# PCR-DGGE analysis after Bioleaching Stimulation by Indigenous Microorganisms in Vineyards Soil and Copper Mining Waste

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**Abstract**
Bioleaching techniques can be used to remove copper ions from contaminated soils and recover polluted areas *in situ* or *ex situ*. Several treatments with HCl, $H_2SO_4$, and $FeSO_4$ were used to stimulation of bioleaching in two experimental conditions applied in vineyard soil and in waste copper mining. The bioleaching treatment using $FeSO_4$ and $H_2SO_4$ mixed solution had more effect on copper lead, and the second condition using controlled temperature at 30ºC and no water addition to collect soil solution, was more efficient than the first condition using room temperature deionized water to extract soil solution. The treatment with $FeSO_4$ and $H_2SO_4$ - in the second condition, bioleached more than 1,100 µg/kg of copper from vineyard soil in one collects. After the bioleaching period (115 days), both substrates were analyzed to evaluate the effect of treatments on soil microbial by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA fragments. A high diversity of bacterial was found in the vineyard site as *Rhodobacter* sp., *Silicibacter* sp., *Bacillus* sp., *Paracoccus* sp., *Pediococcus* sp., *Myxococcales* sp., *Clostridium* sp., *Thiomonas* sp., *Firmicutes* sp., *Caulobacter vibrioides*, *Serratia* sp. and Actinomycetales; in the copper mining waste basically was found *Shingomonas* sp. specie in most of treatments; it also was found *Paracoccus* sp. and *Enterobacter* sp. in this soil sample.

**Key words**
Bioremediation; copper bioleaching; DGGE; microbial community.

## Introduction
Soil pollution with heavy metals by either industrial or agriculture activity has been a serious environmental problem. Copper is an essential nutrient for all organisms. However, an excessive concentration of this metal is toxic for most of all living organisms. Vineyard areas are currently potential areas to copper contamination due long-term treatment diseases with copper based fungicide. Copper mining areas increase the copper contamination of adjacent areas as well as produce high amount of waste. The waste is disposed in areas promoting high copper concentrations and consequently environmental pollution. Contaminated areas by heavy metals must be recovered and a remediation processes are necessary to address environmental pollution. Bioremediation is eco-friendly and costly appropriate to this case. In bioremediation process some techniques as bioleaching and biohydrometallurgy has been used to either heavy metal extraction or decontamination (Halinen *et al.* 2009). Bioleaching is an emerging technology with significant potential to add value to the mining industries so as to deliver attractive environmental and social benefits to all associates (Pradhan *et al.* 2008). The DGGE technique is one powerful and ecology tool that has been successfully used to investigate the predominant microorganisms in different environment sites tool (Halinen *et al.* 2009). Furthermore, identification of these microorganisms as well as characterization of environmental population in these areas might be useful in the future to either bioremediation or decontamination of copper polluted areas by efficient microorganisms. For these reason, an environmental conditions to copper bioleaching was created in two different soils being a 40 years vineyard and a copper mining waste area from Southern Brazil. In these soils were used acids, iron, mixture of treatments and environmental conditions to bioleach copper *ex situ*, and after that, it was evaluated the microbial community by DGGE technique.

## Methods
The soils were sampled from two copper contaminated soils in Southern Brazil. An Inceptisol was sampled from vineyard area located in Bento Gonçalves, RS, and the other substrate was sampled in the copper mining waste area, located in Caçapava do Sul, RS. The soils were sampled, drayed, sieved (3 mm mesh), homogenized and then weighted 140 g into a 200 mL double plastic flasks used as experimental unit. After

the samples weighted, it was added 10 mL of each treatment (Table 1) into soil and the moisture was adjusted to 80% of field capacity with deionized water.

**Table 1. Treatments solutions applied in two periods for copper bioextraction from two different soils.**

| Treat. | Soil | Treatment Solutions | Extraction Solution | |
|---|---|---|---|---|
| | | | *1st condition* | *2nd condition* |
| N1 | Inceptisol | $H_2O$ (deionized) | $H_2O$ | $H_2O$ |
| N2 | Inceptisol | HCl (0.01%) | $H_2O$ | HCl |
| N3 | Inceptisol | $H_2SO_4$ (0.128%) | $H_2O$ | $H_2SO_4$ |
| N4 | Inceptisol | $FeSO_4$ (1.35 mM) | $H_2O$ | $FeSO_4$ |
| N5 | Inceptisol | $FeSO_4$ (1.35 mM) + $H_2SO_4$ (0.128%) | $H_2O$ | $FeSO_4$+$H_2SO_4$ |
| N6 | Inceptisol | No treatment- original soil | - | - |
| R1 | Waste | $H_2O$ (deionized) | $H_2O$ | $H_2O$ |
| R2 | Waste | HCl (0.01%) | $H_2O$ | HCl |
| R3 | Waste | $H_2SO_4$ (0.128%) | $H_2O$ | $H_2SO_4$ |
| R4 | Waste | $FeSO_4$ (1.35 mM) | $H_2O$ | $FeSO_4$ |
| R5 | Waste | $FeSO_4$ (1.35 mM) + $H_2SO_4$ (0.128%) | $H_2O$ | $FeSO_4$+$H_2SO_4$ |
| R6 | Waste | No treatment-original waste | - | - |

The experiment was conducted in two different conditions. In the first condition, the soil moisture of all treatments was adjusted with deionized water and incubated at room temperature. The soil solution was obtained by the displacement column using deionized water. In the second condition, the soil moisture was adjusted with the treatment solutions and incubated at controlled temperature (30ºC ± 1) (Table 1). It was added 30 mL of either water or treatment solutions into the samples; this was realized according to each experiment condition. This process was taken 8 hours. After soil solution collect, the solution pH and copper content in soil solution were measured. After the measurements of all soil solutions, the soil samples were collected and stored in freezer (-4ºC) until the DNA extraction. The DNA extraction of soil was done for all treatments with the UltraCleanTM Soil DNA Isolation Kit (MOBIO, USA), using 0.5 g of soil sample of each treatment. After DNA extraction, it was amplified for PCR reactions with a CG clamp primer 338F (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GACTCCTACGGGAGGCA-3') and 519R (5'-GWATTACCGCGGCKGCTG-3'). The DGGE analysis was performed in a DCDE Universal Mutation Detection System (Biorad, Hercules, CA, USA) apparatus. The PCR product generated by 338F-GC and 518R primers were loaded onto 6% (w/v) polyacrylamide gels in 1x TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) buffer, 1 mm thick and 16 x 16 cm sized. The polycrylamide gel was made with linear denaturating gradient of urea and formamide ranged from 40% to 60% (where 100% denaturat contains 7 M urea and 40% formamide) by gradient Marker (Bio-Rad, Hercules, CA, USA). The electrophoresis conditions were run for 4 h at 200 W in 1x TAE buffer at a constant temperature of 60ºC. The gel plate was cooled in ice water for 10 min., and the gels were stained in an ethidium bromide solution (0.5 µg/mL) for 15 min., and distained in 1 x TAE buffer for more 15 min. The distained gel was placed in a UV trans-illuminator and digitalized using a digital camera. The target band was removed from the DGGE gel and was placed into a 1.5 mL sterile tube containing 20 µL of sterilized water. The freeze-thawing cycle was performed thrice at -80 and 50ºC and 1 µL of eluted DNA was amplified with primers 338F and 519R. The PCR conditions have been described in the preceding section. PCR was performed as previously described and the analysis of DNA sequences and homology searches were completed using the BLAST algorithm for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn). Genbank BLAST (N) was used for homology searches.

## Results

Bioleaching with different treatments in vineyard soil contaminated with copper (Figure 1A) and copper mining waste area was analyzed (Figure 1B) was evaluated. All treatments had the same tendency in the first experimental condition, when they were compared with water treatment (N1). The treatments on the fifth collect that received acid N2, N3 and N5 were better than the other treatments on copper bioleaching with 21.44, 82.56 and 59.91 mg of copper per kg of soil respectively. In addition, the copper bioleaching concentration in all acid treatments in the second condition were superior than 11 mg/kg of soil, and the

highest copper extraction occurred with the fifth collect in the treatment with $H_2SO_4$ (N3) with more than 82.56 mg/kg of copper bioleached from soil after 115 days of incubation. In the other hand, the first condition did not go over 1.42 mg of copper per kg of soil bioleached (N5) (Figure 1A). The treatments with sulfuric acid $H_2SO_4$ (R3) presented copper bioleaching of 2.60 mg/kg in the 4th collection, and the treatment with iron and acid together ($FeSO_4$ and $H_2SO_4$ (R5)) with 15.08 mg/kg and 20.12 mg/kg showed high copper bioleaching in the 4th and 5th collection respectively, when compared with the other treatments in study. Also, the second condition of incubation showed better results in copper bioleaching in copper mining waste (Figure 1B).

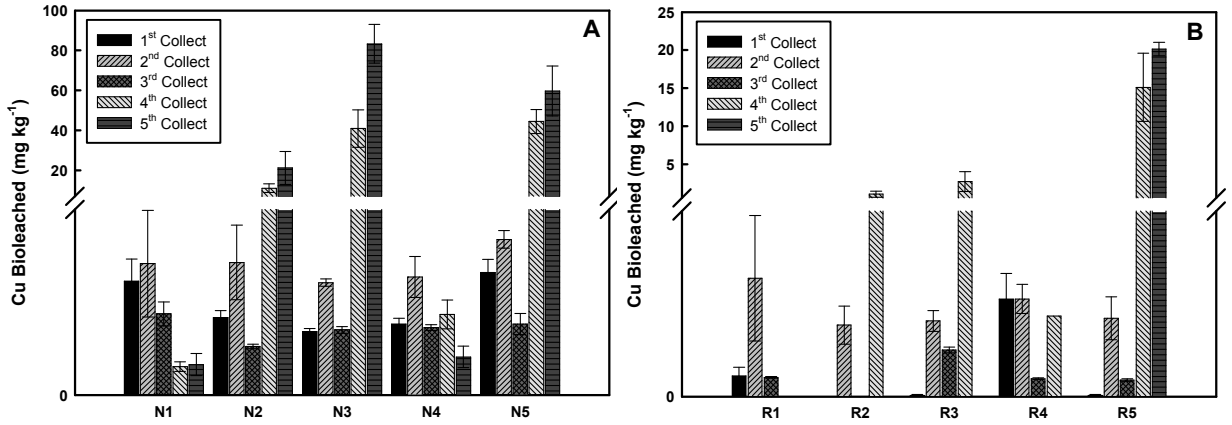


**Figure 1. Extraction of copper from Inceptisol (mg of Cu per kg of soil) with different treatments: N1=$H_20$, N2=HCl, N3=$H_2SO_4$, N4=$FeSO_4$ and N5=$FeSO_4$+$H_2SO_4$ (A); and copper mining waste with different treatments: R1=$H_20$, R2=HCl, R3=$H_2SO_4$, R4=$FeSO_4$ and R5=$FeSO_4$+$H_2SO_4$. Error bars are standard error of the mean.**

Figure 2 shows the DGGE profiles indicating the number of bands detected at each sample and matched bands among lanes. It shows that number of bands detected at each well are 4 in the vineyard soil (1. Inceptisol) and 2 in the in the copper mining waste (2. waste), where just the treatment with HCl (R2) had just the band A in the well and the others treatments had bands A and B.

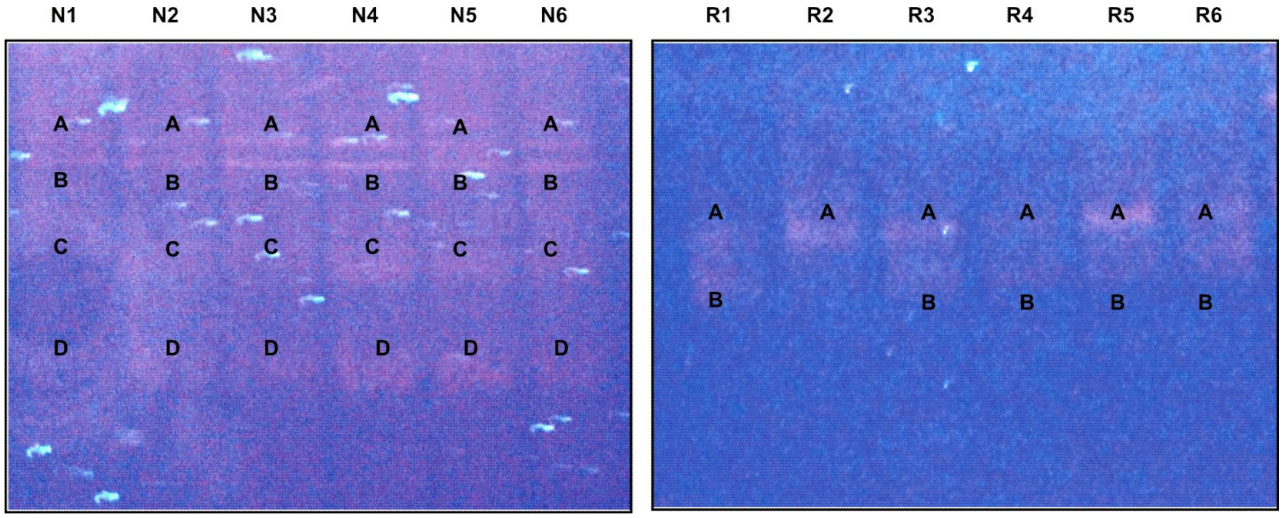


**Figure 2. Representation of the DGGE profiles, where picture 1 is from Inceptisol and picture 2 is from copper mining waste area.**

Thirty-five bands were cut and sequenced from DGGE gels of vineyard soil contaminated with copper and copper mining waste (Figure 2), and twenty-eight of these bands were identified by 16S rRNA sequence analysis. Two isolates from vineyard soil were identified as *Rhodobacter* sp. (in the treatment-band N1-A and N4-A), one as *Silicibacter* sp. (N1-B), one as *Bacillus* sp. (N1-C), four as *Paracoccus* sp. (N1-D; N2-D; N3-D and N4-D) all in the band D, one as *Pediococcus* sp. (N2-A), one as Myxococcales (N2-B), two as *Clostridium* sp. (N2-C and N6-D), one as *Thiomonas* sp. (N3-A), one as Firmicutes (N3-B), one as *Caulobacter vibrioides* (N3-C), one as Sphingobacteria (N4-B), one as *Serratia* sp. (N5-A), and one as

Actinomycetales (N6-C). From the copper mining waste, it was identified almost six isolates as *Sphingomonas* sp. (R1-A; R2-A; R3-A; R3-B; R4-A and R5-A), one as Sphingobacteria (R1-B), two as *Paracoccus* sp. (R4-B and R5-B), and one as *Enterobacter* sp. (R6-B).

**Discussion**

Copper is an essential micronutrient for living microorganisms but at high concentrations is a toxic heavy metal in the environment. Copper microbial bioleaching from contaminated areas (Halinen *et al*. 2009) have been increasing attention in recent years. In this work were used principles of copper bioleaching and biohydrometallurgy to remove copper from two different sites contaminated with copper (40 years of vineyard production and copper mining waste using the stimulation of indigenous community of soil). Furthermore, it was used fingerprinting method (DGGE) to analyze the effect of the treatments on soil microbial community, where it is important to use microbial control methodologies to study in reliable and automatic way the microorganisms associated with different zones of bioleaching process to subsequent optimize their efficiency (Malki *et al.* 2006).

After the treatments, it was extract DNA from samples and used PCR-DGGE method to evaluate the soil microbiota community. It was identified more than one microorganism per band in the PCR-DGGE gel from vineyard soil, i.e. *Silicibacter* sp., Myxococcales, Firmicutes, and Sphingobacteria where found in the band "B" of vineyard soil. Otherwise, it is known that one band could possibly represent more than one species (Heuer and Smalla 1997) and also, some bacteria can produce more than one band on DGGE gel method (Muyzer *et al.* 1993). Comparing our study with other DGGE study with vineyard soil contaminated with high copper concentrations to evaluate the microbial community (Dell'Amico *et al.* 2008), it was verified similar diversity in some species such as *Bacillus* sp. and *Sphingomonas* sp., where they were found in both studies. Also, it was verified that the specie *Sphingomonas* sp. showed in the copper mining waste in abundance in all treatments, less in the control R6 (natural mining waste).

In summary, the second condition applied with controlled temperature and addition of the same treatment to collect soil solution was more efficient to copper bioleaching than the other tratments. In the community of bacteria found after treatments analyzed with DGGE assay, it was found basically gram positive bacteria. Firmicutes filum as *Clostridium* sp. and *Bacillus* sp., was found in the vineyard soil. Other important information was the isolate *Paracoccus* sp., where its pathway there is the oxidation of $SO_4^{2-}$, where it is important to copper bioleaching. Microorganisms identified as *Sphingomonas* sp. was widely found in the copper mining waste in study, known of the characteristics as metal resistant, they can be used for bioremediation studies especially in this are.

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