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Isolation, Characterisation and Detection of Plasmids from Chromium and Copper Resistant Bacterial Strains of Textile Effluents

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Chromium resistant *Bacillus subtilis* NCBT-012, 013 strains and copper resistant *Pseudomonas fluorescens* NCBT-036, 037, 038 indicator bacterial strains were isolated from textile effluents and characterised. These indicator bacterial strains have shown multiple metal as well as antibiotic resistance characters. FTIR spectroscopic analysis of unpolluted control and the strains isolated from textile effluent *B. subtilis* and *P. fluorescens* revealed alteration in the absorption frequencies of main functional carbonyl, carboxyl, amino and hydroxyl groups between them. The effluent bacterial strains *B. subtilis* (NCBT-012) carried a plasmid DNA having a molecular weight of 14.86 kb and *P. fluorescens* (NCBT-036) showed a plasmid DNA with molecular weight of 23 kb suggesting that the plasmid genes detected is responsible for chromium and copper resistant characters of these bacterial strains. Further these isolates can be exploited for safe biodegradation of textile effluents.

Key words: *Bacillus subtilis* (NCBT 012, 013), *Pseudomonas fluorescens* (NCBT-036, 037, 038), FTIR analysis, Plasmid DNA.

Pollution of the environment with toxic heavy metals is spreading throughout the world along with the industrial progress. Cadmium, Copper, chromium, nickel and zinc are known to be the most commonly used and wide spread contaminants of the environment[19]. The heavy metals are classified into two groups essential and non essential: essential metals required for metabolic process are as follows: Na, K, Mg, Ca, V, Se, Fe, Co, Ni, Cu, Zn and Mo. Traces of these heavy metals are necessary as co· factors of the enzymatic reaction, but high levels of them cause extreme toxicity to living organisms due to inhibition of metabolic reactions. Non essential metals such as Cd, Hg, Sn, Pb, Ag, and AI have no metabolic function and are very toxic even at very low concentration. The bioremediation of heavy metals using microorganisms has great deal of attention in recent years, not only as scientific novelty but also for its potential application in industrial effluents[15].

Copper is an essential micronutrient that is required in trace amounts and is involved in various redox reactions. At higher concentrations it can affect the intracellular homeostasis and produce toxic effects on bacterial growth. The

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toxicity of copper is proposed to involve the inactivation of bacterial essential enzymes, which are thio group proteins[24]. Copper resistant bacteria have been isolated from environments where copper levels are elevated due to mining, industrial and agricultural activities[11]. The resistant mechanism and their genetic control are responsive to the requirement to accumulate cations at trace level and at the same time reduce cytoplasmic concentration from potentially toxic levels[9]. In most of the bacterial isolates, the genes responsible for the metal resistance are plasmid mediated and plasmid borne copper resistance have been reported in several plant pathogenic bacteria such as Pseudomonas syringae[12]. Until now two copper resistant operons have been well studied *i.e*, *cop* operon present on plasmid Ppt23D isolated from *Pseudomonas syringae*[4] and *pco* operon on Prj1004 isolated from *E. coli*[8].

Chromium, well recognized for its detrimental effect on the environment, accumulates throughout the food chain, is generated from varied industries especially from leather tanning, ink and paint formation industries. Chromium is present in different oxidative forms. The most important is trivalent and hexavalent chromium. Trivalent naturally occurs in the environment as an essential nutrient[2]. Hexavalent chromium is a well known carcinogen[17]. Besides this it can also cause skin ulcer, convulsions, kidney and liver damage. To avoid such toxic effects of Cr (VI), it is exigent to convert this into Cr(III). The aim of this present work is to interpret the role of plasmids and its potential of metal toxin and antibiotic resistant characters in bacterial isolates from textile effluents.

MATERIALS AND METHODS

The effluent samples were collected from three different textile industries at Tirupur in sterile bottles (Fig. 1a). Reference water sample was collected from a natural pond 2 km away industry area. Samples were transported in an ice box to the laboratory for isolation, identification, characterization, physiochemical and microbiological analyses. A portion of the effluent sample was immediately preserved for heavy metal analysis by acidifying 1.5 ml HNO_3/l so that pH<2 is maintained (Fig.1b and c). After acidifying, the samples were placed at 4° C in the refrigerator to prevent the volume change due to evaporation.

Isolation of bacteria was conducted within 6 hr of collection using a serial dilution technique. The chromium and copper resistant bacteria were isolated on Nutrient agar (Hi-media) (Fig. 2) plates supplemented with different concentration of Cr as $CrCl_3$ and Cu as $CuSO_4$, following the standard pour plate technique[1]. Plates were incubated at 37° ± 2° C for 24-48 hr and the total number of bacteria was determined as CFU/ml. The effluent was diluted to 1-1000 times with sterile double distilled water and used for isolation of chromium and copper resistant bacterial strains.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of the heavy metal resistant bacterial isolates grown on heavy metal incorporated media against respective heavy metal was determined by gradually increasing the concentration of heavy metal, 50 µg/ml each time on Nutrient Agar plates until the strains failed to grow colonies on the plate. The initial concentration was 50 µg/ml and the culture growing on the last concentration was transferred to the higher concentration by streaking on the plate for its confirmation. MIC was determined when the isolates failed to grow on plate[25].

Determination of co-resistance to other heavy metals

Chromium and copper tolerant isolates were also studied for the tolerance to other toxic metals. The fresh overnight Peptone water broth culture of the isolates was inoculated aseptically on Nutrient Agar and *Pseudomonas* Isolation Agar plates supplemented individually with other toxic metals. MIC of the isolates for the other metals was determined. The metal salts used are $CdCl_2$, $NiCl_2$, $CoCl_2$, $ZnSO_4$, $Pb(CH_3COO)_2$, $CuSO_4$ and $CrCl_3$.[20]

Study of antibiotic resistance

Susceptibility to different antibiotics for the chromium and copper tolerant isolates was determined by Disc diffusion method[3]. The antibiotic impregnated disc was placed on freshly prepared lawns of each isolate on Muller Hinton Agar and examined for zone inhibition. The isolates were classified as resistant, intermediate and susceptible following the standard Antibiotic Disc Sensitivity Testing Method. Disc containing the

Table 1. Cr and Cu resistant bacterial strains showing multiple metal resistance.

Bacterial isolates	Minimum inhibitory of concentration (mg)						
	$CdCl_2$	$NiCl_2$	$CoCl_2$	$ZnSO_4$	$Pb(CH_3COO)_2$	$CuSO_4$	$CrCl_3$
Bacillus subtilis NCBT-012	120	200	190	390	90	300	160
Bacillus subtilis NCBT-013	210	250	250	370	170	190	110
Pseudomonas fluorescens NCBT-036	120	180	180	460	130	180	130
Pseudomonas fluorescens NCBT-037	80	110	160	340	110	220	140
Pseudomonas fluorescens NCBT-038	90	140	210	410	90	180	110

Table 2. Antibiotic sensitivity profile of Cr and Cu resistant bacterial isolates.

Antibiotics	Disc content (mcg)	Diameter of inhibition zone (mm) of isolates				
		NCBT-012	NCBT-013	NCBT-036	NCBT-037	NCBT-038
Ampicillin	10	16.0 (I)	10.0 (R)	NI (R)	20.0 (S)	17.0 (I)
Tetracycline	30	40.0 (S)	36.0 (S)	NI (R)	30.0 (S)	33.0 (S)
Streptomycin	10	NI (R)	14.0 (I)	NI (R)	NI (R)	12.0 (I)
Gentamycin	10	10.0 (R)	26.0 (S)	20.0 (S)	6.0 (R)	8.0 (R)
Chloramphenicol	30	14.0 (I)	28.0 (S)	18.0 (S)	20.0 (S)	16.0 (I)
Kanamycin	30	NI (R)	24.0 (S)	20.0 (S)	12.0 (R)	NI (R)

Note: I: Intermittent; R: Resistant; S: Sensitive; NI: No inhibition

following antibiotics (mcg) were tested: Ampicillin [10], Chloramphenicol [30], Gentamycin [10], Kanamycin [30], Tetracycline [30] and Streptomycin [10].

Determination of metals in the effluent sample

For analysis of metal ion concentration in the effluent, method of Clesceri *et al*. (1989)[10] was adopted. Filtrate of the effluent samples (50 ml) was taken in a glass beaker, and 10 ml of concentrated HNO_3 was added and kept on a hot plate for slow boiling. When the volume was reduced to 10-15 ml, 10 ml of 1 + 1 HNO_3 was added and again heated till the volume was reduced to 10 ml. The solution was filtered through Whatman No. 41 filter paper and the volume of the filtrate

Table 3. Concentration of metals in textile effluent.

Metals	Concentration in textile effluent (mg/l)
Cr	2.38
Cu	0.01
Pb	*ND*
Zn	3.43
Co	*ND*
Ni	0.245
Cd	0.018

Note: ND: Not detected

Fig.1. a) Textile effluent sample
b) Pre digested textile effluent sample
c) Digested textile effluent sample

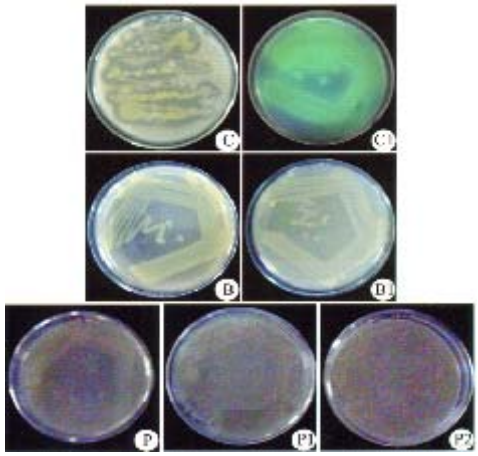

Fig. 2. Copper and Chromium bectrial strains.
C-Control *Bacillus subtilis*
C1-Control *Pseudomonas flurescens*
B-*Bacillus subtilis* strain NCBT-12
B1-*Bacillus subtilis* strain NCBT-13
P-*Pseudomonas flurescens* strain NCBT-036
P1-*Pseudomonas flurescens* strain NCBT-037
P2-*Pseudomonas flurescens* strain NCBT-038

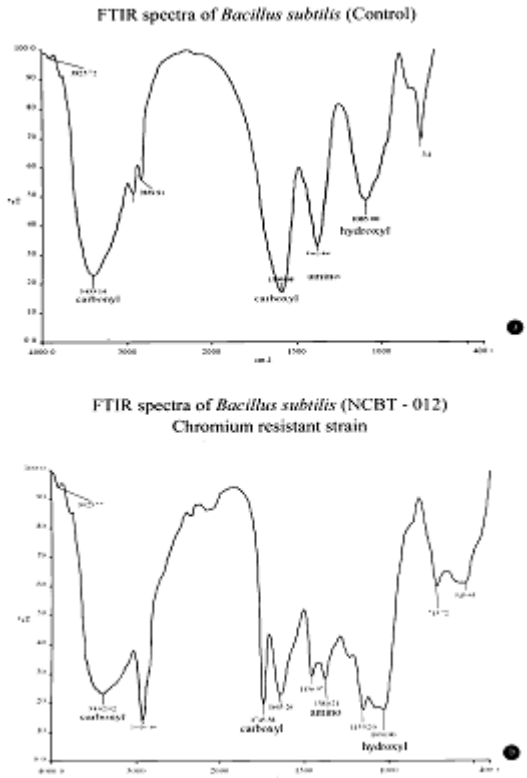

Fig. 3.

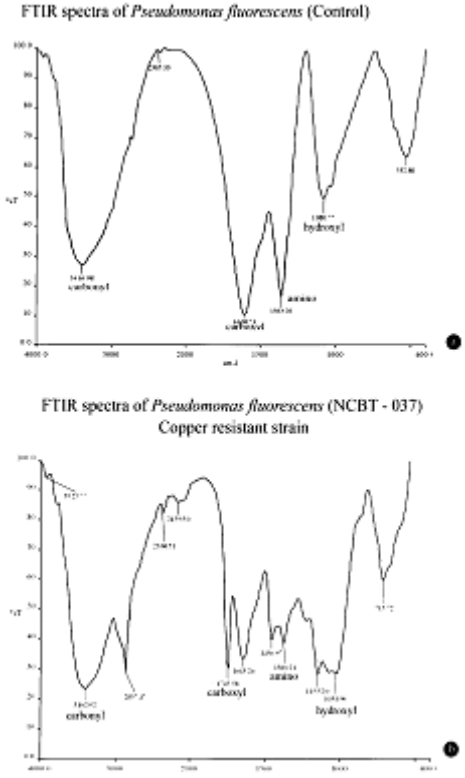

Fig. 4.

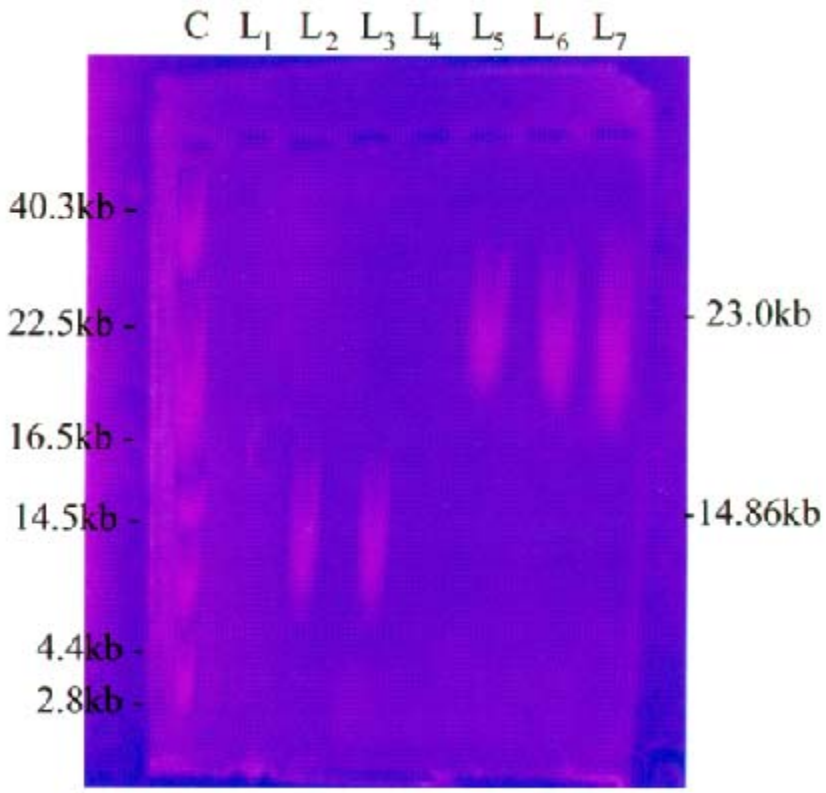

Fig. 5. Textile effluent indicator becteria-Isolated plasmid on agrose gel

C-Control marker *E.coli* V517.
Lane 1-Control *Bacillus subtilis*
Lane 2- *Bacillus subtilis* strain with NCBT-012 plasmid
Lane 3- *Bacillus subtilis* strain with NCBT-13 plasmid
Lane 4-Control *Psedomonus fluorescens*
Lane 5- *Psedomonus fluorescens* Strain with NCBT-036 plasmid
Lane 6- *Psedomonus fluorescens* Strain with NCBT-037 plasmid
Lane 7- *Psedomonus fluorescens* Strain with NCBT-038 plasmid

was made upto 50 ml, by adding deionised distilled water. Digested samples were stored in polypropylene bottles at room temperature till analysis. Metal analysis was carried out using Ion Coupled ac - Optical Emission Spectrometer (ICP - OES).

Fourier transform infra red (FTIR) spectroscopy analysis - FTIR spectroscopy was used to detect vibration frequency changes in *B. subtilis* and *P. fluorescens* of unpolluted control and strains isolated from textile effluent. The spectra were collected by Perkin Elmer spectrometer with the range 4000-400 cm^{-1} using chloroform as mulling agent. The background obtained from the scan of pure chloroform was automatically subtracted from the sample spectra.

Isolation of plasmid DNA

Plasmid DNA was determined using a Modified Alkaline Lysis method[7,22]. In the modified method, alkaline lysate was neutralized with ammonium acetate rather than potassium or sodium salts. This procedure allows the purification of plasmid DNA without the use of toxic organic solvents, CsCl centrifugation or column chromatography. Plasmid DNA was detected by electrophoresis in horizontal 0.7% agarose gel

using TAE buffer (lx). 15µ1 sample was loaded in each well and electrophoresis was carried out for 2 hr at 60V and the gel was stained by 0.5 µg/ml ETBr solution and observed under UV-transilluminator. The size of the plasmid DNA of the isolates was determined with *E. coli* V517 as marker.

RESULTS

The bacterial population decreased with increasing concentration of chromium and copper indicating their sensitivity to higher level of concentration. The effluent has sufficient number of bacteria with its count being 1.5×10^3 cfu/ml. Distinct morphological colonies were selected as copper and chromium resistant. They were identified as *Bacillus subtilis* and *Pseudomonas fluorescens* based on the specific characteristics such as colonial cellular morphology, gram reaction and biochemical test. Based on the frequency of occurrence Chromium resistant *B. subtilis* designated as NCBT-012 and NCBT-013, and three different *P. fluorescens* isolates of copper resistant, designated as NCBT-036, 037 and 038. All the bacterial isolates showed multiple metal resistance and the results were tabulated Table 1.

NCBT-012 showed the highest resistance to $CuSO_4$ (300 µg), $CrCl_3$ (160 µg), while NCBT-013 showed lower resistance, *i.e*., 190 mg of $CuSO_4$ and 110 mg of $CrCl_3$. Among the copper resistant strain NCBT-037 showed the highest resistance towards $CuSO_4$ (220 µg) and $CrCl_3$ (140 µg) whereas NCBT-036 and NCBT-038 showed lower resistance. As toxic metal resistance is linked with antibiotic resistance in microorganisms, the isolated Chromium resistant strain NCBT-012 and copper resistant strain NCBT-037 showed resistance towards Streptomycin, Gentamycin and Kanamycin whereas NCBT-037 showed resistance to Ampicillin; NCBT-036 showed resistance to Ampicillin, Tetracycline and Streptomycin; NCBT-038 against Gentamycin and Kanamycin Table 2.

FTIR functional group analysis

The functional groups involved in absorption of chromium by *B. subtilis* NCBT-012 and the control *B. subtilis* as well as absorption of copper by *P. fluorescens* NCBT-037. The main functional groups involved in absorption process were found to be carbonyl, carboxyl, amino and hydroxyl groups for *B. subtilis* and *P. fluorescens* control organisms (Fig. 3a and 4a). Involvement of these functional group in metal absorption process could be judged from change in frequency of absorbing groups (Fig. 3b and 4b). The absorbance of the peaks of carbonyl frequency 3433.14 is changed to 3402.92, for carboxyl frequency 1599.68 to 1745.58, for amino frequency 1362.84 to 1380.21 whereas for hydroxyl group frequency 1085.00 is changed to 1034.90 for *B. subtilis* (NCBT-012) chromium resistant strain. For *P. fluorescens* the absorbance peaks of carbonyl frequency 3416.98 is changed to 3402.92, carboxyl 1608.73 is shifted to 1745.58, amino frequency 1363.26 is changed to 1380.21 and hydroxyl group frequency 1080.77 is shifted to 1034.90.

A single band of plasmid DNA with the molecular weight of 14.86 kb was isolated from Chromium resistant NCBT-012 and Copper resistant NCBT-037 showed the molecular weight of 23.0 kb. The data of metal concentration in the textile effluent are furnished in Table 3. Analytical results revealed the average concentration of Cr and Cu in the effluent as 2.38 mg/l and 0.01 mg/l Fig. 5.

DISCUSSION

Textile effluents heavily polluted with heavy metals *viz*. chromium, copper, zinc, nickel and cadmium provide a natural source for the isolation of heavy metal resistant bacteria. The present study highlights the prevalent occurrence of a chromium and copper tolerant microbial population in textile effluents and their antibiotic resistance. The metal resistance was studied in a basal medium because the complexation with the heavy metal is minimum: therefore the shown metal concentration is approximately the free metal concentration[14]. The chromium and copper resistant isolates showed multiple metal resistance Table 1 and suggests the prior exposure of these isolates to the metals which are present in the effluent (sampling site) and this phenomenon of multiple metal resistance has been reported by Silver and Misra (1988)[22] in *Pseudomonas* sp. Chromium resistant NCBT-012 and copper resistant NCBT-037 were resistant to antibiotics like Streptomycin, Gentamycin and Kanamycin which matches the study of Duxbury and Bicknell (1983)[13].

The present work reveals a strong relationship between resistance to antibiotics and heavy metals which correlates with the study of Novick and Roth (1968)[17]. Although resistance to metal ions is of less clinical concern than resistance to antibiotics, such association is significant. Knowledge of resistance to metal ions may provide useful information on the mechanism of antibiotic resistance, plasmid genetics, physiology and ecology of microbes in the polluted environment[21]. Genes encoding resistance to metals and antibiotics were located on transmissible plasmids. The prevalence of such metal tolerant microorganisms is ecologically important, particularly if they are also antibiotic resistant. Under environmental condition of metal stress, those resistant populations will adapt faster by the spread of R Factor than by mutation and natural selection, thus leading to a very rapid increase in their number[5]. Thus plasmids also assist the bacteria to acquire tolerance and resistance mechanism against heavy metals and other toxic substances in the polluted environment. For instance, the incidence of 14.86 Kb plasmid in *B. subtilis* and 23.0 Kb plasmid in *P. fluorescens* was significantly higher in textile effluent polluted water than in the same bacteria isolated from non-pollutant water, the study is in agreement with the report of Boronin (1992)[6]. The wide spread use of metal and the discriminate disposal of byproducts and wastes from industries have created serious environmental issues[16]. The industrial effluents are the enriched medium to propagate and spread microbial population which are resistant to multiple metal ions.

From the present study, it is to be concluded that *B. subtilis* strains NCBT-012 and *P. fluorescens* NCBT-37 isolated from textile effluents can be efficiently used for bioremediation process with minimum cost and high efficiency and also to establish more number of genetically modified strains.

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