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RESEARCH ARTICLE

A study of biofilm production by a multi-metal tolerant strain of *Klebsiella* sp.

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Abstract

Heavy metals, released from various industries and mines impart significant adverse effects in the environment. Biofilms, produced by microbes have been found to be suitable for the remediation of pollutants because of their high microbial biomass and ability to immobilize pollutants. In the present study a newly isolated strain of *Klebsiella* sp PRBC 14 showed remarkable efficacy to tolerate heavy metals like Aluminum, Manganese, Zinc, Stannous, Silver, Nickel, Ferric, Ferrous, Lead, Cadmium, Cr(III), Cr(VI), Copper, Cobalt, Arsenate and Arsenite in high concentration. The cells showed a tendency to form biofilm in presence of these metal ions, which varied with the nature of the metal ion present in the medium. The photo-micro-graphic studies revealed that in most cases, the bacterial cells were caught in the sticky slime and managed to escape the toxic effect of heavy metals. In some cases the sequestering of metal ions from the media was accomplished by trapping of the salts in the extra polysaccharide matrix of the biofilm.

Introduction

Heavy metals are ubiquitous and persistent environmental pollutants that are introduced into the environment through anthropogenic activities, such as mining and smelting, as well as through other sources of industrial waste (Gail *et al*, 2003) The known mechanisms of heavy metal toxicity include inducing oxidative stress and interfering with protein folding and function (Nies, 1999). Bacteria have developed a variety of resistance mechanisms to counteract heavy metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Nies,. 1999). Separation of toxic metals may be accomplished by the production of biofilms. A biofilm, sometimes referred to as slime, is a polymeric mixture generally composed of extra cellular DNA, proteins, and polysaccharides (Abdel-Aziz and Aeron, 2014). Bacteria growing in biofilms exhibit increased resistance to antimicrobials and host immune response compared to their freeliving, planktonic counterparts due to several reasons like restricted penetration of antimicrobials into a biofilm, decreased growth rate, and expression of possible resistance genes (Donlan and Costerton 2002). Biofilms are permeated at all levels by a network of channels through which water, bacterial garbage, nutrients, enzymes, metabolites and oxygen move to and from, with gradients of chemicals and ions between micro-zones providing the power to shunt the substances around the biofilms (Paraje , 2011) . Within a biofilm it has been found that EPS, specifically the polysaccharide components, binds heavy metals (Christensen *et al*, 1985). Several researchers have reported that biofilms are capable of removing heavy metal ions from bulk liquid (Ferris, et al, 1989) and the use of biofilms to remove heavy metals from wastewater has been investigated (von Canstein *et al*, 1999). Biofilm are complex communities of micro organisms attached to a surface or interface enclosed in an exopolysaccharide matrix of micro bial and host origin to produce a spatially organized three dimensional structure (Maldonado *et al*, 2007). Bacterial adhesion and biofilm formation are very important concepts in the area of bacterial disease and control. Not only do they aid colonization but also often provide a

degree of protection against outside stresses (Stewart and Castleton, 2001). Several explanations have been proposed for the enhanced resistance of biofilm-associated cells to both metals and antibiotics (Baker-Austin *et al*., 2006).
The present work focuses on the effect of heavy metals on the growth rate, morphological alteration and biofilm production by the working strain *Klebsiella sp* PRBC 14.

Materials and methods

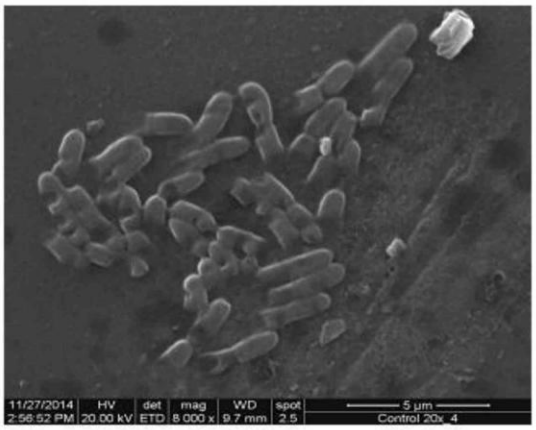
Collection of soil sample Soil samples were collected from the top soil of Khonbond iron mine (at 21°57 min 18.02 sec North and 85°23 min 15.4 sec East) located at an altitude of 676 meter in Keonjhar district of Odisha, India and were taken in sterilized polyethylene bags using sterilized spatula and stored at 4ºC until examination.
Isolation of the bacterial strain The bacterial strain was isolated by cultivating in basal medium (BM) composed of (g/L): peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1 (pH-8.0).and 0.1 glucose at for 24-36 hours.
Identification of the bacterial strain The bacterial strain was characterized by routine tests, for identification and was genetically identified using 16srRNA. It was later submitted to NCIM, Pune, India for confirmation.
Photomicrographic study The crystal violet-saffranine stained bacterial cells were visualized under Axioscop-40 (Zeiss) microscope at 1009.For SEM, paraformaldehyde–glutaraldehyde fixed and totally dehydrated specimens were sputter coated with gold palladium under vacuum and observed and photographed in a scanning electron microscope (FEI Quanta-200 MK 2).
Quantification of biofilm formation The bacterium was grown in normal culture medium for 0-60 hours. Each flask containing growth medium of different hour was diluted (1:100) with fresh medium. The flat bottom tissue culture plates (96 wells) were filled with 200µl of each type of diluted cultures individually. The culture plates were incubated at 37°C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 200 µl of phosphate buffer saline (pH 7.2) four times to remove free-floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a UV visible spectrophotometer (Shimadzu, Japan) at wavelength of 540 nm. The OD values of non inoculated sterile medium were taken as control. (Mathur *et al*, 2006). The data obtained were used to classify the strains as high producers (OD higher than 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD lower than 0.100) (Maldonado *et al*, 2007).
Determination of tolerance to various metal ions Strains were grown on Petri plates on solid basal medium supplemented with different metal ions namely, Aluminum, Manganese, Zinc, Stannous, Silver, Nickel, Ferric, Ferrous, Lead, Cadmium, Cr(III), Cr(VI), Copper, Cobalt, Arsenate and Arsenite of different concentrations. A non inoculated plate without inoculums for each metal ion was maintained as control.
Measurement of turbidity The turbidity of the culture wa**s** measured at 660 nm in a UV visible spectrophotometer (Shimadzu, Japan).
All experiments were done in triplicate and their values were averaged.

Results and discussion

The strain grown in basal medium with no metal additive showed moderate growth and slime production. The scanning electron micrograph indicated that the cells were rod (3.75 µm) .Under compound microscope; the cells appeared as Gram negative elongated rods which showed a tendency to form a cord like arrangement (Fig 1).
Growth of the bacterial strain under the stress of various heavy metals led to the change in cell structure as well brought about trapping of these cells in the biofilm (Harrison *et al.*, 2005) or binding the metal residues with the extracellular polysaccharides to reduce the metal load from the culture medium (Huang *et al***,** 2000). The mechanism of metal –microbe separation varied with the nature and concentration of metal salt used.
In presence of Zinc, Manganese and Lead the growth was poor, cells were short in size but the biofilm production was not reduced. In zinc supplemented medium, patches, formed of aggre-gated cells in slime (Fig 1), representing thick sessile communities were found (Zubair *et al*, 2011). In Alumium, Tin supplemented media; both the growth and biofilm production were increased. The cells were caught in the sticky slime and somehow managed to escape from the direct exposure of heavy metals. In presence of Cu^{2+}, Co^{2+}, $Ni^{2+}$and Ag^{2+}, the growth was severely hampered with lesser amount of detectable slime. But in all the cases, the cells showed a tendency to form chains. Although the growth was better in presence of ferrous salts, biofilm production was higher in presence of ferric salts. In the former salt the cells formed a chain, whereas in the later, the cells were almost trapped in the slime material. Probably ferric salts provided more stress to the bacterial strain than that of ferrous salt that led to the formation of more slime to protect the cells from metal toxicity. The strain showed profuse growth in presence of chromate salt, of which trivalent chromium was proved to have a growth boosting effect. The cells were normal in shape and size with normal amount of slime generation. This revealed the fact that probably the strain could utilize Cr (III) in its metabolic pathway. On the other hand, in presence of hexavalent chromium, a potent toxic metal, the

cells were with decreased size and gradually tend to accumulate. Although the cells were found to produce more slime in presence arsenite salt and cells aggregate within the slime to form a thick cord of slime accumulated cells , the bacterial growth was higher in case of arsenate added medium. Removal of arsenate and arsenite ions through binding with extracellular polysaccharide of biofilm in *Acidithiobacilus ferrooxidans* was described by Chandraprabha and Natarajan, 2011.

Similarly, *Candida albicans* and *Candida tropicalis* were known for high levels of resistance to the water-soluble ions Hg^{2+}, Pb^{2+}, Cd^{2+}, arsenate (AsO_4^{3-}) (Berdicevsky *et al.*,1993) In presence of cadmium the strain produced profuse amount of slime that was clearly found to conglomerate the cadmium particles. Such ability to produce biofilm in presence cadmium was also found in *Pseudomonas* sp (Ching Chien *et al*, 2013).



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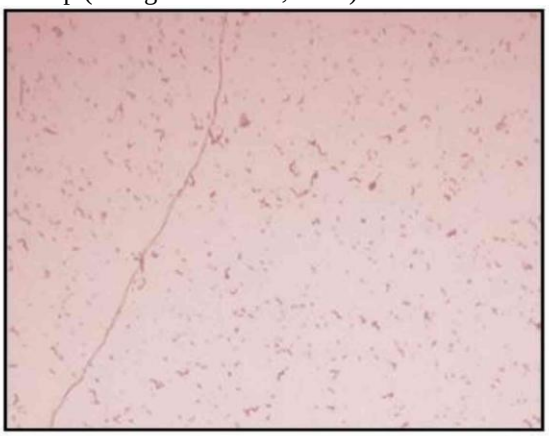

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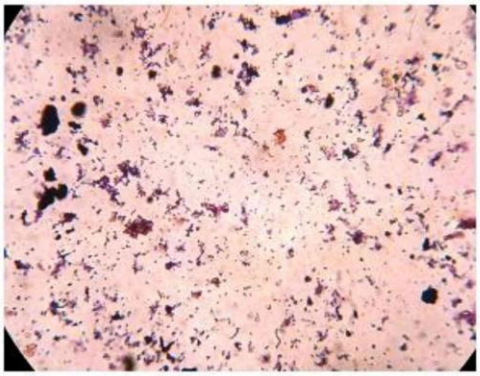

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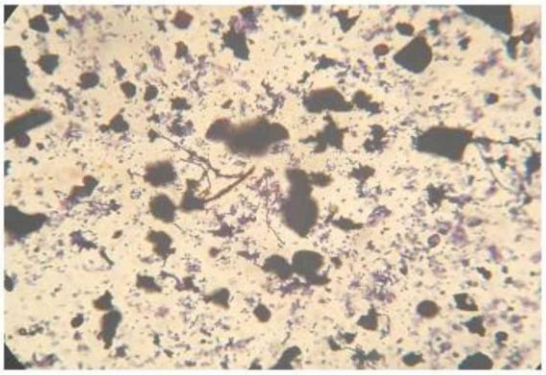

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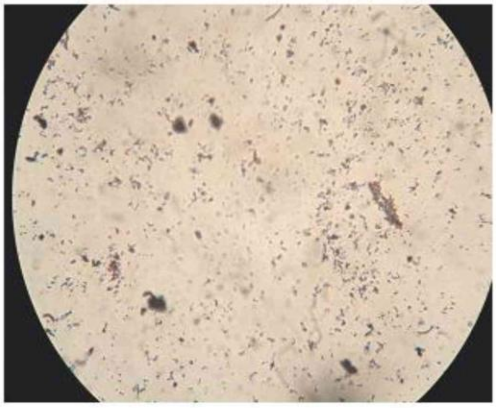

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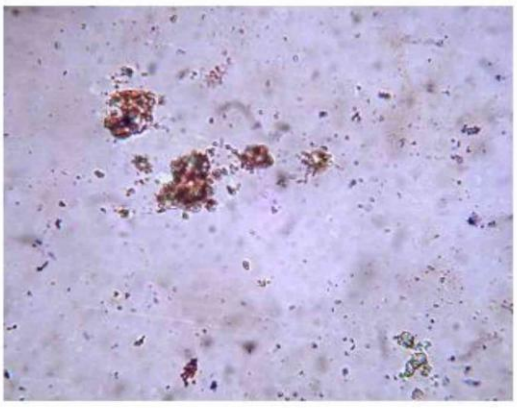

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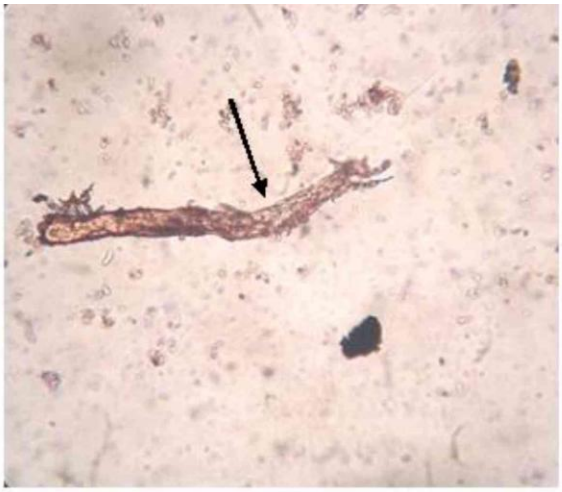

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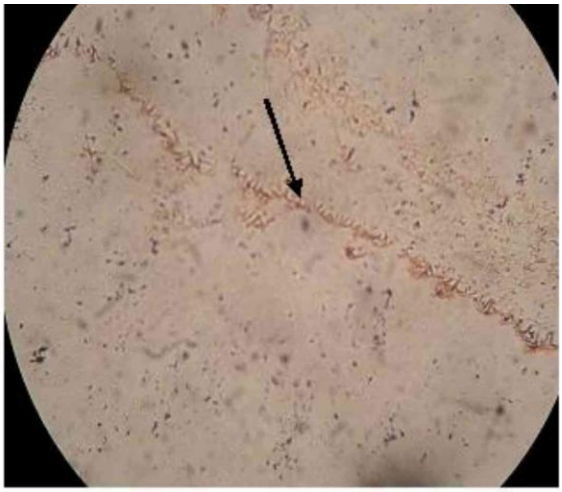

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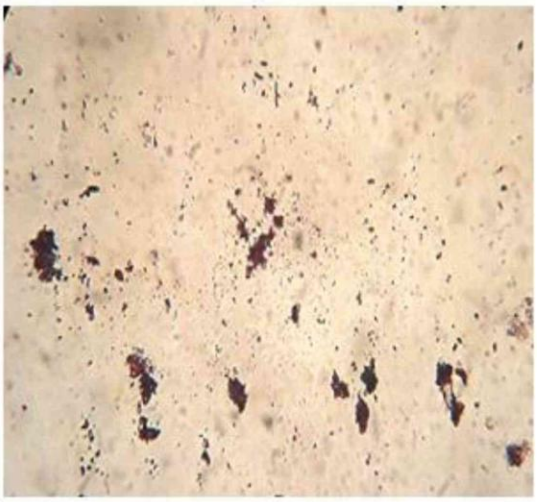

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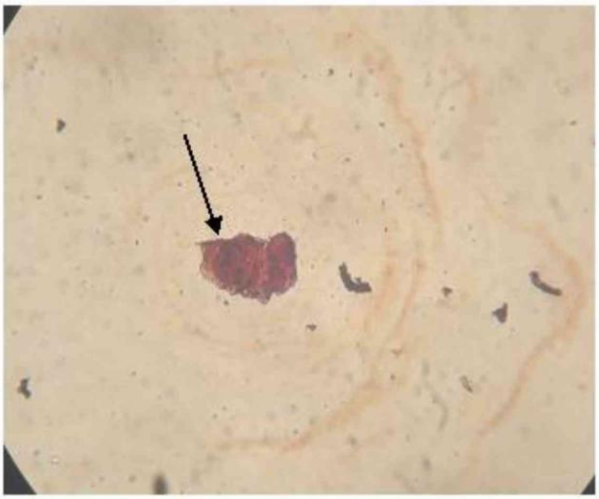

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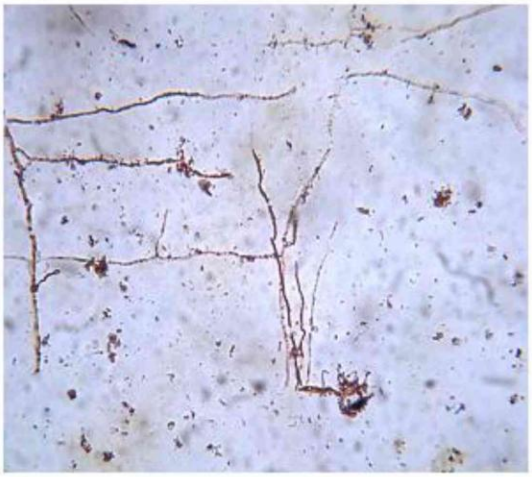

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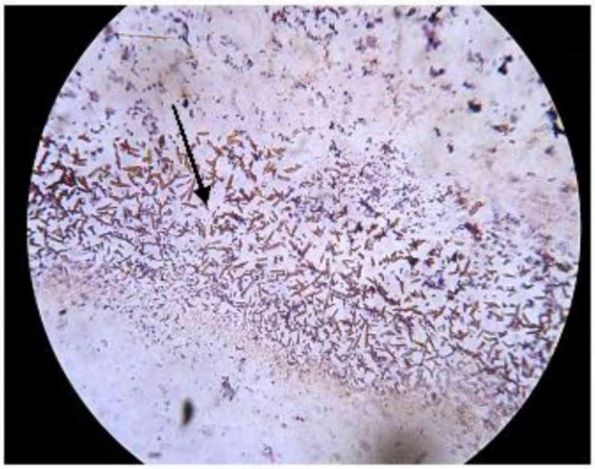

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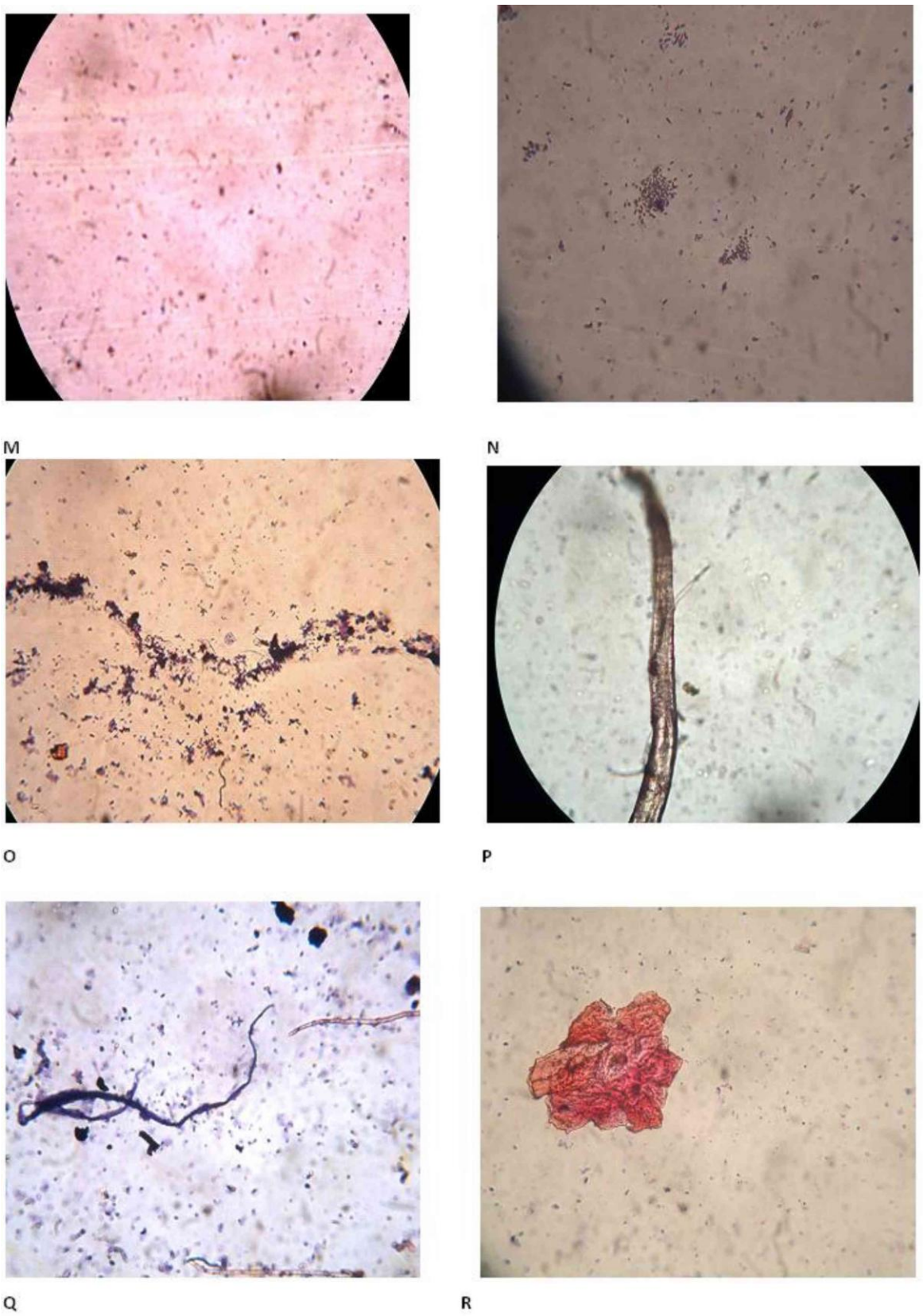

Fig 1. Photomicrographs of *Klebsiella* sp PRBC 14 at different conditions.
A: Electron micrograph, **B** : Photomicrograph of normal strain grown in basal medium.

C- R: Photomicrograph of the strain grown in presence of various metal salts for 22 hrs.
C: Manganese salt, **D**: Zinc salt, **E**: Lead salt, **F**: Aluminum salt, **G**: Stannous salt (Tin), **H**: Silver salt, **I**: Nickel salt, **J**: Copper salt, **K**: Cobalt salt, **L**: Cadmium salt, **M** :Trivalent Chromium , **N** : Hexavalent Chromium, **O** : Arsenate salt , **P** :Arsenite salt, **Q** : Ferrous salt ,**R** : Ferric salt

Table 1: Effect of metal salts on growth and slime production by *Klebsiella* sp PRBC 14.

Metal added (500mg/L)	Growth (at 660nm)	Biofilm production (at 540nm)
None	.102	+++
Aluminum	.130	++++
Manganese	.075	+++
Zinc	.080	+++
Stannous	.120	++++
Silver	.046	++
Nickel	.051	++
Ferric	.070	++++
Ferrous	.103	++
Lead	.072	+++
Cadmium	.020	++++++
Cr(III)	.280	+++
Cr(VI)	167	++
Copper	.040	+
Cobalt	.061	+
Arsenate	.098	+++
Arsenite	.050	++++

Biofilm production : ++++ :> 0.75, +++ : >0.5, ++> 0.3, + :< 0.25 at 540nm.

Conclusion:

The strain showed a remarkable power to resist the toxic effect of the heavy metals and it was attributed by its efficacy to generate biofilm made up of extracellular polymeric substances.
Since biofilm has a profound impact on an array of biomedical, biotechnology and industrial fields including pharmaceutical and surgical applications, food engineering, bioremediation and biohydrometallurgy (Barbara *et al*, 2009), understanding the mechanisms is important for understanding the microbial ecology of heavy metal-affected environments, as well as the basis of biofilm resistance to antimicrobial agents in general (Teitzel and Parsek, 2003).

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