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Partial purification of antibiotic compound from resistant bacteria species isolated from Bhavani river water

A. Saravanan[1*], P.R. Yaashikaa[2], S. Divya[3], R. Keerthana[3], S. Shanmathi[3]

[1]*Department of Chemical Engineering, SSN College of Engineering, Chennai-603110, India*

[2]*Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam-638401 India*

[3]*Department of Biotechnology, Vivekanandha College of Engineering for Women, Namakkal-637205, India*

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ABSTRACT

The river Bhavani is the second largest river in Tamil Nadu, South India and a major tributary of the Cauvery River. The chemical manufactures, sugar mills, textile units, tanneries, paper industries, etc., discharges the treated and untreated wastes into the Bhavani river. The study reveals collection from various sites of Bhavani River, Mettur, Pallipalayam and Kumarapalayam. The microbes were approached by culture-dependent for understanding the physiological, morphological and biological property. Pure colony isolation and analysis of sensitivity by disc diffusion method were done. As the uptake of toxic substances by the microorganisms for its growth, so they are possessing high resistivity. Among 26 isolates 19 isolates showed maximum sensitivity (10 mm - 32 mm) and 8 isolates showed moderate sensitivity (1 mm - 10 mm) to different antibiotic disk. Among all the isolates, isolate 19 showed maximum resistances to all antibiotics (≥ 4mm). Hence the isolate 19 was further purified by column chromatography, fraction 17 showed antimicrobial activities to 5 microorganisms.

1. Introduction

Water is one of the basic needs of life. Water supports all forms of life and also affects our health, lifestyle and economy. The largest available sources of fresh water are underground and river water. The water from these sources is used for domestic, agricultural, industrial applications all over the world. Fresh water is a finite resource, without fresh water of considerable quality and quantity sustainable development will not be possible. Rivers play a vital role in assimilation of municipal, industrial and agricultural wastes that constantly polluting the source[1-3]. The contamination of River water increases due to rapid development in agriculture, mining, urbanization and industrialization activities. Almost 70% of the water has become polluted due to the discharge of domestic wastes, industrial effluents and hazardous wastes into the natural water sources[4-6]. The analysis of water is vital since the quality of the water is directly related to the human health. The portability of the water is decided with the help of physical, chemical and microbiological standards. The river Bhavani is the second largest river in Tamil Nadu, South India and a major tributary of the Cauvery River. The water drains an area of 6,200 kmA^2 spreads over Tamil Nadu (87%), Kerala (9%) and Karnataka (4%). The main course of river is located on entire of the North-Western Erode district of Tamil Nadu[3]. Bhavani rises in the silent valley in Palghat ranges in the state of Kerala, after receiving Siruvani. It flows into Coimbatore District and enters Erode District traversing through Bhavani. It feeds the Bhavani sagar reservoir, it ultimately joins river Cauvery on the Salem borders. The Bhavani River has 12 major rivulets which joins and drains into the northern Nilgiri slopes. At Attappady Mukkali, Bhavani takes an abrupt turn and flows through inter-State border[7]. It gets reinforced by the Kunda River forms the boundary between Kerala and Tamil Nadu. It joins the Bhavani border in western Tamil Nadu at a place called Athikadavu. The Siruvani River a perennial stream of Palakkad district and the Kodungarapallam River, joins the Bhavani at Koodappatti on the inter-state. At coonoor, it meets Coonoor River where it drains into the valley between the northern slopes of the Nilgiris and the southern slopes of the Bilgiri Hills. After the Moyar it is blocked by the Lower Bhavani Dam. The river continues transversing Kodiveri Dam, near Gobichettipalayam and Bhavani taluks and merges with the Cauvery. A small distance before joining Cauvery it feeds Kalingarayan irrigation canal. The large scale industries such as chemical manufactures, sugar mills, textile units, tanneries, paper industries, etc., are discharging the treated and untreated wastes into the river[8,9]. The same river is used for irrigation, drinking and other domestic activities by the nearby villagers. The effluent mixed river water crosses the district Coimbatore, Nilgiris, Erode and merges with the river Cauvery[10]. According to the Tamil Nadu pollution control department, 77 illegal dyeing units are now functioning on both sides of the river in Bhavani, Kadayampatti, Sengadu and Seruvyanarapalayam. Along with 49 authorized dyeing units they discharge toxic effluents directly into the river water, polluting both Bhavani and Cauvery and endangering the lives of more than six million people.

***Corresponding author:** A. Saravanan*, Department of Chemical Engineering, SSN College of Engineering, Chennai-603110, India; E-mail: *sara.biotech7@gmail.com*

Most of the industrial units lack a proper system to treat effluents. The existing units do not have treatment facilities. As a result, the processing units store the chemical effluents in the day and discharge them into the river in the night causing irreversible damage to the ecosystem**[11]**. Thus it has been confirmed that Bhavani is fast becoming one of the most polluted rivers in the country. The groundwater in the number of parts in Erode district is contaminated heavily due to indiscriminate discharge of untreated effluents by the textile and leather processing units. The color of water drawn from many wells is dark red. There is no other option to use the polluted water for all purposes except drinking. A large number of residents suffer from skin-related diseases like albinism, eczema, actinickeratosis and hypoglycemia because of the usage of the polluted water. The toxic substances which are discharged into the water produce dense population and various species of microorganism. The water samples were collected from various sites of Bhavani River, water sample from Mettur dam serves as standard and other two sites are Pallipalayam and Kumarapalayam. Dying industry in Kumarapalayam releases its effluent and the paper industry in Pallipalayam discharges its toxic substances into River Bhavani. The Microorganisms in the river water uses the industrial discharge as substrate for its growth. Due to the uptake of toxic substances the microorganisms possesses high resistivity**[12]**. Analysis of microorganism in Bhavani River is necessary for its portability. Culture-based approach is useful in understanding the physiological, morphological and biological properties of microorganisms that could be cultured. Microorganism cultured from Bhavani River was undertaken for screening. The microorganisms were isolated from the water sample collected from Bhavani River in Tamil Nadu. Serial dilution was done to reduce the number of microbes present in the water samples**[5]** (Figure 1). The isolates were placed on nutrient agar medium and incubated at 37 ºC for 24 h. The pure and single colonies were isolated and plated on another separate nutrient agar plates. The isolates were streaked on muller-hinton agar plates and tested with antibiotic discs by disc diffusion method for its sensitivity. The diameter of zone of inhibition explains the resistivity and sensitivity of the isolates. The antimicrobial compounds were purified by various compound purification techniques. The culture was centrifuged and the supernatant was separated and undertaken for compound purification. Equal volume of ethyl acetate was added to the culture supernatant and the compounds are separated. The upper phase was separated and allowed for evaporation and the dry solid compounds are used for further purpose. The extracts were then column fractionated using silica gel chromatography with different proportions of methanol: chloroform. The fractions were concentrated and used for antimicrobial**[13]**. The concentrated fractions were subjected to Thin Layer Chromatography. Extracts were spotted on silica gel plates and dipped in solvent mixture of methanol, chloroform and acetic acid and the active spots are detected. The extracts were further purified using High Performance Liquid Chromatography for obtaining pure compounds. The culture supernatant was added with ethyl acetate and analysed by HPLC. 10µl of sample was injected to C18 column, at flow rate 0.50 ml/min and analyzed at 220 nm wavelength. The sample was purified the active fraction (methanol: chloroform 20: 80 extract); chloroform was added gradually (drop wise) until formation of the first precipitate, the precipitate was centrifuged to it Ammonium sulphate was also added. Each precipitate was dissolved in water and re-precipitated again by ammonium sulphate**[14]**. The present study reports the isolation of bacterium from Bhavani river water, antibiotic resistivity and compound purification.

Figure No. 1: Sample collection sites (a). Mettur, (b). Kumarapalayam, (c). Pallipalayam

2. Materials and methods

2.1 Sample Collection

Samples were collected from different sites of Bhavani river, Erode District of Tamil Nadu during the month of January 2013.The Bhavani river is located at 11.4333° N and 77.6833°. All samples were collected from different habitats Mettur located at 11.8000°N -77.8000°E, Pallipalayam located at 11.3643°N-77.7623°E, and Kumarapalayam located at 11.4419°N- 77.7088°E. Samples were collected in sterilized plastic bottles and brought to laboratory within 2 hours.

2.2 Physicochemical analysis

Physicochemical properties (pH, temperature, total suspended solid, color) were analyzed**[15-18]**.

pH

pH meter was calibrated by double distilled water, then 30 mL of samples were taken in a beaker, then the pH of the samples was determined by pH meter**[19]**.

Temperature

Temperature is measured at the site of collection by using thermometer**[16]**.

Total suspended solid

The weight of the Whatman filter paper was measured. Then the water sample was filtered through Whatman filter paper and then allowed for air dry. Later, weight of the filter paper was measured**[19]**. Total suspended solid content can be calculated by following equation

Total suspended solids = filter paper weight before filtration - filter paper weight after evaporation

2.3 Isolation of bacterial strains

1ml of water sample was serially diluted (10^{-1} to 10^{-6}) and 0.1 mL of was placed on nutrient agar medium by spread plate method**[9]**. Then the plates were incubated at 37 °C for 24 h. Morphologically distinct colonies from dilutions 10^{-1} to 10^{-6} were selected and were further streaked onto the same medium to obtain pure colonies. All the pure colonies obtained were sub cultured in nutrient agar medium for further purpose. Similar glycerol stock was prepared with 20% glycerol and 80% culture broth and store for further use**[20,21]**.

2.4 Sub culturing of bacterial strains

Morphological distinct colonies from all the plates of were transferred onto nutrient agar media. Then the plates were incubated at 37 °C for 2 days. Single colonies in the above mentioned plates were selected and were restreamed to obtain pure colonies**[22]** (Figure 2,3,4,5**)**.

2.5 Screening of bacterial strains by antibiotic susceptibility test

Pure isolates were selected for antibiotic susceptibility assessment using the disc diffusion method**[18,23,24]**. The isolates were grown in nutrient broth and cultures were swabbed on to the Mueller–Hinton agar. Antibiotic-impregnated discs were placed on Mueller Hinton Agar and incubated at 37 ºC for 24 h. Zones of inhibition were measured. Antibiotics in the panel included Tetracycline (30 µg), Gentamycin (120 µg), Erythromycin (10 µg), Kanamycin (10 µg), Penicillin G (30 µg) were used. Among the isolates which showed more resistant to antibiotic was selected for further purpose.

2.6 Mass cultivation of screened colony

The screened colony was inoculated in 100ml nutrient broth and incubated at 37 °C with shaking for 24 h. This culture was again inoculated in 2 L nutrient broth for mass cultivation of production and incubated at 37 °C with shaking for 24 h**[25]**.

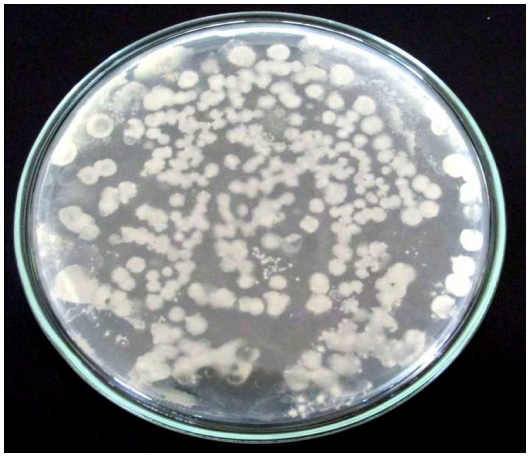

Figure No. 2: Bacterial isolates

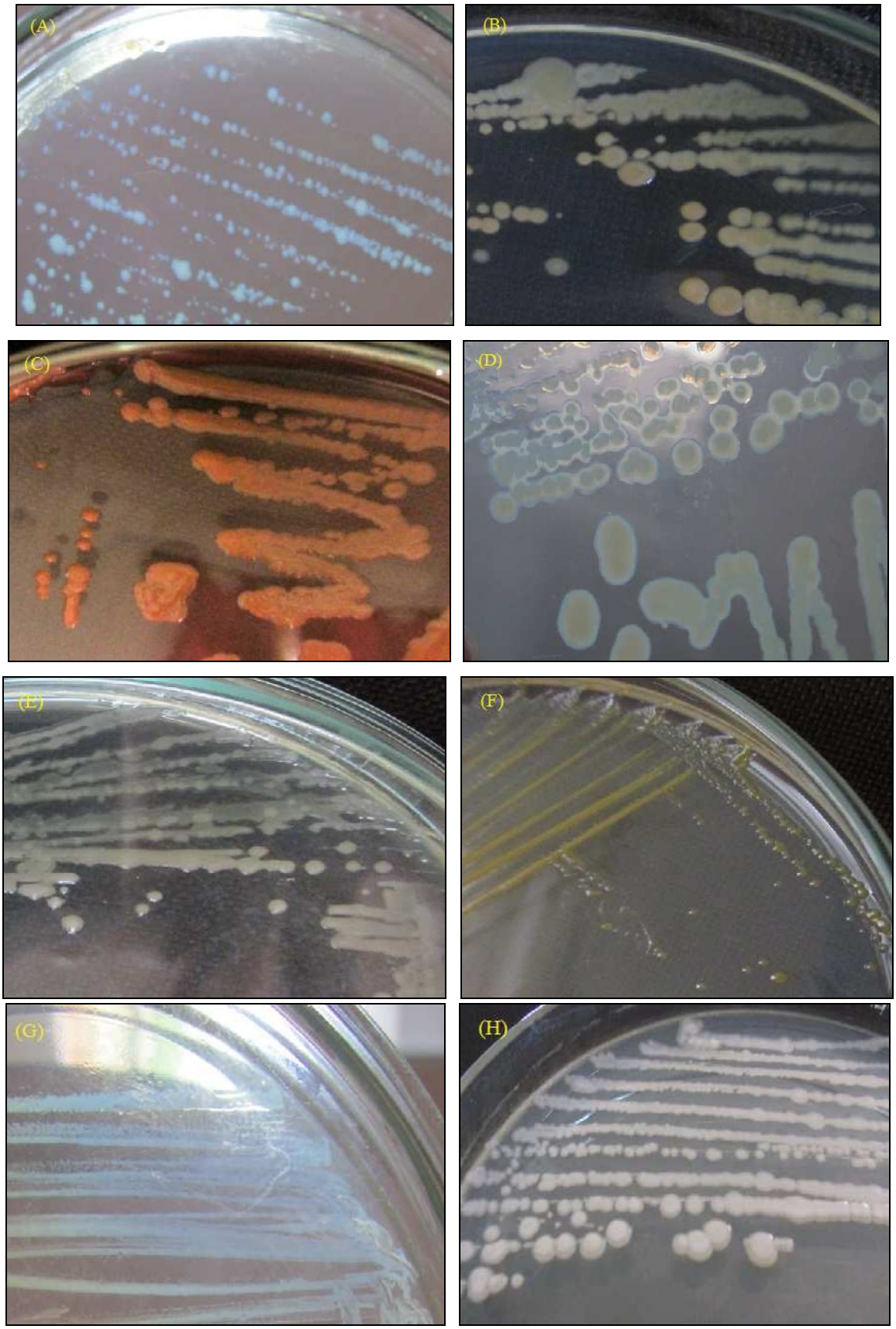

Figure No. 3: Purified colonies (a). Isolate-2, (b). Isolate-4, (c). Isolate-8, (d). Isolate-15, (e). Isolate-19, (f).Isolate-6, (g). Isolate-18, (h). Isolate-22

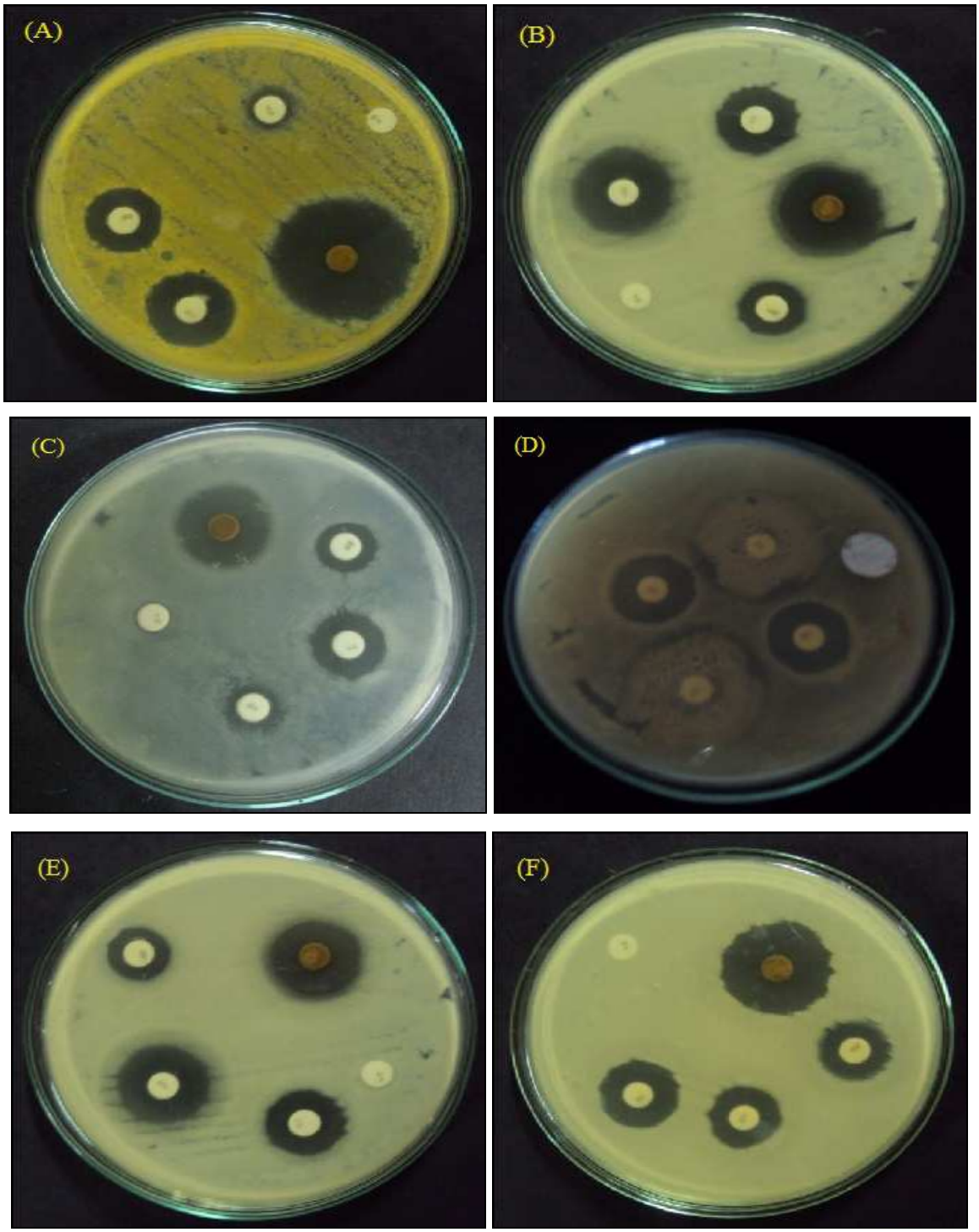

Figure No. 4: Zone of inhibition obtained after 24 h incubation of antibiotic susceptibility test (a). Isolate-17, (b). Isolate-22, (c). Isolate-19, (d). Isolate-7,(e). Isolate-4, (f). Isolate-1

Figure No. 5: Mass cultivated screened colony (a). Control, (b). Culture media after 24 hours

2.7 Partial purification of antibiotic compound

Solvent extraction

From the production media the cells were removed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant was subjected to solvent extraction with ethyl acetate (1:1). The fractional extract was spun-dried and the solvent residue was dissolved in 10 mL of solvent were used as the test extracts for antimicrobial activity assay**[26]** (Figure 6).

Figure No. 6: Solvent extraction

Thin Layer Chromatography

Thin layer chromatography (TLC) plates were prepared by making 30 gm of silica gel-G with 60 mL of distilled water. The silica gel was evenly spread on glass plates (20x20cm) and air dried. Optimization of mobile phase (butanol: acetic acid: water in two ratios of 4:1:5 and 2:1:8) for fractions was done. The plates were dried in an oven for 30 min. Chromatogram was developed by loading 10 µL each fraction and running for half an hour. Spots on the plates were visualized in an UV illuminator.

Column Chromatography

The purification of the antibiotic compound was carried out using silica gel column (1.5 X 25) chromatography with 60-12 mesh and 1ml/min flow rate. Different fractions of Chloroform and Methanol from 99:1 to 1:99 (v/v) were used as an eluting solvent**[13]**. 1ml crude extract to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel (Figure 7).

Figure No. 7: Column chromatography

Antimicrobial activity test for the fractions

The collected fractions were tested for their antimicrobial activities. The fractions can be diluted with distilled water up to

10 mL then 5 mL of this diluted fractions were added to the culture media after that the bacterial strains *E.coli*, *Bacillus*, *Pseudomonas*,*Serratia and Klepsiella* were incubated at 37 °C and optical density at 240 nm was measured at 4 to 24 h intervals. The highly active fraction was selected for further use.

High Performance Liquid Chromatography

The obtained highest active fraction was analyzed by HPLC. 10 µL of sample was injected to C18 column (250mm X 4.6mm X 5mm). The flow rate was 0.50 mL/min. Sample was analyzed at 220 nm wave length[14].

FTIR

Fraction that showed maximum anti-bacterial activity was further subjected to spectroscopic analysis[27]. A known weight of TLC-purified fraction of the extract (1gm) was taken in a mortar and pestle and ground with 2.5 mg dry potassium bromide (KBr). The powder so obtained was filled in a 2mm internal diameter micro-cup and located onto FTIR set at 26°C ± 1°C. The sample was scanned using infrared in the range of 4000-400 cm-1 using Fourier Transform Infrared Spectrometer. The spectral data obtained was compared with the reference chart to identify the functional groups present in the sample.

3. Results and discussion

3.1 Sample collection

Water samples were collected from Mettur, Kumarapalayam and pallipalayam in Bhavani river of Tamil Nadu. Sample collection sites were shown in Fig. 1. The collection sites Kumarapalayam and Pallipalayam has been contaminated by industrial effluents and the microbes grown in this water use the toxic substances as substrate for their growth which was already reported by[7].

3.2 Physicochemical analysis

Physicochemical properties (pH, temperature, total suspended solid, color) were analyzed and the results were shown in Table 1.

Table No. 1 Physicochemical parameters

	SAMPLE - 1	SAMPLE - 2	SAMPLE - 3
pH	6.8	6.65	6.53
Temperature	29˚C	28.5˚C	25˚C
Total suspended solid	0.008g	0.0095g	0.091g
Colour	Colour less	Light Brownish	Yellowish White

3.3 Isolation of bacterial strains

A total of 26 distinct morphological bacterial isolates were obtained from serially diluted water samples on agar plates from 10^{-1} to 10^{-6} [24] and the results was shown in Fig .2. Among these 23 isolates, 6 colonies were isolated from Mettur sample, 9 colonies were isolated from Pallipalayam sample and 11 colonies were isolated from Kumarapalayam sample.

3.4 Purification of bacterial strains

The isolated colonies were sub cultured using same medium by quadrant streaking method. Single colonies obtained from the purification plates were used for further screening. There are 26 morphological distinct colonies which were further plated on nutrient agar medium and the results are shown in Fig .3. Glycerol stock was also maintained with 20% glycerol and 80% culture broth [28].

3.5 Screening of antibiotic resistance bacterial strains

Purified each isolates were inoculated in 100 mL nutrient broth. This culture was swapped on Muller Hinton agar medium and five different antibiotics were placed on the plates. Zone inhibition for all the isolates were measured after 24 h inhibition, the result was shown in Fig .4. Among 26 isolates 18 isolates showed maximum sensitivity to Tetracycline, 8 isolates showed moderate sensitivity to Tetracycline, 17 isolates showed maximum sensitivity to Gentamycin, 9 isolates showed moderate sensitivity to Gentamycin, 16 isolates showed maximum sensitivity to Erythromycin, 8 isolates showed moderate sensitivity to Erythromycin, 19 isolates showed maximum sensitivity to Kanamycin, 7 isolates showed moderate sensitivity to Kanamycin, 18 isolates showed moderate sensitivity to Penicillin G and 8 isolates showed resistant to Penicillin G, the result was shown in Table 2 [29-31]. Glycerol stock was maintained with 20% glycerol and 80% culture broth.

Table No. 2: Zone of inhibition in antibiotic susceptibility test

Antibiotic disk Isolates	Tetracycline (diameter mm)	Gentamycin (diameter mm)	Erythromycin (diameter mm)	Kanamycin (diameter mm)	Penicillin G (diameter mm)
Isolate -1	9	6	10	5	0
Isolate -2	30	12	27	15	2
Isolate -3	26	11	17	17	1
Isolate -4	10	8	8	9	0
Isolate -5	32	12	22	17	1

Isolate -6	30	13	24	10	2
Isolate -7	26	11	23	9	0
Isolate -8	25	13	20	17	2
Isolate -9	29	10	9	14	1
Isolate -10	8	14	22	15	0
Isolate -11	27	11	25	17	1
Isolate -12	26	16	21	16	2
Isolate -13	9	10	10	8	1
Isolate -14	27	12	18	14	1
Isolate -15	8	15	21	10	0
Isolate -16	30	10	23	16	1
Isolate -17	19	1	9	7	0
Isolate -18	32	12	18	17	1
Isolate -19	4	1	3	2	0
Isolate -20	26	13	18	14	1
Isolate -21	28	11	22	16	1
Isolate -22	7	3	6	5	0
Isolate -23	32	14	22	17	2
Isolate -24	28	15	24	15	1
Isolate -25	26	11	8	16	2
Isolate -26	8	14	20	14	2

Table No. 3: Optical density of culture growth

Species name	***E.coli***	***Bacillus***	***Pseudomonas***	***Serratia***	***Klepsiella***
Time interval	**Optical density at 600nm**				
4 hours	0.099	0.097	0.106	0.096	0.112
8 hours	0.096	0.094	0.097	0.094	0.107
12 hours	0.095	0.091	0.095	0.094	0.097
16 hours	0.094	0.091	0.095	0.094	0.097
20 hours	0.094	0.091	0.093	0.092	0.094
24 hours	0.094	0.091	0.093	0.092	0.094

3.6 Mass cultivation of screened colony

The screened colony was sub cultured in nutrient broth (production media), and then this culture was inoculated in 2 L nutrient broth and incubated at 37 ºC for 24 to 48 h. The result was shown in Fig .5. After incubation it was used for further use.

3.7 Partial purification of antibiotic compound

Solvent extraction

The mass cultivated culture was centrifuged and supernatant was collected for solvent extraction. Solvent extraction was done in separating funnel with 1:1 ratio of culture supernatant and ethyl acetate. The result was shown in Fig .6. An amount of 2.14 g crude solvent extract was obtained from 2 L of fermentation broth after defatted with ethyl acetate**[13]**.

Column chromatography

Crude solvent extract was subjected to silica gel column (2.5×50 cm and 120 mesh size). The compound was eluted using different solvent ratios from 99:1 to 1:99 of chloroform and methanol, the result was shown in Fig. 7**[13]**.

Conclusion

Bhavani river is becoming more polluted and it is under major threat. The result obtained in the present study indicates that various microorganisms were populating in the river as they use industrial effluents as their substrate. The population of these organism could increase, if proper measure was not taken could directly or indirectly affect humans as these organisms will become potential human pathogens. Secondary metabolites of these organisms can be used as antibiotic for other pathogen and MRSA. Hence the present study concludes that the partially purified compound of the isolated organism can be used as antibiotic for clinical pathogens and other MRSA etc.

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