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Heavy metal and antibiotic resistant bacteria isolated from soil contaminated by the sugar industry effluent

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Key words: Antibiotic resistance, Heavy metal resistance, 16S rDNA, Multi-Drug Resistant, Sugar industry effluent.

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Abstract

In this study, antibiotic and heavy metal resistant bacteria were isolated from the agriculture field's soil around sugar industry situated in Roorkee, District Haridwar, Uttarakhand (India).The antibiotic and heavy metal resistance profiles of the isolates were determined. Fifteen antibiotics and five heavy metals were used for reference. Four promising isolates were recovered, two gram positive E-2 and E-10 and two gram negative isolate E-7 and E-9. Detailed morphological, biochemical, and molecular characterization was done to identify the bacteria. The isolates E-2 and E-10 were identified as Bacillus licheniformis sp, and Teribacillus aidingensis sp. while E-7 and E-9 were identified as Chryseobacterium indologenes sp., and Enterobacter cloacae sp. respectively. These isolates were found resistant to heavy metals such as Ni^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+} at different concentrations ranging from 0.05 mM to 12 mM. The trend for tolerance of heavy metals by isolates was as follows: E-2:- Ni = Cu >Pb> Zn > Hg, E-7: Ni > Cu = Zn >Pb> Hg, E-9:- Pb = Ni > Cu = Zn > Hg and E-10:- Zn > Ni > Cu >Pb> Hg. The Anti-biogram pattern indicates that all isolates were showing resistance against more than five antibiotics and all are designated as multi-drugs resistant bacteria. These bacteria can be explored for bio-absorption of heavy metals from contaminated sites.

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Introduction

The increase in industrialization, urbanization, and population growth has been progressively increasing environment contaminants. The toxic heavy metals such as lead, copper, zinc, nickel, arsenic, mercury, and cadmium are polluting the environment by industrial solid and liquid wastes, which are generated by various industrial processes such as electroplating, leather tanning, wood preservatives, paper and pulp processing, steel manufacturing, etc.(Rani *et al.*, 2010; Ahemad, 2012). In soil, heavy metals occur naturally at low concentration. These metals are non-degradable and persist in the environment to toxic level at higher concentration (Chopra *et al.*, 2009).

In India, Sugar industry is one of the important agro-based industries and takes second position after textile industry. It plays major role in contribution to country's economy. Besides this, sugar industries also release the effluents with high organic and inorganic waste, and contaminate the receiving sites and possess serious health hazards. (Kumar and Chopra 2010; Saranraj and Stella 2014). The microflora of the soil is sensitive to toxic heavy metals present in the environment. The high concentration of antibiotics and heavy metals in industrial wastes can develop a stress in the soil environment that can lead to mutations in microorganisms which allow them to better survival and multiplications in the changed environment. Due to climate variations and stressed condition, various mechanisms are induced in bacteria like metal sorption, complexation, mineralization, extracellular precipitation and enzymatic oxidation resulting into less toxic form for adaptation in the metal-stressed condition by various transporters and become metal resistant (Nies D.H, 1999; Rajbanshi, 2008; Nanda *et al.*, 2011; Rajkumar *et al.*, 2012; Hookoom and Puchooa, 2013 and Silver, 1996). Microbes interact with metals at great extent and are capable of metal adsorption of cations on the negatively charged cell surface by various forces and interaction (Rajendran *et al.*, 2003). These heavy metal-tolerant bacteria may play significant role in bioremediation. Bioremediation is natural, eco-friendly, and cheaper technology that uses microorganism to degrade or remove toxic pollutants from the environment. Though, this technology is slow and takes longer time but it is better than the conventional chemical process and is also able to retain soil fertility (Wu *et al.*, 2010; Ahemad, 2012, Sanaraj and Stella, 2012).Several studies have been reported for isolation of drug- and metal- resistant bacteria from various polluted sites, but, there are limited reports in reference to sugar mill contaminated sites. In this study we focused on exploring the bacteria of the sites contaminated with sugar mill effluents and find out the potential of the bacteria against selected heavy metals and antibiotics.

Materials and methods

Sampling

Soil sample was collected from the site near the sugar mill industry situated at Iqbalpur, Roorkee; District Haridwar. The geographic coordinates of the sampling site were 29°52'25"N 77°47'40"E. Samples were collected From a depth of 5-10 cm from the surface of the soil in sterile polythene bags and were tightly packed. They were transferred to the laboratory and stored at 4°C for further analysis.

Isolation and Screening of microorganism

For the isolation of heavy-metal-resistant bacteria the soil sample was serially diluted in sterile distilled water and plated on the nutrient agar medium supplemented with 0.1mM of heavy metals such as Cu^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, and 0.01mM µg/ml Hg^{2+} as $CuSO_4$, $NiCl_2$. $6H_2O$, $Pb(NO_3)$, $ZnSO_4$, and $HgCl_2$. The 1M of stock solutions of heavy metals were prepared in double distilled water and sterilized by autoclaving at 121°C, 15psi for 15 minutes. The plates were incubated at 35°C for 48h to screen the resistant colonies. The large and distinct colonies were separated and preserved by sub-culturing in fresh media for further investigations.

Identification and Characterization of the bacterial isolates

Bacterial isolates were characterized on the basis of morphological, physiological, and biochemical

characteristics. These bacteria were identified in accordance with Bergey's Manual of Determinative Bacteriology (Claus and Berkeley, 1968) and were further studied for heavy metal and antibiotic tolerance studies.

Optimization of growth conditions

The optimal growth conditions were determined with reference to pH and temperature in the absence and presence of heavy metals in Nutrient agar broth. The pH range was varied from 4 to 9 and temperature from 25°C to 40°C. The flasks were inoculated with 0.5ml of overnight culture and incubated at different temperature in an orbital shaker at 120 rpm. After 24h incubation, bacterial growth was monitored by measuring the absorbance at 600nm using spectrophotometer (Model–Evolution 201 UV-visible &Fluorescence spectrophotometer) to determine the optimum growth. Experiment was performed in triplicates (Raja *et al.*, 2006).

Determination of MIC

The MIC of five heavy metals (Cu^{2+}, Ni^{2+},Pb^{2+},Zn^{2+}& Hg^{2+}) was determined for the isolated strains using Mueller-Hinton agar containing each heavy metal in concentration ranging from 0.01 mM to 12 mM. The concentration of respective heavy metal was raised in agar plate until the strain failed to grow on the plate. The plates were incubated at 35°C for 24-48h and bacterial growth was observed to evaluate MIC (Raja *et al.*, 2006).

Determination of multi-heavy metal resistant bacteria

On the basis of higher degree of resistance, bacteria were tested against mixture of heavy metals (Cu^{2+}, Ni^{2+}, Pb^{2+}& Zn^{2+}) at concentration of 1mM.The metals were supplemented in 25 ml Muller Hinton broth, inoculated with overnight grown culture of each bacterium. The cultures were incubated at 35°Cfor 24h in orbital shaker at 120 rpm. The bacterial growth was observed by spectro-photometer at 600nm.

Determination of antibiotic sensitivity and resistances

Antibiotic sensitivity of the isolated heavy metal resistance bacteria was determined according to the disc diffusion method (Bauer *et al.* 1996). Fifteen antibiotics (Hi-Media disc 6mm) belonging to eleven classes were placed on newly prepared lawns of each isolates on Muller-Hinton agar and incubated at 35°C for 24h. After incubation, the plates were observed and the diameter of the inhibition zone around the disc was measured. The sensitivity pattern of the isolates was observed according to zone size interpretative chart of Hi Media laboratories Pvt. Ltd. The following antibiotics were used Aminoglycosides (Amikacin AK, 30µg, Streptomycin S, 10 µg and 25µg), Penicillin (Amplicillin AMP, 2 µg), Cephems (Cefotaxime CTX, 10µg, Ceftazidime CTZ, 30 µg,CeftriaxoneCTR,30 µg), Phenicols (Chloramphenicol C,30 µg), Linocosamides (ClindamycinCD,2 µg), Macrolides (Erythromycin E,10 µg), Quinolones (Levofloxacin LE,5µg), Penems (Meropenem MRP, 10µg), Fluroquinolone (Norfloxacin NX, 10µg), Lipopeptides (Polymyxin-BPB,50µg) and Tetracyclines (TetracyclinTE,30µg).

16S rDNA gene Amplification, Nucleotide sequencing and Alignments

Bacterial genomic DNA was isolated using gene O-spin Microbial DNA isolation kit (Mak and Ho, 1991). The primer pair Bacterial 16S region gene was amplified using the standard PCR reaction. The primer pair, 27F forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R reverse primer (5' – TACCTTGTTACGACTT-3') was used in a PCR reaction (Frank *et al.*, 2008). The initial duration for 10 min at 95°C, there were 35 cycles consisting of denaturation at 94°C for 1 minute, and extension at 72°C for 2min, and final extension step consisting of 10 min at 72°C. After completion of PCR, the PCR products were checked on 1%agarose by agarose gel electrophoresis and spiked with ethidium bromide (0.5µg/ml) in 0.5X TBE buffer. Bands were detected under a UV Trans-illuminator. The purified PCR amplicon was sequenced using the gene specific sequencing primers (27F) and ABI Big Dye Terminator v3.1 Cycle Sequencing reaction kit

(Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at BlastN site at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) to compare the sequence with previously published bacterial 16S rDNA sequence in NCBI database. The 16S rDNA sequences of isolates have been deposited in GenBank by using BankIt service.

Statistical analysis

All the experiments were analyzed in triplicate, unless otherwise stated. The data in the tables and figures represent the mean, with all error bars shown (Mean ± 1 standard error of mean) using the statistical package on Microsoft® Excel Version 2007.

Results and discussion

The soil contaminated with sugar industry effluent influences the microflora of the site. Resistance to heavy metals and antibiotics is induced under stressed environment. In this study eight bacteria were isolated from sugar mill effluent contaminated soil sample in the initial screening process. For further studies, four strains E-2, E-7, E-9, and E-10 were selected based on high degree of heavy metal and antibiotic tolerance. Among them, E-2 and E-10 strains were gram positive rod-shaped bacteria while E-7and E-9 strains were gram negative rod-shaped bacteria. On the basis of morphological, biochemical characterizations (Table 1) of isolates and comparative analysis of sequence with database of NCBI it can be conclude that E-2 and E-10 are the members of *Bacillus licheniformis* sp., and *Teribacillus aidingensis,* with 99 % and 100% similarities respectively.

Table 1. Morphological, Physiological and Biochemical properties of bacterial isolates from contaminated soil.

Morphological/Physiological/Biochemical Characteristics	ECS-2	ECS-7	ECS-9	ECS-10
Gram stain	+	-	-	+
Cell shape	Rod	Rod	Rod	Rod
Colony colour	White	Yellow	Cream	White
Culture characteristic on Agar	Rough, Irregular	Smooth, Round	Soft, smooth	Small, circular
Growth at temperature 35°C	+	+	+	+
Growth at pH 7	+	+	+	+
Indole Test	-	-	-	+
Catalase Test	+	+	+	-
Citrate Test	+	-	+	+
Oxidase Test	-	+	-	+
MR- Test	+	-	-	+
VP-Test	-	-	+	-
H_2S Test	-	-	-	-
Motility	-	-	+	-
Starch hydrolysis Test	+	+	+	-
Gelatine liquefaction	-	+	-	-
Fermentation (Triple Sugar Iron Agar) TSI Test Slant/But	K/A	K/K	K/A	A/A
Glucose	+	-	+	+
Lactose	-	-	-	+
Sucrose	-	-	-	+
Gas production	-	-	+	+

Note: + positive; - negative, A= acidic, K= alkaline.

The names of these strains were designated as *Bacillus licheniformis* strain UTUE2, and *Teribacillus aidingensis* strain SPE10. On the other hand E-7 and E-9 is the close members of *Chryseobacterium indologenes* and *Enterobacter cloacae,* respectively, with 99%maximum similarities. The names of these strains were designated as *C.indologenes* strain SSE-7 and *E.*cloacae strain PRE9. The phylogenetic trees are shown in (Fig. 1A-D).

The 16S rDNA sequence of E-2, E-7, E-9 and E-10 were submitted in the NCBI GenBank under

accession number KT251206, KR014101, KR028480 and KR057920, respectively (Table-2). *B. licheniformis*UTUE2 is gram positive, catalase, citrate, starch hydrolysis and MR positive with endospore forming bacteria isolates from soil. Sneath *et al.*, 1986 described about the characteristic of *B. licheniformis* which never reported to be pathogenic for either animals or plants.

Table 2. The percentage of maximum similarity and GenBank accession number of isolates.

Isolates	Organism	Identify (in %)	Accession No.
E-2	*Bacillus licheniformis* strain UTUE2	99% similarity to *Bacillus licheniformis* ATCC 14580, complete genome	KT251206
E-7	*Chryseobacterium indologenes* strain SSE-7	99% similarity to *Chryseobacterium indologenes* strain 100	KR014 101
E-9	*E. cloacae* strain PRE9	99% similarity to *Enterobacter cloacae* strain GGT036	KR028 480
E-10	*Teribacillus aidingensis* strain SPE10	100% *similarity Terribacillus aidingensis* strain MP602, complete genome	KR057920

Table 3. Optimum pH and temperature of isolates in the presence of heavy metals.

Isolates	Copper	Nickel	Lead	Zinc	Mercury
E-2	8[a]	4	5	5	5
	40[b]	35	35	40	35
	1.184 ±0.052[c]	1.101±0.017	1.107±0.021	1.234±0.033	1.136±0.020
E-7	5	5	5	5	5
	30	35	35	35	35
	1.282±0.048	.109±0.019	1.019±0.004	0.991±0.013	0.987±0.012
E-9	6	6	5	5	7
	25	35	25	25-30	40
	1.361±0.054	0.970±0.005	1.056±0.010	0.984±0.013	0.921±0.015
E-10	6	6	8	5	6
	35	35	30	35	35
	1.153±0.004	0.944±0.008	1.080±0.007	0.998±0.047	0.825±0.008

Note: a= pH, b= Temperature in ^{0}C , c= Optical density value at 600nm (O.D Mean ± Standard).

The morphological and biochemical characteristics of *C. indologenes* SSE-7 are similar to the observation of Vandamme *et al.*, (1994) who studied the characteristics of the genus *Chryseobacterium* and their allied bacteria. The species of *Chryseobacterium* are widely distributed in soil, water, and clinical sources, and they are gram negative, aerobic, non-spore forming rod, colonies are translucent-opaque, circular, convex, smooth, yellow colour and shiny with entire edges. Biochemically, *Chryseobacterium* is catalase, oxidase, indole, and gelatinase positive. The isolate *E. cloacae* PRE9 is gram negative rod, circular, soft, and cream-colored colony on medium. It is motile, gas producing bacteria which are catalase, citrate, VP positive but indole, oxidase, and MR negative. Stiles *et al.*, (1981) isolated *Enterobacteriaceae* (86%) from different meat samples. The percentages of positive biochemical and identifying characteristics of seven member of *Enterobacteriaceae* were described. Among all, characteristic of *E. cloacae* was (89.2%) were positive for motility, 95.2% for VP, (92.2%) for citrate, (100%) were positive for acid and (98.8%) for gas production from glucose.

The optimum temperature and pH for E-2 and E-10 in absence of heavy metals were 35^{o}C, pH 7 while for E-7and E-9 in absence of heavy metals were 35^{o}C, pH

7 and 25-30°C, pH 5-6 respectively. Metal uptake mechanisms depend on various factors such as initial metal's concentration, chemistry of heavy metal, temperature, pH, redox potential of metals, and availability of metals in medium. Mostly, microorganisms prefer neutral pH 7 for binding metal cations species on their negatively charged surface, but it is not true for all bacteria species because many bacteria can survive in extreme pH conditions and also tolerate heavy metal concentration by generating various mechanisms Monachese *et al.*, (2012). The bacterial growth in metal containing broth at different pH and temperature was analyzed spectrophotometrically at 600 nm. It was observed that all isolate preferred low pH and temperature range 30-40°C for all selected heavy metals (Table 3). These findings resemble with the study of Suriya J *et al.*, (2013) they reported that *E.cloacae* AB6 uptake metals ions at pH 4-5, and at alkaline pH, the availability of metals ions was decreased. At low pH, metal exist as free ions, but in alkaline pH, the ions precipitate as insoluble hydroxides. Bacteria isolated from soil contaminated with sugar mill effluent showed high degree of resistance against five heavy metals ranging from 0.05mM to 12 mM (Table 4).

Table 4. MIC values of soil isolates against heavy metals.

Heavy Metals	E-2	E-7	E-9	E-10
Copper	6	6	7	5.5
Zinc	4.5	6	7	12
Lead	5.5	5.5	7.5	5
Nickel	6	7.5	7.5	7
Mercury	0.07	0.05	0.1	0.1

Note: Heavy metal concentration in mM (millimolar).

Table 5. Antibiotic sensitivity test of metal resistant bacteria.

Antibiotics (Disc potency)	E-2	E-7	E-9	E-10
Meropenem MRP10	31(S)	23(S)	20(I)	14(R)
Cefotaxime CTX10	N.Z	08(R)	17(R)	12(R)
Ceftazidime CAZ30	N.Z	11(R)	13(R)	11(R)
Ceftriaxone CTR30	N.Z	14(R)	19(R)	17(R)
Tetracyclin TE30	28(S)	15(S)	14(I)	13(I)
Amikacin AK30	19(S)	16(I)	15(I)	13(R)
Levofloxacin LE5	31(S)	24(S)	16(I)	13(R)
Ampicillin AMP2	6(R)	N.Z	N.Z	N.Z
Chloramphenicol C30	N.Z	19(S)	20(S)	16(I)
Erythromycin E10	N.Z	15(I)	N.Z	N.Z
Clindamycin CD2	15(I)	21(S)	N.Z	07(R)
Polymyxin-B PB50	N.Z	N.Z	07(R)	N.Z
Norfloxacin NX10	26(S)	19(S)	18(S)	27(S)
Sterptomycin S 25	16(S)	18(S)	13(I)	13(I)
Streptomycin S 10	14(I)	15(S)	12(I)	11(R)

Note-: S= Sensitive, I= Intermediate, R= Resistance & N.Z= No Zone.

E-2 showed highest MIC against Nickel and copper and E-10 against Zinc. The gram negative bacteria E-7 and E-9 showed highest MIC against Nickel. The resistance patterns of isolates were as follows: E-2:- Ni = Cu >Pb> Zn > Hg; E-10:-Zn>Ni>Cu>Pb>Hg; E-7:- Ni > Cu = Zn >Pb> Hg and E-9, Pb = Ni > Cu = Zn > Hg.

The highest ability of metal tolerance in mixture of metals solution was showed by E-9 bacteria which could be due to the presence of large set of gene and plasmid as compared to other isolates (Fig. 2).

Bacillus species are the most studied bacteria in term of high tolerance to heavy metal toxicity (Ince-Yilmaz 2003). Gupta *et al.,* (2014) were reported about four species of Bacillus species (*B.carotarum*, *B.cereus*, *B. lentus* and *B. licheniformis*) from sewage water. They studied that bacillus sp. can tolerate wide range of metals (lead, chromium and zinc) and antibiotics. No study has been available in our knowledge to describe the metal tolerance capability of *C. indologenes* and *T. aidingensis* species.

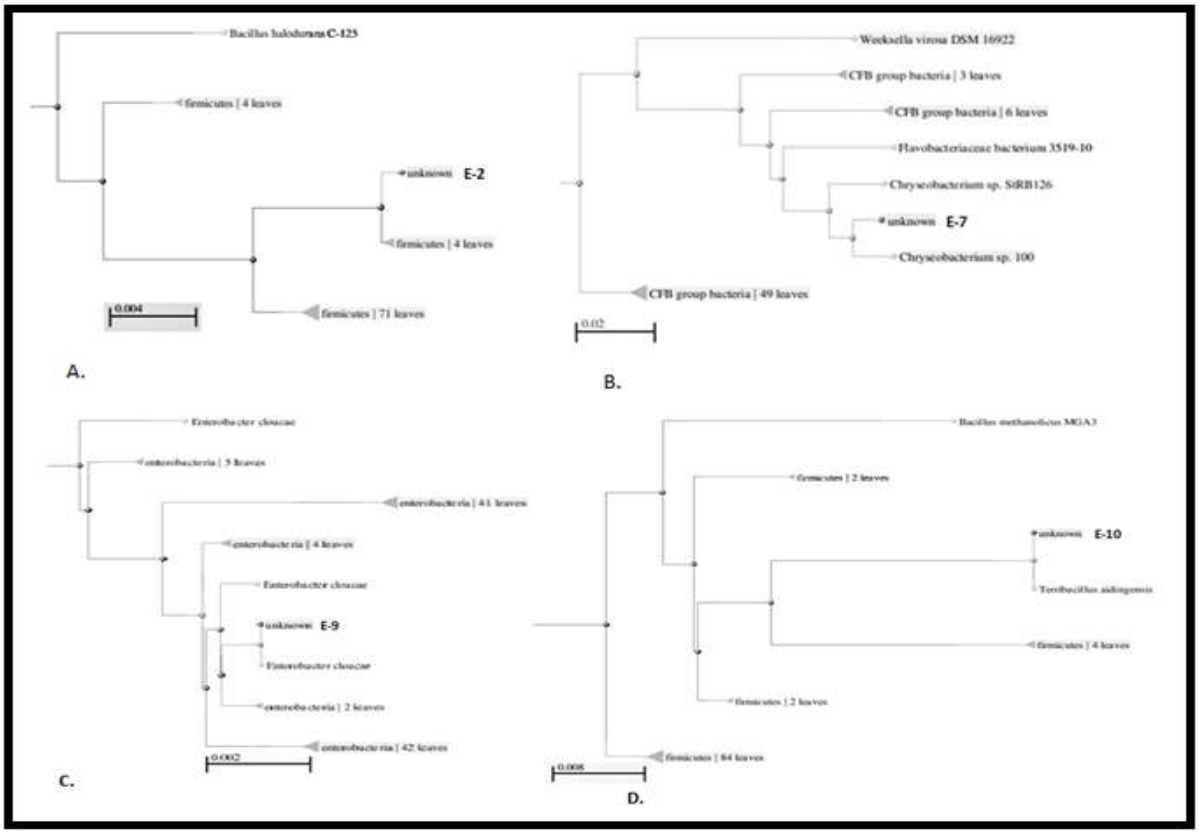

Fig. 1(A-D). Phylogenetic analysis of 16 rDNA gene sequences of isolates E2, E-7, E9 and E-9 by Neighbour joining method.

All heavy metal resistant isolates were tested against selected fifteen antibiotics. They were found to be multi drug resistant (Table 5). Matyar F. (2012) and Pontes *et al.*, (2009)were reported that bacteria resistant to four or more antibiotics were designated as MDR (Multi Drug Resistant). E2 was resistant against seven antibiotics such as ampicillin, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, Erythromycin and polymixin-B, sensitive against Meropenem, tetracyclin, amikacin, levofloxacin, norfloxacin and streptomycin 25, and intermediate response to rest of others.Veith *et al.*, (2004) studied the genome of *B. licheniformis* and reported that bacteria contain genes which secrete exoenzymes protease. This bacterium is used for large scale industrial production of protein. Samata *et al.*, (2012) reported that bacillus species were resistant against kanamycin, ampicillin and methicillin and also tolerate wide range of heavy metals. E-10 exhibited resistance against eleven antibiotics such as Meropenem, ampicillin, amikacin, cefotaxime, ceftazidime, ceftriaxone, clindamycin, erythromycin, streptomycin 25 and polymixin-B, sensitive against norfloxacin and intermediate to rest of others.E-7was resistant against five antibiotics such as ampicillin, cefotaxime, ceftazidime, ceftriaxone and polymixin-B, sensitive against Meropenem, tetracyclin, levofloxacin, clindamycin, chloramphenicol, norfloxacin and streptomycin10, 25 and intermediate response to rest of others. Similar results reported by Zeba *et al.*, (2005, 2009) identified *Chryseobacterium indologens* 597 from

patient's urine sample which showed yellow colonies on plate or in liquid culture. These bacteria produced metallo β-lactamase (MBL) enzyme which is capable to hydrolyse most common β-lactum antibiotics including benzylpenicillin, ampicillin, amoxicillin, cefalotin, cefotaxime, cefuroxime and imipenem except ceftazidime and second generation cephalosporine. *C. indologenes* SSE-7 growth was inhibited by Levofloxacin and Norfloxacin which are members of quinolones antibiotic class.

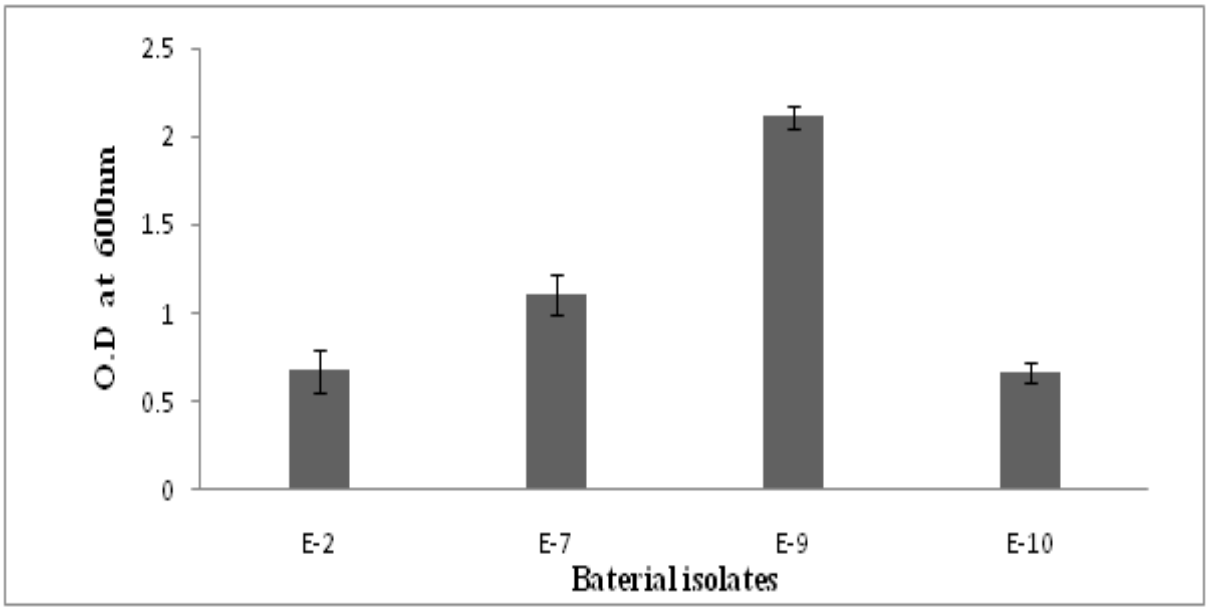

Fig. 2. Growth of isolates bacteria in mixture of heavy metal.

These results confirmed by the study of Kirby *et al*., (2004) reported that new quinolones (garenoxacin, gatifloxacin & levofloxacin) are the most effective antimicrobial agents to treat infections caused by *chryseobacterium sp*. E9 species were reported as heavy metal-and antibiotics resistant bacteria. It exhibited resistance against seven antibiotics such as ampicillin, cefotaxime, ceftazidime, ceftriaxone, clindamycin, erythromycin, and polymixin-B, sensitive against chloramphenicol and norfloxacin and intermediate to rest of others. These properties agree to the findings of Deeb (2009) who had reported the resistant property of endophytic *Enterobacter BN4* species. The bacterium was tolerant to heavy metal in the order: Pb(6mM)>Zn(5mM)>Cd(3mM) and also resistant to tetracycline, kanamycin and ampicillin. Ren *et al*., (2010) reported *Enterobacter* species to be opportunistic pathogen to humans and causative organism for nosocomical infections carry seven operons with heavy metal resistant gene which probably made it possible for them to survive in heavy metal rich environment. Kocis & Szabo (2013) described that the *Enterobacteriacea* protect them by secreting various enzymes for inactivating antibiotics, modifications in their targeting molecules, and use of antibiotics efflux pump systems.

The resistance order of isolates against antibiotics was E-10 > E-9 = E-2 > E-7.In most of the studies, it was founded that metal resistance has been associated with antibiotics resistance (Verma *et al*., 2001; Raja *et al*., 2006) and assumed that the resistance gene to both heavy metals and antibiotics may probably be present closely on same plasmid in bacteria and is more likely to be transferred to other bacteria. Austin *et al*. (2006) had given the concept of co-selection of the resistance mechanism among bacteria and the two environments: agricultural and clinical, in which the proliferation of resistance is of main concern. The resistance of soil bacteria to trace metals and antibiotics is associated with a mechanism of exchange of plasmids. The ability of heavy metal and antibiotic resistance in microorganisms helps

them to adapt faster under stress condition by mutation and natural selection (Bhattacherjee *et al.,* 1988; Silver & Mishra 1988).

Conclusion

In this study, it is revealed that sugar mill effluent-contaminated soil contains bacteria which are resistant to heavy metals and antibiotics. The presence of metal- and antibiotic-resistant properties in bacteria is due to transfer or exchange of the resistant gene. In the present study four different strains B. *Licheniformis* UTUE2, *C.indologenes* SSE-7, *E. cloacae* PRE9 and *T.aidingensis* SPE10 were found to withstand the high concentration of heavy metals and have MDR property. Hence, these bacteria are clinically important and can be employed for bioremediation which is a green and eco-friendly approach to cleanup heavy metal contaminated sites by contaminants like Zinc, Lead, Nickel, Copper and Mercury.

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