

A rapid and simple method for detecting active acidophilic microorganisms in copper bioleaching processes

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ABSTRACT

The whole bioleaching process depends principally on the presence and viability of microorganisms. For this reason, it is imperative to have methodologies that allow rapid, reliable and effective monitoring of the biological activity in commercial-scale, bio-hydrometallurgical processes. The objective of this work was to develop a rapid method for detecting the presence of acidophilic microorganisms in aqueous samples such as bioleaching solutions or acid mine drainages. The method is comprised of the following steps: a) concentrating acidophilic microorganisms from a given volume of aqueous sample; b) removing the agents that were inhibitory for the bioluminescence reaction by washing the previously concentrated acidophilic microorganisms in two treatments with aqueous washing agents; and c) extracting adenosine-triphosphate (ATP) from the acidophilic microorganisms and measuring the light generated by such ATP by means of a bioluminescence detection system. There was a high correlation between the method for detecting the presence of *Acidithiobacillus ferrooxidans* by bioluminescence and the total bacterial count in a Petroff-Hausser counting chamber ($R^2 = 0.9980$) or the viable bacterial count determined by using the floating filter method ($R^2 = 0.9862$). The simplicity of the method allows it to be used without problems by an operator at the mining site. The bioluminescence method shows its ability to discriminate between metabolically active and inactive microorganisms, which represents an advantage over such methods as microscopic count or PCR that measure only total microbial cells. Regarding the methods that determine viability by culture, the bioluminescence method is advantageous because it reduces the analysis time from 14 days to only a few minutes. This last result is very important because detecting metabolically active microorganisms is a relevant parameter for the operation and control of industrial-scale bioleaching processes.

INTRODUCTION

Due to the fact that the entire bioleaching process depends strictly on the presence and viability of microorganisms, it is capital to have methodologies that allow rapid, reliable and effective monitoring of the biological activity in commercial-scale, biohydrometallurgical processes. Therefore, during monitoring of sulphide leaching heaps, the existing biological activity must be known at different sampling points in the leaching heap to ensure efficient metal recovery and to take corrective measures whenever necessary (as for instance when inoculating leaching microorganisms, supplying limiting nutrients for biological activity, avoiding the presence of toxic agents, etc.). However, currently there are no modern methodologies that can effectively replace classic techniques for determining total viable bacteria, which have a very long analysis time (between seven and fourteen days) and therefore do not allow carrying out the required corrective actions in time.

Microorganism detection in bioleaching heaps is currently carried out by using different classic methods for determining total and viable leaching bacteria. They are microscopic counts of microorganisms, culture-dependent viable count of microorganisms and indirect methods.

The total microbial count can be determined by direct count using a phase contrast microscope and a Petroff-Hausser chamber. Alternately, total microbial count can be performed by microfiltration membrane treatment with selective fluorochromes bacteria (*i.e.* acridine orange or DAPI) and fluorescence microscope observation.

The culture-dependent viable count of microorganisms is based on determining microbial growth in different adequate culture media. The most widely known counting technique in a solid medium currently used was created by Harrison [1]. This method uses two agarose layers with different concentration. Johnson [2] subsequently developed an efficient counting procedure, called 'overlay' technique, in which an acidophilic heterotrophic bacterium is incorporated into the underlayer of a two-layer gel. This method has been used successfully to culture all known iron-oxidising [2]. The Most Probable Number (MPN) statistical technique [3, 4] has also been used. This technique is advantageous because it uses a liquid culture medium. However, these media only allow partial recovery of the present microorganisms and require an incubation time of more than two weeks and this fact prevents their use to improve an ongoing bioleaching process. The fastest counting method based on microorganism culture is the floating filter technique, which decreases the incubation time to only five days [5]. This methodology comprises filtrating microorganisms by using a polycarbonate membrane, which is placed in a liquid culture medium and kept in suspension by floating. The recovery of viable ferrooxidising bacteria using this technique is approximately 57%.

Other alternatives have been developed with the object of reducing microbial viability analysis time, such as the determination of Fe(II) oxidising activity [6] and the measurement of oxygen consumption rate [7]. However, these methods have the disadvantage of being indirect measurements and their execution requires large amounts of work and depends on the analyst's skill.

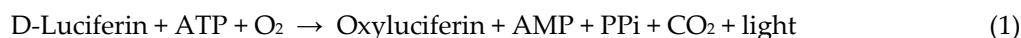
In the last years, the use of molecular biology techniques has become a very powerful tool to identify leaching microorganisms without microorganism culturing. Accordingly, many different techniques have been developed at a research level to identify acidophilic microorganisms present in heap leaching effluents and in acid mine drainages [8-15]. The "fluorescent in situ hybridisation" (FISH) and (CARD-FISH) use specific primers to distinguish and identify bacteria and archaea

directly from other cells present in a mixture. The effective implementation of the FISH technique to bioleaching microorganisms has been reported in recent publications in this area of research [12]. Real-time quantitative PCR offers a rapid and simple method to quantify microbial populations in community and it has been successfully applied to quantify bacteria and archaea in commercial-scale copper bioleaching plant [14-15].

Despite the fact that these techniques give valuable qualitative and quantitative information about the diversity of microbial communities that take part in leaching processes or are responsible for acid mine drainage formation, such techniques are not able to give an account of the activity or viability of such microorganisms.

An interesting potential alternative is the detection of intracellular microbial ATP by means of an enzyme reaction observed from nature, namely, the reaction that causes fireflies to glow. With the assistance of the substrate enzyme system (luciferin-luciferase) of the firefly, ATP can be determined from microorganisms. Luciferase enzyme, in the presence of its co-factor D-Luciferin, generates light in the presence of ATP according to Reaction 1.

Luciferase



The amount of light is proportional to the concentration of ATP in the original sample. Due to the fact that all metabolic active microorganisms show a practically constant concentration of ATP, the reaction has been proven to be proportional to the amount of microorganisms present in solution. This bioluminescence-based methodology has been used for determination of bacteria in wastewater effluents [16], in industrial waters, raw materials, intermediaries and products used in food, pharmaceutical, cosmetics, electronics and other industries [17, 18].

Despite the relevant development of the bioluminescence-based methodology, none of these publications describe a technique that could be applied to detect the presence of active acidophilic microorganisms that are important in bioleaching processes or responsible for the production of acid mine drainage. Measurement of active acidophilic microorganisms present in these samples has always had significant difficulties. The inconveniences that are present in the analysis of the growth of these microorganisms in laboratory culture media, the environment or bioleaching industrial processes, are the result of the diversity of inorganic compounds that are present in these environments (toxic metals, high salt concentration, etc.), the extreme conditions in which these microorganisms proliferate (particularly dominated by very low pH values) and the low cell density attained by these microorganisms in their growth. This has made their detection by means of bioluminescence techniques very difficult.

From the former exposition, it is evident the necessity of a method that allows a rapid, reliable and effective detection of the presence of active acidophilic microorganisms. The objective of this work was to develop a new bioluminescence-based methodology for detection of the presence of bioleaching microorganisms.

METHODOLOGY

Microorganisms and culture conditions

The microorganisms used in this study were *Acidithiobacillus ferrooxidans* strain ATCC 23270, *Acidithiobacillus thiooxidans* strain DSMZ 14887 and *Leptospirillum ferrooxidans* strain DSMZ 2705. *Acidithiobacillus ferrooxidans* strain was grown in ferrous iron-containing modified 9K medium as described previously [19]; *Acidithiobacillus thiooxidans* strain and *Leptospirillum ferrooxidans* strain was cultured in culture medium 71 and 882 in accordance with German Collection of Microorganisms and Cell Cultures (DSMZ) recommendations. 250 cm³ Erlenmeyer flasks containing 100 cm³ of the culture medium were used. Each flask was inoculated with 0.5 cm³ of a culture. Subsequently, the flasks were incubated in an orbital shaker at 30 °C and 120 rpm.

Total bacterial count

Total (viable and dead) bacterial count was determined by microscopy. To that end, a culture sample was placed in a Petroff-Hausser counting chamber and at least 50 independent squares were counted. Counting was performed in a direct sample or in a dilution thereof, depending on the amount observed in a first sample. Average is calculated and the result is multiplied by a conversion factor to obtain the total bacterial count as bacteria/cm³. Microscopic observations were made by using a Carl Zeiss microscope (Germany) equipped with phase contrast and using a 400× magnification.

Viable bacterial count

Total viable bacterial count was determined by the floating filter method described by de Bruyn *et al.* (1990) [16].

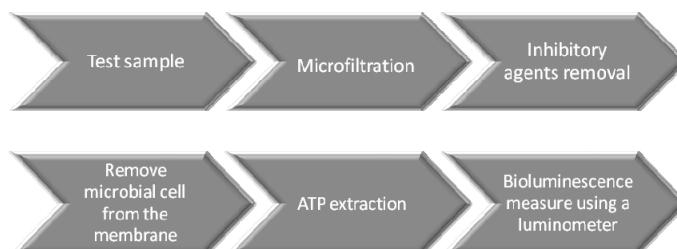


Figure 1 Method overview of the quantification of active bioleaching microorganisms using the LixKit®

Bioluminescence-based bacterial count

The quantification of active bioleaching microorganisms was performed using the LixKit® (Biohidrica, Chile [19]), according to the manufacturer's instructions. Briefly, 10 cm³ of the microorganism culture to be assayed were passed through a filter holder with a 0.22 µm membrane by means of a syringe. In a second stage of this assay, removal of the agents that are inhibitory for the bioluminescence reaction was carried out by sequentially washing the previously concentrated acidophilic microorganisms. To that end, 20 cm³ of solution 1 were passed through the filter. The filtrate was discarded. Then the membrane was rinsed with 20 cm³ of solution 2 and the filtrate was discarded again. Finally, air is allowed to pass through the membrane to remove any water remnant (by using moderate pressure over a syringe plunger). A swab was wetted by immersion in solution 2, and is rubbed against the

surface of the membrane, keeping a constant pressure. In a third stage of the assay, extraction of intracellular adenosine-triphosphate (ATP) from the acidophilic microorganisms and the bioluminescence reaction was performed. Finally, emitted light was immediately measured in a luminometer. The values obtained are expressed in relative light units (RLU). The assay duration can vary from 10 to 30 minutes, depending on the user. Figure 1 shows a schematic overview of the quantification of active bioleaching microorganisms using the LixKit®

RESULTS AND DISCUSSION

Application of LixKit® to detection active microorganisms in cultures of bioleaching microorganisms

Bioluminescence method applied to Acidithiobacillus ferrooxidans at different culture times

Table 1 shows a comparison of the results obtained with the method of microscopic total bacteria counting in a Petroff-Hausser chamber and the bioluminescence method for the detection of *Acidithiobacillus ferrooxidans*. At different culture times, the microscopic total bacterial counts remain substantially constant. However, the same culture analysed by using the bioluminescence method shows high light emission values (RLU) between days three and twelve, when the cultures are more metabolically active and have high intracellular ATP concentrations. In changes starting from day twenty two on, the light emission values (RLU) start to descend markedly, due to culture aging.

Table 1 Comparison of method of microscopic total bacterial counting in a Petroff-Hausser chamber and the bioluminescence method for the detection of *Acidithiobacillus ferrooxidans* at different culture times.

Culture time (days)	Total count method (bacteria/cm ³)	Bioluminescence method (RLU) ¹
3	3.4 x 10 ⁷	21,817
5	1.8 x 10 ⁸	21,000
12	1.2 x 10 ⁸	26,517
22	3.2 x 10 ⁸	2,703
48	2.9 x 10 ⁸	963
70	6.7 x 10 ⁷	849

¹ Triplicate average measurement

This result shows that thanks to the acidophilic microorganism concentration and inhibiting agent removal stages according to this method, it is possible to attain high light emission values from ATP from the acidophilic bacterium *Acidithiobacillus ferrooxidans* despite the existence of different agents or chemical compounds in the culture medium that are inhibitory for the bioluminescence reaction, such as an extremely low pH and high metal concentration such as high iron concentration. The total bacterial count remains almost constant between five and forty eight days, despite the culture age. This method cannot distinguish active and inactive bacteria. In contrast, the bacterial ATP content measured by bioluminescence decreases rapidly after twelve days. Therefore, it is evident the ability of the bioluminescence method to discriminate between metabolically active and inactive bacteria, which represents an advantage over the methods that measure total bacterial count. Regarding the methods that determine viability by culture, the present technique is advantageous because it reduces the analysis time from seven to fourteen days to only a few minutes.

Bioluminescence method applied to *Acidithiobacillus thiooxidans* at different culture times

Table 2 shows a comparison of the results obtained with the method of microscopic total bacteria counting in a Petroff-Hausser chamber and the bioluminescence method for the detection of *Acidithiobacillus thiooxidans*. This result shows that the bioluminescence method is able to discriminate between metabolically active and inactive bacteria, and it is possible to further confirm the advantages of the bioluminescence method when compared to the traditional viable acidophilic microorganism counting techniques, due to the easiness of the analytic technique.

Table 2 Comparison of method of microscopic total bacteria counting in a Petroff-Hausser chamber and the bioluminescence method for the detection of *Acidithiobacillus thiooxidans* at different culture times

Culture time (days)	Total count method (bacteria/cm ³)	Bioluminescence method (RLU) ¹
5	3.3 × 10 ⁸	114,833
12	1.9 × 10 ⁸	53,377
20	2.7 × 10 ⁸	36,637
27	2.0 × 10 ⁸	470

¹ Triplicate average measurement

Bioluminescence method applied to *Leptospirillum ferrooxidans* at different culture times

Table 3 shows a comparison of the results obtained with the method of microscopic total bacterial counting in a Petroff-Hausser chamber and the bioluminescence method for the detection of *Leptospirillum ferrooxidans*. In this result, it is possible to assess that the bioluminescence method is able to discriminate between metabolically active and inactive bacteria, and it is possible to further confirm the advantages of the bioluminescence method when compared to the traditional viable acidophilic microorganism counting techniques, due to the easiness of the analytic technique.

Table 3 Comparison of method of microscopic total bacterial counting in a Petroff-Hausser chamber and the bioluminescence method for the detection of *Leptospirillum ferrooxidans* at different culture times

Culture time (days)	Total count method (bacteria/cm ³)	Bioluminescence method (RLU) ¹
7	5.9×10^8	17,324
13	1.9×10^8	1,163

¹ Triplicate average measurement

Correlation between LixKit® and other methods currently used to determine the count of bioleaching bacteria

The correlation between LixKit® and other methods currently used to determine the count of bioleaching bacteria was studied. To that end, *Acidithiobacillus ferrooxidans* was cultured in Erlenmeyer flasks for four days. Subsequently, determinations of different samples by the bioluminescence method, microscopic total bacterial count determination in a Petroff-Hausser counting chamber and total viable bacterial count by the floating filter method were made in parallel by using different dilutions of an *Acidithiobacillus ferrooxidans* culture.

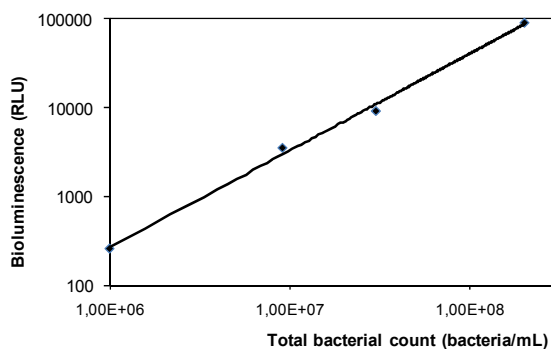


Figure 2 Plot of bioluminescence results (RLU) determined by means of the method for detection of the presence of acidophilic microorganisms by bioluminescence, compared to total bacterial count determined in a Petroff-Hausser counting chamber

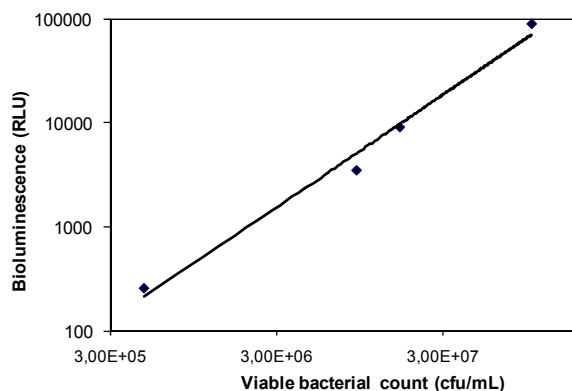


Figure 3 Plot of bioluminescence results (RLU) determined by means of the method for detection of the presence of acidophilic microorganisms by bioluminescence, compared to viable bacterial count (cfu/cm³) determined by the floating filter method

Figure 2 shows a high correlation between the method for detection of the presence of acidophilic microorganisms by bioluminescence and the total bacterial count in a Petroff-Hausser counting chamber ($R^2 = 0.9980$). It is important to note that this is possible because a young, very active culture (four days old) was used.

Figure 3 shows a high correlation between the method for detection of the presence of acidophilic microorganisms by bioluminescence and the viable bacterial count determined by using the floating filter method ($R^2 = 0.9862$). This last result is important because detection of metabolically active microorganisms is a relevant parameter for the operation and control of industrial scale bioleaching processes.

CONCLUSIONS

The LixKit[®] is an innovative technology and a powerful tool for monitoring the activity of microbial populations in a bioleaching heap at the mining plant.

The bioluminescence method demonstrates the ability to measure metabolically active microorganisms. This represents an advantage over the methods like microscopic count or PCR that measure only total microbial cells. Regarding the methods that determine viability by culture, the bioluminescence method is advantageous because it reduces the analysis time from fourteen days to only a few minutes. This last result is very important because detection of metabolically active microorganisms is a relevant parameter for the operation and control of industrial scale bioleaching processes.

The following are the advantages of LixKit[®] for monitoring the activity of microbial populations in a bioleaching plant:

- Rapid determination (10 minutes)
- Easy operation (kit ready for use)
- High reproducibility
- Allows measurements in the field

- Determines the metabolic activity of leaching microorganisms — ‘health’ of the microflora
- Facilitates the metallurgical and operational decisions.

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NOMENCLATURE

AMP	adenosine monophosphate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CARD-FISH	catalyzed reporter deposition fluorescent in situ hybridization
DAPI	4',6-diamidino-2-phenylindole
DSMZ	German Collection of Microorganisms and Cell Cultures
FISH	fluorescent <i>in situ</i> hybridization
MPN	most probable number
PCR	polymerase chain reaction
RLU	relative light units

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