Heavy Metal Bioremediation by Novel Microbial Strains *Proteus Mirabilis* and *Bordetella Avium*

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Abstract

The entitled study focuses on exploring the microbial diversity and its applicability in the remediation of metal contaminated soil using microbes which is a reliable, and cost effective technique.

Present investigation studied microbiota present in tungsten enriched soil of kuhi-Agargaon-Khobna region using culturable approach confirmed by ICP-MS and SEM-EDS analysis. Furthur applicability in Bioremediation and Azo Dye degradation was studied. XRF analyses of soil samples were performed followed by traditional microbiological analysis for isolation of tungsten tolerant bacteria's. Tungsten accumulation was confirmed using ICP-MS and SEM-EDS techniques. Different metals and azo dye tolerance studies were conducted. Antibiotic susceptibility test revealed the antibiotic resistance profile of these microbes.

XRF analyses of soil samples from these regions measured about 300 ppm tungsten/kg of soil at the Agargaon region and 200 ppm tungsten/kg of soil at the Khobna region. The traditional microbiological analysis resulted in two bacterial isolates which were identified for tolerance to tungsten named as *Proteus mirabilis* strain RS2K and *Bordetella avium* strain RS3K through 16S rDNA gene sequencing and phylogenetic analysis. These microbes were found to accumulate tungsten intracellularly as confirmed through ICP-MS and SEM-EDS analyses. In addition to (sodium) tungsten, the microbes were tolerant to tungstic acid, ammonium metaparatungstate, mercuric chloride, cobalt chloride and azo dyes .

Microbes exhibited well-equipped cellular mechanisms for metal tolerance to survive in heavy metal-laden ecology. The novel strains obtained through a culturable approach in this study contain substantial potential in bioleaching of heavy metals and green mining.

Introduction

Tungsten is a heavy metal that was largely substituted for lead, has been identified as an emerging contaminant and its health hazards have been confirmed recently. The heavy metal exists in various oxidative states (W^{2-} to W^{6+}) which are often stable in water. Tungsten mineral ore formations are commonly found in the south of the Bhandara-Balaghat granulite belt of 190 km length in Central India which is proposed to be a part of the Bastor Craton (Ramachandra and Roy, 2001; Santosh et al., 2020) and well known for tungsten mineralization. In the pH range of 4-9, tungsten occurs in several unstable polymeric forms in addition to at least 3 stable forms of polytungstates: paratungstate A, paratungstate B, and metatungstate which is the most soluble. Furthermore, polytungstates like sodium metatungstate ($3Na_2WO_4{\cdot}9WO_3$) that exist in higher concentrations are more toxic than monomeric tungstate (Na_2WO_4) (Strigul et al., 2005). Tungsten is found to impact the nutrient availability in soil and negatively affects the plant-microbe interactions (Shanware and Phadtare 2014). Strigul et al. (2005) reported a statistically significant decrease of *Bacillus subtilis* and *Pseudomonas fluorescens* which are markers of a healthy rhizosphere. The presence of tungsten in soil is known to impact root elongation and phosphate concentration inside the cell that fuel the cell signaling pathways, and plant cell division. Tungsten

particles reportedly also caused breakage of phosphodiester bonds in native DNA at a limited number of sites in wheat embryos after a biolistic transformation. In studies on solubility, sorption, and soil respiration of tungsten (Dermatas et al., 2004) reported that elemental tungsten added to soils above 3% (30 g tungsten/ kg of soil) by mass adversely affected the respiration of soil microbes (Strigul et al., 2009).

The heavy metal contamination in soil is a major environmental problem for which effective in-situ bioremediation techniques have been developed and implemented. The ability of potent microorganisms to tolerate heavy metals and promote transformations that turn a few metals less toxic has immense potential in effective management of heavy metal soil pollution. Development of such soil remediation processes relies on microbes and its metabolic potential.

2. Methods

2.1 Study area and sampling

The geological survey of India report (1994) surveys an area of 220 sq. km. of Sakoli basin falling in parts of Nagpur and Bhandara districts for tungsten mineralization. Tungsten mineralization was observed in the north-western part of the proterozoic Sakoli basin. The BRGL-MECL report (1991) has also confirmed the occurrence of tungsten deposits in the Khobna region. Hence Kuhi-Agargaon and Khobana region of the Sakoli basin were selected for soil sampling in this study. Figure 1 and Supplementary Table 1 depict the location details of the sites where the soil was sampled. USEPA standard protocols were followed for sub-sampling of soil and samples were transported and stored at 4ºC for further analysis.

2.2. XRF analysis

Sampled soils were subjected to X-ray fluorescence analysis for estimation of heavy metal concentration in soils. The energy dispersive X-ray fluorescence spectroscopy (XRF) technique was used for the qualitative and quantitative determination of the elemental composition of samples where fluorescence radiation was measured by the detector (http://xrf-spectroscopy.com/). These analyses were performed at the Indian Bureau of Mines (IBM) laboratory, MIDC, Nagpur, India.

2.3. Physical and chemical characterization of Soil

The physical and chemical characteristics of the soil system influence the transformation, retention, and movement of pollutants through the soil. Hence soil characteristics like pH, electrical conductivity, organic carbon, total nitrogen, phosphorus, and potassium content of the soil samples were analysed using standard protocols (Government of Maharashtra, 2009).

2.4. Microbial isolation and identification

Enrichment techniques and serial dilution resulted in isolated bacterial colonies tolerant to tungsten. Bacterial cultures were grown in M9 minimal media with 6 different concentrations of sodium tungstate (5-30 gm/L) with respective controls were incubated at 37°C for 24-48 hrs in an orbital rotator shaker incubator at 120 rpm and checked for visible growth (Jadeja et al., 2019). Cultures in their exponential growth phase were subjected to increasing concentrations of sodium tungstate for 24 hours.

For identification of strains, extraction of total DNA from bacterial cells for PCR analysis was performed by following a simple and rapid CTAB NaCl lysis protocol (Jadeja et al., 2019). Extracted DNA was amplified in Peltier Thermal Cycler and MJ Research Thermal Cycler, with optimal temperatures with thermo cycling program set for 35 cycles. The thermocycling steps include denaturation at 95ºC for 5 minutes and 35 cycles of steps: denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºC for 1 minute, followed by a final extension at 72ºC for 10 minutes. 16S rDNA gene universal primer with the forward sequence AGAGTTTGATCCTGGCTCAG and reverse sequence AAGGAGGTGATCCAGCCGCA was used in PCR reaction. The PCR product was analysed using horizontal gel electrophoresis systems of standard dimensions from Bangalore Genei with electrophoresis carried out at 100-120 V for 90 min in 1% agarose gel using appropriate dye. The PCR product was sequenced and read in the sequencer with the help of DNA baser software. Sequencing was performed at Triyat Scientific Company. Basic Local Alignment Search Tool search resulted in showing the homologs to the sequences obtained. Phylogenetic tree using closest matches were generated in the MEGA X tool (Kumar et al., 2018).

2.5. Growth curve

Each isolated colony was inoculated in 0.1X LB broth and grown overnight at 120 rpm 37ºC. The next day the cells were harvested by centrifugation, washed with 56 Mm Phosphate buffer and inoculated in 50 ml of M9 medium further incubated at 37°C at 120 rpm in orbital incubator shaker. Absorbance at 600 nm was measured starting from 0 hours to 4 days at the interval of every 8 hours. The growth of isolates was tested in presence of different metals i.e., ammonium metaparatungstate, tungstic acid, cupric nitrate, mercuric chloride, and silver nitrate at different concentrations. All the flask experiments were performed in triplicates.

2.6. Antibiotic susceptibility tests

Antibiotic susceptibility tests were performed by using 24 different antibiotic discs which included ofloxacin (5 µg), nitrofuranoze (100 µg), ciprofloxacillin (30 µg), clindamycin (10 µg), carbenicillin (100 µg), polymyxin b (300 units), fluconazole (10 µg), cefazolin (30 µg), lincomycin (15 µg), amikacin (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 units), norfloxacin (30µg), netillin (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), tetracycine (30 µg), gentamycin (10 µg), kanamycin (30 µg), co-trimoxazole (25 µg), streptomycin (10 µg). The sensitivity and resistance profile was determined by the diameter of the zone of inhibition and further evaluation was done according to National Committee for Clinical Laboratory Standard's charts.

2.7. Quantification of tungsten using Inductively Coupled Plasma Mass Spectrometry

To check the extent of the tolerance, bacterial isolates previously tested for metal tolerance were further studied. The isolates that had visible growth in the presence of tungsten were chosen for further analysis. Inductively coupled plasma mass spectrometry (ICP-MS) was used to detect tungsten concentrations. M9 minimal media was prepared to screen for potential tungsten resistant bacterial strains (TRSBs). 1000 ppm of sodium tungstate was supplemented in the media and 500µl bacterial cells of pure isolates (O.D. value = 0.6) were used as inoculums. The tubes were incubated at 37 ± 1ºC on a rotatory shaker at 120 rpm till the development of moderate turbidity. Cultures with sodium tungstate were centrifuged at 8000 rpm at 20ºC for 10 min in sterilized falcon tubes. The supernatant was filtered, and ICP-MS analysis were performed for quantification of tungsten in samples at Anacon Labs, Nagpur.

2.8. Dye decolorization assay

Microbes found tolerant to tungsten were further tested for decolourization of acid orange 7 dye. The dye decolorization experiments were carried out in 100 ml flasks containing 50 ml of minimal M9 media and acid orange 7 dye (100 mg/l). The pH was adjusted to 7 ± 0.2 using sodium hydroxide and hydrochloric acid solution and the cultures were incubated at 37°C for 4 days. Samples were drawn at 24 hours intervals for observation. 10 ml of the dye solution was filtered and centrifuged at 8000 rpm for 20 minutes. Decolorization was assessed by measuring the absorbance of the supernatant with the help of spectrophotometer at wavelength maxima (λm, here 485 nm) of the respective dye (Shah, 2014). Decolourization at different pH ranging from 3,5,7,9,11,13 was studied using tungsten tolerant isolates. The decolorization assay measured percentage decolorization using UV-Spectrophotometer with the following equation,

% Decolorization = (Initial OD-Final OD*100)/Initial OD

3. Results And Discussion

In the present study soil sampling site at a tungsten contaminated area was performed from where heavy metal tolerant microbes were isolated and analysed.

3.1 Soil characteristics

GSI and BRGL-MECL reported tungsten reserves in the Sakoli basin region, so we selected the Agargaon and Khobna region was selected for sampling (Figure 1). Sampled soil was of brown clayey loam type texture with a high percentage of clay particles and some sand-slit, Table 1 enlists the chemical characteristics of soil samples. X-Ray Fluorescence analysis confirmed the presence of tungsten in both soil samples. Supplementary Table 1 enlists the results of XRF analyses, which indicate the presence of transition metals (period 3, 4, 5 and 6).

3.2. Phylogenetic analysis of TRSBs

Microbial isolates were obtained from sampled soils using serial dilution and enrichment techniques in presence of 300 ppm of sodium tungstate. Colonial, cellular and gram staining characteristics of about 13 isolates were studied as given in Table 2. Two isolates, RS2K and RS3K capable of tolerating 300 ppm of sodium tungstate were further studied for their morphological and biochemical characteristics. Supplementary Table 2 enlists the biochemical characteristics of these 2 isolates. 16S rDNA gene sequencing of RS2K and RS3K and homology search in BLAST revealed that the two isolates were novel strains. RS2K was annotated as *Proteus mirabilis* and RS3K was annotated as *Bordetella avium.*

Figure 2a and b depict the phylogenetic analysis of *Proteus mirabilis* strain RS2K and *Bordetella avium* strain RS3K. The evolutionary history inferred using the neighbor joining method resulted in the sum of branch length 0.00621436 for RS2K and 46.27749999 for RS3K. which are indicative of the evolutionary distances of these strains. The evolutionary distances were computed using the Maximum composite likelihood method and both phylogenetic constructions involved 12 nucleotide sequences. There were a total of 1453 positions and 1359 positions assessed in the RS2K and RS3K 16S rDNA gene sequence and their homologs.

3.3 Physiological assays

Figure 3a and b depict the growth physiology of the two TRSBs in presence of sodium tungstate at varied concentrations and other heavy metals. Bacterial growth exhibited typical logarithmic growth curves when grown on metals and different concentrations. These two pure cultures were also found to tolerate other metals like mercuric chloride, cobalt chloride, and ammonium metaparatungstate along with tungstic acid (Figure 3b).

This is the first report of *Proteus* and *Bordetella* genera for tungsten tolerance. Such catabolic capacities render these microbes an effective candidate for soil bioremediation using microbial consortia. The catabolic capacity of heavy metal tolerance in these microbes can be attributed to the presence of a heavy metal-ABC transporter cassette. Members of *Pseudomonas* genera are the most common heavy metal tolerant microbes (Chellaiah 2018). However diverse microbes like *Thiomonas, Acidithiobacillus* and *Acidithiobacillus* strains are also recognized for their metal solubilizing abilities (Navarro et al., 2013). These microbes possess a variety of genetic mechanisms that facilitate heavy metal sequestration which renders them an ideal candidate for eco-friendly and cost-effective biosensors.

3.4. Antibiotic susceptibility of TRSBs

Before proposing microbial candidates for heavy metal bioremediation, it is critical to test their antibiotic resistance profile. Antibiotic resistance is increasing at alarming levels and is a public health concern (WHO, 2019). Hence, we tested RS2K and RS3K for antibiotic resistance and the test results are shown in Figure 4. RS2K was found susceptible to gentamicin with 40 mm zone of inhibition, nitrofuranzone 38mm, ofloxacin (5 mcg) 38 mm, ofloxacin (2 mcg) with 36 mm zone of inhibition and resistant to antibiotics amikacin and ampicillin showing no zone of inhibition. *Bordetella avium* strain RS3K was

found to be susceptible to nitrofuranoze and ciprofloxacin with 40 mm of zone, gentamicin with a zone of inhibition of 36 mm and resistant to antibiotics like ceftazidime, cefazolin, polymyxin. *Proteus mirabilis* is known to carry class 1 integrons which are responsible for its resistance to antibiotics and therefore further genomic analysis is necessary before considering this microbe for any widespread application in the environment.

3.5. Quantification of tungsten in microbial cells.

Tungsten was being either tolerated or accumulated by RS2K and RS3K as confirmed by ICP-MS analysis. Table 3 shows the accumulation of tungsten by bacteria. The concentration of tungsten was found depleted as compared to the initial concentration in the supernatant. *Proteus mirabilis* strain RS2K was found to be most prominent with 13% of bioaccumulation whereas *Bordetella avium* strain RS3K showed 4% of accumulation as depicted by ICP-MS. EDS results show that the strains were capable of tungsten accumulation which was predicted by observing peaks of tungsten 5.08% atomic weight. SEM analyses in Figure 5 show distortion in the shape of bacterial cells which confirmed the intracellular accumulation of tungsten and being distributed through the cell of both the strains as seen in Figure 5A (a and b) and 5B (a and b). In the *Proteus mirabilis* strain RS2K 5.08% atomic weight of tungsten was observed and in *Bordetella avium* strain RS3K 1.59% atomic weight of tungsten was measured. In a recent study where *Metallosphaera sedula* was supplemented with tungsten polyoxometalate SEM analysis revealed the transformations of appearance of polyoxometalate to low molecular weight tungsten (Milojevic et al., 2019). While there are scarce reports of accumulation of tungsten in microbes, its concentration in soil ranged from 0.05 to 28.48 mg/kg in a study from China proving an increased chance of accumulation of this metal in vegetables that were grown in tested soils (James and Wang 2020).

3.6. Dye Degradation

We used acid orange 7 to check the dye degradation potential in *Proteus mirabilis* strain RS2K and *Bordetella avium* strain RS3K. While both strains showed effective dye decolourization, especially at pH 7, *Bordetella avium* strain RS3K was found to be more efficient. Figure 6 depict the dye decolourization capacities of these two strains. *Proteus* and *Bordetella* are not quite common dye degrading microbes; however, a few studies have found these microbes effective in decolorization of dyes. A strain of laccase enzyme producing *Bordetella bronchiseptica* was found to decolourize synthetic dyes and *Proteus mirabilis* strain isolated from textile wastewaters was identified for its catabolic potential of dye degradation (Madhushika et al., 2019; Unuofin 2020).

3.7. Data availability

The complete 16s rDNA gene sequences for strain *Proteus mirabilis* RS2K and *Bordetella avium* RS3K isolated in this study are available in the NCBI database under accession numbers KJ937078.1 and KJ937079.1 respectively.

Conclusion

Long-term exposure to heavy metals leads to the selection of the microbes which evolve to thrive in stressed environments. The environmental stress caused by heavy metals generally decreases the diversity and activity of soil's microbiome leading to a reduction of the total microbial biomass and loss of diversity. However, such ecologies have a unique environment and are model sites for the discovery of the novel microbes. Although we tested soil samples for tungsten tolerance, the microbes isolated had resistance to other heavy metals and an effective role in dye degradation. The catabolically potential microbial population optimally adapted to contaminated soil and rhizosphere conditions. While we are witnessing the era of high throughput sequencing technologies that have facilitated in-silico analysis of microbes and microbiomes, the culturable approach continues to contribute greatly to our fundamental understanding of soil microbial ecology

Declarations

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Tables

Table 1: Chemical characteristics of Soil samples

No.	Characteristics	Agargaon soil sample	Khobna soil sample
1	pH	8.20	7.9
2	Electrical Conductivity	0.38 deci/m	0.51 deci/cm
3	Organic Carbon content	0.68 %	1.48 %
4	Total Nitrogen (Kjeldahl method)	431.12 kg/ha	489.33 kg/ha
5	Available Phosphorous	27.66 kg/ha.	71.69 kg/ha
6	Available Potassium	430.08 kg/ha.	551.04 kg/ha

Table 2. Morphological characterization of isolates

Sr. No.	Stain Name	Colour	Size/Shape	Gram Nature	Motility
1	RS2K	white	very small rounded	gram negative	motile
2	RS3K	white	very small rounded	gram negative	motile
3	RS7K	whitish yellow	elongated	gram negative	motile
4	RS10K	whitish yellow	rough-edged rounded	gram positive	motile
5	RS12K	yellow	very small rounded	gram positive	non-motile
6	SD3	whitish yellow	very small rounded	gram negative	non-motile
7	EN1	yellow	round	gram negative	non-motile
8	EN2	whitish yellow	very small rounded	gram negative	motile
9	EN3	yellow	rough-edged	gram negative	motile
10	EN4	white	round	gram negative	non-motile
11	EN5	white	round	gram positive	non-motile
12	EN6	white	very small rounded	gram negative	motile
13	EN7	yellow	very small rounded	gram positive	non-motile

Table 3. Quantification of tungsten accumulation by ICP-MS

Bacterial Strain	Initial tungsten Supplemented (g/L)	Residual tungsten (Supernatant) (g/L)	Tungsten Bioaccumulated (%)
Proteus mirabilis strain RS2K	10	8.7	13%
Bordetella avium strain RS3K	10	9.6	4%

Figures

a

b

Figure 1

a. Closed Tungsten mine at Agargaon b. Location of sampling sites at Bhandara and Sakoli regions near Nagpur city.

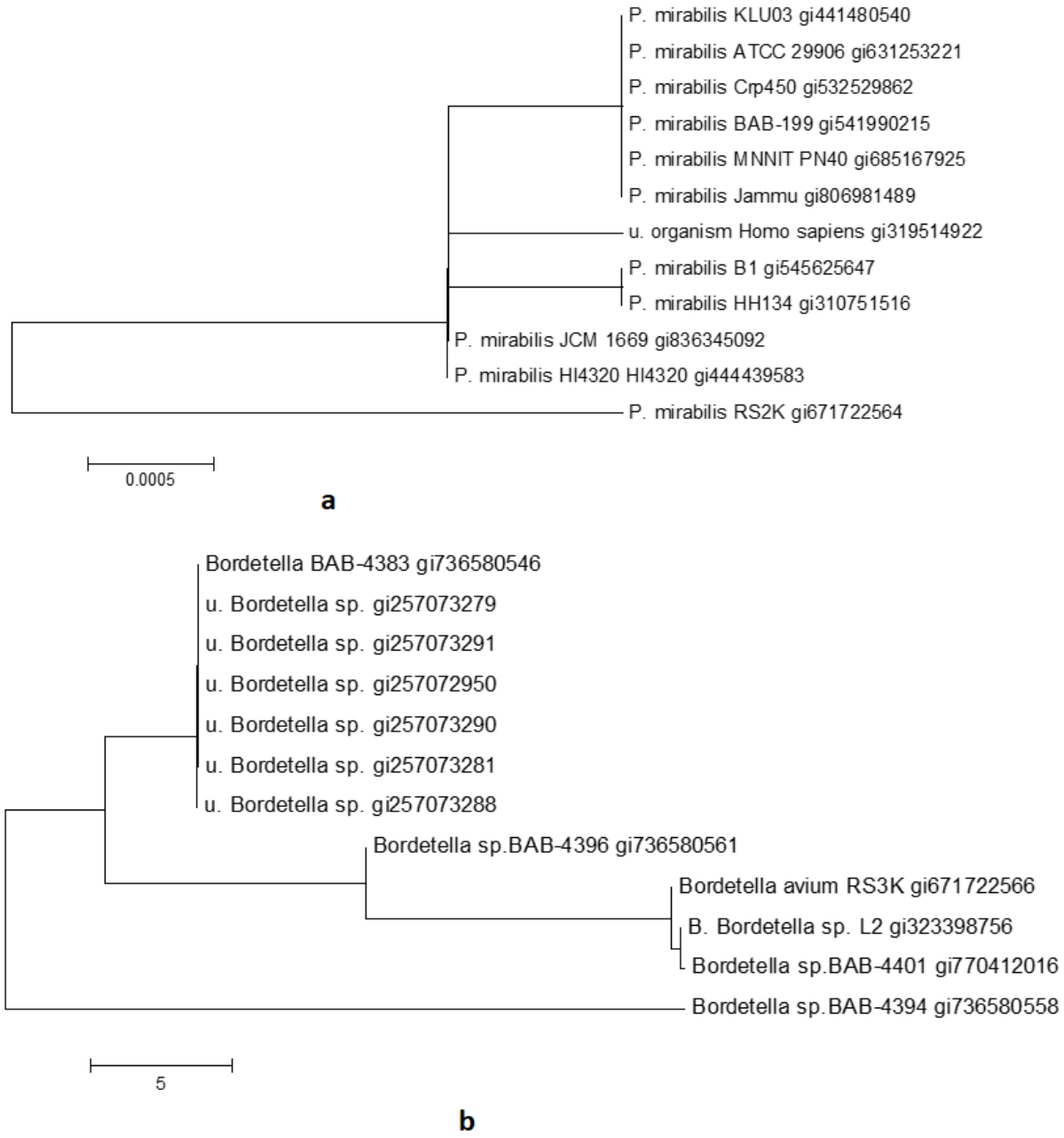
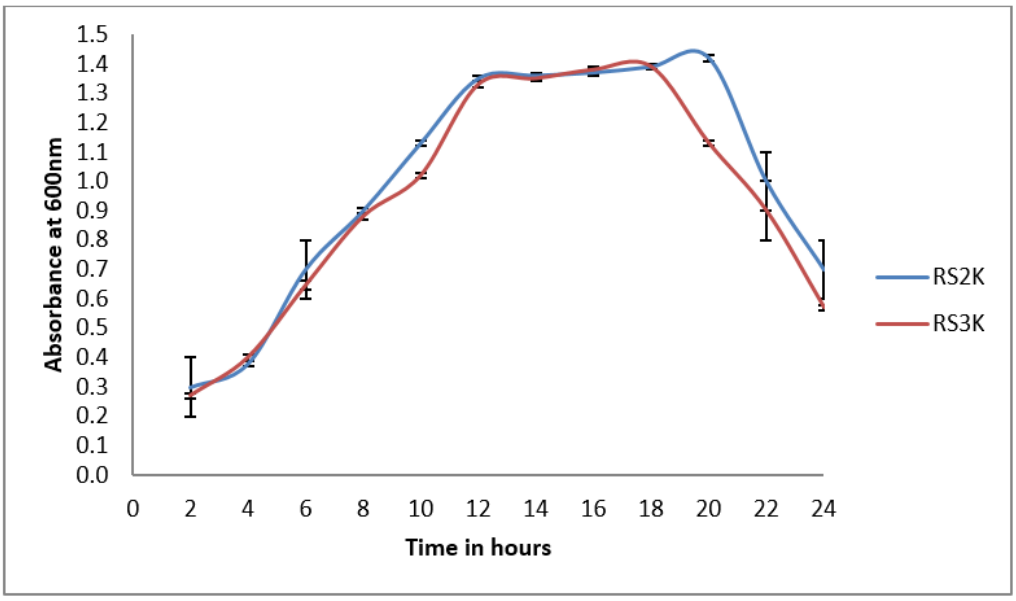
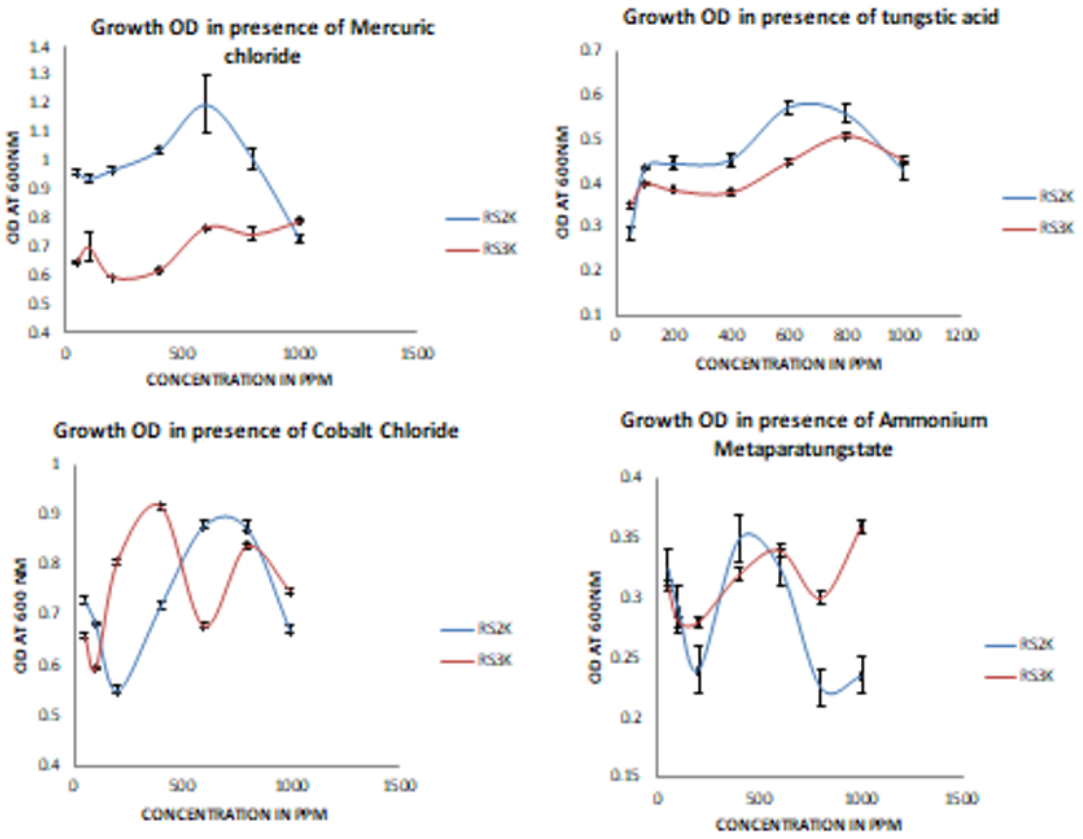


Figure 2

a. Phylogenetic tree of Proteus mirabilis strain RS2K obtained by Neighbour joining method. b. Phylogenetic tree of Bordetella avium strain RS3K obtained by Neighbour joining method.



a



b

Figure 3

a: Growth pattern of RS2K, RS3K isolates b. Growth OD of 2K and 3K in presence of different metal concentration.

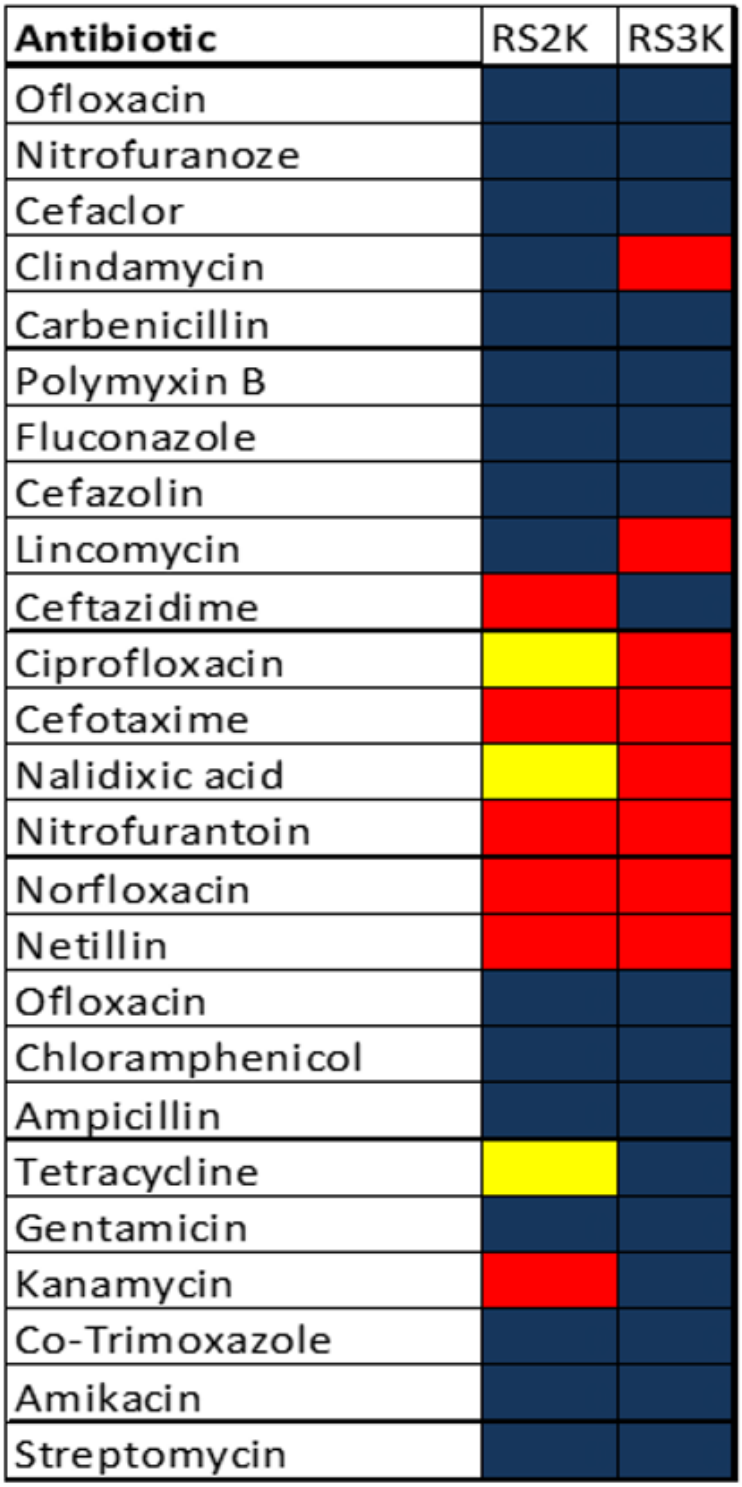


Figure 4

Antibiotic disc test results, blue indicates sensitivity, yellow indicates intermediate and red indicates resistances in the microbial isolate

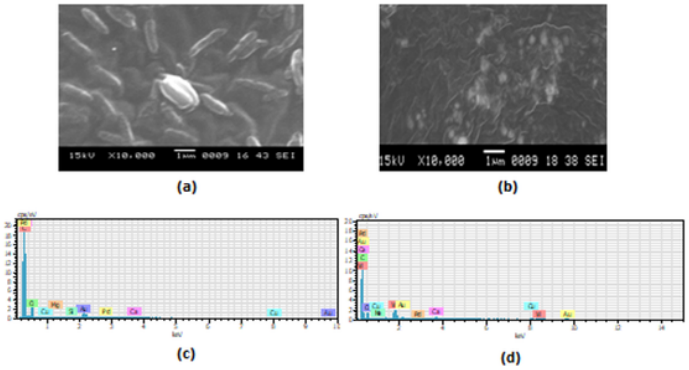


Figure 5A: (a)SEM in absence of sodium tungstate (b) SEM in Presence of sodium tungstate (c) EDS in absence of sodium tungstate (d) EDS in Presence of sodium tungstate of *Proteus mirabilis strain RS2K* in M9 medium

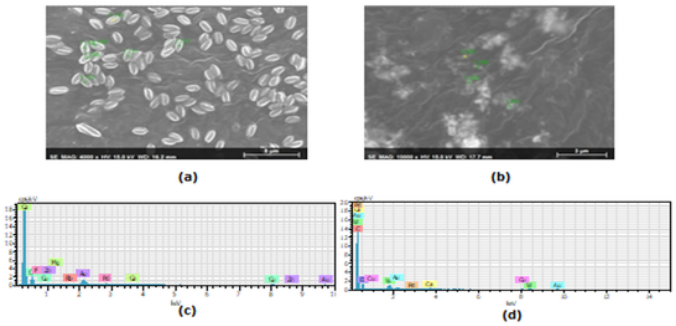


Figure 5B: (a)SEM in absence of sodium tungstate (b) SEM in Presence of sodium tungstate (c) EDS in absence of sodium tungstate (d) EDS in Presence of sodium tungstate of *Bordetella avium strain RS3K* in M9 medium.

Figure 5

See image above for figure legend.

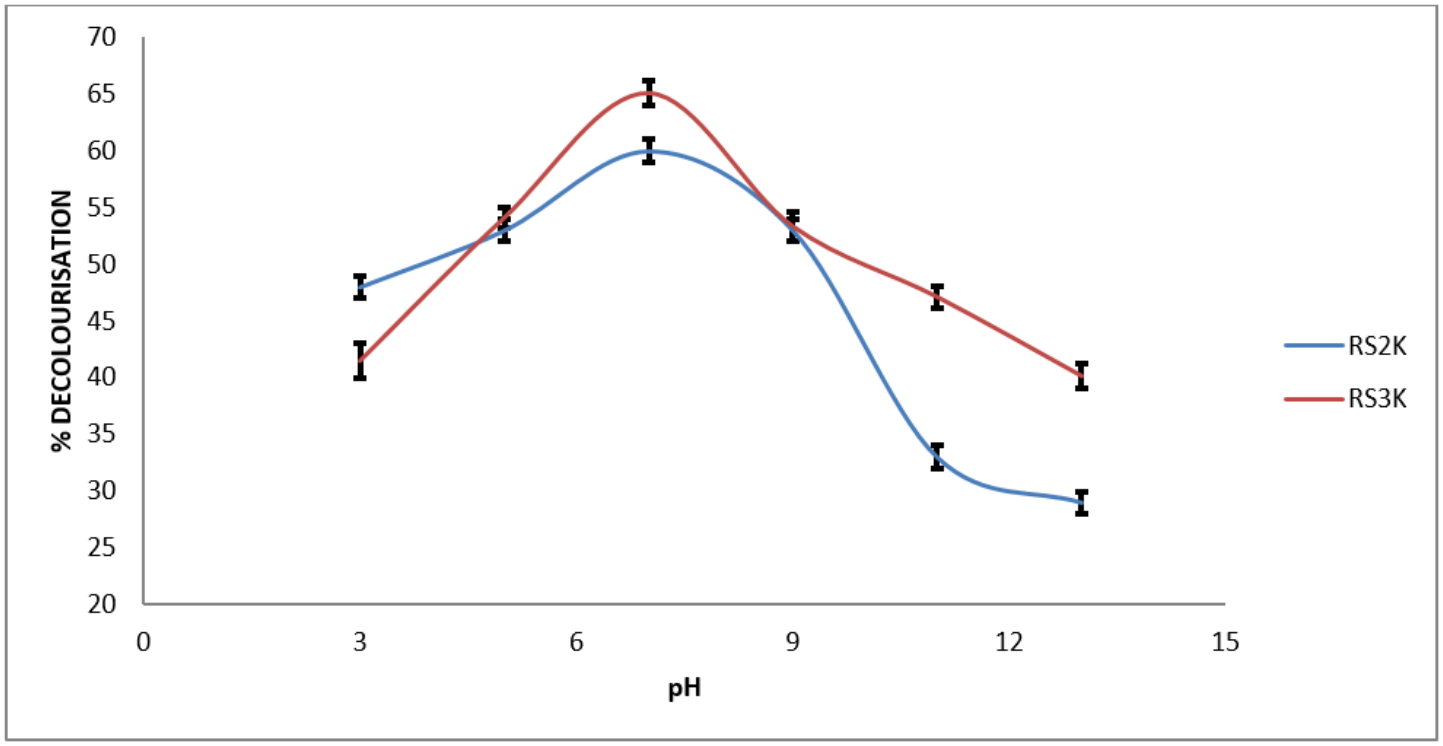


Figure 6

Acid orange 7 dye Degradation of the two strains at different pH

Supplementary Files

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