# Characterization of copper biosorption and bioreduction by copper resistant bacteria isolated from a vineyard soil

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**Abstract**
This study presents factors affecting copper bioreduction and biosorption by a highly copper resistant monoculture of *Pseudomonas putida* strain NA and copper bioremoval from soil. Seven bacteria resistant to high concentration of Cu(II) were isolated from enrichment cultures of vineyard soils and mining wastes. Culture parameters influencing copper bioreduction and biosorption by one monoculture isolate were studied. The isolate was identified by 16S rRNA gene sequence analysis as a *Pseudomonas putida* strain NA (98% similarity). The optimal temperature for growth was 30ºC and bioremoval (bioreduction and biosorption) of Cu(II) was maximal at 35ºC. Considerable growth of the isolate was observed between pH 5.0 and 8.0 with the highest growth and biosorption recorded at pH 6.0. Maximal bioreduction was observed at pH 5.0. *Pseudomonas putida* strain NA removed more than 110 mg/L Cu(II) in water within 24 h through bioreduction and biosorption. More than 23 mg/L of Cu(II) was biologically reduced in 24h at an initial concentration of 100 mg/L. In 12 h, 20 mg/L of Cu(II) was removed through biosorption. Results indicate a great potential for use of *Pseudomonas putida* NA for bioremoval of copper from water and soil.

**Keywords:** Copper (II) resistant bacteria, biosorption, bioreduction, copper bioleaching

## Introduction
Copper pollution of the environment occurs via addition of contaminated waste, mineral fertilizers and pesticides in crop production. In vineyards, sprays of various formulations containing copper as the active ingredient are used to control fungal diseases, including mildew, leaf spots and blights (Mirlean *et al.* 2007). Wastes from copper mining areas containing high concentration of copper are also major source of copper pollution of adjacent environments. High concentration of copper decreases the population of normal soil organisms and promotes microbial resistance to copper in contaminated environments (Atlas and Bartha, 1997). Bioremediation is an important tool for environmental remediation of heavy metals (Okeke, 2008). Biosorption and bioreduction of contaminants are effective bioremediation processes for removal of copper and other toxic heavy metals from the environment. Hence there is increasing interest in copper bioremoval by biosorption in aqueous media. Studies on copper bioremoval are not limited to bacterial isolates; the algae *Gelidium* has been employed for copper biosorption from industrial effluents (Villar *et al.* 2008, 2009). Bioreduction of copper Cu(II) to Cu(I) is catalyzed by copper-reductase. Reduction of Cu(II) to Cu(I) enhances copper mobility and consequently availability of copper to cell wall ATPases (Whiteley and Lee, 2006). ATPases have affinity to Cu(I) and rapidly pumps it into the cell promoting copper biosorption and bioremoval from aquatic and terrestrial environments. We present copper bioreduction and biosorption by a highly copper resistant monoculture, *Psuedomonas* sp strain NA, isolated from vineyard soil contaminated with copper. We examined the environmental factors influencing copper bioreduction and biosorption by the monoculture isolate. Furthermore we studied the capacity of the monoculture isolate to remove copper from soil.

## Materials and methods
A vineyard soil sample was collected from EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) experimental station, Bento Gonçalves, RS, Brazil. The isolate more efficient in copper biosorption and bioreduction was tested and used to characterization of environmental analysis. The isolate was identified by 16S rRNA gene sequence analysis as a *Pseudomonas putida* strain NA (98% similarity). It was studied the Cu(II)-resistance, Cu(II) biosorption and Cu(II) reduction in different pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), time course (0, 2, 4, 6, 8, 10, 12 and 24 h), copper concentrations (0, 50, 100, 150, 200, 250 and 300 mg/L) and temperature (20, 25, 30, 35 and 40ºC), and copper bioleaching from soil contaminated with 500 mg/kg of copper as ($CuSO_4.5H_2O$) after 6 days leached. Total copper was analyzed using an atomic absorption spectrometer. Aliquots of culture supernatant (200 µL aliquots) were diluted 20 times and injected into the

atomic absorption spectrometer. Copper biosorption was calculated as the difference in total copper added to the medium and remaining total copper in the medium after different microbial treatments. (CuBiosor = CuTotal added – CuTotal after growth). Copper reduction was quantified by measuring monovalent copper complex with 1mM neocuproine hydrochloride (Smith and McCurdy, 1952).

**Results**

Cu(II) bioreduction dynamics and their relationship to biomass development and biosorption is presented in Figure 1. Isolate NA grew slowly for the first 8 hours and thereafter grew rapidly until 24 h. An exponential growth pattern was observed from 6 hours to 24 h incubation. Copper bioreduction was rapid in 12 h and continued to increase over the 24 h incubation time. Approximately 20 mg/L and 23 mg/L copper were reduced after 12 and 24 h respectively. Copper biosorption profile for isolate NA similarly increased with biomass development during the 24 hours of incubation. After 12 h and 24 h, 23.33 mg/L and 21.66 mg/L copper were respectively bioremoved.

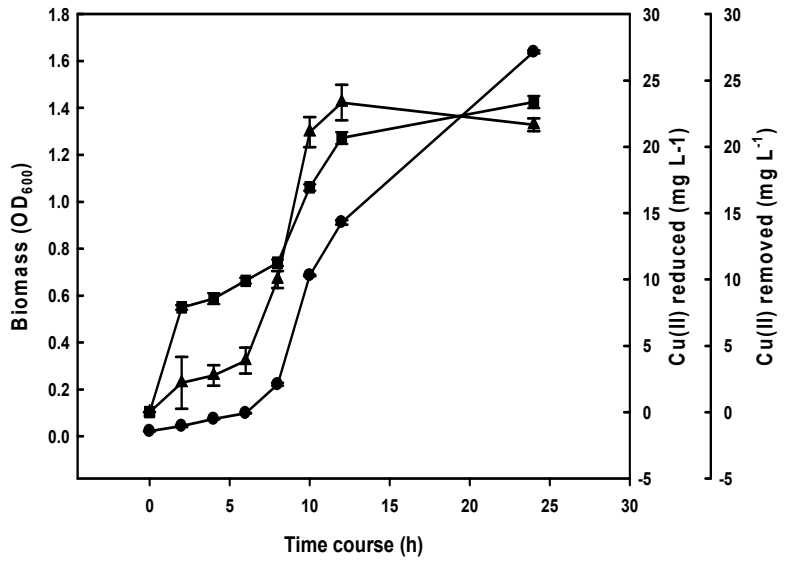


**Figure 1. Time course of Cu(II) resistance (●), Cu(II) reduction (■), and Cu(II) bioremoval (▲) for Cu(II) resistant isolate NA in TSB medium contaminated with 100 mg/L of $CuSO_4$ and incubated at 30ºC for 24 hours. Error bars are standard errors of the means of 3 replicates.**

The effect of pH on Cu(II) resistance, bioreduction and biosorption by NA is summarized in Table 1. Significant copper bioreduction occurred at initial pH range of 5.0 to 7.0 with optimum at pH 5.0 (31.04 mg/L of Cu(II) reduced in 24 h). The isolate substantially removed Cu(II) in the initial pH range of 5.0 to 9.0 and maximal removal occurred at pH 6.0 (26.25 mg/L of Cu(II) removed in 24 h). Substantial growth of the isolate was observed between pH 5.0 and 8.0, and the maximal growth occurred at pH 6.0. A slight change in initial pH occurred with growth of the isolates but it was not substantial. Little or no growth of the isolate was observed at pH 4.0 and no copper reduction was observed at pH 4.0.

**Table 1. Effect of pH on Cu(II) resistance, bioreduction and bioremoval by isolate NA**

| Initial pH | Biomass $OD_{600nm}$ | Cu(II) reduced ---------- mg/L ---------- | Cu(II) removed | Final pH |
|---|---|---|---|---|
| 4.00 | 0.069±0.001 | 00.00±0.001 | 2.29±0.481 | 3.79±0.057 |
| 5.00 | 1.847±0.029 | 31.04±1.042 | 22.92±0.524 | 5.59±0.009 |
| 6.00 | 1.866±0.009 | 20.74±0.228 | 26.25±0.208 | 6.45±0.032 |
| 7.00 | 1.639±0.006 | 16.93±0.327 | 21.67±0.481 | 7.26±0.011 |
| 8.00 | 1.189±0.003 | 7.34±0.208 | 21.25±1.156 | 7.96±0.013 |
| 9.00 | 0.745±0.007 | 4.85±0.097 | 24.79±1.069 | 8.74±0.009 |

Values are the means ± SD (n-1) of 3 replicates.

The relationship between Cu(II) concentration and biomass development are presented in Table 2. There was an inverse relationship between growth of NA and copper concentration in medium. Rates of copper

reduction and biosorption increased with increase in copper concentration to about 111.87 mg/L and 111.25 mg/L respectively. Isolate NA displayed strong bioremoval of Cu(II) with as much as 220 mg/L removed at the highest concentration.

**Table 2. Effect of Cu(II) concentration on Cu(II) resistance (A), Cu(II) reduction (B), and Cu(II) bioremoval (C) in a TSB medium for 24 hours at 30ºC.**

| Cu(II) Concentration | Biomass -------- $OD_{600}$ -------- | Cu(II) Reduction ---------- mg/L ---------- | Cu(II) Bioremoval ---------- |
|---|---|---|---|
| **0** | 1.45±0.0025* | 0.00±0.0100 | 0.00±0.0010 |
| **50** | 1.34±0.0002 | 17.83±0.3205 | 19.58±1.3889 |
| **100** | 1.26±0.0020 | 33.18±0.1715 | 37.08±6.5972 |
| **150** | 1.20±0.0001 | 45.80±0.3267 | 48.33±0.3472 |
| **200** | 1.14±0.0014 | 62.35±9.3750 | 72.50±9.3750 |
| **250** | 1.05±0.0032 | 79.07±1.9506 | 90.42±2.4306 |
| **300** | 0.99±0.0002 | 111.85±15.727 | 111.25±19.791 |

Values are the means ± SD (n-1) of 3 replicates.

Temperature profile for Cu(II) bioreduction and bioremoval by sorption is presented in Figures 2A and 2B respectively. Growth of isolate NA was substantial between 20 and 35ºC. Optimal growth of the isolate in copper medium occurred at 30ºC. Cu(II) was maximally reduced and removed at 35ºC (Figures 2A and B). Isolate NA removed high copper concentrations between 30 and 35ºC. At 35 ºC more than 41 mg/L Cu(II) was removed.

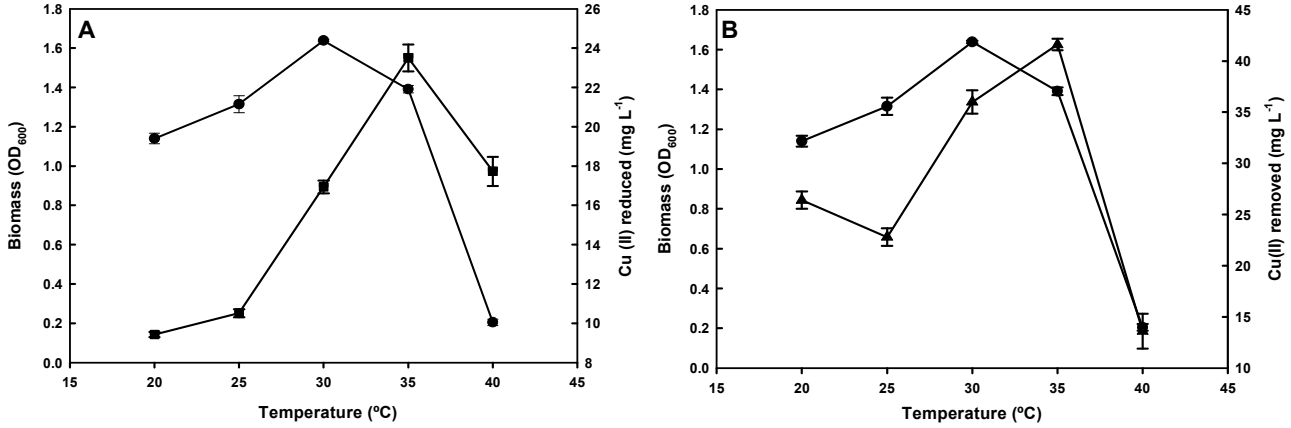


**Figure 2. Effect of temperature on A: Cu(II) reduction (■), and B: Cu(II) bioremoval (▲), in TSB culture of isolate NA contaminated with 100 mg/L of Cu(II) and incubated for 24 hours. Biomass development profile (●). Error bars are standard errors of the means of 3 replicates.**

Figure 3 shows copper bioremoval and biomass in leachate from copper contaminated soil inoculated with isolate NA suspension. In the control treated with distilled water cell density decreased slightly after 2 days and thereafter stabilized. Copper bioleaching in soil treated with distilled water (control) gradually increased during the course of the treatment. In soil cultures of isolate NA, biomass increased exponentially reaching maximum on day 4 and thereafter declined. Copper bioleaching from copper contaminated soil treated with isolate NA, rapidly increased during the course of the treatment and was significantly higher than in the control. Total copper bioleached from soil treated with isolate NA was over 18 mg/kg and only 8 mg/L in the control treated with distilled water.

## Conclusions

Divalent copper is an essential micronutrient for living organisms but negatively impacts them at high concentrations. Consequently removal of high concentrations of copper from soils and aquatic environments is critical. Biological detoxification of pollutants is an attractive technology that is cost-effective and eco-friendly. In this study, *P. putida* NA displayed strong tolerance, bioreduction and bioremoval of Cu(II). Temperature, pH and pollutant concentration had marked influences on copper bioreduction and bioremoval capacity of *P. putida* NA. The isolate strongly promoted copper bioleaching in soil. The high Cu(II) tolerance, Cu(II) biosorption and bioreduction capacity of isolate NA as well as its stability in soil make it a

candidate organism for Cu(II) bioremoval in diverse complex environments contaminated with divalent copper.

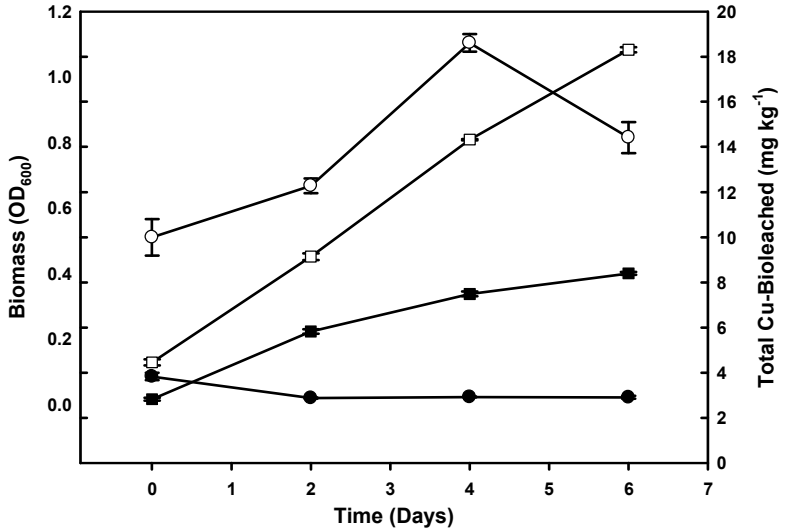


**Figure 3. Bioleaching of Cu(II) from soil contaminated with 500 mg/kg. Cell density in soil treated with water (●), copper bioleached in soil treated with water (○), cell density in soil treated with *P. putida* cell suspension (■) and copper bioleached in soil treated with *P. putida* cell suspension (□). Error bars are standard error of the means.**

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