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Multiple antibiotic resistance patterns of rhizospheric bacteria isolated from *Phragmites australis* growing in constructed wetland for distillery effluent treatment

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Abstract: *Susceptibility patterns of 12 different antibiotics were investigated against rhizospheric bacteria isolated from Phragmites australis from three different zones i.e. upper (0-5 cm), middle (5-10 cm), lower (10-15 cm) in constructed wetland system with and without distillery effluent. The major pollutants of distillery effluent were phenols, sulphide, heavy metals, and higher levels of biological oxygen demand (BOD), chemical oxygen demand (COD) etc. The antibiotic resistance properties of bacteria were correlated with the heavy metal tolerance (one of distillery pollutant). Twenty-two species from contaminated and seventeen species from non-contaminated site were tested by agar disc-diffusion method. The results revealed that more than 63% of total isolates were resistance towards one or more antibiotics tested from all the three different zones of contaminated sites. The multiple-drug resistance property was shown by total 8 isolates from effluent contaminated region out of which 3 isolates were from upper zone, 3 isolates from middle zone and 2 isolates were from lower zone. Results indicated that isolates from contaminated rhizosphere were found more resistant to antibiotics than isolates from non-contaminated rhizosphere. Further, this study produces evidence suggesting that tolerance to antibiotics was acquired by isolates for the adaptation and detoxification of all the pollutants present in the effluent at contaminated site. This consequently facilitated the phytoremediation of effluent, which emerges the tolerance and increases resistance to antibiotics.*

Key words: *Antibiotics, Distillery effluent, Heavy metal, Rhizosphere, Bacteria*
PDF of full length paper is available with author ***(*ramchandra_env@indiatimes.com)***

Introduction

Anaerobically digested distillery effluent is a major source of environmental pollutant due to presence of many inorganic and organic compounds (Nath *et al.,* 2007). Among the effluent's pollutants, the major are phenol, amino-carbonyl complex *i.e.* melanoidins, high sulfates and heavy metals (Cu, Mn, Zn, Ni, Pb and Fe). Pollutants can influence the ecological functioning of soil as well as aquatic systems at virtually all tropic levels. Therefore, there is need for pollutant minimization using a cost effective technique. However, the growth of many plant species on contaminated site gives strong evidence for phytoremediation capability on terrestrial and wetland condition (Leigh *et al*., 2002). The rhizospheric bacteria have been reported to facilitate the tolerance and accumulation of pollutants in plant species (Peverly *et al*., 1995; Hassen *et al.,* 1998). Thus, bioremediation is mediated by very important and unique groups of bacteria associated with roots known as rhizospheric bacteria. These bacterial communities either make bioavailability of the different elements to plants or detoxify them. In addition the bacterial population protects the plant from infectious pathogens by releasing antibacterial compounds against them (Amico *et al.,* 2005; Yilmaz *et al.,* 2006). The wetland plant rhizosphere provides a complex micro-environment for the growth of aerobic and anaerobic both bacterial species and their role and nature is a matter of interest. Thus, effective rhizospheric bacteria have been elaborated for bioremediation of major industrial pollutants. The concept of pollution-induced community tolerance (PICT) has been applied successfully to establish cause-effect relationships between a toxicant and the structure of terrestrial microbial communities (Díaz-Ravina and Baath, 1996; Siciliano *et al*., 2000). This increase in tolerance is thought to reflect three possible toxicant effects: the disappearance of sensitive species through direct intoxication and the proliferation of more tolerant species, physiological changes that render the organisms less sensitive, and genetic changes such as acquiring mobile genetic material encoding for more resistance (Schmitt *et al*., 2005). However, as a natural consequence of selection under the pressure of antibiotics, resistant microorganisms are becoming prevalent in the environment (Mitsuhashi, 1979). Under such circumstances, resistant mutants are selected, because only resistant mutants can grow and the resistant trait can easily be transmitted to susceptible microorganisms by conjugation and transduction, although the frequency of mutation remains unchanged. (Ogawara, 1981). Therefore, microorganisms have conflicting properties, susceptibility and resistance to antibiotics.

Laboratory investigations have shown that many bacteria resistant to the effects of high concentrations of heavy metals are concomitantly resistant to several antibiotics (Foster, 1983). Such studies cannot differentiate intrinsic resistance *i.e.* species-specific resistance from acquired resistance due to either chromosomal mutations or incoming and thus transferable genes mainly carried by plasmids or transposable elements (Rice and Bonomo, 1996). Microorganisms resistant to antibiotics and tolerant to metals make their appearance, as a result of exposure to metal contaminated

environment, which causes co-incidental selection of resistance factors for antibiotics and heavy metals (Dhakephalker *et al.,* 1994). The aim of this study was to isolate the dominant bacterial species and to evaluate acquired antibiotic resistance in rhizospheric bacterial population growing at different zones of distillery contaminated sites. Further this property was compared with non-contaminated site, which showed wide adaptability and stability in diverse environment of bacteria.

Materials and Methods

Constructed wetland designing: The wetland was constructed and designed in 8 x 21 m area located at Industrial Toxicology Research Centre, Gheru campus, Kanpur road, Lucknow, U.P., India. A channel was constructed as a zig-zag structure of length 19 m by cemented material to prevent the seepage. Upstream channel depth/width was kept 50/40 cm and downstream depth/width was kept 58/40 cm maintaining 0.88% slope to maintain the hydraulic flow. Horizontal bed was filled with pebbles, coarse gravel and sand particles each layered 8 cm height respectively (EPA, 1996). The distillery waste for treatment was circulated in channel for about 30 days till the constant degrading parameters. Subsequently, simultaneously a separate constructed wetland system was also run with tap water only as control.

Collection of rhizosphere soil samples: *Phragmites australis* plant growing at constructed wetland distillery effluent contaminated and non-contaminated sites were taken out along with its roots and adhered soil in it. The roots were cut into three equal parts, upper zone (0-5 cm), middle zone (5-10 cm) and lower zone (10-15 cm). Samples were taken from different zones to get diverse group of bacterial population from surface of soil till the depth of root. Simultaneously contiguous soil and distillery effluent samples were also collected for physico-chemical analysis. Effluent sample was collected from Unnao distilleries, Unnao (U.P.) India. Samples were safely brought to the laboratory and 0.9% NaCl solution was added to each flask and subjected to gyro-rotatory shaker (Innova 4230, New Brunswick, USA) for an hour. Later 1 ml of sample was serially diluted and by spread plate method (Aneja *et al*., 1996) bacteria were isolated and purified on plate count agar.

Physico-chemical analysis: Soil and effluent samples were tested for parameters such as pH, total solids, sulphate, sulphide, phenol, BOD and COD (APHA, 2005; EPA, 1996) and heavy metals by inductively coupled plasma spectroscopy and elements such as nitrogen, chloride, magnesium, sodium and potassium by ion meter (Orion autoanalyzer, 960, USA).

Isolation and characterization of bacteria: Morphologically different and single growing colonies were picked up and purified by re-streaking. Further cell shape, Gram-staining, motility, oxidase, catalase, growth aerobic/anaerobic and oxidative/fermentative and other biochemical tests were performed for bacterial characterization (Barrow and Feltham, 1993).

Table - 1: Physico-chemical characteristics of contaminated water and soil in wetland ecosystem

Parameters	Effluent in wetland system	Sediments in constructed wetland
Color appearance	Dark brown	Brown
Color intensity	15000±600	-
pH	7.3±0.293	8.2±0.30
BOD	860±43	3800±38.63
COD	5900±118	8200±59.42
TS	404.98±20.25	547.6±16.80
TDS	233.89±9.33	410±6.95
TSS	171.19±8.56	137.6±5.73
Sulphide	0.32±0.016	2.63±0.13
Sulphate	1610±16.10	1926±3.13
Phenol	360±14.4	407.4±16.6
Nitrogen	275.66±11.026	292.6±10.5
Chloride	675.48±40.529	750.69±22.85
Magnesium	67.50±1.025	74.8±5.82
Potassium	300±15.00	449±18.0
Sodium	268±12.45	371±21.50
Heavy metals	**(mg l^{-1})**	**(µg g^{-1})**
Fe	7.64±0.28	10.58±0.14
Cr	1.31±0.27	2.56±0.11
Ni	0.21±0.026	1.20±0.02
Pb	0.53±0.12	1.76±0.09
Cu	0.75±0.19	2.15±0.05
Zn	0.43±0.07	1.86±0.027
Mn	0.41±0.04	1.23±0.06

All values are in ppm except pH and color intensity
n = 3 ± SD, BOD = biological oxygen demand, COD = chemical oxygen demand, TS = total solids, TDS = total dissolved solids, TSS = total suspended solids

Determination of antibiotic susceptibility: Twelve antibiotic discs (Hi-Media Ltd, India) chloramphenicol 30 µg, gentamicin 10 µg, kanamysin 30 µg, ampicillin 10 µg, tetracycline 30 µg, streptomysin 10 µg, penicillin 10 U, amoxycillin 30 µg, norfloxacin 10 µg, erythromycin 15 µg, vancomycin 30 µg and neomycin 30 µg were obtained. The response of organisms to antibiotic discs were determined by spreading ~ 1×10^6 cells in broth cultured suspensions on Mueller Hinton agar (Hi-Media Ltd., India) plates and antibiotic discs were placed with the help of disc dispenser (Aneja *et al*., 1996). All the plates were incubated at 37ºC for 24-48 hr. On the basis of zone diameter, the isolates were classified as resistant, intermediate and sensitive (Bauer *et al*., 1966). All the experiments were carried out in triplicate with both contaminated and control site isolates.

Heavy metal resistance: Heavy metal resistance of rhizosphere isolates was determined by agar-dilution method. Plates containing 20 ml of one-half strength nutrient agar and different concentrations of metal were poured. The selections of heavy metals were done on the basis of metals present in the distillery effluent. Eight-fold concentrations of original effluent were used in the study and metal

tolerance was determined by the plate count method with Cu, Zn and Fe at the highest levels tested and with Ni, Pb and Mn at medium levels. The compounds used were $NiCl_2$. $6H_2O$, $ZnSO_4$. $7H_2O$, $Pb(CH_3COO)_2$. $3H_2O$, $CuSO_4$. $5H_2O$, $FeSO_4$. $6H_2O$ and $MnSO_4$. $7H_2O$. Plates were inoculated with 0.1 ml from exponentially grown cultures and incubated at 37°C for 48 hr. Plates containing media with no added metal were inoculated in the same manner to serve as controls. All tests were carried out in duplicate.

Plasmid extraction and curing: The treatments were tested to evaluate curing efficiency. The strains were grown in LB broth at 37°C for 24 hr. One set of 10 ml test tubes were prepared with 2 ml of LB broth with final concentration of 50, 100, 150 and 200 mg ml^{-1} of ethidium bromide. All the tubes were inoculated with 200 ml of the cultured bacterial broth and incubated at 37°C for 24 hr under constant agitation. Serial dilutions in normal saline solution 0.8% were done and 100 ml plated in LB agar, the plates were incubated at 37°C for 24 hr. The colonies were selected from the treatments with the highest concentrations of curing agent (Molina-Aja *et al*., 2002) and antibiograms were performed for the antibiotics to which they were originally resistant. Colonies that showed change in the size of the inhibition zone were submitted to plasmid extraction with the Wizard plus SV Minipreps DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's instruction, using Luria-bertani (LB) as incubation medium. The extracted DNA was electrophoresed in a 1% agarose gel at 80 V for 45 min.

Statistical analysis: One-way ANOVA was used to examine the hypothesis that resistance was greater in contaminated rhizosphere isolates than in the non-contaminated rhizosphere isolates (control). All percentage data were transformed before analysis. One-way paired *t*-test was used to compare multiple-drug resistance (MDR) patterns from bacterial isolates in contaminated and control samples.

Results and Discussion

The sediment and distillery effluent water quality parameters in constructed wetland system are shown in Table 1. The sediments of constructed wetland showed higher levels of BOD, COD, heavy metals, phenol and sulfate compared with effluent in constructed wetland system. This was observed due to deposition and binding of different elements with sediment particles. The basic biochemical characteristics of all the isolates were tested and the identified bacteria were listed (Table 2, 3). We have already characterized fast growing rhizospheric isolates from *P. australis* contaminated site by 16S rRNA gene sequencing in the previous study (Chaturvedi *et al.,* 2006). A total of 22 bacterial strains were isolated from effluent contaminated

Table - 2: Biochemical properties of bacterial isolates from the rhizosphere of *Phragmites australis* growing in distillery contaminated site

Isolates	Biochemical properties									
	Gram-stain	Motility	Cell shape	Oxidase	Catalase	Cellulase	Lipase	Urease	O/ F	Citrate utilization
SUF1	+	-	R	+	+	-	-	+	-	-
SUF2	-	+	R	+	+	+	-	-	-	+
SUF3	+	-	R	W^+	+	-	-	-	F	+
SUF4	+	ND	R	-	+	-	-	+	O	+
SUF5	-	+	R	+	+	ND	ND	ND	F	ND
SUF6	+	-	R	-	+	-	-	-	O	W^+
SUF7	-	+	R	+	+	ND	ND	ND	F	ND
SUF8	-	+	R	+	+	ND	ND	ND	F	ND
SMF1	+	+	R	W^+	+	-	-	+	F	+
SMF2	-	+	R	+	+	-	W+	-	-	+
SMF3	-	+	R	+	+	-	W^+	-	-	+
SMF4	-	+	R	+	+	ND	ND	ND	F	ND
SMF5	+	+	R	W^+	+	-	W^+	-	F	+
SMF6	+	-	R	-	+	-	-	+	F	+
SMF7	+	+	R	-	+	+	-	-	F	+
SMF8	-	+	R	+	+	ND	ND	ND	F	ND
SLF1	+	-	S	-	+	-	-	+	F	-
SLF2	-	+	R	-	+	-	-	-	O	-
SLF3	-	-	R	-	+	ND	-	-	O	ND
SLF4	-	+	R	+	+	-	-	ND	F	-
SLF6	+	+	R	-	+	-	ND	+	O	W^+
SLF7	-	-	R	+	+	ND	ND	ND	F	ND

+ = Positive, - = Negative, W^+ = Weakly positive, R = Rod, S = Spherical, F = Fermentative, O = Oxidative, ND = Not determined

SUF1 = *Microbacterium hydrocarbnoxydans,* SUF2 = *Achromobacter xylosoxidans,* SUF3 = *Bacillus subtilis,* SUF4 = *Bacillus megaterium*, SUF5 = *Aeromonas* sp, SUF6 = *Bacillus* sp, SUF7 = *Vibrio* sp, SUF8 = *Vibrio* sp, SMF1 = *Bacillus licheniformis*, SMF2 = *Achromobacter xylosoxidams,* SMF3 = *Achromobacter xylosoxidams*, SMF4 = *Vibrio* sp, SMF5 = *Bacillus thuringiensis*,SMF6 = *Bacillus licheniformis*, SMF7 = *Bacillus subtilis*, SMF8 = *Aeromonas* sp, SLF1 = *Staphylococcus epidermidis*, SLF2 = *Pseudomonas migulae*, SLF3 = *Alcaligenes faecalis,* SLF4 = *Vibrio* sp, SLF6 = *Bacillus cereus* and SLF7 = *Pasteurella* sp

Table - 3: Biochemical properties of bacterial isolates from the rhizosphere of *Phragmites australis* growing in non-contaminated site

Isolates	Biochemical properties							
	Gram-stain	Motility	Cell shape	Growth aerobic	Growth anaerobic	Oxidase	Catalase	O/F
SFCU1	+	+	R	+	-	+	+	F
SFCU4	-	-	R	+	+	+	+	F
SFCU5	-	+	R	+	-	+	+	O
SFCU7	-	-	R	+	-	-	+	F
SFCU10	-	+	R	+	+	+	+	F
SFCU11	-	+	R	+	+	+	+	F
SFCM1	-	-	R	+	+	+	+	F
SFCM2	-	+	R	+	+	+	+	F
SFCM3	+	+	R	+	+	-	+	F
SFCM5	-	+	S	+	+	+	+	F
SFCM6	-	+	S	-	+	+	+	O
SFCL1	-	-	R	+	+	+	+	F
SFCL2	-	+	R	+	+	+	+	F
SFCL3	-	-	R	+	+	+	+	F
SFCL4	+	-	R	+	+	-	+	F
SFCL5	+	+	R	+	+	+	+	F
SFCL6	-	-	R	+	-	+	+	O

R = rods, S = spherical, F = fermentative, O = oxidative

SFCU1 = *Bacillus cereus*, SFCU4 = *Actinobacillus* sp, SFCU% = *Achromobacter* sp, SFCU7 = *Acinetobacter* sp, SFCU10 = *Vibrio* sp, SFCU11 = *Aeromonas* sp, SFCM1 = *Actinobacillus* sp, SFCM2 = *Aeromonas* sp, SFCM3 = *Bacillus* sp, SFCM5 = *Vibrio* sp, SFCM6 = *Achromobacter* sp, SFCL1 = *Pasteurella* sp, SFCL2 = *Vibrio* sp, SFCL3 = *Actinobacillus*, SFCL4 = *Bacillus mycoides*, SFCL5 = *Bacillus thuringiensis* and SFCL6 = *Flavobacterium*

Table - 4: Antibiotic sensitivity profile and zone size interpretative chart of rhizosphere isolates (mm) from contaminated site

Isolates	Antibiotic[a]											
	Chl	Gen	Kan	Amp	Tet	Str	Pen	Amo	Nor	Ery	Van	Neo
SUF1	S^{25}	S^{25}	S^{30}	S^{24}	S^{27}	S^{19}	R^{24}	S^{30}	S^{36}	S^{17}	S^{28}	S^{27}
SUF2	S^{23}	S^{20}	S^{20}	R^{11}	S^{16}	S^{20}	R^{10}	S^{13}	S^{20}	S^{28}	S^{19}	S^{19}
SUF3	S^{31}	S^{24}	S^{26}	S^{21}	S^{22}	S^{18}	R^{24}	S^{26}	S^{31}	S^{27}	S^{21}	S^{22}
SUF4	S^{37}	S^{32}	S^{32}	S^{30}	S^{36}	S^{24}	S^{30}	S^{34}	S^{26}	S^{23}	S^{22}	S^{27}
SUF5	S^{31}	S^{23}	S^{22}	S^{17}	S^{20}	S^{17}	R^{10}	S^{16}	S^{24}	R^{10}	S^{19}	S^{20}
SUF6	S^{36}	S^{27}	S^{22}	R^{18}	S^{23}	S^{17}	R^{10}	S^{32}	S^{21}	S^{18}	S^{29}	S^{25}
SUF7	S^{29}	S^{25}	S^{20}	R^{15}	S^{26}	S^{19}	S^{19}	S^{19}	S^{27}	S^{28}	S^{25}	S^{35}
SUF8	S^{33}	S^{24}	S^{26}	R^{22}	S^{27}	S^{36}	S^{21}	S^{22}	S^{32}	S^{27}	S^{27}	S^{31}
SMF1	S^{25}	S^{30}	S^{34}	S^{25}	S^{36}	S^{22}	S^{25}	S^{33}	S^{38}	S^{20}	S^{28}	S^{29}
SMF2	S^{26}	S^{21}	S^{24}	S^{16}	S^{27}	S^{21}	S^{13}	S^{16}	S^{23}	S^{18}	S^{22}	S^{20}
SMF3	S^{23}	S^{26}	S^{28}	S^{16}	S^{29}	S^{19}	S^{18}	S^{20}	S^{21}	S^{26}	S^{18}	S^{25}
SMF4	R^{10}	S^{27}	S^{30}	R^{8}	S^{17}	S^{20}	S^{11}	R^{19}	S^{34}	R^{10}	R^{11}	S^{26}
SMF5	S^{35}	S^{28}	S^{26}	S^{34}	S^{31}	S^{23}	S^{37}	S^{44}	S^{36}	S^{25}	S^{22}	S^{21}
SMF6	S^{27}	S^{33}	S^{31}	S^{24}	S^{35}	S^{23}	S^{27}	S^{35}	S^{37}	R^{13}	S^{28}	S^{28}
SMF7	S^{19}	S^{25}	S^{29}	R^{12}	S^{25}	S^{19}	S^{33}	S^{18}	S^{34}	R^{9}	S^{24}	S^{23}
SMF8	S^{29}	S^{24}	S^{28}	R^{11}	S^{27}	S^{20}	S^{10}	R^{12}	S^{22}	S^{17}	S^{18}	S^{19}
SLF1	S^{27}	S^{25}	S^{28}	S^{30}	S^{35}	S^{28}	S^{35}	S^{30}	S^{26}	S^{28}	S^{22}	S^{24}
SLF2	S^{24}	S^{19}	R^{9}	R^{2}	S^{23}	S^{14}	R^{2}	S^{14}	S^{23}	S^{2}	S^{2}	S^{17}
SLF3	S^{33}	S^{25}	S^{24}	S^{25}	S^{33}	S^{23}	S^{26}	S^{27}	S^{32}	S^{30}	S^{28}	S^{31}
SLF4	S^{30}	S^{25}	S^{25}	S^{20}	S^{30}	S^{15}	S^{22}	S^{26}	S^{30}	S^{25}	S^{20}	S^{16}
SLF6	S^{28}	S^{22}	S^{20}	S^{19}	S^{22}	S^{17}	R^{10}	R^{13}	S^{20}	S^{14}	S^{19}	S^{20}
SLF7	S^{28}	S^{19}	S^{18}	S^{14}	S^{26}	S^{16}	R^{12}	S^{16}	S^{21}	S^{19}	S^{18}	S^{17}

S = sensitive, R = resistant

Zone size diameters are in superscript, n = 3±SD, ANOVA ($p<0.05$)

[a]Chl = chloramphenicol, Gen = gentamycin, Kan = kanamycin, Amp = ampicillin, Tet = tetracycline, Str = streptomycin, Pen = Penicillin, Amo = amoxicillin, Nor = norfloxacin, Ery = erythromycin, Van = vancomycin, Neo = Neomycin, SUF = upper zone isolates, SMF = middle zone isolates, SLF = Lower zone isolates

Table - 5: Antibiotic sensitivity profile and zone size interpretative chart of rhizosphere isolates (mm) from non-contaminated site

Isolates	Antibiotic[a]											
	Chl	Gen	Kan	Amp	Tet	Str	Pen	Amo	Nor	Ery	Van	Neo
SFCU1	S^{25}	S^{35}	S^{32}	S^{25}	S^{40}	S^{25}	I^{27}	S^{32}	S^{40}	R^{12}	S^{25}	S^{29}
SFCU4	S^{20}	S^{27}	S^{18}	S^{22}	S^{26}	S^{15}	I^{22}	S^{25}	S^{18}	I^{17}	S^{15}	I^{16}
SFCU5	S^{40}	S^{28}	S^{27}	S^{35}	S^{24}	S^{14}	S^{20}	S^{23}	S^{29}	S^{25}	S^{27}	S^{28}
SFCU7	S^{45}	S^{45}	S^{38}	S^{17}	S^{38}	S^{30}	I^{19}	S^{28}	S^{28}	I^{17}	S^{30}	S^{35}
SFCU10	S^{38}	S^{42}	S^{35}	S^{40}	S^{45}	S^{35}	S^{52}	S^{25}	S^{36}	S^{40}	S^{38}	S^{37}
SFCU11	S^{37}	S^{35}	S^{36}	S^{45}	S^{39}	S^{28}	S^{45}	S^{50}	S^{32}	S^{30}	S^{28}	S^{30}
SFCM1	S^{30}	S^{23}	S^{22}	S^{21}	S^{24}	S^{18}	I^{25}	S^{28}	S^{28}	I^{22}	S^{22}	S^{22}
SFCM2	S^{24}	S^{25}	S^{25}	I^{15}	S^{26}	S^{16}	R^{11}	I^{15}	S^{21}	I^{17}	R^{14}	S^{16}
SFCM3	S^{24}	S^{25}	S^{28}	S^{34}	S^{34}	S^{22}	S^{39}	S^{42}	S^{30}	S^{26}	S^{20}	S^{25}
SFCM5	S^{20}	S^{26}	S^{27}	S^{27}	S^{30}	S^{19}	S^{29}	S^{34}	S^{29}	S^{26}	S^{21}	S^{21}
SFCM6	S^{40}	S^{30}	S^{28}	S^{35}	S^{30}	S^{21}	R^{10}	I^{10}	S^{35}	S^{28}	S^{25}	S^{23}
SFCL1	S^{28}	S^{22}	S^{21}	S^{24}	S^{30}	S^{17}	I^{25}	S^{25}	S^{26}	I^{21}	S^{18}	S^{20}
SFCL2	S^{30}	S^{26}	S^{28}	S^{29}	S^{31}	S^{20}	I^{27}	S^{32}	S^{30}	S^{23}	S^{20}	S^{21}
SFCL3	S^{25}	S^{33}	S^{33}	S^{44}	S^{31}	S^{21}	S^{36}	S^{40}	S^{28}	I^{16}	S^{21}	S^{25}
SFCL4	S^{35}	S^{28}	S^{28}	S^{28}	S^{28}	S^{26}	S^{30}	S^{27}	S^{22}	I^{21}	S^{20}	S^{23}
SFCL5	S^{35}	S^{30}	S^{28}	S^{25}	S^{33}	S^{22}	I^{20}	S^{27}	S^{35}	S^{30}	S^{22}	S^{24}
SFCL6	S^{38}	S^{31}	S^{30}	S^{32}	S^{37}	S^{20}	S^{35}	S^{38}	S^{30}	S^{30}	S^{25}	S^{25}

S = sensitive, R = resistant, I = intermediate

Zone size diameters are in superscript, n = 3±SD, ANOVA ($p<0.05$)

[a]Chl = chloramphenicol, Gen = gentamycin, Kan = kanamycin, Amp = ampcillin, Tet = tetracycline, Str = streptomycin, Pen = Penicillin, Amo = amoxicillin; Nor = norfloxacin, Ery = erythromycin, Van = vancomycin, Neo = Neomycin, SFCU = upper zone isolates, SFCM = middle zone isolates, SFCL = Lower zone isolates

rhizosphere samples and 17 bacterial isolates were obtained with a predominance of Gram-ne gative organism from control site. Among various bacteria isolated from different depth of study were found 8, 8 and 6 in contaminated site while 6, 5 and 6 were found in control site of upper, middle and lower zone respectively. The difference in the concentration of pollutants on soil surface and towards depth alter the soil properties due to differential percolation of different pollutants, hence the number and the bacterial species varies in different zones (Girvan *et al.,* 2003). Among them, *Bacillus* sp. was found more prevalent in contaminated as well as control followed by *Vibrio* sp in all the three zones due to the motile nature of the organism. While *Achromobacter* and *Aeromonas* were dominant in upper and middle zone and *Pasteurella* in lower zone of both contaminated and control sites.

All bacterial strains were assayed for antibiotic resistance. Among the tested strains from effluent contaminated site maximum resistance was observed 36% for Pen and minimum resistance was shown as 4% for Chl, Kan and Van. Out of 22 isolates 8 were susceptible to all of the twelve antibiotics tested. Among those sensitive isolates one was from upper zone, 4 from middle zone and 3 from lower zone of contaminated site while 5, 3 and 6 isolates showed sensitivity from upper, middle and lower zones of control site respectively (Table 4). More than 63% of isolates had shown resistance towards one or more antibiotics tested from all the three different zones. Strains with a single resistance (27.2%) were principally Pen, Amp and Ery in contaminated samples (Table 4). Among the non-contaminated (control) site isolates only 3 bacterial species showed resistance towards Pen, Ery and Van (Table 5). In particular, total resistance for antibiotics tested was significantly higher in bacteria isolated from distillery-contaminated samples (ANOVA, $p<0.05$) compared to the non-contaminated (control) bacterial samples. Among those, Gram-negative bacterial isolates displayed the greatest incidence of resistance to Amp and Pen (ANOVA, $p<0.05$).

A total of 11 different multiple-drug resistance (MDR) pattern was shown by 8 isolates from effluent contaminated region. Out of which 3 were from upper zone, 3 were from middle zone and 2 were from lower zone of rhizosphere (Table 6). The comparative resistance between contaminated and control was found in upper zone 87.5% and 16.6%, in middle zone 50% and 40% and in lower zone 50% and 40% respectively (Fig. 1). The numbers of resistant strains decreased considerably from upper zone to lower zone as upper layer has higher concentration of pollutants on the surface of sediments. Hence, the numbers of pollutant tolerant and reducing bacteria were dominant which were having single or multiple resistance property with antibiotics. In contrast less number of resistant bacteria were found in non-contaminated site. This indicated that antibiotic resistance property is directly proportional to the pollutant concentration. This finding corroborated with the previous studies (Parveen *et al*., 1997; Asthana and Chandra, 2001).

The antimicrobial activity and resistant to antimicrobials of bacteria from a rhizosphere habitat can help and explain the selection

Table - 6: Multiple-drug resistance (MDR) patterns of strains isolated from contaminated and control samples by one way paired t-test and comparison of heavy metal tolerance and antibiotic resistance among isolates of contaminated and non-contaminated sites

Resistance marker(s)[a]	Antibiotic resistance pattern				Isolates resistant or tolerant to heavy metals					
	No. of resistant strains		Plasmid size (Kb)		Cu^{2+}	Fe^{3+}	Mn^{2+}	Ni^{2+}	Zn^{2+}	Pb^{2+}
	C	NC	C	NC						
Pen	3	1	1.3	1	$+^1 +^2 +^3$	$+^1 +^3$	$+^1$	$+^1 +^2 +^3$	$+^C +^2$	$+^2$
Amp	2	0	4.6, 6.6	0	$+^1 +^2 +^C$	$+^1 +^C$	$+^1$	$+^2$	$+^1 +^2$	$+^2$
Ery	1	1	7.7	2.8	$+^1$	-	$+^1$	-	$+^C$	-
Amp/Pen	2	0	8	0	-	$+^1 +^2$	$+^1 +^2$	$+^1$	$+^1 +^2$	$+^2$
Ery/Pen	1	0	5	0	$+^1$	$+^1$	-	$+^1$	$+^1$	$+^1$
Amo/Pen	1	0	5	0	$+^1$	-	-	$+^1$	$+^1$	$+^1$
Van/Pen	0	1	-	1.4	$+^C$	-	-	-	$+^C$	-
Amp/Ery	1	0	3.6	0	$+^1$	-	$+^1$	-	-	$+^1$
Amp/Amo	1	0	5.3	0	$+^1$	-	-	-	$+^1$	$+^1$
Kan/Amp/Pen	1	0	32	0	$+^1$	-	-	-	$+^1$	$+^1$
Amo Ery Van	1	0	7.7	0	-	-	$+^1$	-	$+^1$	$+^1$
Chl/Amp/Amo/Ery/Van	1	0	4.3	0	$+^1$	$+^1$	$+^1$	$+^1$	$+^1$	-

[a]Pen = penicillin, Amp = ampicillin, Ery = erythromycin, Amo = amoxycillin, Van = vancomycin, Kan = kanamycin, Chl = chloramphinacol, C = contaminated, NC = non-contaminated. $+^1$ = first isolate of contaminated site resistant to adjacent antibiotic, $+^2$ = second isolate of contaminated site resistant to adjacent antibiotic, $+^3$ = third isolate of contaminated site resistant to adjacent antibiotic, $+^C$ = isolate of control site resistant to adjacent antibiotic

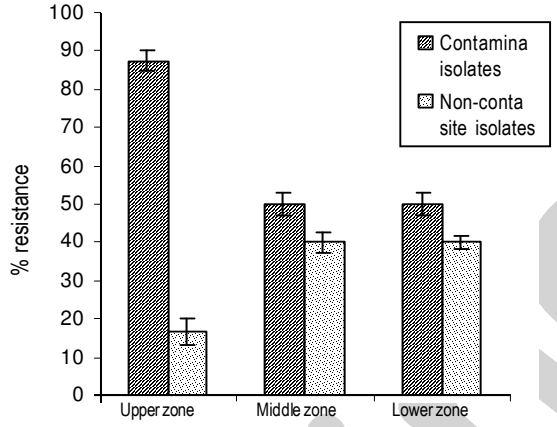

Fig. 1: Rhizosphere isolates showing total resistance with antibiotics

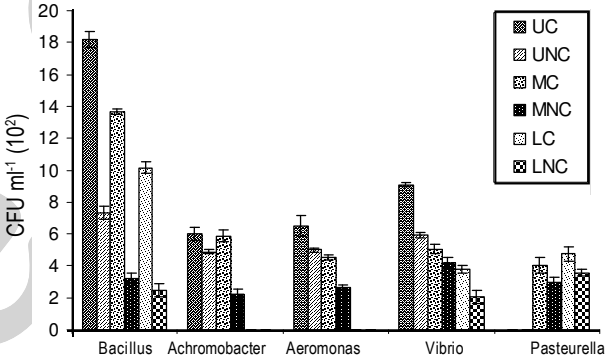

Fig. 2: Comparison of dominant bacterial genera among the contaminated and non-contaminated sites at different zones of rhizosphere (UC = upper contaminated, MC = middle contaminated, LC = lower contaminated) zones. (UNC = upper non-contaminated, MNC = middle non-contamonated and LNC = lower non-contaminated) zones

and persistence of such isolated strains in the particular contaminated ecological niche (Demain, *et al.,* 1983). The tolerance in eight-fold concentration of the heavy metals found in the effluent was tested with rhizospheric isolates from contaminated and control sites during the study. It was observed that the isolates from contaminated site showed more tolerance to heavy metals when compared with the control site isolates. Previous studies (Goni-Urriza *et al.,* 2000; Stepanauskas *et al*., 2005 ; Wright *et al.,* 2006) have often revealed that bacteria isolated from environment with elevated levels of pollutants such as heavy metals exhibit greater resistance towards antibiotics. The ecological value of the sensitivity or resistance of rhizosphere isolates to metals lies in its ubiquitous presence in many habitats, in its capacity to grow in the presence of a relatively high heavy metal concentration. On the other hand when correlating the heavy metal tolerance with the antibiotic resistance property, we found that those isolates showing multiple antibiotic resistance were having tolerance properties with more number of heavy metals tested (Table 6) both in contaminated and control site isolates. The significance of metal tolerance may play a role in maintaining retention of antibiotic resistance once resistance is attained.

Duxbury (1981), reported that generally Gram-negative soil species appeared to be more tolerant than Gram-positive and thus supported our study. In most of the 22 isolates from contaminated sites, plasmids of different molecular size were found (Table 6). Strains susceptible to antibiotic were negative for plasmid. Although 14 isolates from contaminated and 3 isolates from control sites contain plasmid. One of these strains from contaminated site harbored a

plasmid (32 Kb) and rest contain (1.3-8.0 Kb) smaller plasmids. There was a consistent relationship between plasmid content and antibiotic resistance pattern. 14 strains were treated with ethidium bromide at 200 μg ml^{-1} and only 8 were found to tolerate at higher concentration, which were further cured for plasmid. The antibiotic resistance property was found to be lost this indicated the above property is plasmid born. Many antibiotic resistance genes are plasmid-borne and in many isolates tend to be present on the same plasmids as resistance factors for metals such as mercury (Christon *et al.,* 1997). The presence of R-plasmids among different bacterial isolates from contaminated and non-contaminated sites has been previously documented (Foster, 1983; Bhattacharjee *et al.,* 1988; Thavasi *et al.,* 2007) and acquired resistance to β-lactams, aminoglycosides, macrolides, glycopeptides and amphenicols *etc.* Further, the dominating bacterial isolates belonged to the genus *Bacillus, Achromobacter, Aeromonas, Vibrio* and *Pasteurella* in contaminated and normal rhizosphere. The comparative CFU analysis of contaminated and control microflora revealed that dominating genera in both sites showed a decreasing pattern of distribution. Subsequently, there was also a decline in the ratio of upper, middle and lower zone of bacterial density. This indicated that bacterial density is concentration dependent of heavy metals and other pollutants due to their descending concentration during the percolation (Fig. 2). Therefore, the observed occurrences reflected the descending concentration of distillery pollutants with the soil/ sediment depth, which could act as a gradient filter.

Our findings are supported by the recent study (Edwards 2002; Schmitt *et al*., 2005; Espigares *et al.,* 2006), which showed that the industrial pollutants can influence the ecological functioning of aquatic as well as soil systems and increase the proliferation of more tolerant species showing a multiple antibiotic resistance property. The study revealed that rhizosphere isolates from contaminated site had distinguishable property of multiple-drug resistance and tolerance towards heavy metals due to this reason they were able to tolerate and reduce the toxic nature of the distillery waste.

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