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Research Article

TOLERANCE OF *PSEUDOMONAS AERUGINOSA* TO SELECTED CONCENTRATIONS OF METALS AND SODIUM CHLORIDE

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ABSTRACT
The presences of heavy metals at concentrations that exceed their permissible limits are toxic to humans, animals and aquatic life. This study was aimed at determining the tolerance of *Pseudomonas aeruginosa* to concentrations of selected metals (Zn, Cu, Cr, Fe and Ni) and sodium chloride. To achieve this aim, the effects of metal concentration, pH, temperature and initial inoculum size on viable counts of the isolate in presence of the respective metals were investigated. The results revealed that in presence of Zn, Cu, Cr, Fe and Ni, no growth of the isolate was observed at concentrations above 100 mg/L while in the presence of the sodium chloride, remarkable growth was observed at the different concentrations investigated. At the different incubation temperatures studies, the highest counts of 5.8×10^2 cfu/mL and 5.5×10^2 cfu/mL were observed in the presence of sodium chloride and iron, respectively at 35^{o}C. In the case of pH, highest counts of 1.7×10^3 cfu/mL, 1.6×10^3 cfu/mL, 1.5×10^3 cfu/mL, 1.3×10^3 cfu/mL and1.2×10^3 cfu/mL were observed at pH 6 in the presence of Cu, sodium choride, Zn, Cr, and Fe respectively. In the presence of Fe, highest microbial count of 1.2×10^3 cfu/mL was observed at pH 8. The study was able to reveal the effect of the test metals on growth of the test bacterial isolate.

Keywords: *Bacteria, Growth, Heavy Metals, Metal Tolerance*

INTRODUCTION
A number of industrial wastewater streams may contain heavy metals, such as Cd, Sb, Cr, Cu, Pb, Zn, Co, Ni, etc. Because these metals are toxic, when present in either high or low concentrations, they must be effectively removed from the wastewater (Ozbek and Akman, 2012). The presence of heavy metals at concentrations higher that the permissible limits can lead to a variety of health and environmental impacts (Gakwisiri *et al.*, 2012).

Although heavy metals can be removed from water by conventional means, such removal is known to have some intrinsic drawbacks. These drawbacks include the requirement of skillful operators, multiple basin configurations, a large area of land, and a sludge dewatering facility. Recently, some modern processes have been advanced for the elimination of heavy metal from wastewater, which include precipitation, ion exchange, bio-sorption, and neutralization. Each of these processes has numerous merits and demerits (Ozbek and Akman, 2012).

Despite the fact that some heavy metals are indicated to be useful, as trace elements, at high concentrations, they can be toxic to the life of microorganisms and other forms of life. Because heavy metals are progressively found in microbial environments due to natural and industrial processes, microorganisms have devised a variety of mechanisms (efflux, complexion, or reduction of metal ions) to tolerate their presence. In some cases, they can use such metals as terminal electron acceptors in anaerobic respiration (Spain, 2003).

Since some heavy metals are vital for enzymatic functions and bacterial growth, there is the presence of uptake mechanisms that allow for the entrance of metal ions into the cell. In microorganisms, two types of uptake mechanisms are indicated. The mechanisms are the quick and unspecific one, which is driven by a chemo-osmotic gradient across the cell membrane (do not require ATP) while the other is slower and more substrate-specific (driven by energy from ATP hydrolysis). In order to survive in metal-stressed environments, it is reported that bacteria have evolved several types of mechanisms to tolerate the uptake

of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, the accumulation and complexion of metal ions inside the cell and the reduction of heavy metal ions to a less toxic state (Nies, 1999; Nies and Silver, 1995).

Microorganism such as bacteria, fungi and protozoa carry out important roles in biological elimination of nutrient, the association of bacteria and fungi plays a vital function in the cycling of nutrient in marine environments (Johannes 1965). As a result of the metal toxicity, most living cell systems utilized to date have been used for decontamination of sewage containing metals at concentrations below toxic levels. These may make use of a combination of microorganisms as well as higher plants. The aim of this study was to investigate the effect of some selected metals on the viable count of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

The test bacterium for this experiment was *Pseudomonas aeruginosa*. The isolate was part of the laboratory stock at the Department of Biological Sciences, Landmark University, Omu-Aran Nigeria. Prior to use, the bacteria was first streaked on nutrient agar plates to ascertain its purity. After ascertaining its purity, it was sub-cultured into the nutrient broth and incubated for 24 h at a temperature of 37^{o}C. The broth culture was then centrifuged at 5000 rpm for 30 mins before suspending the cells in sterile normal saline (0.85 % NaCl, w/v). The suspended cells in normal saline were then stored in a refrigerator until when needed.

The following metal salts were used for the study: copper (VI) sulphate pentahydrate ($CuSO_4.5H_2O$), chromium (III) chloride, $ZnCl_2$, iron (II) chloride tetrahydrate ($FeCl_2.14H_2O$), nickel sulphate ($NiSO_4.H_2O$) and sodium chloride (NaCl). The salts were to serve as sources of copper, chromium, zinc, iron, nickel and sodium chloride, respectively. A stock solution of each metal salts was prepared by calculating the mass of each metal salts using the molecular weight of the metals and weighing the mass of each metal salts into 500mL of deionized water. Prior to use, the stock solution of each metal was stored in 500 mL amber bottles and then sterilized in an autoclave at 121^{o}C for 15min.

In the present study, the effects of initial inoculum size, metal concentration, temperature and pH on metal tolerance by the test bacteria isolate were investigated. In determining the effect of initial inoculum on tolerance limit of the test isolate, a known metal concentration was added to a flask containing nutrient agar and sterilized in an autoclave at 121^{o}C for 15 min. After sterilization, the respective flasks were allowed to cool before a predetermined inoculum size of the test bacterial isolate suspended in sterile normal saline (0.85 % NaCl w/v) was inoculated into each of the flasks under aseptic conditions. The inoculum was mixed uniformly with the molten agar by swirling gently, before dispensing into sterile petri dishes to solidify. After solidifying, the respective petri dishes were inverted and incubated at 37^{o}C for 24 h. After 24 h, the growth of the isolate that was observed on the petri dishes were counted and recorded. In this study, the concentrations of the test bacterium isolate of the inoculum sizes used were 2.50×10^5 cfu/mL, 1.02×10^5 cfu/mL, 8.30×10^4 cfu/mL, 6.25×10^4 cfu/mL and 5×10^4 cfu/mL.

The metal concentrations used for this study were 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L, 600 mg/L, 700 mg/L, 800 mg/L, 900 mg/L, 1000 mg/L for the different metals. In all the experiments for each metal and concentration, two controls (one containing inoculum but no metal and one containing metal but no inoculum) were set up together with the main experiment.

A total of five different incubation temperatures that was used for this study were 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. In the investigation of the effect of the optimum pH on the growth of the isolates, the study was carried out using pH 4, 6, 8 and 10.

All experimental procedures and analysis were carried out in duplicates for each of the metals. Generally, all the reagents used for the study were of analytical grades.

RESULTS AND DISCUSSION

Results

As shown in Table 1, there were no observable growth of the test isolate at concentration of zinc, iron and chromium that were greater than 100 mg/L. In the presence of copper and nickel, none of the concentrations investigated supported the growth of the test isolate. In the presence of sodium chloride, growth was

observed at all the concentrations investigated. At 100 mg/L, viable counts for the *Pseudomonas aeruginosa* were observed to be 1.40×10^2 cfu/mL, 1.50×10^2 cfu/mL, 6.4×10^2 cfu/mL and 7.6×10^2 cfu/mL, in the presence of zinc, iron, chromium and sodium chloride, respectively (Table 1).

Table 1: Effect of metal concentrations on growth of the *Pseudomonas aeruginosa* at pH 7 and incubation temperature of 37 ºC

Concentration	Zn	Cu	Fe	Ni	Cr	NaCl
0 mg/L	1.7×10^2 (±70.71)	3.2×10^2 (±113.14)	2.2×10^2 (±28.28)	4.3×10^2 (±35.36)	3.2×10^2 (±113.14)	7.6×10^2 (±56.57)
100 mg/L	1.4×10^2 (±56.57)	0	1.5×10^2 (±14.14)	0	6.4×10^2 (±226.27)	7.6×10^2 (±395.98)
200 mg/L	0	0	0	0	0	1.0×10^3 (±282.84)
300 mg/L	0	0	0	0	0	6.0×10^2 (±56.57)
400 mg/L	0	0	0	0	0	1.4×10^3 (±282.84)
500 mg/L	0	0	0	0	0	6.8×10^2 (±56.57)
600 mg/L	0	0	0	0	0	7.6×10^2 (±56.57)
700 mg/L	0	0	0	0	0	7.6×10^2 (±56.57)
800 mg/L	0	0	0	0	0	7.6×10^2 (±56.57)
900 mg/L	0	0	0	0	0	1.2×10^3 (±565.69)
1000 mg/L	0	0	0	0	0	1.1×10^2 (±169.71)

All values are averages of duplicate analysis. Numbers in parenthesis represent ± standard deviation

When investigating the effect of pH on growth of the *Pseudomonas aeruginosa* at metal concentration of 150 mg/L, the results revealed no growth at the pH of 4 in presence of the respective metals used for investigation. Although no growth was observed in the presence of nickel at the different pH used for investigation, in the presence of chromium, iron and sodium chloride, growths were observed at pH 6, 8 and 10. In the presence of zinc and copper, growths were only observed at pH 6 and 8. In all, a highest growth of 1.5×10^3 cfu/mL, 1.7×10^3 cfu/mL, 1.3×10^3 cfu/mL and 1.6×10^3 cfu/mL were observed at pH 6 in the presence of the zinc, copper, chromium and sodium chloride, respectively. In the presence of the iron, highest growth of 1.2×10^3 cfu/mL was observed at pH 8 (Table 2).

Table 2: Effect of pH on growth of the *Pseudomonas aeruginosa* at metal concentration of 150 mg/L and incubation temperature of 37 ºC

pH	Zn	Cu	Cr	Ni	NaCl	Fe
4	0	0	0	0	0	0
6	1.5×10^3 (±141.42)	1.7×10^3 (±141.42	1.3×10^3 (±141.42)	0	1.6×10^3 (±282.84)	5.0×10^2 (±353.55)
8	1.1×10^3 (±141.42)	1.4×10^3 (±212.13)	8.5×10^2 (±212.13)	0	1.3×10^3 (±424.26)	1.2×10^3 (±70.71)
10	0	0	7.5×10^2 (±212.13)	0	8.0×10^2 (±282.84)	9.0×10^2 (±141.42)

All values are averages of duplicate analysis. Numbers in parenthesis represent ± standard deviation

As shown in Table 3, no growth of *Pseudomonas aeruginosa* was observed in the presence of the zinc, copper, chromium and nickel. In presence of the iron and sodium chloride, remarkable growths were observed at the different temperatures. The highest growths of 5.8×10^2cfu/mL and 5.5×10^2 cfu/mL were observed at incubation temperature of 35ºC, in the presence of sodium chloride and iron, respectively. Growth was only observed in the presence of chromium at incubation of 40ºC (Table 3).

Table 3: Effect of incubation temperature on growth of the *Pseudomonas aeruginosa* at metal concentration of 150 mg/L and pH 7

Temperature	Zn	Cu	Cr	Ni	NaCl	Fe
25ºC	0	0	0	0	2.8×10^2 (±35.36)	2.3×10^2 (±35.36)
30ºC	0	0	0	0	3.8×10^2 (±35.36)	4.3×10^2 (±35.36)
35ºC	0	0	0	0	5.8×10^2 (±35.36)	5.5×10^2 (±70.71)
40ºC	0	0	2.8×10^2 (±35.36)	0	4.8×10^2 (±35.36)	4.5×10^2 (±70.71)
45ºC	0	0	0	0	3.8×10^2 (±106.10)	3.8×10^2 (±35.36)

All values are averages of duplicate analysis. Numbers in parenthesis represent ± standard deviation

As shown in Table 4, no growth of the *Pseudomonas aeruginosa* was observed at the different initial inoculum sizes in the presence of copper and nickel. In the presence of the zinc, iron, sodium chloride and chromium, remarkable growth was observed at the different initial inoculum sizes. In the presence of the metals that showed growth, there was a steady increase in growth with decrease in initial inoculum size. At inoculum size of 5×10^4, the highest growth of 1.9×10^3 cfu/mL, 2.3×10^3 cfu/mL, 1.8×10^3 cfu/mL and 1.9×10^3 cfu/ML, were observed in the presence of the zinc, iron, sodium chloride and chromium, respectively (Table 4).

Table 4: Effect of initial inoculum size on growth of the *Pseudomonas aeruginosa* at metal concentration of 150 mg/L, pH 7 and incubation temperature of 37ºC

Inoculum size	Zn	Cu	Fe	NaCl	Ni	Cr
2.50×10^5 cfu/mL	5.0×10^2 (±141.42)	0	3.6×10^2 (±56.57)	3.8×10^2 (±28.28)	0	3.0×10^2 (±141.42)
1.02×10^5 cfu/mL	9.0×10^2 (±141.42)	0	7.0×10^2 (±141.42)	5.6×10^2 (±56.57)	0	7.0×10^2 (±141.42)
8.30×10^4 cfu/mL	1.3×10^3 (±141.42)	0	1.3×10^3 (±353.55)	8.4×10^2 (±56.57)	0	1.1×10^3 (±141.42)
6.25×10^4 cfu/mL	1.7×10^3 (±141.42)	0	1.8×10^3 (±353.55)	1.3×10^3 (±141.42)	0	1.5×10^3 (±141.42)
5.00×10^4 cfu/mL	1.9×10^3 (±141.42)	0	2.3×10^3 (±353.55)	1.8×10^3 (±282.84)	0	1.9×10^3 (±141.42)

All values are averages of duplicate analysis. Numbers in parenthesis represent ± standard deviation

Discussion

In this study, the effect of zinc, iron and chromium concentration greater than 100 mg/L shows no observable growth of *Pseudomonas aeruginosa*, while at concentrations less than 200 mg/L viable counts for *Pseudomonas aeruginosa* were observed. In a study carried out by Nies (1999) and Li *et al*., (2004), it was reported that at high concentrations, heavy metals are known to exert inhibitory action on microorganism by blocking essential functional groups or modifying the active conformations of biological

molecules while at low concentrations, they provide vital co-factors for metalloproteins and enzymes, which are vital for microbial growth.

From the results it was observed that at lower concentration (100mg/L) of chromium, the growth of *Pseudomonas aeruginosa* was not affected. This result point out the fact that *Pseudomonas* sp. possess an enzyme known as chromium reductase which reduced Cr(VI) to Cr(III) as reported by Kathiravan *et al*., (2010). It is evident from the result that the growth of *Pseudomonas aeruginosa* was decreased as Cr(VI) concentration was increased in the medium. Anyanwu and Ezaka (2011) observed the inhibition of growth of *Pseudomonas aeruginosa*, at chromium concentration of 200 µg/mL and higher.

In the presence of copper and nickel, none of the concentrations investigated supported the growth of test isolate. Gopinath *et al.*, (2011) indicates that growth of *Pseudomonas aeruginosa* was depleted severely at even lower concentration of copper and copper ions affect the transcription mechanism in cell which in turn affect the cell division and ultimately have effect on its activities. Many researchers have concluded that copper is more toxic than other metals. Seesuriyachan *et al.*, (2007) also reported that Ni at 100 mg/L concentration had significant negative influence as it affect the cell viability and inhibiting the enzyme activity by denaturing enzyme therefore no growth was observed at 100 mg/L of nickel, thus growth was inhibited at higher concentrations of nickel.

In the presence of zinc, iron, sodium chloride and chromium, remarkable growth was observed at the different initial inoculum sizes. In similar investigations, *Pseudomonas aeruginosa* was reported to accumulate significantly higher amounts of the respective metals at all tested initial concentrations, a trend that was irrespective of the initial biomass size (Al-Asheh and Duvnjak, 1995; Sampedro *et al*., 1995; Al Garni, 2005). In the presence of copper and nickel no growth of the *Pseudomonas aeruginosa* was observed at the different initial inoculum sizes. Heavy metals like copper and nickel have been reported as one of the most toxic pollutants (Cameron, 1992). These heavy metal pollution is of great concern, as these hazardous pollutants are accumulated in all living organisms and microorganisms (Ogbo and Okhuoya, 2011). They are also responsible for numerous metabolic and physiological disorders, which may lead to growth inhibition (Rani and Goel, 2009; Matyar *et al*., 2010).

With respect to pH, the present study revealed an optimum of 6 in presence of the test isolates. pH is said to play a major role in nutrient removal by affecting different factors, which in turn affects growth and activity of the organisms involved. Studies carried out by earlier workers have shown that pH affect enzymes of interest, affinity for the substrate, substrate availability, effects of inhibitory compounds, and substrate or product production (Prosser, 1989; Antoniou *et al.*, 1990; Groeneweg *et al.*, 1994).It was revealed that in the presence of chromium, growths were observed at pH 6 and 8. Silva *et al*., (2009) revealed the chromium level sorbed by *Pseudomonas aeruginosa* was in pH range of 7-7.2.

In the presence of zinc, chromium and iron, growths were observed at pH 6, 8 and 10. It was reported that zinc, chromium and iron tends to sorb more readily at a high pH (pH >7) than at a low pH. The pH of the wastewaters was acidic, ranging from 4.3 to 5.1. However, a pH near 7.0 (neutral) plays a part in determining both the qualitative and quantitative abundance of the isolate (Edward, 1990; Federov *et al*., 1993). The results of the present study revealed that the number of *Pseudomonas aeruginosa* was affected by the introduced metal concentrations. The response of the bacteria was influenced by the level and type of heavy metal amendment.

When water is polluted by heavy metal, it causes a pressure on sensitive microorganisms and so changes the diversity of soil microflora according to Zaguralskaya (1997). In the media amended with metal concentration of 100mg/L, no significant negative effects of the metals on the bacterial growth were observed when compared with the control without metal amendment. Thus, zinc and chromium at that concentration had no significant inhibitory effects on the growth responses of the bacteria. However, such inhibitory effect was significant at higher metal concentrations. The microbial load decreased with increase in the concentration of heavy metal indicating toxic effect of the heavy metals on the growth of microorganisms.

In the present study, sharp decreases in growth were observed at increased metal concentrations. This was irrespective of the metal used for investigation. The no growth or lower values of microbial load at higher

metal concentrations could indicate that the bacterial growth was affected due to the presence of heavy metal in the growth medium. This observation was in agreement with a report by Kikovic (1997).

Conclusion

This study, which was aimed at investigating the effect of heavy metals on viable counts of *Pseudomonas aeruginosa* was able to reveal the following:

- The effect of zinc, iron, chromium and sodium chloride on *Pseudomonas aeruginosa* at the concentrations of 100 mg/L did not inhibit the growth of the test isolate while there was an inhibition of growth of the isolate at concentrations above 100 mg/L. Also, copper and nickel at concentrations of 100 mg/L and above inhibited the growth of the test isolate whereas, sodium chloride has no effect in inhibiting the growth of the test isolate at concentrations at 100 mg/L and above.
- With respect to pH, no observable growth of the test isolate was seen at the pH of 4 for the respective metals used for investigation. Although growth was inhibited in the presence of nickel at the different pH used for investigation, in the presence of chromium, iron and sodium chloride growth of the test isolate were observed at pH of 6, 8 and 10. Whereas growth of the test isolate were only observed at pH 6 and 8 in the presence of zinc and copper. In all, a highest growth of the test isolate was observed at pH 6 in the presence of zinc, copper, chromium and sodium chloride, and at pH 8 in the presence of iron respectively.
- The test isolate showed no remarkable growth in the presence of zinc, copper, chromium and nickel at different temperatures (25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C). At different temperatures the presence of nickel and sodium chloride shows remarkable growth of the test isolate. Also, the highest growths of the test isolate were observed at incubation temperature of 35^{o}C in the presence of sodium chloride, iron and growth was only observed in the presence of chromium at the incubation of 40^{o}C.
- The study revealed that copper and nickel inhibited the growth of the test isolate at the different initial inoculum sizes whereas growth of the test isolate was not inhibited at the different initial inoculum sizes in the presence of zinc, iron, sodium chloride and chromium, respectively. Also it was observed that at the inoculum size of 5x10^{4}cfu/mL, the highest growth of test isolate was observed in the presence of zinc, iron, sodium chloride, and chromium.

Despite the fact that the present study cannot be considered to be exhaustive, it has still provided valuable information on the tolerance limits of the isolate to the selected metal concentrations under the experimental conditions investigated.

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