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Isolation and Characterization of Heavy Metal Tolerant Bacterial Isolates VITNJ12 and VITNJ13 from Paper Mill Effluent, Erode District, Tamilnadu, India.

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Abstract:
In this present study total six bacterial isolates were obtained from paper mill effluent collected from Erode district, Tamilnadu, India. Out of these, two isolates VITNJ12 and VITNJ13 has shown the resistance to heavy metals at maximum concentration of 300mg/ml. Then, the isolates were further characterized by morphological, biochemical and molecular technique and the isolates were found to be *Acinetobacterschindeleri* and *Lysinibacillussphaericus*. Both the isolates have shown the maximum resistance to antibiotics such as Rifampicin, Miconazole, Fluconazole, Ceflazidime and Methicillin. The heavy metal and antibiotic resistant bacteria isolated from paper mill effluent could be further explored for bioremediation purpose.

Keywords: Effluent, heavy metal resistance bacteria, antibiotic resistance, 16SrRNA.

Full Length Original Research Paper

Introduction

Paper pulp industries are one the largest effluent generating industries of the world [1]. The paper mill industry consumes water nearly 275-455 cum for the production of one tone of paper which forms effluents and needs reclamation and disposal [2]. The disposal of effluents directly to the fresh water bodies affects the quality of the water and since most of the water applied for irrigating the agricultural land and seriously affects the nature of the soil [3]. This effluent contains 700 organic and inorganic compounds [4] most of them are heavy metals. Heavy metals are group of metals whose density is greater than 5g/cm^{3}contaminates the environment due to their toxicological effect to living organisms [5]. They are very stable, cannot be destroyed or degraded and so persistent in nature [6]. Heavy metals influence the microbial population by affecting the growth and biochemical activity leads to decrease in biomass. Therefore, microbes have developed many mechanisms to tolerate the heavy metals for their survival [7]. These main mechanisms include the efflux of metal ions outside the cell [8]. An emerging problem for the treatment of infectious disease is bacterial resistance to antibiotics. It is thought that there is relationship exists between metal tolerance and antibiotic resistance in bacteria because of the similarity of that resistance genes to antibiotics and heavy metals might be closely together on the same plasmid in bacteria [9]. Many studies were reported on isolation of antibiotic resistance and heavy tolerance bacteria from the highly polluted environment [10]. Hence, this study was

carried out to isolate and characterize the antibiotic resistance and heavy metal tolerance bacteria from the paper mill effluent near the Kaveri river basin, Erode district, Tamilnadu.

MATERIALS AND METHODS

Sample collection site:

Samples were collected from the contaminated site of Erode district, Tamilnadu in a sterile plastic container, transported to the microbiological laboratory, VIT University and they were stored in the refrigerator at 4°C. The physico-chemical properties of the effluents were estimated using the standard methods.

Isolation and identification of heavy metal tolerant bacteria:

Bacteria were isolated by serially diluting sample and plated in Nutrient agar plate were supplemented with individual heavy metals in the form of salts $K2Cr2O_7$, $CdCl_2$, $CuCl_2$ and NiSO4 respectively at the concentration of 50ppm. The plates were incubated for 24 hrs at 37°C for their bacterial growth. Then, the distinct colonies were sub cultured continuously on the suitable medium for the isolation of pure culture. Pure cultures were further identified on the basis of their morphology and biochemical tests. The isolated colonies were characterized on the basis of gram staining, hanging drop motility test, oxidase test, catalase test and IMVIC test. To isolate the heavy metal tolerant strains, it is mandatory to standardize the cultural and physiological conditions of the selected organisms. Among these, temperature, pH and salt concentration are of importance for the growth of bacteria. The molecular characterization of the bacteria was done by 16s r RNA sequencing. [11]. The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers: 518F 5' CCAGCAGCCGCGGTAATACG3'and 800R 5'TACCAGGGTATCTAATCC3' .Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). 16S rRNA sequences of bacterial isolates have been deposited in the Gen Bank database under the accession no.KM047497 and KM047498. The partial 16SrRNA bacterial sequences isolated from paper mill effluent samples were analysed using BioEdit software [12]. The sequences were compared with the available sequences against 16S ribosomal RNA sequences (Bacteria and Archaea) database using NCBI's BlastN. Sequences were aligned using CLUSTALW program in Mega 6.0. A Neighbour-joining tree assessing the phylogenetic diversity of the sequences was constructed using Jukes and Cantor method. Bootstrap analysis was performed on 1000 random samples taken from the multiple sequence alignment.

MIC of bacterial isolatesto heavy metal tolerance:

The isolates were tested for metal tolerance using five various concentrations of four different metals. The salt and its concentrations used were shown in the Table 1. The minimum inhibitory concentration of the metal ions at which no growth was determined by the broth dilution method [13].

Table 1: Various salts and its concentration

Salt	Concentration (mg/l)
Pentahydrated cupric sulfate	100,150,200,250,300
Nickel (II) sulfate crystals	100,150,200,250,300
Potassium dichromate	100,150,200,250,300
Cadmium	100,150,200,250,300

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Antibiotic resistance test

The isolates were screened for antibiotic sensitivity according to the Kirby-Bauer disc diffusion method [14] with 8 antibiotics. The concentrations of the disc used were Streptomycin (10 mcg), Ampicilin (10 mcg), Rifampicin (15 mcg), Miconazole (30mcg), Methicillin (5mcg), Fluconazole (10 mcg), Ceflazidime (30 mcg) and Ciprofloxacin (10 mcg). 0.1 ml of bacterial culture was uniformly spread on a sterile Muller Hinton agar plate. The culture was allowed to dry on the plate for 5-10 min at room temperature, antibiotic impregnated disc were placed on the plates aseptically on the bacterial colonies on agar plates. The plates were incubated for 18 -24 hrs at 37°C. The diameter of the inhibition zone was measured and the isolates were classified as resistant (R) and Susceptible (S) following the standard method.

Results

The result of the physical and chemical properties of the paper mill effluent as presented in the Table 2.

Table 2: Physical and chemical property of effluent

Parameters	Untreated effluent
Colour	Brown
BOD (mg/l)	256
COD (mg/l)	64
Turbidity	78 NTU
pH	8.42
Chromium (mg/l)	0.1416 mg/l
Zinc (mg/l)	0.026 mg/l
Copper (mg/l)	0.067 mg/l
Nickel (mg/l)	0.045 mg/l
Lead (mg/l)	0.083 mg/l
Fluoride (mg/l)	0.047 mg/l
Silicate (mg/l)	1.723 mg/l
Iron (mg/l)	0.032 mg/l
Calcium (mg/l)	0.043 mg/l
Magnesium (mg/l)	0.247 mg/l
Sodium (mg/l)	0.432 mg/l

A total of six bacterial isolates (J1, VITJ12, J3, J4, J5 and VITNJ13) from paper mill effluents were isolated and tested for resistance against certain heavy metals such as chromium, cadmium, copper and nickel. Out of six, two bacterial isolates (VITNJ12, VITNJ13) have shown resistance towards the heavy metals as shown in Table 3 and they were selected for further studies. The morphological and biochemical characteristics of the two isolates (VITNJ12, VITNJ13) were examined and the results are depicted in Table 4.

Table 3: Heavy metal resistance of bacterial isolates from paper mill effluent

Heavy metal	J1	VITJ 12	J3	J4	J5	VITNJ 13
Pentahydrated cupric sulfate	2mM	4.8mM	1mM	1mM	2mM	5.4mM
Nickel (II) sulfate crystals	1mM	5.3mM	-	-	1mM	4.7mM
Potassium dichromate	2mM	4.2mM	-	-	-	5.8mM
Cadmium	1mM	5mM	2mM	-	2mM	5.3mM

Table 4: Characteristics of heavy metal resistant bacterial isolates from paper mill effluent

Morphological and Biochemical characteristics	VITN1J12	VITNJ13
Gram staining	Gram negative	Gram positive
Catalase test	Positive	Positive
Oxidase test	Negative	Positive
Indole test	Negative	Negative
Methyl red	Negative	Negative
Voges-Proskauer test	Negative	Negative
Citrate utilization test	Positive	Negative
Hydrogen sulphide production test	Negative	Negative
Triple sugar ion test	Negative	Negative

PCR amplification of 16S r RNA using a set of primers 518 F and 800R yielded 1,400 bp.

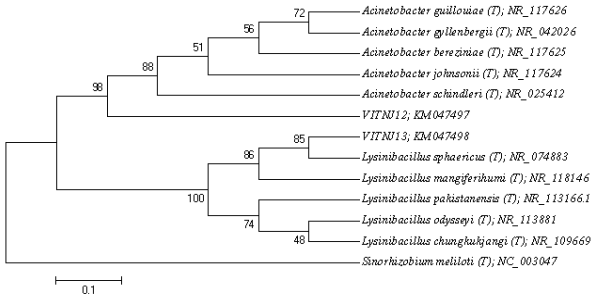

Figure 1: Phylogenetic relationships of 16s RNA sequences of the isolates from paper mill effluent to closely related sequences from Gen Bank

Both the isolates VITNJ12 and VITNJ13 were found to be multi-antibiotic resistant as shown in the Table 5. The presence of small amount of antibiotics and heavy metals in the paper mill effluent induce the emergence of antibiotic and heavy metal resistant bacterial isolates. Both the isolates in the present study showed multiple tolerances to both heavy metals and antibiotics.

Table 5: Antibiotic sensitivity and resistant activity of heavy metal resistant bacterial isolates

Bacterial isolates	Sensitive to	Resistant to
VITNJ12	Ampicillin, Rifampicin	Miconazole, Fluconazole, Ceflazidime, Methicillin
VITNJ13	Ampicilin Streptomycin	Rifampicin, Miconazole, Fluconazole, Ceflazidime, Methicillin

The microbial resistance to heavy metal is attributed to a variety of detoxifying mechanism developed by resistant microorganisms such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux etc. In the present study, it is suggested that the microorganisms resistant to antibiotics and tolerant to metals appear to be the result of exposure to metal contaminated environment which is fairly consistent with the finding of Remeteke [15]. The fact was also established by other researchers that multiple metal resistance bacterial isolates exhibits high resistance towards a group of antibiotics [16]. Based on the MIC values and antibiotic pattern of the isolate strains and as studied by Bruins *et al.,*[17]*Pseudomonas sp*.Shows resistance to a variety of toxic substances, heavy metal and antibiotics which has generated a high degree of interest in the area of environment bioremediation. Further studies are required to understand the role of these two isolates VITNJ12 and VITNJ13 in bioremediation.

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