Study of Multiple Metal Resistant Citrobacter Sp. Isolated from Industrial Effluent

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Abstract

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Microbe related technologies may provide an alternative or addition to conventional method of metal removal or metal recovery. The present study deals with isolation, identification and characterization of heavy metal resistant bacteria from industrial effluent. a total58 isolates were screened for heavy metal resistant from industrial effluent. The GOZPRN isolate show multiple metal resistant and antibiotic resistances. On the basis of morphological, biochemical, partial 16S rRNA gene sequencing revealed that this isolates belongs to *Citrobacter sp.*. The isolate showed optimum growth at 30°C and pH 7.0. This isolate was found to be resistant to nickel (Ni), lead (Pb), zinc (Zn), Chromium (Cr). The minimal tolerance concentration (MTC) of this isolate against Cr, Ni, Pb, and Zn was 2.8 mM, 80 mM, 800 mM, and 500 mM respectively. It also exhibited resistance against tetracycline. This *Citrobacter Sp.* has potential to be useful for the bioremediation of heavy metals.

Keywords: Heavy metal resistant, Antibiotic resistance, 16S rDNA Sequence, Industrial effluent.

1. Introduction:

Heavy metal is a general term assigned to metallic elements having molecular weight above 40.04 (atomic mass of Ca) [1]. Metals play a vital role in biological systems as a living cell cannot exist without metal ions. Trace amounts of heavy metals are also required by living organism including copper, cobalt, iron. Excessive levels of essential metals however can be toxic to the organism [2]. Among the pollutants which are discharged in to water bodies, heavy metals are of most concern because other pollutant may be degraded by some microorganisms but metals cannot be degraded. Presence of heavy metals even in traces is toxic and detrimental to all living organisms [3].The presence of heavy metals in industrial effluents is known to have major hazard to natural water, animal and human health. High concentrations of heavy metals have deleterious effect on the environment [4].Over the years, scientists had been trying to eradicate the heavy metals from environment but no efficient technique suffices the need so far. Principally, there are two modes of eradication viz. physical and biological. The physical mode chiefly includes ion exchange methods, precipitation, sorption, electrochemical treatment etc. but these are not very cost effective. On the contrary, biological approach incorporates the usage of plants or microbes for the detoxification of soil[5].

Aim of the present study was to isolate heavy metal resistant bacteria and its characterization and its application for removal of heavy metals from industrial effluent. In this study, Isolation and characterization of heavy metal resistant bacteria from the industrial effluents of different G.I.D.C. Area such as Ankleshwar G.I.D.C, Panoli G.I.D.C, Dahej G.I.D.C, Vagra G.I.D.C were attempted. These

strains were investigated in respect of the minimum tolarance concentrations (MTC) of heavy metals (Cr, Pb, Ni, Cu and Zn) and Sensitivity and resistance to antibiotics (ampicillin, tetracycline, chloramphenicol, vancomycin, Erythromycin, Bacitracin etc.).

2. Materials and methods

2.1 Isolation of heavy metal (Cr, Zn, Cu, Pb and Ni) resistant bacteria:

For isolation, the samples were serially diluted in sterile distilled water, and 100μl form each dilution was plated on minimal salt agar supplemented with individual heavy metals Cr, Zn, Cu, Pb and Ni in the form of their salts, $K_2Cr_2O_7$, $ZnSO_4$, $CuSO_4$, $Pb[(C_2H_3O_2)]_2$ and $NiCl_2$ respectively at concentration 0.4mM. The plates were incubated at 30º C for 24-48 hours. After incubation a number of morphologically different colonies were picked and isolated after successful purification process on the same medium. For further screening of multi metal resistant bacteria, the isolates were inoculated on minimal salt agar media containing 0.4mM of rest of the four metals separately and incubated at 30º C for 24 h. Isolates grow on these plates were transferred on minimal salt agar plate and subsequently purified[3].

2.2 Biochemical and molecular characterization of selected isolate:

Molecular identification of selected isolate was carried out by extracting genomic DNA. 16S rRNA sequencing reaction of PCR amplicon was carried out with using BDT v3.1 Cycle sequencing kit on ABI 3730*1 genetic analyser. The partial sequence of 16S rRNA of bacterial isolates were amplified using 27F – 5'AGAGTTTGATCMTGGCTCAG-3' and 1391R–5'GACGGGCGGTGWGTRCA3' as forward and reverse primer respectively. Identification of isolates were carried out by using BLAST(Basic Local Alignment Search Tool) with the database of NCBI(National Centre for Biotechnology Information) gene bank database based on maximum identity score.

2.3 Phylogenetic tree analysis of selected isolate:

The phylogenetic analysis of selected isolate was carried out on the basis of partial 16S rRNA sequence. The nucleotide sequences of related organisms was obtained from the NCBI database and used for alignment and calculating the homology level. Clustal W programme was used to align the sequences. The Phylogenetic tree was constructed by the neighbor-joining method using MEGA 11 (Molecular Evolutionary Genetics Analysis) software[6].

2.4 Determination of MTC of heavy metals:

The minimum tolerance concentration of metal, which inhibits the bacterial growth was determined by agar dilution method. Stock solutions of 1000mM of heavy metals were prepared by dissolving the exact quantities of the following metal salts: $CuSO_4$, $ZnSO_4$, $Pb[(C_2H_3O_2)]_2$, $NiCl_2$, and $K_2Cr_2O_7$ in sterile distilled water. Minimal salt agar medium was sterilized at 121 ºC for 20 min. After sterilization, heavy metal stock solution was added to get desire final concentration of metal in the medium using following equation.

$$C_1 \times V_1 = C_2 \times V_2$$

where C_1 is the metal concentration in stock solution,

V_1 is the volume of stock solution used,

C_2 is the concentration of metal in agar medium,

V_2 is the volume of Minimal salt Agar medium.

15ml of minimal salt agar supplemented with heavy metal pour into a petriplate. Thereafter, the bacterial isolate was streaked onto the medium-containing increasing concentrations of metal salts using sterile

loops. Plates were sealed and incubated at 30 ºC for 24hr.Plate-containing only minimal salt agar was also inoculated and incubated to act as control. The lowest concentration of each metal at which no growth occurred when compared to the control plates was considered as the MTC [7].

2.5 Determination of optimal temperature and pH for growth:

The optimal growth conditions with reference to pH and temperature were determined. The isolates were grown in minimal salt broth medium with different pH values (5, 6, 7, 8, and 9) set using 1.0M NaOH or 1.0M HCl and incubated at 30º C for 24 h. For temperature, isolates were inoculated into minimal salt broth medium and incubated at different temperature (10º C, 20º C, 30º C, 37º C, and 50º C). A culture of isolates were prepared having absorbance of 0.1 at 600nm (Equivalent to 0.5 McFarland scale). The growth is observed by monitoring optical density of the culture grown in the medium at 600 nm [8].

2.6 Determination of antibiotic susceptibility of isolate:

Resistance to antibiotics was determined on Mueller-Hinton agar plates (Hi Media, India). 1 ml suspension of isolate was spread on a plate. Sterile forceps were used to place the multiple antibiotic discs (PBL, Bio-Disc-12) on the media. Plates were incubated at 30º C for 24h.Inhibition zone was noted and resistance was recorded as positive. Strains were considered susceptible when the inhibition zone was 12 mm or more in diameter [7].

3. Results and Discussion

3.1 Sample collection and Isolation of heavy metal resistant bacteria:

Samples of different industrial effluents were collected from G.I.D.C area of Ankleshwar, Panoli and Dahej of south Gujarat, India. Initially 58 morphologically different heavy metal resistant that can tolerate 0.4mM metal concentration were isolated from total 12 sample. Isolate GOZPRN was found to exhibit resistance against zinc, lead, chromium and nickel and identified as multi heavy metal resistances. This strain was selected for further study.

### 3.2 Result of biochemical and molecular characterization of selected isolate:

Table 1: Biochemical characterization of GOZPRN

Name of the biochemical test	Result	Name of the biochemical test	Result
Indole test	+	Nutrient glucose broth	A & G
Methyl Red test	+	Nutrient sucrose broth	A & G
Vogus-Proskauer test	+	Nutrient lactose broth	A & G
Citrate utilization test	+	Nutrient mannitol broth	A & G
H_2S production test	-	Nutrient maltose broth	A&G
Nitrate reduction test	+	TSI agar Slant	A
Urea hydrolysis test	-	Butt	A
Oxidase	+	CO_2 Production	+
Catalase	+	H_2S Production	-
+ Positive, - Negative, A-Acid production, G- Gas production			

Isolate GOZPRN was further identified by performing the 16S rRNA sequencing by using Sanger dideoxy sequencing method, the selected isolate number GOZPRNobtained from the cosmetic industry effluent sample and it was identified as *Citrobacter sp.* by using BLAST tool (Basic Local Alignment Search tool) of NCBI (National Center for Biotechnology Information). The partial sequence of 16S

rRNA of *Citrobacter sp.*1302 nucleotide long. This partial sequence of *Citrobacter sp.* (GOZPRN) has been submitted to NCBI (National Center for Biotechnology Information) having accession number **PP622667.**The phylogenetic tree was constructed for isolate GOZPRNusing MEGA software (version 11). Evolutionary tree analysis of isolate GOZPRN along with set of organisms or group of organisms (taxa) was shown in Figure 1. Evolutionary history was inferred using the Neighbor-Joining method.

Figure 1: Phylogenetic tree of isolate GOZPRN:
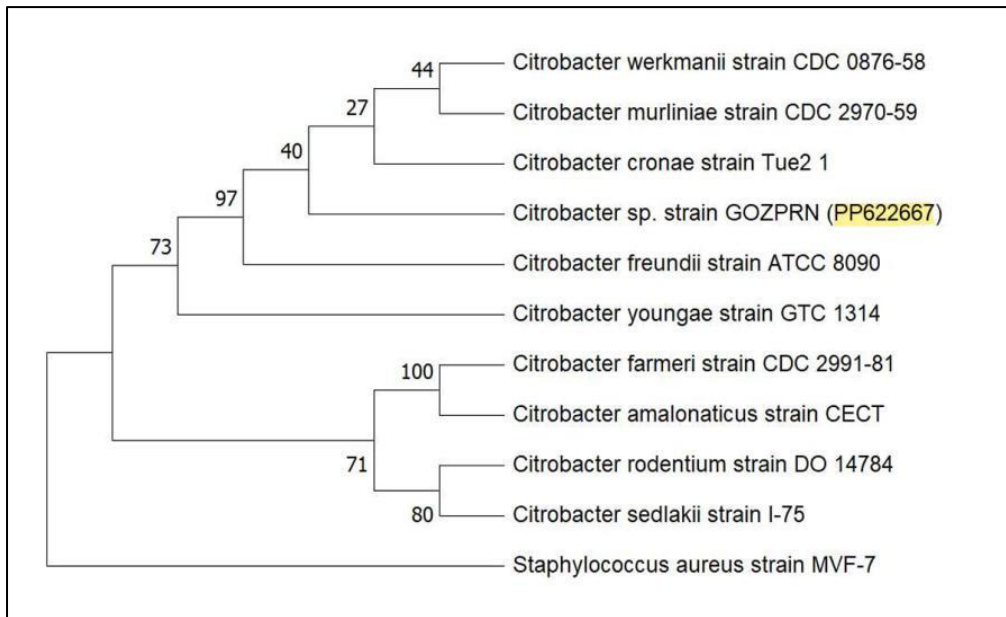
3.3Result of MTC of heavy metals:

GOZPRN isolate showed high degree of resistance to all the heavy metals except Cu. Isolate was resistant to 4 heavy metals under investigation, ranging from 10-1000 mM. Shown in table 2:

Table 2: MTC of GOZPRN again different heavy metal:

Isolate GOZPRN	Concentration in mM										
	10	100	200	300	400	500	600	700	800	900	1000
Chromium (Cr)	+	+	-	-	-	-	-	-	-	-	-
Zinc (Zn)	+	+	+	+	+	-	-	-	-	-	-
Lead (Pb)	+	+	+	+	+	+	+	+	+	-	-
Nicke (Ni)	+	+	-	-	-	-	-	-	-	-	-
+ Growth, - No growth											

3.4 Determination of optimum growth condition:

Optimum growth condition was determined in order to large scale biomass production for further application. GOZPRN showed maximum growth at pH 7 and very moderate growth were observed at pH 5. That mean acidic pH retard the growth. Graph also shows.

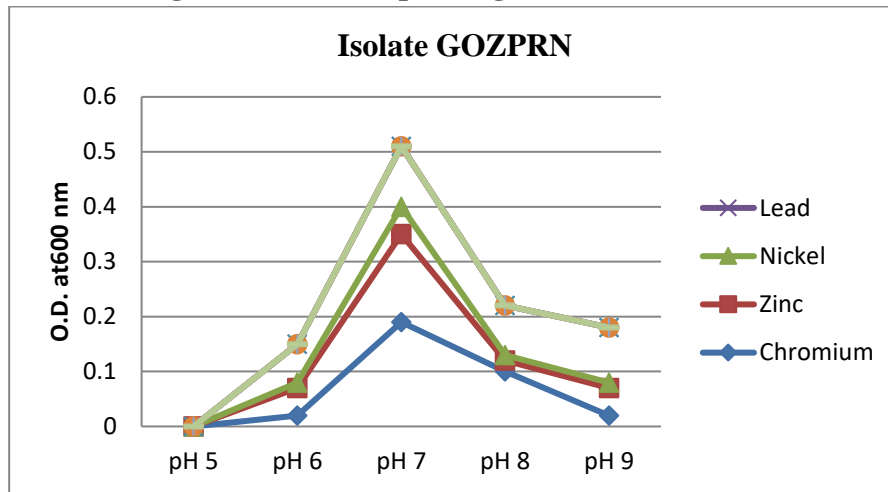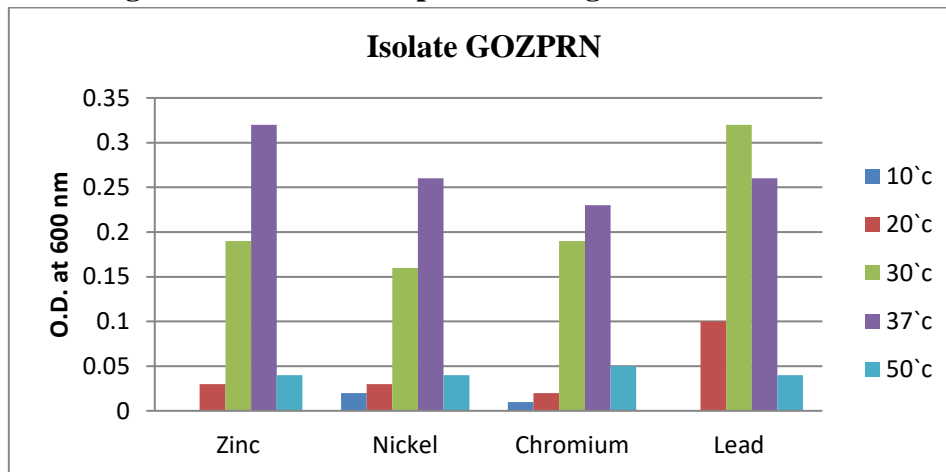## Figure 2: Effect of pH on growth of GOZPRN

Figure 3: Effect of Temperature on growth of GOZPRN

3.5 Determination of antibiotic susceptibility of GOZPRN:

Antibiogram of the GOZPRN was carried out by Kirby-Bauer disc diffusion method using a combi-Disc (PBL, Bio-Disc-12). It contains 12 different antibiotics. Isolate found to be susceptible to 11 antibiotics and only resistant to tetracycline.

Figure 4: Antibiotic susceptibility of GOZPRN

4. Conclusion:

In this study, Heavy metal resistant bacteria were isolated from industrial effluents in south Gujarat region. This isolate was identified as *Citrobacter sp.* which exhibited high resistance level. Optimal growth condition was found to be at pH 7 and 30° C. The study also revealed a correlation between Heavy metal and antibiotic resistance, suggesting potential candidate for bioremediation of heavy metal.

5. References:

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