

## *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* Behaviour in a bioleaching process from three Colombian coals

### Evaluación del Comportamiento del Consorcio Bacteriano *Acidithiobacillus ferrooxidans* y *Acidithiobacillus thiooxidans* en un Proceso de BIODESULFURIZACIÓN DE TRES CARBONES COLOMBIANOS

Mosquera, Y. A.<sup>I</sup>; Gómez, J.<sup>I</sup>; Caicedo, G.<sup>a,II</sup> y Márquez M.<sup>b,II</sup>

**Resumen.** Se evaluó el efecto del tamaño de partícula de tres carbones colombianos sobre el comportamiento del consorcio bacteriano *Acidithiobacillus ferrooxidans* y *Acidithiobacillus thiooxidans* en fracción líquida y sólida de un proceso de biodesulfurización. Se analizó la adherencia bacteriana a la superficie azufrada mediante la presencia de células en sólido y líquido a lo largo del proceso a través de conteo en cámara de Neubauer. Se estableció la mineralogía del proceso mediante análisis químicos y a través del uso de técnicas de caracterización como Difracción de rayos X (DRX), Espectroscopía de Infrarojo con Transformada de Fourier (FTIR) y Microscopía Electrónica de Barrido (SEM/EDX). Los resultados muestran un incremento en la oxidación de la pirita para el menor tamaño de partícula (0.074 mm), obteniendo eficiencias de hasta un 94.74% de pirita removida, así como el comportamiento bacteriano durante el proceso fue favorecido con el mismo tamaño de partícula de carbón alcanzando hasta 2.7E+09 células por mililitro.

**Palabras clave:** *Acidithiobacillus*, Carbón, Comportamiento Bacteriano, BIODESULFURIZACIÓN.

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I Biologist, Sciences Faculty, Tolima University, Ibagué – Colombia  
 II School of Mines, Applied Mineralogy Group (GMAB), National University of Colombia, Medellín  
 a Chemical Engineer. Biotechnology MSc, PhD candidate.  
 b Geologist Engineer, Msc, PhD. Director of GMAB

**Abstract.** The particle size effect from three Colombian coals over the behaviour in solid and liquid fractions of a bioleaching process by an *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* consortium was investigated. Through cells presence monitoring in solid and liquid during the process by counting in Neubauer chamber, the bacteria adherence to the sulphur surface was studied. Characterization techniques such as X-ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM/EDX) and chemical analysis were used in order to establish the mineralogy of the process. The results showed an increase in pyrite oxidation for the smaller particle size (0.074 mm), giving efficiencies up to 94.74% of pyrite off, and the same way the bacterial behavior in the process was enhanced with the smaller particle size reaching up to 2.7E+09 cells per milliliter.

**Keywords:** *Acidithiobacillus*, Coal, Bacterial Behaviour, Bioleaching.

## 1. INTRODUCTION

Coal is one of the most important non-renewable energy sources of fossil origin. The sulphur dioxide generated during coal combustion is the major air pollutant in acid rain formation, which has received environmental attention throughout the world. For environment protection it is mandatory to reduce these gases concentrations, also because sulphur present in coals affects their energy yield (Acharya et al., 2005). This way the research on alternatives for pyrite bioleaching has been increased in order to find new atmosphere protection developments in hydrometallurgy.

The role of the bacterial action is to oxidize  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , this suggests that the indirect mechanism plays a major role (Lin et al., 2008). It takes place by the interaction of the mineral with intermediate or final products of the bacterial metabolism (Rodriguez et al., 2001). However it is consider that in the bioleaching process can appear another mechanism that supposed the direct attach to the sulfur minerals by the bacteria through exopolysaccharides (EPS) that results in the dissolution of the mineral as a product of the electrochemical processes that take place in the interface between microorganism cell wall and the sulfide surface (Watling, 2006). But additional to these mechanisms, there is another one named as “cooperating leaching” that involves the simultaneous presence of the two mechanisms above, that can be understood as an optimum supply of chemical energy from a limited sulfur surface favoring the survival of the bacteria (Ballester, 2005).

The genus *Acidithiobacillus* have been designated by Donovan and Wood in 2000. These bacteria are aerobic, obligately acidophilic, growing in an optimum pH less

than 4.0, they are Gram-negative rods with a length from 0.4 to 2.0  $\mu\text{m}$ , they are motile by one or more flagella, using reduce sulfur compounds to support autotrophic growth (Donovan and Wood, 2000).

*A. thiooxidans* and *A. ferroxidans*, obligately chemolithoautotrophic bacteria that use inorganic sulfur compounds as energy sources, are both capable of oxidizing elemental sulphur to sulphuric acid under aerobic conditions, whose sulfur oxidation mechanism has been studied with sulfur grown cells to show the pathway of oxidation:



In the oxidative pathway which occurs via reduced - oxidized glutathione, colloidal polythionates are produced, which are oxidized to sulphate by bacteria (Gargarello et al., 2010). Some of the most important features that influence the biodesulfurization process are pH, redox potential (Eh), temperature, pulp density, particle size, content and distribution of pyrite in coal, microorganisms, coal rank, etc (Cardona and Márquez, 2009)

For the present work, three coals from diverse regions of Colombia with different sulfur content and two particle sizes were biodesulfurized using a consortium of native microorganisms *A. ferroox* and *A. thioox* for a period of 28 days.

The main objective was to evaluate the microbial behavior of the process for the specific coals and conditions to generate basis for industrial processes. The bacteria preference for attaching or not attaching directly to the sulfur surface by monitoring the cells presence in solid and liquid throughout the process by counting in Neubaüer chamber was also studied. In order to identify mineralogical transformations occurring during the process and how they affect the sulfur-removal efficiency, characterization techniques as XRD, FTIR, SEM/EDX and chemical analysis were applied for the solid fraction. Chemical measurements such as pH, Eh, and sulphates and iron concentration in solution, were periodically monitored.

## 2. MATERIALS AND METHODS

### 2.1 Mineral

Three coal samples with different sulfur content were collected from Cauca, Córdoba and Valle del Cauca states of Colombia, named Mina Vieja, Guacamaya and Manto Grande respectively. Proximate analyses were performed according to stan-

standard techniques. In all samples, the content of total, pyritic and sulphate sulfur was determined by the ASTM D4239 and ASTM 2492 methods. The mineralogical composition of the samples was established by XRD, FTIR and SEM/EDX.

## 2.2 Strains and media

*Acidithiobacillus ferrooxidans* ATCC 2327 and *Acidithiobacillus thiooxidans* ATCC 2379 were used throughout the study. The bacterial consortium was cultured at initial pH of 1.38 in T&K modified medium containing (g/L):  $\text{NH}_4\text{Cl}=0.4$ ;  $\text{MgCl}_2=0.4$ ;  $\text{KH}_2\text{PO}_4=0.4$ ;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  33,3% w/v = 2 ml was added at inoculating time. The medium was autoclaved at 120 °C for 20 min. The  $\text{FeSO}_4$  was separately sterilized through a 0.2  $\mu\text{m}$  filter and was added aseptically to the iron free medium.

## 2.3 Coal biodesulfurization

The bioleaching processes were performed in 500 ml Erlenmeyer. The working volume was 200 ml containing 180 ml of modified T&K medium, 20% v/v of the bacterial solution composed by equal proportions of *A. ferroox.* and *A. thioox.* at the starting time. The experiments were performed for the three coals, Mina Vieja, Guacamaya and Manto Grande with two particle sizes: -60 Tyler mesh and -200 Tyler mesh, having 12 total conditions. 20% w/v pulp density was used. The process was carried out in an orbital shaker incubator for 28 days, with a temperature of 33 °C and 180 rpm. For all experiments the initial pH was adjusted to 1.38. The pH and the Oxidation-reduction potential (ORP) of all the flasks was measured with a pH and Eh meter SCHOTT Handylab, with an Eh electrode of Ag/AgCl and a pH electrode with a KCl electrolyte. 5 ml sample were taken from each flask to analyze iron and sulphate tenors in solution by UV visible spectrophotometry with a GENE-SYS 10 spectrophotometer, every three days. Evaporation and sampling losses were compensated by the addition of distilled water.

At the end of the process, the solid fraction were dried at environmental temperature and analyzed by XRD, FTIR and SEM/EDX in order to establish mineralogical transformations in the coals.

## 2.4 Chemical analysis

The sulfur forms were determined by standard methods (ASTMD 2492). For pyritic sulfur, the iron concentration in nitric acid solution was measured by colorimetry.

Iron and sulphate concentrations were measured with a UV–visible spectrophotometer GENESYS 10. Ferrous and total iron were measured with O-phenantroline. Sulphate production was quantified photometrically by precipitation with  $\text{BaCl}_2$  according to the Standard Methods for water analysis.

## 2.5 Bacterial Behaviour

5 ml samples of each treated coals flasks were taken in order to calculate bacterial growth in the process by counting in Neubäuer Chamber (0,100 mm depth and area of  $1/0,0025 \text{ mm}^2$ ) with an optical microscope OLYMPUS every three days.

It was first measured the suspension fraction. After that, in order to separate solid and liquid fraction, samples were centrifuged at 3000 rpm for 10 minutes and small parts of the liquid were taken in order to follow the bacterial behaviour by separate.

Bacterial growth was also monitoring with pH and Eh measurements.

## 2.6 Statistical analysis

The data was processed with SPSS 18 Statistical Package, with a multivariate analysis of variance with repeated samples: two and three factors intra-subjects and one factor between subjects. In order to value the statistical meaning of the obtained discriminating functions there was used Wilks' lambda statistic test to analyze if there were differences between the identified groups of subjects on the combination of the dependent variables like the bacterial growth and the leaching values obtained. For all the treatments was obtained a reliance interval of 95%.

# 3. RESULTS AND DISCUSSION

## 3.1 Kinetics of the process

In order to investigate the effect of the particle size on the microbial kinetic in a bioleaching process of pyrite, two sizes were selected, -200 and -60 Tyler mesh. The microbial population showed a lag phase during the first four days, after of those it had a considering growth in the logarithmic phase that had time over the next 5 days, after this, it had its stationary phase within the next 9 days. Some difference between the treated samples was shown, according to the numeric data obtained from the statistic analysis, where the value of the inter-subject test showed significant differences, it becomes evident that the tyler mesh size does affect the microbial growth, having  $p < 0,05$  values under 5%, which means that with the finest particle

size there is a faster bacterial growth as it is shown in figure 1 for a) Mina Vieja coal, b) Guacamaya coal, c) Manto Grande coal.

### 3.2 Bacteria vs. Particle Size

The cell growth was enhanced by the conditions of -200 tyler mesh in Mina Vieja (MV) coal suspension, where it shows the highest values of individuals in the time as it is graphed in fig.1; whereas the smallest cell growth was for Manto Grande (MG) coal in liquid at -60 tyler mesh followed by Guacamaya (G) coal in the same circumstances. For the three coals there were similar growth behaviors, having better results in number of cells with the suspension treatments than with the liquids ones with 0,074 mm than with 0,250 mm particle size respectably.

Acharya et al. (2001) affirm that the size of the coal particles is an important factor in the microorganism ability to desulphurise coal, it determines the accessibility of the pyrite and exerts a considerable influence on the rate of oxidation of pyrite and thereby, sulphur removal.

However Nemati et al. (2000) observed no bacterial activity in the presence of fine pyrite particles, despite a general trend of increase in oxidation rate with decreasing particle size noted, they concluded that the fine particles caused damage to the bacterial cells resulting in loss of oxidising activity.

The results in this study agree with Acharya et al. (2001) having the biggest growth with the smallest fractions as a factor of response. This behavior is directly correlated with the efficiency of the biodesulfurization process, having the highest removal for Mina Vieja -200 tyler mesh with a removal percentage of 94.74% and the lower leaching for Manto Grande -60 tyler mesh with a removal percentage of 63.97%, see table 1. These results agree with Cardona et al. (2009) who affirm that they achieved the highest pyritic sulfur removal with the finest particle size, because of the higher surface area of pyrite that is exposed and which is related to the bacterial growth due to the substrate disponibility as it is describe above.

**Table 1.** Sulphur forms in MV, G and MG coals after 28 days of biodesulfurization process.

ASSAY	S <sub>sulfate</sub> (%)	S <sub>pyritic</sub> (%)	S <sub>organic</sub> (%)	S <sub>total</sub> (%)	Pyrite oxidation (%)
Original MV	1,59	3,32	1,24	6,16	0
MV (-60)	1,79	0,35	1,22	3,36	89,57
MV (-200)	1,82	0,17	1,24	3,24	94,74
Original G	0,19	1,16	1,03	2,39	0
G (-60)	0,29	0,30	1,07	1,67	74,24
G (-200)	0,32	0,28	1,04	1,65	76,06
Original MG	0,09	1,54	0,27	1,90	0
MG (-60)	0,13	0,19	0,28	0,60	63,97
MG (-200)	0,14	0,14	0,27	0,56	73,25

### 3.3 Bacteria vs. solid or liquid presence

It has been evident the cells attachment on the mineral matrix which is represented in the highest solid values of the microbial growth, established by the difference in the number of cells counted in suspension and in liquid (Figure 1). One of the reasons for this behavior is that the mineral-grown cells of *A. ferrooxidans* have an exposure of hydrophobic regions of proteins that may help in their adhesion to mineral surfaces when grown on those, where adhesion is required for growth. In mineral-grown conditions, since the substrate is insoluble, the adhesion of bacteria is required for its enzymatic machinery to come into close contact for the dissolution of the mineral (Devasia et al., 2010).

In the other hand, *A. thiooxidans* have the same behavior as *A. ferrooxidans*, this has been demonstrated by Liu et al., 2003 through SEM and AFM images of pyrite surfaces after bioleaching by this bacterium but they also affirm that the direct attachment onto the pyrite surface probably accounts for the initial stage of pyrite oxidation, and at some point during bioleaching, when the concentration of EPSs on the pyrite surface is sufficiently high, the indirect mechanism probably becomes principally responsible for the dissolution of  $Zn^{2+}$  and  $Cu^{2+}$ .

This could suggest for the present results that most of the bacteria founded in liquid were *A. thiooxidans* without excluding *A. ferrooxidans* absolutely, because Fowler et al. (1998) have demonstrated a non-necessary direct mechanism for the last one, argued that the indirect mechanism plays a central role in the leaching of zinc sulfide,

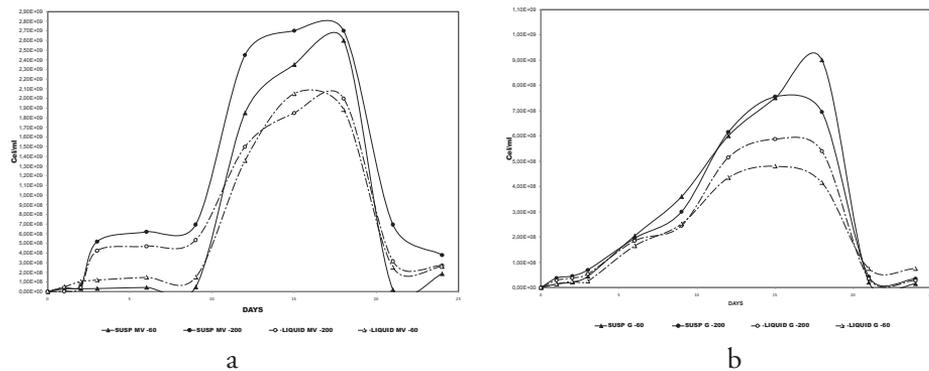
founding no evidence that there is a direct mechanism over a wide range of conditions.

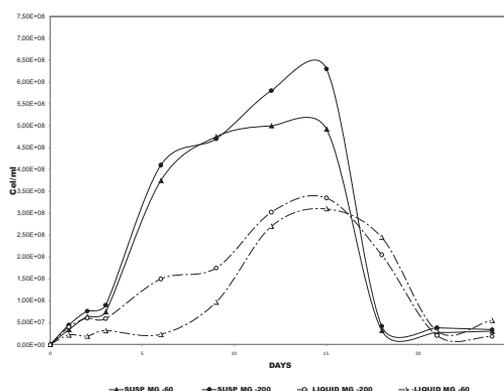
According with authors above, it is possible to have both mechanisms implicated in the bioleaching process, having a direct attachment first and then acting by an indirect way. Since this have been concluded of the individual research of this microorganisms, it becomes necessary to make future research in order to identify by chemical or molecular methods if as a consortium they assume one or another mechanism according to the stage where they act and the type of job they make.

### 3.4 pH and Eh behavior

Figure 2, shows an increase in pH values in the first 24 hours for all the assays in a) for -60 Tyler mesh and b) for -200 Tyler mesh treatments; after this time, all the test showed a gradual pH decrease associated with a bacterial production of sulfuric acid from the sulphur content in the mineral. For the three coals there was a typical behavior, getting pH of 1.7 – 1.9 in the first hours as a result of carbonates dissolution, followed by a gradual decrease and stabilization near 1.3 – 1.4. There were no statistic significative differences between both particle size fractions.

Figure 3, shows an increase in redox potential (Eh) values over the time, which evidence changes in the  $Fe^{3+}/Fe^{2+}$  ratio due to the increase of the concentration of trivalent iron generated in the media. More than 650 mV in treated samples indicate a favorable medium for pyrite leaching showing that the bacterium consortium is active in the oxidation system, regenerating  $Fe^{3+}$  from  $Fe^{2+}$  ions, while its controls maintain Eh values below 300 mV during the process indicating a low oxidizing medium.





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**Figura 1.** Bacterial behavior during the bioleaching process. a) Mina Vieja coal, b) Guacamaya coal, c) Manto Grande coal. For the three graphics: suspension and liquid with -60 and -200 tyler mesh.

### 3.5 X-Ray Diffraction (XRD)

Figure 4, shows for the three coals a considerable decrease on the pyrite associated peaks intensity as well as quartz and kaolinite peaks after 28 days of treatment with the bacterium consortium. This data agree with Pandey et al. (2005) about removal or partial reduction of mineral content in coals.

For Mina Vieja coal it can be seen Gypsum which is formed as a result of the carbonates dissolution, especially calcite ( $\text{CaCO}_3$ ), and a posterior calcium precipitation in sulphate form.

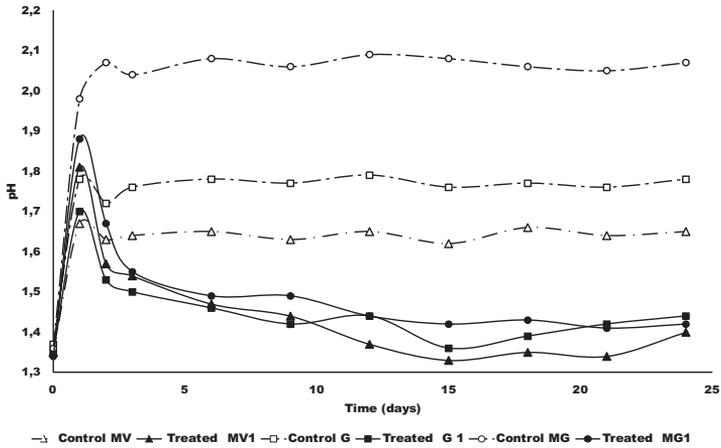
For all the coals it can be seen also montmorillonita, another kind of carbonate, iron sulphates as jarosite, associated with the microbial oxidation in acid medium of pyrite an iron sulfide, as well as melanterite, that probably explain the high content of sulphate found in the chemical analysis for this coal, (Caicedo, 2008).

### 3.6 FTIR measurements

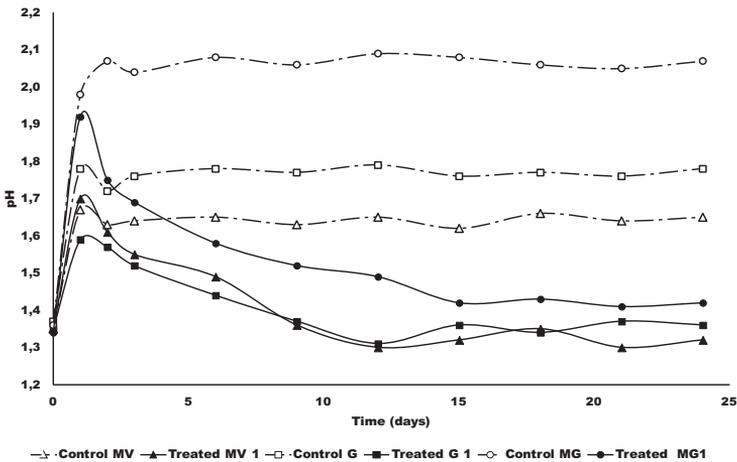
FTIR spectrums for MV, G and MG raw coals and biodesulfurized samples are shown in Fig.4. a) -60 mesh G coal. b) -60 mesh MV coal. c) -60 mesh MG coal.

The general banding pattern of all FTIR spectrums is very similar, having small

changes in the intensities of bands related to minerals but almost none variations in those related to organic compounds of coal. This is because of the ability of the microorganisms employed to interact only with the inorganic phase of coal reported by some authors before (Acharya et al., 2001; Valdés et al., 2008).

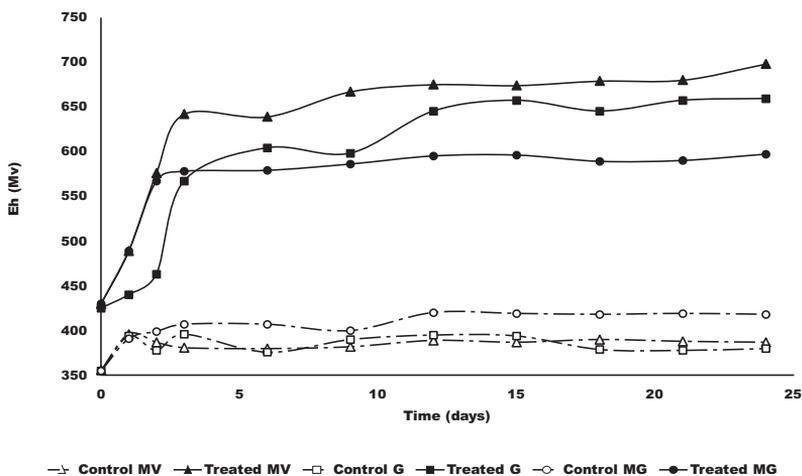


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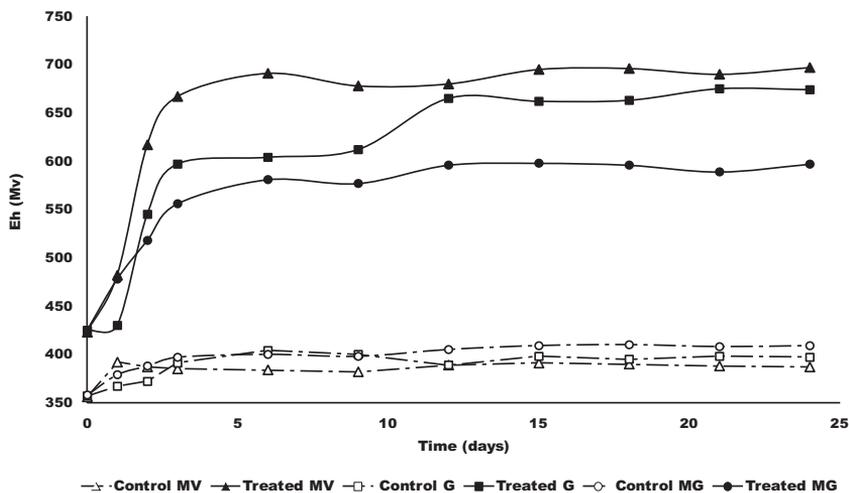


b

**Figura 2.** pH evolution graphs for the biodesulfurization process. a) MV, G and MG coals in -60 Tyler Mesh treated and with their respectably controls. b) MV, G and MG coals in -200 Tyler Mesh, treated and with their controls.

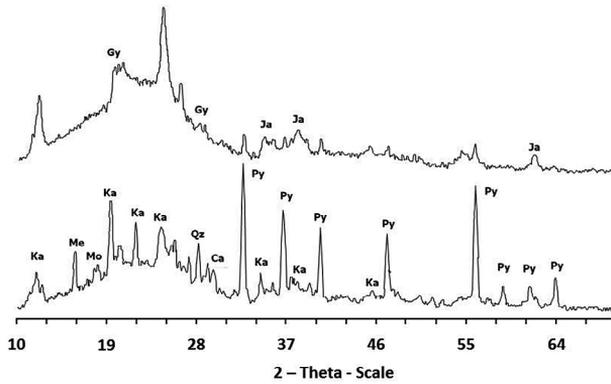


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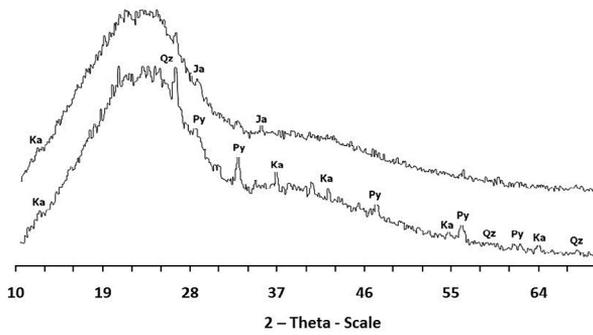


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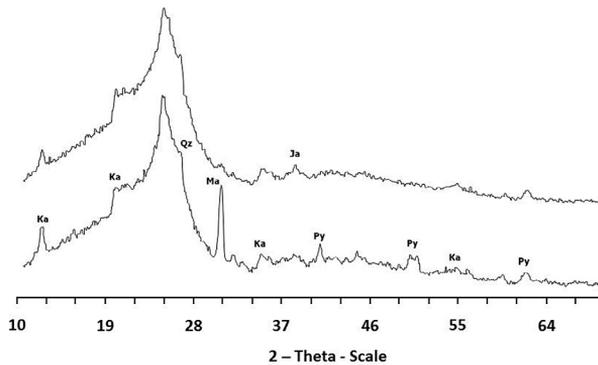
**Figura 3.** Eh evolution graphs for the biodesulfurization process. a) MV, G and MG coals in -60 Tyler Mesh treated and with their respectively controls. b) MV, G and MG coals in -200 Tyler Mesh, treated and with their controls.



a



b

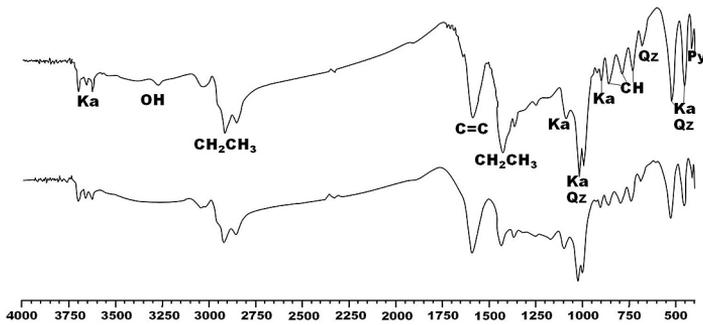


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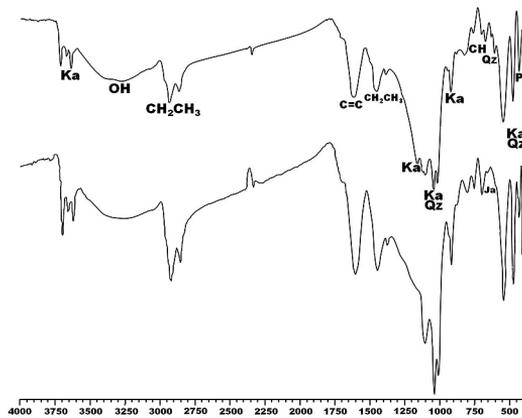
**Figure 4.** XRD spectrums for a) MV, b) G and c) MG raw coal and biooxidated sample with a particle size of -60 mesh. Ka: kaolinite, Py: pyrite, Qz: quartz, Ja: Jarosite, Gy: Gypsum, Ca: Calcite, Me: Melanterite, Mo: Montmorillonite, Ma: Maghemite.

The three coals presented peaks at  $1699\text{ cm}^{-1}$  which indicates the presence of carbonyl ( $\text{C}=\text{O}$ ) content (Saikia et al., 2007). They also showed bands at  $630\text{ cm}^{-1}$  with an small increase related to jarosite, eventhough it is a way to see sulphur removal, it is important not having a great increase of this mineral because the efficiency of the process would be affected due to the return of the sulphur removed to the solid fraction of the coal and this kind of compounds can act as a physic barrier that prevents the later oxidation of the mineral (Cardona, 2008).

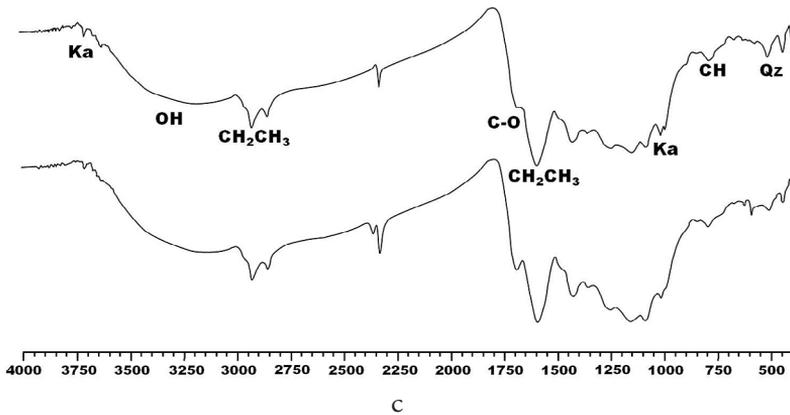
The Py band present near to  $420\text{ cm}^{-1}$  doesn't have big changes but its leaching became evident with DRX technique. Peaks from  $1200\text{ cm}^{-1}$  to  $400\text{ cm}^{-1}$ , are related with clay minerals, specially quartz and kaolinite. Peaks at  $2850\text{ cm}^{-1}$  are attributed to alkyl with  $\text{CH}_2$  groups and  $2925\text{ cm}^{-1}$  to alkyl with  $\text{CH}_3\text{-CH}_2$  groups. Can be observed bands at  $1400\text{ cm}^{-1}$  and in the region  $3100\text{ -}3500\text{ cm}^{-1}$ , that are assigned to N-H stretching vibrations of ammonium ion formed by amine (Ungureanu et al., 2006).



a



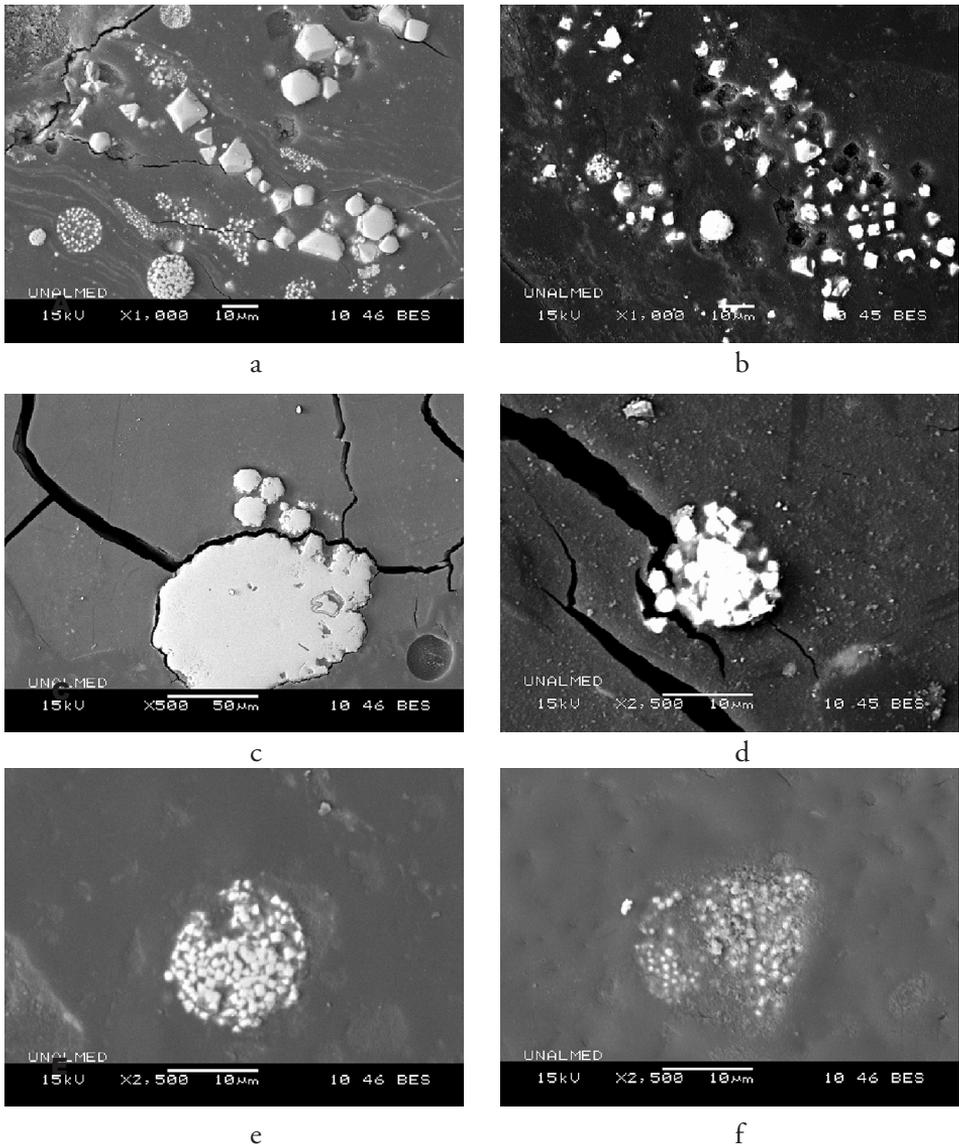
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**Figura 5.** FTIR spectrums for raw coal and biooxidated samples. a) particle size -60 mesh G coal. b) particle size -60 mesh MV coal. c) -60 mesh MG coal. Ka: kaolinite, Qz: quartz, Do: dolomite, Py: pyrite, Lo: loncreekite, S<sup>o</sup>: elemental sulfur, Ja: jarosita.

### 3.7 SEM images

Scanning electron micrographs of biooxidated coal samples (Fig. 6, (A) MV raw coal. (B) MV treated coal. (C) G raw coal. (D) G treated coal. (E) MG raw coal. (E) MG treated coal) show typical corrosion features such as gulfs or holes in the surface of pyrite crystals. For all the coals, pyrites crystals lose their typical shape and get irregular forms after the bioleaching process. In Mina Vieja treated coal, can be seen irregular edges in pyrite crystals the same as in Guacamaya treated coal; additionally for this last one there are sulphates product of the oxidation rounded the pyrite surface, Cardona et al. (2009) affirm that these coatings impede the complete oxidation of pyrite because they constitute a physical barrier for the interaction of the mineral with microorganisms and/or leaching medium. The framboidal pyrite of Manto Grande coal was partially attacked remaining irregular shapes of pyrite coated of sulphates the same as in Guacamaya coal, because of the bacterial oxidizing at the end of the process.



**Figure 6.** SEM micrographs of raw and biooxidized coal samples. (A) MV raw coal individuals and framboids crystals of pyrite. (B) MV coal after 28 days of treatment, partial oxidation and corrosion of framboids pyrite. (C) G raw coal, pyrite crystals. (D) G coal treated, it is shown the fragmented pyrite corroded and a sulphate layer coating the crystals. (E) MG raw coal framboids pyrite. (F) MG coal after the microbial treatment, framboids pyrite partially attacked by the bacterium.

#### 4. CONCLUSION

The coal particle size determined the access grade of the microorganisms and/or leaching medium to interact with the mineral, even though some authors affirm that small particle sizes affects the bacterial growth, in this biodesulphurization process the bacterial consortium growth was enhance with the smallest tyler mesh used, probably because this one is significantly larger than the size of bacterial cells used in the current study, and therefore, the mechanical shear effects on the bacterial cells would probably be minimal.

For the analyzed conditions, best results were reached with the finest particle size in all the three coals, having results up to 94.74% of sulphur leaching. This shows that the effect of the coal particle size on the microbial behavior in a bioleaching process is related to a higher growing cell in smaller coal particles due to a better use of the iron and sulphur source in the carbonaceous matrix.

It was also demonstrated that not only the size but the solid and liquid phase of the bioleaching process also plays an important role since it was found greater cell number in the carbonaceous matrix that in the leaching media by the methodology used which indicates a cooperating leaching preference in these species.

FTIR, XDR and SEM techniques supported the results of the biodesulphurization in order to establish a comparison of the microbial behavior with the efficiency of the process. It is important to continue investigating the cell adhesion to the carbonaceous matrix with molecular methods that help to clarify if one or another microorganism is in charge of the adhesion or if that conduct is assume for both and the stage in what they are involved.

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