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Research Article

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Effect of copper sulphate on "*Citrobacter*"

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ABSTRACT

The quality of life on earth is linked undeniably to the overall quality of the environment. Pollution of the biosphere by heavy metals due to industrial, agricultural and domestic activities has created a serious problem for the safe and rational utilization of soil. Industrial inputs and the agronomic application of fertilizers, pesticides and metal-contaminated sewage continue to contribute the metal accumulation in the soil . The pollution of the ecosystem by heavy metals is a real threat to the environment because metals cannot be naturally degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain. Here effect of copper sulphate on Citrobacter has been discussed on its growth. These findings will be very beneficial to reveal the causes behind soil pollution.

Keywords: sulphates, heavy metals, Citrobacter , ecosystem, fertilizers, pesticides

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INTRODUCTION

Increasing amounts of discharged sewage, progressing urbanization , the chemicalization of agriculture and industry, as well as anthropogenic activities all effect the quality of underground waters. The final effect of water degradation are the limits as to the use of drinking water reservoirs . Frequently this state is coupled with microbiological contamination resulting in the penetration of potentially pathogenic bacteria or microorganisms detrimental to underground waters through the soil [15]. Hence these bacteria may become the source of various diseases the intensity of which would largely depends on microorganisms pathogeneity and disease potential [13]. Some of the bacteria like *Pseudomonas* may be a threat to human health due to their ability to multiplying in drinking waters [7]. Bacterial contamination is most dangerous in the case of shallow reservoirs of underground water originating from the quaternary [3]. Here sulphates have been used on *Citobacter*. Sulphides can be chemically or biologically oxidized into metal sulphate when exposed to oxygen and water[8,1]. This lead to acid mine drainage (AMD) and this results in severe environmental stress [11]. In the biological sulphate reduction process, sulphate reducing bacteria (SRB) are used [14]. As copper sulphate has been used here, copper has been used as a biocide for centuries. These compounds have been shown to effectively kill a wide range of yeast, fungi and bacteria [2,4,6,9,10,12]. Copper treatments always disrupt biological life in a dugout ecosystem. Copper treatment work by dissolving copper into the water. When dissolved into the solution , free copper ions will kill the target organisms [5].

EXPERIMENTAL SECTION

Collection of strain: The pure strain of *Citrobacter* was collected from Department of biotechnology, Jiwaji University Gwalior (M.P.). This strain was used in this study to evaluate the metal stress effect on growth and metal ions accumulation by the strain.

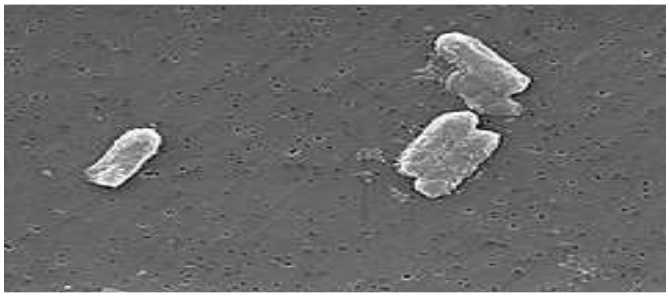


Figure: *Citrobacter*

Scientific classification	
Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	***Citrobacter*** Werkman and Gillen, 1932

Turbidity standard for inoculums preparation:
To standardize the inoculum density for susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard (e.g., latex particle suspension), was used**.**

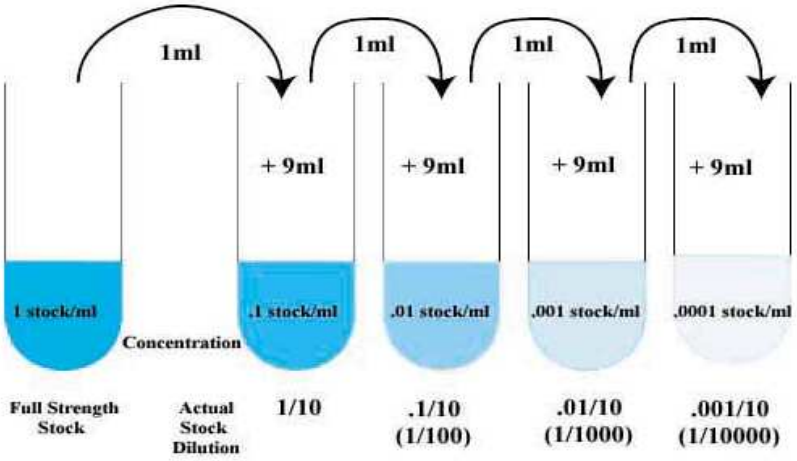
A $BaSO_4$ 0.5 McFarland standard was prepared as follows:
1. 0.5ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard was verified by using a spectrophotometer. The absorbance at 625nm was 0.008 to 0.10 for the 0.5 McFarland standard.
3. The barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before use each time and inspected for a uniformly turbid appearance.

Inoculum preparation:
The strain of *Citrobacter* bacteria was standardized at $1*10^8$ CFU/ml(colony forming units) using Mc Farland standard. The turbidity of overnight grown cultures of *Citrobacter* was measured spectrophotometrically at 600nm and compared with 0.5 McFarland as standard. The cells were further diluted in respective culture media to obtain a concentration of $1*10^5$ CFU/ml for susceptibility tests.

This concentration was achieved by serial dilution of *Citrobacter* strain. The serial dilutions were 2-fold, 3-fold or 10-fold depending upon the requirement. These were performed as follows: One ml of *Citrobacter* culture was added in 9 ml of sterilized media, the concentration of cells then reduced to 10^7 cells/ml. The step was repeated two or more times so that the concentration of culture reached to $1*10^5$ cell/ml required for experiment.

Revival and Sub-culturing of pure *Citrobacter* culture:
The test strains were streaked on Muller Hilton agar (MHA) (Himedia Pvt. Ltd) medium plates and kept overnight at 37°C to isolate pure colonies of bacterial strains. Muller Hinton Broth (MHB) (Himedia Pvt. Ltd) was used for sub-culturing the *Citrobacter*. Six 50 ml flask were taken and 25 ml of sterilized MHB media was poured. 4-5 well grown *Citrobacter* colonies were picked and inoculated into MHB with help of inoculating loop and kept overnight at 37ºC in incubator to achieve optimum growth. After 24 hours the growth kinetics was observed

spectrophotometrically at 600nm to ensure that cultures reached their exponential phase. The turbidity was checked with 0.5 McFarland standard.



Representation of serial dilution

Metal ions as antimicrobial agents:
The sulphate salts of heavy metals were obtained from Department of Biotechnology, Madhav Institute of Technology and Science, procured from Himedia Pvt. Ltd. The metal used as antimicrobial agents were taken in various concentrations viz., 0, 20, 40, 60, and 80mg/ml and dissolved in sterile media.

Preparation of metal ion stock solutions: The powders of metal salts were accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile glassware. Stock solutions were frozen at 4ºC for future experimental work.

Stock solution preparation:
Stock solutions were prepared by using the formula

(1000/P)*V*C=W

Where;
P=Potency given by the manufacturer in relation to the base
V= Volume in ml required
C=Final concentration of solution (multiples of 1000)
W= Weight of the antimicrobial to be dissolved in the volume V

Determination of effect of metal ion stress on *Citrobacter*
Susceptibility testing:
Mueller-Hinton agar for susceptibility test: Mueller-Hinton Agar (MHA) medium is the only susceptibility test medium that has been validated by NCCLS. MHA (Himedia Pvt. Ltd.) was used for disk diffusion susceptibility testing, according to NCCLS and international guidelines. Two standard methods were used to check the susceptibility of various bacterial and fungal strains.

a. The effect of metal ions concentrations were determination by Agar Well/Disk diffusion method
b. The effect of metal ions concentrations were determination by Broth Dilution Method

Determination of metal stress effect on bacterial and fungal growth by Agar Well/Disk diffusion method:
The Agar Well/Disc Diffusion method was used to test metal stress effect on growth of bacterial and fungal species. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The dried surface of a MHA plates were inoculated by streaking the swab over the entire sterile agar surface. The lid was left ajar for 5 minutes to allow for any excess surface moisture to be

absorbed before impregnate well in agar. The wells were filled with each metal (20µl). After 24hrs at 37°C of incubation each plate was examined for *Citrobacter* growth. The diameter of the zones of complete inhibition was measured, including the diameter of well with a ruler .

Determination of effect of metal ions concentrations were determination by Broth Dilution Method:
2ml of sterile MHB was taken in test tubes for *Citrobacter* growth. 2ml of metal ion stock was added in one test-tube and was diluted 2-fold upto 10 times. 20µl of *Citrobacter* culture at $1*10^5$ CFU/ml was added in each test-tube. The test tubes were incubated for 24 hrs at 37ºC. After incubation the test-tubes were observed for visual growth of *Citrobacter*. The test-tube without turbidity was considered as test-tube containing metal ion at the concentration that inhibits the growth of *Citrobacter* and is called as minimum inhibitory concentration of metal ion for a *Citrobacter*.

RESULTS

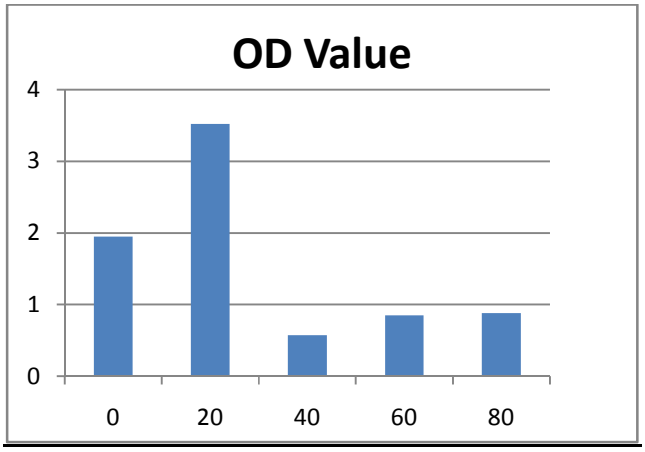
Effect of metal stress on growth of *Citrobacter* by Agar Well Disc Diffusion method (diameter in mm).

Metal ion	Test organism	20mg/ml	40 mg/ml	60 mg/ml	80 mg/ml
Copper	*Citrobacter*	R	12	22	24

Well Diameter (mm) ≤12 Resistant, 13-17 Intermediate, ≥18 Susceptible

Effect of Copper sulphate

Concentration(mg/ml)	Optical Density
0	1.72
20	0.63
40	0.78
60	0.98
80	1.30



Representation of the growth of *Citrobacter* in presence of copper sulphate

DISCUSSION

After performing these experiments, it is concluded that growth is maximum at 20 mg/ml but it varies upto some extent according to other sulphates which are not tested. This is going to be helpful in determining the causes behind soil pollution. The growth of bacteria may be varies from the exact graph given in this report because of so many reasons like concentration, environmental condition etc.

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