PLASMID PROFILING AND EFFECT OF DIFFERENT PHYSIOLOGICAL PARAMETERS ON THE CHROMIUM REDUCTION POTENTIAL OF MICROBES

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Abstract

Chromium is toxic for both human and aquatic life. It is recommended to eradicate from wastewaters or to alter its oxidation state to less toxic level. The purpose of current research was to isolate heavy metal (Cr) resistant bacteria from different industrial effluents (soil and waste water), to determine their potential for chromium reduction (CRP) at different parameters (time period, pH, temperature and concentrations of chromium) and to determine the plasmid profiles of Cr (VI) resistant bacterial isolates. The growth of chromium resistant bacteria was determined by checking the influence of pH, concentration of chromium, time period and temperature on isolates using UV spectrophotometer, while chromium reduction potential was also investigated using Deleto and Ehrlich method. Plasmid profiling was performed and analyzed using agarose gel electrophoresis (0.8%) to determine the number, size and relationship of plasmid with heavy metal resistance. Results showed that the identified bacterial isolates (*S. aureus* and *S. epidermidis*) were resistant to heavy metal (Cr) confirmed by resistance profiling. The maximum growth of bacterial isolate was recorded after 24-hour incubation period (1.154), at pH 8 (1.512), temperature 37°C (1.615) and 500 µg/mL chromium concentration (1.978), while suitable conditions observed for chromium reduction potential was 24-hour incubation period (57%), pH 7 (62.6%), temperature 30°C (60%), and 500 µg/mL concentration of chromium (60%). The plasmid profiles revealed that plasmids were randomly distributed among the bacterial isolates with average plasmid number (2.9) ranging from 0-5 and molecular size (100-12000bps). Overall, no defined relationship was observed among resistance pattern and plasmid mediated profiles.

Keywords: Chromium reduction potential, plasmid profiles, chromium resistant bacteria, heavy metal, % age removal of chromium.

I. Introduction

Heavy metal contamination in soil and water, is a serious environmental issue and of global health concern, particularly in developing countries (Kleckerova and Doeekalova, 2014; Akoto *et al.*, 2014). Heavy metals are generally poisonous and can accumulate in living tissues causing various diseases (Abdominal pain, Nausea, Vomiting, Fatigue, Dehydration, Abnormal heart beat, Weakness, Skin and Lung irritability, etc.) and disorders (Lesions on Kidneys and Liver, Nervous system damage, Myocardium, Edema, etc.) in body posing a great threat to public health (Fu and Wang, 2011).

Chromium (Cr^{+6}) a well-documented toxin and carcinogen (Stearns, 2007) is one of the most potent heavy metal. Maximum tolerance level of chromium for the drinking water is 0.05mg/L (Becquer *et al.* 2003). Hazards due to chromium contamination depend on its oxidation state and solubility (Shrivastava *et al.*, 2002).

Many bacteria have specific genetic mechanisms of resistance to toxic metals (Mindlin, 2001) such as *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus thuringiensis*, *Micrococcus* sp. (Agostinho, 2012). Chromium-resistant microbes are *Pseudomonas aeruginosa*, *Bacillus* sp., *Pasteurella* sp., *Pseudomonas* sp., *E. coli* and *Staphylococcus* sp. (Benmalek, 2012). Major illnesses caused by chromium resistive microbes include diarrhea, abdominal pain, fever, pneumonia (Melton, 2014), musculoskeletal infections, tissue necrosis, scalded skin syndrome, abscesses, impetigo, cellulitis etc. (Schlecht, 2015). The heavy metals degradation capacity of *Micrococcus* and *Staphylococcus* sp. might be plasmid mediated (Silver, 1988).

Plasmids are self-replicating, extra-chromosomal DNA molecules that exist in bacteria, allow parallel gene (heavy metal resistance) transmission by conjugation, transduction and alteration (Alam *et al.*, 2010; Raj, 2012) between bacteria of identical or different genera (Wonglumsom *et al.*, 2011).

Connubial transmission of plasmids is substantial in dispersal of resistant genes among bacterial strains (Cumhur and Ismet, 2014). Bacterial resistance to heavy metals and antibiotics was affirmed to be plasmid mediated (Seget *et al.*, 2005; Jayaprakashvel *et al.*, 2015). Former authors (Tewari, 2013; Al-Deeb, 2009) have reported plasmid mediated antibiotic resistance and heavy metal tolerance among the clinical as well as environmental (soil and water) bacterial isolates (Saumendra *et al.*, 2016).

Plasmid profiling determines the number and size of plasmid in bacteria signifying the correlation between heavy metal resistances in bacteria with plasmid profile (heavy metal resistance genes). Detailed information about pathogenicity of the bacteria have been established through the plasmid profiling technique (Growther *et al.*, 2012). To regulate the transmission structure of heavy metal resistance genes in bacterial cultures plasmid profiling was used widely as an epidemiological tool (Wonglumsom *et al.*, 2011).

II. Methodology

Isolation and screening of isolates

Water and soil samples were collected from different industrial effluent sites, bacteria were isolated on L-agar plates which were purified and screened for further process.

Resistance Profile

Different concentrations of chromium were made in L-broth media (to find their effect on the growth of bacterial strains) poured in test tubes, inoculated with (0.5 McFarland standard) purified test organisms and 24-hours incubation was given to the tube cultures with the two replicates prepared in the same manner at 37°C. After 24-hours, growth rate was measured by examining the volume of the isolates in tubes. The highly grown isolates were used for further process.

Biochemical Characterization

The bacterial isolates were identified microscopically, macroscopically and biochemically. Results were observed and documented.

Physiological Characterization

To examine the physiological characteristics of all the isolated cultures, Luria-broth was prepared, inoculated with chromium resistant isolates and incubated for different Time Periods (4, 8, 12, 16, 20, 24 hours), at different temperatures (24°C, 30°C, 37°C, 42°C), different pH (5, 6, 7, 8) levels and different chromium concentrations (100 µg/mL, 500 µg/mL, 700 µg/mL, 1000 µg/mL), to find their effects on the growth of isolates by measuring their optical density at 600 nm in spectrophotometer.

Chromium Reduction Potential

Chromium reduction potential of isolates was checked by Deleo and Ehrlich, (1994) method by using spectrophotometer. Cultures were grown in Luria-broth microfuge tubes with 500 µg/mL of hexavalent chromium for 24 hours and centrifuged for 10 minutes at 14,000 rpm. Then, 100 µL of supernatant formed in cultural broth tubes were taken in autoclaved test tubes (having 10 mL of distilled water, 1 mL of Di-phenyl carbazide, 1 drop of phosphoric acid), O.D was taken at different time periods, temperature, pH and different concentrations of chromium using spectrophotometer (540 nm).

Plasmid Profiling Technique

Plasmid Extraction

After confirmation of isolates, plasmid was extracted using Phenol: chloroform: Isoamyl alcohol (25:24:1) method. The selected bacterial isolates were inoculated in N-broth followed by incubation at 37°C for 24 hours. After incubation, overnight cultures (0.5 mL) were taken in a microfuge tube and projected to freeze thaw cycles (3 times). 0.5 ml of phenol: chloroform: Isoamyl-alcohol (25:24:1) was added in broth containing microfuge tube and vortexed (14,000 rpm for 1 minute). TE buffer (10 Mm TrisCl, 7.5, 1 Mm EDTA and pH 7.4) was used for saturating phenol prior to mixing with isoamyl alcohol and chloroform. Followed by the centrifugation at 12,000 rpm for 5 minutes, the upper aqueous phase (0.45 mL) was taken and moved to another microfuge tube containing 0.5 mL isopropanol, leaving the interphase undisturbed. The microfuge tube was thoroughly mixed before being centrifuged at 12,000 rpm for 5 minutes.

After, discarding supernatant, the pellet was reconstituted with 0.5 mL of 70% ethanol by carefully adding along the side of the microfuge tube, and centrifugation was performed for 10 minutes at 14,000 rpm. The supernatant

was again discarded, and the pellet was dried at room temperature. Then, 25 µL of deionized water was added to the dried pellet and stored at -20°C until required (Zhang and Cahalan, 2007).

Preparation of Agarose Gel

0.8% of agarose gel was prepared by dissolving 0.8 gm of agarose powder in 1X TAE (Tris-acetate-EDTA) buffer, making the final volume of 50 L. In a microwave oven, the solution was boiled in short shots of around 20, 10, and 10 seconds. After allowing it to cool at 55°C, 3 µL of ethidium bromide (50 µg/mL) was added to gel. Then the gel was poured into casting tray with combs already in place to make wells and it was allowed to solidify at room temperature.

Plasmid Profiling

The gel was removed carefully from casting tray after polymerization and placed in TAE buffer containing horizontal gel electrophoresis apparatus. Plasmid DNA (7 µL) was combined with 3 µL of loading dye to load the wells. To evaluate the size and number of plasmids in bacterial isolates, a DNA ladder (3 µL) of 1 Kbps was also loaded into the well. The electrophoresis apparatus was connected to a power supply, and current of 80 V was applied at room temperature for 45 minutes. The gel was removed from electrophoresis apparatus after the run time, and visualized under UV trans-illuminator and gel documentation system. The size and number of plasmid was determined by comparing with 1 kbps DNA ladder and results were recorded for further use (Farshad *et al.*, 2012; Wonglumsom *et al.*, 2011).

III. Results

Isolation and Screening of isolates

20 bacteria were isolated and purified; among them 10 bacteria were screened positive for heavy metal (chromium).

Resistant profile

Isolate n=10 (33.3%) M1, M2, M3, M4, M5, M6, M7, M8, M9 and M10 showed 100% (maximum) good growth in all concentrations of chromium.

Biochemical Characterization

Isolates (Chromium resistant) were identified biochemically, microscopically and macroscopically. All bacterial isolates (100%) were gram positive purple cocci (chains, clusters and diplococci), whereas presence of *Staphylococcus aureus* and *Staphylococcus epidermidis* species were identified microscopically and macroscopically.

Physiological Characterization

Physiological characterization of chromium resistant isolates was done by checking the effect of different time periods (4th, 8th, 12th, 16th, 20th and 24th hours) (Figure 1), different temperatures (24°C, 30°C, 37°C, 42°C) (Figure 2), different pH (5, 6, 7, 8) (Figure 3) and different concentrations of chromium (100 µg/mL, 500 µg/mL, 700 µg/mL, 1000 µg/mL) (Figure 4) on the growth of chromium resistant isolates. Almost all of the isolates (100%) showed

maximum growth at 24th hour of incubation, 37°C of temperature, 8 pH and 500 µg/mL concentration of chromium at 600 nm.

Figure1: Percentage growth of isolates with chromium at different time periods

Figure 2: Percentage growth of isolates at different temperatures

Figure 3: Percentage growth of isolates at different pH

Figure 4: Percentage growth of isolates at different concentrations of chromium (VI)

Chromium Reduction Potential

Percentage removal of chromium metal at different time periods (4, 8, 12, 16, 20 and 24 hours) was done by following Deleo and Ehrlich, 1994 method (Figure 5). Reduction potential of chromium was highest at 24th hour with average percentage of 43% followed by 20th (39%) and 16th hour (35%) and lowest at 4th hour of incubation, as the time passes the reduction rate increased to several folds. Results under mass spectrophotometer indicated that all of the isolates (under different temperatures and specific concentrations of chromium) showed maximum reduction percentage (average percentage of 58%) at 30°C followed by 37 °C (49%) as shown in Figure 6. Maximum removal % age of chromium among isolates (under different pH and specific concentration of chromium) was at pH 7 (56%) followed by pH 8 (44%) (Figure 7). Maximum reduction was determined under 500 µg/mL (average percentage =44%) concentration of Cr⁺⁶ followed by 700 µg/mL (average percentage =37%), under 1000 µg/mL of chromium, the reduction % age was also noticeable (average percentage =38%) (Figure 8).

Figure 5: Percentage chromium reduction at different time periods

Figure 6: Percentage chromium reduction at different temperatures

Figure 7: Percentage chromium reduction at different pH

Figure 8: Percentage chromium reduction at different concentrations of chromium

Plasmid Profiling

Plasmid profiling of bacterial isolates of industrial waste water and soil samples were performed and analyzed using agarose gel electrophoresis. The separation showed plasmid bands in different size and number as mentioned in Table 1.

Table 1: Plasmid profiles (number and size) of *Staphylococcus* sp.

Sample ID	No. of plasmids	Sample Size (base-pairs)				
		1	2	3	4	5
1	02	200	-	-	-	12,000
2	04	200	800	1,200	-	12,000
3	03	150	900	1,200	-	-
4	02	300	-	-	-	11,000
5	01	-	-	-	-	12,000
6	03	200	800	-	-	11,000
7	05	100	900	1,200	9,000	12,000
8	00	-	-	-	-	-
9	05	200	800	1,200	10,000	11,000
10	04	150	800	1,200	-	12,000

Selected bacterial isolates (n=10) were subjected to plasmid mediated analysis and out of 10 bacterial isolates, 9 (90%) were harboring the plasmid DNA. Many isolates were containing the same number and size of plasmid. Average plasmid number in selected bacterial isolates was 2.9 ranging from 0 to 5. The highest number of plasmid was recorded in isolate 9 and 7 which was 5. Only one isolate (10%) was harboring only 1 plasmid, two isolates (20%) had 2 plasmids, two isolates (20%) showed 3 plasmids, two isolates (20%) had 4 plasmids, two isolates (20%) harbored 5 plasmids and just one isolate (10%) had no plasmid.

The size of plasmid was determined by comparing with DNA ladder (1 kbps). Plasmid DNA of different sizes ranging from 100-12000 bps were determined in selected isolates. The smallest plasmid size was 100 bps while the largest was 12000 bps which was observed in many bacterial isolates. A plasmid of 12000 kb was found in about half (50%) of the isolates, either alone or combined with other plasmid bands of different sizes. The plasmid bands of sizes 150, 9,000, 10,000 and 11,000 kb were also analyzed. Almost 50% isolates had plasmids of similar size i.e. 200, 1,200 and 12,000 bps. About 60% isolates showed small size bands of plasmids (i.e. 100 bps, 200 bps or 300 bps). Plasmid bands of 800 bps, 900 bps, and 1200bps sizes were also found in various combinations in the isolates.

Relationship of Plasmid Mediated Studies with Heavy Metal Resistance

The relationship of current plasmid profiles and heavy metal resistance of selected bacterial isolates against different concentrations of chromium was determined, using current data collected on heavy metal resistance. Out of 10 *S. aureus* and *S. epidermidis* isolates, plasmid number 0 (10%) and 1 (10%) was observed in two isolates separately, showing moderate to high resistance profiles respectively. Two isolates (20%) were highly resistant to different concentration of chromium (100 µg/mL, 500 µg/mL, 700 µg/mL, 1000 µg/mL) and were harboring two plasmids. Bacterial isolates (20%) were containing 3 plasmids and resistant to high concentration of chromium (500 µg/mL).

The heavy metal resistance was in elevation against heavy metals (Cr^{+6}) in only two bacterial isolates (20%) and plasmid copy number was 4 while bacterial isolates (20%) were resistant to different concentrations of chromium and plasmid number was 5. The plasmid copy number in current study was varying from isolate to isolate while the resistance pattern of heavy metal (Chromium) was almost same. All these bacterial isolates had different plasmid size (bps).

Overall plasmid profiles revealed that heavy metal resistance was plasmid or may be chromosomal mediated in some isolates. However, no significant relation was observed in heavy metal resistance with plasmid profiles.

IV. Discussion

Chromium present in numerous oxidation statuses (+2 to +6), but the most constant forms are Cr (0), Trivalent and Hexavalent chromium. Its toxicity makes it a primary environmental pollutant certainly present in air, water, soil and foodstuff (Avudainayagam *et al.*, 2003). Cr (VI) is a potent skin irritant and carcinogenic (Park *et al.*, 2004) for living organisms (Gonzalez *et al.*, 2003), poisonous for herbs (plants), aquatic creatures (Velma *et al.*, 2005) and microbes.

Hexavalent Chromium is 1000 times more toxicant than Trivalent Chromium due to its impermeability into cell membrane. The biological reduction of Cr (VI) to Cr (III) has been measured as a substitute procedure for the handling of Cr (VI) contaminated wastes due to its insolubility, which allows for its precipitation and elimination (Cervantes *et al.*, 2001). Range of bacterial species have been stated, accomplished to reduce Hexavalent Chromium to Trivalent Chromium, comprising *Bacillus* sp. (Elangovan *et al.*, 2006; Liu *et al.*, 2006), *E. coli* (Bae *et al.*, 2005), *Microbacterium* sp. (Pattanapitpaisal *et al.*, 2001a), *Staphylococcus* sp. (Rajbanshi *et al.*, 2008) etc.

Staphylococcus sp. have been found abundantly in both water and soil, resistant to several classes of antimicrobial agents (Tavares and A.L., 2014). Antibiotic and heavy metal resistance genes can be found on plasmids and transposons, and they can be passed from one *Staphylococcal* species to another, as well as among Gram-positive bacteria (Werckenthin *et al.*, 2001). Using a group of enzymes known as chromium reductases, *Staphylococcus* has ability to reduce chromium in both aerobic and anaerobic environments. Under aerobic conditions, Cr (VI) reduction is accomplished by soluble elements which use NADH as electron donor (Ackerley *et al.*, 2004).

In recent study, the screened isolates were positive for chromium reduction potential (CRP). The CRP was determined by 1, 5-diphenylcarbazide method (Park *et al.*, 2000). The categorization and optimization of different parameters such as different chromium concentrations, time period, pH and temperature was carried out to maximize growth and CRP of each isolate individually. During the present study the effect of different time periods, temperature, pH and different concentrations of chromium on the growth of chromium resistant bacteria was also checked. The maximum growth of chromium resistant isolates was observed at 24th hour of incubation period, 37°C temperature with pH 8 and 500 µg/mL concentration of chromium.

Current study also estimated reduction potential of chromium resistant bacteria with varying pH, temperature, time period and concentrations of chromium. Results of present study indicated that the preferable time period for chromium reduction potential was 24 hours of incubation, notable temperature recorded for CRP was 30°C, and 7 was the suitable pH for chromium reduction potential, whereas 500 µg / mL was the desirable concentration of chromium for chromium resistant bacteria.

Plasmid profiles of present study showed average plasmid number 2.9 ranging from 0-5. Highest plasmid number harboring bacteria was 5, while the molecular size was determined using DNA ladder, ranging from 100-12000bps Overall, no consistent relation was found among plasmid profiles with heavy metal resistance.

V. Conclusion

Current study indicated that to reduce chromium (VI) to chromium (III) the preferable time period was 24 hours of incubation, notable temperature recorded for CRP was 30°C, 7 was the suitable pH, whereas 500 µg/mL was the desirable concentration of chromium for chromium resistant bacteria. Whereas, plasmid profiles revealed that plasmids were randomly distributed among the bacterial isolates. Overall, no defined relationship was observed among resistance pattern and plasmid mediated profiles. The current data is consistent with limited number of samples and bacterial isolates, more study and research is required to reveal the patterns.

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