Heavy Metal Tolerance of *Klebsiella pneumoniae* Kpn555 Isolated from Coffee Pulp Waste

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ABSTRACT

Microorganisms gain the ability to tolerate complex pollutants such as heavy metals, pesticides and polyaromatic hydrocarbons on continual exposure to them. The ability of indigenous microorganisms in tolerance and degradation of persistent xenobiotics has been frequently exploited. Coffee pulp waste is known to contain trace amounts of heavy metals attributing to residues from agro industrial activities. In the present study, a Gram negative non motile rod shaped bacterium, isolated from coffee pulp waste, showed tolerance to heavy metals such as cadmium (Cd), lithium (Li) and mercury (Hg). It was identified as *Klebsiella pneumoniae* Kpn555 by 16s RNA sequencing. The bacterium showed a minimum inhibitory concentration of 150 mg/L, 250 mg/L and 10 mg/L of Cd, Li and Hg, respectively. When the bacterium was grown in nutrient broth supplemented with concentrations of Hg (5 and 10 mg/L), Cd (50 and 100 mg/L) and Li (100 and 200 mg/L), it was observed that the growth reduced with the increase in concentration of heavy metals. The residual heavy metal concentration in the cell free supernatant was determined and the percentage of reduction in the concentration of heavy metals was calculated. It was observed that the cell free broth had a reduction of 54.8%, 50.6% and 40.6% of Li, Cd and Hg, respectively. This means that the bacterium has adsorbed the heavy metals from the medium onto their cell wall or inside the cytoplasm. This study revealed that *K. pneumoniae* Kpn555 has multi heavy metal resistance which could be utilised for bioremediation of soil and water polluted with multiple heavy metals.

Keywords: *K. pneumoniae* Kpn555, cadmium, lithium, mercury, bioremediation

INTRODUCTION

Land and water are precious natural resources that rely on the sustainability of agriculture and the civilization of mankind. Unfortunately, they have been subjected to maximum exploitation and are severely degraded or polluted due to anthropogenic activities. In a developing country like India, water pollution is a grave issue that affects the population as more than 70% of the available water is polluted by various organic and inorganic pollutants (Goyal *et al.,* 2015). Contamination by heavy metals is the most serious because of their persistence in the environment which leads to bioaccumulation, toxicity and carcinogenicity in all forms of life (Jaishankar *et al.,* 2014).

Heavy metals are commonly defined as those having a specific density of more than 5 g/cm^3. Heavy metals pollution is an environmental problem of worldwide concern because most of them can be toxic even at low concentrations.

Industrialized societies are responsible for increasing environmental contamination by trace metals produced as wastes from industrial and agricultural processes and household activities (Li *et al.,* 2014). Some of these metals include Cd, lead (Pb), Hg, Li, arsenic (As), silver (Ag) etc. Exposure to increased concentration of heavy metal ions such as Pb, As, Cd, Hg can cause immediate effects like cell toxicity, oxidative stress, lipid peroxidation and DNA damage (Beyersmann & Hartwig, 2008). The current need is to find an economical, eco-friendly, safe method for removal of these heavy metal contaminants from the environment.

The potential of bioremediation using plants and microbes is currently being exploited for the removal or inactivation of heavy metals. However, phytoremediation of heavy metals has many disadvantages such as location dependency, occupation of large surface area of land and accessibility of heavy metals to roots (Kopstik, 2014). Bioremediation by microorganisms is

preferred over other methods as it is ecofriendly, efficient, cost effective and fast. Microorganisms when continuously exposed to heavy metals acquire resistance and develop mechanisms for their detoxification (Ezaka & Anyanwa, 2011). The wastes generated from coffee processing industries such as coffee waste pulp, coffee processing waste water and husk contain heavy metals because of machinery enamel and the use of metal alloys that make up the machinery tools use in processing of coffee beans (Degefe *et al.,* 2016; Dadi *et al.,* 2019). Hence, isolation and characterisation of indigenous microbes that have the ability to detoxify multiple heavy metals is very important.

The present study deals with the investigation of the heavy metal tolerance of *Klebsiella pneumoniae* Kpn555, isolated from coffee pulp waste. The growth of *K. pneumoniae* Kpn555 was studied by supplementing nutrient broth with various concentrations of Cd, Li and Hg. The percentage reduction of heavy metals in the broth was carried out by atomic absorption spectrophotometer (AAS).

MATERIALS AND METHODS

Collection of Coffee Waste Pulp

The coffee waste pulp was collected from processing site of a coffee production industry situated in Chikmagalur, Karnataka in sterile containers. They were stored in the laboratory at refrigerated conditions (4 °C) until further use.

Isolation of Bacterium and Culture Maintenance

One gram of coffee waste pulp was added to 15 mL of sterile saline and the mixture was mixed properly using a vortex. The solution obtained was appropriately serially diluted and spread plated onto nutrient agar. The bacterium under study was isolated and sub cultured in nutrient broth and stored as glycerol stocks in nutrient broth at -20 °C.

Preliminary Characterisation and Identification of Bacterium by 16s RNA Sequencing

The bacterium was sub cultured in nutrient broth and Gram staining was performed to determine the shape and type of bacterium. The identification of the isolated bacterium was carried out by 16sRNA sequencing (Zafra *et al.,* 2016). The 16s sequence was analysed by homology analysis using Blast program (www.ncbi.nlm.nih.gov/blast) which also gave a phylogenetic tree using neighbour considering sequences showing >98% homology.

Determination of Minimum Inhibitory Concentration (MIC) of Heavy Metals

Minimum inhibitory concentration (MIC) of Cd, Hg and Li was determined by supplementing nutrient broth (NB) (Hi-Media Lab. Ltd., India) with the concentrations of 5-300 mg/L at intervals of 5 mg/L. The medium was inoculated with 1% (v/v) of 16 h old culture of *K. pneumoniae* Kpn555 (1×10^6 cells) and incubated for 24 h at 37 °C. The experiments were performed in triplicates. MIC was defined as the minimum inhibitory concentration of the heavy metal that did not show visible growth at 600 nm after 24 h of incubation. The OD at 600 nm was measured using a UV-visible spectrophotometer (Shimadzu, Japan).

Growth Kinetics of *K. pneumoniae* Kpn555 in Presence of Heavy Metals

The NB medium (100 mL) was supplemented with different concentrations of Cd, Hg and Li based on MIC results. The medium was inoculated with 1% (v/v) of 16 h old culture of *K. pneumoniae* Kpn555 (1×10^6 cells). The experiments were performed in triplicates. The effect of these heavy metals on the growth bacterium was observed by measuring the OD of bacterium at 600 nm for 48 h (6 h interval).

Heavy Metal Reduction Studies

The residual concentration of heavy metals in the broth at periodic time intervals was measured by analysing the cell free acid digested broth by an atomic absorption spectrophotometer (AAS – GBS – AVANTA). The broth was centrifuged at 10000 rpm for 15 mins. The cell free supernatants were then mixed with twice the volume of concentrated sulphuric acid. The mixtures were then digested on a hot plate at 100 °C until 1/3rd of the volume remained. The sample was then filtered and appropriately diluted and analysed for heavy metal content. The percentage reduction in heavy metal concentration was calculated following Eq. (1).

$$\frac{\% \text{ heavy}}{\text{Metal utilised}} = \frac{\text{heavy metal added to broth - heavy metal at the end of culture}}{\text{heavy metal added to broth}} \times 100\% \quad (1)$$

RESULTS AND DISCUSSION

Preliminary Characterisation and Identification of Bacterium by 16s rRNA Sequencing

The isolated bacterium was Gram negative and rod shaped. On analysis of the 16s RNA sequence by homology using BLAST tool, it was observed that the bacterium showed 100% similarity to *K. pneumoniae* DSM 30104 (Figure 1). The 16s RNA gene sequence is deposited in NCBI GenBank with accession number KX570899.1.

Determination of Minimum Inhibitory Concentration (MIC) of Heavy Metals

The MIC of Cd, Hg and Li in *K. pneumoniae* Kpn 555 was found to be 150 mg/L, 10 mg/L and 250 mg/L respectively. *Pseudarthrobacter oxydans* strain MM20 and *Pseudomonas frederiksbergensis* strain SS18 isolated from tundra ecosystem of Ny-Ålesund, Svalbard showed an MIC of 2 mg/L and 1.5 mg/L, respectively, for Hg (Balan *et al.,* 2018). The MIC of Cd in *Pseudomonas sp.* M3, isolated from wastewater collected from the industrial area of Penang, Malaysia, was found to be 550 mg/L (Abbas *et al.,* 2014). *Klebsiella pneumoniae* isolated from Soltan Abad river sediments showed a Cd tolerance of 10 mg/L (Kafilzadeh *et al.,* 2013).

Growth Kinetics of *K. pneumoniae* Kpn555 in the Presence of Heavy Metals

The concentrations of Hg (5 and 10 mg/L), Cd (50 and 100 mg/L) and Li (100 and 200 mg/L) added were chosen on the basis of MIC obtained. The growth was found to decrease as the concentration of added heavy metal increased. It was observed that in the presence of heavy metals, some fluctuation occurred in the bacterial growth in the stationary phase, which was absent in the control (Figure 2). It could be presumed that, since heavy metals offers stress to the growth of bacterium in the stationary phase, it could activate some genes which may produce certain products that inhibit the stress hence reducing the toxic effects on the bacteria. Khan *et al.* (2015) have reported the production of metallothionein, a thiol-containing, cysteine rich, metal binding protein induced by the exposure of Cd to *Klebsiella pneumoniae*, isolated from industrial waste water.

Residual Heavy Metal Concentration in the Broth

It was observed that there was a periodic decrease in the concentration of metals from the medium. This could probably mean that the metals were either bioabsorbed on the bacterial cell surface or accumulated into the cell cytoplasm (Figure 3). However, in the case of Hg in the medium, it was observed that the concentration of Hg in the broth increased at 22 h and then there was a slight decrease with increase in incubation time. This could probably mean that when the biosorbed or bio accumulated Hg offered heavy stress to the bacterial growth, hence some amount of Hg was released back into the broth (Figure 3). This response also coincided with the fluctuations in the bacterial growth in the stationary phase. *Bacillus* sp. isolated from a gold mining site in Karnataka showed that on exposure to Cd and As the stationary phase reduced to a bell shaped curve (David *et al.,* 2016).
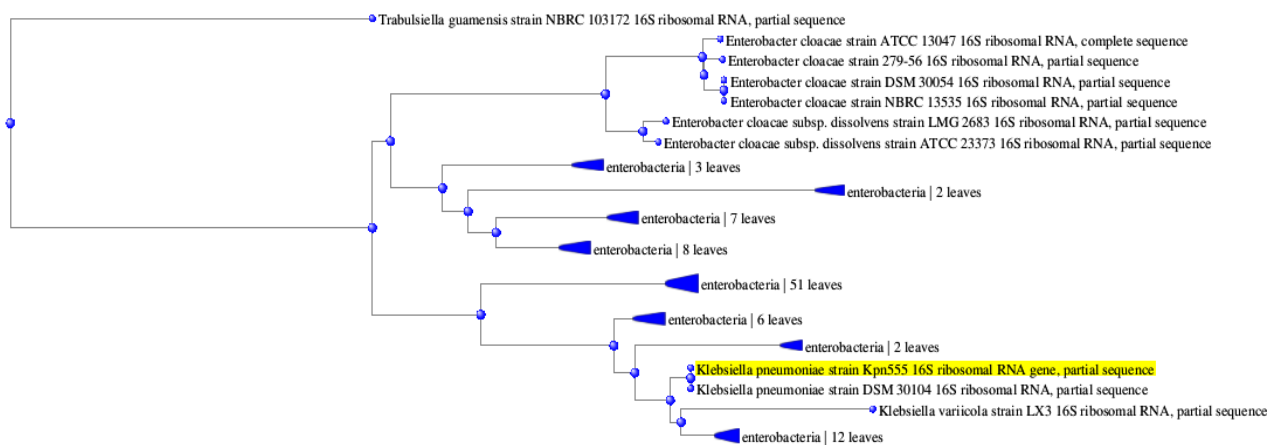

Figure 1. Phylogenetic tree showing the relationship of identified *Klebsiella pneumoniae* Kpn555 with related species.
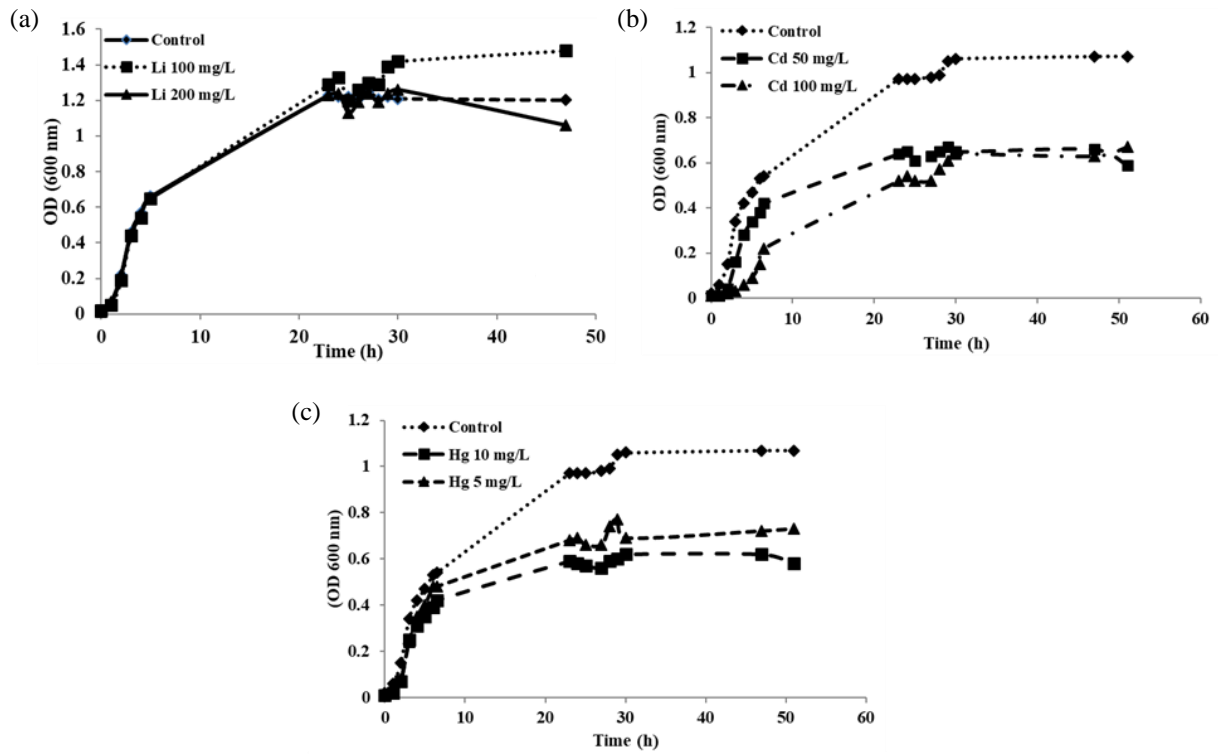
Figure 2. Difference in growth pattern of *Klebsiella pneumoniae* Kpn555 in the absence and presence of Li (a); Cd (b) and Hg (c).
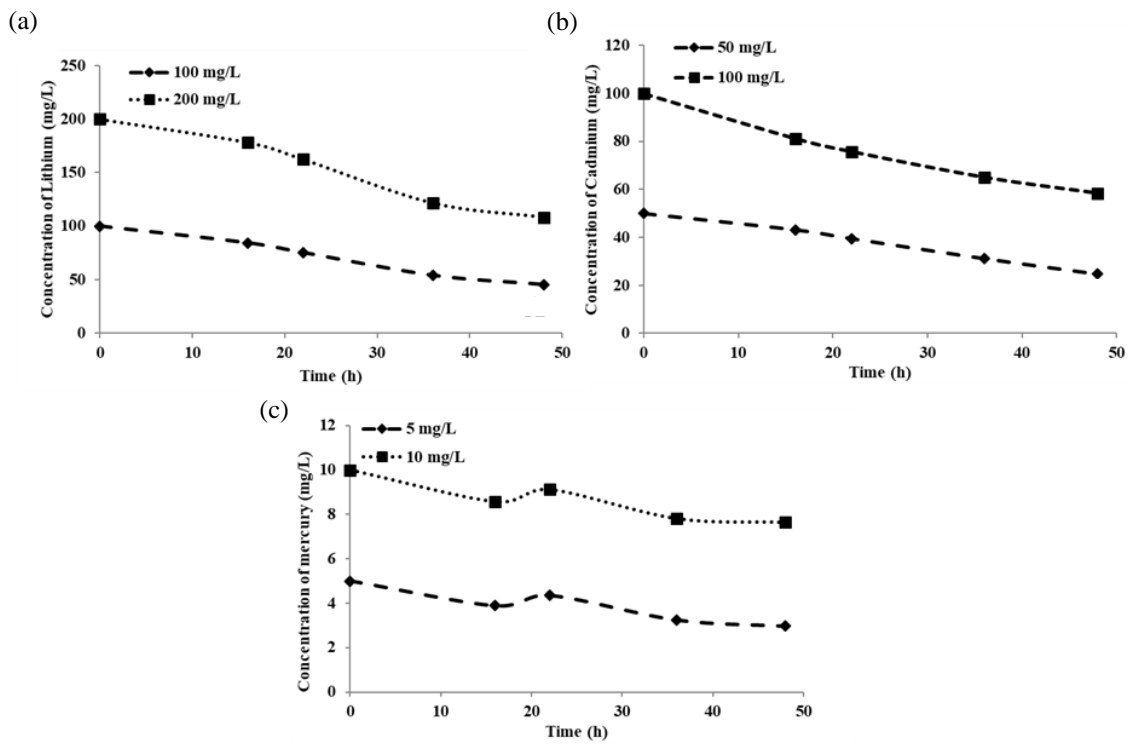

Figure 3. Decrease in concentration of Li (a); Cd (b) and Hg (c) from NB supplemented with heavy metals, on growth of *Klebsiella pneumoniae* Kpn555 for 48 h.

The bacterium on growth in NB supplemented with Li showed the highest reduction in concentration of Li (54.8% and 45.95% in 100 mg/L and 200 mg/L, respectively) whereas the lowest reduction was observed in concentration of Hg (40.6% and 23.5% in 5 mg/L and 10 mg/L, respectively). The reduction in the concentration of Cd in NB supplemented with Cd was found to be 50.6% and 41.7% in 50 mg/L and 100 mg/L, respectively. Degradation of Cd by *Gemella* sp. and *Micrococcus* sp., isolated from tannery effluent showed 50.99% and 38.64%, respectively (Marzan *et al.,* 2017).

CONCLUSION

Klebsiella pneumoniae Kpn555 isolated from coffee pulp waste could tolerate and reduce the concentrations of heavy metals such as Cd, Hg and Li. A few bacteria are capable of tolerating Hg up to concentrations as low as 10 mg/L as Hg is very toxic to the growth of all life forms. Since *K. pneumoniae* is pathogenic and cannot be used for removal of heavy metals from polluted sites, further studies need to be carried out to ascertain if this bacterium has a plasmid and if the heavy metal resistance is plasmid mediated. In that case bacteria transformed with the plasmid from *K. pneumoniae* Kpn555 could be exploited as a good ecofriendly source for the removal of these heavy metals from the polluted sites.

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