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Impacts of different concentrations of Copper and Zinc on *in vitro* responses of *Azotobacter chroococum* in biomass and nitrogen fixing outputs

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Abstract

The responses of *Azotobacter chroococum* for copper and zinc were surveyed in this study. The impacts of different concentrations of copper and zinc on biomass formation and nitrogen fixation of *Azotobacter chroococum* were investigated. Batch trials were performed under continuous airflow using Jensen's nitrogen free broth, at ambient room temperatures for seven days. Maximum biomass yields of 0.068 OD_{600} units and 0.131 OD_{600} units were recorded at 25 mg L^{-1} and 200 mg L^{-1} for copper and zinc amended trials, respectively. Maximum nitrogen fixations of 1.446 ppm and 1.507 ppm were also recorded for copper and zinc amended trials at 12.5 mg L^{-1}, respectively. Statistical analysis revealed strong significant correlations between metal concentrations and nitrogen fixation for copper and zinc amended trials, respectively. Leudeking-Piret modeling showed that nitrogen fixations of *Azotobacter chroococum* in both copper and zinc amended trials were associated with biomass density.

Keywords: Bio-stimulation, kinetic modeling, metal tolerance, micro-nutrients, secondary metabolite, toxicity.

INTRODUCTION

Metals are a natural part of most environments. However, elevated concentrations of metals arise mainly due to anthropogenic activities, such as: mining, smelting, printing, electroplating, and battery manufacture and disposal. Copper, for instance, is extensively used in herbicides and fungicides. Many of the essential elements required by microbes in low concentrations become toxic to the same microbes at elevated amounts (Nriagu, 1990). Metal toxicity in microorganisms usually manifest in growth inhibition and impairment of specific metabolic processes (Montuelle *et al*., 1994).

Metals are critical components of many biochemical systems. The lighter metal ions (Na^+, K^+, Ca^{2+}, and Mg^{2+}) are used as enzyme co-factors and are important components of ion pumps that drive oxidative phosphorylation and help to maintain osmotic balance within cells (Mills, 2002). Many of the heavy metals are also essential components of biological systems; they are required at low concentrations for growth and biochemical processes. Microorganisms have evolved mechanisms that vary in capacity and specificity to accumulate and tolerate different heavy metals, such as: Cu, Zn, Ni, Mn, and Co in the environment (Gadd, 1990). Metal requirements and tolerance of microbes have been extensively studied by many researchers (Wyszkowska *et al*., 2013). However, the impact of heavy metals depends on duration of exposure, dose, and type of metal (Paudyal *et al*., 2007).

Copper is an essential micro-nutrient required as a co-factor for a number of cupro-enzymes, including: amine oxidases, cytochrome c oxidases, laccases, methane monooxygenases, nitrate oxidases, superoxide dismutases, and so on. These proteins are involved in diverse cellular processes, such as energy transduction, iron mobilization, and oxidative stress response (Huertes *et al*., 2014). Antibacterial properties and toxicity of copper to microorganisms have also been widely documented by researchers (Merroun, 2007).

Zinc is another essential micronutrient, and a component of alcohol dehydrogenase, carbonic anhydrase, and

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carboxypeptidase enzymes. Zinc has been reported by Hassen *et al* (1998) to be toxic to some bacterial strains even at very low doses, and at the same time highly tolerant by other bacteria. Slater and Capone (1984) and Ahmad *et al* (2005) in their separate studies reported zinc to be of relatively low toxicity to indigenous soil microbial population.

Azotobacter species are free-living obligate aerobic bacteria dominantly found in soils, which can also grow under low oxygen concentration (Shridhar, 2012). Their population ranges from negligible to 10^4 per gram of soil. However, actual numbers depend upon the physico-chemical and microbial properties of the soil. *Azotobacter chroococum* is the most prevalent species found, but other species also occur (Kizilikaya, 2009). Other species of *Azotobacter* include*: A. vinelandii*, *A. beijerinckii*, *A.paspali*, *A. armeniacus*, *A. nigricans*, and *A. salinestri*. *Azotobacter species* are heterotrophic, and their main property is their ability to fix nitrogen non-symbiotically (Jimenez *et al*., 2011). Wyszkowska *et al* (2013) indicated that nitrifying bacteria, symbiotic nitrogen fixing bacteria, and *Azotobacter spp*. are the most susceptible microorganisms to heavy metal toxicity.

This study investigates the *in vitro* effects of different concentrations of copper (as $CuSO_4.5H_2O$) and Zinc (as $ZnSO_4.7H_2O$) on the biomass and nitrogen fixation yields of *Azotobacter chroococum*. Hence, this study will provide information on levels of copper and zinc tolerances of *Azotobacter chroococum* as it affects the aforementioned essential bio-activities. Though the concentrations of copper and zinc salts used in this study are unlikely to be found in the natural environment, such concentrations may build up in soils due to different anthropogenic activities, such as agricultural, industrial, and mining activities.

MATERIALS AND METHODS

Preparation of Azotobacter chroococum inoculum

Stock samples of *Azotobacter chroococcum* (Mac12638:11142017) were obtained from MacCliff Research Laboratories (MRL) Ltd., Owerri, Nigeria. This stock sample was authenticated and re-vitalized in Jensen's broth. Stock broth culture of *A. chroococum* was prepared. To prepare stock broth culture of *A. chroococum*: a sterile wire loop was used to introduce *A. chroococum* from the Jensen's agar slant stock to sterile Jensen's (nitrogen free) broth (SRL, India), and incubated at prevailing room temperatures for 5 – 7 days under continuous airflow. Biomass growth was indicted by increased turbidity of the broth. From this stock broth, 0.5 McFarland standards were prepared (Kizilkaya, 2009).

Toxicity tests

200 mg L^{-1}, 100 mg L^{-1}, 50 mg L^{-1}, 25 mg L^{-1}, 12.5 mg L^{-1}, and 0 mg L^{-1} concentrations of sterile $CuSO_4$ and $ZnSO_4$ solutions, respectively, were prepared in half strength Jensen's broth (Mills, 2002). The initial concentration of 12.5 mg L^{-1} was chosen arbitrarily, while subsequent concentrations were double folds of the previous concentration. Then 0.1 ml aliquot of 0.5 McFarland standard of *A. chroococcum* was added to 9.9 ml of each of the above sterile $CuSO_4$ and $ZnSO_4$ concentrations, respectively. This set up was incubated for 7 days at prevailing room temperatures under continuous airflow in a sterile chamber plugged to a Airfree T800 Filterless Air Purifier (USA). The optical density at 600nm (OD_{600}) (using model 722 visible spectrophotometer, manufactured by Shanghai Third instrument Factory, China), nitrate-N concentration, and amino-N concentration of each test were measured at days 0 and 7, respectively (Paudyal *et al*., 2007).

Estimation of nitrate nitrogen and amino nitrogen

Broth culture experiments were analyzed for nitrate nitrogen (NO_3-N) and amino nitrogen (Amino-N) at the beginning (Day 0) and at the end (Day 7) of the trials. Nitrate-N concentration was determined by Cataldo's method as described by Cataldo *et al* (1974): 2.5 µl of sample solution was taken into a 1.5 ml Eppendorf tube, and 10 µl of salicylic acid-sulfate solution (500 mg of salicylic acid was dissolved in 10 ml of concentrated sulfuric acid) was mixed and kept for 20 minutes. Then, 250 µl of 2M NaOH solution (8.00 g of NaOH was dissolved in 100 ml of water) was mixed and kept for 20 minutes. 200 µl of the reaction solution was put in a 722 visible spectrophotometer and the absorption at 410 nm was measured. Standard solution was made by dissolving 42.5 mg of $NaNO_3$ in 100 ml of water, which contains 5 mM nitrate (70 mg N l^{-1}). Diluted solutions (0, 1, 2, 3, 4, 5 mM) were used for the calibration and plotting of standard curve.

Amino-N concentration was determined by ninhydrin method (Herridge, 1985): 2.5 µl of sample solution was taken into a 1.5 ml Eppendorf tube, and 75 µl of citrate buffer (5.6 g of citrate and 2.13 g of NaOH was dissolved in 100 ml of water) was mixed. Afterwards, 60 µl of ninhydrin solution (958 mg of ninhydrin and 33.4 mg of ascorbate was dissolved in 3.2 ml of water and filled up to 100 ml with methoxyethanol in a flask. The flask was secured with its cork and heated at 100°C for 20 minutes in a hot air oven (Quincy Hydraulic Gravity Convection Oven, USA.). Then, 300 µl ethanol was added and cooled to room temperature for 10 minutes. 200 µl of the reaction solution was put in a 722 visible spectrophotometer and the absorption at 570 nm was measured. Standard solution was made by dissolving 66.1 mg of asparagine (or 70.1 mg of asparagine monohydrate) plus 73.1 mg glutamine in 100 ml of water, which contains 5 mM asparagine + 5 mM glutamine (280 mg N L^{-1}). Diluted solutions (0, 28, 56, 84, 112, 140 mg N L^{-1}) were used for the calibration and plotting of standard curve.

Determination of specific growth rate

The specific growth rate of *Azotobacter chroococum* was determined using the formula given by Stanier *et al* (1985):

$$\text{Specific growth rate} = \frac{\text{Log OD}_1 - \text{Log OD}_0}{T_1 - T_0} \times 2.303$$

Where,

Log OD_1 = Log value of optical density (OD) of culture at time T_1 days

Log OD_0 = Log value of optical density (OD) of culture at time T_0 days.

Estimation of fixed nitrogen

The amount of nitrogen fixed was estimated with the formula:

Nitrogen fixed = $N - N_0$

Where,

N = the total concentrations of (nitrate nitrogen + amino nitrogen) in culture medium after incubation.

N_0 = concentrations of (nitrate nitrogen + amino nitrogen) in culture medium before incubation.

Estimation of nitrogen fixation rate

Nitrogen fixation rate was estimated using the formula:

$$\text{Nitrogen fixation rate} = \frac{N - N_0}{T - T_0}$$

Where,

$N - N_0$ = Nitrogen fixed

$T - T_0$ = incubation period

Statistical analysis

All measurements were made in triplicate, and values reported as means of triplicate values. Student t tests and Pearson's correlation analysis for all possible variable pairs were estimated using Minitab 17 software. Significance was taken at 5% level of significance ($p \leq 0.05$).

Mathematical modeling

The Luedeking-Piret model was applied to analyze product synthesis (nitrogen fixation) kinetics, using Curve Expert Professional 2.4.

RESULTS AND DISCUSSION

After 7 days incubation, the control experiment (trial without any metal amendment) gave a biomass yield of X_o = 0.042 OD_{600} units and a nitrogen fixation yield of P_o = 0.847 ppm. Maximum biomass concentration (X_{max}) of 0.068 OD_{600} units and 0.131 OD_{600} units occurred at 25 mg L^{-1} $CuSO_4$ and 200 mg L^{-1} $ZnSO_4$ concentrations, respectively (Figure 1). While, maximum nitrogen fixations (P_{max}) of 1.446 ppm and 1.507 ppm occurred at 12.5 mg L^{-1} $CuSO_4$ and 12.5 mg L^{-1} $ZnSO_4$ concentrations, respectively (Figure 2).

Copper enhanced biomass growth of *A. chroococum* progressively at 12.5 mg L^{-1} and 25 mg L^{-1} $CuSO_4$ concentrations, while at 50 mg L^{-1}, 100 mg L^{-1}, and 200 mg L^{-1} $CuSO_4$ concentrations progressively inhibited biomass growth (Figure 1). At 50 mg L^{-1} $CuSO_4$ concentration, though biomass yield was less than that of the control experiment, nitrogen fixation yield surpassed that of the control experiment. This indicates that increased biomass yield does not always imply increased bio-product output, as also reported by Orji *et al* (2018). A maximum growth rate (μ_{max}) of 0.076 OD_{600} units day^{-1} at 25 mg L^{-1} $CuSO_4$ concentration was observed for *A. chroococum* in these trials. Similar inhibitory activities of copper on biomass growth and nitrogen fixation of diazotrophs have been also been reported by: Slater and Capone (1984), El-Ghamry *et al* (2000), Zhan and Sun (2012). Wyszkowska *et al* (2013) reported that *Azotobacter species* are among the most susceptible microorganisms to heavy metals.

As all five zinc metal concentrations tested enhanced the biomass of *A. Chroococum* progressively (Figure 1), a maximum growth rate (μ_{max}) of 0.170 OD_{600} units day^{-1} (at 200 mg L^{-1} of $ZnSO_4$) was observed for *A. Chroococum*. Zinc (as $ZnSO_4$) tremendously improved biomass growth of *A. Chroococum*. Its specific growth rate was increased approximately 20 times (when compared to the control) at the lowest $ZnSO_4$ concentrations (12.5 mg L^{-1}) tested. On the other hand the copper increased specific growth rate of *A. Chroococum* only about three times (when compared to the control) at 12.5 mg L^{-1} of $CuSO_4$. Though the specific growth rate of *A. Chroococum* progressively increased from 0 mg L^{-1} to 200 mg L^{-1} $ZnSO_4$ concentrations, the rate of increments of specific growth rates progressively waned between consecutive increments of $ZnSO_4$ concentrations. Student's t test revealed significant difference ($p < 0.05$) in the specific growth rates between trials amended with copper and zinc. Pearson's correlation revealed a very strong positive correlation (r = 0.878) between the specific growth rates of the copper and zinc amended trials. Though, *A. chroococum* significantly differed in its biomass value responses to copper and zinc, its pattern of responses was basically similar to both copper and zinc.

Nitrogen fixation by *A. chroococum* was elevated non-progressively at 12.5 mg L^{-1}, 25 mg L^{-1}, and 50 mg L^{-1} $CuSO_4$ concentrations, and abated progressively at 100 mg L^{-1} and 200 mg L^{-1} $CuSO_4$ concentrations (Figure 2). For $ZnSO_4$, nitrogen

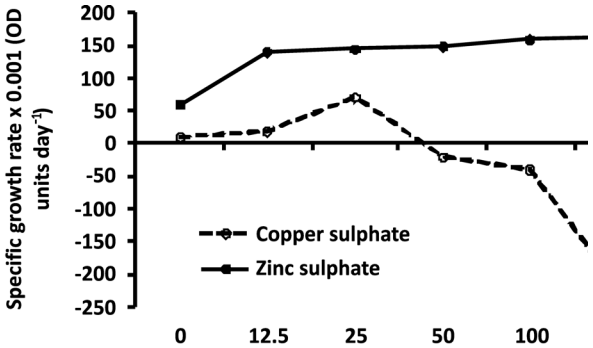

Figure 1. Growth rates of *Azotobacter chroococum* at different concentrations of copper and zinc salts.

fixation was enhanced only at 12.5 mg L^{-1} concentration, while subsequent higher concentrations tested showed progressive reductions in the amounts of nitrogen fixed during the trial.

Pearson's correlation analysis at 5% level of probability revealed significantly ($p<0.05$): very strong indirect correlation between copper concentrations and biomass yields ($r = -0.826$); very strong indirect correlation between copper concentrations and nitrogen fixations ($r = -0.911$); very strong and indirect correlation between copper concentrations and specific growth rates ($r = -0.927$); and a fairly strong and indirect correlation between copper concentrations and nitrogen fixation rates ($r = -0.642$). For zinc trials: a strong direct correlation was seen between zinc concentrations and biomass growths ($r = 0.705$); a very strong indirect correlation between zinc concentrations and nitrogen fixations ($r = -0.869$), and fair positive but insignificant ($p>0.05$) correlations between zinc concentrations and specific growth rates ($r = 0.565$); and a very weak direct and insignificant correlation between zinc concentrations and nitrogen fixation rates ($r = 0.208$).

Kinetic associations between cell mass and nitrogen fixation of *Azotobacter chroococum* at different metal (copper and zinc) concentrations were analysed using the Leudeking-Piret model.

$$\mathbf{r_{fp} = \alpha r_{fx} + \beta x} \qquad \mathbf{(1)}$$

Where,

r_{fp} = rate of product formation

r_{fx} = rate of biomass formation

α = coefficient of proportionality between the rate of product formation and growth rate (ppm//OD-units)

β = coefficient of proportionality between the rate of product formation and biomass concentration (ppm/OD-units day^{-1}).

According to this model, the product formation rate (nitrogen fixation rate) depends linearly upon the growth rate and the cell mass concentration. A linear regression plot of nitrogen fixation rate against specific growth rate generated $\alpha = 0.0010156$ and $\beta = 0.14032$ for copper amended trials (Figure 3). Since, $\alpha > 0$ and $\beta > 0$, then it implies that in this trial, nitrogen fixation was associated with both biomass growth and biomass concentration of *A. chroococum* (Ramakrishnan *et al*., 2015). However, since β (0.14032) is far greater than α (0.0010156), and also since the value of $\alpha = 0.0010156$ is not significant at 0.01 level of significance, one may infer that nitrogen fixation yields in these trials were more significantly influenced by the non-growth associated constant (β), or otherwise insignificantly determined by the growth associated constant, α (Ahmad *et al*., 2011; Thierie, 2013). A linear regression plot of nitrogen fixation rate against specific growth rate for zinc trials gave $\alpha = -0.00054363$ and $\beta = 0.15508$ (Figure 4). Since, $\alpha < 0$ and $\beta > 0$, it implies that nitrogen fixation in the zinc amended trial was associated with biomass density rather than with biomass growth.

The Leudeking-Piret linear regression plots gave $R^2 = 0.8337$ ($p = 0.01$) for copper amended trials, and $R^2 = 0.1524$ ($p = 0.44$) for zinc amended trials (where, R^2 = coefficient of determination). The high R^2 and low p ($p<0.05$) values of the copper amended trials indicate that the Leudeking-Piret model explains about 83.37% of variation in the biomass growth and nitrogen fixation by *A. chroococum*. Since R^2 values lies between 0 and 1, and a value ≥ 0.75 indicates the level of *goodness of fit* of the model (Keur and Satyanarayana, 2005), hence one conclude that the Leudeking-Piret model is an appropriate kinetic model for analyzing the copper amended trial. However, the low R^2 and high p ($p>0.05$) values of the zinc amended trial plot reveal that Leudeking-Piret model does not sufficiently explain how biomass of *A. chroococum* accounts for the amount of nitrogen fixed. Hence, in the zinc amended trials other factors (probably not monitored in this study) such as the presence of other substances, for example nutrients (whether micro or macro),

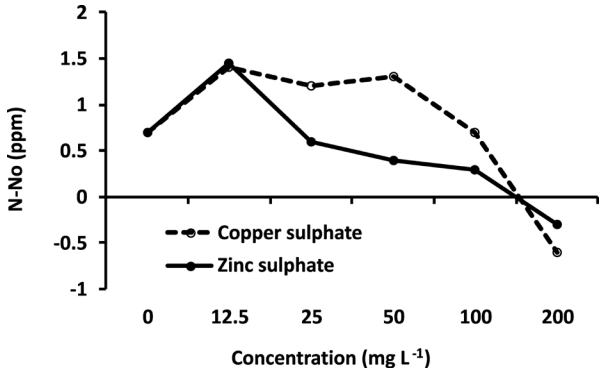

Figure 2. Nitrogen fixation of *Azotobacter chroococum* at different concentrations of copper and zinc salts.

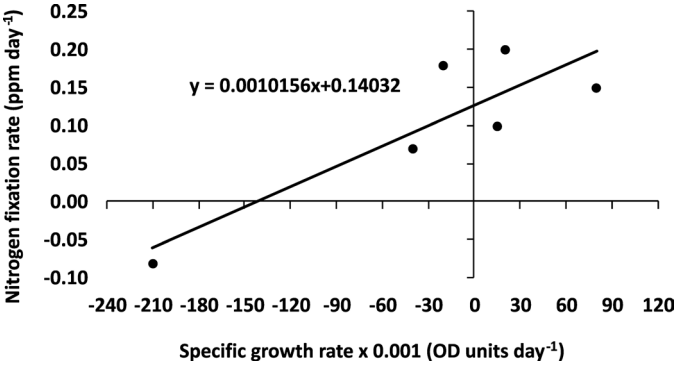

Figure 3. Leudeking-Piret model of *Azotobacter chroococum* for copper sulphate concentrations.

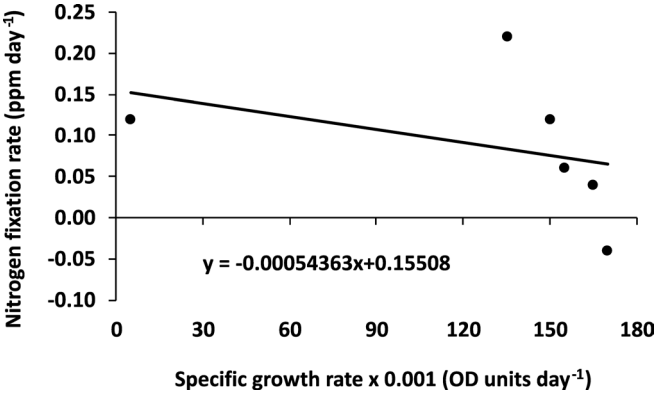

Figure 4. Leudeking-Piret model of *Azotobacter chroococum* for zinc sulphate concentrations.

inhibitors, etc. may have been significant extrinsic factors in determining the quantity of nitrogen fixed by *A. chroococum*. Furthermore, the insignificant correlations ($p>0.05$) between $ZnSO_4$ concentrations and: biomass growth, specific growth rate, and nitrogen fixation, of *A. chroococum*, (though, does not explain why the Leudeking-Piret model falls short in accounting for the kinetics of the zinc amended trial) go on to affirm the disparities observed between the said parameters.

CONCLUSIONS

Data obtained in this study indicate that copper at low concentrations enhanced both biomass formation and nitrogen fixation of *A. chroococum*; however at higher concentrations copper abated both biomass formation and nitrogen fixation. For zinc, all the concentrations assayed in this study enhanced biomass progressively, however only 12.5 mg L^{-1} $ZnSO_4$ concentration enhanced nitrogen fixation. Nitrogen fixations in both copper and zinc trials were significantly associated with biomass density.

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