4
Materials and methods

4.1.
Mineral sample preparation

Pure hematite was used in this study. The sample was provided by a local supplier, *Estrada* Mining, Belo Horizonte, Minas Gerais State. Originally, the sample was delivered in a medium particle size of 3mm. After dry grinding in a porcelain mortar and wet screening in Tyler mesh sieves, were obtained the desired size fractions (Table 9).

Table 9 - Particle size range for each experiment

Test	Particle size (µm)	Tyler mesh
Microflotation	-90+75	170/200
	-75+53	200/270
Adsorption/ Microflotation	-53+38	270/400
Zeta potential	-38+20	400/635

The ground hematite was washed with HCl 0,25M, and later with double-distilled water until the pH of the mineral suspension was the same as initially. Finally, all samples were stored in a desiccator.

In order to reaffirm the composition of hematite sample, it was carried out a quantitative analysis by X-ray diffractometry on Bruker AXS equipment (See Appendix) and Energy dispersive X-ray spectroscopy (EDS) in a Quantax 70 TM-3000 by Hitachi.

4.2.
Bioreagent obtainment – Bacterial cultivation

Rhodococcus ruber (*R.ruber*) was used as the bacterial strain from which was obtained the flotation reagent. It was supplied by the Culture collection of Fundação Tropical de Pesquisa e Tecnologia André Tosello - Campinas, São Paulo.

The bacteria was sub-cultivated in the laboratory using the culture medium TSB (*Tryptic Soy Broth)* from Himedia® (composed from 15 $g.L^{-1}$de tryptone, 5 $g.L^{-1}$digesting enzyme of soybean meal e 5 $g.L_{-1}$ of sodium chloride). According to Borges (2011), this culture medium presents the best conditions for the growth of *Rhodococcus ruber* cells.

To reproduce the bacteria colonies on solid medium, it was taken 3 $g.L^{-1}$ TSB and 2 $g.L^{-1}$ Agar-Agar for 100mL. Stocks of the bacteria were prepared and renewed periodically using this medium in Petri plates and saving them for 48 hours at 20°C in a bacteriological stove.

For the culture in liquid medium, it was dissolved 6 $g.L^{-1}$ of TSB in 200mL of double-distilled water, in a 500mL Erlenmeyer flask. Twelve of these flasks were used to contain the liquid medium (kept as constant pattern of bacterial growth), and later were sterilized in an autoclaving device before *Rhodococcus ruber* inoculation. After the bacteria cultivation in the liquid medium (pH 7.2), the flasks were disposed on a rotary shaker, maintained at 200rpm and 28°C, for 24 hours according to the Growth curve of *Rhodococcus ruber* (See 4.2), time after which the bacterial suspension was centrifuged, twice washed in deionized water and finally concentrate in 100mL of 10^{-3}M NaCl solution. To avoid further bacterial development, the concentrate was inactivated in the autoclave.

From now on, the cellular suspension will be called as Bioreagent, and its concentration was quantified first by Dry weight on an oven at 60°C for 24 h, and by optical density in UV-1800 Shimadatzu Spectrophotometer, at a wavelength of 620nm.

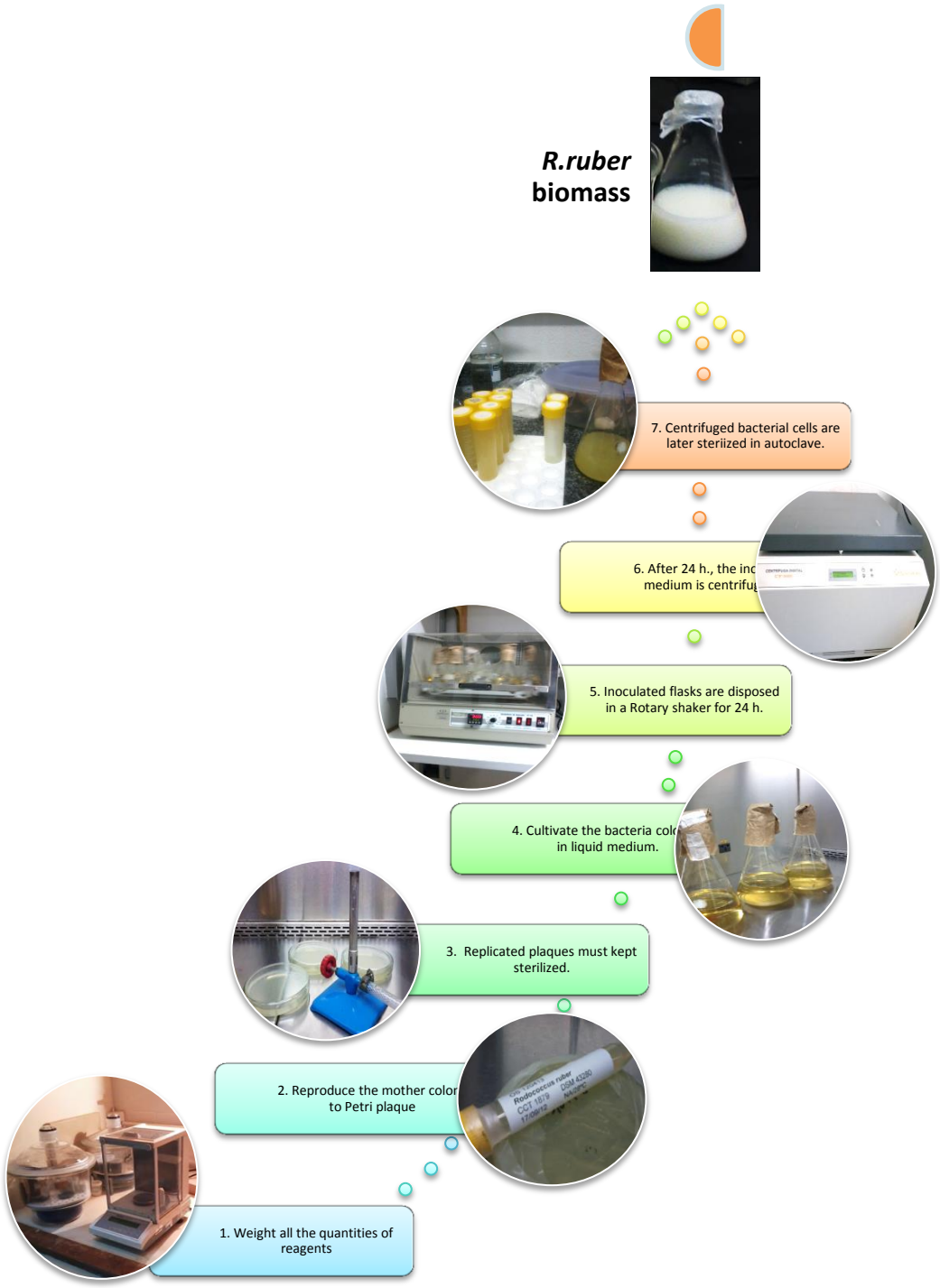

Figure 15 - Sequential diagram of *R.ruber* growth and bioreagent obtainment

Figure 15 shows a sequential diagram of *Rhodococcus ruber* growth by stages, obtaining finally the bioreagent used for the fundamental studies of hematite bioflotation.

4.2.1.*Rhodococcus ruber* growth curve

Rhodococcus ruber growth curve was established in order to determine each of the stages of bacterial growth as well as the time needed to reach the stationary phase.

The procedure was as follows: 100uL of *Rhodococcus ruber* bacteria concentrate was placed in 200 ml of liquid culture medium, inoculated in 10 Erlenmeyer flasks, which served as new growth medium of the bacteria. Each of the flasks is a determined time (1,2,4,6,8,10,12,18,24,48hrs) . After growth of bacteria within the allotted time, they were centrifuged for 8 min at a speed of 4000 rpm and after series of washes needed, was suspended in deionized water and brought to a volume of 250 mL. This was done for each of the points in the study. The concentration of the bacterial suspension was made as explain in Figure 22. The growth curve was established as cell number versus time of fermentation.

4.2.2.*Rhodococcus ruber* cell counting

For *Rhodococcus ruber* cell counting, it was taken samples of 0.1mL from 8 dilutions of biomass suspension concentrate, and measured at Neubauer chamber coupled with Optic microscope. Each of the dilutions was used also for absorbance measurements with the aim of correlate Absorbance (at 620 nm) and the number of cells. The dilution ratios are explained in Table 10.

Table 10 - Dilution ratios for *Rhodococcus ruber* cell counting

Ratio of dilutions	Volume biomass suspension (mL)
1	100
1/10	10
1/100	1
1/1000	0,1
1/10000	0,01
1/100000	0,001

4.3. Adsorption measurements

For cell adhesion experiments, 0,25 g of mineral sample was added to 25 mL of cellular suspension with a fixed initial concentration of 0,6 $g.L^{-1}$, and conditioned for 10 min after adjusting the pH (2,3,5,7,9), biomass concentration (0,15; 0,30; 0,45; 0,60 and 0,75 $g.L^{-1}$) and contact time (0,5,10,20,30 min), at 25ºC with 10-3M NaCl as a supporting electrolyte. It was observed that the adhesion between the bacteria and both minerals was complete after 5 min. An additional time of 1 min was allowed for settling of the mineral particles, after which 5 ml of the supernatant was collected for absorbance measurements to estimate the amount of adsorbed cells.

After conditioning, absorbance measurements (UV Shimadatzu Spectrophotometer) were carried out with the supernatant in order to estimate the amount of adsorbed cells.

4.4. FTIR measurements

The size fraction used for adsorption measurements was also used for FT-IR studies. The spectra of *Rhodococcus ruber* biomass, of the hematite fines and of the system Hematite-*Rhodococcus ruber* were recorded. The biomass and the mineral were collected, washed, and dried separately at 40°C in an oven for 24 hours, at pH 7. Hematite was conditioned with *Rhodococcus ruber* cell suspension for 5 minutes using intensive stirring at constant pH around 3. Later were washed with deionised water to remove the loosely holding cells and were air dried. To remove the excess water but to protect the organic material, drying was done at room temperature and the samples were stored in a desiccator.

For recording the spectra, the diffuse reflectance IR method was used, with KBr matrix. For the biomass, 5% w/w (% weight) concentrations was used, for hematite 2%w/w and for the hematite-biomass system 2%w/w were found to be accurate for FT-IR measurements. Measurements were done with 32 scans using a Nicolet FTIR Spectrometer.

4.5.
Zeta potential measurements

Zeta potential measurements were made using the Zeta Meter 4.0+ apparatus (Zeta-Meter, Inc., Staunton-USA), using the electrophoresis technique. A size fraction of -38+20µm was used for these measurements.

The evaluation of the zeta potential profiles for the minerals was carried out before and after interaction with the *Rhodococcus ruber* biomass suspension, and the pH was adjusted using 0,25M HCl and 0,25M NaOH.

The zeta potential measures of mineral and bacterial suspension were carried out using as aqueous medium the indifferent electrolyte 10^{-3} M NaCl, both at a concentration of 0,2 $g.L^{-1}$for hematite and 100 $g.L^{-1}$ for the bioreagent. For the case of the system Hematite-*R.ruber*, it was used 0,25 $g.L^{-1}$ of hematite in a biomass suspension of 0,15 $g.L^{-1.}$

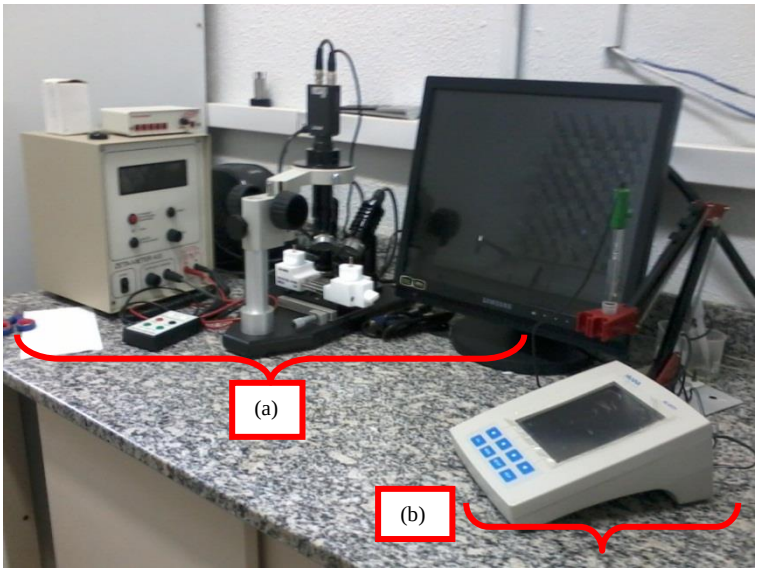

Figure 16 – Zeta meter4.0+ equipment (a) and pH meter (b)

4.6.
Flotation experiments

Microflotation experiments where performed in a modified Partridge-Smith flotation cell. For each of the evaluations were varied the pH of the solution, the biomass concentration, and the particle size range.

An amount of 0,5 g of hematite was added to 0,25 L total volume suspension of known bacterial concentration, with pH adjusted with 0,25M HCl and 0,25M NaOH solutions. The mineral was conditioned with the biomass suspension inside the Partridge-Smith cell under constant stirring with a magnetic agitator for 5 minutes in aqueous medium using the indifferent electrolyte 10^{-3} M NaCl, and then the mineral flotation tests were carried out using air at a flow rate (supplied by a Vacuum pump) of 15mL.min^{-1} (See Appendix for Flowmeter calibration) for 5 minutes. The floatability was calculated as the ratio of the weights of floated related to the total weighted sample.

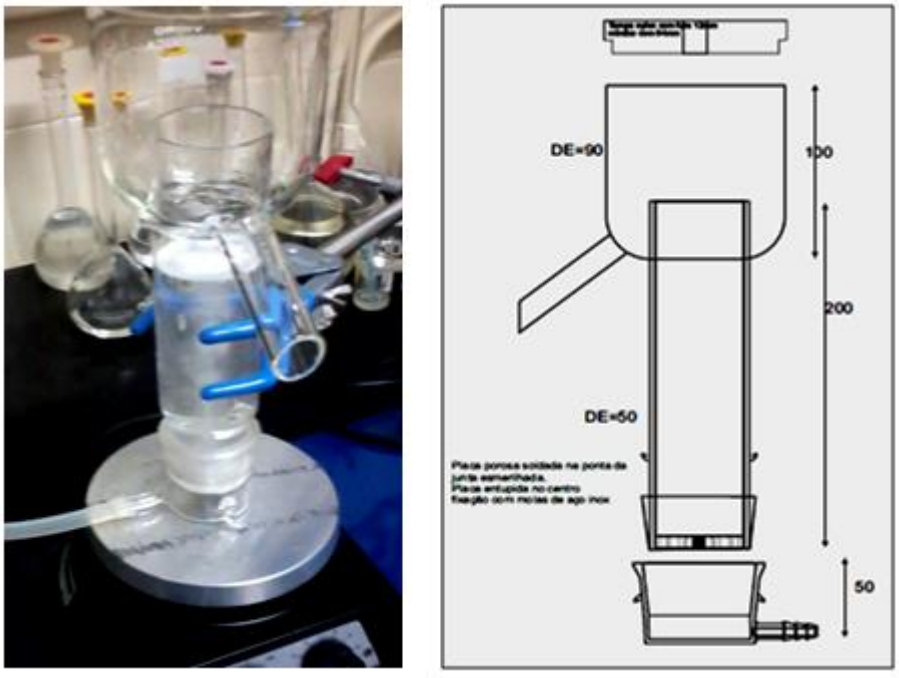

Figure 17 - Modified Partridge-Smith flotation cell

4.7.
Scanning electronic microscope (SEM) micrographic analysis

In this stage, samples of *Rhodococcus ruber* biomass, hematite and *Rhodococcus ruber* adhered to hematite were selected for SEM micrographic analysis.

A sample of biomass *Rhodococcus ruber* was centrifuged at 3000 rpm for 5 minutes and then chemically fixed for 24 hours at 40°C using glutaraldehyde. After that, the sample was rinsed in distilled water to remove traces of glutaraldehyde; and finally the samples were dehydrated in graded series of acetone (from 30%w/v [2]to 100%w/v), and dried under vacuum. A sample of hematite after interaction with the biomass suspension was centrifuged at 4000 rpm for 5 min. After that, water remains were removed and later were dehydrated in graded series of ethanol or acetone, and air dried under vacuum. Dry samples were loaded for SEM studies in a Quantax 70 TM-3000 by Hitachi.

[2]%w/v stands for %weight on determined volume