

Flow Cytometry—A New Tool for Direct Control of Fermentation Processes

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ABSTRACT

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With flow cytometric cell cycle analysis, fermentation processes become transparent. This implies that we can control the process, by determining growth phases of the yeast population at specific times during the fermentation process, and that the process can be optimised and regulated directly.

Key words: Cell cycle analysis, flow cytometry, yeast.

INTRODUCTION

In the last decade, molecular biology was successfully used to decode the genome of *Saccharomycetaceae*. However, despite this important molecular biotechnology event, much still remains to be discovered about the cell kinetics and the cell physiology of brewing yeast during the fermentation process. Today the brewing industry has numerous commercial and technological problems. The commercial imponderables include:

1. Consumer behaviour has changed
2. Dumping philosophies affect beer pricing
3. Insolvency of smaller breweries leads to a concentration of brewery groups.

However, one of the most important technological problems is the rapid fermentation of beer in cylindroconical tanks. Today, cylindroconical fermentations are monitored by indirect methods (Fig. 1). These methods only provide limited data about the fermenting process and yield no information about the physiological status of the yeast population.

“The need for more specific and direct procedures have to be recognized and should be integrated in breweries’ laboratory control.” This statement of principle by Quain⁶, which is also my opinion, is only accepted by a small segment of the bigger breweries in Germany, and they have integrated this principle into their yeast management process using molecular techniques and flow cytometric and image analysis.

In former times, conventional fermentations were controlled empirically by studying the development of the yeast’s cover during the process. Corresponding to the view of the cover, brewmasters knew the growth state of the population (Fig. 2).

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In 1992, at Eichbaum breweries AG, we introduced the fluorescence-optical technique of flow cytometry to control young beer fermentations, and image analysis to score contaminants in filtered beer for final product quality³⁻⁵.

MATERIALS AND METHODS

A mono-dispersed yeast cell suspension is forced through a nozzle or a quartz glass chamber by air pressure and hydrofocussing. Cells are arranged in a laminar stream, like a string of pearls, one behind the other. Figure 3 is a schematic of a flow cytometry system.

The yeast cells are fluorochromized, corresponding to intracellular macromolecules such as DNA or glycogen, or extracellularly labelled by a fluorescence antigen-antibody reaction. The yeast cells then flow through a focussed area

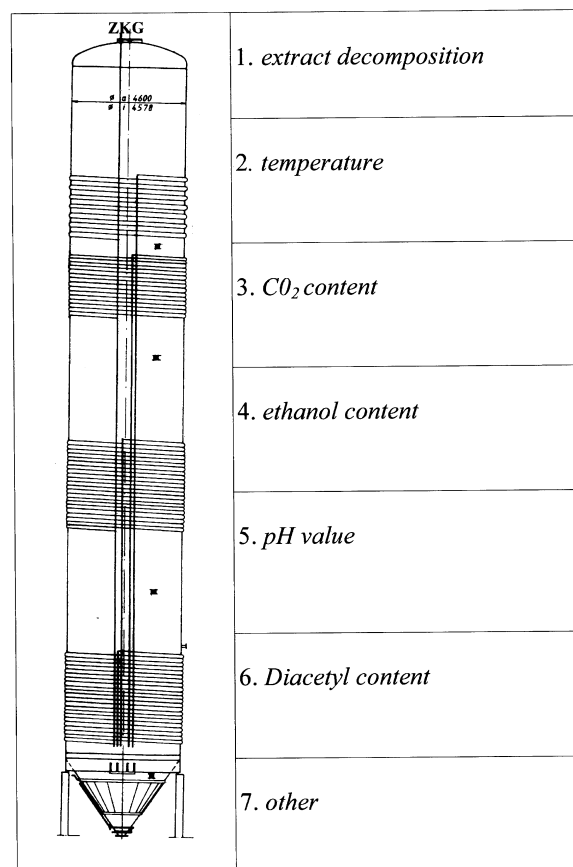


FIG. 1. Factors affecting the growth state of the yeast population in a cylindroconical fermenter.

(2 μm^2) of a laser beam or a mercury high pressure lamp (light sources vary and correspond to the flow system used). After excitation of the treated cells, the emission of fluorescence light is directed over a filter- and dichroic mirror-system, to several photomultipliers according to their spectral colours (green fluorescence is detected by PMT1, orange fluorescence by PMT2, while red fluorescence is detected by PMT3, etc.). Scatter signals give information about cell volume (forward light scatter, FLS) and cell granularity (side scatter, SS, measured at a 90° angle).

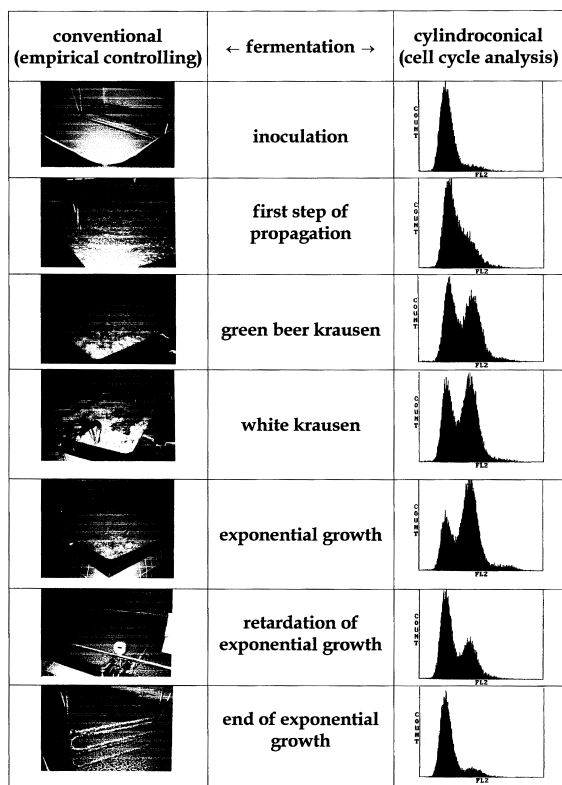


FIG. 2. Biomonitoring of fermentations: conventional fermentation monitoring and monitoring by cell cycle analysis of cylindroconical fermentations. Left column: Process control on the basis of empirical observations of the yeast cover. Right column: Flow cytometric frequency distributions according to growth phases of the yeast population during a closed cylindroconical fermentation.

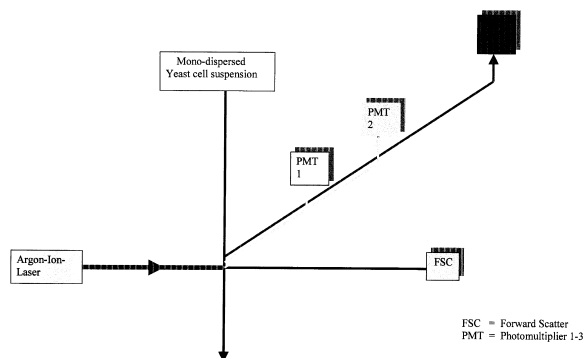


FIG. 3. Schematic of a flow cytometric device.

RESULTS AND DISCUSSION

The advantage of flow cytometric analysis is ultra rapid measuring of a high cell rate (1,000 to 10,000 cells per second). This can yield data of high statistical significance. Modern flow cytometers are equipped with the feasibility to differentiate eight fluorochromes and two scatter signals. This gives a flood of distinct information about each yeast cell, and is presented in a split second.

For our biomonitoring, DNA content is the most important macromolecule to study the cell cycle and the cell kinetics of the yeast in process and this paper will only discuss DNA content. The flow cytometric DNA profile of a lager and an ale yeast can be distinguished (see Figs. 4 and 5). Lager yeast have an approximate diploid DNA content. According to Howard and Pelc² the cell cycle of eucaryotes (mammalian cells and lower eucaryotes such as *Saccharomyces* yeasts) can be divided into three growth phases. Using flow cytometry, the DNA content of these

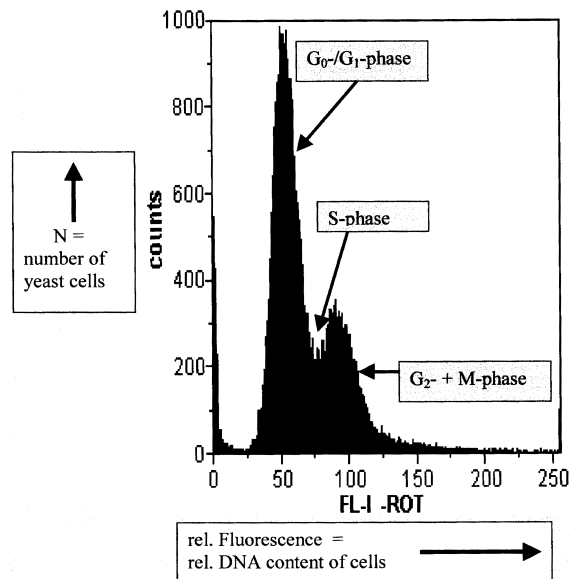


FIG. 4. DNA distribution of a lager yeast. The diploid state of yeast cells is indicated by characteristic G_0-/G_1- , S-, and $G_2- + M$ -phase.

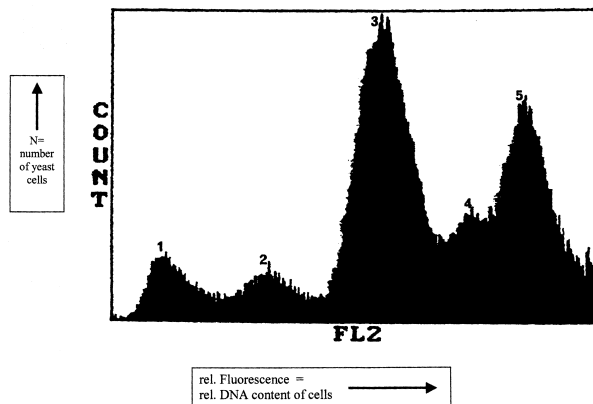


FIG. 5. Multi peak distribution of an ale yeast. The single DNA peaks of different clones lead to an overlap so it is impossible to identify the cell cycle profiles of each individual clone.

three cell fractions of an unsynchronised population are arranged into two peaks and there is a cell fraction between both of them¹.

The cell fractions represent:

1. G_0 -/ G_1 -phase-cells are recruited under the first peak. These are non-proliferating quiescent (G_0 -) and proliferating (G_1 -) cells with uniform diploid DNA content.
2. Under the second peak, yeasts are assembled with uniform tetraploid DNA content, so called G_2 - + M-phase cells. These large budding yeasts divide in a short time span (mitotic cells).
3. The cell fraction between $G_1 = gap1$ and $G_2 = gap2$ consists of replicating cells. These synthesizing S-phase cells have a non-uniform DNA content.

During the fermentation process yeast have optimal growth conditions. Starting with cropped yeasts, cells are in their quiescent, non-proliferating growth phase. That means viable cells of the population nearly simultaneously start their individual cell cycle. The cell cycle course in the fermentation process is shown in Fig. 6. The figures shows two lager yeast strains, a non-flocculent (Staubhefe, left column) and a flocculent (Bruchhefe, right column) strain. At the beginning, with the inoculation of the cropped yeast (left column = non-flocculent strain) all cells were in the quiescent and G_1 -phase respectively. After 8 h (see arrow) a small cell fraction starts DNA replication. At this

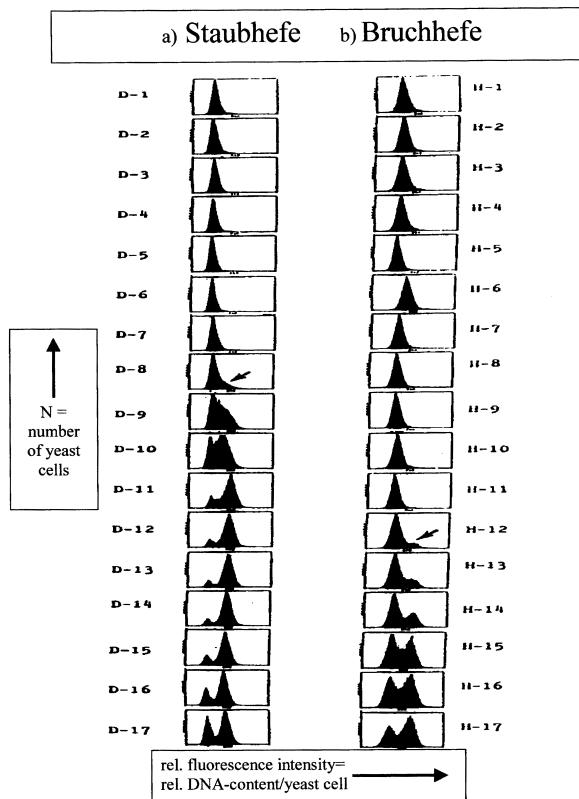


FIG. 6. a: Flow cytometric cell cycle course of a non-flocculent (Staubhefe) lager yeast strain. Beginning of DNA replication after 8 h (arrow). b: Flow cytometric cell cycle course of a flocculent (Bruchhefe) lager yeast strain. A retardation occurs at all stages of the cell cycle of the flocculent lager strain. Beginning of DNA replication after 12 h (arrow).

time cells with a small bud are noticeable. After 12 h, cells enter the exponential growth phase and most of the cells now have a daughter cell. At the end of the G_2 -phase the yeast cells divide into two cells. After mitosis both daughter cells will start their individual cell cycles. Cell cycle growth phases of the flocculent lager yeast require an adaptation phase of 12 h to start DNA replication, and a further 3 h to enter the exponential growth phase. All further stages of growth are delayed for several hours.

Ale yeast strains are usually polyploid or aneuploid. The DNA frequency distributions have more than the two peaks shown with the diploid lager yeast strains. That suggests that ale yeasts have two or three clones with different DNA contents (Fig. 7). Each clone has its own individual cell cycle with characteristic G_1 -, S- and G_2 + M-phases. But during the fermentation process, the different clones start their cell cycle with a time delay. This phenomenon is called diauxie. Because of the overlapping of cell cycle phases of these clones, an identification of DNA content of all distinct clones is not possible. DNA profiles with typical one or two peak distributions, according to the cell cycle phase, can be recognized as diploid yeast strains, while DNA profiles concerning three or more peaks, suggest typical ale yeast strains.

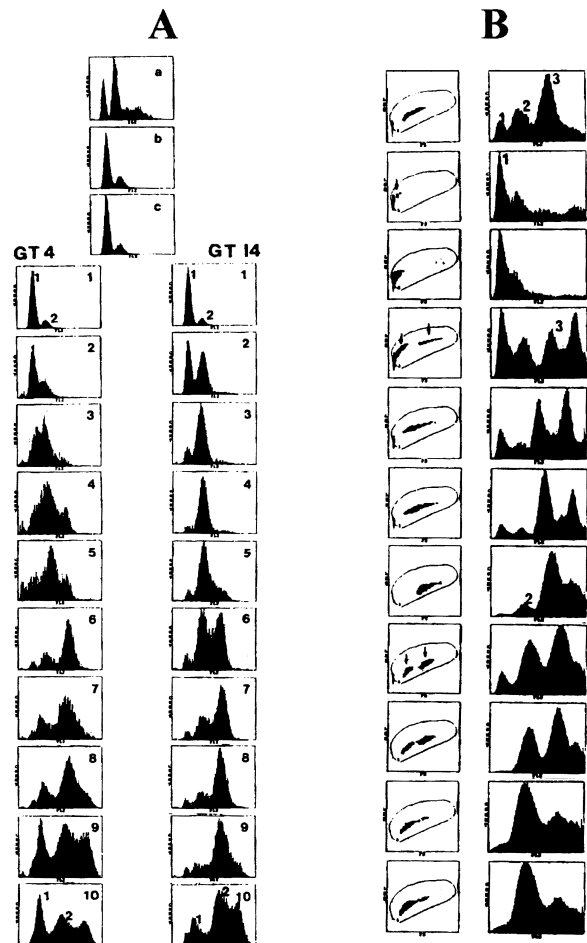


FIG. 7. Flow cytometric biomonitoring of different ale yeast fermentations. Both columns show different clonal growth. This is noticeable in the histogram by the accumulation of fluorescent counts (i.e., DNA content of single cell counts).

In process, ale yeasts are fast growing cells. One clone of this mixed population starts with cell proliferation, while the others persist in the quiescent state. When the first clone has finished aerobic cell growth, the next is following the cell cycling, etc. The first one has now already started with anaerobic cell growth. Anaerobic growth of yeast cells is accompanied by retardation of G₁-, S-, and G₂- + M-phases. During G₁-phase yeast cells metabolise extract. In this manner all clones alternate to carry out their individual cell cycles.

In conclusion, with flow cytometric cell cycle analysis, fermentation processes become transparent. That implies that we can control the process by determining growth phases of the yeast population at specific times during the fermentation process and that the process can be optimised and regulated directly.

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