APPLICATION NOTE

Evaluation of Yeast Viability and Concentration during Wine Fermentation Using Flow Cytometry

MICROBIAL CYTOMETRY BD FACSCalibur, BD FACSort Flow Cytometers

Introduction

Wine production depends on a controlled fermentation of grape juice by a known variety of yeast. The concentrations of total and viable yeast are important indicators of the health of the fermentation. Historically, these parameters have been measured using methylene blue staining of the yeast population, a hemacytometer, and a microscope, but this method is subject to error and operator fatigue. Agar-based growth assays are slow, requiring 24 to 48 hours of incubation. Flow cytometry provides a rapid and accurate means to monitor the concentration and viability of yeast throughout the fermentation process, as well as to detect the presence of spoilage organisms such as *Zygosaccharomyces, Dekkera (Brettanomyces)* and *Lactobacillus*.[1-3] The technique of flow cytometry offers the possibility of near real-time monitoring of microbial populations in industrial fermentations.

The brewing industry has long had an interest in applying flow cytometry to the microbiology of the brewing process. Hutter, et al[4-7] determined the purity of yeast cultures using immunofluorescence and flow cytometry, as well as using antibodies to discriminate the presence of other organisms in fermentations. They also monitored yeast cell cycle in hourly samples to evaluate the state of a fermentation.[8] Fluorescent dyes and flow cytometry have been used to distinguish between stressed and non-stressed populations of brewing yeasts.[9-10] Yeast starters have been brought to the optimal proliferative state for inoculation by monitoring the cell cycle using flow cytometry.[11]

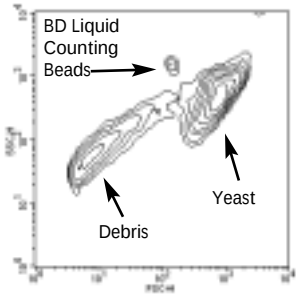

Figure 1A

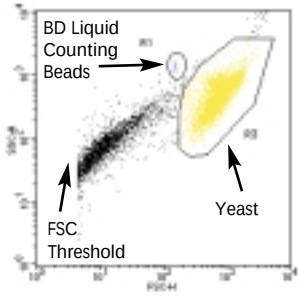

Figure 1B

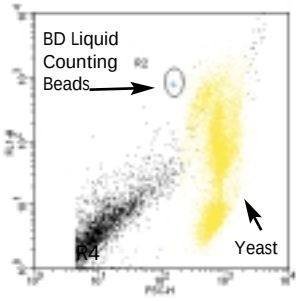

Figure 1C

Figure 1 Gating strategy for yeast and beads in a fermentation culture

A. FSC vs SSC contour plot showing the expected positions of yeast and beads

B. FSC vs SSC dot plot showing the regions defining beads (R1) and yeast (R3)

C. FSC vs FL1 dot plot showing the region defining beads (R2), with beads identified by gating on R1 AND R2, and yeast identified by gating on R3

Also, the health of a yeast population has been monitored by determining the glycogen content by flow cytometry.[12] More recently, flow cytometry has been applied in the wine industry to quantify wine yeast viability,[13-14] bacteria viability,[15] and to sort wine yeast populations.[16]

The use of flow cytometry with DNA-reactive dyes to quantify viable and total yeast populations during the fermentation of grape juice is reported in this note. Live cells have intact membranes and are impermeable to dyes such as propidium iodide (PI), which leaks only into cells with compromised membranes. Thiazole orange* (TO) and SYTO™16†[17] are permeant dyes and enter all cells, live and dead, to varying degrees. Thus a combination of PI and a permeant dye provides a rapid and reliable method for discriminating live and dead yeast. If enumeration of the yeast is important, BD Liquid Counting Beads (BD Biosciences, San Jose, CA), a flow cytometry bead standard, can be used to accurately quantify the concentrations of live, dead, and total yeast in a sample.

Materials and Methods

Cells

Grape juice from a commercial crush (E & J Gallo Winery, Modesto, CA) was inoculated with *Saccharomyces cerevisiae* and maintained at 20°C to obtain fermentation samples for method development. For cytometry, a sample of the fermentation was diluted 200- to 1,000-fold in staining buffer. Alternately, a 500-µL juice sample was centrifuged in a 1.7-mL microfuge tube at 7,000 x *g* for 3 minutes; then the supernatant was decanted. The pellet was resuspended with 500 µL of either staining buffer or phosphate-buffered saline (PBS), pH 7.4, and then further diluted 100-fold in PBS to be stained for flow cytometry. Samples and balling (sugar concentration) data from commercial-scale fermentations were provided by E & J Gallo Winery.

Reagents

1. Thiazole orange solution, 17 µM (BD Biosciences Catalog No. 349483 or 349480), or equivalent at 8.1 µg/mL (FW 476.6) in dimethyl sulfoxide (DMSO)
   Or
   1 mM SYTO16 (Molecular Probes, Eugene, OR, Catalog No. S-7578), diluted 50-fold to 20 µM in DMSO

2. Propidium iodide solution, 1.9 mM (BD Biosciences Catalog No. 349483 or 349480), or equivalent at 1.3 mg/mL (FW 668.4) in water

3. BD Liquid Counting Beads (BD Biosciences Catalog No. 349480), optional (for counting)

4. Staining buffer: phosphate-buffered saline, 1 mM EDTA, 0.2% Pluronic™ F-68 (BASF Corporation, Mount Olive, NJ, Catalog No. 51554728), 0.1% sodium azide, pH 7.4
   Tween-20 at 0.01% can be substituted for Pluronic F-68. The staining buffer should be passed through a 0.22-µm filter and used within two weeks.

* US Patent Nos. 4,883,867 and 4,957,870
† US Patent Nos. 5,436,134 and 5,534,416

Equipment

1. Disposable 12 x 75-mm capped BD Falcon™ polystyrene test tubes (BD Biosciences Discovery Labware, Bedford MA, Catalog No. 352052), or equivalent
2. Vortex mixer
3. Micropipettor with tips (Pipetman™, Rainin Instrument Company, Emeryville, CA) or equivalent
4. 1.7-mL microfuge tubes
5. Centrifuge
6. BD FACS™ brand flow cytometer (BD FACSCalibur™ flow cytometer or equivalent) equipped with 488-nm laser excitation

Staining with Cell-Reactive Fluorophores

Cells in 400 µL of buffer, prepared as described previously, were labeled with permeant dye by adding either 40 µL of 17 µM TO for a final concentration of 4.2 µM, or 8 µL of 20 µM SYTO16 for a final concentration of 400 nM. To label dead cells, 10 µL of 1.9 mM PI was added for a final concentrations of 48 µM. To obtain cell concentrations, BD Liquid Counting Beads were added to the tube. Cell preparations were stained for 5 to 20 minutes.

The fluorescent beads were gated separately, and the assigned bead count was used to determine the sample volume.

Flow Cytometer Setup

A BD FACSort™ flow cytometer was optimized manually using BD CaliBRITE™ Beads and BD FACSComp™ software (BD Biosciences) to verify instrument performance. Equivalent performance is observed for a BD FACSCalibur flow cytometer.

PMT voltages and threshold levels were adjusted using an unstained suspension of yeast. The yeast population was positioned so that it was entirely on scale on an FSC vs SSC plot (Figure 1A). For fluorescence measurements, FL1, FL2, and FL3 PMT voltages were adjusted to place the unstained yeast population on-scale in the lower left quadrant of two parameter plots (data not shown).

Instrument settings for the following results were:

Threshold—FSC

FSC—E00, logarithmic amplification

SSC—375 V, logarithmic amplification

FL1—535 V, logarithmic amplification

FL2—520 V, logarithmic amplification

FL3—600 V, logarithmic amplification

Compensation—none used

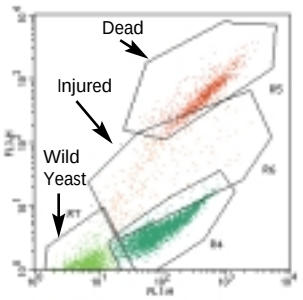

Figure 2A

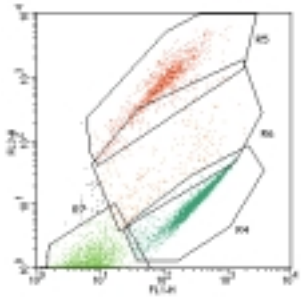

Figure 2B

Figure 2 Analysis strategy discriminating live, dead, and injured yeast

A. FL1 vs FL3 for a fermentation culture stained with SYTO16 and PI, gated on yeast (R3, defined in Figure 1)

B. FL1 vs FL3 for a fermentation culture stained with TO and PI, gated on yeast (R3), with live yeast (green) identified by gating on R3 AND R4, dead yeast (red) by gating on R3 AND R5, injured yeast (orange) by gating on R3 AND R6, and distinguishing wild yeast (yellow) in the grape juice using R3 AND R7

Data Acquisition and Analysis

Data files were acquired and analyzed using BD CellQuest™ software (BD Biosciences). Fifty thousand events (ungated) were acquired per sample in Acquisition-to-Analysis mode. The yeast population and beads were identified using gates in FSC vs SSC and FSC vs FL1 (Figures 1B and 1C). The contour plot (Figure 1A) shows where breaks occur between populations while the multicolor dot plots better display population identities. Live and dead organisms were discriminated in an FL1 vs FL3 plot, gated on yeast (Figure 2).

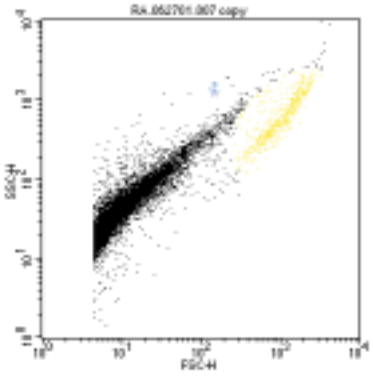

Figure 3 Appearance of a yeastlike population in grape juice that might represent wild yeast. FSC vs SSC dot plot showing position of wild yeast (yellow), analyzed as in Figure 2

Results and Discussion

Figure 1 shows the resolution of yeast populations from background in a grape-juice fermentation using FSC and SSC. Fermentations typically contain a large amount of debris from grape residue and yeast, especially in the early phase of fermentation where the yeast is present in a lower concentration. Yeast can generally be defined by a region in FSC vs SSC (R3, Figure 1B). Because of the size range of the yeast relative to other components in a fermentation, it was advantageous to use logarithmic amplification with FSC. Although yeast can be adequately analyzed in PBS alone, addition of surfactant (Pluronic F-68 or Tween-20), as in the defined staining buffer, can improve resolution from debris. Maintaining an event rate below 1,000 per second can further improve resolution. If there is unacceptable contamination of the stained yeast by debris, further gating using FL1 or setting an FL1 threshold can improve yeast identification.

To determine cell concentration, BD Liquid Counting Beads can be resolved from background using FSC, SSC, and FL1. Figures 1B and1C show how beads can be defined by gating on R1 AND R2. Cell concentration can be determined as follows:

$$\frac{\text{\# events in region containing cell population}}{\text{\# of events in bead population}} \times \frac{\text{\# of beads per test*}}{\text{test volume}} \times \text{dilution factor} = \text{concentration of yeast population}$$

Yeast shows considerably slower uptake kinetics of permeant dyes than mammalian cells. The recommended concentrations of TO and SYTO16 are 5- to 10-fold higher than would be expected for other cell types and give adequate staining of *S. cerevisiae* within 5 minutes although brightness continues to increase for reaction times up to about 20 minutes. PI enters dead cells rapidly.

Figure 2 shows the region strategy for identifying live, injured, and dead yeast. When gated on the region defining yeast by scatter (R3), the live and dead populations are well resolved on FL1 vs FL3, with an intermediate population representing injured or dying cells. Figure 2A shows the results obtained with SYTO16 and PI; Figure 2B shows the results obtained with TO and PI. Although results are very similar, slightly greater resolution of populations could be achieved with the TO and PI mix.

A fourth population (R7) in Figure 2 could be resolved as well. This population has the scatter properties of yeast, but much lower uptake of either TO or SYTO16. The population can be observed in the starting grape juice (Figure 3), and is assumed to represent wild yeast present in the crush.

When a commercial-scale fermentation is followed, dramatic changes can be observed in the yeast population. Figure 4 shows yeast viability vs balling (sugar content) across a 19-day stirred fermentation of Chardonnay juice. Viability was found to be uniformly high following some early

* This value varies with each lot and is provided with the reagent.

fluctuation, probably because conditions had not yet become very stressful for the yeast at the end of the test period. Yeast concentration from the same fermentation (Figure 5) climbs dramatically during early fermentation, after the initial drop while juice was added to the starting culture.
The concentrations determined using a flow cytometer agreed closely with hemacytometer values (data not shown). Yeast concentration leveled off after about day 8.

Flow cytometry provides a unique window on the quantification of individual cells in fermentation. Permeant and impermeant dye combinations are readily adapted to viability assays, as discussed previously. Other fluorescent reagents can be used to identify cell type or metabolic state. Evaluation of viability and concentration by flow cytometry is sufficiently rapid to allow the near real-time assessment of cultures and can assist in the understanding of problems that arise during fermentation.

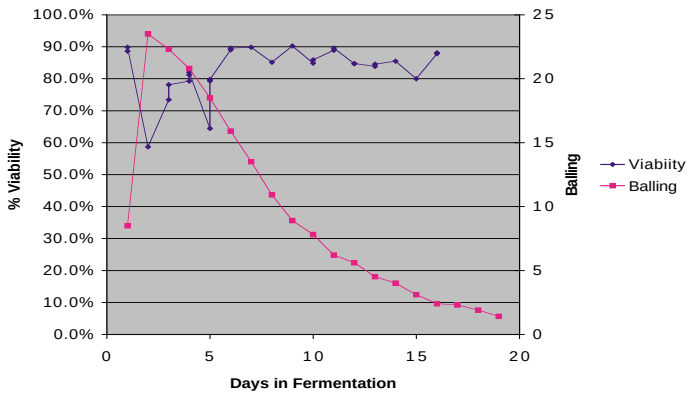

Figure 4 Yeast viability and sugar concentration over time in fermentation. Samples stained with SYTO16 and PI, and analyzed after the addition of BD Liquid Counting Beads

Hints

- TO and SYTO16 fluoresce primarily in FL1 and FL2; PI primarily fluoresces in FL3. Therefore, the best discrimination of live and dead populations is on an FL1 vs FL3 plot. This method can be applied with a variety of buffer systems, but optimal resolution requires some surfactant to be present in the staining buffer.
- In practice, staining protocols must be adjusted to the organisms being analyzed.
- TO and SYTO16 are hydrophobic. Solutions should be maintained in DMSO. Dye concentration will decrease over time in aqueous solution due to adsorption to surfaces although the effect is more pronounced for SYTO16 than for TO.
- SYTO16 staining is adequate for analysis at 5 to 10 minutes but requires at least 20 minutes to achieve maximum intensity. PI stains very quickly while SYTO16 enters the cells more slowly.
- Setting FSC and SSC on logarithmic amplification assures that a wide range of cell sizes, yeast to bacteria, can appear on-scale and helps present recognizable populations for gating.
- An event rate of ≤1,000 events per second minimizes the chance of coincidence and improves population resolution. High event rates can be corrected either by dilution or by decreasing the sample flow rate on the instrument.
- At least 1,000 bead events should be collected to provide reliable concentration data.
- If the population of interest cannot be adequately resolved using an FSC threshold alone, a secondary threshold on FL1 can be used. An FL1 threshold alone might not be adequate due to the large amount of small fluorescent debris that could be present in a stained sample.
- As the concentration of yeast decreases, background noise will become progressively more prominent.

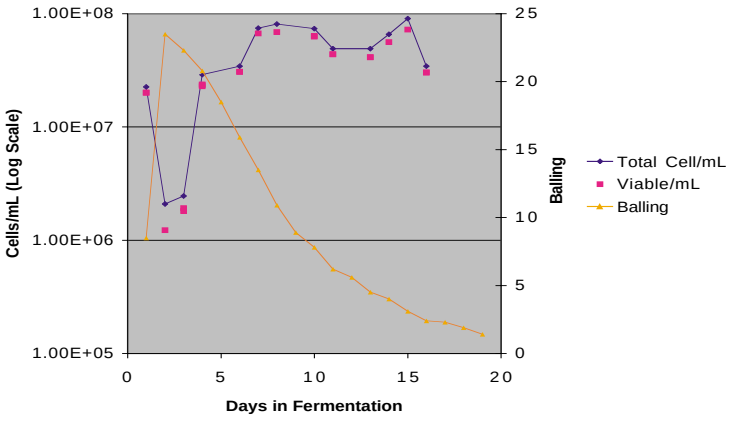

Figure 5 Total and viable yeast cell number and sugar concentration over time in fermentation. Samples stained with SYTO16 and PI, and analyzed after the addition of BD Liquid Counting Beads

BD Biosciences publishes this method as a service to investigators. Detailed support for non-flow cytometric aspects of this procedure might not be available from BD Biosciences.

- If high background counts are observed on an instrument, the staining buffer and sheath fluid should be checked for particles. An instrument cleaning cycle and a drain/fill cycle can also reduce noise.
- Samples can be checked by fluorescence microscopy to confirm that the target organisms are stained.
- Stained samples and extra dye solution should be disposed of according to local regulations.

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