ORIGINAL ARTICLE

Effect of Lead on Microorganisms with Respect to Antibiogram, Glucose and Amino Acid Metabolism

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Abstract:

Background: Lead poisoning is a prevalent health hazard in today's world of industrialization and is gaining the concern of medical professionals globally. The first organisms in the biosphere to be affected by this are the microorganisms. Many studies have established that metal tolerance is accompanied by antibiotic resistance as both the genes are present on plasmids. *Aims and Objectives:* The study was conducted to identify the concentrations of lead at which the microbial growth and antibiotic sensitivity was affected and also to identify whether any of the key metabolic activities were influenced. Microorganisms like *Escherichia coli, Staphylococcus aureus* and *Candida albicans. Pseudomonas aeruginosa* were chosen due to their increasing importance as a potent hospital acquired pathogen. *Material and Methods:* American Type Culture Collection (ATCC) strains were chosen and exposed to varying concentrations of lead acetate ranging from 1 to 1000 ppm. The growth was quantitatively analyzed spectrophotometrically at 600 nm. The antibiogram was done using disk diffusion method. The sugar fermenting property and the amino acid utilization was studied as they are the basic requirements for growth of any microorganism. *Results:* On exposure to lead, a decrease in the growth was seen with the three organisms but the growth pattern was different with *Pseudomonas* as it showed a sudden increase at 100 ppm accompanied by the production of H_2S at certain concentrations. The antibiotic sensitivity tests which were carried out after exposure to lead, showed a resistance pattern to the β lactam group of antibiotics, hence implying that tolerance to the heavy metal affected the sensitivity of these organisms to the antibiotics. The biochemical tests showed no change in the presence of lead. Lead may exist in the soil in various concentrations but may exert a selective pressure only at certain concentrations. It has been established that a pattern exists between the antibiotic resistance and tolerance to lead, but since there seems to be no effect on the constitutional properties of the microorganisms, it can be concluded that heavy metal tolerance may have an effect only on plasmid conferred properties for the microorganisms.

Keywords: Antibiogram, Amino Acid Utilization, Lead Poisoning, Microorganisms, Sugar Fermentation

Introduction:

Heavy metal pollution has been a problem that has plagued mankind for a number of years. However, now, the need to understand and contain the effects of this health and environmental hazard has only increased manifold. Lead toxicity is one of the most widely studied heavy metal poisoning [1]. Accumulation of lead and other heavy metals by microbial communities is just one of the many ways by which they enter into the food web, ultimately accumulating in humans. The biogeochemical cycling of various compounds by these microorganisms combined with human activities, such as mining, agriculture and industrialization, results in local and global pollution [2]. Thus, to fully understand the molecular level at which the heavy metal toxicity is seen, it is necessary to analyze the genetic changes as well as changes in

the protein systems of organisms like bacteria and fungi as they are first in line to be exposed to varying concentrations of the metals in the environment.

A study was conducted to identify the concentrations of lead at which the growth of the microbes was affected and also to identify whether any of the key metabolic activities were influenced by the presence of lead. Also, the antibiograms of each of the organisms were studied to identify and establish a pattern of resistance, if any, and its correlation to the concentration of lead to which the microorganisms had been exposed. Common human pathogens were chosen for the study such as *Escherichia coli, Staphylococcus aureus* and *Candida albicans. Pseudomonas aeruginosa* was also chosen due to its increasing importance as a potent hospital acquired pathogen and its recent inclusion as a potential bioremediation agent [2]. American Type Culture Collection (ATCC) strains of all the microorganisms were chosen as all the biochemical properties are known and any changes in the properties of the microorganisms could be attributed to the presence of lead in the *in-vitro* growth environment. Other studies have been carried out by isolating organisms from environments such as marine water, estuaries and drinking water samples [3-5] but no reports are found related to the use of the laboratory strains of these specific microorganisms.

Material and Methods:

The microbial cultures were adjusted according to the 0.5 McFarland standards. Four to five identical colonies were picked up from the culture plates of each organism, inoculated into Nutrient Broth and incubated at 37°C for 2 hours and then the turbidity was matched with that of the standard.

Exposure of the Microorganisms to Various Concentrations of Lead Acetate Trihydrate from the Stock Solution:

From the stock solution of 1500ppm, different aliquots were pipetted out into sterile test tubes. Calculations were done as per standard formulae ($C_1V_1=C_2V_2$).

The lead salt solution and the broth culture were added in such a way that the concentration of lead remained the same as required per 5ml as shown in (Table 1).

Table 1: Proportions of Lead Solution and Broth Cultures to Make up the Volume to 5ml

PPM	Volume of Broth culture (µL)	Volume of Lead solution (µL)
0	5000	0
1	4996.7	3.3
10	4966.7	33.3
50	4833.4	166.6
100	4666.7	333.3
250	4166.7	833.3
500	3333.4	1666.6
1000	1666.7	3333.3

Spectrophotometric Measurement of Growth of Microorganisms after 24 hrs Exposure to Lead:

The growth of the microorganisms, which were exposed to lead for 24 hrs, was measured at 600nm using a spectrophotometer. The readings were taken at two intervals of 2 hours and 24 hours after inoculation into the lead solution and graphs were plotted using appropriate software tools.

Antibiotic Sensitivity Tests of (AST) Micro-organisms after 24 hrs Exposures to Lead

The cultures those were exposed to lead for 24 hrs were tested with discs of the standard antibiotics which are used clinically to treat diseases caused by these organisms. The method was followed according to standardized Kirby Bauer method of disc diffusion (Table 2).

Table 2: Antibiotics and their Concentrations used for AST of the Lead Exposed Microorganisms

Organism	Antibiotic	Concentration (µg)
Escherichia coli	Ampicillin	10
	Cefotaxime	30
	Gentamicin	10
	Netillin (Netilmicin Sulphate)	30
	Ciprofloxacin	05
Staphylococcus aureus	Ciprofloxacin	05
	Penicillin	10
	Chloramphenicol	30
	Gentamicin	10
	Co-Trimoxazole (Sulpha/Trimethoprim)	25
Pseudomonas aeruginosa	Piperacillin	100
	Ceftriaxone	30
	Ceftazidime	30
	Ciprofloxacin	30
	Amikacin	30
Candida albicans	Fluconazole	25

Gram Staining of Cultures to Determine the Purity of Inocula:

Modified Hucker's Method - To establish the purity of the cultures obtained from the test plates, Gram staining was done of the test cultures and the pure cultures and compared under the microscope.

Determination of Sugar Fermenting Property of Microorganisms after Lead Exposure:

The microbial cultures, which were exposed to various concentrations of lead, were checked for their sugar fermenting ability. The medium used

was that of 1% glucose broth with bromothymol blue as an indicator. For *Candida albicans*, the percentage of glucose was increased to 2% [6], [7] and incubated overnight at 37°C.

Determination of Amino Acid Decarboxylase Activity of Lead Exposed Microorganisms:

The amino acid decarboxylase activity of the microorganisms was done to determine the amino acid utilization potential. Suitable inocula of the lead exposed cultures were added to Moeller's Decarboxylase broth containing the amino acids Lysine, Arginine and Ornithine individually and incubated overnight at 37°C.

Results:

Exposure of the Microorganisms to Various Concentrations of Lead Acetate Trihydrate from the Stock Solution:

As seen in (Fig. 1), a variation in the turbidity was seen as the concentration of lead increased. The tubes containing the *Pseudomonas* inocula showed the presence of H_2S being produced in the tubes containing only 10, 50, 100 and 250ppm of lead. H_2S reacts with lead acetate to form a black precipitate of lead sulfide. None of the very high or very low concentrations of lead elicited such a response. Even among the positive tubes, a gradation was seen in the density of the color as seen in (Fig. 2).

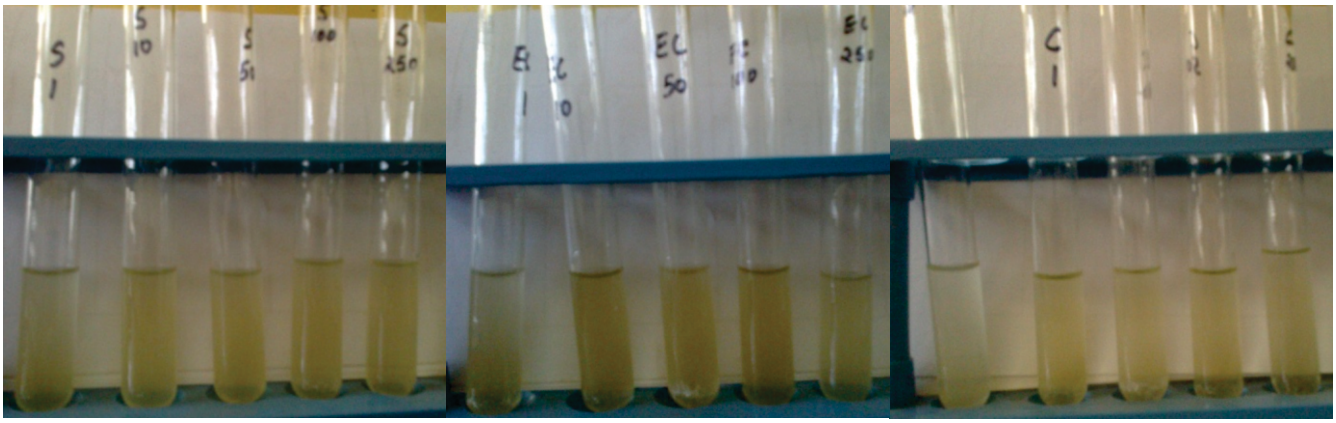


Fig. 1: Tubes Containing Inocula Exposed to Various Concentrations of Lead and Incubated at 37°C for 24 hrs

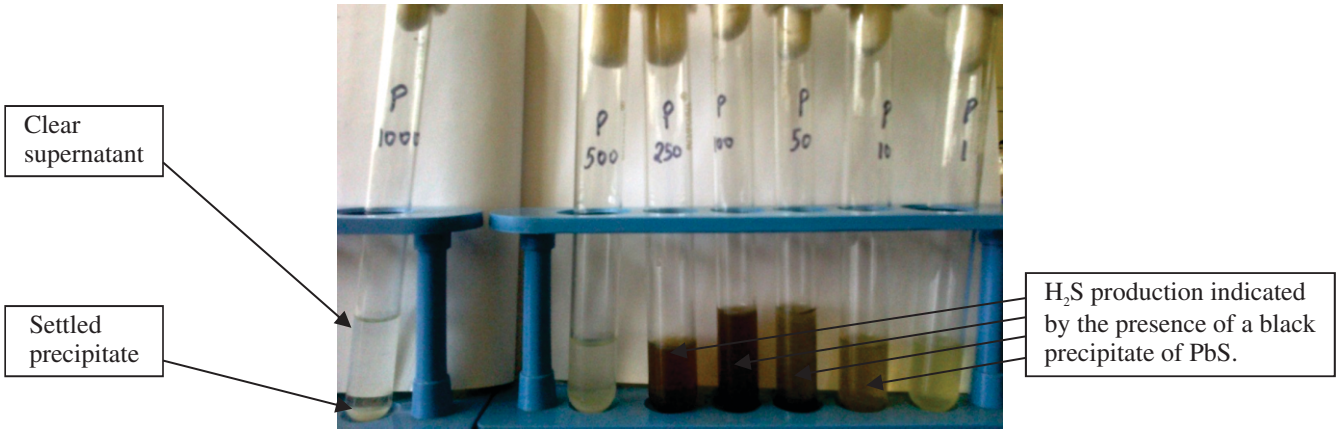
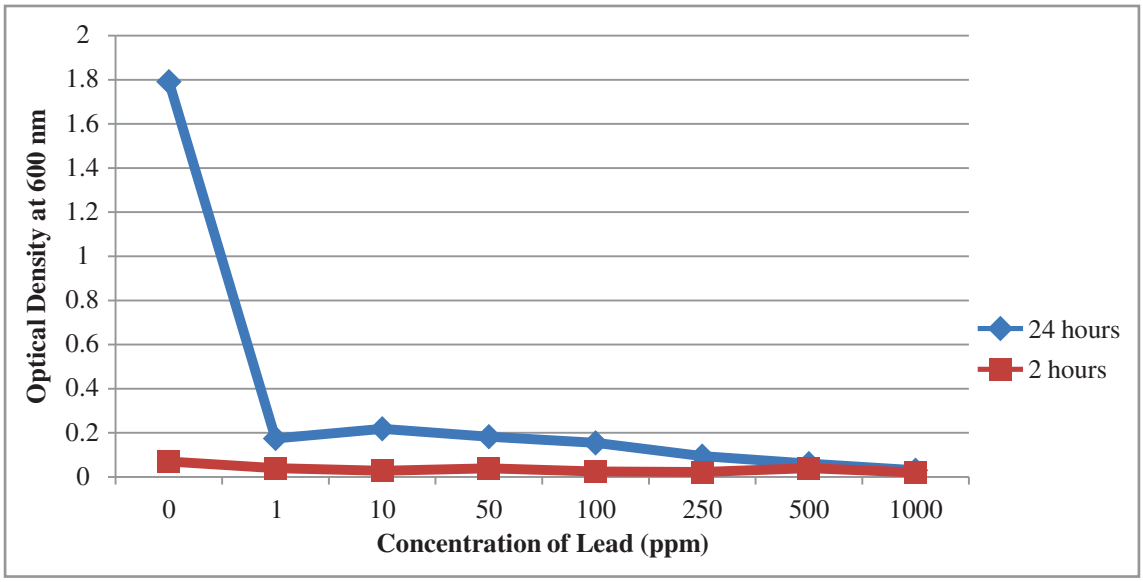


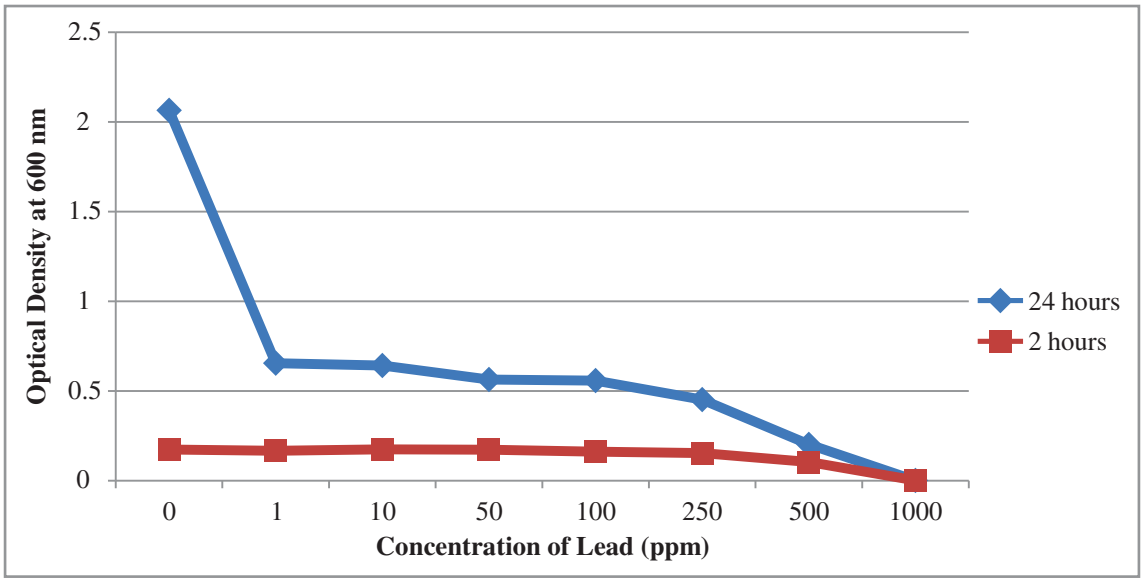
Fig. 2: Tubes Containing the Pseudomonas Inoculum Exposed to Various Concentrations of Lead and Incubated at 37°C for 24 hrs

Spectrophotometric Measurement of Growth of Microorganisms after 24 hrs Exposures to Lead:

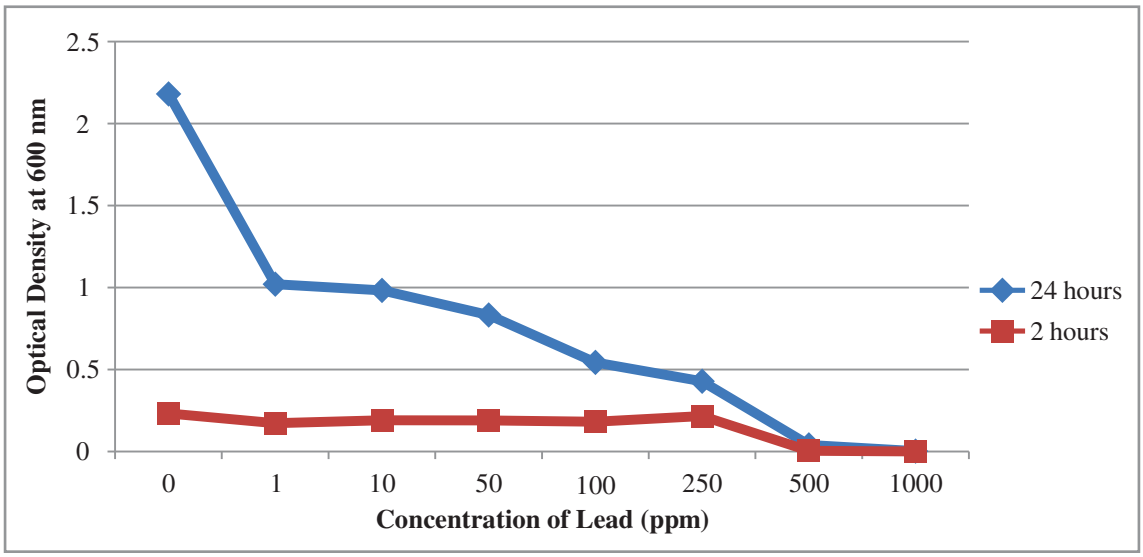
The graphs labeled 1-4, display the variation of optical density with time. The OD values were taken at 600 nm to monitor the growth and a consistent decrease was seen with the three organisms but the growth pattern was different with *Pseudomonas* as it showed a sudden increase at 100ppm accompanied by the production of H_2S at certain concentrations.



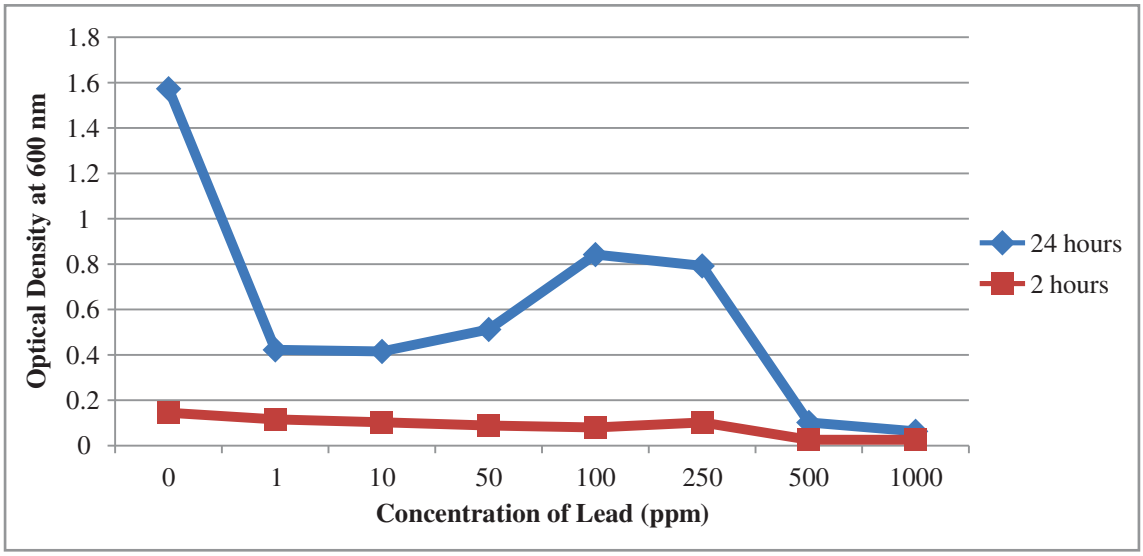
Graph 1: Graph Indicating the Growth Pattern of *Candida albicans* when exposed to Various Concentrations of Lead (ppm) with OD Values taken at 2 hrs and 24 hrs after Exposure to Lead



Graph 2: Graph Indicating the Growth Pattern of *Escherichia coli* when Exposed to Various Concentrations of Lead (ppm) with OD Values taken at 2 hrs and 24 hrs after Exposure to Lead



Graph 3: Graph Indicating the Growth Pattern of *Staphylococcus aureus* when Exposed to Various Concentrations of Lead (ppm) with OD Values taken at 2 hrs and 24 hrs after Exposure to Lead



Graph 4: Graph Indicating the Growth Pattern of *Pseudomonas aeruginosa* when Exposed to Various Concentrations of Lead (ppm) with OD Values taken at 2 hrs and 24 hrs after Exposure to Lead

Gram Staining of Cultures to Determine the Purity of the Cultures:

Gram staining was carried out for the cultures and the purity of the culture was determined. The Gram positive organisms were *Staphylococcus aureus* and *Candida albicans* and the Gram negative organisms were *Pseudomonas aeruginosa* and *Escherichia coli.*

Antibiotic Sensitivity tests of Microorganisms after 24 hrs Exposure to Lead:

The MHA plates were examined after incubation at 37°C for 24 hours. The diameters of the zones of

inhibition were measured and the sensitivity/ resistance patterns were determined using the standard information provided by Himedia from where the antibiotic discs were sourced. All the organisms except *Pseudomonas aeruginosa* showed some resistance to antibiotics. However, *Pseudomonas aeruginosa* showed sensitivity to all the antibiotics. The antibiotic sensitivity/ resistance pattern was as depicted in (Table 3) and (Fig. 3a, b, c & d).

Table 3: Response to Antibiotics used in the AST of the Lead Exposed Microorganisms

Organism	Antibiotic	Concentration of lead (ppm)	Response of lead exposed microorganisms
Escherichia coli	Ampicillin	1,10,50,100,250,500	Resistant
	Cefotaxime	1,10,50,100,250,500	Sensitive
	Gentamicin	1,10,50,100,250,500	Sensitive
	Netillin (Netilmicin Sulphate)	1,10,50,100,250,500	Sensitive
	Ciprofloxacin	1,10,50,100,250,500	Sensitive
Staphylococcus aureus	Ciprofloxacin	1,10,50,100,250,500	Sensitive
	Penicillin	1,10,50,100,250,500	Resistant
	Chloramphenicol	1,10,50,100,250,500	Hetero resistant colonies seen
	Gentamicin	1,10,50,100,250,500	Sensitive
	Co-Trimoxazole (Sulpha/Trimethoprim)	1,10,50,100,250,500	Sensitive
Pseudomonas aeruginosa	Piperacillin	1,10,50,100,250,500	Sensitive
	Ceftriaxone	1,10,50,100,250,500	Sensitive
	Ceftazidime	1,10,50,100,250,500	Sensitive
	Ciprofloxacin	1,10,50,100,250,500	Sensitive
	Amikacin	1,10,50,100,250,500	Sensitive
Candida albicans	Fluconazole	1,10,50,100,250,500	Resistant

Note: All ATCC strains of these microorganisms are sensitive to all the above mentioned antibiotics.

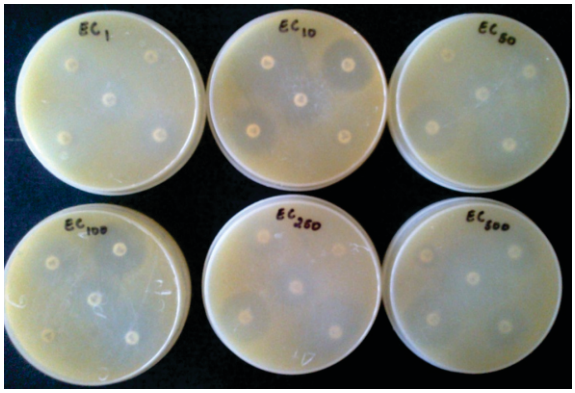


Fig. 3a: MHA Plates Showing the Antibiotic Sensitivity test of *Escherichia coli* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24hrs

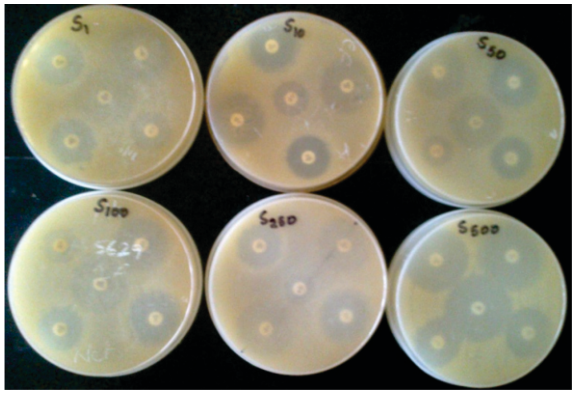


Fig. 3b: MHA Plates Showing the Antibiotic Sensitivity test of *Staphylococcus aureus* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24hrs

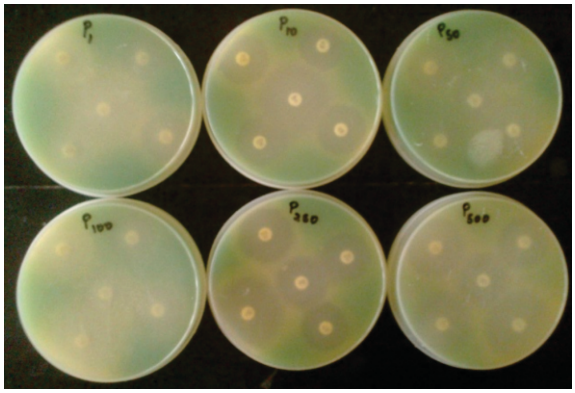


Fig. 3c: MHA Plates Showing the Antibiotic Sensitivity test of *Pseudomonas aeruginosa* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24hrs

Fig. 3d: MHA Plates Showing the Antibiotic Sensitivity test of *Candida albicans* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24hrs

Fig 4: MHA Plate Showing the Hetero Resistant Colonies of *Staphylococcus aureus to* Chloramphenicol

Staphylococcus aureus showed the presence of hetero resistant colonies in the zone around Chloramphenicol as seen in (Fig. 4).

Determination of Sugar Fermenting Property of Microorganisms after Lead Exposure:

The sugar fermenting property was studied using the standard Hugh Liefson sugar solution. The sugar fermentation test showed the presence of acid production in accordance with the property associated with ATCC strains of all the microorganisms as seen in (Table 4) and (Fig. 5a, b, c & d).

Table 4: Sugar Fermentation Test Result of Control Organisms and after Exposure to Various Concentrations of Lead

Microorganism	Concentration of lead (ppm)	Colour Change Observed	Sugar Fermentation Result
Staphylococcus aureus	ATCC Control	Blue-green to yellow	+
	1,10,50,100,250,500	Blue-green to yellow	+
Escherichia coli	ATCC Control	Blue-green to yellow	+
	1,10,50,100,250,500	Blue-green to yellow	+
Pseudomonas aeruginosa	ATCC Control	Blue-green to yellow	+
	1,10,50,100,250,500	Blue-green to yellow	+
Candida albicans	ATCC Control	Blue-green to yellow	+
	1,10,50,100,250,500	Blue-green to yellow	+

Fig. 5a: Tubes Showing the Fermented Sugar Solution by *Escherichia coli* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24 hrs

Fig. 5b: Tubes Showing the Fermented Sugar Solution by *Staphylococcus aureus* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24 hrs

Fig. 5c: Tubes Showing the Fermented Sugar Solution by *Pseudomonas aeruginosa* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24 hrs

Fig. 5d: Tubes Showing the Fermented Sugar Solution by *Candida albicans* after Exposure to Various Concentrations of Lead and Incubated at 37°C for 24 hrs

Determination of Amino Acid Decarboxylase Activity of Lead Exposed Microorganisms

The amino acid utilization tests for lysine, arginine and ornithine showed the same results as characterized for the ATCC strains for all the microorganisms. However, this test was not done for *Candida albicans. Staphylococcus aureus* was arginine positive and negative for the other two amino acids. *Pseudomonas aeruginosa* was positive for arginine and negative for lysine and ornithine and *Escherichia coli* was positive for all the amino acids as shown in (Table 5) and (Fig. 6a, b, c).

Table 5: Amino Acid Decarboxylase Test Result of Control Organisms and after Exposure to Various Concentrations of lead

Microorganism	Concentration of lead (ppm)	Colour Change Observed	Lysine Decarboxylase	Arginine Dihydrolase	Ornithine Decarboxylase
Staphylococcus aureus	ATCC Control	L- Yellow	-	+	-
		A- Violet/purple			
		O- Yellow			
	1,10,50,100, 250,500	L- Yellow	-	+	-
		A- Violet/purple			
		O- Yellow			
Escherichia coli	ATCC Control	L- Violet/purple	Variable	+	+
		A- Violet/purple			
		O- Light/purple			
	1,10,50,100, 250,500	L- Violet/purple	+	+	+
		A- Violet/purple			
		O- Light/purple			
Pseudomonas aeruginosa	ATCC Control	L- No Colour	-	+	-
		A- Violet/purple			
		O- No Colour Change			
	1,10,50,100, 250,500	L- Yellow	-	+	-
		A- Violet/purple			
		O- Yellow			

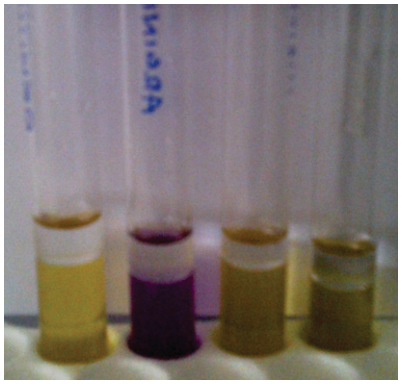


Fig. 6a: Tubes showing the LAO Reactions by *Staphylococcus aureus*

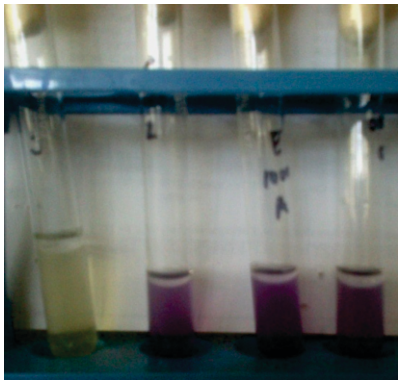


Fig. 6b: Tubes showing the LAO Reactions by *Escherichia coli*

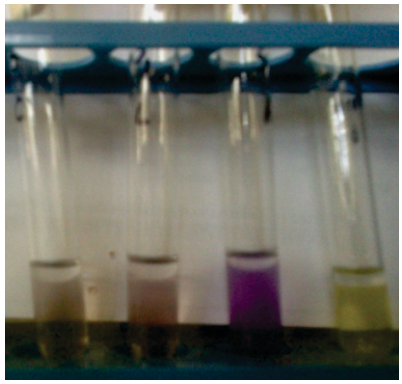


Fig. 6c: Tubes showing the LAO Reactions by *Pseudomonas aeruginosa*

Discussion:

The genetic material in a microorganism is made up of two components namely, the chromosomal and extra chromosomal DNA. The extra chromosomal DNA is also referred to as plasmid DNA [8]. The parameters that were investigated were selected based on their location on microbial genetic material. Two chromosomal and two extra chromosomal properties were investigated to study the effect of lead on the organism. The plasmid associated properties investigated were that of metal tolerance and antibiotic resistance and the chromosomal properties were that of sugar fermentation and amino acid utilization.

Survival of the microorganisms in an environment containing heavy metals such as lead is a relative phenomenon and different patterns of tolerance can be attributed to variations that may be present in the genes on the extra chromosomal material, namely, plasmids. On exposure to lead at various concentrations, *in vitro*, the growth of the microorganisms was sufficiently affected as the lead present in the culture media could have asserted a selective pressure on the organisms, thus making the culture medium a stressful environment for the microorganisms. A sudden increase in the optical density indicated a spurt in the growth of *Pseudomonas aeruginosa* at 50, 100 and 250ppm. The growth at 10, 50, 100 and 250ppm was accompanied by the production of H_2S gas, which was identified by the formation of a black precipitate upon reaction with lead acetate.

A steep decrease was seen in the growth from 250 to 500ppm. This whole phenomenon can be possibly explained from studies that have shown that H_2S is produced in the presence of a stress factor. The H_2S further adds to the stress in the environment of the organism, thus inducing a sudden spurt in the growth as a strategy for survival [9].

At lower concentrations, the presence of the metal may not be a limiting factor for the growth of the microorganisms but at higher concentrations, the metal is lethal. Metal tolerance genes and genes for antibiotic resistance are often congregated on self transmissible plasmids that can be transferred across various genera in a given microbial community. Hence, many studies have established the correlation between the two phenomena.

The microorganisms have been sensitive to all the antibiotics except to the ones that target cell wall synthesis. This leads us to believe that the expression of some cell-wall protein channel blocker is induced in the presence of lead. This parameter

however, needs to be studied in detail and the exact mechanism of action of lead on the cell wall synthesis inhibiting antibiotics needs to be investigated further. Lead has no effect on the chromosomal properties of the microorganisms as the sugar fermentation tests showed no difference from the standard ATCC characterization [10, 12, 13]. The indicator used was bromothymol blue which makes the medium blue-green. Upon inoculation with the organism of interest, the acid produced lowers the pH to the acidic range and the indicator changes to yellow, thus, indicating a positive result [7]. Similarly, the amino acid utilization tests also showed no deviation from the characterized properties [10, 12, 13].

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