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Use of the Direct Epifluorescent Filter Technique for the Enumeration of Viable and Total Acetic Acid Bacteria from Vinegar Fermentation

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A rapid epifluorescence staining method using the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit was applied to differentiate both viable and total counts of acetic acid bacteria in vinegar fermentation. The results obtained were compared with those from other measurement techniques: 4′,6-diamidine-2phenyl indole (DAPI) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and colony counts. *Bac*Light total counts were comparable to DAPI (differing by <3.5%). *Bac*Light viable counts were similar to CTC counts but considerable higher than colony-forming cells in plates.

KEY WORDS: Vinegar; acetic acid bacteria; acetators; *Bac*Light; DAPI; CTC; viability staining.

INTRODUCTION

Accurate methods of measuring biomass and microbial activity are essential in understanding microbial characteristics at different stages of industrial vinegar production. Difficulties have been encountered in growing these organisms in culture media, especially when isolated directly from vinegar. Even when isolation is possible, the process is often too long to be useful.

The limited knowledge about the bacteriology of this process is mainly due to the difficulties in isolation and cultivation of the active organism. A suitable bacterial population in nonsterile acetators is maintained only by the steady selective conditions in the reactor, which are high concentration of acetic acid and ethanol. In addition, cells from the economically important submerged fermentations die quickly by interruption of the intense aeration in the reactor [1].

To better understand the ecology of microorganisms in acetators and to quantify concentrations of microbial population, methods are needed to differentiate among metabolically active and inactive microorganisms as they exist in situ. Conventional culture-based detection methods are incapable of identifying nonviable or nonculturable microorganisms and result in understimation of the microbial concentrations. For practical purposes, the rapid method studied seems to be a good alternative to traditional cultural methods as part of quality control programmes in vinegar making.

Epifluorescent direct count techniques are frequently the methods of choice, yielding more accurate estimates of total and viable cell numbers in a wide variety of situations as alternative to plate counts. These methods have been applied successfully in water treatment [2–6], in food technology [7–12] and in bioremediation research [13].

In the present report, LIVE/DEAD *Bac*Light Bacterial Viability kit and DAPI and CTC stains were used for direct microscopic counting of live and dead bacteria, to evaluate their efficiency in the enumeration of the bacterial population, several reference strains, and acetic acid bacteria from industrial acetators.

MATERIALS AND METHODS

Bacterial Strains

To assess the suitability of the DEFT methodology, three reference strains and strains from industrial acetators

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(Frings-acetators, 20 m^3) were used. The reference strains, *Acetobacter aceti* (CECT 298), *Acetobacter pasteurianus* (CECT 474), and *Gluconobacter oxydans* ssp. *suboxydans* (CECT 360T) were obtained from Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain). These strains were grown in the media recommended by CECT.

Cells were collected by centrifugation at 8000 × *g* for 10 min and resuspended in water or phosphate buffer (pH = 7), depending of the dyes used. Different suspensions were prepared by dilution (10^5–10^{11} cells·ml^{-1}).

LIVE/DEAD *Bac*Light Bacterial Viability Kit

Direct viable and total counts of bacteria were realized with the LIVE/DEAD *Bac*Light Bacterial Viability kit provided by Molecular Probes, Inc. This test evaluates the structural integrity of the bacterial membrane employing the fluorescence dyes SYTO 9 and propidium iodide. Equal volumes of the two stains were combined in a microfuge tube and 3 μL of this solution was added to 1 ml bacterial suspension (the concentration of dyes for cell staining was 5 and 30 $\mu m \cdot L^{-1}$ for SYTO 9 and propidium iodide, respectively). These were mixed thoroughly and incubated at room temperature in the dark for 15 min as recommended by the manufacturer. After staining the sample was filtered through a black polycarbonate membrane with a pore size of 0.2 μm and 13 mm diameter (Millipore Corp., Madrid, Spain); the filter was mounted in *Bac*Light mounting oil and immediately observed under epifluorescence microscope. The viable cell count was performed by counting the green fluorescing cells and the number of nonviable by the red fluorescing cells. Formaldehyde-treated cultures (3.7% final concentration, treated for 30 min) were used as killed controls.

DAPI Direct Count Method

The enumeration of the total bacteria cells by the DNA intercalating agents DAPI (4′,6-diamidine-2phenyl indole 2 HCl) was performed using a procedure modified from Boulos *et al.* (4). A stock solution of DAPI was prepared by dissolving 1 mg of DAPI in 10 ml of ultrapure water. Cell suspensions were mixed with the stain at a final concentration 10 μg/ml and then incubated for 30 min at room temperature in the dark. After the incubation, the samples were filtered on a black polycarbonate membrane; the membrane was air-dried and mounted on glass slide in *p*-phenylendiamine solution. The bacteria on DAPI-stained membranes were enumerated by counting the total number of blue fluorescing bacteria.

CTC Reduction Assay Method

Direct viable counts of actively respiring bacteria were measured by the reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Polysciences Europe, Eppelheim, Germany). CTC staining was performed using a procedure modified from Rodriguez *et al.* (2). A stock solution (50 m*M*) was prepared in deionized water and filtered on a 0.22 μ*M* GS Millex filter (Millipore Corp., Madrid, Spain) and used at a final CTC concentration of 5 m*M*. This concentration was based on an optimization assay conducted with pure cultures. Cell suspensions were incubated for 1 h, in the dark, at 30°C and mixed at 200 rpm. After incubation, the bacteria were fixed with formaldehyde (final concentration 2%, v/v) and collected on a black polycarbonate membrane. The membrane was air-dried and mounted on glass slide in *p*-phenylendiamin solution. Under epifluorescence microscopy, the CTC-formazan granules produced by respiring bacteria appeared red. Formaldehyde-treated (3.7% final concentration, treated for 30 min) cultures were used as killed controls. No CTC-formazan was detected in killed controls.

CTC/DAPI Staining

CTC was used in conjunction with DAPI to differentiate metabolically active cells from inactive cells. CTC staining was performed using the procedure described above. Following the CTC incubation, DAPI solution was added to a final of 10 μg/ml and samples were incubated for 1 h, in the dark, at 30°C. Different epifluorescent filter sets for visualizing CTC-stained cells and DAPI-stained cells were used.

Enumeration of Microorganisms

Microscope slides were examined under oil immersion in a Zeiss axioplan microscope equipped with an HBO-50 W mercury lamp and appropriate filters for *Bac*Light, CTC, and DAPI. The images were taken with Hamamatsu color chilled 3CCD camera and developed by Raster Ops videocaptor and treated by Adobe PhotoShop 6 program. The number of bacteria were estimated from counts of 20 microscopic field (at × 1000). An eyepiece with a graticule calibrated was used for all bacterial counting.

For *Bac*Light-stained cells, the viable cell count was performed by counting the green fluorescing cells and

the number of nonviable by the red fluorescing cells in 20 random fields of vision. Estimation of number of cells in each sample was calculated as follows:

$$N = \frac{C \times A}{a \times V} \times D$$

where N is the number of cells per ml^{-1}; C is the number of cells per observation field; A is the filtration area (mm^2); a is the observation field area (mm^2); V is the volume of sample filtered (ml); and D is the dilution factor.

Standard Plate Count Method

The viable cells were counted by plating the appropriate sample dilutions onto the media recommended by CECT. The growth conditions were the most appropriate ones for each strain.

RESULTS AND DISCUSSION

Application of BacLight, DAPI and CTC to Acetic Acid Bacteria

The LIVE/DEAD *Bac*Light Bacterial Viability kit was developed to differentiate live and dead bacteria based on plasma membrane permeability. Thus, bacterial cells with compromised membranes fluoresce red and those with intact membranes fluoresce green (Fig. 1 A). Formaldehyde-treated (3.7% final concentration, treated for 30 min) cultures were used as killed controls (Fig. 1 B).

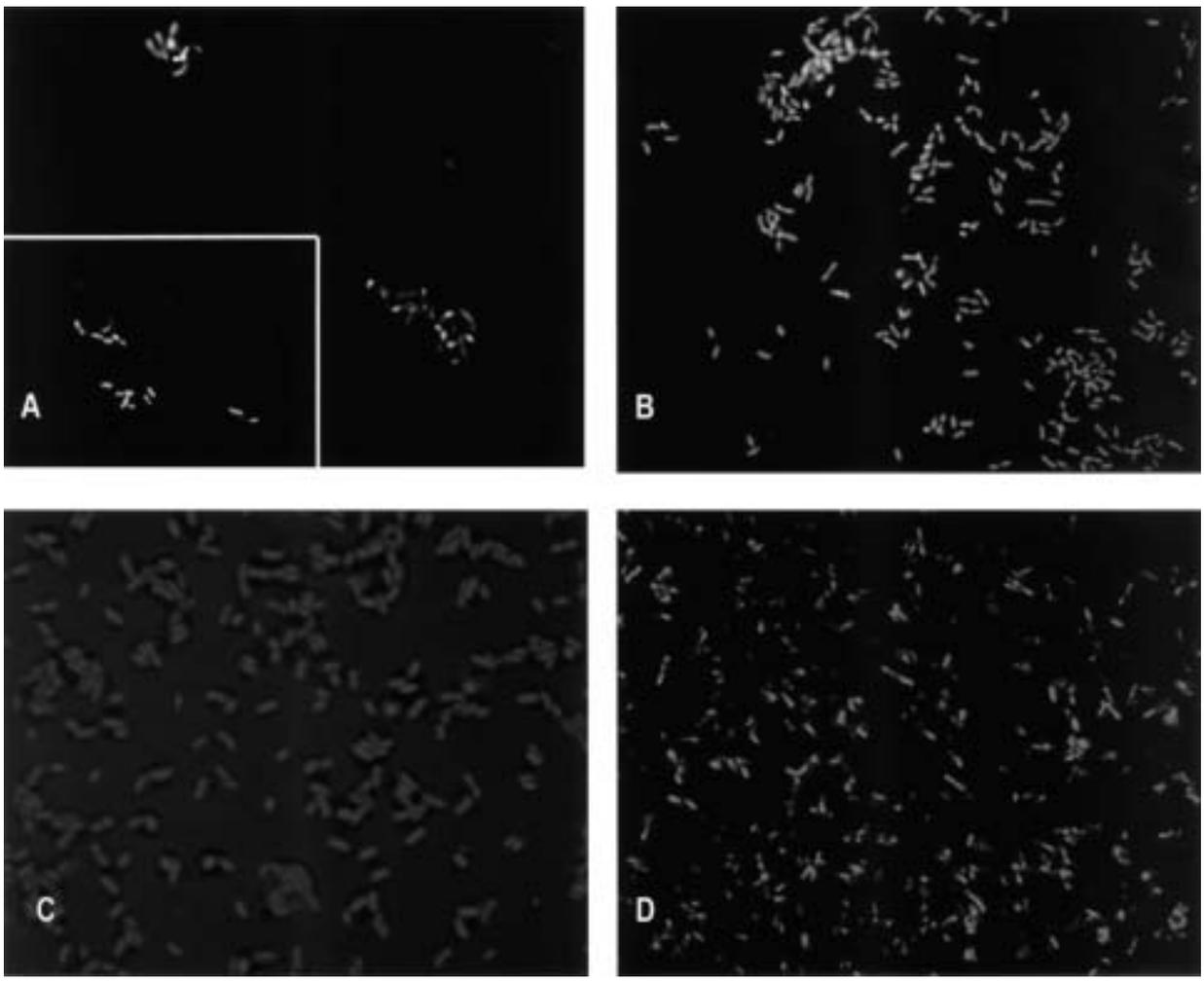

Fig. 1. Bacterial cells recovered from industrial acetators by membrane filtration, stained with different dyes, and revealed by epifluorescent microscopy. Bacteria are 1 to 2 μM long. (A) *Bac*Light. (B) *Bac*Light, killed controls. (C) DAPI. (D) CTC.

DAPI was used at concentrations of 0.1, 1, 10, and 20 μg/ml. Very few bacteria could be counted when DAPI concentrations were 0.1–1 μg/ml. But with 10 μg/ml, more bacteria were seen and could be distinguished easily from the background. When used at 20 μg/ml, excessive background fluorescence was observed. Therefore for routine counting, DAPI was used at a final concentration of 10 μg/ml for 30 min (Fig. 1 C).

The impact of CTC concentration (1–5 m*M*) on both the abundance and the fluorescence of respiring cells was tested and finally used at a final concentration of 5 m*M*. This concentration was chosen on an optimization assay conducted with several pure cultures (Fig. 1 D).

The combined use of DAPI and CTC presented some complications and the microscopic visualization of formazan precipitates within bacteria was very difficult. The intercalation of DAPI with bacterial nucleotids has been reported to dampen the fluorescent signal emitted from CTC-formazan precipitates (13). Consequently the developed method did not allow the enumeration of active and total bacteria.

Comparison of Viable and Total Counts Obtained with *Bac*Light, DAPI, CTC, and Plate Count Method

The relationship between the direct count methods was determined using regression analysis. Correlation coefficients (r) and standard errors were calculated from the data, and results are presented in Figs. 2 and 3.

A linear relationship was found between the *Bac*Light and DAPI ($r^2 = 0.99$; standard errors ± 3.5%) (Fig. 2). No significant difference between the two methods was observed for all strains studied.

As observed in Fig. 3, similar counts were observed by *Bac*Light and CTC. Data from this study suggest that a variety of acetic acid bacteria are capable of reducing CTC to CTC-formazan. CTC reduction was observed during all phases of growth, and no CTC reduction was observed in killed controls. These results suggest that CTC can be used to quantify the metabolic activity in different stages during the fermentative process.

However, these investigations point out that discrepancies exist between the fluorescence optical counting methods and the standard plate count. Results obtained with *Bac*Light and CTC were similar to those obtained with plate counts using reference strains. But, when comparing viable direct counts to those obtained with plate counts of bacteria from acetators, the results differed considerably. Plate counts were also approximately 4 orders of magnitude lower than active bacterial counts as determined by *Bac*Light or CTC staining.

The large differences between the CTC-positive counts or *Bac*Light and the plate counts may have been due to problems in the handling of the acetic acid bacteria outside of the fermentation environment, cultivation outside of the bioreactor, and transfer from solid to liquid medium (14–17).

In summary, the use of CTC or *Bac*Light for evaluating metabolic activity of acetic acid bacteria during the acetification process is promising. *Bac*Light staining has several advantages. It is a reliable, rapid, and easy to use test and yields both viable and total counts in one step. The preparations are easy to read because of the high degree of contrast between the green color of the viable bacteria and the red color of the dead cells and background fluorescence is minimal. However, more studies are needed to completely evaluate the degree of accuracy of the assessment of bacterial viability.

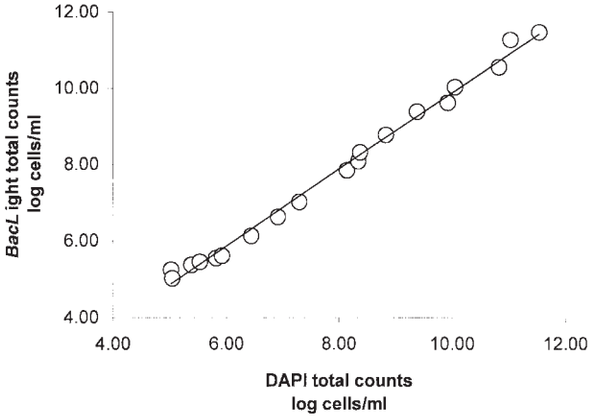

Fig. 2. Relationship between enumeration of total bacteria using and DAPI (strain 474).

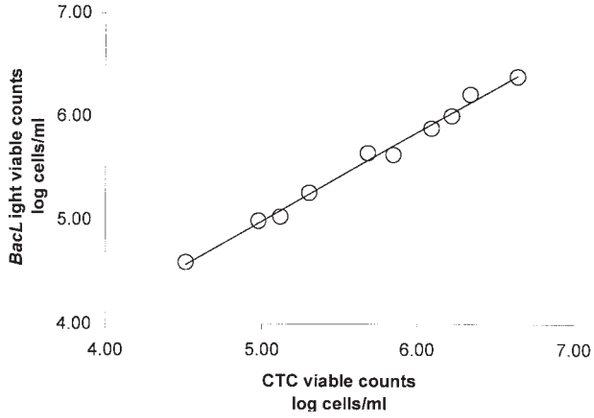

Fig. 3. Relationship between enumeration of viable bacteria using *Bac*Light and CTC (samples from industrial acetators).

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REFERENCES

1. M. M. Mesa, I. Caro, and D. Cantero (1996) Viability reduction of *Acetobacter aceti* due to the absence of oxygen in submerged cultures. *Biotechnol. Progr.* **12**, 709–712.
2. G. G. Rodriguez, D. Phipps, K. Ishiguro, and H. F. Ridgway (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* **58**, 1801–1808.
3. J. Coallier, M. Prévost, and A. Rompré (1994) The optimization and application of two direct viable count methods for bacteria in distributed drinking water. *Can. J. Microbiol.* **40**, 830–835.
4. L. Boulos, M. Prévost, B. Barbeau, J. Coallier, and R. Desjardins (1999) Live/Dead® *Bac*Light™: Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods.* **37**, 77–86.
5. R. Ramalho, J. Cunha, P. Teixeira, and P. A. Gibbs (2001) Improved methods for the enumeration of heterotrophic bacteria in bottled mineral waters. *J. Microbiol. Methods* **44**, 97–103.
6. J. Vollertsen, A. Jahn, J. L. Nielsen, T. Hvitved-Jacobsen, and P. H. Nielsen (2001) Comparison of methods for determination of microbial biomass in wastewater. *Water Res.* **35**, 1649–1658.
7. G. L. Pettipher, R. J. Fulford, and L. A. Mabbitt (1983) Collaborative trial of the direct epifluorescent filter technique (DEFT), a rapid method for counting bacteria in milk. *J. Appl. Bacteriol.* **54**, 177–182.
8. U. M. Rodrigues and R. G. Kroll (1986) Use of the direct epifluorescent filter technique for the enumeration of yeast. *J. Appl. Bacteriol.* **61**, 139–144.
9. J. T. Holah, R. P. Betts, and R. H. Thorpe (1988) The use of direct epifluorescent microscopy (DEM) and the direct epifluorescent filter technique (DEFT) to asses microbial population on food contact surfaces. *J. Appl. Bacteriol.* **65**, 215–221.
10. G. Duffy and J. J. Sheridan (1998) Viability staining in a direct count rapid method for the determination of total viable counts on processed meats. *J. Microbiol. Methods* **31**, 167–174.
11. J. Couto and T. Hogg (1999) Evaluation of a commercial fluorochromic system for the rapid detection and estimation of wine lactic acid bacteria by DEFT. *Lett. Appl. Microbiol.* **28**, 23–26.
12. M. Hermida, M. Taboada, S. Menéndez, and J. L. Rodríguez-Otero (2000) Semiautomated direct epifluorescent filter technique for total bacterial count in raw milk. *J. AOAC Int.* **83**, 1345–1348.
13. V. K. Bhupathiraju, M. Hernández, P. Krauter, and L. Alvarez-Cohen (1999) A new direct microscopy based method for evaluating in-situ bioremediation. *J. Hazard. Mater.* **67**, 299–312.
14. M. Kittelmann, W. W. Stamm, H. Follmann, and H. G. Trüper (1989) Isolation and classification of acetic acid bacteria from high percentage vinegar fermentations. *Appl. Microbiol. Biotechnol.* **30**, 47–52.
15. M. Sievers, S. Sellmer, and M. Teuber (1992) The microbiology and taxonomy of *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermentors in central Europe. *Syst. Appl. Microbiol.* **15**, 610–616.
16. S. J. Sokollek and W. P. Hammes (1997) Description of a starter culture preparation for vinegar fermentation. *Syst. Appl. Microbiol.* **20**, 481–491.
17. S. J. Sokollek, C. Hertel, W. P. Hammes (1998) Cultivation and preservation of vinegar bacteria. *J. Biotechnol.* **60**, 195–206.