Practiced Gram negative bacteria from dyeing industry effluents snub metal toxicity to survive

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ABSTRACT

Isolation and characterization of metal-tolerant bacteria from dyeing industry effluent was the prime drive of the present investigation. Results yielded during screening and isolation, noted the dominance of bacteria verses fungi; in addition, metal tolerance ability of six out of twenty five bacterial isolates were observed. The six selected isolates were gram's negative, and were identified as *Stenotrophomonas maltophilia* UOMGNS116, *Pseudomonas* sp. UOMGNS216, *Pseudomonas stutzeri* UOMGNS316, *Pseudomonas stutzeri* UOMGNS416, *Citrobacter freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616 by 16s rDNA analysis. This investigation also evidenced the varied resistance capabilities by *Achromobacter* sp. UOMGNS616 and *Citrobacter freundii* UOMGNS516 against chromium and lead by overproduction of external polysaccharides especially against lead.

1. INTRODUCTION

Sinking up of inorganics especially heavy metals into the ecosystem is due to the disturbing and accelerating nature's slow geochemical cycle by anthropogenic events [1-3]. Expanding industrial areas, mine tailing, disposal of high metal wastes, paints, leading gasoline, application of fertilizers [4], pesticides [5], sewage sludge, waste water irrigation [6, 7], spillage of petroleum chemicals, coal combustion residues [8-10]. Altered biological balance of every living organism because of the enormous complexity of the ecosystem and varying complexity of contaminants from case to case made researchers tough to detail the behaviour of every pollutant. Inability of majority of microbes to oxidize inorganic contaminants similar to organic contaminants [11] and its solubility in aquatic environment increases its ability of accumulation in the food chain [12]. In addition, non-economical physico-chemical or conventional treatment techniques have major shares in increased flow of bulk effluents with complexing organic and metal contaminations to the ecosystem [13]. Survival ability of microbes in harsh environment has attracted the attention of the researchers worldwide who are exploring the possibility of addressing the adverse effects of metal pollution by developing simple and comprehensive treatment models [14] employing microbes as work horses to reconstruct the environmental damage posed by heavy metal accumulation. By considering the advantages of understanding native microbial species to design effective biological tool [15, 16].

2. MATERIALS AND METHODS

2.1 Collection of effluent samples

Industrial effluent samples were collected from effluent treatment plant of dyeing industry situated in Bengaluru, India. The samples were collected in pre-sterilized acid washed Pyrex bottles and transported to the laboratory for microbiological assessment.

2.2 Bacterial isolation and pure culture

All the three samples were serially diluted up to 10^{-6} and inoculated by spread plate method. On LB for bacterial growth, PDA for fungal growth. The LB agar plates were incubated at 35^0c temperature for 24-48 hours, and PDA plates were incubated at room temperature for 2-3 days. Individual plates were observed and morphologically differentiated colonies were isolated. The isolated colonies were maintained on the slant LB medium.

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2.3 Determination of heavy metal acceptability of isolates

All the 25 bacterial isolates were coded serially as UOMGNS01 to UOMGNS25. Individual isolates were inoculated on LB amended with different samples **(Table 1)** at 0.2mM of concentration and plates were incubated at 35^0 C for 48 hours. Based on colonizing ability of bacteria on agar plates, isolates were selected for further experiments.

2.4 Biochemical characterization of heavy metal tolerant bacteria

2.4.1 Gram's reaction

Gram staining was carried out according to the protocol of Vincent 1970 [17]. Results were confirmed by KOH solubility test, was performed according to [18].

The other biochemical test includes, Catalase, Urease, starch hydrolysis, DNA hydrolysis (DNase test) and Lipase as describe by Hankin and Anagnostakis 1970 [19].

2.4.2 Catalase

A loopful of bacterium of 24h old culture was mixed with a toothpick on a clean glass slide with a drop of 3% Hydrogen peroxide solution and observed for the presence or absence of air bubbles/froth formation on the slide.

2.4.3 Urease

Urease test was done using Stuart's Urea broth (SUB) (Yeast extract-0.1g, potassium phosphate monobasic-9.1g, potassium phosphate dibasic-9.5g, urea-20g, phenol red-0.01g). To 3ml of SUB a loop full of isolated cultures were inoculated and incubated at 35 ˚C with 75rpm. The broth was observed for the colour change at 12, 24 and 48hrs.

2.4.4 DNA hydrolysis (DNase test)

The production of extracellular enzyme: DNase by bacterial isolates was tested by inoculating the cultures on Difco DNAase agar (tryptone 20g, DNA 2g, NaCl 5g, & agar 14g; pH 6). After 3days of incubation, plates were flooded with 1N HCl and observed for clear zone around the colonies which indicates the hydrolysis of DNA in test medium.

2.4.5 Starch hydrolysis

The capacity of isolated bacterial cultures to degrade, indicates the ability to produce amylolytic enzyme. This was determined by streak inoculating on to starch agar media (0.5% peptone, 0.3% beef extract, 0.5% NaCl, 1.5% agar, 0.2% starch-heat to boiling) were incubated at 37 ˚C for 72hrs. The hydrolysis of starch was determined by the observation of clear yellow zone around a colony upon addition of Iodine reagent.

2.4.6 Lipase test/Lipolytic activity

The test was carried out as described by Sierra (1957). The medium to contain: Difco peptone, 10g; NaCl, 5g; CaCl2 2H2O, 0.1g; agar, 20g, pH 6. The Tween 20 per liter was sterilized separately by autoclaved and 1ml added per 100ml of sterile and cooled basal medium. The formation of lipolytic enzymes by a colony was seen as either visible precipitate due to the formation of crystals of the calcium salt of the lauric acid liberated by the enzyme or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid.

2.5 Molecular characterization of metal tolerant bacteria
2.5.1 Molecular characterization of metal-tolerant bacterial isolates DNA isolation

Genomic DNA extraction and PCR amplification procedures were carried out by following previous standardised protocol of Doyel and Doyel's [20]. Molecular characterization studies were carried out by following protocols detailed previously by Sunil *et al.,* (2015), Total DNA from selected six isolates were used as template to amplify variable region of bacterial 16s rDNA by PCR using the universal primers 6sF 5'-CCAGACTCCTACGGGAGGCAGC-3' and 16sR 5'-GCTGACGAGAGCCATGCAGCACC-3' (Sigma Aldrich). The PCR reaction system of 50μl included 5μl of 10x Taq buffer, 1μl of 0.2mM dNTPs, 0.15μl of forward and reverse primers (10pmol), 10μl of DNA dilution 100ng/μl, 1.75μl of Taq polymerase (1U) and 37.45μl of nuclease free water and the system was programmed with 30 cycles at 94 °C for 4 mins, 94 °C for 45s, 55 °C for 45s, 72 °C for 1min and 74 °C for 10mins (Bio Rad). The results were analyzed by 1.2% agrose gel electrophoresis.

2.5.2 nBlast analysis of nucleotide sequence

All sequences were identified using NCBI nucleotide blast as the selected algorithm, except highly somewhat similar sequences algorithm was chosen to identify the sequence and the Metal tolerant bacterial isolates were identified.

2.6 Determination of minimal inhibitory concentration of heavy metal tolerant isolates

Six isolates were inoculated with load of measures inoculum 0.2 optical density measures at 600 nm using spectrophotometer in 100 ml of pre-autoclaved LB broth amended with 0.2, 0.5 and 1mM of concentration of Heavy metal salts (Table 1). Culture flasks were incubated at 37 °C for 74 hrs, and turbidity was measured at the time interval of 24hrs in order to record the growth rate at 600nm wave length using spectrophotometer (Beckman coulter DU 700, Germany)

2.7 Assessing lead acceptability of *Achromobacter* sp. against Lead

Achromobacter sp. UOMGNS616 with five other test strains inoculated in 100ml of pre-sterilized LB amended with 0.2 to 0.5mM of lead in duplicates were incubated for three days at its optimal conditions and after the incubation period cultures were centrifuged and wet weight of pellets were measured in grams and tabulated against the control flask incubated without any metal stress.

3. RESULTS AND DISCUSSION

3.1 Determination of microbial load in effluent sample
3.1.1 Biochemical characterization of metal tolerant isolates
3.1.1.1 Determination of optimal growth conditions

      Though it is well known that microbes cannot destroy metals, interest among researchers towards understanding the ability of microbes in altering chemical properties of metal is being increasing never than before due to their incredible potentials to survive in harsh metal contaminated environments by employing surprising array of mechanisms [21], which can be exploited for bioremediation of contaminated sites [22]. This investigation was taken up with the intention of evaluating heavy metal tolerance and resistance ability of bacterial consortium habituated in effluents of dyeing industries. Primarily, bacterial dominance especially Gram-negative strains have been observed during the screening studies compared with the fungal load in the effluents. This might be because of increased size of fungal cells with lower density and lower mechanical strength and rigidity when compared with bacterial cells [23]. This result supports the earlier reports on inability of fungal cells to develop equal competence against bacterial cells in heavy metal contaminated sites [24]. Amongst twenty five bacterial isolates only six have been chosen for further studies based on heavy metal acceptability in which all showed to be negative for Gram's staining (Table 1). Results reveal the failure of many bacterial isolates to disclose the adaptive response though they have resided in samples with excessive metals. Further, using partial sequence of their 16s rDNA gene and phylogenetic analysis, the isolates were identified and coded as *Stenotrophomonas maltophilia* UOMGNS116, *Pseudomonas* sp. UOMGNS216, *Pseudomonas stutzeri* UOMGNS316, *Pseudomonas stutzeri* UOMGNS416, *Citrobacter freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616.
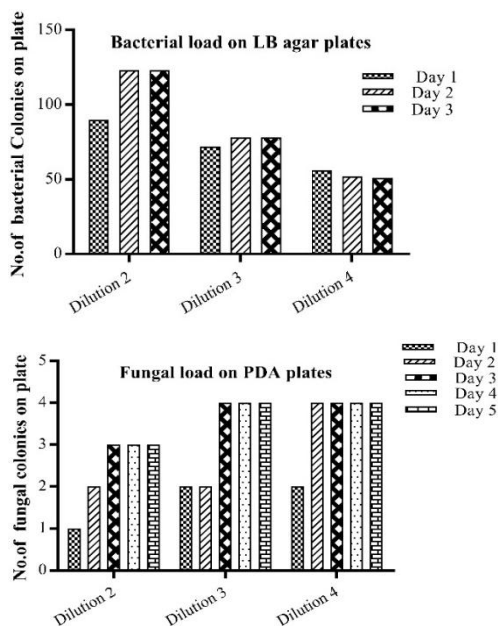
Table 1: Biochemical characterization of multi-metal tolerant bacterial isolates.

Isolates	Gram's test	Starch hydrolysis	DNase hydrolysis	Urease Test	Catalase test
Stenotrophomonas maltophilia UOMGNS116	-	+	-	-	+
Pseudomonas sp. UOMGNS216	-	-	-	-	+
Pseudomonas stutzeri UOMGNS316	-	-	-	-	+
Pseudomonas stutzeri UOMGNS416	-	+	+	-	+
Citrobacter freundii UOMGNS516	-	-	+	-	+
Achromobacter sp. UOMGNS616	-	+	+	-	+

      All the six isolates have proved their efficiency to multiply in both acidic and alkaline conditions and recoded their individual pH to grow optimally though they have habituated in neutral pH environment(Fig: 2).
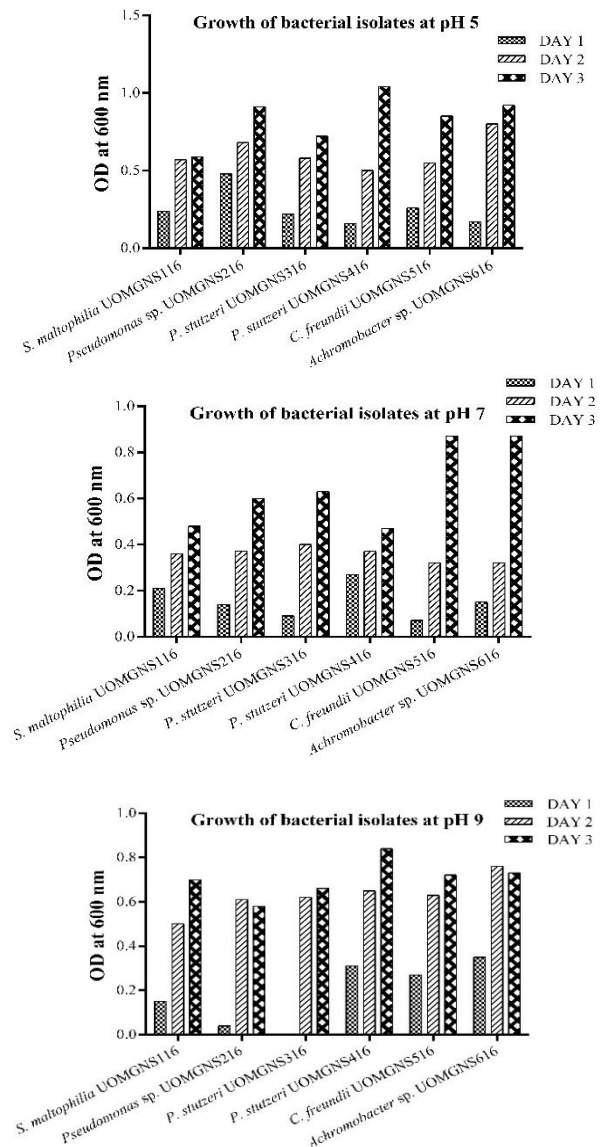

Fig. 1: Bacterial and fungal load in effluent sample on LB agar and PDA plates.

Fig. 2: Determination of optimal pH of heavy metal tolerant isolates.

It is important to note the ability of bacteria to survive in alkaline pH environment which is of widespread importance in remediation in industrial setting [25]. Shifting to an alkaline and acidic environment is stressful for bacteria which involves the responses of key biological molecules [26, 27]. In addition to survival ability, capacity of metal tolerant isolates to grow at optimal rate in both acidic and alkaline conditions extends the interest to study the influence of pH in gaining tolerance particularly against lead and chromium in future studies. It is also interesting to note that though all the isolates recorded their optimal growth at 35 °C the survival ability or reproducibility of bacteria at thermostatic environment promises participation in bioremediation at different physiological status (Fig: 3).
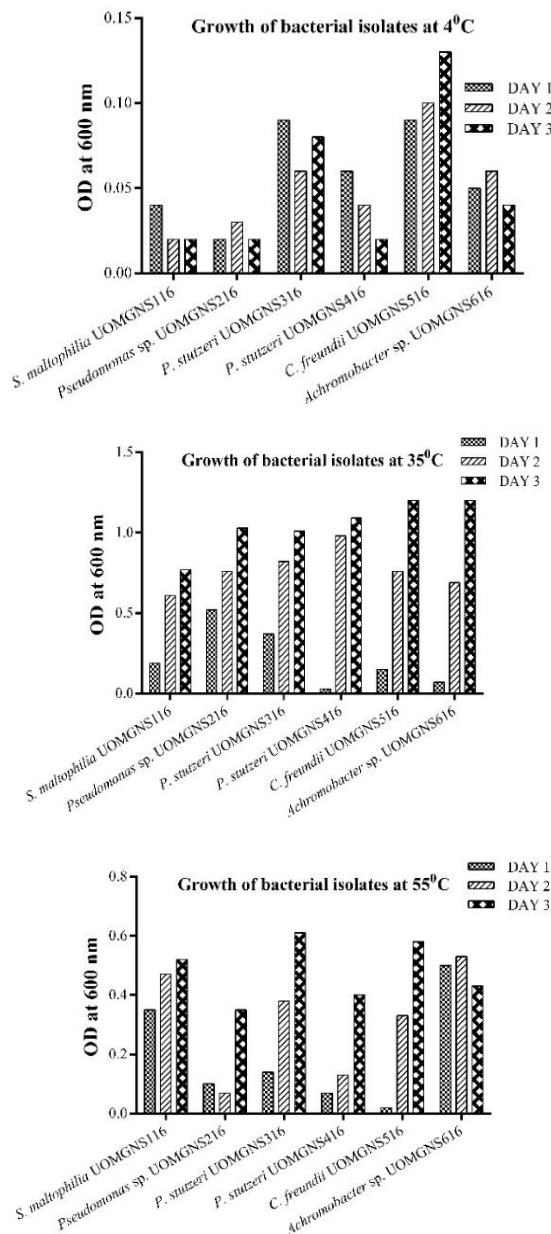
Selected isolates were further subjected to assess their heavy metal tolerance at their optimal growth conditions at increasing metal concentrations. Growing ability of all the test isolates were determined by measuring turbidity of medium due to bacterial growth, all the six isolates failed to survive at low concentrations of cadmium and mercury (data not shown), but exhibited varied tolerance ability against chromium and lead at different concentrations ranging from 0.2 - 1mM (Fig: 4 & 6).
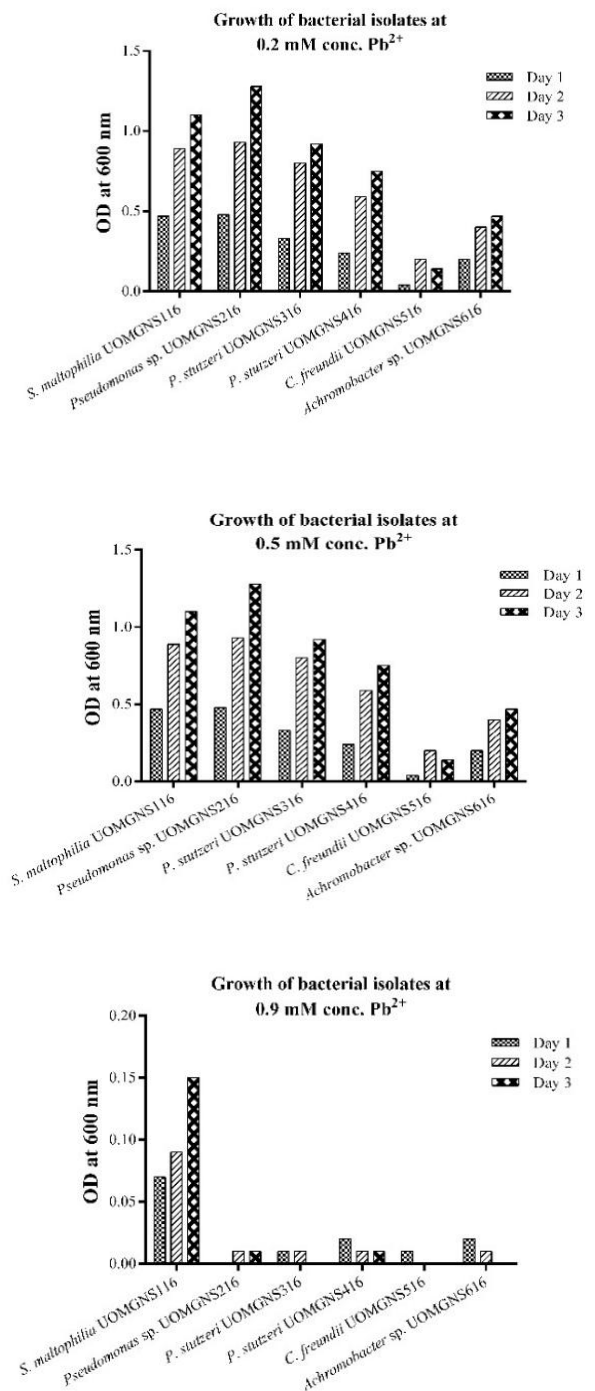

Fig. 3: Determination of optimal temperature for the growth of heavy metal tolerant isolates.

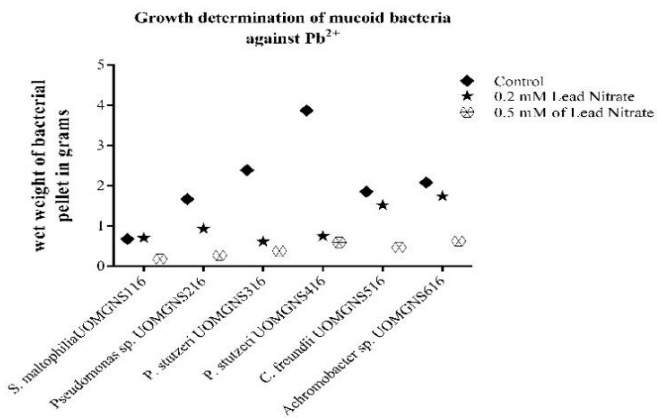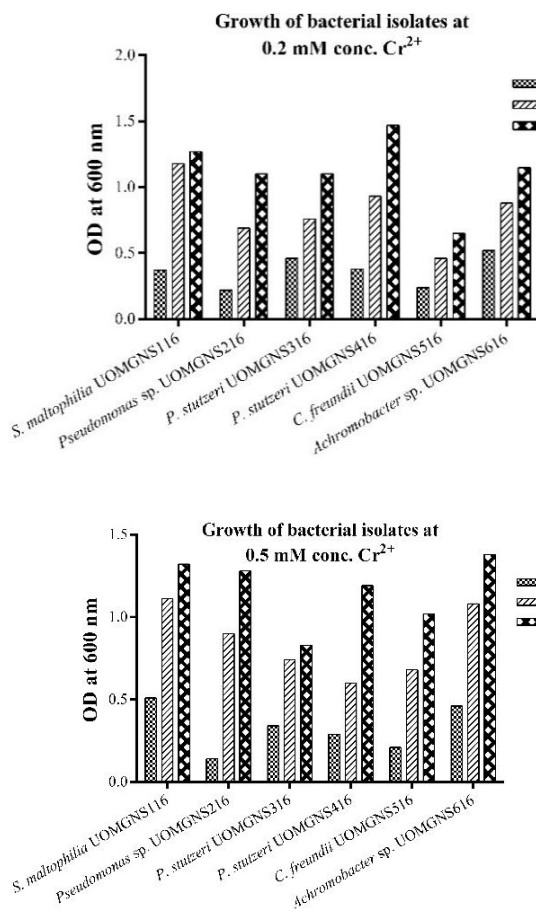**Fig. 4:** Determination of minimal inhibitory concentration against different concentration of lead.

Fig. 5: Determination of growth rate of metal tolerant isolates against lead by measuring wet weight of pellets.

Pseudomonas stutzeri UOMGNS316, *Pseudomonas stutzeri* UOMGNS416 to multiply at both 0.2 and 0.5mM conc (Fig: 4). Only *C. freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616 failed to grow at these concentrations when growth determined by measuring turbidity. It is possible that bacteria acquire resistance against metals by preventing the access of metals to sensitive cellular components or by altering them to reduce the sensitivity [28]. In the present investigation mucoid phenotype was noticed in *C. freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616 which is indicative of production of external polymeric substances (EPS) by these isolates [29]. In order to determine growth of mucoidal phenotype produced by *C. freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616 the wet weight was measured and compared with all other test isolate at 0.2 and 0.5mM conc. In supports evidenced the significant tolerance ability of *C.freundii* UOMGNS516and *Achromobacter* sp. UOMGNS616 (Fig: 5). Results of the present study evident the potentiality of *C. freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616 in heavy metal tolerance. Further characterization of these isolates by biochemical and molecular tools is required before they can be employed for lead clean-up activites of industrial effluents. Further studies are also required to understand the varied metal tolerant potency of *S. maltophilia* UOMGNS116 compared with other co-habitats.
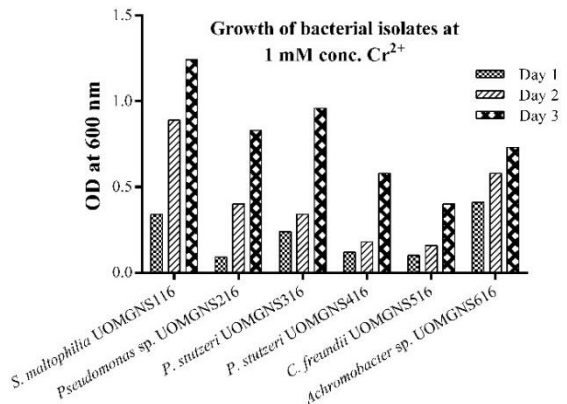

Fig. 6: Determination of minimal inhibitory concentration of metal tolerant isolates against chromium

4. CONCLUSION

A result of the present investigation reveals varied behaviour of bacterial consortium isolated from effluents and strongly highlights the importance of understanding acquired resistance of *C. freundii* UOMGNS516 and *Achromobacter* sp. UOMGNS616 against lead. In future, studies on communication of bacteria to detect specific metal stress in the external environment and communication with its own neighbour cells to lock and unlock the adaptive responses may reveal new clues towards developing sustainable biological clean-up tools. In addition, results also support the importance of understanding biochemical and molecular abilities of *S. maltophilia* UOMGNS116 acquired in order to sustain in different harsh environments.

All the six isolates showed gradual increase in their growth measured at every 24hrs time interval and proved to be equally potent to multiply at 0.2mM concentration of chromium. At higher concentrations of 0.5 and 1mM chromium all the isolates except *Achromobacter* sp. UOMGNS616 and *C. freundii* retained their tolerance ability (Fig: 6). Minimal inhibitory concentration of lead was estimated for all the six isolates by incubating at different lead concentrations ranging from 0.2 – 0.9mM. This study revealed the ability of *Stenotrophomonas maltophilia* UOMGNS116, *Pseudomonas* sp. UOMGNS216,

5. ACKNOWLEDGEMENT

Conflict of Interests: There are no conflicts of interest.

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