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Isolation and Identification of Bacterial Strains and Study of their Resistance to Heavy Metals and Antibiotics

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Abstract

The main objective of the work was to isolate and identify the bacterial strains and their resistance. A total of 9 strains could be isolated from the sample collected from the oxidation pond stage of waste water treatment of NIT Warangal. From all nine strains isolated, the maximum resistance was shown for chromium (490µg/ml for the strain1) and minimum for nickel (80µg/ml for strain6). The maximum resistance was shown for amoxilcillin (330 µg/ml for the strain8) and minimum for kanamycin (15µg/ml for the strain9). The metal resistant strains isolated from the waste water sample can be used for bioremediation process by construction of bioreactors where the strains can be immobilized for treating waste water effluents from industrial or domestic sources.

Keywords: Heavy metal resistant bacteria; Antibiotic resistance; NITW waste water treatment plant

Introduction

Sewage treatment is the process of removing contaminants from wastewater and household sewage, both runoff (effluents) and domestic. It includes physical, chemical, and biological processes to remove physical, chemical and biological contaminants. Its objective is to produce a treated effluent and a solid waste or sludge suitable for discharge or reuse back into the environment. This material is often inadvertently contaminated with many toxic organic and inorganic compounds. The main object of treatment units is to reduce the sewage contents (solids) from the sewage and remove all the nuisance causing elements and change the character of the sewage in such a way that it can be safely discharged in natural water course applied on the land. Screening is the very first operation carried out at a sewage treatment plant and consists of passing the raw sewage through different types of screens so as to trap and remove the floating matter such as tree leaves, paper, gravel, timber pieces, rags, fibre, tampons, cans, and kitchen refuse etc. Microorganisms present in sewage can tolerate metal concentrations toxic to human beings, such as cobalt, lead, nickel, chromium etc., and also antibiotics. Various experiments are to characterize and identify the microorganisms present in the domestic sewage water collected from the sewage treatment plant at NIT Warangal. This characterization aids in the further use of these strains for bioremediation of waste water.

Characterization of experiments

Four staining techniques were used to characterize the bacteria isolated.

1. Gram Staining
2. Morphology
3. Metal resistance
4. Antibiotic Resistance

Gram staining: It is a differential staining technique used to characterize bacteria as Gram positive and Gram negative. The fixed bacterial smear is subjected to Crystal Violet, Iodine Solution, Alcohol (decolorizing agent) and Safranin respectively. Gram-positive bacteria retain crystal violet and hence appear deep violet in color, while Gram-negative bacteria lose the crystal violet and are counterstained by the Safranin. Hence they appear red in color.

Morphology: Bacteria are generally spherical (cocci), rod-shaped (bacilli), comma shaped (vibrio) or spiral-shaped (spirilla). After Gram staining bacteria are observed under a Light Microscope.

Metal resistance: Bacterial strains present in sewage are resistant to heavy metals such as cobalt, nickel, chromium, lead etc. Here six metal salts are used in order to identify bacteria corresponding to them. Six nutrient agar plates were prepared, each consisting of the respective salts of a particular metal.

Antibiotic resistance: Incomplete metabolism in humans has resulted in release of large amounts of pharmaceutical drugs into municipal wastewater treatment plants. The presence of antibiotics in sewage water can lead to the development of antibiotic resistant strains. Hence tests were performed to detect the presence and tolerance for various antibiotics.

Materials and Methods

Preparation of culture media and stock solutions

The nutrient agar (NA) medium is prepared as follows: (see Table1).

The following components are weighed (except for agar) and dissolved in 600 ml of DDW.

Metal stock solutions were prepared as follows:

1. Metal salts were used to prepare stock solutions of concentration 10 mg/ml.
2. Weight of the salt required to make stock solution is calculated in the following Table 2.

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Sl. No.	Component	Percentage	Amount for 1 litre (in grams)
1	Beef Extract	0.3	3
2	Peptone	0.5	5
3	Sodium Chloride	0.8	8
4	Agar	1.5	15

The pH was set to 7.0 and volume was made up to 1 litre.15 grams of agar was added then the medium was autoclaved.

Table 1: NA medium composition.

Sl. No.	Metal	Salt used	Mol wt	Atomic weight	Weight %	Weight for 100 ml stock solution (in g)
1	Cr	$K_2Cr_2O_7$	294.2	51.99	17.67	5.65
2	Cd	$CdCl_2$	183.32	112.41	61.32	1.63
3	Pb	$(CH_3COO)_2Pb$	325.29	207.2	63.7	1.57
4	Cu	$CuSO_4$	159.61	63.55	39.82	2.51
5	Co	$CoCl_2$	129.84	58.93	45.39	2.20
6	Ni	$NiCl_2$	129.6	58.69	45.28	2.21

Table 2: Stock solutions of metals.

Isolation of bacteria to get pure culture

NA medium was used to isolate the bacterial strains from the samples. The cell counting technique in agar plates was followed to determine the population density in colony forming units per ml (cfu/ml) for each sample. A series of dilutions were made to reduce the cells in the samples. One ml of diluted sample was spread onto the surface of NA medium in the petri dishes and incubated at 37ºC and allowed to grow for 24 h. Single developed colony was picked on the NA plates and subcultured in nutrient broth and plated again on NA medium after dilution to obtain pure cultures. Pure bacterial strains were obtained after successive transfer of individual colony in NA plates and incubated for 24 h at 37ºC temperature. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial agents or metal ion concentration that will inhibit the visible growth of a micro-organism after overnight incubation.

MIC for heavy metals commonly found in waste water

Waste water from domestic sources has various heavy metal ions in varying concentration. In order to isolate bacteria tolerant of heavy metal ions, we checked for their respective MICs using various metal ions like chromium(Cr), cadmium(Cd), lead(Pb), copper(Cu), cobalt(Co) and nickel(Ni). The pure cultures isolated above were plated on NA medium with metal concentrations ranging between 50 µg/ml and 500µg/ml with an increment of 50µg/ml. Then the procedure was repeated with an increment of 10µg/ml in the range where visible growth was absent to determine the MIC for each metal (Shakoori et al., 1998). NA medium was prepared. 20 ml NA was poured into each Petri plate and the volume of metal stock solutions were calculated by the formula: $C_1xV_1=C_2xV_2$, where C_1 is the metal concentration in stock solution, V_1 is the volume of stock solution used, C_2 is the concentration of metal in agar and V_2 is the volume of agar.

The isolated strains were then streaked onto the NA medium containing metal salts using sterile loops. The plates were sealed and incubated for 7 days at 37ºC. The plates were checked for bacterial growth. The concentration of metal ion at which there was no growth observed is the MIC for that salt for that strain.

MIC for various antibiotics

The isolated strains were also checked for antibiotic resistance with Kanamycin, Streptomycin and Amoxicillin with concentrations ranging between 10 and 250µg/ml the results have been tabulated (Table 5). NA medium is prepared. 20 ml NA is poured into each petri plate and the volume of antibiotic stock solutions were calculated. The isolated strains were then streaked onto the NA medium using sterile loops. The plates were sealed and incubated for 7 days at 37ºC. The plates were checked for bacterial growth. The concentration of antibiotic at which there was no growth observed is the MIC for that salt for that strain. Antibiotic sensitivity and resistance of the isolated heavy metal resistant isolates were assayed according to the Kirby-Bauer disc diffusion method given by Bauer et al. (1996) (Mackey and McCartney, 1996).

Physical characterization

Physical characteristics like shape and staining properties play an important role in identification of strains. Isolated strains were observed under microscope and shapes were noted. They were stained using Gram stain by the following procedure: Smears were prepared and heat-fixed. The slides were stained as follows: The crystal violet stain was flooded for one minute. Excess dye was poured off and gently washed in tap water and the slide drained against a paper towel. The smears were exposed to Gram's iodine for one minute by washing with iodine, then added more iodine and left it on the smear until the minute was over. The slide was washed with tap water and carefully drained. The slide was washed with 95% alcohol for 30 seconds. The slide was washed with tap water at the end of the 30 seconds to stop the decolonization and the water was drained. The bacteria were counterstained with 0.25% safranin for 30 seconds. It was then washed, drained, blotted.

Results and Discussion

Physical characterization

A total of 9 strains could be isolated from the sample collected from the oxidation pond stage of waste water treatment. The density of microorganisms was found to be $9x10^3$ cfu/ml. After pure cultures were obtained they were checked for the Gram staining reactions and the morphology noted and the results are tabulated (Table 3).

Metal tolerance

Maximum resistance was shown for chromium and minimum for nickel. The strains were found to be resistant to various heavy metal ions in varying concentrations as shown as follows (Table 4).

Antibiotic tolerance

Maximum resistance was shown for amoxilcillin and minimum for kanamycin. The strains showed antibiotic resistance to the above antibiotics and their respective MICs are tabulated as follows (Table 5).

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S.No.	Strain / Species	Gram staining	Morphology
1	Strain 1	positive	cocci
2	Strain 2	negative	rod-shaped
3	Strain 3	negative	rod-shaped
4	Strain 4	negative	cocco-bacillary
5	Strain 5	negative	rod-shaped
6	Strain 6	negative	rod-shaped
7	Strain 7	positive	rod-shaped
8	Strain 8	negative	rod-shaped
9	Strain 9	negative	rod-shaped

Table 3: Physical Characterization Results.

Sl. No.	Strain / Species	MIC for various metals in µg/ml					
		Cu	Co	Ni	Cr	Pb	Cd
1	Strain 1	90	140	160	490	160	140
2	Strain 2	Nil	130	110	200	160	100
3	Strain 3	Nil	170	90	140	Nil	Nil
4	Strain 4	210	180	140	200	140	170
5	Strain 5	220	200	120	180	190	310
6	Strain 6	Nil	100	80	130	100	210
7	Strain 7	140	120	190	220	140	170
8	Strain 8	320	140	90	250	160	120
9	Strain 9	140	260	120	150	110	100

Table 4: MIC values for various heavy metals.

Sl. No.	Strain / Species	MIC for various antibiotics in µg/ml		
		Kanamycin	Streptomycin	Amoxicillin
1	Strain 1	90	80	180
2	Strain 2	85	75	125
3	Strain 3	20	35	055
4	Strain 4	40	90	105
5	Strain 5	80	65	95
6	Strain 6	85	60	115
7	Strain 7	70	85	220
8	Strain 8	65	250	330
9	Strain 9	15	35	65

Table 5: Results for Antibiotic Resistance.

Conclusion

The effluent in NIT Warangal waste water treatment plant includes waste water from a variety of sources like hostels, mess and staff quarters. In addition to that there are various laboratories situated in the campus like chemistry, biochemistry, microbiology and molecular biology laboratories which contribute a large number of chemicals including heavy metals to the effluent. The waste water treatment plant was setup in NIT Warangal campus so as to recycle the water and use it for irrigation of lawns. Sample collected from the NIT Warangal waste water treatment has a lot of bacterial species which help in the breakdown of organic compounds. Apart from that these bacterial species have been found to reduce the levels of toxic metals present in sewage and industrial waste. The metal resistant strains isolated from the waste water sample can be used for bioremediation process by construction of bioreactors where the strains can be immobilized for treating waste water effluents from industrial or domestic sources. Further selection of strains can result in isolation of strains with higher resistance which could serve as an effective means of treating waste water.

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