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Rapid method for total, viable and non-viable acetic acid bacteria determination during acetification process

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Abstract

A rapid epifluorescence staining method using the LIVE/DEAD® *Bac*Light Bacterial Viability Kit (*Bac*Light™) and direct counts in Neubauer chamber were applied to estimate both viable and total counts of bacteria in different stages of vinegar making. *Bac*Light kit is composed of a mixture of two nucleic acid binding stains: SYTO 9™ and propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate viable bacterial cells. SYTO 9 penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells. Optimal dilution and incubation conditions were found to be 1:5 and 15 min at room temperature in dark respectively. Total (red plus green) and viable (green) cells can be obtained in one staining step and hence counted simultaneously. Results obtained with this technique were compared with those from other measurement techniques (colony counts and flow cytometry).

Keywords: Acetic acid bacteria; Vinegar; Viability; Epifluorescence; Neubauer chamber; LIVE/DEAD®

1. Introduction

Vinegar is the result of growth and metabolism of acetic acid bacteria (AAB) in which, alcohol produced from different agricultural raw material as a carbon source, is oxidized to acetic acid. Modern industrial vinegar production is carried out by submerged fermentation process. The state of art is a batch aerobic process with a partial renewal of the fermentation medium at the end of each cycle [1]. In any industrial process it is relevant the identification and quantification of different species and strains involved in the biotechnological transformation. It is also important to monitor the likely presence or absence of micro-organisms in the final product after the manufacture process. So, a quick and accurate procedure for detection and enumeration of these bacteria is important from the industrial point of view. Acetic acid bacteria have traditionally been enumerated by quantifying viable colonies by plating on solid culture media. Some works have been done using selective media [2,3], but the limitations of cultural methods are: the time required and the inability to detect viable but non-culturable bacteria. Nowadays, in spite of the high technology involved in industrial vinegar production, only little is known about the microbiology of this oxidation process. The low level of knowledge is mainly due to the difficulties encountered in growing these micro-organisms on solid culture media, especially when isolated directly from vinegar [1,4–6]. Even when the isolation is efficient, the process is too long for the growth of the active micro-organisms involved in this bio-process. To overcome these disadvantages, we have developed epifluorescence direct count methodology, which yields more accurate estimates of total and viable cell number as an alternative to plate counts. Similar methods have been applied successfully in water treatment [7,8], in food technology [9] and in bioremediation [10]. Mesa et al. [6] studied the use of direct epifluorescent techniques for enumeration of viable and total

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AAB from vinegar fermentation. They concluded that LIVE/DEAD *Bac*Light bacterial viability test and CTC staining method appear to be good to this aim. Though the above technique is reliable and easy to use, it is possible to go forward in order to simplify the method and short the whole time needed for.

In the present report a new quicker method, which joins a direct counting in a special Neubauer chamber with LIVE/DEAD *Bac*Light bacterial viability kit, has been used to evaluate its efficiency in the counting of acetic acid bacteria from acetators.

2. Material and methods

2.1. Laboratory fermentation

Wine vinegar fermentation under laboratory conditions was performed in 10 l pilot acetator (Frings Co., Bonn, Germany) with a working volume of 8 l. Aeration was at 60 l/h and fermentation temperature at 31 °C. The fermentation was carried out semi continuously and started with seed vinegar from an industrial bioreactor. When a minimum ethanol concentration of 1–3% (v/v) was reached, a percentage of total medium volume was discharged and be replaced with new mash, containing 12% (v/v) ethanol.

2.2. Media and growth conditions

Acetic acid bacteria were isolated and enumerated by plating 100 μl of various dilutions of fermentation broth at different stages of process onto a solid M10 medium (0.5% yeast extract, 0.3% peptone, 2.5% mannitol and 2% agar). Plates were incubated at 30 °C for 2–7 days.

2.3. Direct epifluorescence method (DEM)

DEM was performed using LIVE/DEAD *Bac*Light bacterial viability kit (L-7012) provided by Molecular Probes (Eugene, OR, USA) (http://www.probes.invitrogen.com/). This test uses SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. SYTO 9 stain generally labels all bacteria in a population, those with intact and damaged cytoplasm membrane. In contrast, propidium iodide penetrates only bacteria with damaged membrane, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Using an appropriate mixture of these two dyes, bacteria with intact cytoplasm membrane stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. One millilitre of sample at different stages of acetification process, with appropriate dilution was incubated in dark with 1.5 μl of each dye for 15 min at room temperature, and then 5 μl of this bacterial suspension was placed in a special Neubauer chamber and examined under Olympus microscope or treated for flow cytometry (see below).

2.4. Enumeration of micro-organisms

Samples were removed at distinct times and diluted in sterilized distilled water at 1:5 for cell counting in a special Neubauer chamber with 0.02 mm depth and rhodium-coated bottom. Though the chamber is subdivided into 25 groups, composed of 16 squares each, only five groups (0.04 mm^2 each) on the diagonal were used for counting.

Neubauer chamber was examined under oil immersion in Olympus BX51 microscope equipped with an U-RFL-T-100W mercury lamp and appropriate filters for LIVE/DEAD *Bac*Light bacterial viability kit. The images were taken with Olympus CF70 camera and Olympus C4040-zoom. For *Bac*Light-stained cells, the viable cell counts were performed by counting green cells and the number of non-viable by red fluorescent cells.

2.5. Flow cytometry

In order to check the obtained results by the above method, flow cytometry has been used. All experiments were performed using a Beckman coulter flow cytometer equipped with an argon-ion laser, emitting at a fixed wavelength of 488 nm. Fluorescent filters and detectors were all standard with green fluorescence collected in the FL1 channel (525 nm), red fluorescence collected in the FL3 channel (620 nm). The dyes described on Section 2.3 were used. All parameters were collected as logarithmic signals and data were analysed using the software EXPO32 Analysis (ver. 1.1.c).

3. Results

3.1. Bacterial enumeration from different stages of acetification process

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 plasma membranes stain fluorescent red while those with intact membranes stain fluorescent green; so, initially the first one may be considered as non-viable cells and the green ones as viable cells. In any case, it is important to point out that these conclusions must be checked for every case. Fig. 1A shows

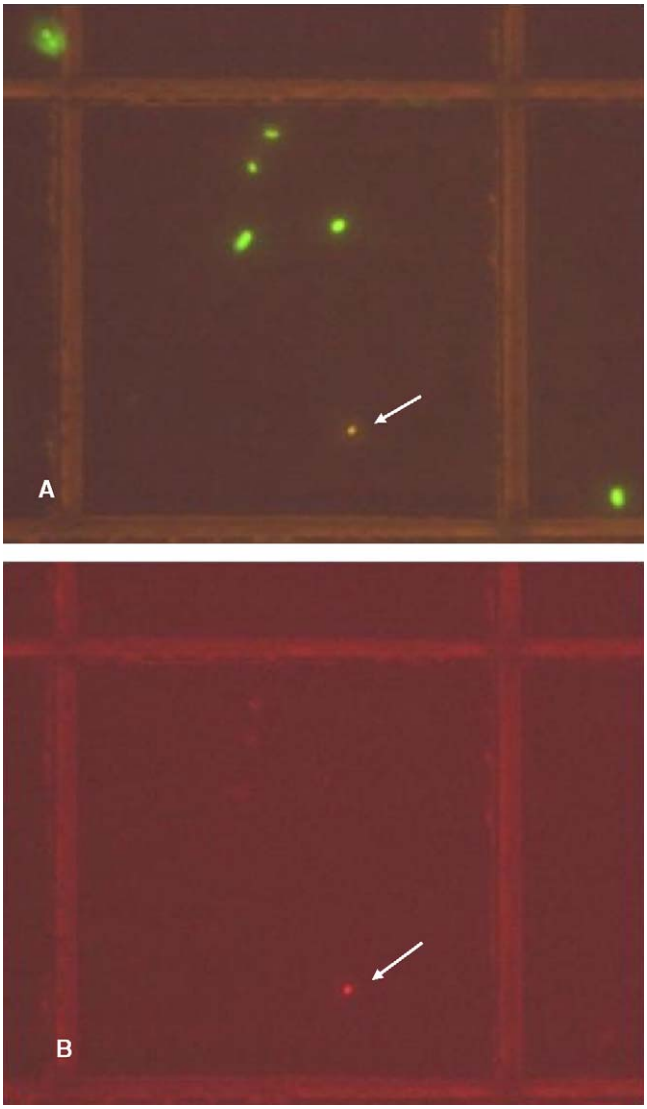

Fig. 1. View of bacterial cell recovered from acetator, stained with LIVE/DEAD *Bac*Light: (a) under filter Olympus MNB-2 and (b) under filter Olympus MNG-2.

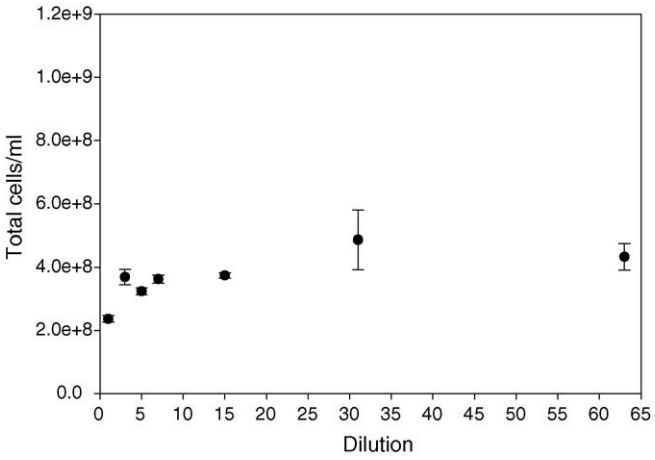

Fig. 2. Influence of the dilution on the total cell concentration. Bars represent standard error ($n = 4$).

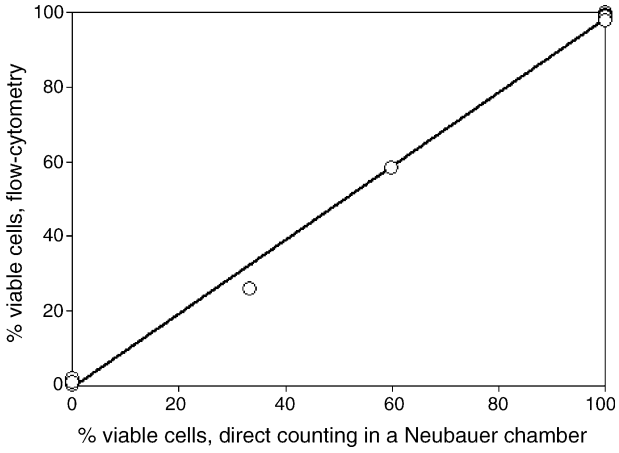

Fig. 3. Comparison between flow cytometry and direct counting in a Neubauer chamber for estimating the percentage of viable cells using *Bac*Light viability test.

a view of the bacteria with the filter Olympus MNB-2. This filter allows to see all the cells but with different colours, some of them as green and other as yellowish. Beside, on Fig. 1B the view with the filter Olympus MNG-2 is shown; only cells with red fluorescence can be seen. A control for killed cells using glutaraldehyde 4% effectively resulted in a red fluorescence for all bacteria (data not showed).

Fig. 2 shows the influence of the dilution on the total cell concentration. Several samples were prepared by adding water to 1 ml of medium from the bioreactor up to the total volume shown on abscise. Each sample has been repeated four times; the standard error is indicated on the figure. As it can be seen, the errors were not significant up to dilution values of 64; further high dilutions imply higher errors. Based on these results a 1:5 dilution was used for subsequent experiments.

3.2. Comparison of viable and total counts obtained with BacLight, flow cytometry and colony-forming unit count

In order to check the validity of the results obtained with *Bac*Light kit and direct counts on Neubauer chamber, flow cytometry has been used as an additional method. For instance, on Fig. 3, a comparison amongst 12 samples with different percentage of viable cells obtained by both methods is shown; a linear relationship with a correlation regression of 0.99 was found. For all samples tested, both the *Bac*Light kit staining and flow cytometry analysis gave similar results.

Also, a range of 16 vinegar samples from repeated cycles (see Section 3.3) at similar environmental conditions (time of cycle) were analysed for numbering viable cells by direct count and culturable acetic acid bacteria on solid media. The results of these analyses are shown in Fig. 4. As can be seen, variable results are found. While some cases (samples 13, 14, 15 and 16) gave similar results, the rest differ, in general, in several order of magnitude and the number of bacteria capable of forming colonies on solid media is less than those actually present and metabolically active. On the same figure, acidity of the different samples is also shown, values close to 5 where used in all samples.

3.3. Monitoring of total, viable and no viable bacteria during vinegar production cycle

The developed method has been applied to follow a semi-continuous wine vinegar production cycle. The next results coming from eight repeated experiments under the same experimental conditions:

- discharge volume, 6 l (75% of total volume);
- charging flow rate, 2 l/h;
- final ethanol concentration, 2°GL;
- air flow rate, 7.5 l air/l medium/h;
- ethanol concentration in feed wine, 12°GL.

Fig. 5 shows the evolution of viable cells, measured by the direct epifluorescence method referred on material and methods, as well as the acetic acid concentration; time zero corresponds to the beginning of charging period. Viable cells decreased during the charging period due to the effect of dilution. Also, the cell growth can be followed through the cycle by the increasing values of cell concentrations. As expected, the raise of acidity in the medium followed a pattern similar to that of viable cells.

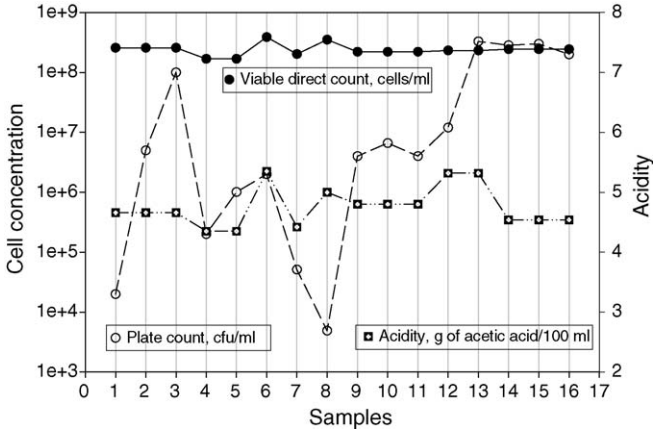

Fig. 4. Comparison between plate counting and direct microscopic enumeration on Neubauer chamber.

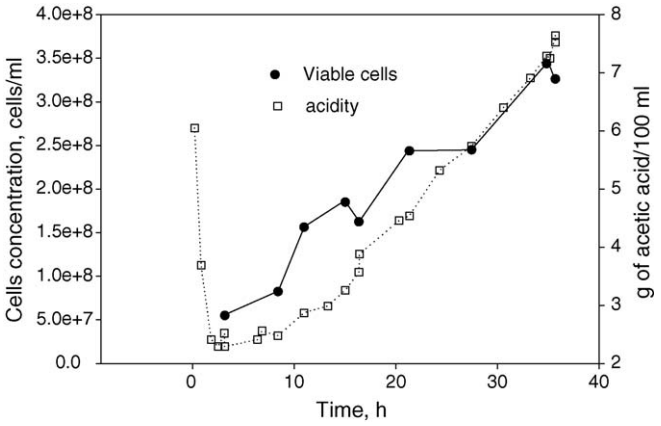

Fig. 5. Viable cell concentrations during a wine acetification semi-continuous process. Values from eight repeated experiments are shown.

4. Discussion

The proposed method has been successfully applied to quantify total microbial biomass and evaluate viable and non-viable cell concentrations. Total (red and green) and viable (green) cells can hence be counted simultaneously in only one staining step.

The LIVE/DEAD *Bac*Light bacterial viability kit, developed by Molecular Probes, has been widely used to assess the activity of bacteria [11,12]. Membrane integrity demonstrates the protection of cell constituents and its potential to generate gradients that endow ability to live, but does not guarantee cell replication. Cells with a damaged membrane cannot sustain any electrochemical gradient and are normally classified as dead cells. With respect to total counts, *Bac*Light counts with direct microscopic enumeration were equal to those obtained with flow cytometry (see Fig. 3). In contrast, analyses of the bacterial population in vinegar samples by direct microscopic enumeration, with direct epifluorescence techniques and colony counting, gave very variable results. Comparison between results of plate counting, direct Neubauer chamber enumeration with *Bac*Light stain showed that the number of bacteria capable of forming colonies on solid media was, in general, less than the number actually present and metabolically active, often by several orders of magnitude in vinegar samples (see Fig. 4). Since the principle of each method is different this observation was not surprising. However, what was striking is the fact that the results varied so much. In any case, for the referred repeated experiments (same conditions in all cases), meanwhile the direct bacteria counts seem to be according to the observed steady behaviour (see acidity, Fig. 4) the colony counting does not.

Furthermore, to survive in their natural environments, bacteria must constantly adapt themselves to changing environmental conditions and shift between growth and non-growth states [13]. These transitional stages between culturable and viable, or non-culturable and non-viable bacteria are indistinct and yet poorly understood [6,14,15]. On the other hand, direct microscopic counts of samples and directly stained with the fluorophores do not provide reliable estimates of viability, i.e. the ability of the bacteria viewed by this procedure to grow, metabolize, respire or divide. The difficulty in defining a clear line between the dead and live state requires new methods for detection of physiological stages in addition to the conventional plate count method (cfu), which is based on the bacteria's ability to reproduce. Meanwhile, the direct epi-fluorescence technique used in this work yields more reliable results than traditional plate count methods.

The differences between the Neubauer direct counts or *Bac*Light viability kit or flow cytometry and plate counts are due to difficulties in the handling of acetic acid bacteria outside of acetification process, cultivation outside of bioreactor and transfer from liquid to solid medium [4,6,16].

The method proposed to evaluate and follow biomass of vinegar samples during acetification process has several advantages: (i) it is a reliable, rapid, easy and yields both viable and total bacteria in only one step; (ii) samples are easy to prepare and easy to differentiate because of the high degree of contrast between the green colour of the viable bacteria and the red colour of the dead cells; (iii) *Bac*Light stain does not produce background fluorescence. The method described in this study is quicker and easier to use than described by Mesa et al. [6]; it is not necessary a centrifugation and new suspension in order to stain the cells. Also, the sample is not filtered through a black polycarbonate membrane; this step results in a non-homogeneous distribution of acetic acid bacteria throughout the filter, so the counting is not very precise.

In conclusion, the method proposed here can be considered, for vinegar samples, as a replacement to the conventional plate counts, which yields variable results and often underestimate the real number of viable bacteria present during different stages of acetification process.

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References

[1] Arnold S, Becker T, Delgado A, Emde F, Enenkel A. Optimizing high strength acetic acid bioprocess by cognitive methods in an unsteady state cultivation. J Biotechnol 2002;97:133–45.

[2] Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K. Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg 1906) comb. nov., *Acetobacter lovaniensis* (Frateur 1950) comb. nov., and *Acetobacter estunensis* (Carr 1958) comb. nov. J Gen Appl Microbiol 2000;46:147–65.

[3] Lisdiyanti P, Kawasaki H, Widyastuti Y, Saono S, Seki T, Yamada Y, et al. *Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the alpha-Proteobacteria. Int J Syst Evol Microbiol 2002;52:813–8.
[4] Sokollek SJ, Hertel C, Hammes WP. Cultivation and preservation of vinegar bacteria. J Biotechnol 1998;60:195–206.
[5] Millet V, Lonvaud-Funel A. The viable but non-culturable state of wine micro-organisms during storage. Lett Appl Microbiol 2000;30:136–41.
[6] Mesa MM, Macías M, Cantero D, Barja F. Use of the direct epifluorescent filter technique for the enumeration of viable and total acetic acid bacteria from vinegar fermentation. J Fluorescence 2003;13:261–5.
[7] Boulos L, Prévots M, Barbeau B, Coallier J, Desjardins R. LIVE/DEAD *Bac*Light™: application of new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microbiol Methods 1999;37:77–86.
[8] Hoefel D, Grooby WL, Monis PT, Andrews S, Saint CP. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. J Microbiol Methods 2003;55:585–97.
[9] Hermida M, Taboada M, Menéndez S, Rodríguez-Otero JL. Semi-automated direct epifluorescent filter technique for total bacterial count in raw milk. J AOAC Int 2000;83:1345–8.
[10] Bhupathiraju VK, Hernandez M, Krauter P, Alvarez-Cohen L. A new direct microscopy based method for evaluating in-situ bioremediation. J Hazard Mater 1999;67:299–312.
[11] Haugland RP. The handbook: a guide to fluorescent probes and labeling technologies. Web edition of the handbook. 10th ed. 1999. http://www.probes.invitrogen.com/handbook/ [Chapter 15, Section 3].
[12] Vives-Rego J, Lebaron P, Nebe-von Caron G. Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol Rev 2000;24:429–48.
[13] Trainor VC, Udy RK, Bremer PJ, Cook GM. Survival of *Streptococcus pyogenes* under stress and starvation. FEMS Microbiol Lett 1999;176:421–8.
[14] Joux F, Lebaron P, Troussellier M. Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. FEMS Microbiol Ecol 1997;22:65–76.
[15] Decker E-M. The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral Streptococci. Lett Appl Microbiol 2001;33:188–92.
[16] Kittelmann M, Stamm WW, Follmann H, Trüper HG. Isolation and classification of acetic acid bacteria from high percentage vinegar fermentations. Appl Microbiol Biotechnol 1989;30:47–52.