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Identify healthy and dead cells simultaneity of *Bacillus subtilis* MZ through to use of propidium iodide and acridine orange

Identificación de células vivas y células muertas simultáneamente de *Bacillus subtilis* MZ mediante el empleo de ioduro de propidio y naranja de acridina

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Abstract

The use of microorganism in a lot of process brings the development of new technologies. For guarantee the effect and the impact of the microorganism, even, the production rate when these microorganisms are involved in the production of a particular compound, become necessary know the number and condition of this organism. In the actuality, there are a lot of tools to identify this condition, since kits of staining cells, until, the best microscopy technology. But the use of this tools could become inaccessible due the high cost and in the case of de staining kits had a limited test. Then emerge the necessity to search and implement news methodologies to help us to identify health and death cells in different inoculs or products. In this work, we proposed a new technology for the simultaneous observation of healthy and dead cells trough the staining samples with propidium iodide and acridine orange using an optic microscopy with incandescent light and ultraviolet light.

Healthy and dead cells, Microorganism staining, Acridine orange, Propidium iodide

Resumen

El uso de los microorganismos en un sin número de procesos e investigaciones, ha sido el estímulo fundamental para el desarrollo de nuevas tecnologías. En la búsqueda de garantizar el efecto que tendrán los microorganismos, su impacto o inclusive su taza de producción, cuando se emplean para la obtención de un compuesto en particular, es necesario conocer la cantidad y la condición de los organismos presentes. Actualmente existen un sinfín de herramientas para identificar esta condición, que van desde realizar la tinción de células, hasta, microscopios de última tecnología. Sin embargo, el uso de estos instrumentos puede volverse inaccesible debido a los altos costos de los equipos y en el caso de los kits se manejan para un número limitado de muestras. Por lo que surge la necesidad de buscar e implementar nuevas metodologías que ayuden a determinar células vivas y células muertas en diferentes inóculos o productos. En el presente trabajo se propone una metodología para la observación simultanea de células vivas y muertas, mediante la tinción de las muestras con ioduro de propidio y naranja de acridina y su observación en un microscopio óptico con luz incandescente y luz ultravioleta.

Células vivas y muertas, Tinción de microorganismos, Naranja de acridina, Ioduro de propidio

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Introduction

Currently, the potential that microorganisms represent is studied and exploited every day. The microorganisms are used as an alternative to obtain compounds used for the production of emerging medicines and even in the agricultural industry they are used as biocontrol agents for pests and as biofertilizers.

The wide range of application of microorganisms is due, in the case of bacteria, to the different bacterial interactions that they stimulate by combining different factors such as the activation of different metabolic pathways, production and secretion of signaling compounds, siderophores , antibiotics, plant protection against pathogens, plant growth promotion [Molina et at. 2019].

Microorganisms are currently the subject of many investigations, only in processes or analyzes of the environment, such as the detection of pathogenic bacteria in the environment [Singh et at. 1989], the identification of bacteria present in soils such as Pseudomonas spp. isolated from agricultural crop soils [Albarado et at. 2008],

An important element in the various investigations carried out in the aforementioned environment, whether with the objective of generating basic knowledge or its application, is the need to identify the viability of microorganisms, determine their location, their quantification, among others. The need to determine the viability of cells is important in many applications [Singh et at. 1989].

For the identification of microorganisms and even for the determination of the condition in which they are found, there are currently a wide range of methods to differentiate the viability of bacteria, to identify whether they are alive or not. These methods are based on differential staining, microcolony culture, microautoradiography or measurement of cellular respiration [Singh et at. 1989].

The staining methods have a wide range of application, they are not only used for the staining of microorganisms, but also, they have application in studies carried out with human cells for example for the monitoring of colon cancer cells in human cell cultures, during the incursion of new compounds to alter the condition of the cells [Kim et at. 2013].

They are also widely used for in vivo localization of subcellular differentiation [Gerhart et at. 2012]. There are different commercial kits that allow distinguishing between viable gram positive and gram negative bacteria. [Vázquez et at. 2010].

An alternative for bacterial observation is fluorescence microscopy. The fluorescence microscope is an optical microscope capable of illuminating the sample with a light whose wavelength may vary due to its passage through an excitation filter. This filter transmits exclusively the excitation light of the sample that has the selected wavelength. [Vázquez et at. 2010].

Propidium iodide is an impermeable fluorophore, which only crosses the membrane of dead cells. It interacts with nucleic acids, emitting red fluorescence (Emission: 617 nm) [Vázquez et at. 2010]. In contrast, acridine orange is a very permeable fluorochrome that acts as an intercalating agent, allows nucleotide staining, causing green fluorescence, and interacts with RNA causing orange fluorescence [Albarado et at. 2008; Kim et at. 2013, Vázquez et at. 2010]. Despite the progress and development of the methods currently used for the determination of cell viability, the development of methodologies or innovation in them, it is an indispensable tool for the development of new projects, it also represents an option to be able to perform an analysis in which less time is spent and even that represents a lower economic expense.

In the present work, a methodology for the simultaneous observation of living and dead cells is proposed, using an optical microscope with incandescent light and ultraviolet light, by staining the samples with the dyes of propidium iodide and acridine orange using a variation of the Kenneth and James methodology [Kenneth et at. 1985], simultaneously with the methodology of Hobbie et al. [Hobbie et at. 1977].

Objective

Develop a methodology that can simultaneously identify the presence of live cells and dead cells of Bacillus subtilis MZ in a modified starch matrix, using an optical microscope using incandescent light and ultraviolet light.

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Methodology

This project was developed in Cinvestav Unit Irapuato. The samples were observed under a microscope (BX50, Olympus, Japan) using 4X / 0.10 Plan (α - -) and 20X / 0.50 objectives, UPlan-FL (α - 0.17). The lighting methods were incandescent light whose source was halogen lamp (IOUSH, Japan) and ultraviolet light with mercury vapor lamp (OSRAM, Mexico) and excitation filter at 350 to 400 □m. Image acquisition was performed with a high sensitivity camera for Infinity3 fluorescence (Lumenera, Canada) synchronized through the Image Pro Premier 9.1 program (Media Cybernetics, USA).

The biological material or the samples analyzed were three different conditions of Bacillus subtilis MZ. The first condition was Bacillus subtilis MZ in a modified starch matrix, which is the objective of the investigation.

The other two conditions were implemented as a positive control of Bacillus subtilis MZ in the present methodology. Which consisted of samples of the same strain of the bacteria, but using vegetative cells and spores of our strain.

Staining of the samples was performed as described below:

1. Preparation of "stock" solutions

0.1% solution of acridine orange in sterile distilled water.

2% solution of propidium iodide in saline phosphate regulator as mentioned by Kenneth and James [1985].

2. Staining of Bacillus subtilis MZ in the modified starch matrix

1.- A solution called "staining solution" was prepared with the "stock" solutions in a 1: 1 ratio. This solution was prepared at the time each of the observations was made.

2.- To a sample of our study subject, mounted on a slide, 10 µl of the staining solution was added.

3.- Samples were observed under a microscope (BX50, Olympus) at two wavelengths, the first of 480-510 ηm m and the second of 530-550ηm.

4.- A splicing of the photographs taken for the observations at both wavelengths was made, to appreciate live cells and dead cells in the same image and see if they co-locate.

3. Staining of Bacillus subtilis MZ "Positive Control 1"

As a positive control, Bacillus subtilis MZ cells (in the absence of the modified starch matrix) were observed, to make the observations the cells were obtained as follows:

1.- Bacillus subtilis MZ was grown in potato infusion, incubated at a temperature of 23 ° C for 24 hours.

2.- To eliminate background noise in microscopic observations caused by the culture medium, the cells were separated by centrifugation at 10,000 rpm for a time of 5 minutes at 4 ° C.

3.- Two rinses were made with sterile distilled water, under the same centrifugation conditions. The supernatant was discarded and the pellet was resuspended in sterile distilled water.

4.- To harvest the cells, it was centrifuged again under the same operating conditions. The pellet was resuspended in 200 µl of the staining solution, allowed to stand for 3 minutes. For staining, 200 µl of the staining solution was used for every 100 µl of culture sample.

5.- The cells were separated from the dye by centrifugation, in the same way as in step 1. The pellet was resuspended in 200 µl of sterile distilled water.

6.- A 7 µl aliquot of the sample was mounted on a slide, observations were made with the microscope at two wavelengths, 480-510 □m and 530-550ηm m.

7.- A splicing of the photographs taken for observations at both wavelengths was made to see live cells and dead cells in the same image and see if they co-locate.

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4. Staining of Bacillus subtilis MZ spores "Positive control 2"

As a second positive control, Bacillus subtilis MZ spores were observed, the samples were obtained in the manner described in the previous paragraph, unlike the culture of B. subtilis MZ was incubated for 4 days to allow the greatest number of spores.

For each sample three stains were made of which, a total of 5 fields were observed at both mentioned wavelengths.

Results and Discussion

The samples of Bacillus subtilis MZ stained with acridine orange were observed simultaneously with propidium iodide, using the methodology previously described. All photographs were taken at the different lengths specified, using the Image Pro Premier 9.1 program.

Our main subject of study was Bacillus subtilis MZ in a modified starch matrix. It was possible to observe and identify clearly and quickly the living cells in green, the dead red cells. The analysis of the splicing of the two conditions was carried out to verify that they do not co-locate and to confirm that the selectivity of the dyes used for staining (Figure 1).

Green fluorescence was observed in living cells, because the orange acridine dye binds to the bacterium RNA by electrostatic interactions. Likewise, the dead cells were observed red, by the passage of the propidium iodide through damaged membranes since this is interspersed with DNA chains, in addition, this dye is excluded from intact membranes, which makes it a dye effective to identify non-viable cells. The interaction of the bacterial genetic material, DNA, RNA with the two dyes simultaneously causes fluorescence in the different colors [Albarado et at. 2008; Kenneth et at. 1985].

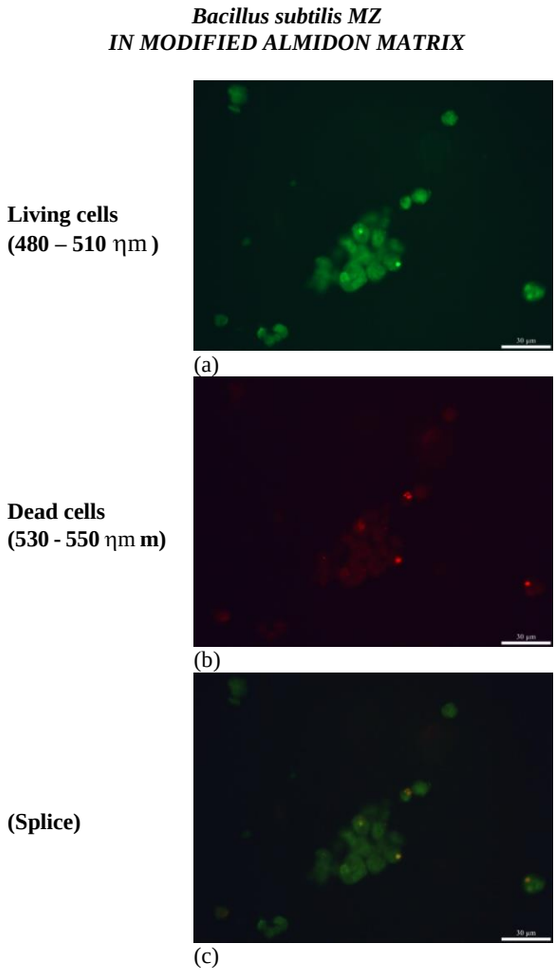

Figure 1 Bacillus subtilis MZ in a modified starch matrix. Photographs of the results obtained by simultaneous staining of acridine orange with propidium iodide

The proposed methodology based on simultaneous staining with acridine orange and propidium iodide, in this work is presented as a tool for the qualitative analysis of the viability of bacteria. This methodology, with the necessary complementary equipment, can be used to determine the viability of the cells quantitatively, by measuring the intensity of the emitted fluorescence [Hussain et at. 2019].

The propidium iodide also allows staining of damaged cells [Kirchhoff et at. 2017]. A culture of Bacillus subtilis MZ in a vegetative state was established as the first positive control (Figure 2).

For the first positive control, the presence of living cells was corroborated (Figure 2a), as well as the presence of dead cells (Figure 2b), splicing of the images was performed to analyze the co-location of the bacteria (Figure 2c).

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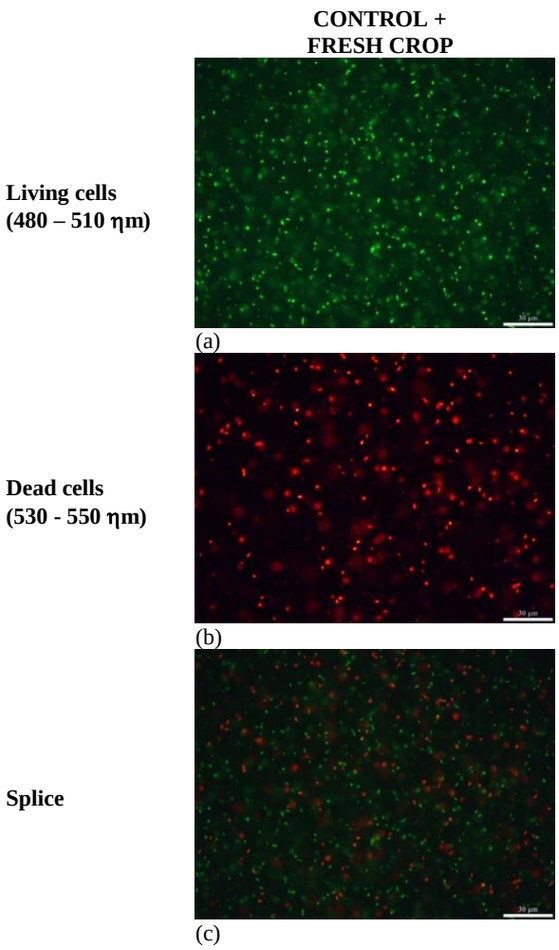
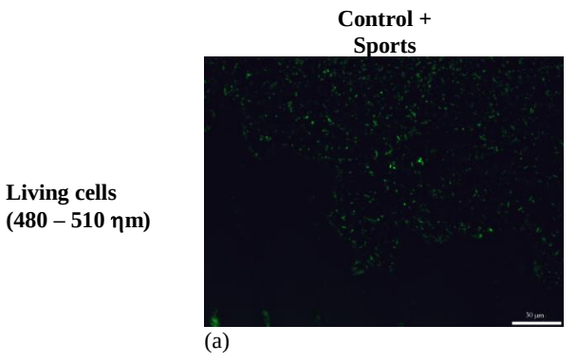


Figure 2 Vegetative cells of Bacillus subtilis MZ. Photographs of the results obtained by simultaneous staining of acridine orange with propidium iodide

A culture of Bacillus subtilis MZ spores was evaluated as a second positive control (Figure 3). The spores were observed in the length of living cells, due to their interaction with the dye (Figure 3a), as well as the presence of dead cells in the sample (Figure 3b). The same co-location analysis used for the other samples was performed (Figure 3c). These results indicate that part of the cells that are stained with propidium iodide as dead cells may be spores of B. subtilis MZ.



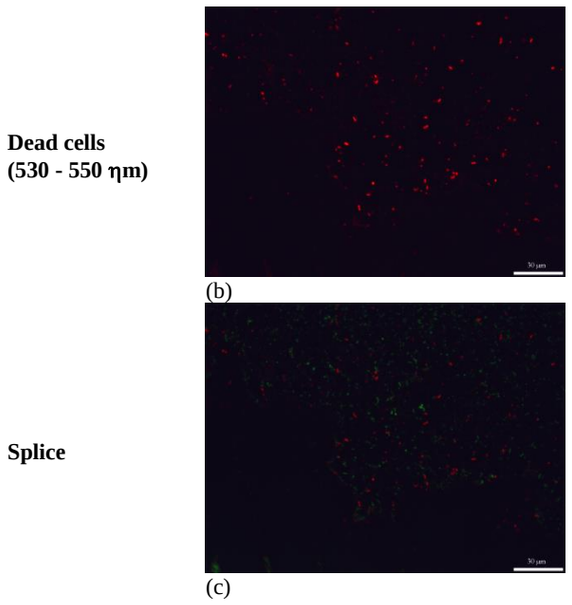
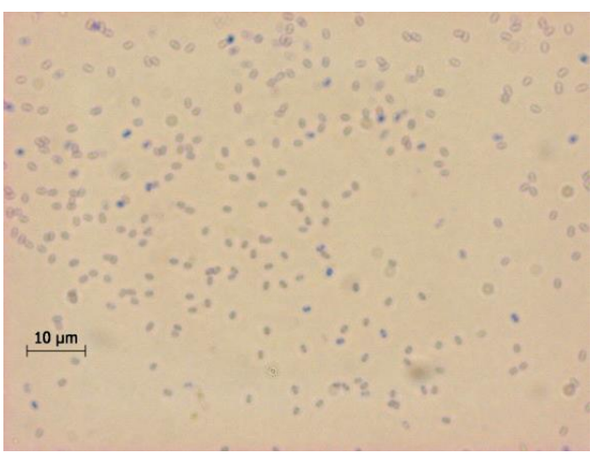


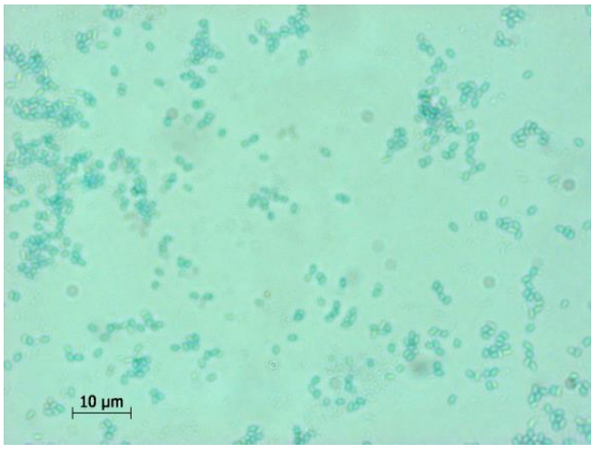
Figure 3 Bacillus subtilis MZ spores. Photographs of the results obtained by simultaneous staining of acridine orange with propidium iodide

The second positive control was also analyzed by Gram staining (Figure 4a) and malachite-safranine green staining (Figure 4b). The green Bacillus subtilis Mz spores were successfully identified because the malachite green penetrates the spore membrane, the use of safranine is to obtain a contrast with the cells in a vegetative state.

The spores present multiple aspects of interest, represent an important process of cell differentiation or morphogenesis perfectly regulated and controlled by the bacteria, [Vázquez et at. 2010]. Staining with malachite-safranine green allows the bacterial endospora to be revealed, a form of resistance produced by some genera of gram-positive bacteria. [Vázquez et at. 2010].



(a)



(b)

Figure 4 Photograph of (a) Gram staining, (b) staining with malachite green- safranine both Bacillus subtilis MZ

Conclusions

The proposed methodology allowed to observe simultaneously live cells and spores of the bacterium B. subtilis MZ. It is an option in which less time and economic resources are invested to identify the viability and the location of microorganisms. As mentioned by Ortega et al. [Ortega et at. 2010], one of the advantages of the tests, is not only its ease and cost, but also that the information it provides is very useful for the analyzed factor.

Albarado et al. [Albarado et at. 2008], also conclude that modified fluorescence differential coloring methods can be used as a complementary technique in clarifying the molecular mechanisms involved in the cell cycle. The use of the present methodology coincides with that proposed by Vázquez et al. [Vázquez et at. 2010] who express that for the observation of bacteria various general fluorochromes can be used both in crops, as in samples from natural environments (soil, water, interior of living beings) or artificial (sludge digesters, composting piles, biological reactors , etc.). [Vázquez et at. 2010]. Hussain and food collaborators [Hussain et at. 2019] have used this type of dyes to determine chemical and microbiological profiles in food samples.

The scope of the work developed could be unlimited, because the methodology developed can be used for any bacterium with the certainty of the results obtained in the present study.

Only the existing interaction with the vehicle in which the bacteria to be evaluated would have to be evaluated, in our case the starch matrix did not represent an impediment to the observation of the condition of Bacillus subtilis MZ.

An important factor that must be considered for the use of the established methodology is the solubility of the dyes used with the medium in which the bacterium to be analyzed is found, in the case of the present study the modified starch matrix is soluble in the work solutions used.

As a perspective, we propose the analysis that has the effect of the amount of RNA and DNA present in the cell according to the phase of its cell cycle, in the staining of the bacteria [Albarado et at. 2008]. It is also proposed to evaluate the membrane potential, because it has been reported that it is a parameter that affects cell staining [Kirchhoff et at. 2017].

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