected from sewage regions, the *Vigna radiata* seedlings were grown in soil supplemented with 10 mM hexavalent chromium. Mixed microbial culture of East Kolkata Wetland, Circular Canal, Kestopur Khal and pure culture of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018, and the *Bacillus xiamenensis* 26K018 were applied at 24 hours intervals to contaminate the experimental soils. A control set was maintained where plants grown in hexavalent chromium supplemented sterile soil.

Figure 4.2.5: *Vigna radiata* grown in experimental conditions [in 10 mM Cr^{6+} chromium containing sterile soil contaminated with mixed and pure cultures of our isolated rhizosperic soil microbe]

4.2.3. Metal estimation in soil and plant sample:

The pallets were formed with 150 mg to 160 mg lyophilized soil or plant sample and inserted into the sample holder of EDXRF (Energy Dispersive X-ray Fluorescence) spectrometer with the help of scotch tape, to determine the metal content [Sudarshan et al. 2011].

Five grams of pulverized plant samples and one gram of pulverized soil samples were digested with HNO_3 and H_2SO_4 (in 3:1 ratio) on a hot plate accordingly to estimate metal content by Atomic Absorption Spectrophotometer (APHA 23rd Ed., 3030E) model Variant AA140.

4.2.4. Calculation of plant Bio-absorption factor (BCF):

The BCF of plant rhizospheric soils is the ratio of the metal concentration in plants to the metal concentration in the soil supporting that vegetation. A <1 BCF indicates that the plant likely absorbs the heavy metal, but do not accumulate it within the cell.

4.2.5. Study microbial indole compound production:

To monitor Indole compound formation, the bacteria were cultured in 1 mg/ml of L-tryptophan supplemented Luria Bertani (LB) broth media. After 24 hours of incubation, 1 ml of bacterial culture was centrifuged, and ferric chloride-perchloric (Salkowski reagent; $FeCl_3$-$HClO_4$) [Gordon and Weber 1951] was added in 1:1 ratio to the supernatant and incubated for 30 minutes in dark. The colour intensity of the supernatant was measured by a spectrophotometer using the 530 nm wavelength.

The amount of Indole compound produced was measured by a standard curve provided by Sigma Aldrich. The concentrations of different indole-containing compounds (IAA and IBA) were confirmed by HPTLC (High-performance thin-layer chromatography) using isopropanol, ammonia, and distilled water in the ratio 8:1.4:0.6 as the mobile phase, with 10 cm/10 cm silica plates of 200 µl layer thickness, and was executed on TLC scanner 3.

1000 ng/µl standard solution of IAA and IBA was prepared and applied in triplicate on the silica gel plate of 20mm×10mm×0.2mm thickness (E. Merck, Germany) in 5mm width with the help of Camag microlitre syringe. The different volumes of standard solutions were applied on the TLC plate to get 100 ng to 1000 ng concentration of standard IAA and IBA solution. Calibrate the peak area against the concentration of IAA and IBA to obtain the regression value and was compared it with sample solutions.
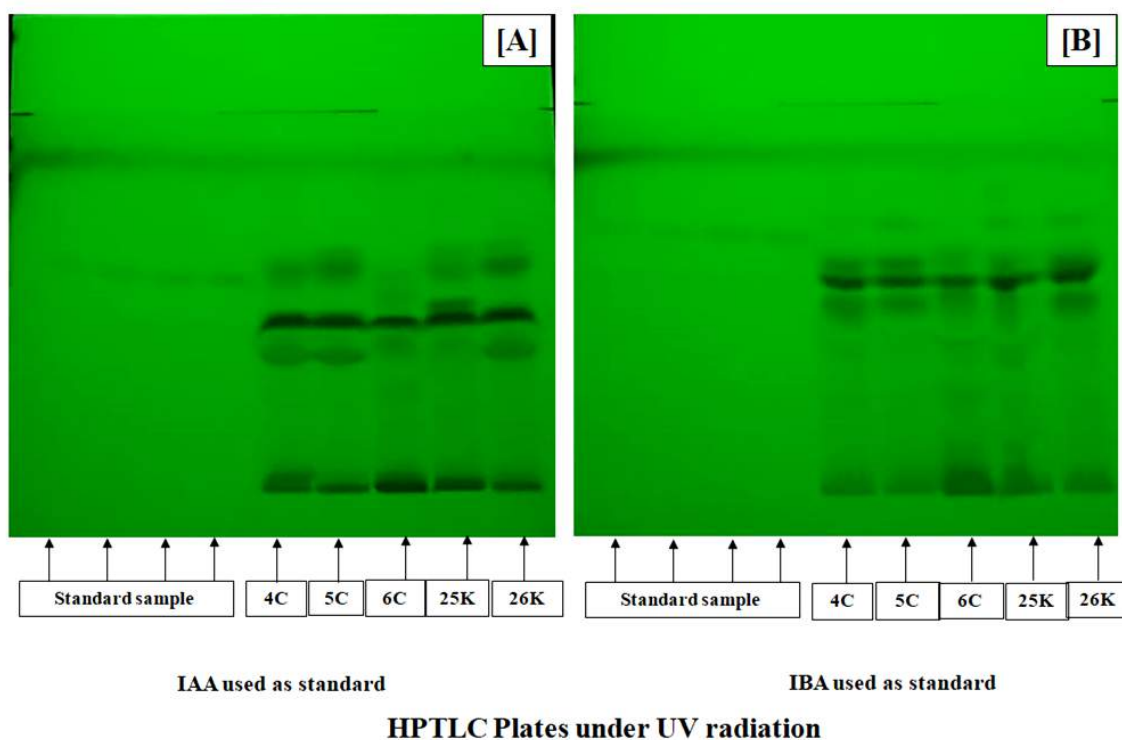
IAA used as standard   **IBA used as standard**

HPTLC Plates under UV radiation

Figure 4.2.6(A-B): Image of the thin layer chromatography plate speculated the presence of [A] IAA and [B] IBA within Salkowski reagent treated bacterial supernatants in compared to standard solution under UV radiation (254 nm) chamber.

4.2.6. Study microbial siderophore production:

Bacterial siderophore production was measured by the Chrome Azurol S agar (CAS agar) test, $FeCl_3$ test, and estimated the proportion of siderophore production in comparison to bacterial total cell protein content by Lowry method.

CAS agar plates were prepared by dissolving Chrome Azurol S and hexadecyltrimethyl ammonium bromide in King's B base. The disappearance of the blue colour of Fe^{3+} and the formation of orange pigment due to the extraction of siderophore by the iron-starved microbes were observed after 2 to 7 days of incubation.

The $FeCl_3$ test was performed by adding 1 ml (to detect hydroxamate siderophore) to 3 ml (to detect catechol siderophore) of freshly prepared 2% $FeCl_3$ to 1 ml of cell free culture filtrate, and the intensity of the purplish brown colour was detected by the appearance of 420-450 nm spectrophotometric peaks [Jalal and Vander 1990].

Bacterial total cell protein content was estimated by the conventional Lowry method [Lowry 1951] to calculate the amount of hydroxamate siderophore produced per mg of cellular protein.

4.2.7. Study microbial phosphate-solubilizing enzyme production:

To estimate microbial phosphate-solubilizing enzyme producing ability, inspect the appearance of the clear zone surrounding the bacterial colonies that were retained on Pikovskaya agar plates [Rao and Sinha, 1963] after five days of incubation.

4.2.8. Study microbial exopolysaccharide (EPS) production:

To extract microbial EPS, overnight bacterial culture containing YEM media [Loper and Ishimaru, 1991] was centrifuged at 1000 rpm for 10 minutes and three times chilled ethanol (Microbiology grade) was added to the supernatant (V/V). The ethanol containing supernatant was incubated at 4^oC for 20 minutes to study the formation of intense white precipitate due to EPS formation. After formation of the white precipitate, the ethanol containing supernatant was left for overnight at 6^oC. Next day, dialyzed the centrifuged crude EPS [through dialysis bag containing >14,000 KDa retaining capacity] overnight after freezing drying and got the purified polysaccharide after lyophilization. The concentration of produced EPS was estimated by mixing 5% phenol (V/V) and 95% sulfuric acid (V/V) with the lyophilized EPS and incubating the mixture for 25 minutes. After completion of incubation tenure, measured the intensity of developed brown colour in the spectrophotometer at 490 nm and the O.D. values were compared with the glucose standard curve to calculate the formed EPS concentration. [Bhattacharyya et al. 2017].

4.2.9. 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 a P ≤ 0.05 significance level (and p <0.01 high significance level) and r value between +1 and -1 using Microsoft Excel and SPSS software.

4.2.10. Composition of used media:

4.2.10.1. Chromazurol S Agar [after Louden et al. 2011]:

Ingredient			Amount
Blue Dye	Solution one		0.06 g of CAS (Fluka Chemicals) in 50 ml of ddH_2O
	Solution two		0.0027 g of $FeCl_3$-6 H_2O in 10 ml of 10 mM HCl
	Solution three		Dissolve 0.073 g of HDTMA in 40 ml of ddH2O
	Mix 50ml solution one + 9 ml solution two + 40ml solution three		
Mixture Solution	Minimal Media 9 (MM9)	Potassium hydrogen phosphate	15 g in 500 ml double distilled water
		Sodium chloride	25 g in 500 ml double distilled water
		Ammonium chloride	50 g in 500 ml double distilled water
	Glucose stock		20%
	NaOH stock		25 g in 150 ml double distilled water (pH 12)
	Casamino acid Solution		3 g Casamino acid in 27 ml double distilled water
			Extract with 3% 8-hydroxyquinoline in chloroform
CAS agar preparation	MM9		100 ml in 750 ml double distilled water
	PIPES [piperazine-N, N'-bis (2-ethanesulfonic acid)]		32.24 g (pH 6.8)
	Bacto-agar		15 g
	Sterile casamino acid solution		30 ml
	20% glucose in PIPE/MM9		10 ml
	Blue dye		100 ml

4.2.10.2. YEM Broth:

Ingredient	Amount
Yeast extract	1.00 g/liter
Mannitol	10.00 g/liter
Dipotassium hydrogen phosphate	0.50 g/liter
Magnesium sulphate	0.20 g/liter
Sodium Chloride	0.10 g/liter
Final pH (at 25°C)	7.0±0.2

4.2.10.3. Pikovskayas Agar:

Ingredient	Amount
Yeast extract	0.50 g/liter
Dextrose	10.00 g/liter
Calcium phosphate	5.00 g/liter
Ammonium sulphate	0.50 g/liter
Potassium chloride	0.20 g/liter
Magnesium sulphate	0.10 g/liter
Manganese sulphate	0.0001 g/liter
Ferrous sulphate	0.0001 g/liter
Agar	15 g/liter

4.3 Results:

4.3.1. The role of rhizospheric microbe in decreasing plant heavy metal uptake from the surrounding environment:

The average chromium content of edible plants collected from East Kolkata Wetland, Kestopur Khal, and Circular Canal was 751.92 ± 0.7 ppm, 16.08 ± 0.05 ppm, and 16.08 ± 0.05 ppm, respectively (Figure 4.3.1). Despite the alarmingly high concentration of chromium in the soil (20864 ± 0.28 ppm, 187.1 ± 0.41 and 187.1 ± 0.15 ppm) and high solubility of chromium [Sharma et al. 2020], the amount of soil chromium absorption by plants and the BCF value was quite low which might be due to the presence of rhizospheric soil microbes. The BCF (chromium) of the plants collected from East Kolkata Wetland, Circular Canal, and Kestopur Khal was 0.036 ± 0.009, 0.028 ± 0.01, and 0.085 ± 0.01, respectively (Figure 4.3.2A).

The BCF of *Vigna radiata* plants grown in the presence of a mixed microbial culture of East Kolkata Wetland, Circular Canal, Kestopur Khal and pure culture of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018, and the *Bacillus xiamenensis* 26K018 were 0.74 ± 0.01, 0.58 ± 0.09, 0.22 ± 0.5, 0.88 ± 0.01, 0.28 ± 0.05, and 0.26 ± 0.1, respectively. *Vigna radiata* grown in microbe free soils (control set) shows a BCF value greater than one (1.05 ± 0.02). These data indicated that the soil rhizospheric microbes play a critical role in controlling plant's chromium absorption in chromium-rich soil (Figure 4.3.2B).
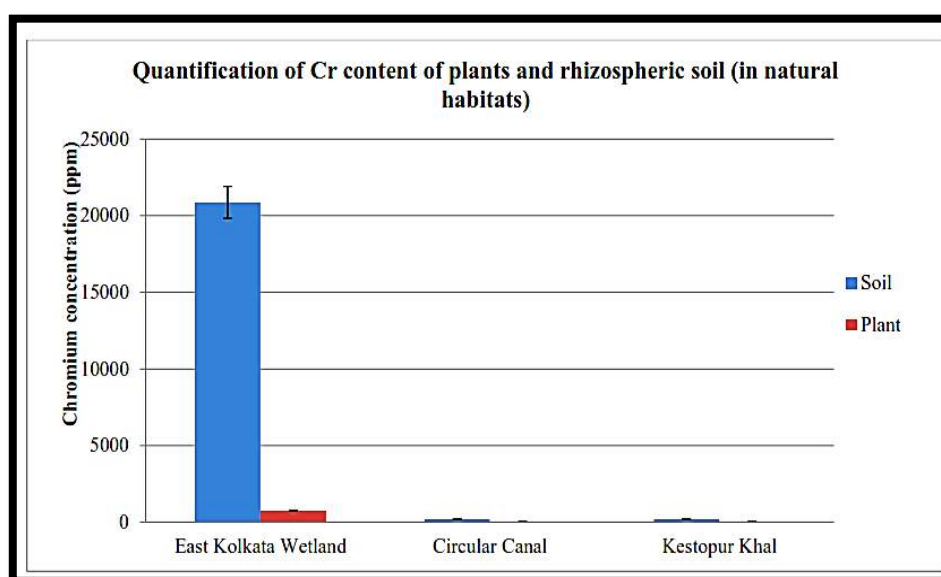

Figure 4.3.1: Quantification of the heavy metal content of the plants and soil collected from heavy metal contaminated rhizospheric soil
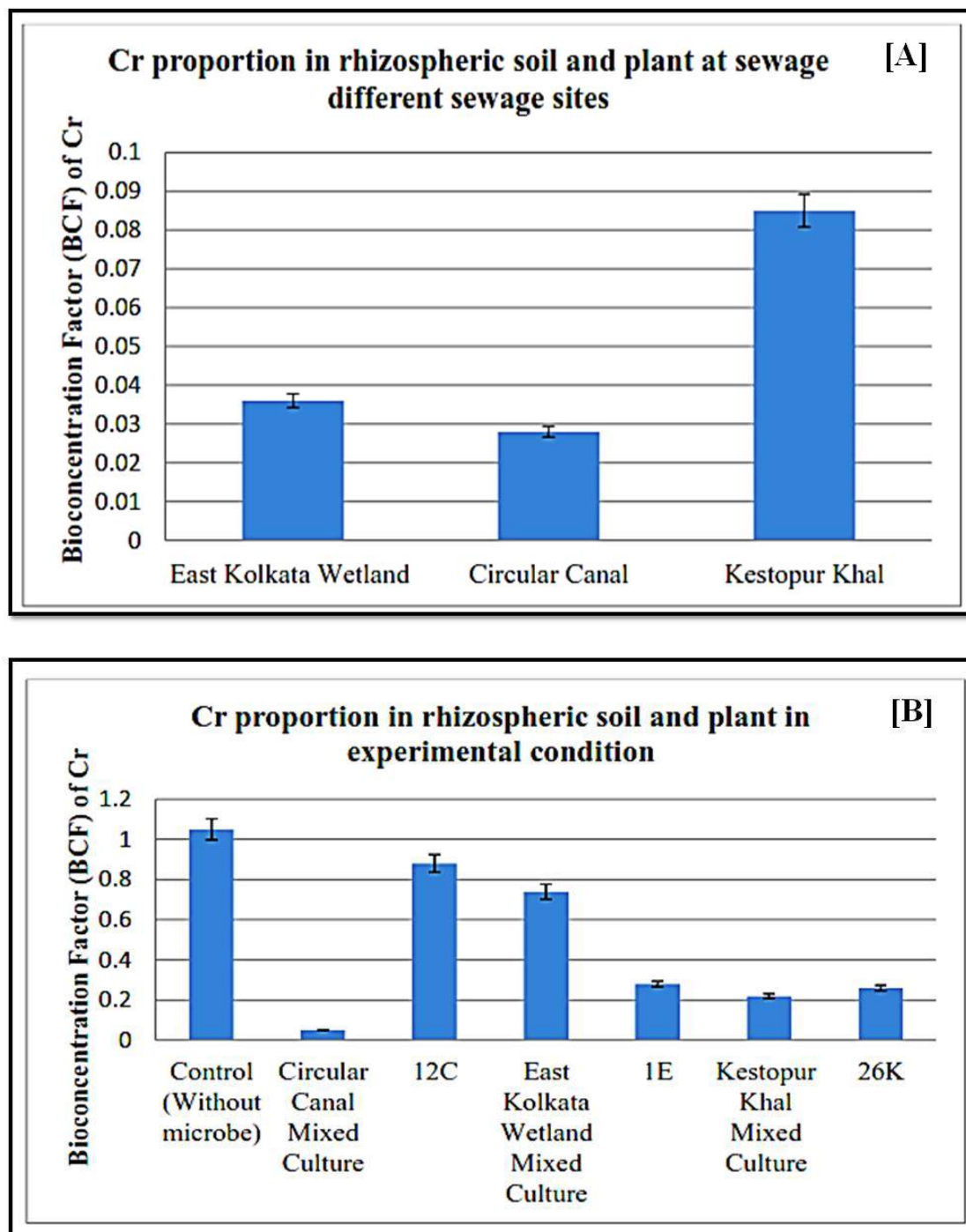
Figure 4.3.2: Chromium content [A] in sewage sites and [B] in experimental condition represented by the proportion of same in rhizospheric soil and plant

The low chromium BCF values of the edible plants collected from the sewage regions indicated that these plants don't accumulate the absorbed soil chromium and therefore soil chromium is unable to enter the food chain to cause bioaccumulation and biomagnification.

4.3.2. Inspection of microbial Indole compounds production:

Most of our isolated rhizospheric soil bacteria produced Indole compounds (Table 4.3.1).

Table 4.3.1: Concentration of Indole compounds produced by rhizospheric soil bacteria (implemented using standard curves provided by Sigma-Aldrich). The experiments were repeated in triplicate and the values were calculated with the help of standard solutions (± standard deviation)

Bacterial Strain	Indole compounds formed (in μg) in per ml of surrounding bacterial media
1C	37.5±0.2
2C	25±0.28
3C	37.5±0.11
4C	43.75±0.16
5C	43.75±0.1
6C	37.5±0.2
7C	37.5±0.2
8C	31.25±0.3
9C	37.5±0.05
10C	24.5±0.11
11C	25±0.5
12C (*Microbacterium radiodurans* K12016)	25±0.36
1E (*Bacillus xiamenensis* strain 1E0018)	13±0.06
2E	27±0.12
3E	25±0.24
4E	25±0.09
5E	27±0.07
6E	15±0.3
7E	14±0.08
8E	28±0.24
9E	21±0.09
10E	14±0.24
11E	16±0.32
12E	21±0.06
13E	35±0.21
15E	29±0.04
1K	31±0.2
2K	27±0.7
3K	26±0.28

4K	21±0.09
5K	32±0.3
6K	31±0.09
8K	28±0.04
9K	37±0.07
10K	35±0.32
11K	32±0.34
12K	31±0.09
13K	33±0.32
16K	22±21
18K	33±0.09
25K	29±0.01
26K (*Bacillus xiamenensis* strain 26K018)	39±0.04

The red coloration developed due to addition of Salkowski reagent (0.5M $FeCl_2$ and 35% perchloric acid) to the L-tryptophan supplemented media indicated the presence of Indole compounds (IAA, IBA). (Figure 4.3.3).
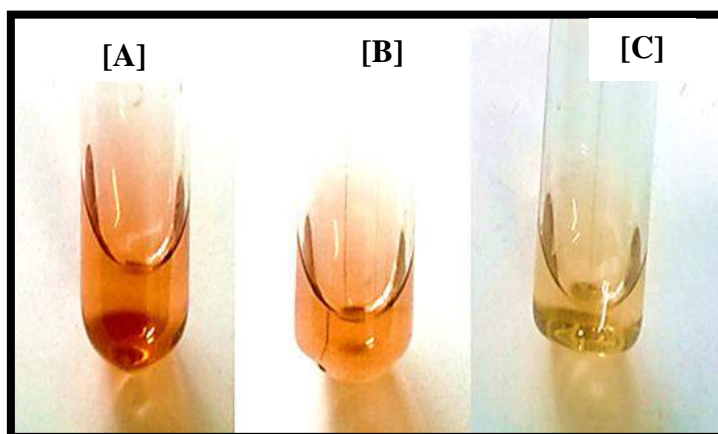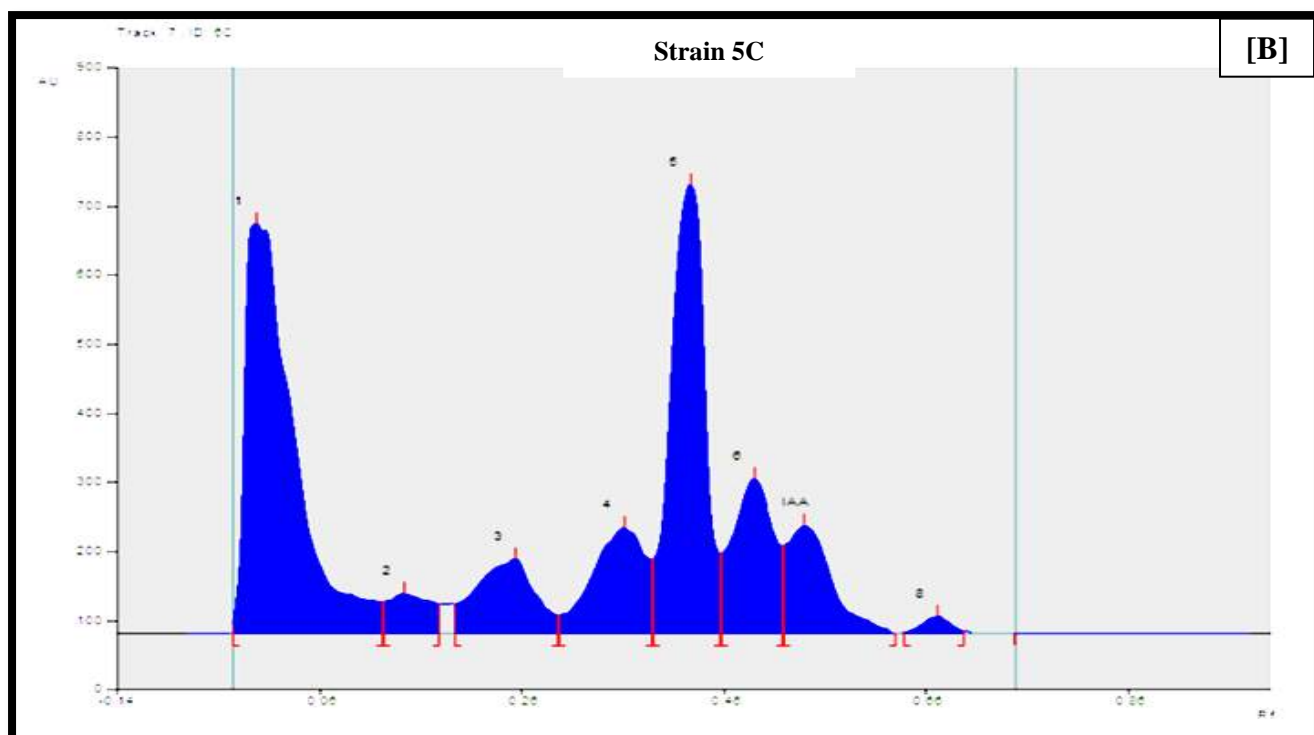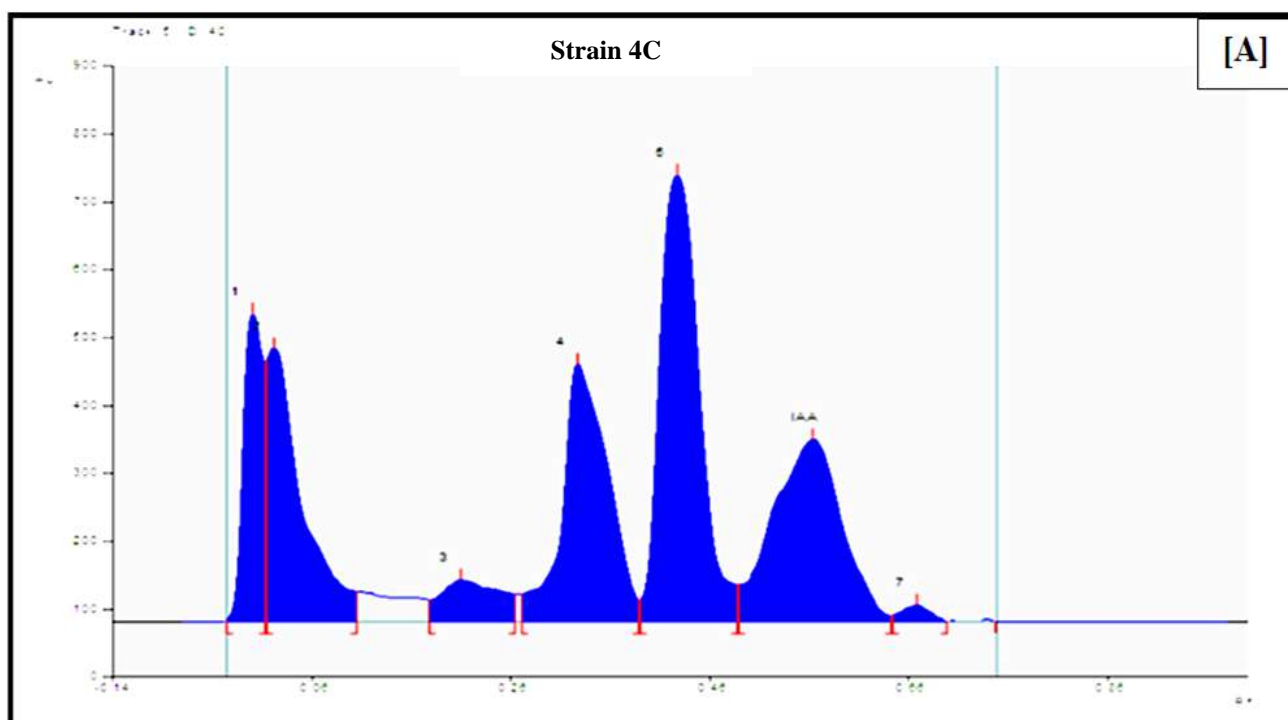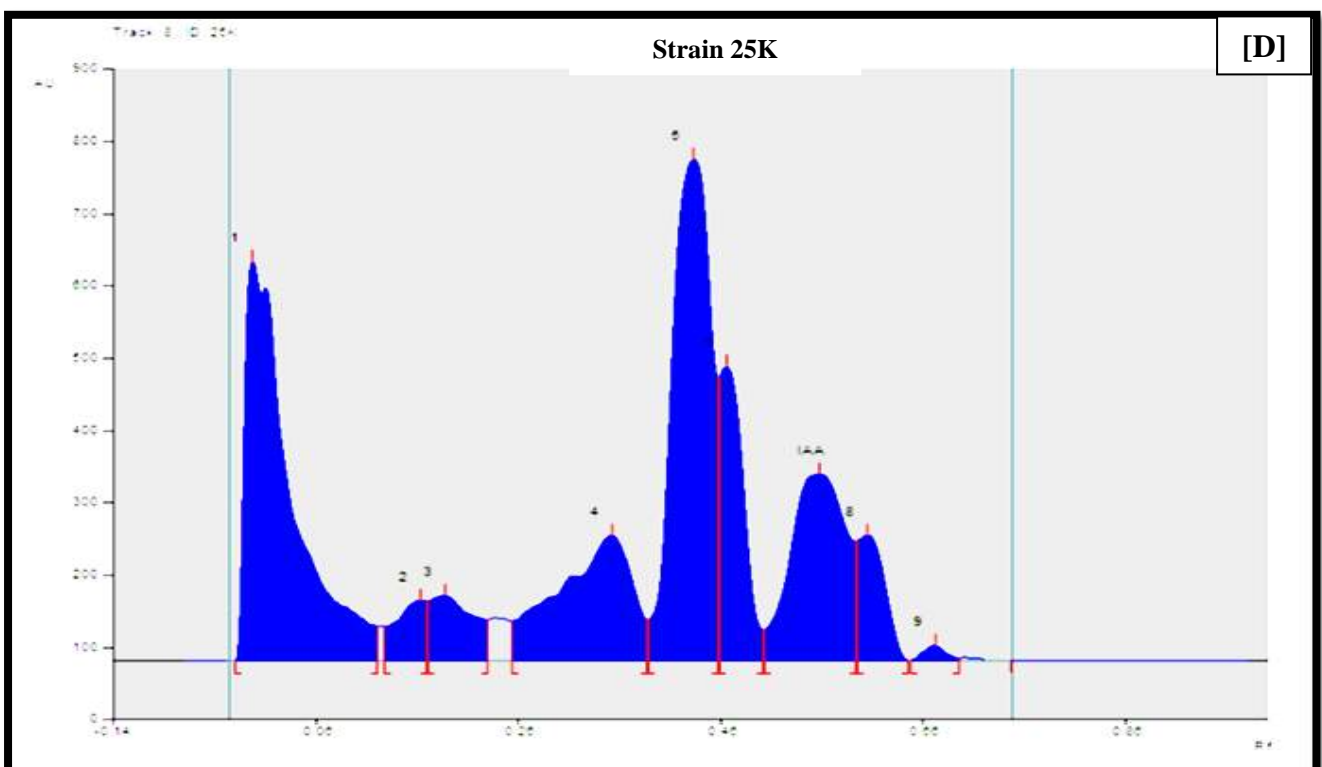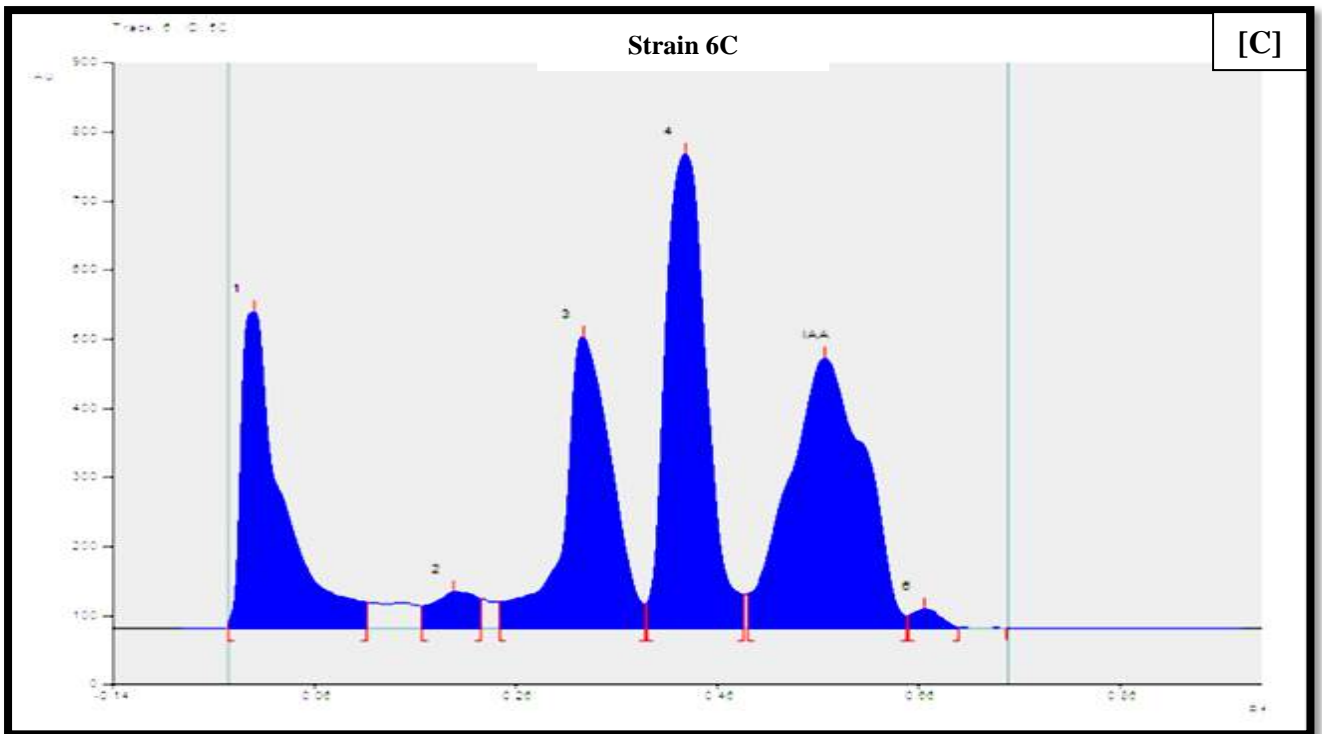

Figure 4.3.3 (A-C): The culture tubes shown different gradation of red colour after adding Salkowski reagent to L-tryptophan supplemented cell free bacterial media due to formation of roslindale dye in different concentration. The dye had formed by the reaction between Salkowski reagent and microbial isolate produced Indole compounds formed from precursor L-tryptophan.
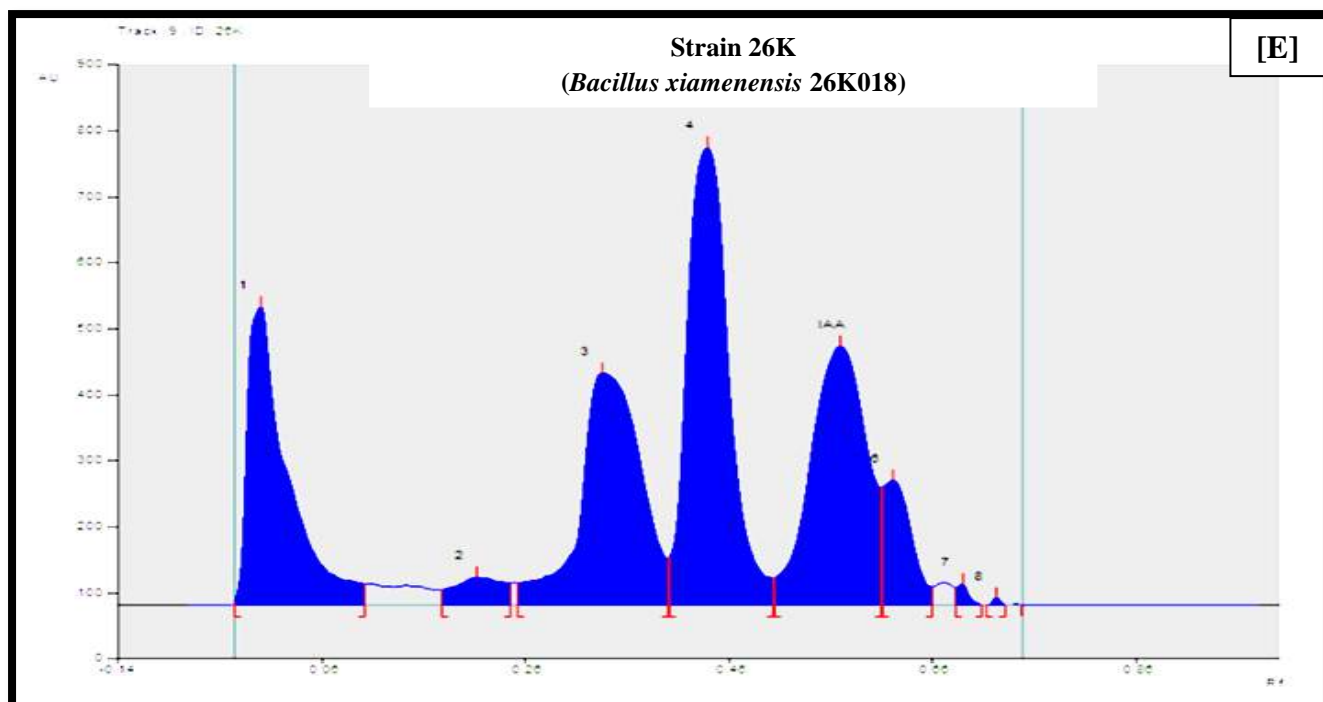
The cell free extracts of some selected microbes were subjected to HPTLC to confirm the presence and concentration of IAA and IBA. The co-chromatogram plots showed the

presence of IAA and IBA peaks in all the samples compared with standard solutions (Figure 4.3.4; Figure 4.3.5).
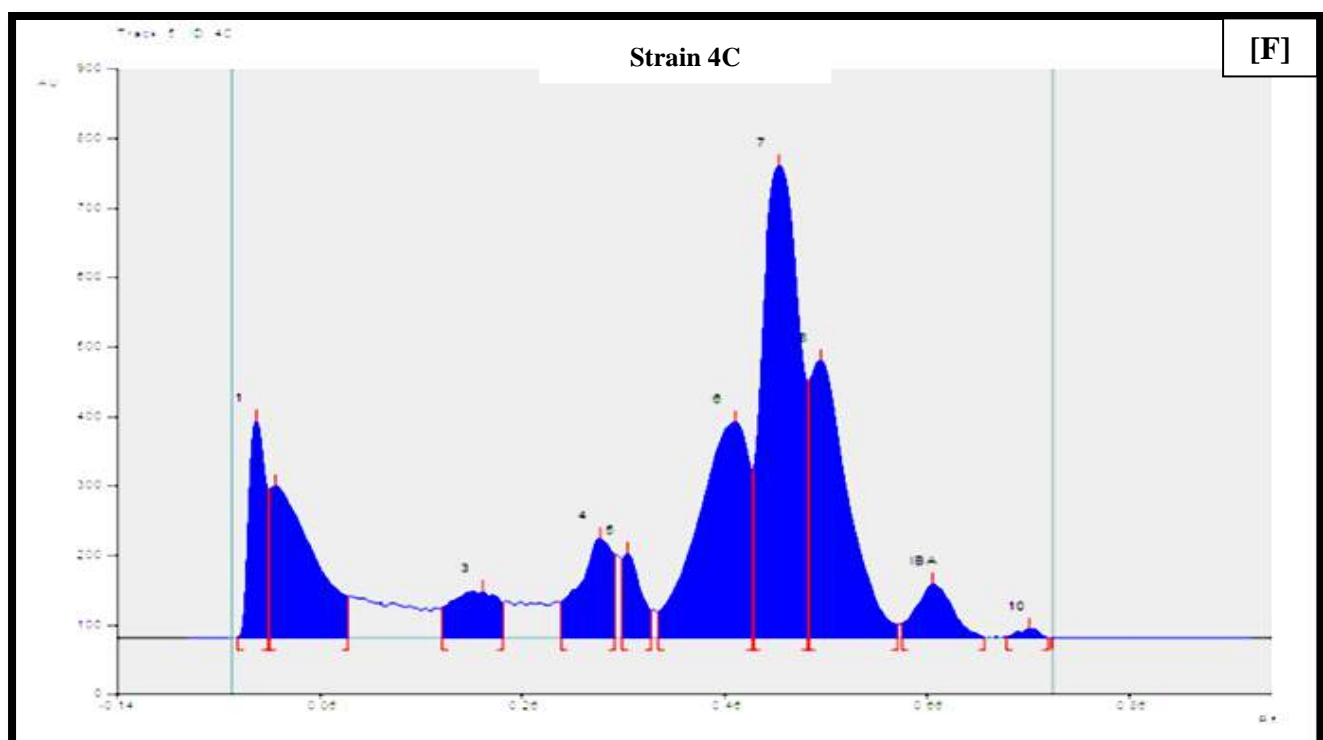
I.   **HPTLC chromatograms of IAA:**

Strain 4C [A]

Strain 5C [B]

Strain 6C [C]

Strain 25K [D]

Strain 26K
(*Bacillus xiamenensis* 26K018)
[E]

II. HPTLC chromatograms of IBA:

Strain 4C
[F]

Strain 5C

[G]

Strain 6C
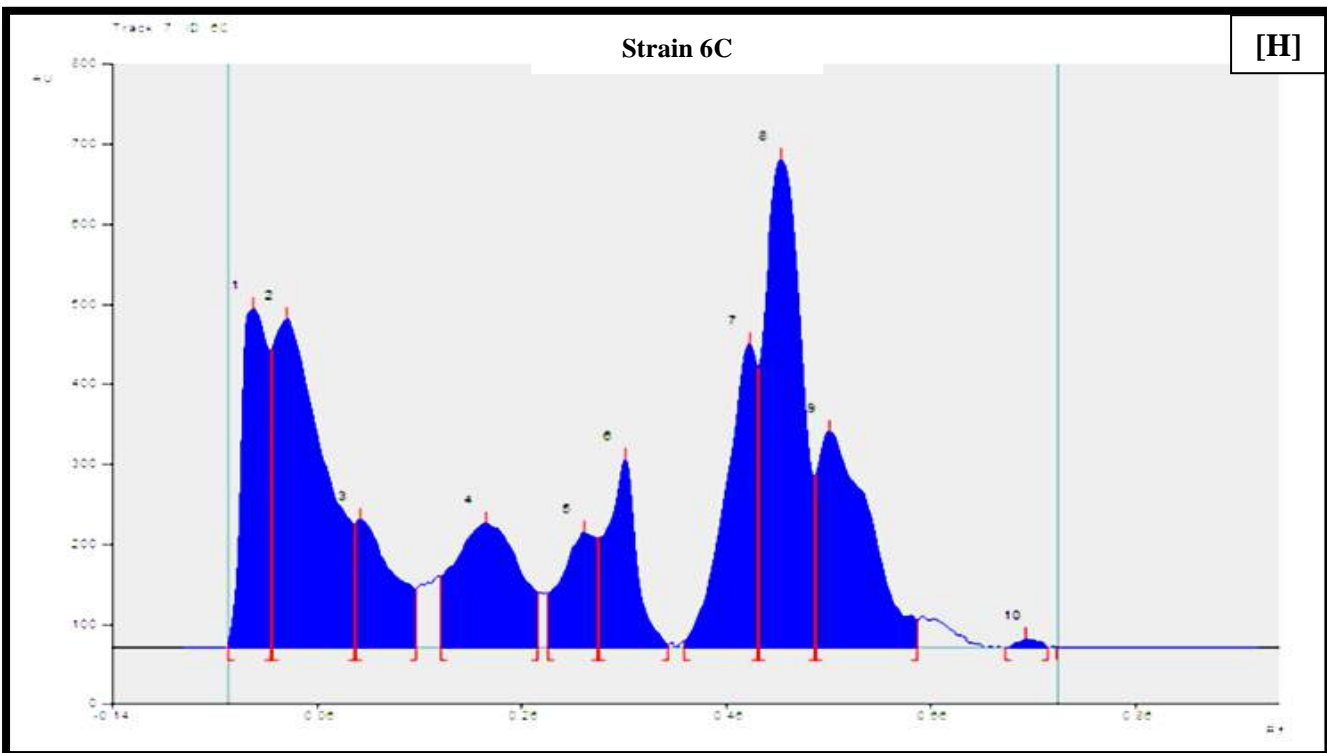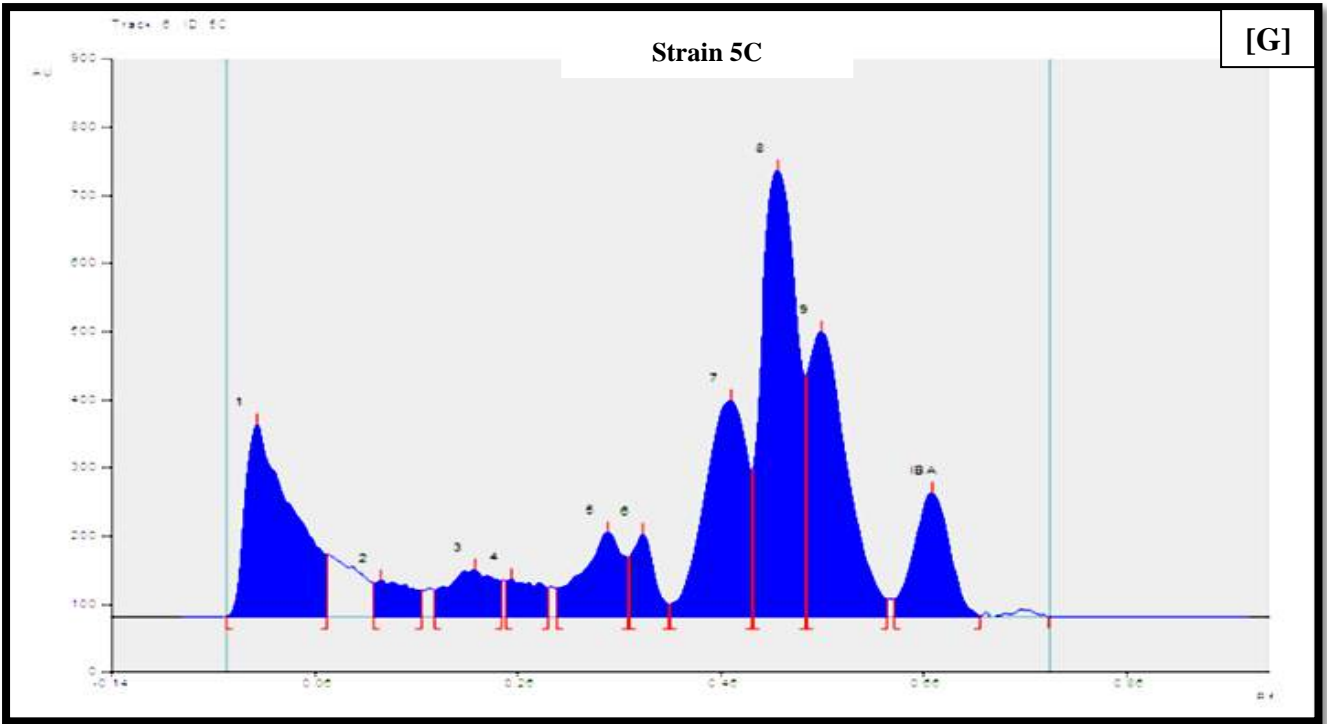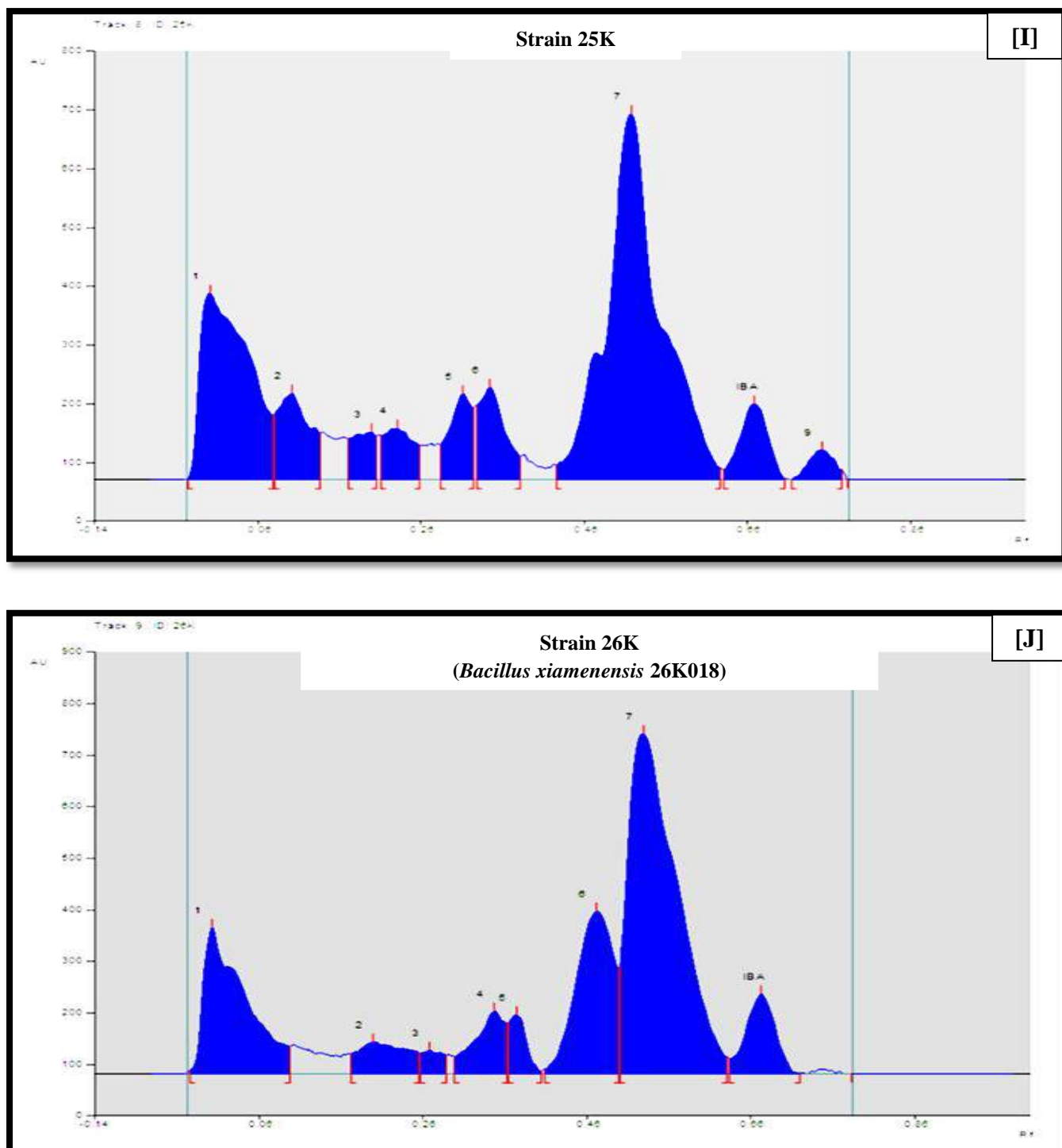
[H]

Figure 4.3.4 (A-J): HPTLC chromatogram of IAA (RF-0.47±0.02) and IBA (RF-0.69±0.05) compared with chromatograms of different Salkowski reagent treated bacterial supernatants
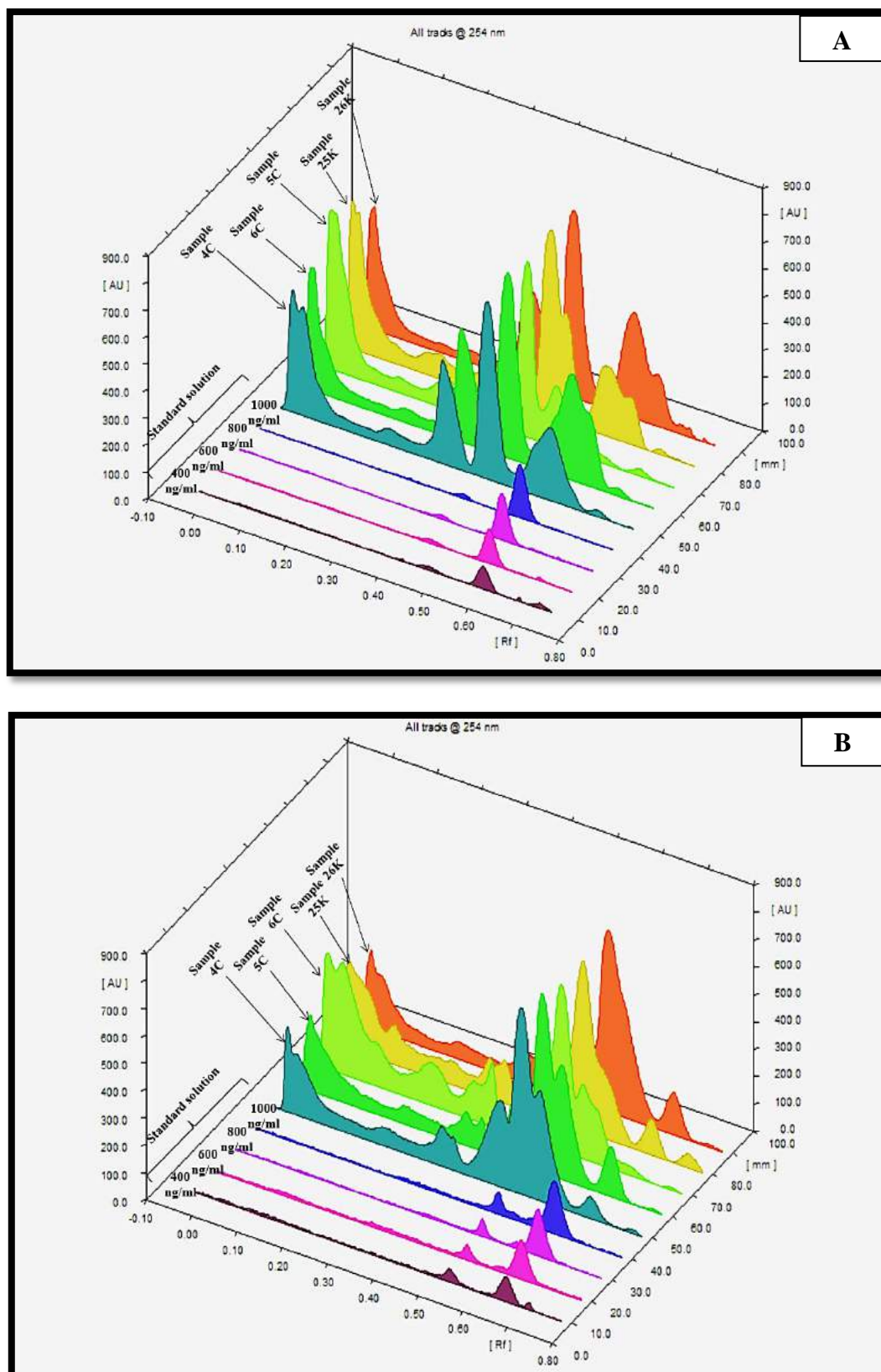
Figure 4.3.5: HPTLC chromatogram plot of IAA [A] and IBA [B] showing linearity

The calibration curves prepared with standard solutions in the range of 400ng/ml, 600ng/ml, 800ng/ml and 1000ng/ml for IAA and IBA are linear. Comparison of the peaks of standard IAA and IBA of known concentrations revealed the identity of IAA and IBA present in all the samples. Using the standard curve, the concentrations of IAA and IBA within our samples was calculated and it can be stated that amongst the selected Indole producing microbe, the majority produced IBA (Table 4.3.2).

Table 4.3.2: Concentration of IAA and IBA produced by different microbial isolates measured by HPTLC technique:

Strain	IAA (ng/ml)	IBA (ng/ml)
4C	764.69±2	380.79±1.6
5C	-	923.04±0.9
6C	-	-
25K	-	640.78±2.6
26K	-	806.09±3.6

4.3.3. Inspection of microbial siderophore production:

In the present study, results of the CAS agar test (Figure 4.3.6, Table 4.3.3) and $FeCl_3$ test (Table 4.3.3) revealed that almost all of the isolated soil microbes produced siderophores which help with plant growth and metal bioremediation.

Day 1   **Day 6**   **Day 12**
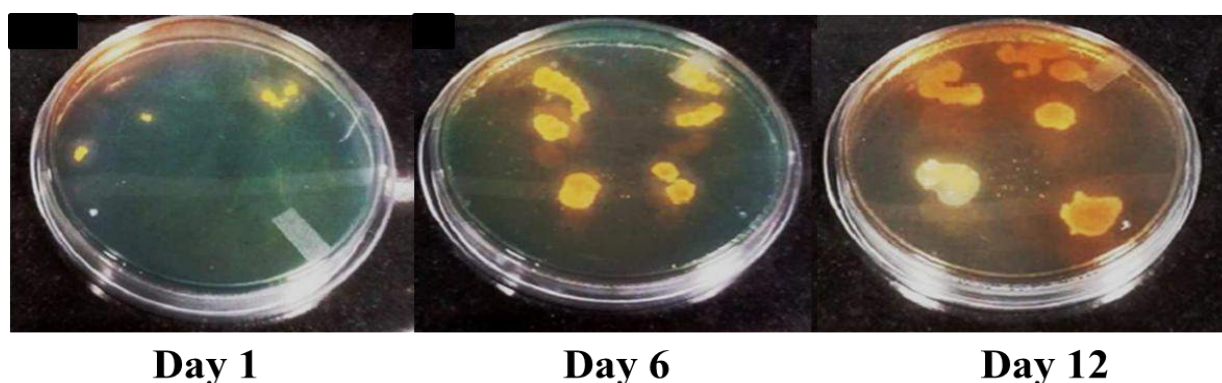
Figure 4.3.6: Detection of siderophore production on CAS agar plate after first, sixth and twelfth days of incubation. The disappearance of the blue colour of CAS agar media and the formation of orange pigment indicated that siderophore had been produced because siderophore producing organisms produce orange depletion zones by extracting iron from the blue Fe-CAS complex.

Table 4.3.3. The results of the qualitative and quantitative assays for estimating siderophore production by the bacterial isolates:

Bacterial Strain	CAS Assay	FeCl₃ Test	Arnow's test for Catechol	Tetrazolium test for hydroxamate	Hydroxamate Siderophore (μg)/ protein(mg) (using Lowry method)
1-C	+++	+	-	+	0.03
2-C	+++	+	-	+	--
3-C	+++	+	-	+	0.03
4-C	+++	+	-	+	0.01
5-C	+++	+	-	+	0.01
6-C	+	+	-	+	0.03
7-C	+	+	-	+	--
8-C	+++	+	-	+	0.01
9-C	++	+	-	+	0.03
10-C	+++	+	-	+	--
11-C	+++	+	-	+	0.05
12-C (*Microbacterium radiodurans* K12016)	+++	+	-	+	0.01
1-E(*Bacillus xiamenensis* strain 1E0018)	-	+	-	+	0.03
2-E	+++	+	-	+	0.02
3-E	+++	+	-	+	--
4-E	+++	+	-	+	0.03
5-E	+++	+	-	+	0.03
6-E	+++	+	-	+	0.03
7-E	++	+	-	+	0.05
8-E	+++	+	-	+	0.03
9-E	+++	+	-	+	0.05
10-E	+++	+	-	+	0.01
11-E	+++	+	-	+	--
12-E	+++	+	-	+	0.05
13-E	+++	+	-	+	0.03
15-E	-	+	-	+	0.05
1-K	+++	+	-	+	0.01
2-K	+++	+	-	+	0.03

3-K	+++	+	-	+	--
4-K	+++	+	-	+	0.03
5-K	+	+	-	+	0.01
6-K	+++	+	-	+	0.02
7-K	+++	+	-	+	0.01
9-K	++	+	-	+	0.03
10-K	+++	+	-	+	0.01
11-K	+++	+	-	+	0.05
12-K	+++	+	-	+	0.03
13-K	+	+	-	+	--
16-K	+++	+	-	+	0.01
18-K	+++	+	-	+	0.05
25-K	+++	+	-	+	0.02
26-K (*Bacillus xiamenensis* **strain 26K018**)	+++	+	-	+	0.03

4.3.4. Study microbial phosphate-solubilizing enzyme production:

No considerable change had taken place on Pikovskaya agar medium after 7 days of incubation at 30°C, indicated that the isolated microbes didn't formed phosphate solubilizing enzyme which mineralized phosphorus to make it accessible for plants (Figure 4.3.7).
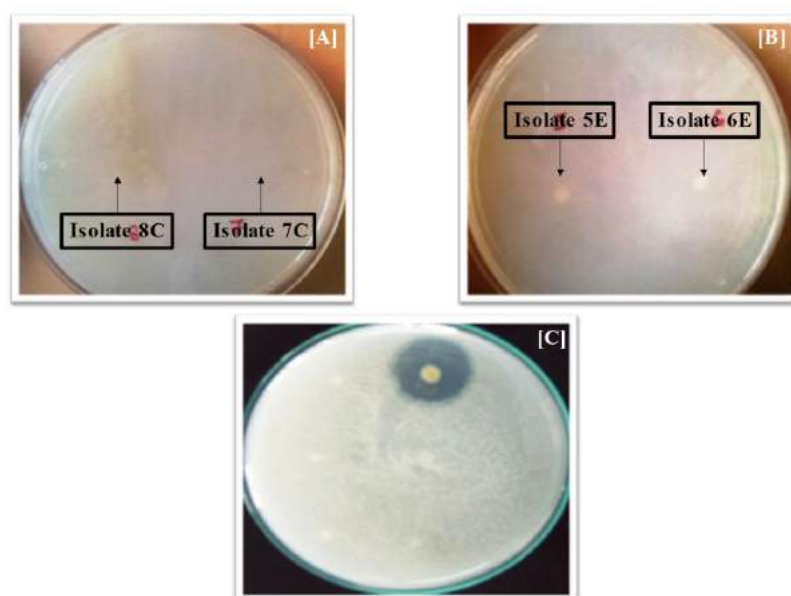

Figure 4.3.7(A-B): No observable change within the inoculated Pikovskaya agar medium after 7 days of incubation indicated that the isolated soil microbes did not take

part in phosphate solubilizing enzyme production. Figure 4.3.7(C): The *Bacillus* species used as positive control where the zone of clearance surrounding the microbial colony was formed due to the break-down of media-incorporated tricalcium phosphate to soluble phosphorus

4.3.5. Estimation of microbe formed Exopolysaccharide (EPS):

Most of the microbial isolates were produced EPS. The concentration of produced exopolysaccharide was remained within the range of 96 µg/ml to 159 µg/ml. (Figure 4.3.8; Table 4.4.4).

Figure 4.3.8: The intense white precipitate indicates the presence of exopolysaccharide (EPS) in chilled ethanol treated bacterial supernatant

Table 4.3.4: Concentration of exo-polysaccharide (EPS) produced by different microbial isolates compared and measured by calibrated glucose curve*:*

Bacterial isolate	Concentration of produced EPS (µg/ml)
5C	147±0.02
6C	148±0.09
7C	121±0.09
8C	148±0.01
12C (*Microbacterium radiodurans* K12016)	123±0.09
1E (*Bacillus xiamenensis* 1E0018)	131±0.06
2E	97±0.02
3E	109±0.01

4E	149±0.08
7E	120±0.9
5K	126±0.09
6K	104±0.01
7K	96±0.8
25K	159±0.05
26K (*Bacillus xiamenensis* 26K018)	147±0.09

4.4. Discussion:

Due to unintended industrialization and urbanization, the municipality sewage sites of the city Kolkata, become contaminated with hazardous heavy metals and surprisingly, no considerable modern sewage management system have been executed in this metro city [Vicziany, 2017]. The largest urban wetland ecosystem of this city, East Kolkata Wetland, also a declared Ramsar site, carry a load of both solid and liquid waste products of the tannery industry for a long time [Chattopadhyay et al. 2000; Vicziany, 2017]. In consequence of that, a huge amount of chromium has been accumulated in the water body as well as the adjoining soil and the region became highly polluted with chromium contaminants. The presence of battery manufacturing industry, organic dye-forming industry, electroplating industry in the near vicinity of different sewage lands of Kolkata and their direct untreated waste-water removal to the sewage-body is a legitimate source of chromium pollutants. Another major source of chromium contaminant is the compost containing leached-out soil from agricultural land situated adjacent to the wetlands.

Some sewage plants like *Eichornia crassipes* (water hyacinth), edible *Pistia stratiotes* (water lettuce) can sustain at different sewage sites and have successfully taken part in chromium bioaccumulation and removal until a certain limit [Mishra and Tripathi, 2008; Giri and Patel, 2011; Chakraborty et al. 2015]. Beyond the sustainability threshold concentrations, heavy metals like chromium generated many deleterious effects on the plants. [Hadad et al. 2011]. The plant-accumulated metal also affects human health adversely through biomagnification at each trophic level [Cervantes-Ramírez et al. 2018]. The polluted wastewater nourished aquaculture ponds and agricultural crop-lands of Kolkata, make used for fish and vegetable production for economic purposes, are the frightening source of heavy metal bio-accumulation and bio-magnification [Vicziany, 2017; Chattopadhyay et al. 2000]. Bhattacharya et al (2006) and Sanyal et al. (2017) had reported an alarmingly high concentrations of chromium bio-accumulation inside the commercially cultivated fish tissues in Gangetic West Bengal. Besides large-scale commercial production, some edible plants namely, *Colocasia esculenta*, *Ipomea aquatica*, *Coccinia cordifolia*, *Amaranthus viridis* etc. had found to grow within the slurry sewage soil by the poor local residents. At the time of field visits, the local residents informed us that they have consumed and also sold the sewage grown vegetables on a regular basis. So, there is a great threat of trophic transfer, accumulation and biomagnification of chromium in different sewage sites of Kolkata.

As per our investigation, the waste-water bodies of Kolkata have been adversely polluted with various chromium contaminants. We had noticed through reviewing the previous literature that soil chromium has become a threat for the whole world because of its adverse effects on plant growth, animal and human wellness, as well as microbial diversity [Shrivastava et al. 2002; Cefalu and Hu, 2004; Oliveira, 2012; Stambulska et al. 2018]. Keeping the situation in mind, we wanted to investigate the role of our chromium tolerant rhizospheric microbial isolates in the well-being of the polluted sewage soil inhabitant plants by promoting plant growth along with decreasing plant chromium accumulation. To study their role in decreasing plant chromium accumulation, we calculated the bioconcentration factor (BCF) values of the plant collected from different chromium polluted sewage regions and the plants grown in experimental conditions. The Bioconcentration Factor (BCF), is the ratio of the metal concentration measured in the vegetation to the concentration in the soil supporting that vegetation, can predict if the organism has accumulated the absorbed substance within their cell or not. A lesser than one BCF value in plants indicates that they probably only absorb, but do not accumulate heavy metals within the cell [Glick and Bashan, 1997]. On the other hand, greater than one BCF indicates that the metal is absorbed faster than it is removed and concentration builds up within the tissue i.e., bioaccumulation occurs. The BCF of some edible plants grown at the near vicinity of the sewage regions and consumed by local people should be measured to investigate bio-accumulation and bio-magnification of heavy metals through the food chain. We also calculated the BCF values and henceforth observed the metal absorption and accumulation capacity of leguminous plant *Vigna radiata* in presence of the mixed and pure culture of the microbial isolates of three different wastelands of Kolkata and found that all of the microbial isolate reduced chromium accumulation in experimental conditions. The same trend was observed in field conditions too. We had chosen *Vigna radiata* of family Papilionaceae (formerly known as Leguminosae) as our plant of interest because, several earlier studies reported the adverse effects of hexavalent chromium like plant morphology alteration, growth depletion, seed germination retardation or seedling growth inhibition on the leguminous plants. Most importantly, hexavalent chromium affects leghemoglobin like pigment formation by inducing oxidative stress. But, the symbiotic association of plant roots and microbes has reduced the toxic effects of chromium due to the metal accumulating capability of microbe [Stambulska et al. 2018]. Formerly, Wani and Khan (2010) reported the potent role of *Bacillus* species in the growth promotion of economically important leguminous plant, *Cicer arietinum* at chromium contaminated soil by enhancing nodulation and leghemoglobin formation and decreasing plant-soil chromium

absorption. Oves et al (2013) also described the role of one *Pseudomonas aeruginosa* strain in enhancing the growth of chickpea (*Cicer arietinum*) plant in chromium polluted soil.

The presently isolated chromium tolerant strains *Microbacterium radiodurans K12016, Bacillus xiamenensis 1E0018* and *Bacillus xiamenensis 26K018* promote plant growth by producing plant growth stimulating substances like indole compounds, siderophore. The plant growth-promoting strains also reduced plant chromium absorption by implying soil cation mobility. Low molecular weight iron-chelating compound siderophores are secreted by bacteria under iron starvation and make iron available for microbial and plant cells. Siderophores act as bio-remediators and chelation agents due to their heavy metal mobilization properties. Many previous studies had demonstrated the role of siderophore in soil metal mobility [Renshaw et al. 2002; Dahlheimer et al. 2007; Schalk et al. 2011]. The low molecular weight organic acids produced by the soil microbes have also taken part in ion mobility in soil. Though, the effectiveness of siderophore in soil mineral mobility is three to four times greater than the low molecular weight organic acid because siderophore formed a more stable compound with iron synergistically [Perrin, 1979; Matzanke, 1991; Reichard et al. 2007]. Organic acids which are produced by the anion decarboxylation activity or digestion of organic waste by soil microbe, [Yan et al. 1996; Tang et al. 1999; Binkley and Fisher, 2012] play a significantly important role in soil mineral mobility and make nutrients available for plants [Adeleke et al. 2017]. Our isolated decarboxylation activity exhibiting (by forming extracellular enzyme urease) and organic acid-forming (MR-VP positive) soil microbes also showed the same trend. The most relevant reason behind the microbial role in lowering plant heavy metal absorption can be soil mineral mobility.

In spite of assisting plant growth in several ways, our isolated soil microbes didn't produce phosphate solubilizing enzymes and can't help in soil phosphorus mineralization. This finding indicated that the microbes didn't take part in the mobilization of soil phosphorus to make phosphorus bio-available for plants. Instead of that, the soil microbes incorporated the immobile organic phosphorus within their living cell and utilized for their own nutritional welfare. High concentration phosphorus accumulation within our microbial cell pellet (316000 ± 2.0 ppm) manifested evidence of microbial phosphorus absorption. It doesn't mean that the phosphorus remains permanently unavailable for the plants because the microbe-incorporated phosphorus comes back to the soil after the death and decaying of microbial bio-mass and the phosphorus is again available to the plant through hydrolysis. This kind of phenomenon indicated that the soil phosphorus content had significantly high due to the

presence of a huge amount of organic matter and the plants got an ample amount of mineralized soil phosphorus to conduct the metabolic activity. The higher phosphorus content (11,489±0.8 ppm to 14,862.08±0.03 ppm) in the sewage grown plants indicated that the plants absorb adequate amount of soil phosphorus and that's why the soil phosphorus pool surrounds the root got disrupted [Marschner, 1995]. Another reason behind plant phosphorus pool depletion is the presence of a high concentration of hexavalent chromium (Cr^{6+}) within soil [Ali et al. 2011]. Some stress adopting plants, also have developed their own phosphorus solubilizing mechanism by secreting organic acids which form a chelate with phosphorus, and are not dependent on soil microbe for that [Schachtman et al. 1998]. The rising temperature, neutral to slightly alkaline pH, clay containing weathered sludge [Aishah et al. 2019] and presence of other minerals like calcium increased phosphorus affinity of soil microbes at the sewage regions [Channarayappa and Biradar, 2019]. We had deduced that the clay containing weathered sludge, high calcium content (77,303±0.05 ppm to 26, 514 ± 0.18 ppm), neutral to slightly alkaline soil pH (varies from 7.18±0.25 to 8.05±0.02) and the hot and humid climate [annual average temperature and related humidity varied between 28°C to 38°C and 55% to 85% respectively according to Calcutta (Alipur/Dumdum) Climatological Table, 2020] of different sewage sites of Kolkata might enhance phosphorus immobilization and helped in phosphorus incorporation within microbial tissues. In this manner, the soil surface resident sewage microbes disposed of the waste through decomposing organic matter, transforming them into inorganic molecules and assimilating nutrients [Gerba, 2005]. Thus, the consortia of plant root and their adjacent rhizospheric bacteria developed a microbiome which possesses significant role in the biogeochemical cycling of different important minerals like phosphorus and helps in environmental waste management by implementing several strategies to survive in stress-induced environment. One of the most important strategies acquired by the soil microbe is, to protect plants in the hazardous environments by producing different plant growth inducers and lowering the absorption of high-risk substances like heavy metals by plants. In this way, the microbe help in cropping and at a time reduces the risk of chromium bio-magnification. This type of complex ecological relationship and its interaction with environmental abiotic factors should play a key role when implemented in environmental management.