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Metal resistance and bactericidal activities of a new strain of *Klebsiella variicola* isolated from iron mine

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Abstract

A bacterial strain was isolated from the top soil of an iron mine and was identified as *Klebsiella variicola.* The bacterial strain was found to achieve its maximal growth at 34°C and at a pH of 8.5 and attained stationary phase of growth at 43 hours. It showed resistance against a number of antibiotics that directly affect bacterial translation. The working strain was found to tolerate a variety of metal salts including $Cr^{6+}Cr^{3+}$,As^{2+},As^{3+}, Al^{3+},Ag^{2+}, Zn^{2+},Mn^{2+},Sn^{2+},$Fe^{2+}.Fe^{3+}$,Co^{2+}, Cu^{2+},Cd^{2+},Pb^{2+} and Ni^{2+} at a high concentration. The multi-metal tolerance of the strain might have been attributed by the sticky slime produced by it, and the highest biofilm production was found in presence of lead salt followed by aluminum in the growth medium. The active compound secreted by the bacterial strain (bacteriocin) was found to check the growth of several Gram positive and Gram negative bacteria. The newly isolated strain of *Klebsiella variicola* could be effectively used in bioremediation of metal contamination and extension of shelf life of food materials.

Key words : Metal resistance, Bioremediation, *Klebsiella* sp, Bacteriocin

INTRODUCTION

Metal contamination, mainly developed by anthropogenic activities [1] is a significant environmental problem because of the toxicity, persistence and nondegradable nature of the metal [2, 3]. The toxic effects of these metals result mainly from the interaction of metal ions with proteins (enzymes) and inhibition of metabolic processes. The high concentration of metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium when accumulate in soils, water bodies, impart toxic effects to plants, animals, humans and aquatic life. Hence their removal becomes a challenging task and a need of the hour.

Since different metals are increasingly found in different habitats due to natural and environmental processes, microbes have fund to evolve several mechanisms to tolerate these metals. The interests in utilization of the biological potential of microorganisms in metal removal have been increased considerably in recent years [4].

Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic metals [5] and this property enables these microorganisms to combat the stress and survive in metal contaminated sites [6]. This may be accomplished through the efflux of metal ions outside the cell, accumulation of the metal ions inside the cell [7] with simultaneous reduction of the toxic metal ions to a less toxic state [8] or sequestration placing metals into compartments that spatially remove them from vital tissues [9] or processes.

Moreover, the bacteria growing in ecological niche with limited nutrient availability due to abundance of toxic metals produce a variety of antimicrobial substances to compete against other bacteria which are present in the same ecological niche [10]. These include antimicrobials like bacteriocins, a protein substance which exhibits antibacterial activity against closely related species [11] and can inhibit very restricted spectrum of bacteria.

Although the majority of *Klebsiella* strains reported were isolated from purely clinical isolates and only a few was obtained from other ecological niches like coal mine [12], sewage treatment plant [8] and River Estuary [13] ,almost no report was available on the multi metal tolerant and bacteriocin producing *Klebsiella* strain from iron mine.

The present study reports the multi- metal tolerance and bactericidal properties of a newly isolated bacterial strain obtained from dust of an iron mine.

MATERIALS & METHODS

Random 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. 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. The bacterial strain was characterized by routine tests. It was genetically identified using 16S rRNA and was later submitted to NCIM, Pune, India for confirmation. The crystal violet-saffranine stained bacterial cells were visualized under Axioscop-40 (Zeiss) microscope at 1009. For SEM, paraformaldehydeglutaraldehyde 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).

The strain was cultivated in culture flasks (100 ml), each with 20 ml medium at various temperature (4-45°C), pH (1-10) and in presence of various carbon sources for 24-48 hours. A culture flask without innoculum was maintained as control. Bacterial growth was measured by turbidometric method at 660 nm in a UV visible spectrophotometer (Shimadzu, Japan). Strains were grown in culture flasks , supplemented with different salts of following metal ions namely Fe^{3+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Sn^{2+}, Ag^{2+}, Hg^{2+}, Ni^{2+}, Al^{3+}, As^{2+}, As^{2+}, Cr^{6+}, $Cr^{3+,}$ $Pb^{2+,}$ Co^{2+} at a concentration of 500 mg/L under optimum temperature and pH. An extra flask

without inoculum for each metal ion was maintained as control.

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) for 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 the 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 [14]. 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) [15].

Antibiotic sensitivity and resistance of the isolated bacterial strain was assayed according to the Kirby-Bauer disc diffusion method [16].

To determine the bacteriocidal effect of the working strain, different Gram positive and Gram negative bacteria, yeasts and filamentous fungi were tested. The test strains were cultivated on solid plates by spread plate method. The culture broth of the working strain of *Klebsiella variicola* after cultivation of 30 hours was centrifuged at 10,000 rpm for 10 minutes. The sterile filter paper disc of 0.5 mm diameter, soaked with the supernatant was placed at the centre of each plate containing test organism. After an incubation period of 24-48 hours under optimum conditions, the bactericidal activity was estimated by measuring the diameters of the inhibition zones around the soaked filter paper disc [17].

Table 2: QEffect of antibiotic on the growth of *Klebsiella variicola* PRBC 14

Antibiotic (25µg)	Growth on plate
Streptomycin	+
Kanamycin	+
Doxycilin	++
Amoxicillin	++
Azithromycin	++
Lincomycin	++
Novobiocin	++
Tetracyclin	++
Norfloxacin	++
Clavum	++
Cefuroxime	++
Rifampicin	-
Ethambutol	+
Pyrazinamid	-

(+: resistance, -: susceptibility)

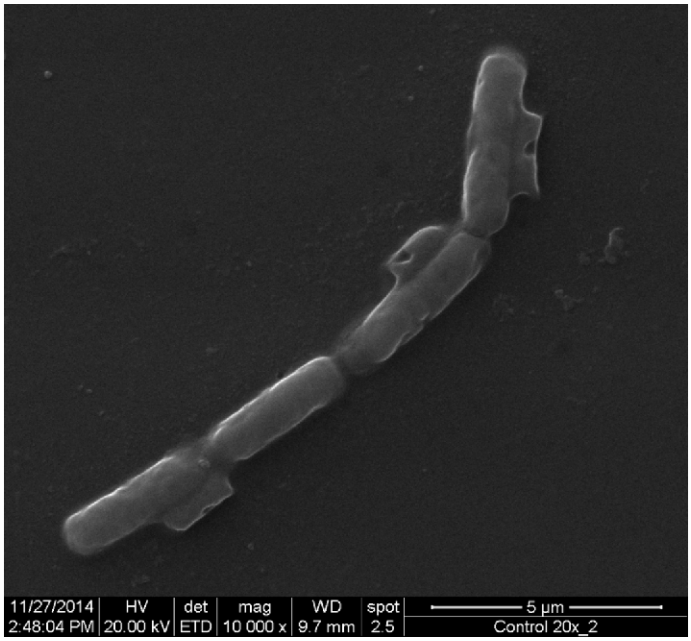

Fig 1: Scanning electron micrograph of cells of *Klebsiella variicola* PRBC 14

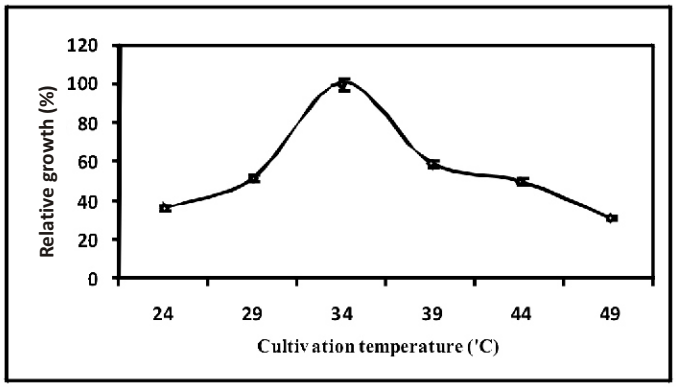

Fig 2: Temperature optima for growth of *Klebsiella variicola* PRBC 14

All the experiments were done in triplicate and their values were averaged.

RESULT

A single strain of bacterium could be isolated from the iron rich red soil dust on the agar plate, even after incubating for 5-6 days. The morphology of the strain was depicted by its photomicrograph (Fig 1).

The isolated bacterial strain PRBC 14 was catalase negative and Gram negative, non motile rods. Based on 16S rRNA analysis, the strain was identified as *Klebsiella variicola* by National Collection of Industrial Microorganisms (NCIM), NCIM, Pune, India. The Sequence text in FASTA format with nearest similarity search NCBI showed maximum homology with *Klebsiella variicola.*

The optimal temperature for the growth of the working strain was found to at 34°C (Fig 2) and about 50% growth was found to

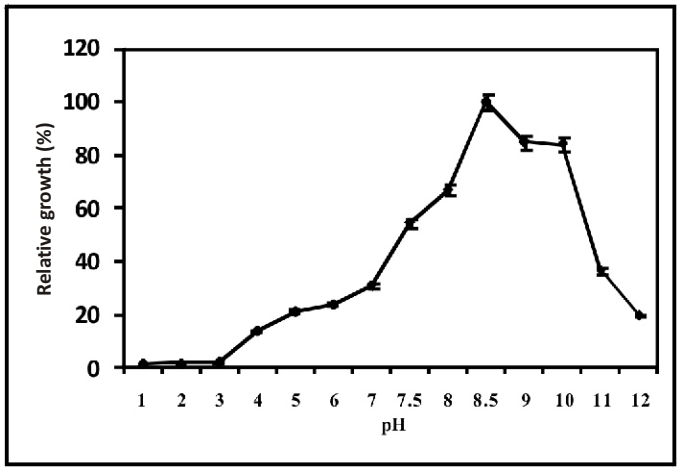

Fig 3: pH optima for growth of *Klebsiella variicola* PRBC 14

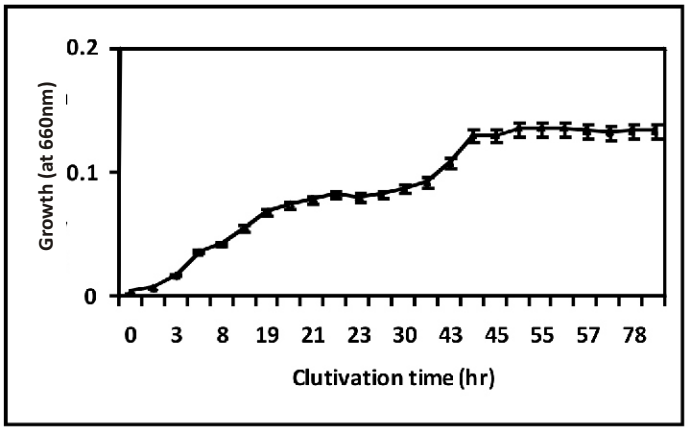

Fig 4: Kinetics of growth under optimized conditions in *Klebsiella variicola* PRBC 14

Table 2: Bactericidal activities of *Klebsiella variicola* PRBC 14

Indicator organism	Source	Inhibition
Bacillus subtilis	soil	+++
Staphylococcus aureus	patient	+++
Lactobacillus sp	milk	+++
Lactobacillus plantarum	milk sample	+++
Eschrishia coli EIII	septicemic neonates, NICED, Kolkata	+++
Eschrishia coli	patient	+++
Acinetobacter baumanii	septicemic neonates, NICED, Kolkata	+++
Klebsiella pneumonia	patient, NICED, Kolkata	+++
Candida albicans	NCIM, Pune	++
Pichia sp	NCIM, Pune	+
Saccharomyces cerevisae	NCIM, Pune	+
Rhizopus oryzae	soil	-
Penicillium janthinellum	soil	-
Trichoderma sp	soil	-

Inhibitory activity the diameter of the inhibition zones around the indicator colonies
+++: 4.5 mm; ++: 3 mm, +: 1mm, -: no inhibition

be retained above a temperature of 44°C. The optimum temperature for growth of the present strain was found to be 34°C. The strain was found to be strictly alkaliphilic , since it showed optimal growth at pH 8.5 and retention of 80% growth at pH 10 (Fig 3). The growth kinetics indicated that highest growth was achieved at about 43 hrs of cultivation and persisted till 80 hrs (Fig 4).

The susceptibility test of strain PRBC 14 against different antibiotics indicated that the strain was resistant against novobiocin, tetracyclin, norfloxacin, clavum, amoxicillin, azithromycin, lincomycin but showed susceptibility against Rifampicin and Pyrazinamid (Table 1).

This strain was found to tolerate a variety of metal salts, as evident from their growth on agar plate (data not shown). The strain could utilize tin, nickel, arsenic and iron for their growth in early log phase. The highest growth found in presence of Cr (III) ,

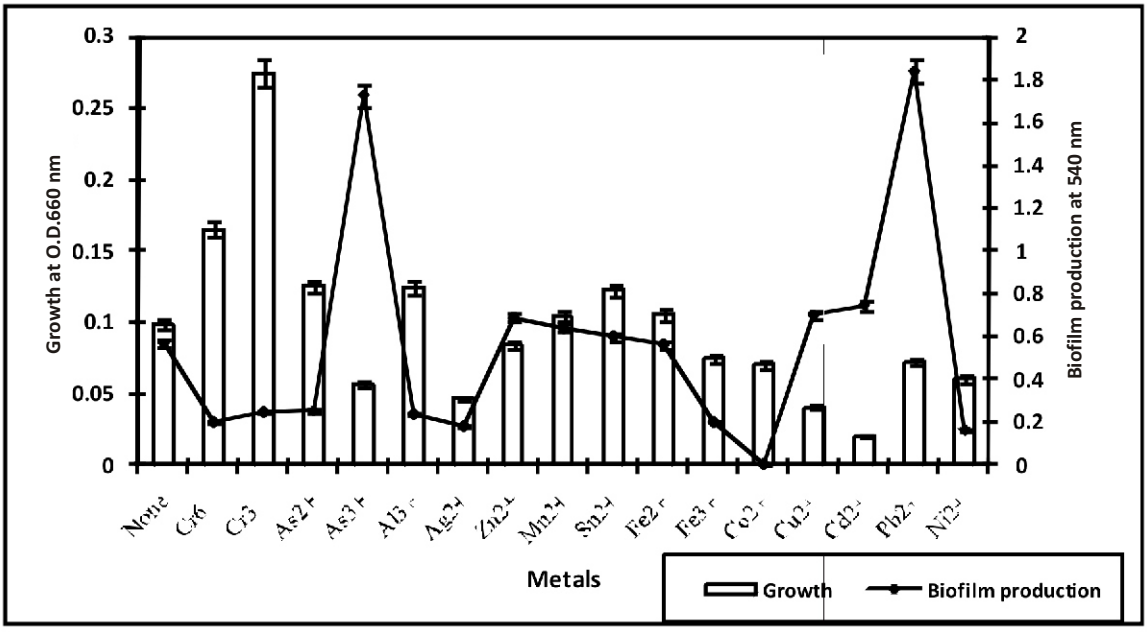

Fig 5: Effect of metal ions (500 mg/L) on growth and biofilm production by *Klebsiella variicola* PRBC 14.

although enhanced in presence of Cr (VI), arsenate, aluminium, ferrous, manganese and tin (Fig 5).

The strain produced significant amount of biofilm both in normal and metal supplemented culture medium. Highest biofilm production was found in presence of lead salt followed by aluminium (Fig 5).

The working strain was found to check the growth and colony formation of several Gram positive (*Staphylococcus aureus, Bacillus subtilis, Lactobacillus sp*) and Gram negative bacteria (*Eschrishia coli, Klebsiella pneumonia, Acinetobacter sp*). The strain, although exerted some static effect on the growth of yeast strains but failed to kill the filamentous fungi tested (Table 2).

DISCUSSION

The iron enriched soil sample was devoid of any cultivable microorganism. The significant absence of other microbes revealed the toxic and stressful nature of the soil. The isolated strain, identified as *Klebsiella variicola* was regarded as a novel species generally found in association with plants and also in clinical isolates [18].

The temperature preference of the working strain was similar to that of some reported strains of *Klebsiella* [19], isolated from some clinical samples, having temperature optima of 30 -35°C. Although a few acidophilic strains were reported [20], the report of alkalophilic strain of *K. variicola* was not available so far. This implies that the present strain represents some unique features which enable them to thrive in the adverse condition of iron mine.

The present strain was found to be susceptible against the antibiotics affecting trans- translation (Pyrazinamide) and inhibiting DNA-dependent RNA synthesis (Rifampicin). The strain showed resistance against almost all the antibiotics that directly interfered with bacterial protein synthesis. Almost similar type of antimicrobial drug resistance was found in *Klebsiella* isolates from Hilla. Iraq [21].

The multidrug and metal-resistance were reported not only in *Klebsiella sp* [22,23] , but also in other bacterial strains [24, 25] and there must be a correlation between metal tolerance and antibiotic resistance in bacteria [26] . This co-tolerance might be exerted by different resistance determinants present on the same genetic element, more precisely by a plasmid.

In the present strain, the biofilm production might have been related with the metal tolerance, since the bacterial cells were caught in the sticky slime and managed to escape the toxic effect of the metals [27].

The bactericidal activity of the strain was surely attributed by the bacteriocin, synthesized by the strain. Possibly due to the synthesis and secretion of bacteriocin, that has been found to play a defining role in the control of undesirable flora [28], the soil sample collected was devoid of any other microorganism. Unlike bacteriocin reported from other *Klebsiella* strains with narrow spectrum of action [21], the present strain showed bactericidal activities against a variety of bacterial strains.

CONCLUSION

The newly isolated strain of *Klebsiella variicola* showing tolerance against a number of metals including few heavy metals may have actual and potential application in bio-remediation of metal pollution. The bactericidal activities of the strain hold a great potential for extension of shelf-life and improvement of microbiological safety of vegetable raw materials and final products. Hence extensive research is needed to exploit the uniqueness of the strain for successful bioremediation and food preservation.

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