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Isolation and Screening of Heavy Metal Resistant Ammonia Oxidizing Bacteria from Soil and Waste Dump: A Potential Candidate for Bioremediation of Heavy Metals

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Abstract

The study was undertaken to examine the response of ammonia oxidizing bacteria to different heavy metal salt in an elevated concentration. Surface soil samples at depth of 0-15 cm were collected at random from Akwa Ibom State University in Akwa Ibom State, soil sample from University of Nigeria, Nsukka and from solid waste disposal site in Uyo, Akwa Ibom State. The response of heavy metal salt on Ammonia Oxidizing bacteria(AOB) isolated from soil samples were investigated by supplementing different heavy metal salts namely, copper(Cu),nickel (Ni), lead (Pb) and cadmium (Cd) at four loading rates(100,200,500,1000 µg/ml) in mineral salt broth with Ammonia Oxidizing Bacteria (AOB) isolate. The cultures were incubated for 7 days. Growth of AOB was measured by withdrawing samples from the medium every 24 hours and absorbance of the turbidity measured at 600 nanometre using spectrophotometer. All bacteria showed high tendency to decrease optical density while increasing metal concentration in the medium. Tolerance for the metal ions was dependent on concentration, time and the isolate tested. All the Ammonia oxidizing bacterial (AOB) showed a high level of tolerance for the metals tested, and exhibited good growth at all metal salt concentrations tested. Nitrifying bacteria; *Achromobacter xylosoxidans* and *Achromobacter insolitus*, were able to carry out biosorption of copper, nickel, lead and cadmium as seen in this study. *Achromobacter insolitus* had the highest biosorption capacity for copper and cadmium at 90.04% and 89.21%, respectively within a period of 28 days. *Achromobacter xylosoxidans* had the highest biosorption capacity for nickel and lead at 96.51% and 92%, respectively within a period of 28 days. These make the nitrifying bacteria attractive potential candidates for further investigations regarding their ability to remove metals from contaminated soil.

Keywords: Heavy metal, Tolerance, Nitrification, Ammonia Oxidizing Bacteria.

Aim of the Study

To study tolerance of ammonia oxidizing bacteria growth to heavy metal

Significance of the Study to the field

Nitrifying bacteria remain good option for bioremediation of soil and waste dump, since it is regarded as eco-friendly and efficient in biosorption of heavy metal. The study is significant to the field of Environmental microbiology by adding to knowledge in bioremediation

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Introduction

Environmental pollution has been on the rise in the past few decades owing to increased human activities on energy reservoirs, unsafe agricultural practices and rapid industrialization [1]. Amongst the pollutants that are of environmental and public health concerns due to their toxicities are: heavy metals, nuclear wastes, pesticides, greenhouse gases, and hydrocarbons. These toxic pollutants are discharged from specific locations worldwide and thus pollute specific regions. These pollutants have different toxicity and chemical behaviour [2]. Environmental contamination caused by heavy metals (HM) has received increased attention worldwide [3-6]. Heavy metal (HM) is any metallic element that has a relatively high density and is toxic or poisonous at low concentrations. Heavy metals are elements with atomic number higher than 20, an atomic mass greater than 40 g and a specific weight of more than 5 g/cm^3 [7,8]. These elements often find their way into soil through environmental contaminants including the atmospheric pollutants in industrial regions (emissions from the rapidly expanding industrial areas), unlimited use of agricultural fertilizers, mine tailings, disposal of high metal wastes, leaded gasoline and paints, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, atmospheric deposition, municipal and industrial sewage systems in a nonreturnable fashion [9-12].

Activities such as the use of agrochemicals and long-term application of urban sewage sludge, industrial waste disposal, waste incineration, and vehicle exhausts are the main sources of HM in agricultural soils [13]. Heavy metals in the soil include mercury (Hg), lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), uranium (U), selenium (Se), silver (Ag), gold (Au) , copper (Cu) and nickel (Ni) [11]. The danger of heavy metals is intensified by their almost indefinite persistence in the environment due to their absolute nature which cannot be degraded [14]. Metals are non-biodegradable but can be transformed through sorption, methylation, complexation and changes in valence state [15].

Toxic metals apply their toxicity in the displacement of essential metals from their normal binding sites of biological molecules, inhibition of enzymatic functioning and disruption of nucleic acid structure, oxidation stress, genotoxicity and interfering with signalling pathways [11,16]. Ecologically, the accumulation of heavy metals in soils is extremely hazardous because soil is a major link in the natural cycling of chemical elements; it is also a primary component of the trophic chain [17-19].

Industrial operations such as electroplating, steel manufacturing, leather tanning, wood preservation, ceramics, glass manufacturing, chemical processing and fertilizer applications release alarmingly higher amounts of heavy metals into the natural environment [12]. Pollution by heavy metal is a threat to the environment and it remediation is a major challenges to environmental research. Heavy metal pollution is a serious global environmental problem as it adversely affects biotic and abiotic components of the ecosystem and alters the composition and activity of soil microbial communities [5]. The non-biodegradability of heavy metals makes it hard to remove them from contaminated biological tissues and soil and this is a major concern for global health because of their lethal nature.

Nitrification is a biochemical process of oxidation of ammonia (NH_4^+) to nitrite (NO_2^-), then finally to nitrate (NO_3^-) by nitrifying bacteria. Nitrification is catalysed by two types of reactions. The first type of reaction is the oxidation of ammonia to nitrite by ammonium oxidizing bacteria (AOB). The second type of reaction involves the oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) [20]. Ammonium-oxidizing microorganisms are organism that carries out the first step in nitrification reaction (biochemical process of oxidation of ammonia (NH_4^+). They include ammonia oxidizing bacteria (AOB) *(Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus, Nitrosovibrio)*, ammonia-oxidizing archaea (AOA), and heterotrophic bacteria (*Arthrobacter globiformis, Aerobacter aerogenes, Thiosphaera pantotropha, Streptomyces grisens,* and various *Pseudomonas* spp*) and* fungi (*Aspergillus flavus)* [8]. Recent research on the metabolic pathways of heterotrophic ammonia oxidation has been conducted using *Paracoccus denitrificans* [21], *Alcaligenes faecalis*, *Pseudomonas putida* [22], and a few other bacterial species. Some studies have suggested that the biochemical mechanisms of heterotrophic nitrification differ among strains [23]. *Nitrospira* in the Nitrogen Oxidizing Bacteria group have been reported as complete ammonia oxidizing bacteria (comammox) that perform the complete nitrification of ammonia to nitrate [24,25] Ammonia oxidizing microbes (AOM) obtain their energy by oxidation of ammonia (NH_3) to nitrite (NO_2^-). These organisms utilize a few key enzymes such as ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) to bring about the conversion. The presence of ammonia mono-oxygenase subunit-A gene (amoA) encodes ammonia mono-oxygenase (AMO), a key enzyme that catalyses the first step in ammonia oxidation. AOB was first reported in 1890 by Winogradsky and several groups began isolating and cultivating AOB from a variety of environments such as marine waters, estuarine soils and waste water treatment systems [26].

Nitrogen is an essential element for plants [27]. Nitrifying bacteria play important role in soil fertility, make available nitrate nitrogen to plants (common soil nutrient element required in large quantity by plants), aid in waste treatment plant,biogeochemical cycling of nitrogen compounds and purification of the air. Nitrifying bacteria in polluted soil initiate a syntrophic pathway that provides intermediates for heterotrophic bacterial activity and thus are excellent candidates for remediation [28], with bacteria and fungi being the most important organisms for reclamation, immobilization or detoxification of metallic and radionuclide pollutants. Some bio minerals or metallic elements deposited by microbes have catalytic and other properties in nanoparticle, crystalline or colloidal forms [29].

Microorganisms are very sensitive; they react quickly to any kind of changes (natural and anthropogenic) in

the environment, and quickly adapt themselves to new conditions. Microorganisms take heavy metals into the cell in significant amounts. This phenomenon leads to the intracellular accumulation of metal cations of the environment and is defined as bioaccumulation [30]. Some bacterial plasmids contain specific genes for resistance to toxic heavy metal ions [4,31-33] and ability to solubilize phosphate (biofertilizers) [3,34]. Some microorganisms can adjust their metabolic activity or community structure to adapt to the harmful shock loadings. Microorganisms play important role in stress environment and the derived ecosystem functions [27,35].

Microorganisms can mobilize or immobilize metals by biosorption, sequestration, production of chelating agents, chemoorganotrophic and autotrophic leaching, methylation and redox transformations. These mechanisms stem from prior exposure of microorganisms to metals which enable them to develop the resistance and tolerance useful for biological treatment [36]. Microbe-metal interaction in soil/waste disposal is of interest to environmentalists in order to use adapted microorganisms as a source of biomass for bioremediation of heavy metals [4,35,37]. Metals detoxification through resistance and tolerance, this resistance can be attributed to mechanisms of exclusion or tolerance [38]. In an endeavour to safeguard the susceptible cellular components, a cell is capable of building up resistance to metals. Bruins *et al.* [39] hypothesized five mechanisms for resistance to metal toxicity. These are: (1) active or dynamic transport, (2) development of a permeability barrier, (3) enzymatic detoxification, (4) reduction in sensitivity and (5) sequestration. Microbes are capable of using one or more of these methods to eliminate nonessential metals and normalize essential metals concentrations in their cells. AOB can adapt to energy stress conditions, including low nitrogen levels and low pH [40]. Ammonia oxidizers groups are often considered as models for unravelling the significance of microbial diversity on the responses of soil processes to environmental stress [41,42].

Materials and Methods

Sample collection

Surface soil samples at depth of 0-15 cm were collected at random from Akwa Ibom State University in Akwa Ibom State, and soil sample from University of Nigeria, Nsukka. And from solid waste disposal site in Uyo, Akwa Ibom State. The soil was collected using sterile auger borer and into sterile polyethylene bag, merged to form a composite soil sample and transferred to the laboratory for analysis.

Microbiological Analysis

Preparation of samples for analyses: Precisely, 5 g of the sieved soil sample was suspended in 45 ml of sterile phosphate buffer containing 139 mg of K_2HPO_4 and 27 mg KH_2PO_4 per litre (pH 7.0) and shake at 100 rpm for 2 hrs in order to liberate the organisms into the liquid medium [28,43].

Preparation of media: Media preparation was carried out using Winogradsky broth medium for serial dilution of soil samples and Winogradsky solid medium for the inoculation of serially diluted soil suspension.

Preparation of Winogradsky broth: Winogradsky broth medium phase 1 (used for the isolation of nitrifying bacteria responsible for oxidizing ammonium to nitrite) was prepared with the following composition (g/l) in sterile distilled water: $(NH_4)_2SO_4$, 2.0 ; K_2HPO_4, 1 ; $MgSO_4.7H_2O$, 0.5; NaCl, 2.0 ; $FeSO_4.7H_2O$, 0.4 ; $CaCO_3$, 0.01. Each of ten test tubes filled with 9 ml of the Winogradsky broth media 1, autoclaved at 121°C at 15 psi for 15 minutes and allowed to cool. The test tubes was used to carry out ten-fold serial dilutions of the soil suspension [28].

Preparation of Winogradsky agar media: Winogradsky agar media for nitrification phases I was prepared by adding 15.0 g agar to 1000 ml of fresh broth and sterilized at 121°C at 15 psi for 15 minutes and allowed to cool to about 45°C before dispersing into sterile Petri dishes [28].

Isolation of nitrifying bacteria from soil sample

All the plates were aseptically inoculated with 0.1 ml of the appropriate dilution of the soil suspension using spread plate technique. All the inoculated Petri dishes were incubated aerobically at room temperature (28 + 2°C) for 1week and examined for growth.

Purification of isolates

Discrete colonies that developed on Winogradsky agar media for nitrification phases 1 after 1week of incubation was aseptically sub-cultured repeatedly on corresponding freshly prepared Winogradsky agar medium. All the inoculated Petri dishes were incubated aerobically at room temperature (28 ±2°C) for 3-5 days. The pure isolates was transferred to Winogradsky agar slants and stored in the refrigerator for further use.

Identification of isolates

Pure isolates from the corresponding agar slants was characterized and identified using morphological (cell and colonial morphology, shape, motility, and gram reaction), biochemical and physiology attributes [44,45]. The molecular characterization was based on 16SrDNA sequencing [46].

Physiological characterization of the isolate

Nitrite determination by Griess method: *Griess Ilosvay reagent was be prepared as follows:* Solution A: 0.6 g of sulphanilic acid was dissolved in 70 ml of hot distilled water, cooled, and 20 ml of concentrated HC1 was added and volume was made up to 100 ml with distilled water [47].

Solution B: 0.6 g of a-naphthylamine was dissolved in 10 ml of distilled water containing 1 ml of concentrated HC1 and the volume was made up to 100ml with distilled water. Solution C: 16.4 g of sodium acetate was dissolved in 70 ml of distilled water and the volume made up to 100 ml with distilled water. The three solutions (A, B and C) was stored in dark bottles and mixed in equal parts before use.

Ammonium oxidation test for determination of nitrite: Five millilitres of Winogradsky mineral basal medium

was prepared. The tubes was sterilized by autoclaving at 121°C at 15 psi for 15 minutes and allowed to cool. One loopful of each ammonium oxidizing bacteria isolate was added into each tube and incubated aerobically for 5 days at room temperature. At the end of the incubation period, the presence of nitrite was tested using Griess Ilosvay reagent. The reagent was added and observed for the development of purplish red/pink colouration within 5 minutes [48,49].

Inoculum preparation and standardization: Inocula were prepared by inoculating isolates onto prepared nutrient agar plates and incubating at 30ºC for 24 h. After incubation, colonies were suspended in test tubes containing sterile normal saline solution. The tubes were vortex for 2 min, and then transferred into a sterile test tube. The cells suspension was adjusted to a 0.5 McFarland standard (Optical density of 0.5 -1.0 at 600nm) using sterile normal saline to give a concentration of 10^8 cfu/ml, to get the final inocula.

Tolerance study

Mineral salts medium of the following composition (g/l): $(NH_4)_2SO_4$, 1.0 ; KH_2PO_4, 1.0g; K_2HPO_4, 1.0g; $MgSO_4$, 0.2 ; $CaCl_2$, 0.02 ; $FeCl_3.6H_2O$; 0.004 for ammonium oxidizing bacteria. Sterilized by autoclaving at 121°C at 15 psi for 15 minutes and allowed to cool.

Salts of Cupper (Cu), Nickel (Ni), Cadmium (Cd), and Lead (Pb) was used as $CuSO_4.5H_2O$, $NiSO_4.6H_2O$, $CdCl_2.H_2O$ and $Pb(CH_2COO)_2)_2$. $(OH)_2$, respectively.

Primary Screening of heavy metal resistant nitrifying bacteria

An amount (0.1 ml) of the standard inoculum was plated into mineral salts agar medium supplement with metal salt concentrations of 100 mg/L. The plates were incubated at room temperature for 3 - 5 days [50,51]. Ammonia oxidizing bacterial isolates was selected for tolerance study.

Experimental Set up

Analytical grades of metal salts was used to prepare stock solutions. The mineral salt medium for ammonia oxidizing bacteria was amended with the appropriate aliquot of the stock solution of the metal salt concentrations of 100 mg/L, 200 mg/L, 500 mg/L and 1000 mg/L.

Effect of heavy metal on the growth of nitrifying isolates

Changes in population of the nitrifying isolates were monitored following their exposure to heavy metals. About 1 ml of the standard inoculum was introduced into each flask containing heavy metal salts amended in mineral salt medium (MSM). The cultures were incubated aerobically at room temperature (28 ±2ºC) for 7 days. The growth was measured by withdrawing samples from the medium every 24 hours and absorbance of the turbidity measured at 600 nanometre using spectrophotometer [47].

Statistical analysis

Result reported as mean ± standard deviation. All data were subjected to statistical analysis by analysis of variance (ANOVA). The means were separated with least significant difference. The result consider significant at $P < 0.05$. Least significant difference test (LSD) was also being performed between each treatment and the control. Correlation (association) and regression (changes) analysis was done using statistical product and service solution (SPSS) for windows version 20.

Results

Morphological and biochemical characterization of ammonia oxidizing bacterial isolates

Table 1 shows the morphological and biological characteristic of ammonia oxidizing isolate, the five potential heavy metal tolerance Ammonia oxidizing bacteria were characterized based on their cultural, morphological and biochemical characteristic. Isolate were identified as Gram negative; Catalase positive; Indole negative; Methylene red negative; Voges proskauer negative; Urease negative and Oxidase negative. The isolates were compared with Standard description of Bergey's Manual of determinative bacteriology. A1, A2, A3, A4 and A5 represent different Ammonia oxidizing bacteria.

Tolerance of ammonia-oxidizing bacteria to heavy metal salts

All the bacterial isolates showed high tendency to decrease in optical density with increasing metal concentration in the medium. Heavy metal salts had effects on nitrifying bacteria growth. Tolerance for the metal ions was dependent on metal salt concentration, time and the isolate tested. Toxicity of the heavy metal salts was in the order of Cd > Cu > Ni > Pb. There was a significant ($p < 0.05$) difference in the nitrifying bacterial response rates to heavy metal salts.

The results represented in Figure 1 shows the rate of tolerance of different copper salt concentrations by the ammonia-oxidizing bacteria (A1 to A5) at different days. As recorded at OD 600nm, the growth of A1 ranged from 0.01 ± 0.002 to 0.07 ± 0.002. Growth was highest in the absence

Table 1: Morphological and Biochemical Characterization of Ammonia Oxidizing Bacterial Isolates.

Suspected organism	Colony	Cell shape	Gram stain	Spore stain	Cat	H_2S	Ind	Cit	MR	Vp	Ur	Mot	Oxid	Nit red
A1	Colourless mucoid raise,	Rod	-	-	+	-	-	+	-	-	+	+	+	+
A2	White mucoid, raise	Rod	-	-	+	-	-	+	-	+	-	+	+	+
A3	White Mucoid raise,	Rod	-	-	+	-	-	+	-	-	-	+	+	+
A4	Raise, brownish	Rod	-	-	+	+	-	+	-	+	-	-	-	+
A5	Colourless mucoid raise,	Rod	-	-	+	+	+	-	+	-	+	+	+	+

+ present (positive)–absent (negative), Cat-Catalase, Ind-Indole, Cit-Citrate, MR-Methyl red, Vp-Voges proskauer, Ur-Urease, Mot-Motility, Oxid-Oxidase, Nit red-Nitrate reduction.

of metal salts (0 mg/l) at 120 hours. However, increasing copper concentration upto 1000 mg/l caused a decline in growth by more than 90% at 168 hours of cultivation. Isolates A2 exhibited related trends in copper tolerance as their growth was not adversely affected even at 200 mg/l but as concentration was increased to 500 mg/l beyond, bacterial growth significantly reduced ($p \leq 0.05$). For A3 to A5 accordingly, growth range values (± 0.002 standard deviation) were; 0.02 to 0.09, 0.02 to 0.10 and 0.02 to 0.08. Thus, for all isolates, the least and peak values for growth were recorded at 1000 mg/l and 0 mg/l respectively between 120 to 168 hours of cultivation and the tolerance of the isolates to copper was in the order; A1 < A4 < A3 < A10 < A2. All the isolates showed the high sensitivity to high concentration (500 and 1000 mg/l) of copper (Figure 1).

Tolerance of ammonia-oxidizing bacteria isolates to nickel

The result represented in figure 2 shows the effect of different nickel concentrations on ammonia oxidizing bacteria A1 to A5 at different days. Isolate A1 growth ranged from 0.06 ± 0.001 to 0.09 ± 0.007 within 120 hours and decreased to 0.07 ± 0.0021 after 168 hours at 0 concentrations. Isolate A1 growth ranged from 0.05 ± 0.000 to 0.06 ± 0.008 within 120 hours. Growth was highest in the absence of nickel at 120 hours. Figure 13 shows the tolerance of the A2 isolates for different concentrations of Ni. The growth of A2 range from 0.0655 ± 0.002 to 0.0835 ± 0.002 within 72 hours, increase to 0.095 ± 0.001 within 120hrs and decrease to 0.072 ± 0.013 after 168 hours at 0 concentration. A2growth range from 0.057 ± 0.003 to 0.071 ± 0.01 within 72 hours; increase to 0.064 ± 0.004 within 120hrs and increase to 0.072 ± 0.011 after 168 hours at 100ug/ml concentration. A4 growth range from 0.0305 ± 0.001 to 0.023 ± 0.007 within 72 hours, decrease to 0.0195 ± 0.006 within 120hrs and decrease to 0.014 ± 0.004 after 168 hours at 1000ug/ml concentration. A2 growth within 120 hours was observed to be; 0.095 ± 0.001; 0.064 ± 0.004; 0.0195 ± 0.006 within 120 hours at 0, 100 and 1000 mg/l concentration, respectively.

For A3 and A4 isolates accordingly, growth range values were; 0.07 ± 0.002 to 0.10 ± 0.001; 0.07 ± 0.000 to 0.09 ± 0.001 and 0.07 ± 0.002 to 0.10 ± 0.001 within 120 hours concentration. Growth of isolate A5 ranged from 0.06 ± 0.0001 to 0.08 ± 0.001 within 120 hours Growth was highest in the absence of nickel at 120 hours. However, increasing nickel concentration upto 1000 mg/l caused a decline in growth by more than 75 % at 168 hours of cultivation. Thus, for all isolates, the least and peak values for growth were recorded at 1000 and 0 mg/l, respectively, between 120 to 168 hour of cultivation and the tolerance of the isolates to nickel was in the order; A1 > 5> A2 > A4 > A3. All the isolates showed high sensitivity to high concentration (1000 mg/l) of Nickel (Ni). A1, A2 and A5 exhibited the great ability to tolerate the metal salts than A4, and A3 (Figure 2.)

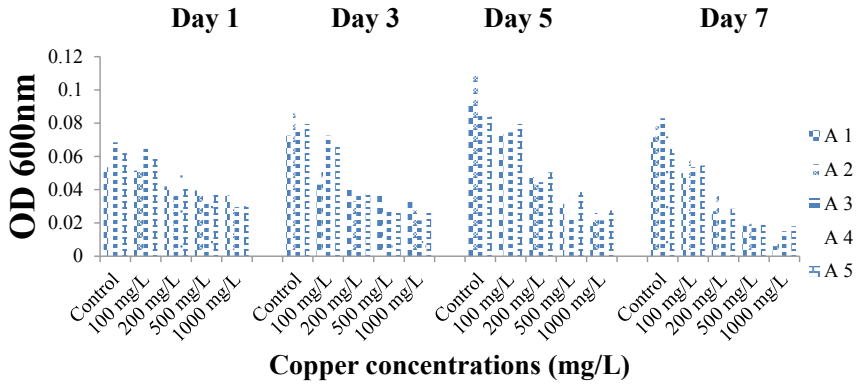

Figure 1: Tolerance of isolates to the Copper.

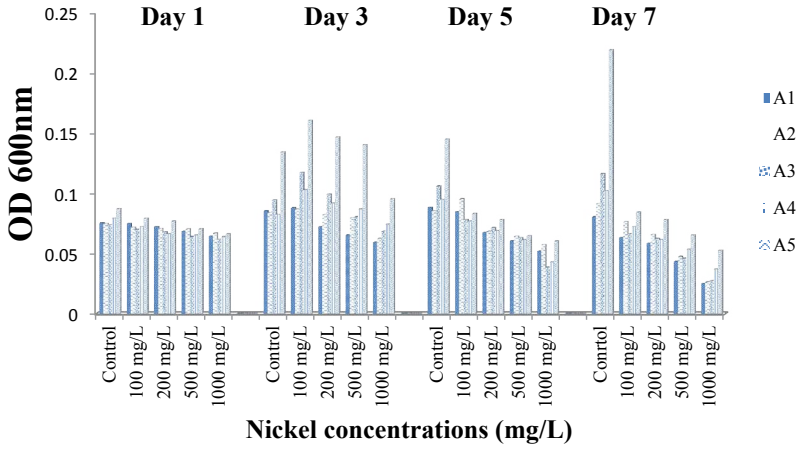

Figure 2: Tolerance of isolates to Nickel.

Tolerance of ammonia oxidizing bacteria growth to lead

Figure 3 shows tolerance of ammonia oxidizing bacteria isolates to different lead concentrations at different day intervals. Growth was highest in the absence of metal salts at 120 hours. However, increasing lead concentration up to 1000 mg/l caused a decline in growth by more than 68 % at 168 hours of cultivation. Growth of A1 ranged from 0.05 ± 0.001 to 0.09 ± 0.001 within 120 hours. At 1000 mg/l concentration, growth for isolate A1 ranged from 0.034 ± 0.001 to 0.052 ± 0.001 within 120 hours and decreased to 0.004 ± 0.0028 after 168 hours.

Growth of A2 and A5 isolates ranged from 0.06 ± 0.000 to 0.11 ± 0.003 and 0.06 ± 0.000 to 0.08 ± 0.001 within 120 hours, respectively. The least and peak values for growth of ammonia-oxidizing bacterial isolates, the least and peak values for growth were recorded at 1000 mg/l and 0 mg/l respectively between 120 and 168 hours of cultivation and the tolerance of the isolates to lead (Pb) was in the order; A1 < A3 < A4 < A2 < A5 **(Figure 3).**

Tolerance of ammonia-oxidizing bacteria isolates to cadmium

The result represented in Figure 4 shows the level of cadmium effect on the ammonia- oxidizing bacteria isolates at different day intervals. All the isolates showed high sensitivity to all the concentration (100, 200, 500 and 1000 mg/l) of cadmium. Growth was highest in the absence of cadmium (0 mg/l) at 120 hours. However, increasing metal salt concentration to 1000 mg/l caused a decline in growth by more than 97% at 168 hours of cultivation. The growth of A1 isolate ranged from 0.08 ± 0.006 to 0.09 ± 0.007 within 120 hours at 0 mg/l concentration. At 100 and 1000 mg/l concentration of cadmium, A1 growth ranged from 0.08 ± 0.010 to 0.08 ± 0.001 and 0.06 ± 0.006 to 0.05 ± 0.001, respectively. Isolate A2 growth range from 0.08 ± 0.008 to 0.08 ± 0.001 within 72 hours, increased to 0.09 ± 0.001 after 168 hours at 0 concentrations. At 100 mg/l concentration A2 growth ranged from 0.07 ± 0.015 to 0.09 ± 0.008 within 72 hours, increased to 0.10 ± 0.001 within 120 hrs and decreased to 0.08 ± 0.001 after 168 hours at 100 mg/l concentration. The growth of A2 ranged from 0.08 ± 0.001 to 0.06 ± 0.002 within 72 hours and reduced to 0.03 ± 0.002 after 168 hours.

For isolates A3 and A4 accordingly, growth ranged values were; 0.08 ± 0.003 to 0.12 ± 0.001; 0.08 ± 0.008 to 0.09 ± 0.002; 0.08 ± 0.005 to 0.12 ± 0.003; 0.07 ± 0.014 to 0.11 ± 0.003 and 0.08 ± 0.005 to 0.10 ± 0.000. Growth of A5 ranged from 0.09 ± 0.002 to 0.13 ± 0.002 within 72 hours, increased to 0.15 ± 0.001 within 120 hours and increased to 0.22 ± 0.02 after 168 hours at 0 concentrations. Isolate A5 growth ranged from 0.08 ± 0.001 to 0.16 ± 0.004 within 72 hours, decreased to 0.08 ± 0.001 within 120 hours and increased to 0.08 ± 0.006 after 168 hours at 100 mg/l concentration. A5 growth ranged from 0.08 ± 0.005 to 0.10 ± 0.020 within 72 hours; decreased to 0.05 ± 0.000 after 168 hours at 1000 mg/l concentration. Tolerance of the ammonia-oxidizing bacterial isolates to cadmium was in the order; A3 < A4 < A1 < A2 < A5 **(Figure 4).**

Biosorption of copper, nickel, lead and cadmium by different nitrifying bacteria

Biosorption of copper, nickel, lead and cadmium by different nitrifying bacteria *Achromobacter xylosoxidans* and *Achromobacter insolitus.* The results of assessment of the two isolates represented as AOB 2 and AOB 5 for biosorption of selected heavy metals via shake flask method at different day intervals for 28 days under controlled environmental conditions.

Biosorption of copper by ammonia-oxidizing bacteria (AOB 2 and AOB 5). Isolate *Achromobacter xylosoxidans* (AOB 2) biosorption capacity of copper range value was; 10 to 90.1. AOB 2 biosorption of nickel were; 12 % on day 1; 48% on day 7; 77% on day 14; 84% on day 21 and 96.51% on 28 days. AOB 2 mitigation of lead range from 15 to 67, 74 to 81.5 and 92 % at 1, 7, 14, 21 and 28 days, respectively. Isolate AOB 2 biosorption of cadmium was 5 % on day 1; 41.796% on day 7; 52% on day 14; 61% on day 21 and 84.82% on 28 days as presented in figure 5. *Achromobacter insolitus* (AOB 5) mitigation of copper ranged from 9 to 52.67, 68 to 86 and 90.04% at 1 , 7, 14, 21 and 28 days, respectively. AOB 5 biosorption of nickel was 7% on day 1; 49.64 % on day 7; 67% on day 14; 80% on day 21 and 94.67% on 28 days.

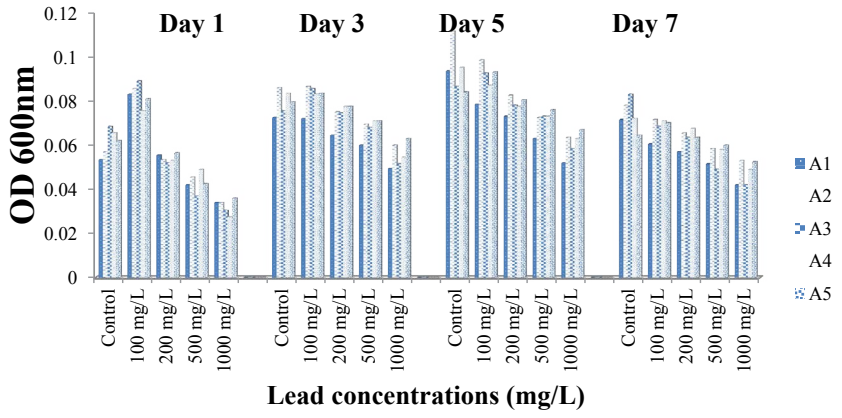

Figure 3: Tolerance of isolates to the lead.

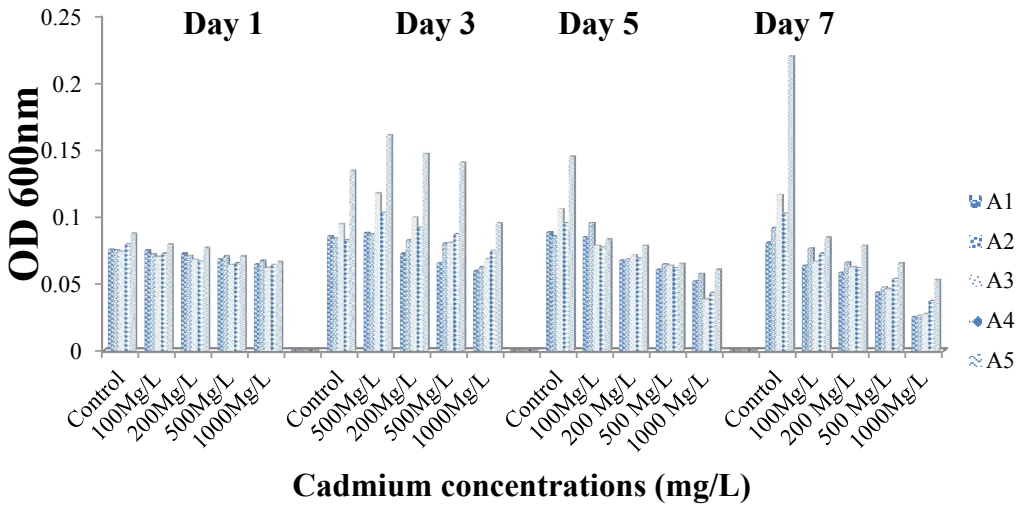

Figure 4: Tolerance of isolates to the cadmium.

Isolate AOB 5 mitigation of lead ranged from 11 to 62.75, 68 to 79 and 90.25% at 1, 7, 14, 21 and 28 days, respectively. AOB 5 biosorption of cadmium was 6 % on day 1; 48.56% on day 7; 54% on day 14; 73% on day 21 and 89.21% on 28 days as presented in figure 6.

Discussion

Effects of copper, nickel, lead and cadmium on the growth of nitrifying bacteria were *evaluated in a different medium formulation*; all the bacterial isolates demonstrated ability to grow in mineral salt agar medium incorporated with different heavy metal salt at different concentrations (100 , 200, 500 and 1000 mg/l).

Growth of ammonia-oxidizing and nitrite-oxidizing bacteria increased successively throughout the period of five days of exposure to various concentrations of the heavy metal salts but decreased at day 7. Increasing heavy metal concentration up to 1000 mg/l decreased the bacterial growth significantly ($p \leq 0.05$). However, maximum growth was recorded for all the isolates, in the absence of heavy metal salts. In addition, there was significant ($p < 0.05$) difference in the nitrifying bacteria response rates to heavy metal salts (copper (Cu), nickel (Ni), lead (Pb) and cadmium (Cd) at four loading rates (100, 200, 500, 1000 mg/l).

Tolerance of ammonia-oxidizing bacterial isolates to copper, nickel, lead and cadmium was in the order; A1 < A4 < A3 < A5 < A2; A1 > A5> A2 > A4 > A3; A1 < A3 < A4 < A2< A5 and A3 < A4 < A1 < A2 < A5 respectively.

Among the ammonia-oxidizing bacterial, isolates A2 and A5 exhibited the greater ability to tolerate the metal salts than A3, A4, and A1. Results obtained showed that the addition of heavy metal pollutants in the environment had different impacts on eco-physiological microorganisms. Adding Cu, Ni, Pb and Cd in low concentration had stimulatory or slightly inhibitory effect, while application of high concentrations of heavy metals had a strong inhibitory effect. Most nitrifying bacteria were susceptible to heavy metals; some bacteria were resistant to the pollution, maintaining themselves even in the presence of elevated concentrations of metals (Cu, Ni, Pb and Cd). Nitrifying bacteria were more sensitive to Cd than for Pb, Cu, and Ni. Harmful effect of cadmium was very obvious in all eco-physiological.

Toxicity of heavy metals not only depends upon the concentration, but also the chemical structure, time of exposure, and the source of the metal contamination [5,52]. The study agrees with the work of Mertoglu *et al*. [53] which reported population shifts in an enriched nitrifying system under gradually increased cadmium loading. Frey *et al.,* Lee *et al.,* and Vasileiadis *et al.* also reported a sensitive response of the AOB community to heavy metals. Heavy metals can affect diversity of certain microbial communities and related soil processes [54-57].

Conspicuous responses of different AOB communities to metal pollution stress have been observed in agricultural soils amended with metals and industrial effluents [26,51,58]. Okpokwasili and Odokuma, demonstrated that nitrifying bacteria (*Nitrobacter*) predominant in waste environments were sensitive to various toxicants [59]. In another study, John and Okpokwasili, studied crude oil degradation and plasmid profile of nitrifying bacteria isolated from oil impacted mangrove sediment in the Niger Delta and found that nitrifying bacteria were able to carry out degradation of crude oil [28].

Alternatively, Frey *et al.* reported that soil metal contamination does not decrease the abundance of AOB while the research of Stefanowicz *et al.* and Qu *et al.* demonstrated that bacterial functional diversity significantly decreased with increasing soil pollution [54,60,61]. Bermudez *et al*. hypothesized that high organic matter contents of soil can bind metals and decrease their toxicity [62]. Yeung *et al.* studied the adaptation of nitrifying microbial biomass to nickel in batch incubations [63]. Wu *et al*. [64] investigated he long-term effect of field fertilization on the community structure of ammonia-oxidizing bacteria and reported no significant effect of metals on AOB abundance, this suggests that metal concentration was not the main factor affecting the

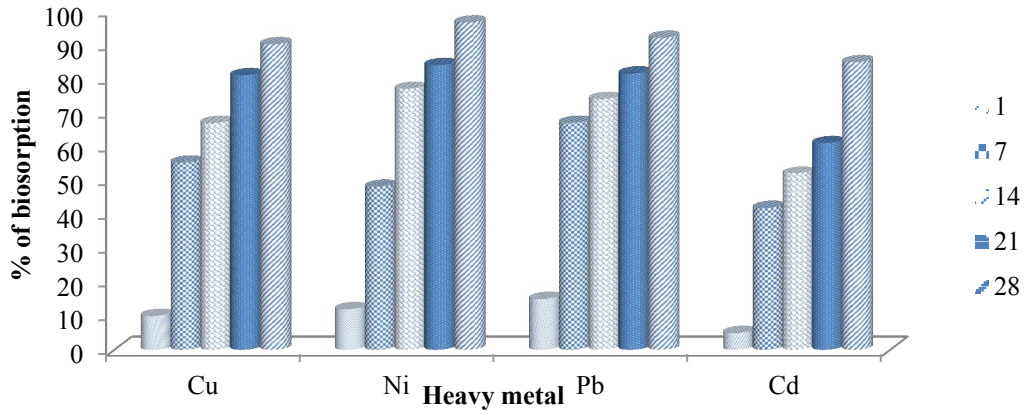

Figure 5: Biosorption of copper, nickel, lead and cadmium by ammonia oxidizing bacteria (AOB 2).

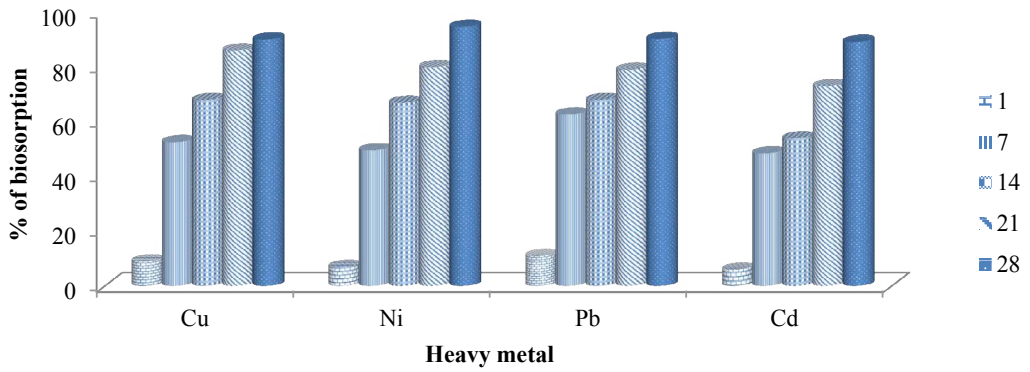

Figure 6: Biosorption of copper, nickel, lead and cadmium by ammonia oxidizing bacteria AOB 5.

abundance of ammonia-oxidizing bacteria. Moreover, Kris *et al.* had demonstrated that the nitrifying community display tolerance to long-term Zn stress. Some microorganisms are able to produce inducible proteins that confer tolerance to metal pollutants [65,66].

Several other studies have particularly emphasized the response of nitrifying bacteria to heavy metals. Some of these studies reported a sensitive response of AOB and NOB community to Heavy metals [54-56]. Additionally, Mertens *et al.,* Liu *et al.,* Lee *et al.,* and Vasileiadis *et al.,* particularly emphasized the response of ammonia oxidizing bacteria to heavy metals such as Zn, Cu and Hg [55,56,67,68]. Heavy metals used in greater amounts result in metabolic disorders and suppress the growth of most microorganisms. Roane *et al.,* [69] inferred that heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities, ultimately resulting in decreased biomass and diversity. According to Khan *et al.*, Siokwu and Anyanwu, Subrahmanyam *et al.,* Vashishth *et al.*, and Wang *et al.,* tolerance of nitrifying bacteria to heavy metal salts is dependent on concentration, time and the isolate tested [66,70-73].

Nitrifying bacteria; *Achromobacter* x*ylosoxidans* and *Achromobacter insolitus*, were able to carry out biosorption of copper, nickel, lead and cadmium as seen in this study. *Achromobacter insolitus* had the highest biosorption capacity for copper and cadmium at 90.04% and 89.21%, respectively within a period of 28 days. *Achromobacter* x*ylosoxidans* had the highest biosorption capacity for nickel and lead at 96.51% and 92%, respectively within a period of 28 days.

Statistical analysis ascertains that there is a significant ($p<0.05$) difference in biosorption rates between medium with the bacteria isolates and control. Biosorption of heavy by nitrifying bacteria gave a positive result. Biosorption of heavy metals by different nitrifying bacteria is dependent on the characteristics of the pollutant (heavy metal). Several researchers have also reported the excellent bioremediation potentials of various soil microorganisms [74-84].

Conclusion

All the Ammonia Oxidizing bacteria were able to adapt and grow under various extreme conditions show a high level of tolerance for heavy metal tested. A2 and A5 exhibited the greatest ability to tolerate the metal salts than the others which makes the organism an attractive potential candidates for further investigations regarding their ability to remove heavy metal in bioremediation. It may be a good option for bioremediation of soil and waste since it is regarded as an eco-friendly and efficient. The understanding of microbial tolerance and adaptation to the presence of metal in the environment is critical in determining the management and potential long-term effect of that part of the environment receiving metal contamination.

The nitrifying bacteria studied showed efficient oxidizing ability for ammonium and nitrite. Nitrification in the presence of heavy metal salt depended on the heavy metal concentration, time and the isolates tested. *Achromobacter xylosoxidans and Achromobacter insolitus* demonstrated nitrification ability even at high concentrations of heavy metal salts. Thus, they may be used to remove high strength ammonium from digested sludge. The organisms were able to carry out biosorption of copper, nickel, lead and cadmium. The understanding of heavy metal toxicity to microorganisms in the environment is crucial in determining its long-term effects and possibility of remediation from the contaminated environments by the adapted microbial strains.

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