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Physico-Chemical and Microbiological Profile of Effluents from Common Effluent Treatment Plants (CETPs)

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ABSTRACT

Metal remediation through conventional technologies in Common Effluent Treatment Plants (CETPs) is often inefficient or very expensive, whereas, bioremediation by using heavy metal resistant microorganisms has received a great deal of attention for its potential application in industry. The present study was envisaged to check the physico-chemical properties of effluents being treated at CETPs and to isolate indigenous heavy metal tolerant bacteria for their subsequent utilization for heavy metal uptake. Effluent samples were collected from two CETPs located in Ludhiana and Jalandhar and were analyzed for their physico-chemical parameters (dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD) and pH) and heavy metal profile using Inductively Coupled Argon Plasma-Emission Spectroscopy (ICAP-AES). In both the samples Cr was found to be the dominant metal contaminant. Out of twenty one bacterial isolates obtained from effluent samples, ten morphologically distinct isolates were tested for minimum inhibitory concentration (MIC) against six different heavy metals *i.e.* As, Cr, Hg, Ni, Pb and W by supplementing the Luria Bertani (LB) agar media with increasing dose of heavy metal salts (5-100 ppm). Four isolates (HM 2, HM 3, HM 15 and HM 16) showed maximum tolerance to selected five different heavy metals except mercury (Hg). These isolates were biochemically characterized, whereby, HM 3 and HM 16 were closely related to *Enterobacter* and HM 2 was found to be related to *Klebsiella*. Optimum growth of selected isolates was obtained at pH 9.0 and temperature 40°C. Of these four isolates, only HM 2 and HM 16 showed comparable growth rate and in-vitro compatibility and were selected as potent candidate for consortium.

Keywords

Common Effluent Treatment Plants, bioremediation, heavy metal tolerant, ICAP-AES.

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Introduction

Due to rapid industrial progress and urbanization, environmental pollution with toxic heavy metals has become one of the major concerns in today's world (Kinare and Shingadia 2014). Environmental pollution due to chemicals and heavy metals is spreading throughout the world and is a problem that may have negative consequences on the hydrosphere. Metal contaminated aquifers pose serious threat to health and ecosystems.

Copper, chromium, cadmium and nickel are known to be the most commonly used heavy metals and are the more widespread contaminants of the environment (Aksu 1998, Doenmez and Aksu 1999). Some heavy metals are essential in trace amount, but high levels of them may cause extreme toxicity to living organisms due to inhibition of metabolic reactions. These are considered to be more persistent and stable than organic

contaminants such as pesticides or petroleum by-products and are non-biodegradable (Lasat 2002, Kavamura and Esposito 2010). Their presence in soils and water can be from natural (Ernst 1998) or anthropogenic origins (Alloway 1995).

Removal of excesses of heavy metal ions from wastewaters is essential due to their extreme toxicity towards aquatic life and humans. These metals can become mobile in soils depending on soil pH and their speciation and finally a fraction of the total mass can leach to aquifer or can become bioavailable to living organisms (Santona *et al.*, 2006, Khan *et al.*, 2008). Heavy metals can accumulate in biological systems and ultimately be introduced into food web via different mechanisms (Giller *et al.*, 1998).

It is well known that heavy metals can be extremely toxic as they damage nerves, liver and bones, and also block functional groups of vital enzymes (Moore 1990, Ewan and Pamphlett 1996). Heavy metal contamination thus considered a serious threat to both the ecosystem and human and requires expensive clean-up costs. Inhibitory effect of heavy metals is a common phenomenon that occurs in the biological treatment of waste water and sewage (Filali *et al.*, 2000, Kongtae *et al.*, 2013).

The bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its potential application in industry. Though the applications of genetically engineered microorganisms (GEMs) in bioremediation have received a great deal of attention, As GEMs have higher degradative capacity and have been demonstrated successfully for the degradation of various pollutants under defined conditions. However, ecological/environmental concerns and

regulatory constraints are major obstacles for testing GEMs (Menn *et al.*, 2008).

Punjab is a hub of small and medium-scale industries. For minimizing environmental pollution by these industries a cleaner production technology and waste minimization has to be reinforced. A collective treatment of this waste at a centralized facility known as Common Effluent Treatment Plants (CETPs) can go a long way in minimizing the constraints associated with effluent treatment in small to medium enterprises. Till 1990, only one CETP at Jeedimetla, Hyderabad was in operation. But in 1991, Ministry of Environment and Forests (MoEF), GoI initiated an innovative financial support scheme for CETPs to ensure the growth of the small and medium entrepreneurs (SMEs) in an environmentally compatible manner (Anonymous, 2010). The level and type of metal contaminants in the effluents collected from a CETP depend upon the type of industries it is catering for. Major sources of chromium pollution include effluents from leather tanning, chromium electroplating, wood preservation, alloy preparation and nuclear wastes (Thacker *et al.*, 2006). The CETPs selected in this study include the one dealing with electroplating industry waste and other with the tannery effluents.

Metal remediation in these CETPs involve the use of conventional technologies, such as ion exchange, chemical precipitation, reverse osmosis and evaporative recovery which are often inefficient or very expensive (Volesky 1990). Compared with the above methods the removal of heavy metals with indigenous microorganisms has advantages such as low cost and without secondary pollution (Jun-xia *et al.*, 2007) and biosorption is an ideal candidate for the treatment of high volume and low concentration complex wastewaters (Wang and Chen 2006). There is a need for

innovative treatment technologies for the removal of heavy metal ions from wastewater. Different microbes have been proposed to be efficient and economic alternative in removal of heavy metals from water (Waisberg *et al.*, 2003). Bioremediation can be used to effectively reduce contaminant toxicity, mobility or volume to levels that are innocuous to human health and ecosystem (Toroglu and Dincer 2009).

Studies on bacterial diversity in heavy metal contaminated sites have demonstrated the presence of a variety of strains (Ellis *et al.*, 2003). These indigenous organisms have not only adapted to the new environments but have also flourished under them (Haq and Shakoori 2000, Roane and Pepper 2000). So exploiting these organisms can be advantageous over the introduction of some foreign/alien microbial culture. The survival of the newly introduced bacterial species under the conditions existing at contaminated sites may be doubtful while intrinsic flora or resident microbes can be far well acclimatized and can have better survival rate and faster growth. Application of a judicious consortium of growing metal-resistant cells can ensure better removal through a combination of bioprecipitation, biosorption and continuous metabolic uptake of metals after physical adsorption.

Recent studies have shown that the strains (bacteria, yeast and fungi) isolated from contaminated sites possess excellent capability of metal scavenging. The multiple metal resistance is often correlated with antibiotic resistance. Some bacterial strains, possessing high tolerance to various metals and antibiotic, may be the potential candidates for metal clean-up from wastes. This work mainly focuses on the isolation of heavy metal resistant strains of bacteria for their applicability in treatment of metal-rich effluents.

Study area

Samples were collected from Ludhiana Electroplaters Association CETP, Focal Point, Ludhiana and Punjab Small Industries and Export Cooperation (PSIEC), Leather Complex CETP, Kapoorthala road, Jalandhar, Punjab. Samples were collected in sterile plastic container.

Materials and Methods

Analysis of physico-chemical parameters

The collected samples were analyzed for normal physico-chemical parameters such as pH, dissolved oxygen (DO), biological oxygen demand (BOD) and chemical oxygen demand (COD) within 12 hours of collection using standard methods (APHA 2001). For determination of pH of the samples a pH meter (S.D. fine-chem limited) was used.

Heavy metal profile of effluent

The concentration of heavy metals such as arsenic, chromium, mercury, nickel, lead etc. present in both the effluent samples was estimated using Inductively Coupled Argon Plasma-Atomic Emission Spectroscopy (ICAP-AES) (Thompson and Walsh 1989).

Isolation and maintenance of bacterial isolates

Isolation of indigenous metal resistant bacteria was carried out using standard microbiological techniques by which Luria Bertani Agar plates supplemented with 5mg/l concentration of six different heavy metals i.e. As, Cr, Hg, Ni, Pb and W were used. The plates were incubated at 37°C for 3-5 days. Individual bacterial colonies on LB agar plates which varied in shape and color were picked up and purified by repeated subculturing on the same medium. Pure

cultures of bacterial colonies were preserved at 4°C as slant cultures for further analysis.

Determination of Minimum Inhibitory Concentration (MIC) of different selected heavy metals

Maximum resistance of the isolates to six heavy metals (As, Cr, Hg, Pb, Ni and W) was evaluated in LB agar plates amended with each heavy metal in concentration ranging from 5ppm to 100ppm.

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 37°C for 24-48 h and bacterial growth was observed to evaluate MIC.

Biochemical characterization of selected bacterial isolates

Biochemical characterization of bacterial isolates was done on the basis of oxidase, catalase, VP-test, MR-VP test, starch hydrolysis and gelatin hydrolysis, indole production and citrate utilization as per Bergey's Manual of Systematic Bacteriology (Claus and Berkeley 1986).

Optimization of growth parameters for selected isolates

Effect of pH on the growth of selected isolates

Optimization of the growth of the four selected isolates (HM 2, HM 4, HM 15 and HM 16) with reference to pH was done. For each of these bacterial strains, 250ml flasks containing 50ml of Luria Bertani broth were adjusted at different pH values i.e. 6, 7, 8, 9 and 10 and autoclaved at 120°C for 20 min. After autoclaving, the broth was inoculated with 0.5 ml of 12 h old culture of selected isolates. The optical density of log phase

growing cultures (8-10h old) was measured at 540 nm.

Effect of temperature on the growth of selected isolates

Optimization of the growth of the four selected isolates (HM 2, HM 4, HM 15 and 16) with reference to temperature was done. For each of these bacterial strains, 250ml flasks containing 50ml of Luria Bertani broth were autoclaved at 120°C for 20 min. After autoclaving, the flasks were inoculated with 0.5 ml of 12 h old culture of selected isolates and incubated at different temperatures i.e. 25°C, 30°C, 35°C, 45°C and 50°C. The optical density of log phase growing cultures (8-10h old) was measured at 540 nm.

Results and Discussion

In Punjab, a total of six CETPs are located (Table 1). Out of six CETPs, only two were working and samples were collected from these two CETPS i.e. *Ludhiana Electroplaters Association* CETP, Ludhiana and *Punjab Small Industries and Export Corporation* (PSIEC) Leather complex CETP, Jalandhar. Samples inoculated in media supplemented with 5 mg/l of different heavy metals led to the isolation of total of 21 isolates, out of which only 10 were found to be morphologically distinct from each other.

Physico-chemical characterization of effluent samples collected from common effluent treatment plants CETP

Results revealed that sample 1 was found to be highly acidic (pH=2.5) because this CETP caters the need of electroplating industries, whereas sample 2 was alkaline (pH=9.0) because it caters the need of tannery industry (Table 2). The BOD of sample 1 (17.2 mg/l) was less as compared to sample 2 (69.8 mg/l), but the COD of sample 1 (390 mg/l) was

more as compared to sample 2 (372 mg/l), these results revealed that the sample 2 was rich in organic matter content with lesser load of heavy metal contents. Earlier also Alam *et al.*, (2011) also reported a high BOD of the tannery effluent irrigated soil and the level of organic matter of tannery effluent irrigated soil was found higher than the control soil. Kamika and Momba (2013) reported that the average value of COD of industrial wastewater effluent was higher than 100 mg/l and the value of DO between 5.76 to 6.81 mg/l.

Comparative heavy metal profile of effluents from CETPs

The heavy metal profile of both the effluent samples was determined by using Inductively Coupled Argon Plasma-Emission Spectroscopy (ICAP) analysis. Whereby, Cr was found to be the dominant metal contaminant with a concentration of 238 ppm, followed by nickel-92 ppm, lead-18.5 ppm, cadmium-0.3 ppm, arsenic-0.04 ppm in sample 1. Similarly, in sample 2 concentration of Cr was 23.20 ppm followed by Pb (20.61 ppm) and Ni (6.65 ppm). Level of heavy metal contaminants in sample 1 was higher in comparison to sample 2 and was above permissible limits in both the samples.

The concentration of Cr was 2380 times higher than the permissible limit (0.1 ppm) in sample 1. This is in accordance with the earlier study by Verma *et al.*, (2001), who analysed tannery effluents for the content of the various heavy metals and found that the total chromium (28.96 ppm) and nickel concentrations (1.08 ppm) in the effluent exceeds the permissible limits (Table 3).

Morphological characterization of isolated strains

A total of 21 strains were isolated, 10 were found to be Gram -ve and 11 were Gram +ve. These isolates were morphologically characterized, 10 isolates HM 1, HM 2, HM 3, HM 5, HM 8, HM 9, HM 12, HM 14, HM 15 and HM 16 were found to be morphologically distinct. These 10 isolates were selected for determination of Minimum Inhibitory Concentration (MIC) against six different heavy metals i.e. As, Hg, Ni, W, Pb and Cr. Table 4 revealed the colony morphology and the Gram’s character. Several investigators have reported high level of metal resistance in bacteria isolated from wastewater. Shakoori and Muneer (2002) isolated bacteria from wastewater and found that all isolates were resistant to Cr, Cd, Hg and Zn.

Table.1 Location and address of common effluent treatment plants (CETPs) located in Punjab

S.No.	Name	Address	Remarks
1.	Punjab Small Industries and Export Corporation (PSIEC) Leather Complex CETP	Kapoorthala Road. Jalandhar, Punjab	Currently under stabilization as it was closed for over 2 years
2.	Malerkotla Electroplating Association CETP	Malerkotla, Distt. Sangrur, Punjab	Yet not working
3.	Ludhiana Electroplaters Association CETP	D-260-261, Focal Point Phase-8, Ludhiana	Capacity to cater to nearly 900 units
4.	Dr. Ambedkar Leather Association CETP	Ramdaspora Noormahal Road, Phillore, Distt. Jalandhar, Punjab	Yet not working
5.	Textile units CETP	Tajpur Road, Ludhiana	Work was stalled due to a very high project cost
6.	Textile units CETP	Bahdurke Road, Ludhiana	CETP Project was delayed and not yet started

Table.2 Comparative physico-chemical parameters of effluent samples collected from CETPs

Sample no. and location	pH	DO mg/l	BOD mg/l	COD mg/l
1. Ludhiana Electroplaters Association CETP	2.5±0.3	47.8±0.5	17.2±0.2	390±0.4
1. Punjab Small Industries and Export Corporation (PSIEC) Leather Complex CETP	9±0.2	90.3±0.3	69.8±0.8	372±0.5

Table.3 Comparative heavy metal profile of effluents from CETPs

S.No	Metals	Sample no. 1	Sample no. 2	Permissible limit
Concentration (ppm)				
1.	Arsenic (As)	0.04	N.D*	0.2
2.	Boron (B)	24.8	N.D	N.D
3.	Calcium (Ca)	183	N.D	N.D
4.	Cadmium (Cd)	0.30	0.32	2.0
5.	Chromium (Cr)	238	23.20	0.1
6.	Copper (Cu)	18.9	0.0075	3.0
7.	Iron (Fe)	5840	0.023	3.0
8.	Potassium (K)	265	N.D	N.D
9.	Magnesium (Mg)	45	N.D	N.D
10.	Sodium (Na)	809	N.D	N.D
11.	Nickel (Ni)	92	6.65	3.0
12.	Phosphorous (P)	146	N.D	N.D
13.	Lead (Pb)	18.5	20.61	0.1
14.	Sulphur(S)	2538	N.D	N.D
15.	Zinc (Zn)	266	0.0063	5.0

*Not determined

Table.4 Morphological characterization of bacterial isolates from effluent waste

Isolate no.	Colony Morphology	Gram character
HM 1	Mucoid, cream	-ve
HM 2	Buttery, cream	-ve
HM 3	Sindoori	-ve
HM 4	Mucoid, off white	-ve
HM 5	Light brown, causing brownish discoloration of media	-ve
HM 6	Gummy, causing brownish discoloration of media	+ ve
HM 7	Dirty white	+ ve
HM 8	Yellowish orange	-ve
HM 9	Light orange with raised centre	-ve
HM 10	Cream	+ ve
HM 11	Mucoid, light brown discoloration of media	+ ve
HM 12	Dirty yellow	+ ve
HM 13	Cream, irregular colonies	+ ve
HM 14	Whitish	-ve
HM 15	Shinning yellow	-ve
HM 16	Lemon yellow, buttery	-ve
HM 17	Budding colonies with raised centre	+ ve
HM 18	Creamish white	+ ve
HM 19	Orange with metallic shine	+ ve
HM 20	Peach with shine	+ ve
HM 21	Orangish red with metallic shine	+ ve

Table.5 Minimum inhibitory concentration (MIC) of morphologically distinct isolates

Isolate No.	Cr	Pb	As	Hg	Ni	W
	Concentration (ppm)					
HM 1	30	60	20	5	50	20
HM 2	120	100	110	-	60	50
HM 3	100	90	100	-	40	40
HM 5	10	-	-	-	-	-
HM 8	30	30	10	-	30	-
HM 9	20	30	20	-	20	-
HM 12	70	60	30	10	30	15
HM 14	30	50	20	-	-	-
HM 15	110	100	90	-	70	30
HM16	100	100	80	10	90	50

Table.6 Biochemical characterization of selected isolates

Test	HM 2	HM 3	HM 15	HM 16
Oxidase	-	-	-	+
Catalase	+	+	+	-
MR-test	-	-	+	-
VP-test	+	+	+	+
Starch hydrolysis	-	+	-	-
Indole production	-	-	-	-
Citrate utilization	+	+	+	+

Fig.1 Comparative growth profile of selected isolates at different pH

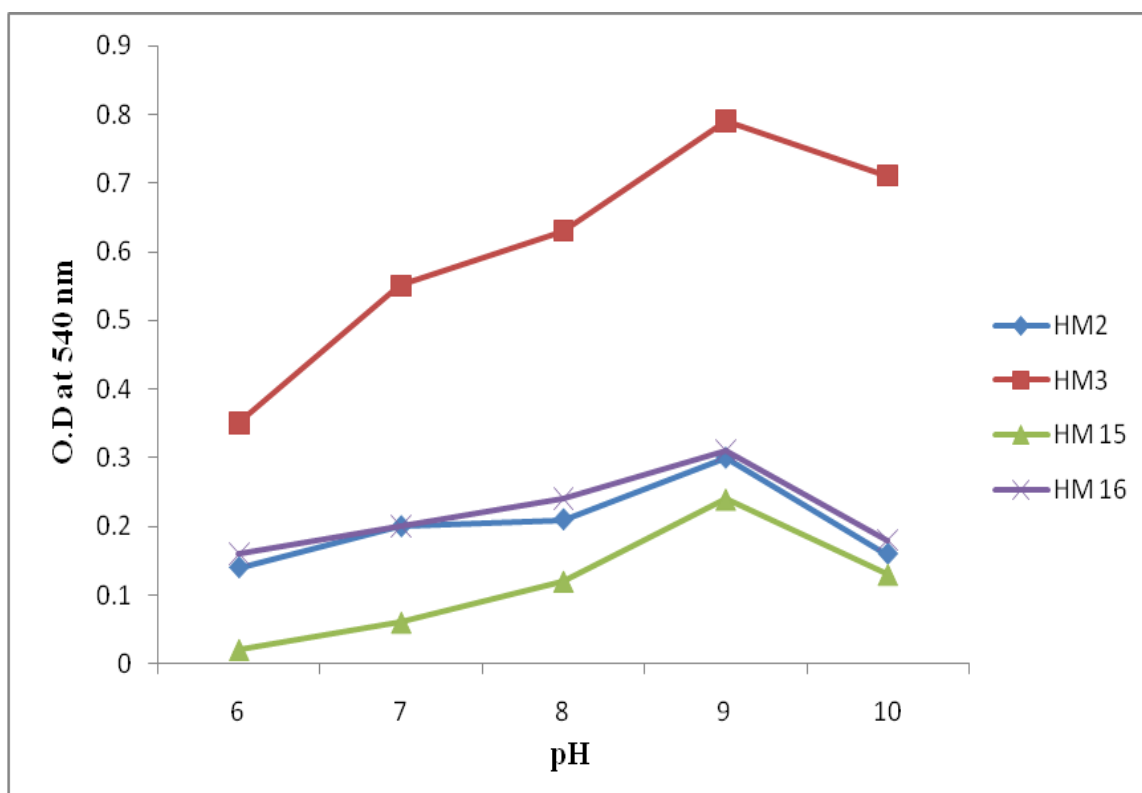
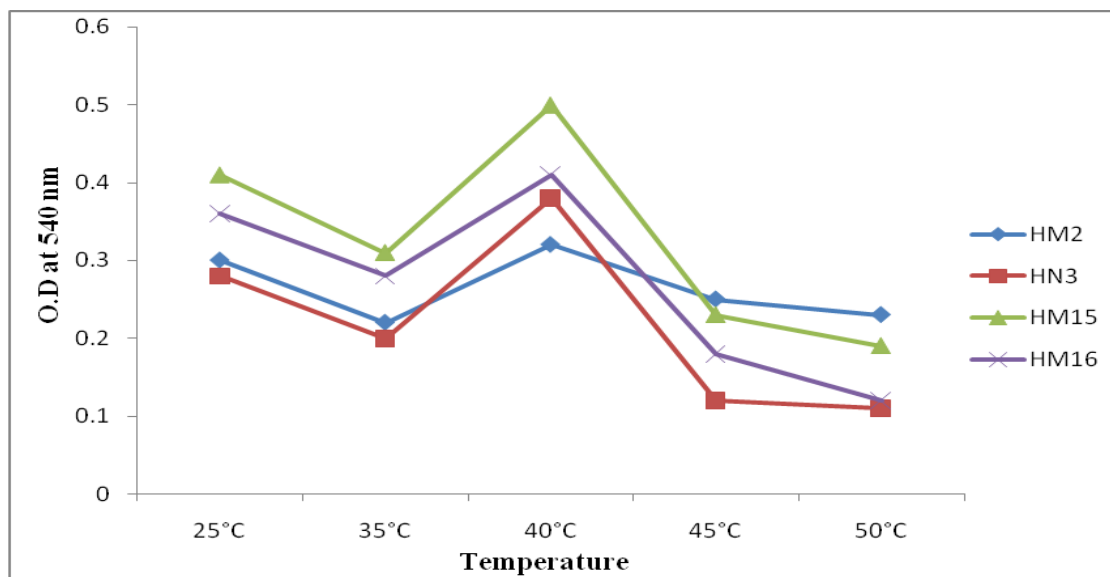


Fig.2 Comparative growth profile of selected isolates at different temperature



Determination of Minimum Inhibitory Concentration (MIC) of different selected heavy metals

Seven isolates HM 2, HM 3, HM 5, HM 8, HM 9, HM 14 and HM 15 were sensitive to mercury, two were sensitive to nickel HM 5 and HM 14 and four were sensitive to tungsten HM 5, HM 8, HM 9 and HM 15. Four isolates i.e HM 2, HM 3, HM 15 and HM 16 shows maximum tolerance to five different heavy metals except mercury. These four isolates were selected for their biochemical characterization. In another study, some workers isolated 53 different species from the sediment samples collected from Krishna Godavari basin. Of these isolates, 79.24% were found to be resistant to 350ppm of Mercury (11.53%), 250ppm of Cadmium (3.77%), 700ppm of Chromate (50.94%) and 250ppm of Zinc (13.20%) (Gunaseelan and Ruban 2011) (Table 5).

Biochemical characterization of selected isolates

On the basis of biochemical characterization HM 3 and HM 16 were more closely related

to genera *Enterobacter*. Whereas, HM 2 was found to belong to genera *Klebsiella*. Same was confirmed by their typical colony color on selective media. In a recent study, bacteria capable of accumulating heavy metals were isolated from soil samples of Mauritius and identified by standard biochemical test. Out of the 113 isolates, twelve were capable of growing in the presence of mercury, lead, silver, zinc and copper at varying concentrations (1.0-5.0mM) and were identified to belong to the *Bacillales*. This was confirmed by the sequenced 16S rDNA genes of all isolates (Hookoom and Puchooa 2013) (Table 6).

Optimization of growth parameters for selected isolates

Optimum growth conditions of selected bacterial isolates were determined with respect to pH and temperature (Figs. 1 and 2). To determine optimum pH, isolates were grown in LB broth adjusted at different pH i.e., 6, 7, 8, 9 and 10 and their absorbance was measured at 540 nm after 8 hr of incubation @ 100 rpm. All the selected isolates showed maximum growth at pH 9. Out of four

selected isolates, HM 3 showed fastest growth and HM 15 showed slowest growth whereas HM 2 and HM 16 showed comparable growth at pH 9. In vitro compatibility analysis by spot plate inoculation revealed HM 2 and HM 16 to be compatible with each other. Hussein *et al.*, (2003) examined the effect of pH on growth of Ni and Cr resistant four selected strains and found that optimum pH for strain 9 and 12 was 5.5. Whereas, strains 2 and 16 have corresponding optimum pH close to 6.

To determine optimum temperature, selected isolates were grown in LB broth at different incubating temperatures i.e 25°C, 35°C, 40°C, 45°C and 50°C absorbance was measured at 540 nm after 8 hour of incubation @ 100 rpm. All isolates showed maximum growth at 40°C.

The present study revealed the capacity of four (HM 2, HM 3, HM 15 and HM 16) heavy metal tolerant bacterial isolates, from effluents, to tolerate and grow at different concentrations (10-120 ppm) of five heavy metals viz. As, Cr, Pb, Ni and W but were sensitive to Hg. On the basis of biochemical characterization HM 3 and HM 16 were more closely related to genera *Enterobacter*.

Whereas, HM 2 was found to belong to genera *Klebsiella*. Further, optimization of growth conditions of selected bacterial isolates with respect to pH and temperature showed maximum growth at pH 9 and temperature 40°C. Out of four selected isolates, HM 3 showed fastest growth and HM 15 showed slowest growth, whereas HM 2 and HM 16 showed comparable growth at pH 9 with *in vitro* compatibility and were selected as potent candidate for consortium. This study elucidated the potential of resident microbes of effluents/sludge for heavy metal removal, as these bacterial species were well acclimatized to the ecology of metal contaminated aquifers.

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