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Ecotoxicological Studies on Heavy Metal Tolerant Microbes Isolated From Marine Ecosystem

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ABSTRACT

The entry of heavy metals into the ecosystem causing high pollution to the water bodies and the surrounding environmental soil has become a major problem which is increasing day by day. Due to the anthropogenic activities of the heavy metals on human health systems and unlike other pollutants, they cannot be degraded from the environment easily; bioremediation process finds its way necessary for removal of heavy metals from environment which is cost effective. The present study includes the isolation and characterization of heavy metal tolerant microbes from marine water. The four different marine samples are taken to have a comparative study of heavy metal tolerance in different environment of microbial habitat. The samples were collected at a distance of maximum ten kilometres away from each sampling site. The tolerance of heavy metal is studied through minimum inhibitory concentration (MIC).Along this study bio chemical tests and morphological characterization were conducted to characterize the isolated microbes. The results are interpreted accordingly.

Keywords: Heavy metals, remediation, anthropogenic, metal uptake.

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INTRODUCTION

Nowadays many micro-organisms have been isolated from many polluted areas and are found to be resistant to different types of heavy metals and they have been focussed on transformation in bacteria and the reduction property of bacteria on metal ions have been studied well [11][15].Metal resistant bacteria can be a potential intoxifying agent. Detoxification of heavy metals is found to be an important concern since they are not biodegradable, highly toxic and are found in many polluted areas in our surrounding environment.

Bioremediation has its main scope even though there are many physiochemical methods due to their cost effectiveness and their ability in removal of heavy metal [19][3]. The Hg2+, Pb2+ and Cd2+ are included as top pollutants in the list of Environmental Protection Agency. U.S [20].

It is evident that the microbial community from marine habitat are found to be resistant for many heavy metals which are seemed to be common pollutant in marine ecosystem [1] [4]. These cells are found to be more sensitive against industrial waste effluents due to their toxicity due to heavy metal, higher pH and other pollutants. The bacterial community have ability to convert toxic metals to intoxic forms due to their ability of volatilization and precipitate the metal ions [8].

The present study includes the isolation and characterization of the heavy metal tolerant microbes from four different marine ecosystems and characterization of isolated micro-organisms. The comparative study on the activity of bacterial cells with presence of heavy metals Cu, Pb, Cr, Ag, Co, Hg was studied.

MATERIALS AND METHODOLOGY

- SAMPLE COLLECTION.
- PHYSICAL CHARACTERISTICS OF WATER SAMPLES.
- EVALUATION OF HEAVY METAL TOLERANT BACTERIA IN PRESENCE OF MIXED HEAVY METAL SAMPLE.
- ISOLATION OF HEAVY METAL TOLERANT BACTERIA.
- MORPHOLOGICAL CHARACTERIZATION OF THE ISOLATED COLONIES.
- BIO CHEMICAL CHARACTERIZATION OF THE ISOLATED COLONIES.
- PREPERATION OF HEAVY METAL STANDARDS.
- DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION FOR HEAVY METALS FOR THE ISOLATED COLONIES

SAMPLE COLLECTION:

The water samples were collected from four different sites of three different sea shores (marina beach, triplicane beach, kasimedu beach and interior sea water of kasimedu beach). The sample collecting sites were of ten kilometres away from each other. The four samples were collected to do the comparative study for the level of tolerance for heavy metal since the microbial species differ in different ecosystem.

SAMPLES

- SAMPLE 1-MARINA BEACH SHORE: and isolates are named as M1X,Y,Z and M2X,Y,Z
- SAMPLE 2-TRIPLICANE BEACH SHORE: and isolates are named as T1X,Y,Z and T2X,Y,Z
- SAMPLE 3-KASIMEDU BEACH SHORE: and isolates are named as K1X,Y,Z and K2X,Y,Z
- SAMPLE 4-INTERIOR SEA WATER OF KASIMEDU BEACH: and isolates are named as I1X,Y,Z and I2X,Y,Z

METHOD OF COLLECTION:

The samples were collected in plastic bottles which was sterilised before sample collection. The samples were acidified with concentrated nitric acid. The samples were then stored in refrigerated condition till the further studies were started.

PHYSICAL CHARACTERS OF WATER SAMPLE:

The physical characters of water are the colour, odour, p^H and turbidity. The physical characters vary with different environment which in turn has effect on microbial growth.

EVALUATION OF HEAVY METAL TOLERANT BACTERIA IN PRESENCE OF MIXED HEAVY METAL SAMPLE BY CALCULATING THE TOTAL VIABLE COUNT:

The heavy metal tolerant bacteria was isolated from the broth culture which contained 80ml of nutrient broth, supplemented with 10ml of heavy metal sample, 10ml of marine water samples and 10grms of nacl(to increase the salt concentration). The ability of microbe to growth in this media was determined. The broth enriched with mixed heavy metal sample was inoculated with marine sample and was incubated for three days and the total viable count was determined at 24, 48 and 72 hrs using spectrophotometer at 620nm.

ISOLATION OF HEAVY METAL TOLERANT BACTERIA:

The 72 hrs broth culture was used for isolation of bacteria. About 0.5ml of broth culture was taken and plated on sterile nutrient agar plates (spread plate technique was followed). The isolation was done in duplicates that is PLATE 1 and PLATE 2. The plates were incubated at 30^{o}C for 24 hours. The isolated colonies were further sub cultured for selected colonies to get the

pure cultures. The pure cultures obtained were stored for further studied. For the minimum inhibition concentration studies the pure cultures were isolated again in nutrient broth media.

MORPHOLOGICAL CHARACTERIZATION OF THE ISOLATED COLONIES:

COLONY MORPHOLOGY:

The colony morphology characterization includes the size, shape, texture, elevation, pigmentation and their effect on growth medium. The pure cultures obtained are evaluated for colony morphology.

CELL MORPHOLOGY:

1. GRAMS STAINING
2. ENDOSPORE STAINING
3. CAPSULE STAINING OR NEGATIVE STAINING

BIO CHEMICAL TESTS FOR THE ISOLATED COLONIES:

Bacterial tests provide scope to test the production of certain enzymes which help in breaking down the complete substrate like sugars, proteins, and lipids into simpler substance. The produced product so formed assayed regarding the gas production, changes in pH of the media using indicators, chemicals which could change colour after interacting with the product. These metabolic reactions are specific to bacterial species depending upon their mode of nutrition. This has been made use to identify individual genera and species, along with preliminary identification by other methods like-morphological character, gram staining etc.

The presence of capsule was determined by performing negative staining method.
The Endospore staining was done using malachite green as spore stain and saffranin as counter stain.

The common bio chemical tests included are

- Starch hydrolysis
- Catalase test
- Oxidase test
- IMViC test

The different organisms can be identified based on the characterization depending on IMViC tests. IMViC series of tests include

- Indole test
- Methyl red test

- Voges proskauer test
- Citrate utilization test

INDOLE TEST:

Tryptophan is an essential amino acid that can undergo oxidation by the enzymatic activities of certain bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase.

The ability to hydrolyze tryptophan with the production of indole is not a characteristic feature of all micro-organisms and therefore, serves as a bio-chemical marker. Production of indole in the medium can be detected by adding Kovac's reagent which produces a cherry red layer. Kovac's reagent is composed of para-di-methyl amino benzaldehyde, butanol and hydrochloric acid. Hence when reacts with indole gives a colored quinoidan complex which appears as cherry red color.

METHYL RED TEST:

To determine the ability of microorganisms to oxidase glucose with the production glucose with the production and stabilization of high concentration of acid end products.
The hexose mono-saccharide: glucose is the major substrate oxidized by all enteric organisms for energy production. The end product of this process will vary depending on the specific enzymatic pathways present in the bacteria. In this test process will vary depending on the specific enzymatic pathways present in the bacteria. In this test the Ph. indicator methyl red detects the presence of large concentration of acid end product Methyl red indicator in the (acidic) ph. range of 4 will turn red, which indicates the positive test. At a Ph of 6, still indicating the presence of acid but with a lower H+ ion concentration the indicator turns yellow and is a negative test when these are further incubated acids may be converted to non-acidic products i.e.. Glucose fermentation may result in 2, 3 butandial and acetoin (acetyl methyl carbine). These products can be detected by the Voges-Proskuer test which is performed simultaneously with Methyl red test.

VOGES PROSKAUER TEST

To determine the capability of some organisms to produce non acidic or neutral end product from organic acids that results from glucose metabolism.This test determines the capability of some organisms to produce non acidic or neutral end products like acetyl carbional from organic acids that results from glucose.

The reagents used in the test (Barrel's reagent) are a mixture of alcoholic alpha napthol and 40% KoH solution. Detection of acetyl methyl carbinol (acetoin) requires that this end product be oxidized to a di acetyl compound. This reaction occurs in the presence of alpha napthol catalyst and the guanidine group i.e. Present in peptone of MRVP medium. As a result pink complex is formed, imparting a rose color to the medium.

CITRATE UTILIZATION TEST:

This test is used to differentiate among enteric organisms on the basis of tier ability to utilize a ferment citrate as the sole carbon sources. The ability of some microorganisms to utilize citrate as a sole carbon sources in the absence of glucose, lactose depends on the presence of citrate permease that facilitates the transport of citrate in the cell citrate is the first major intermediate in the Krebs's cycle and is produced by the condensation of active acetyl with oxalo acetic acid; citrate is acted upon by the enzyme citrase which produce oxalo acetic acid and acetic acid which in turn are enzymatically converted to pyruvic acid and carbon di oxide.

During this reaction medium becomes alkaline carbon dioxide that is generated combines with sodium and water to form sodium carbonate an alkaline product. The presence of sodium carbonate changes the bromothymal blue indicator incorporated into the medium from green to deep Prussian blue.

STARCH HYDROLYSIS TEST:

Starch is a high molecular weight, branching polysaccharide composed of glucose molecules linked by glycosidic bonds. The degradation of this macromolecule requires the presence of extra cellular enzyme amylase for its hydrolysis into monosaccharides like dextrose, maltose and glucose. This degradation process/ability of the enzyme to degrade starch can be demonstrated by a simple experiment in which its hydrolysis is tested by adding iodine solution as an indicator. Iodine gives dark blue coloration on contact with starch.

CATALASE TEST:

During aerobic respiration, micro-organisms produce H_2O_2 and in some case an extremely toxic superoxide. Accumulation of these substances will result in the death of the organism unless they are enzymatically degraded. These substances are produced when the micro-organisms use the aerobic respiratory pathway in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Organisms capable of producing catalase rapidly degrade H_2O_2 by giving out two molecules of water and oxygen as end product. Superoxide dismutase is the enzyme responsible for degradation of toxic superoxides in catalase negative aerobic organisms.

OXIDASE TEST:

Oxidase enzymes play a vital role in the operation of the electron transport system during aerobic respiration. Cytochrome oxidase catalyses the oxidation of a reduced cytochrome by molecular oxygen (0_2), resulting in the formation of H_20 or H_2O_2. The ability of the bacteria to produce cytochrome oxidase can be determined by the addition of the test

reagent tetramethyl –paraphenyl-diamine-dihydrochloride or can also be tested using readily available oxidase discs.

PREPERATION OF HEAVY METAL STANDARDS:

Stock solution of different heavy metals for 1000mg/ltr was prepared by dissolving the calculated amount of required metal compound salts in distilled water and the volume was made to 100ml using standard flask. The stock solution was further made into different concentrations of 100,200,300,400 and 500mg/L. This is used for AAS analysis and Minimum Inhibitory Concentration tests.

The formula used for calculating the required amount of compound salts is as follows

$$\frac{\text{Molecular weight of the compound}}{\text{Atomic weight of the element}} = x \text{ grams/ltr}$$

This is equal to 1000mg/L.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF HEAVY METALS FOR ISOLATED COLONIES BY AGAR DIFFUSION OR WELL DIFFUSION METHOD:

For the determination of Minimum Inhibitory Concentration, the pure cultures isolated were used for this test. Using sterile swabs cultures were lawn cultured on sterile nutrient agar medium and wells were made using sterile cork borer. The heavy metals solution of different concentration was pipetted into the wells using micropipette. About 20µl of heavy metal solution was used and the was incubated for 24hrs at 30^{o}C. After incubation the plates were observed for zone of clearance.

The zone of inhibition can be measured using the formula:

$$\frac{\text{Zone diameter in mm-Well diameter in mm}}{2} = x \text{ mm}$$

RESULTS AND DISCUSSION

The isolated colonies showed small sized, medium round shaped colonies with flat elevation and no margins. The surface of the colonies was smooth. The colour of the colonies was white to pale yellow colour.

ISSN: 0975-8585

TABLE 1: The physical characteristics of the water sample is tabulated below

SAMPLES	COLOUR	ODOUR	Ph
MARINA	CLEAR &COLOURLESS	ODOURLESS	8.15
TRIPLICANE	CLEAR &COLOURLESS	ODOURLESS	8.10
INTERIOR SEA	SLIGHTLY BROWN	ODOURLESS	7.68
KASIMEDU	CLEAR & COLOURLESS	ODOURLESS	6.50

TABLE 2: The total viable count was determined at 620nm and results are tabulated below

SAMPLES	24 hrs	48 hrs	72 hrs
Marina	0.61	0.68	0.74
Kasimedu	0.63	0.63	0.73
Interior sea	0.61	0.66	0.75
Triplicane	0.50	0.69	0.74

The heavy metal bacterium was isolated from the broth cultures on nutrient agar medium. The single isolated colony was selected randomly and was sub cultured in nutrient agar medium and also in nutrient broth.

TABLE 3: BIO CHEMICAL TESTS FOR ORGANNISMS ISOLATED FROM PLATE 1

ORGANISMS	GRAM STAINING	ENDOSPORE STAINING	CAPSULE STAINING	INDOLE TEST	METHLY TEST	VP TEST	CITRATE TEST	CATALASE TEST	OXIDASE TEST	STARCH HYDROLYSIS TEST
M1X	Gram –ve rods	+	+	+	+	-	-	+	-	-
M1Y	Gram +ve cocci	-	-	+	+	-	+	-	-	+
M1Z	Gram +ve rods	+	-	+	-	-	-	-	-	-
T1X	Gram +ve cocci	-	-	+	+	+	-	+	-	-
T1Y	Gram +ve cocci	-	-	+	-	-	+	+	-	+
T1Z	Gram +ve rods	-	+	+	+	+	+	+	-	+
I1X	Gram +ve cocci	-	-	+	+	+	+	+	+	-
I1Y	Gram –ve rods	+	+	+	-	-	-	-	+	-
I1Z	Gram +ve cocci in chain	-	-	+	-	-	+	-	+	-
K1X	Gram +ve	-	-	+	-	-	-	+	-	+

ISSN: 0975-8585

	cocci in clusters									
K1Y	Gram +ve cocci in clusters	-	-	+	-	-	+	+	-	+
K1Z	Gram –ve rods	-	+	+	+	+	-	-	-	-

TABLE 4: BIO CHEMICAL TETS FOR ORGANISMS ISOLATED FROM PLATE 2

ORGANISMS	GRAM STAINING	ENDOSPORE STAINING	CAPSULE STAINING	INDOLE TEST	METHLY TEST	VP TEST	CITRATE TEST	CATALASE TEST	OXIDASE TEST	STARCH HYDROLYSIS TEST
M2X	Gram +ve rods	-	-	-	+	+	+	+	+	-
M2Y	Gram –ve rods	+	+	-	-	-	+	-	+	+
M2Z	Gram +ve rods	-	-	-	-	-	+	-	-	-
T2X	Gram +ve rods	-	+	-	-	-	-	+	-	+
T2Y	Gram +ve rods in chain	-	-	-	+	+	+	+	+	-
T2Z	Gram +ve rods	+	-	-	+	+	-	-	-	+
I2X	Gram –ve rods	+	-	+	-	-	+	-	-	+
I2Y	Gram +ve rods	-	+	+	+	-	+	+	+	+
I2Z	Gram –ve rods	-	-	+	-	-	+	-	+	+
K2X	Gram +ve cocci in clusters	-	-	+	+	+	+	+	+	-
K2Y	Gram +ve cocci	-	-	+	+	-	-	+	-	-
K2Z	Gram +ve cocci in clusters	-	-	+	-	+	+	+	-	-

MINIMUM INHIBITORY CONCENTRATION BY PAPER DISC AND WELL DIFFUSION METHOD TEST

CALCULATED VALUES FOR REQUIRED HEAVY METALS TESTED:

Potassium di-chromate-0.28grms/100ml
Copper sulphate-0.3grms/100ml

ISSN: 0975-8585

Mercuric chloride-0.135grms/100ml
Lead nitrite-0.15grms/100ml
Cadmium chloride-0.23grms/100ml
Silver nitrate-0.15grms/100ml

MINIMUM INHIBITORY CONCENTRATION BY PAPER DISC AND WELL DIFFUSION METHOD TEST:

TABLE 5: TEST FOR Ag METAL

TEST ORGANISMS	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
M1X	-	-	-	-	2mm
M1Y	-	-	-	-	-
M1Z	-	-	-	1mm	3mm
T1X	-	-	1mm	2mm	4mm
T1Y	-	-	-	-	1mm
T1Z	-	-	-	2mm	3mm
K1X	-	-	-	-	-
K1Y	-	-	-	-	-
K1Z	-	-	-	-	-
I1X	-	-	-	1m	4mm
I1Y	-	-	-	-	-
I1Z	-	-	-	-	-

TABLE 6: TEST FOR Hg METAL

TEST ORGANISMS	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
M1X	-	-	-	-	-
M1Y	-	-	1mm	2mm	4mm
M1Z	-	-	-	2mm	5mm
T1X	-	-	1mm	4mm	6mm
T1Y	-	-	3mm	2mm	3mm
T1Z	-	-	1mm	1mm	3mm
K1X	-	-	-	-	1mm
K1Y	-	-	-	-	-
K1Z	-	-	-	-	-
I1X	-	-	-	-	1mm
I1Y	-	-	-	-	-
I1Z	-	-	-	-	-

ISSN: 0975-8585

TABLE 7: TEST FOR Co METAL

TEST ORGANISMS	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
M1X	-	-	-	-	-
M1Y	-	-	-	-	-
M1Z	-	-	-	-	-
T1X	-	-	-	-	-
T1Y	-	-	-	-	-
T1Z	-	-	-	-	2mm
K1X	-	-	-	-	-
K1Y	-	-	-	-	-
K1Z	-	-	-	-	-
I1X	-	-	-	-	-
I1Y	-	-	-	-	-
I1Z	-	-	-	1mm	2mm

TABLE 8: TEST FOR Cr METAL

TEST ORGANISM	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
M2X	-	-	-	-	-
M2Y	-	-	-	-	-
M2Z	-	-	-	-	-
K2X	-	-	-	-	-
K2Y		-	-	-	-
K2Z	-	-	-	-	-
T2X	-	-	-	-	-
T2Y	-	-	-	-	-
T2Z	-	-	-	-	1mm
I2X	-	-	-	-	-
I2Y	-	-	-	-	-
I2Z	-	-	-	-	1mm

ISSN: 0975-8585

TABLE 9: TEST FOR Pb METAL

TETS ORGANISMS	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
M2X	-	-	1mm	2mm	4mm
M2Y	-	-	-	-	1mm
M2Z	-	-	-	-	1mm
K2X	-	-	-	1mm	4mm
K2Y	-	-	2mm	3mm	5mm
K2Z	-	-	-	-	1mm
T2X	-	-	-	1mm	2mm
T2Y	-	-	-	-	-
T2Z	-	-	-	-	-
I2X	-	-	-	-	1mm
I2Y	-	-	-	-	-
I2Z	-	-	-	-	-

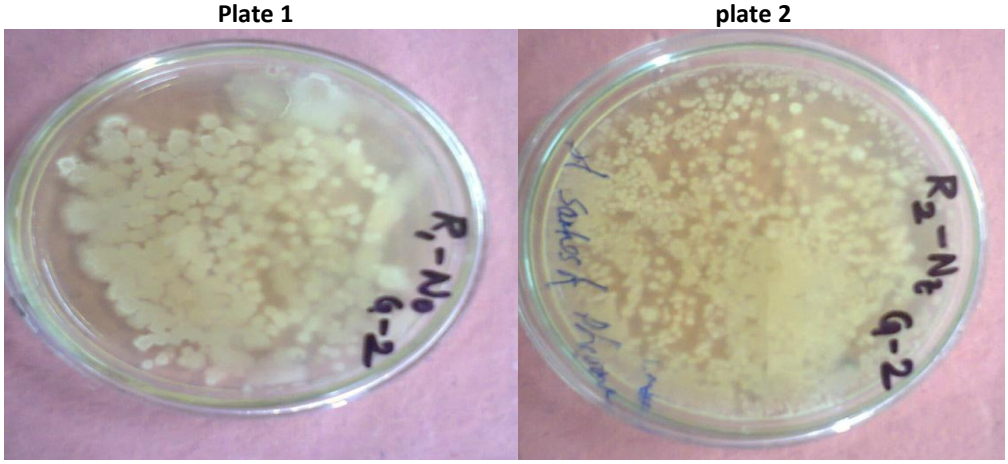
TABLE 10: TEST FOR Cu METAL

TEST ORGANISMS	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
M2X	-	-	-	-	
M2Y	-	-	-	-	
M2Z	-	-	1mm	3mm	3mm
K2X	-	-	-	-	-
K2Y	-	-	-	-	-
K2Z	-	-	-	-	-
T2X	-	-	-	-	1mm
T2Y	-	-	-	-	-
T2Z	-	-	-	-	-
I2X	-	-	-	-	-
I2Y	-	-	-	-	2mm
I2Z	-	-	-	-	-

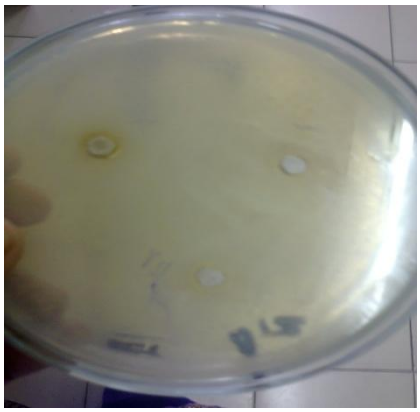
Plate 1 cultures were used to determine MIC for Co, Hg and Ag metals. The plate 2 cultures were used to determine MIC for Cu, Pb, and Cr metals. The zone of inhibition was not observed at the concentration of 100 and 200mg/L of heavy metal sample. Hence the test concentration was further increased to 300,400 and 500mg/L. At this concentration few organisms showed slight zone of inhibition whereas most of the organisms did not show the inhibitory zone. The most of the isolated organisms did not show any significant zone of inhibition in case of heavy metals like Co, Cu and Cr even at 500 mg/L. The inhibition was seen slightly more when compared to other metals in case of heavy metals like Ag, Hg and Pb. The zone of inhibition was seen slightly more at 500mg/L concentration of heavy metals.

ISSN: 0975-8585

Plate 1 plate 2

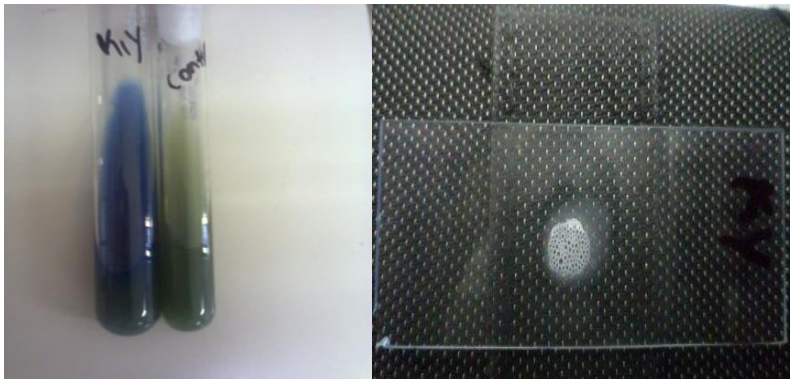


Isolation of microbes from the broth culture



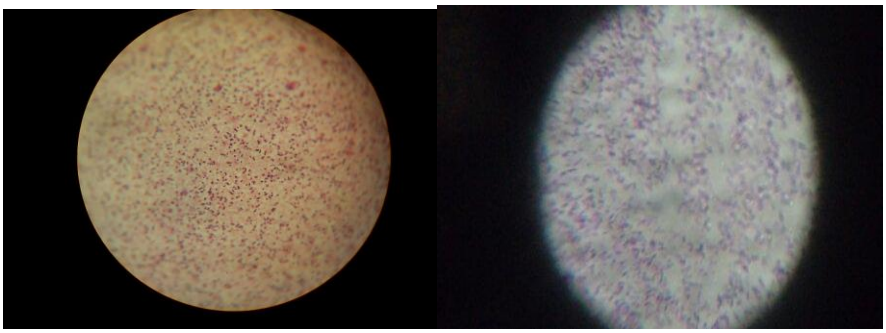
Minimum inhibition test plate (MIC)

Grams staining and other bio chemical test photos



Citrate utilization test result **Catalase test**

ISSN: 0975-8585



Gram's staining

Starch Hydrolysis test

Methyl red test Indole test

CONCLUSION

From the above results we can conclude that the microbes vary with different environment [10]. The organism isolated from one marine ecosystem did not show the same characteristics of the organism isolated from other marine ecosystem in case of tolerance to heavy metals. This may be due to the difference in diversity of microbes with reference to their capability to tolerate the heavy metal concentration and physicochemical characteristics of marine water like presence of organic salts, salinity, organic matter, BOD and COD etc. Every organism adapts them self to the particular environment and differ in their characteristics. The ability to tolerate heavy metal differed in isolated organisms where the sampling site remained same. We also conclude that the some of the marine microbes isolated had the ability to tolerate multiple heavy metals.[2][18] another important fact with the isolation was all the microbes were isolated and cultured on nutrient broth and agar medium with enrichment only with higher concentration of salt. The microbes were able to grow under these conditions other than marine environment. Hence they can be usefully employed in bio remediation and bio

degradation process. The further studies can be done to determine the exact reason for the variation in adaptability among the micro-organisms in different environment by studying physiochemical nature of marine water and also the pathway involved in intake of heavy metals in case of bacterial isolates such as binding ability to organic materials present, precipitation ability of the microbe, ionic interaction or complex formation which may also have key role in heavy metal tolerance [19][15][16]. Thus these microbes can also be used in treatment plants for better efficiency.

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