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Flow cytometry, a sustainable method for the identification and quantification of microorganisms in enology - Part 1/2 Review of the usual methods applied in wine microbiology and the principle of flow cytometry

Cédric Longin[1], Rémi Laforgue[1], Marie-Laure Badet-Murat[1], Hervé Alexandre[2]

1 OENOTEAM, 17 Chemin de Verdet, 33500 Libourne/7 Rue de l'Industrie, 33250 Pauillac, France, info@oenoteam.com
2 UMR Procédés Alimentaires et Microbiologiques, Equipe VAlMiS (Vin, Aliment, Microbiologie, Stress), Institut AgroDijon, Université de Bourgogne/Franche-Comté, IUVV, Rue Claude Ladrey, BP 27877, 21000 Dijon, France

Flow cytometry (FC) is a powerful technique for the detection, characterization and quantification of microbial populations in enology. Depending on the fluorescent markers and specific probes used, FC can provide information on the physiological state of the cell and allows the quantification of a microorganism of interest, or spoilage microorganisms such as *Brettanomyces bruxellensis*, within a mixed population.

Specifications for a microbiological analysis

To guide decision-making in the field, the analysis must provide answers to the following questions:

1/ WHICH ONES? i.e. which yeasts or bacteria and which species are present.

2/ HOW MANY? The goal being to quantify a given population.

3/ Is this population ACTIVE? This concept of VIABILITY (= metabolic activity) is an essential factor.

Review of the usual methods

Among the usual methods, two are used routinely: culture on a nutrient agar medium and quantitative PCR (qPCR).

Long considered the reference method for the enumeration of cultivable microorganisms, culture on a nutrient agar medium has many drawbacks (Table 1). For this reason, operators have turned to "culture-free" methods based on DNA quantification (qPCR), made possible through better knowledge of yeast genomes (and that of *B. bruxellensis* in particular) and the continuous improvement of techniques in molecular biology.

Table 1 summarizes the advantages and disadvantages of these two techniques.

To overcome the many limitations of these methods, some laboratories have turned to flow cytometry (FC), a technique with many advantages.

Principle and performance of FC

FC is an analytical technique that can identify and count cells in suspension in a liquid, individually, quantitatively and qualitatively. When coupled with the use of fluorescent markers and specific probes, FC can provide information on the physiological state of the cell and allows for species-specific quantification within a mixed population.

FC has the decisive advantage that the analysis is performed directly on the sample (Figure 1). The fluid system aligns the cells which then flow past one or more lasers making it possible to determine the size and the relative granularity of each cell, as well as its fluorescence. As a result, the analysis is fast (a few minutes to a few hours depending on the labeling protocols). Combined with the short sample preparation time, FC is thus inexpensive and particularly well suited to monitoring population dynamics in the cellar.

TABLE 1. Advantages and disadvantages of the usual methods.

	Advantages	Disadvantages
Culture on a nutrient agar medium	Low cost	Underestimation due to non-quantification of "viable but non-culturable" (VNC) populations
		Lack of specificity despite the use of inhibitors
		Hazardous due to the use of antibiotics
		Slow due to incubation times
qPCR	Specificity	Underestimation due to DNA loss during extraction* (*Improvement: use of an extraction control, rarely implemented in practice*)
	Speed of response	Overestimation due to the quantification of dead cells* (*Improvement: Use of propidium monoazide or ethidium monoazide, rarely implemented in practice*)
		Poor reproducibility due to the technical complexity of extraction* (*Improvement: Automated extraction, rarely implemented in practice*)
		No information on cell viability
		No comprehensive view of the populations present

*Longin *et al.*, 2016[1].

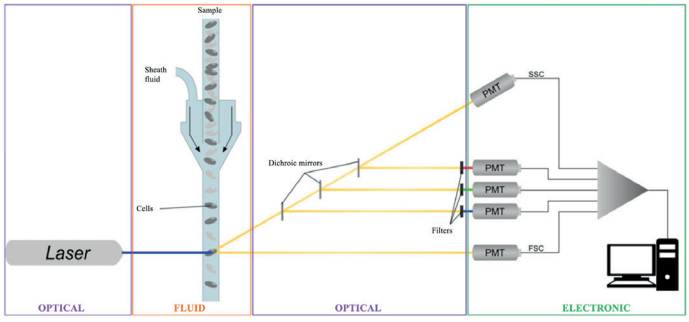

FIGURE 1. Schematic diagram of an FC instrument (Longin *et al.*, 2017)[2]. PMT: photomultiplier tube. FSC: forward scatter (size). SSC: side scatter (granularity).

These time and cost savings only apply to laboratories capable of making up the labeling solutions and buffers. The major drawback of FC is that reading cytograms requires a certain level of operator expertise and is therefore not easily accessible to evervone.

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Quantification of total active yeasts and bacteria

Given that bacteria are smaller and have lower granularity than yeasts, they can easily be distinguished using FC. On a wine matrix, a fluorescent DNA marker is needed to distinguish bacteria from other particles.

For a given population, FC coupled with the use of viability markers also makes it possible to identify cells with metabolic activity, i.e. to distinguish between active and non-active cells[3]. As each cell or set of linked cells is analyzed directly, this technique provides excellent sensitivity. A limit of quantification of 1 cell/mL can thus be achieved, depending on operating procedures and the instrument used, with excellent repeatability (Figure 2, supplementary data).

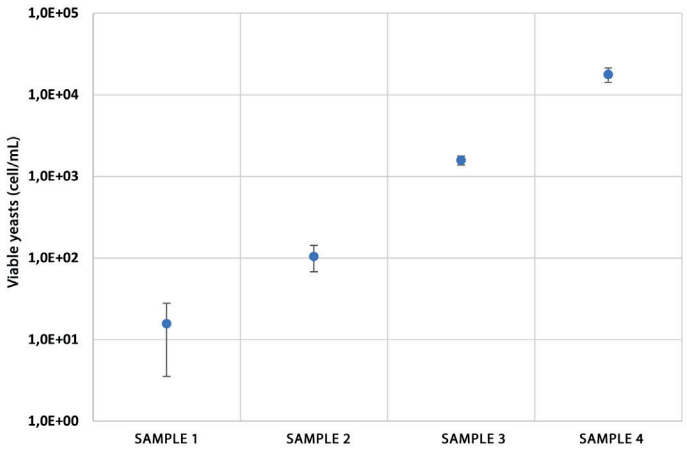

FIGURE 2. Repeatability of our method for quantification of total active yeasts by FC (4 samples with theoretical populations of 1.7^{E+1}; 1.7^{E+2}; 1.7^{E+3} and 1.7^{E+4} yeast cells/mL with 20 repetitions using 2 instruments and performed by 2 operators).

The greater the cellular metabolic activity, the more the fluorescent molecule accumulates in the cell[2]. This allows discrimination between yeasts with high viability (very active or in the multiplication phase, for example), and yeasts with low viability (in decline or under stress). However, no study has demonstrated discrimination between yeasts at species level based on these non-specific markers and these morphological criteria (size/granularity), in particular in the case of *B. bruxellensis*. For this reason, specific quantification methods have been developed.

Species-specific quantification of *B. bruxellensis*

Different cellular targets can be labeled, and different specific markers can be used.

The use of specific antibodies allows quantification via dual antibody/viability marker labeling[4]. However, adsorption of wine compounds by the cell wall can bias the labeling.

Other work has led to the development of a more robust method, using fluorescence in situ hybridization (FISH) with a peptide nucleic acid (PNA) specific to *B. bruxellensis* coupled with fluorescence microscopy[5]. Although extremely specific, this technique has the major drawback that the quantification threshold is too high (in the order of 10^4 cell/mL) to be able to take action before spoilage of the wine. In addition, it is very expensive compared with alternative methods, whatever they may be.

More recent work has resulted in the development of fluorescent probes that are specific for the ribosomal RNA (rRNA) of *B. bruxellensis*[6, 1]. The specific fluorescent probe is brought into contact with the cells after permeabilization. If the target rRNA is present in the cell, i.e. if the cell belongs to the species *B. bruxellensis*, hybridization takes place between the endogenous cellular rRNA and the exogenous fluorescent probe. Due to the specificity of the probe, only cells belonging to the species *B. bruxellensis* fluoresce. The use of these probes, coupled with FC, therefore guarantees specific quantification of this species within a mixed population.

In addition to this major advantage, the abundance of rRNA combined with the fact that CMF analyzes each cell individually, makes it possible to achieve very high sensitivity. This varies depending on the flow cytometer used. Our method, which is available for use under license, can detect down to 5 cell/mL with an analysis time of 24 hours.

Conclusions

The microbiological analysis methods routinely applied in wine microbiology (culture, qPCR) only provide a partial, and sometimes erroneous, view of the microbial population present. Coupled with the use of different markers, FC allows the precise quantification of cells and provides information on their physiological state. The many resulting technical applications will be presented in a second article which also sheds new light on the environmental impact of the various methods presented. ■

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