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Original Article

Effect of nickel on biofilm formation of Halophilic bacteria isolated from *Achyranthus aspera* from salt range Pakistan

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Abstract

When exposed to heavy metals or toxic compounds, bacteria have multiple survival strategies involving several mechanisms of resistance and tolerance. Biofilm formation is one such resistance mechanism for bacterial survival. This paper provides the basis for use of bacteria isolated from saline environment and to check their biofilm forming ability under nickel stress. Our previous results showed that these bacteria are capable of producing proline, glycine betaine, choline and other osmolytes under NaCl stress. On the basis of those results it is hypothesized that this osmolyte production ability may help them in nickel resistance under Ni salt stress. This study was centered on assessing the adherence, motility, hydrophobicity, aggregation and biofilm formation of two salt tolerant strains [*Bacillus subtilis* and *Halomonas aquamarina* (1)] in the presence of nickel. Two concentrations of nickel were used *i.e.,* 500µg/ml and 1000µg/ml for subsequent tests. The results revealed that at low/ sub-inhibitory concentration of nickel (500 µg/ml), motility is inhibited; adhesion, hydrophobicity, aggregation, EPS production and biofilm formation of two strains are increased. This biofilm forming ability can be employed in purification of waste waters and treating contaminated soils.

Key words: Nickel, biofilms, Aggregation, SAT (Salt aggregation test), BATH (Bacterial adhesion to hydrocarbons)

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INTRODUCTION

Biofilm is a well-organized community of microbes attached on a solid material and sheathed in an exopolysaccharide matrix. Biofilm can be formed by single bacterial species or can be made from aggregation of different microbes. Biofilm is formed in aquatic environment by microbes attaching to submerged surfaces and to the air/liquid interface and to each other. Biofilms are attached by microbial appendages like flagella and fimbriae or by the production of exopolysaccharide, proteins and DNA (Donlan, 2016). Biofilm formation is prevailing in bacterial lifestyle. It plays substantial roles such as providing shelter in the harsh environment, attaining unique genetic traits, nutrient accessibility and resistance to antimicrobial compounds and heavy metals. EPS acts as a barrier to physically prevent the entry of various antimicrobial compounds into biofilms (Alexander and Schiesser, 2017).

Both Gram positive and negative bacteria are known to form biofilms. The mechanisms of biofilm formation vary among species. Biofilms of halophilic microorganisms have been known to be employed in the treatment of hyper-saline waste waters. The studies have demonstrated the heterotrophic, halophilic organisms used to remove phenol from synthetic waste brine containing 15% salt and for the degradation or transformation of organic pollutants (Murray *et al.*, 2016).

Heavy metals are widely distributed in the environment due to un-controlled anthropogenic actions, like mining and smelting, and also through industrial waste and irrigation practices (Nocelli *et al.*, 2016). People are not well aware of nickel, as it is majorly employed in preparing alloys which are useful in chemical and food industry, because of heat and corrosion resistance. Nickel metal is found two states *i.e.,* in the Ni(0) or Ni(II) state because they are water stable (Macomber and Hausinger, 2011). Nickel is found to be toxic to humans, plants and microorganisms (Macomber and Hausinger, 2011; Blewett and Leonard, 2017). In microorganisms, nickel toxicosis is problematic to such cells which are either

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natural inhabitant of nickel-contaminated soils or are exposed to industrial pollution (Azizi *et al.*, 2016; Blewett and Leonard, 2017). At trace levels, however, nickel is one of the important micronutrients for microorganisms where it plays a significant role in a variety of cellular mechanisms such as metalloregulation, structural stabilization, electron transfer, substrate/cofactor coordination, and catalysis (Oorts *et al.*, 2007; Macomber and Hausinger, 2011). Microorganisms containing nickel-dependent proteins have evolved the mechanisms of nickel uptake (Oorts *et al.*, 2007; Macomber and Hausinger, 2011; Zeer-Wanklyn and Zamble, 2017). They also contain nickel efflux and detoxification mechanisms to maintain the homeostasis of nickel metals (Costa and Tavares, 2016; Zeer-Wanklyn and Zamble, 2017). Although cells try to maintain the low intracellular concentrations of nickel, this homeostasis can be disrupted by the excess of nickel and it leads to the displacement of necessary metals (Zhang *et al.,* 2016).There are four general hypotheses of nickel toxicity to microorganism: first is replacing metal from metallo-proteins, second is allosteric inhibition by attaching to catalytic site of enzymes, third is binding to non-metallo-enzymes, fourth is damaging molecular machinery of cells (DNA, proteins and lipids) by creating oxidative stress (Aslan and Sozudogru, 2017). The purpose of the current study was to demonstrate the influence of Ni(II) on adherence, motility, hydrophobicity, aggregation and biofilm formation of previously isolated and identified salt tolerant bacterial strains *i.e., Bacillus subtilis* and *Halomonas aquamarina* (1). In addition, combined stress of NaCl and Ni(II) on bacterial strains was also evaluated.

MATERIALS AND METHODS

Bacterial strains and growth requirements

Two previously isolated and identified salt tolerant bacterial strains, (*Bacillus subtilis,* accession no. GU057987; Tolerate NaCl 2M. (Mehr *et al.*, 2002) and (*Halomonas aquamarina* (1) accession No. HQ107978 1m (Hasnain, 1998) were used in this study. L-agar containing 0.5 M NaCl was used for maintaining bacterial cultures at 37℃.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is the minimum concentration of metal or any toxic compound which inhibits the growth of bacteria. MIC of nickel metal was determined by inoculating 100 µl of 24 hours old standardized culture (adjusted to 0.5 absorbance at 600 nm) to 5ml LB-broth tubes containing different concentrations of nickel (0, 100µg-10,000 µg Nickel concentrations). The positive control of each strain, containing no metal stress was prepared by inoculating the medium with 100 µl of standardized cultures. All the tubes were incubated at 37℃ for 24 hours and their optical density was measured spectrophotometrically (IRMECO model 2020 UV visible spectrophotometer) at 600nm by using respective negative control as a blank.

Combined effect of nickel and salt (NaCl)

The combined effect of nickel and salt (NaCl) on the growth of both bacterial strains was observed by inoculating 100 µl of the 24h old incubated standardized bacterial cultures (OD adjusted to 0.5 A at 600nm). Varying concentrations of NaCl (0.08, 0.5, 1, 1.5 and 2M) and $NiCl_2$ (0, 500 µg/ml) were used. The tubes were incubated at 37^0C for 48 hours and their optical densities measured spectrophotometrically at 600nm against respective negative controls.

Biofilm studies

Slime production assay

The two bacterial strains were checked for their slime producing potential by using Congo-red medium (Kaiser *et al.*, 2013). Along with test strains, positive control (*Halomonas campaniensis* GU057990) (Hasnain, 1998) was also streaked, and the plates were given an incubation of 24 hours at 37℃. The bacteria producing slime layers give black colonies with Congo-red.

Cell motility assays

Swimming, swarming and twitching motilities were investigated in the absence and presence of nickel (1000 µg/ml Ni) followed by Di Bonaventura (Di Bonaventura *et al.*, 2008) with some modifications. The individual colonies from an agar growth were transferred to the surface of swimming agar and swarming agar by using a sterile inoculating needle. After incubation for 48 h, diameter (mm) of the halo of growth formed around the point of inoculation was measured. Twitching agar was inoculated, by using a sterile needle, to the bottom of the Petri plate. After incubation for 48 hours, the diameter (mm) of migration and growth at the

agar/Petri dish interface was measured by staining with crystal violet.

Cell Aggregation Assay

Co-aggregation assay

The Nagaoka co-aggregation assay was performed (Nagaoka *et al.*, 2008) using two strains *i.e., B. subtilis* (HAa2) and *H.aquamarina* (RT-2). To demonstrate the effect of nickel on co-aggregation ability of the each strain, 500 and 1000µg/ml nickel was added. The co-aggregation was calculated in percentage by using following formula:

$$Co-aggregation(\%) = \frac{Absorbanceatt0 - Absorbanceatt90}{absorbanceatt0} \times 100$$

Auto-aggregation

In order to determine the aggregate forming abilities of dissimilar cells of *B. subtilis and H. aquamarina***,** auto-aggregation assay (Arenas *et al.*, 2015) was carried out. For examining the consequence of nickel on auto-aggregation potential of the strains, 500 and 1000µg/ml nickel chloride was added. The auto-aggregation was calculated in percentage by using the following formula:

$$Auto-aggregation(\%) = \frac{Absorbanceatt0 - Absorbanceatt90}{absorbanceatt0} \times 100$$

Biofilm formation Assays

Microtiter plate assay

Biofilm formation was monitored by 96 well microtiter plate assay with modifications (Alonso *et al.*, 2014). Both strains were grown in LB broth and their OD was adjusted to an absorbance of 0.5 at 600nm. Nickel concentration of 1000µg/ml was used for each strain. In 96 well PVC -microtiter plates, each well was inoculated with 200µl of metal and cell suspension. L broth and L broth+ nickel were used as controls and the plates were given an incubation of 2, 3, 5 and 7 days at 37℃.

Fluorescent Microscopy

Biofilm formation of *B. subtilis* and *H. aquamarina* on glass slides was investigated by fluorescent microscopy using the method followed by Liaqat and Sabri (2008) with slight modifications. The glass slides were immersed in the flasks containing culture media and 1000µg/ml concentration of Ni^{+2} and were incubated for 48, 72, 120 and 168 hours.

Cell surface hydrophobicity tests

Bacterial adhesion to hydrocarbons (BATH):

BATH test was performed following the protocol of Di Martino with few modifications (Di Martino *et al.*, 2014). In this test, xylene was added to standardized bacterial culture and their hydrophobicity was calculated by using the following formula:

$$Adherence(\%) = \frac{Absorbance\ 0 - Absorbance\ 1}{absorbance\ 0} \times 100$$

The strains were assessed as follows: >50% strongly hydrophobic; 20-50% moderately hydrophobic; <20% Hydrophilic

Salt aggregation test (SAT)

SAT test was performed following the methodology of Rosenberg using a series of ammonium sulphate concentrations (0.1, 0.2, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 M) on microscope slides (Rosenberg, 2006). The results were expressed as the lowest molarity of ammonium sulfate causing bacterial aggregation. The strains were assessed as follows: <0.1 M Strongly hydrophobic; 0.1-1 M Moderately hydrophobic, >1 M Hydrophilic.

Effect of varying nickel concentrations on planktonic (PC), loosely bound cells (LBC) and tightly bound cells (TBC):

The effect of nickel stress on PCs, LBCs and TBCs was qualitatively (ring test) and quantitatively (crystal violet quantification) assayed using a method followed by (Liaqat and Sabri, 2009).

Isolation and purification of exopolysaccharide (EPS):

EPS extraction:

EPS produced by the two bacterial strains was extracted following the procedure of Verhoef (Verhoef *et al.*, 2005). The influence of nickel on EPS production was evaluated by growing the bacterial cultures in the medium supplemented with 500 and 1000 µg/ml Ni^{+2}.

FT-IR spectroscopy of EPS

The purified EPS samples were analyzed via Fourier transform infrared spectrophotometer (Beacon field Buckinghamshire HP9 1QA).The EPS powder (2mg) was mixed with potassium bromide (200mg) and the mixture was pressed in the frame of 3mm. The discs obtained were placed under infrared spectra in the frequency range of 400– 4000cm^{-1}at 4 cm^{-1} resolution and16 scans.

Protein estimation of EPS

Bradford assay (Zhou *et al.*, 2014) was performed for the estimation of protein content present in the EPS of *Bacillus* (HAa2) and *Halomonas* (RT-2) under controlled and nickel stressed conditions. Standard curve of Bovine serum albumin (BSA) was used to calculate the amount of protein content in EPS samples.

Carbohydrate estimation of EPS

The phenol-sulfuric acid method (Hall, 2013) was performed to determine the total carbohydrate content present in EPS samples both in the presence and absence of nickel stress. The amount of total carbohydrate present in the EPS was estimated via glucose standard curve.

RESULTS

Minimum Inhibitory Concentration

The lowest nickel concentration that inhibited the growth of *H.aquamarina* and *B. subtilis* in LB broth was 2000 and 3000µg/ml, respectively (Fig.1).

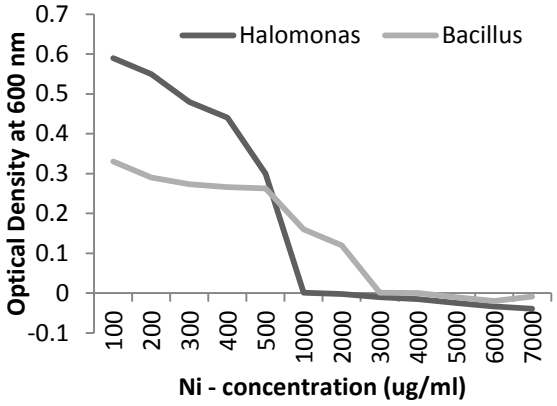

Figure 1: Minimum inhibitory concentration of nickel for *B. subtilis* (HAa2) and *H. aquamarina* (RT2).

Combined effect of nickel and NaCl

Under combination of nickel and NaCl, both strains (i.e. *H. aquamarina* and *B. subtilis*) showed best growth at 0.5M NaCl and 500 µg/ml Ni^{+2}. Further increase in combined stress had an antagonistic effect on bacterial growth (Fig. 2).

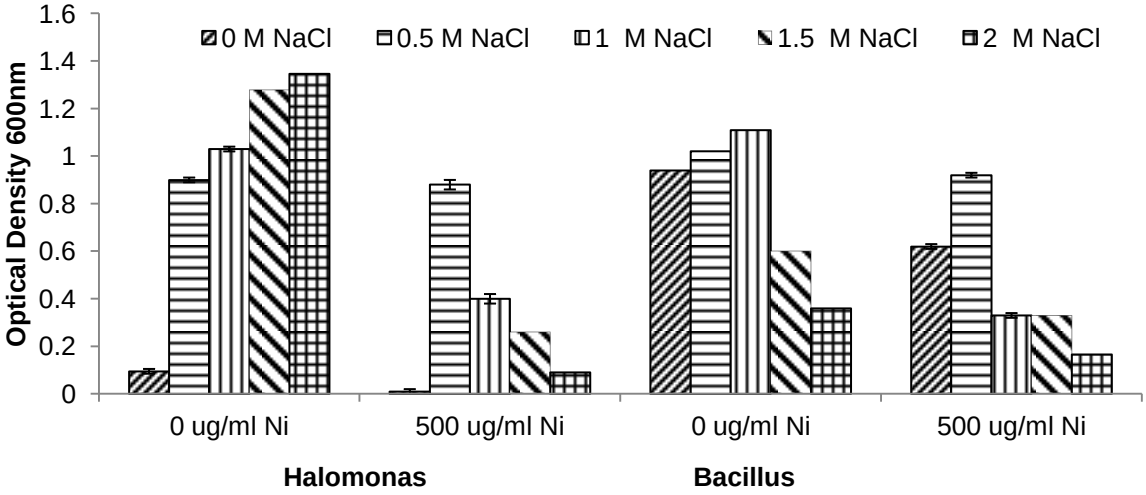

Figure 2: Growth of *H.aquamarina* (RT2) and *B. subtilis* (HAa2) under double stress of NaCl and 500µg/ml Ni+2

Slime production assay

Both test bacterial strains (i.e. *H. aquamarina* and *B. subtilis*) formed red colored mucoid colonies on the Congo red medium whereas the control (*Halomonas campaniensis* GU057990) showed black colored dry colonies on the medium. Presence of nickel did not seem to affect the slime producing ability of both the strains.

Cell motility assays

Both *H. aquamarina* and *B. subtilis* showed significant swimming, swarming and twitching motilities in the absence of nickel stress but in the presence of 1000 µg/ml nickel, swimming and swarming motilities totally vanished whereas the twitching motility was detected but at reduced intensity (Fig. 3).

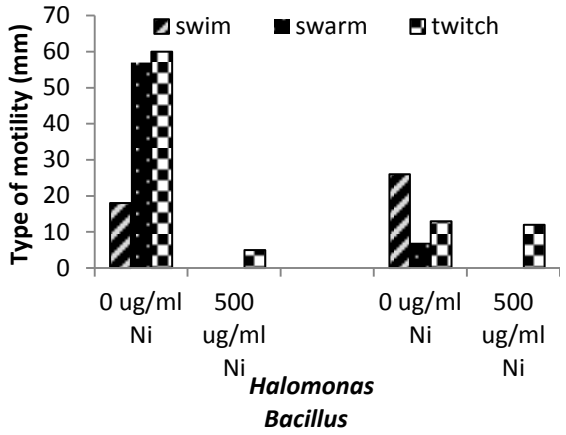

Figure 3: Swimming, swarming and twitching motilities of *H. aquamarina* (RT2) and *B. subtilis* (HAa2) in the absence and presence of nickel stress (500µg/ml Ni(II))

Co-aggregation assay

When *H. aquamarina* and *B. subtilis* were cultured in the absence of nickel, 12% of the cells co-aggregated. While, when the concentration of nickel was raised to 1000µg/ml, the percentage of co-aggregation increased up to 16%.

Auto-aggregation

In case of *H. aquamarina*, autoaggregation of cells was 0.9% but under stress of 500µg/ml nickel it increased up to 12.9% while decreasing at 1000µg/ml to 6%.Auto-aggregation in case of *B. subtilis* was 0.84%, 16.5% and 5.04% at 0,500 and 1000 µg/ml of Nickel, respectively.

Microtiter plate assay

Biofilm formation of *B.subtilis* was much thicker as compared to *H.aquamarina* both under control and nickel stressed environment but 500 µg/ml nickel was found to promote the biofilm formation of both strains up to 120 hours after which it started deteriorating. At 1000 µg/ml nickel, biofilm formation of both strains was decreased (Fig. 4).

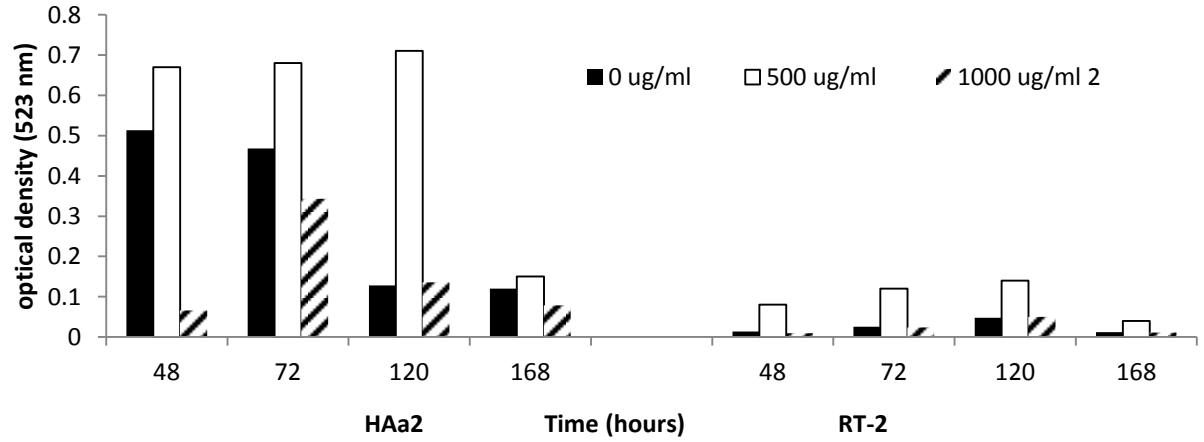

Figure 4: Biofilm formation of *H. aquamarina* (RT2) and *B. subtilis* (HAa2) after 48, 72, 120 and 168 hours of incubation with and without nickel stress

Fluorescent Microscopy

Biofilm formation of both the strains increased with incubation time and was much thicker at 120 hours. The biofilms formed under 500 µg/ml nickel were thick compared to those under 0 and 1000µg/ml nickel (Fig. 5).

Bacterial adhesion to hydrocarbons (BATH)

Both the strains showed hydrophobic nature in BATH test and nickel did not seem to affect their hydrophobicity.

Salt aggregation test (SAT)

Both strains were found to be hydrophobic in nature as they showed aggregations at concentration of 0.1M $(NH_4)_2SO_4$ irrespective of nickel treatment.

Effect of varying nickel concentrations on planktonic, loosely attached and tightly bound cells/biofilm growth

Qualitative test (ring test)

Ring test indicated that in both the strains the rings formed by biofilm-forming cells

were more profound and intense in the presence of 500 µg/ml nickel stress as compared to control. After 72 hours and 120 hours the width of rings decreased in *H. aquamarina* and *B. subtilis* respectively. No rings were observed in 1000µg/ml nickel stress.

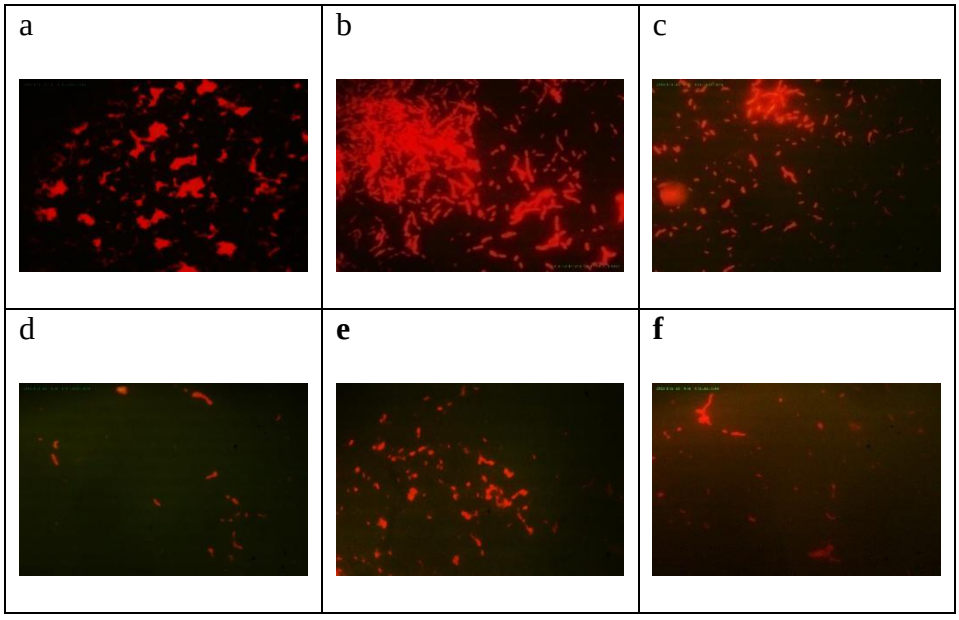

Figure 5: Fluorescent microscopy of *B. subtilis* (HAa2) (a, b, c at 0, 500 and 1000 µg/ml nickel at 120 hours) and *H. aquamarina* (RT2) (d, e, f at 0, 500 and 1000 µg/ml nickel at 120 hours)

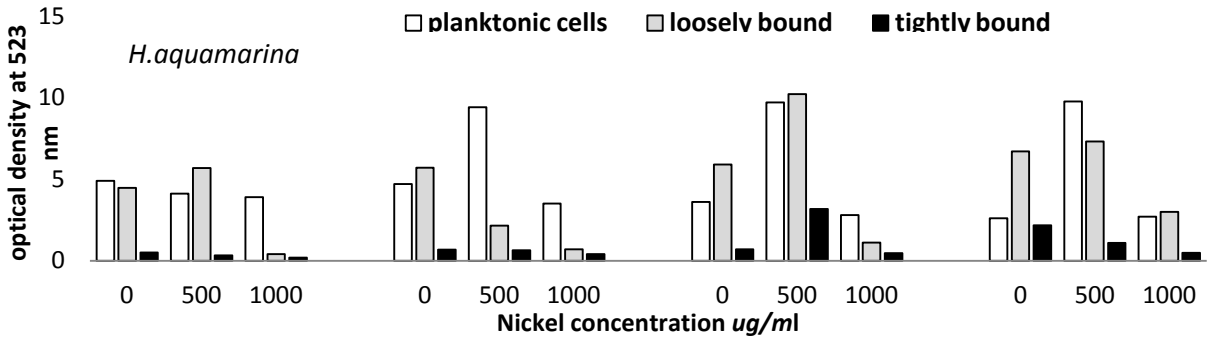

Figure 6: Crystal violet quantification of planktonic, loosely attached and tightly bound cells of *H.aquamarina* (RT2)

Quantitative test (Crystal violet quantification of planktonic, loosely attached and tightly bound cells)

PCs were larger in number as compared to LBCs and TBCs in case of *B. subtilis*. However, in *H. aquamarina*, loosely attached cells were more than planktonic and tightly adhered cells. Tightly bound cells, which are true biofilm formers showed a decline pattern after 72 hours in *B.subtilis* and 120 hours in *H.aquamarina* under 500 µg/ml nickel stress (Fig. 6-7).

EPS extraction

The dry weight of EPS was more in *B. subtilis* than that of *H. aquamarina*. The weight of the pellet under 500 µg/ml nickel concentration was more as compared to that of 0 and 1000 µg/ml.

FTIR of EPS

In the two strains, four significant peaks were observed and analyzed in the frequency range of 700 -4000 cm^{-1} (Fig.8). No or insignificant variation in functional groups or size/shape of peaks was observed in all the three systems (0, 500 and 1000 µg/ml nickel) of both strains

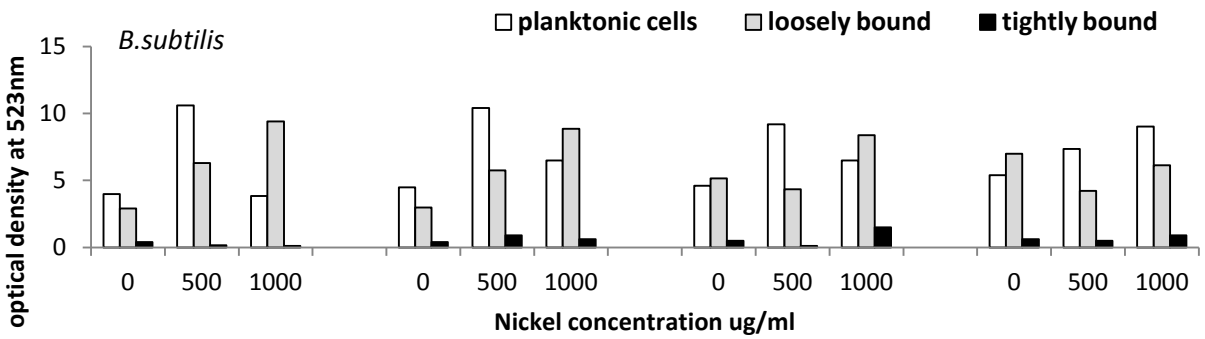

Figure 7: Crystal violet quantification of planktonic, loosely attached and tightly bound cells of *B. subtilis* (HAa2)

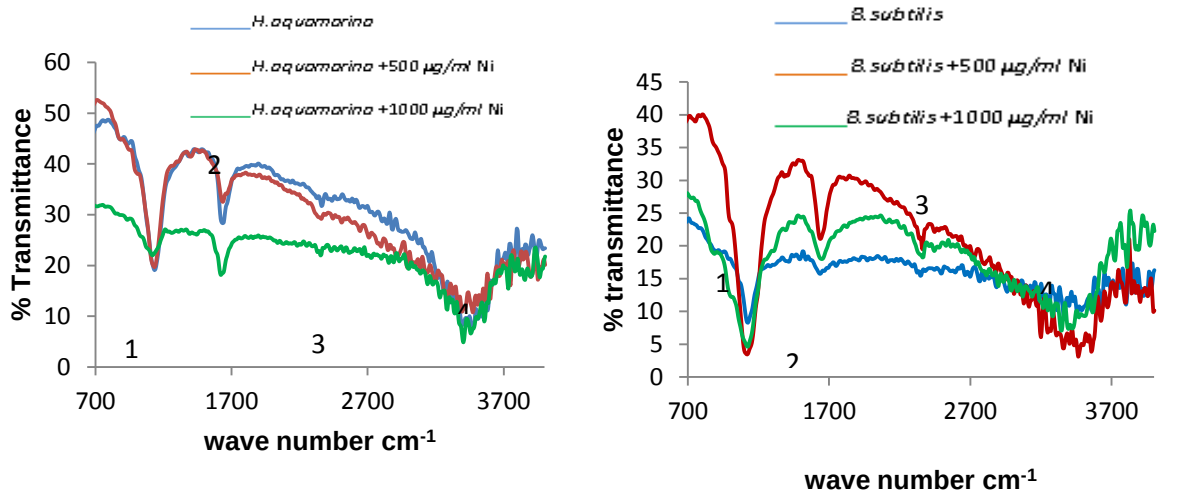

Figure 8: FTIR spectroscopic analysis of EPS of *H. aquamarina* (RT2) and *B. subtilis* (HAa2) with and without nickel stress

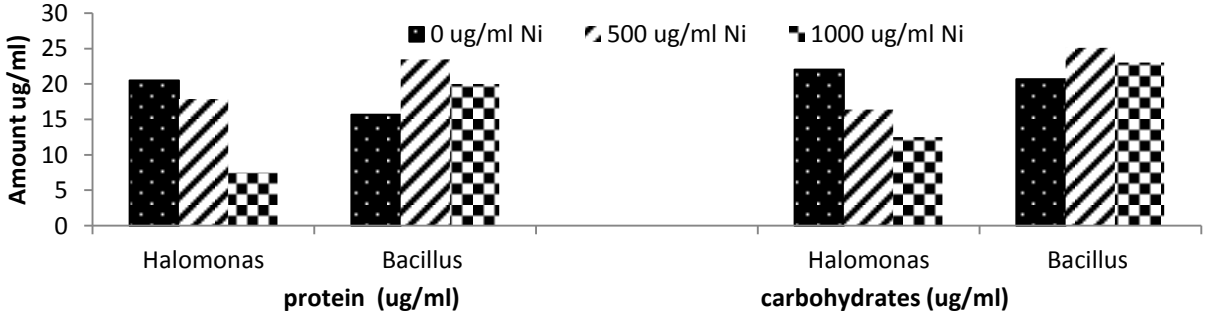

Figure 9: Carbohydrate and protein content in EPS of *H. aquamarina* (RT2) and *B. subtilis* (HAa2) without nickel stress

Protein estimation of EPS

Bradford analysis of protein quantification indicated that the protein content of EPS of *H. aquamarina* decreased with increasing nickel stress. Whereas in case of *B. subtilis*, the protein content was found to be more in the presence of 500 µg/ml nickel stress as compared to controlled and 1000 µg/ml nickel stressed conditions (Fig.9).

Carbohydrate estimation of EPS

By comparing the absorbance values of carbohydrate of EPS sample with glucose standard curve indicated that the carbohydrate content in *H. aquamarina* was less as compared to that of *Bacillus* both under control and stress conditions (Fig. 9).

DISCUSSION

Heavy metals are ubiquitous in the environment due to various industrial processes and human activities. Heavy metals not only affect the plants, animals and humans but also the microbes. Bacteria have developed many resistance and tolerance mechanisms in order to cope with the toxic effects of heavy metals such as efflux systems, changes in membrane permeability and reduction to less toxic form. Biofilm formation is also one of the resistance mechanisms for survival in such toxic environments (Sabri *et al.*, 1995; Yu *et al.*, 2017a). In order to check the effect of nickel on halophilic bacteria, we choose two previously isolated and identified bacterial strains *B. subtilis* and *H. aquamarina* that have the ability to thrive in saline environment. The MIC test is a powerful assay to determine the resistance of microbes to antimicrobial agents. The results indicated that the lowest nickel concentration that inhibited the growth of *H. aquamarina*and *B. subtilis* was 2000 and 3000 µg/ml, respectively. It has been reported elsewhere that high metal concentration inhibits the microbial growth (Yu *et al.*, 2017b). Nickel is a trace element required by many microorganisms at low concentrations for the activation of enzymes. At higher concentrations, nickel is proved to be toxic to microorganisms as it replaces the essential metal of metallo-proteins and allosterically inhibits the enzymes (Macomber and Hausinger, 2011).The combined effect of nickel metal and NaCl on the growth of both bacterial strains showed marked growth at 0.5M NaCl and 500 µg/ml Ni^{+2}. Several studies have indicated that metal toxicity is influenced by both abiotic (pH, osmotic pressure, temperature, ionic and chemical composition) and biotic factors (pigmentation, encapsulation, physiological age, morphological state and nutritional state of bacteria). In this study, we have observed that by increasing the salt concentration upto 0.5M, nickel toxicity in bacterial strains decreased which might be due to the osmoregulation mechanism present in these bacteria under salt stress. As it is previously reported (Qurashi and Sabri, 2012) these bacteria are capable of producing endogenous osmolytes (proline, gylcine, gylecin betain) in presence of NaCl this might provide resistance against nickel as well.

Biofilm formers stick to the nearby surfaces by self-made mucoid layers, which gives dark black color with Congo red (Qurashi and Sabri, 2012). Both the test bacterial strains (*i.e., H. aquamarina* and *B.subtilis* formed red colored mucoid colonies on the Congo-red medium whereas the control showed black colored dry colonies on the medium. Presence of nickel did not seem to affect the slime producing ability of both the strains. Presence of mucoid red to orange or pink colonies indicates that both of the strains are weak slime producers. On contrary, these strains were previously reported to be good biofilm formers. Congo-red agar method was used to check the slime producing ability of the strains, which is considered as a less sensitive method for detecting slime production (García *et al.*, 2004). In addition, it has been found that many microorganisms require specific nutrients such as glucose for the efficient production of slime (Christensen *et al.*, 1982; Singh *et al.*, 2014).

Biofilm formation is influenced by cell surface properties and appendages but it is not always co-related with motility. It has been reported that in *Bacillus, Pseudomonas, Vibrio*, and *Escherichia,* motility to biofilm transition involves either the inhibition of flagellar rotation or flagellar gene transcription (Guttenplan and Kearns, 2013). Motility assays revealed that both the strains showed significant swimming, swarming and twitching motilities without nickel stress but with nickel, swimming and swarming motilities were totally vanished whereas the twitching motility was still there in case of nickel stress but with reduced intensity. So collectively we can say that nickel affected the motility of both the bacterial strains. These results are in accordance with many research studies which indicated that reactive oxygen species produce by heavy metals reduces the motility in microorganisms (Yu *et al.*, 2017a). It might also be due to the inhibition of motility genes as mentioned above (Kao *et al.*, 2014).

Auto-aggregation is the adherence between genetically identical bacterial species. The present work revealed that in case of *H. aquamarina*, percentage auto-aggregation was maximum at the nickel concentration of 500 µg/ml which was reduced to half when the nickel concentration increased to 1000µg/ml. In the

absence of nickel stress the auto-aggregate forming ability was very low. In case of *B. subtilis*, almost same trend of aggregates forming ability was observed as that of *H. aquamarina*. Lower nickel concentrations were reported to encourage the adhesion properties of bacteria by activating various genes expressing appendages (Perrin *et al.*, 2009). Co-aggregation is a cell to cell adherence between different bacterial species. Bacterial cell surface contain many adhesion proteins and carbohydrates which promote co-aggregation between different cells (Moons *et al.*, 2009).

The present study revealed that in the absence of nickel, percentage co-aggregation was 12% which was decreased up to half in the presence of 500µg/ml nickel stress. Furthermore, the percentage co-aggregation was increased up to 16% by increasing the concentration of nickel to 1000µg/ml. Many researchers reported that under stress conditions, bacterial co-aggregation increases in some cells, whereas decreases in others depending upon the architecture of their cellular membrane (Sato and Nakazawa, 2014). So increase in co-aggregation by increasing the nickel stress may favor the two cells two adhere each other by altering their membrane architecture and or their quorum sensing genes because co-aggregation and multi specie biofilm formation are caused by synergistic interactions between different species (Burmølle *et al.*, 2006; Moons *et al.*, 2009) .

Microtiter plate assay and fluorescent microscopy revealed that biofilm formation of *B. subtilis* was much thicker as compared to *H. aquamarina* both under control and nickel stressed environment but 500 µg/ml nickel was found to promote the biofilm formation of both strains up to 120 hours after which it started deteriorating. It shows that nickel tolerance by biofilm formation act in a time-dependent manner. And 500µg/ml nickel was optimum concentration for biofilm formation of strains. When the concentration increased to 1000 µg/ml, biofilm formation was decreased. It has been reported by Perrin that lower (sub-inhibitory concentration) of nickel favors the biofilm formation in *E.coli.* Whereas when the nickel concentration increases (closer to inhibitory concentration), bacteria shut down their biofilm forming mechanism and uses other mechanism of metal resistance like efflux pumps (Perrin *et al.*, 2009).BATH and SAT tests were performed in order to check the hydrophobicity of the two strains. Both strains showed the hydrophobic nature in BATH and SAT test and nickel stress was found to increase their hydrophobicity. It has been reported with some bacterial species such as *Listeria monocytogenes* that cell surface hydrophobicity increases as the pH in the medium decreases (Briandet *et al.*, 1999). As nickel chloride is acidic in nature it renders the growth medium acidic and hence increased the cell surface hydrophobicity of *H. aquamarina* and *B. subtilis.* EPS consists of macromolecules such as polysaccharides, proteins, nucleic acids.EPS production is found to increase under many stress conditions which enable the bacteria to form biofilms by adhering to surfaces and other bacterial cells. FTIR analysis was performed to explore the functional groups of EPS. The FTIR spectra of *B. subtilis* and *H. aquamarina* with and without nickel stress have been shown in Fig. 7. The amide I and amide II groups are usually used as universal probes for proteins, whereas alcoholic, aldehyde, ketone, ether and carboxylic acid functional groups are usually used as representatives of polysaccharides (Wadood *et al.*, 2015).

The FTIR spectra of *B. subtilis* showed four prominent *peaks* in all the three systems. The first peak (1) is between 1000-1300 cm^{-1} wavelength which is specific for alcohols, ethers or phosphorylated proteins (C–OH, C–O–C) (Wadood *et al.*, 2015). Another peak (2) was observed between 1500–1690cm^{-1} which suggests the presence of amide-1 (secondary amides) (C=O) (Wadood *et al.*, 2015). Moreover peak (3) between 2300 and 2800 cm^{-1} was also observed which indicates the presence of carboxylic acid (COO–H) in EPS. The fourth peak observed was in the range of 3000 – 3600 cm^{-1} which indicates the presence of alcohols and absorbed water molecules (Wadood *et al.*, 2015; Tareb *et al.*, 2017).

In case of *H. aquamarina,* in all the three systems, the first peak (1) is between 1000-1300 cm^{-1} wavelength which is specific for alcohols, ethers or phosphorylated proteins (C–OH, C–O–C). Another peak (2) was observed between 1500–1690cm^{-1} which suggests the presence of amide-1 (secondary amides) (C=O) (Tareb *et al.*, 2017). Moreover peak (3) between 2300 and 2800 cm^{-1} was also observed which indicates the presence of carboxylic acid (COO–H) in EPS (Tareb *et al.*, 2017). The fourth peak observed was in the range of 3300 – 3500cm^{-1} which indicates the presence of Amides, amines or adsorbed water molecule NH/OH (Wadood *et al.*, 2015; Tareb *et al.*, 2017). In both bacteria,

carbohydrate and protein functional groups were more prevalent.

It has been reported in cyanobacteria and *E.coli* that under low (sub-inhibitory) nickel concentration, EPS production increases which allows the bacteria to adhere to each other and make bacterial communities. Further increase in nickel concentration resulted in decrease EPS production in cyanobacteria and *E.coli.*(Singh *et al.*, 1999; Perrin *et al.*, 2009; Ohki *et al.*, 2014).

Bradford analysis of protein quantification indicates that the protein content of EPS of *H. aquamarina* decreased with increasing nickel stress. Whereas in case of *B.subtilis*, the protein content was found to be more in the presence of 500µg/ml nickel stress as compared to controlled and 1000µg/ml nickel stressed conditions. The phenol-sulfuric acid method indicated that the carbohydrate content in *H. aquamarina* was less as compared to that of *B. subtilis* both under control and stress conditions. In *Halomonas,* the carbohydrate content was found to be decreased with increasing nickel stress. In case of *Bacillus*, the nickel stress was found to increase the carbohydrate contents of EPS.

It has been testified that the quantity and composition of EPS can be changed by heavy metal toxicity (Pereira *et al.*, 2011). Crystal violet quantification revealed that both *B. subtilis* and *H. aquamarina* have low affinity with glass as compared to PVC microtiter plate, because the biofilm formation at plastic surface was much profound as compared to that of glass both in the absence and presence of nickel stress. It has been reported that hydrophobic bacteria have much affinity to bind to the PVC microtiter plate. Hydrophobic interactions are considered the main forces for hydrophobic surfaces such as PVC, and, thus, cell wall hydrophobicity of strains plays a major role (Rendueles *et al.*, 2014).

Conclusion

In conclusion we can say that at low/ sub-inhibitory concentration of nickel (500 *µg/ml*), motility is inhibited; adhesion, hydrophobicity, aggregation, EPS production and biofilm formation are increased. This suggests biofilm as an early cell adaptation to sub-inhibitory concentration of Nickel. The future perspective of this study is to increase the amount of EPS of these two halotolerant strains and utilize them in waste water treatment and metal contaminated sites for the removal of heavy metals.

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Conflict of interests

The authors declare that they have no conflict of interest

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