Flow Cytometry for Age Assessment of a Yeast Population and its Application in Beer Fermentations

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ABSTRACT

J. Inst. Brew. 115(3), 253–258, 2009

An expeditious method of yeast age estimation was developed based on selective bud scar staining (Alexa Fluor 488-labelled wheat-germ agglutinin) and subsequent fluorescence intensity measurement by flow cytometry. The calibration curve resulting from the cytometric determination of average bud scar fluorescence intensities vs. microscopically counted average bud scar numbers of the same cell populations showed a good correlation and allowed routine cell age estimation by flow cytometry. The developed method was applied for yeast age control in traditional batch and continuous beer fermentations. At the pitching rates used in industrial beer fermentations, our results support former findings by locating a gradient of increasing yeast age from the top to the bottom zone of the fermenter cone. The results also indicate that in continuous beer fermentation, the increasing bud scar fluorescence of immobilized cells could help to schedule the replacement of aged biomass, prior to loss of viability or deterioration of process performance and product quality.

Key words: aging, beer, bud scar, flow cytometry, staining, yeast.

INTRODUCTION

The high quality (vitality) of a living biocatalyst strongly influences the course of a cell catalyzed process. The term vitality describes a group of characteristics and capabilities of microorganisms related to their metabolic activity and stress resistance[12]. Aging is a gradual process characterized by degenerative changes that weaken the functioning of an organism. Therefore the performance of cell catalyzed processes may gradually decrease as a result of biocatalyst aging.

The yeast *Saccharomyces cerevisiae* is known to have a genetically determined replicative lifespan, which is influenced by culture conditions. For instance brewing yeast are capable of only a finite number of divisions (10–30 divisions) prior to entering a non-replicative state termed senescence, terminating in cell death and autolysis[15]. During aging, yeast display a whole array of changes including increase in size, cell surface wrinkling, increase of generation time, increasing bud scar number and decreased metabolic activity[1,10,16].

From a brewer's perspective, however, it is the changed flocculation characteristics and fermentation performance of old yeast that is of considerable importance[17]. Industrial beer fermentation is carried out in vertical tanks with a conical bottom section. Towards the end of fermentation, yeast begins to sediment in the form of large cell clumps (flocs) into the fermenter cone. This biomass is not discarded, and it can be reused (re-pitched) several times. Since incorrect yeast harvesting may select for a population with an imbalance of young or aged individuals, a suitable cropping mechanism and a method of rapid monitoring is requisite.

The question of yeast aging is also important in continuous fermentation processes taking into consideration the long periods of time (several months) that immobilized cells spend in a continuous reactor[21]. The viability and fermentation capacity (vitality) of immobilized brewing yeast in continuous fermentations have been reported to decrease over time[9]. Hence, a rapid and sufficiently accurate method of yeast age determination could ensure stable fermentation performance of bioreactors with immobilized yeast.

In the case of a yeast cell population, a decrease in the proportion of living cells is only one of the parameters that can be used to assess yeast aging. The number of dead cells, a parameter frequently used in the brewing industry, does not give a complete and timely picture of the changes that take place in an aging yeast cell population. The number of bud scars present on the yeast surface is directly related to the number of times a cell has divided and thus represents a biomarker for replicative cell age estimation. Traditional techniques of staining and microscopic counting of bud scars are tedious, labor demanding and do not provide real-time information on a large number of cells. Conversely, flow cytometry offers the prospect of real-time analysis of a yeast population's characteristics[6,13]. The feasibility of a flow cytometric method of yeast bud scar determination was verified in beer fermentation technology.

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Publication no. G-2009-1022-1013

MATERIALS AND METHODS

Microorganisms and medium

A bottom fermenting brewing yeast strain (*Saccharomyces uvarum subsp. carlsbergensis*) no. 96 obtained from the culture collection of brewing yeast (Research Institute of Brewing and Malting, Plc., Prague, Czech Republic) was used throughout the experiments. The yeast were grown either on a complex medium (CM) with the following composition (in g L^{-1}): 5.0, KH_2PO_4; 2.0, $(NH_4)_2SO_4$; 0.4, $MgSO_4.7H_2O$; 2.0, yeast extract (Merck, Darmstadt, Germany); 20, glucose; or on all malt wort of original extract 10% (w/w) prepared from powder wort concentrate (Research Institute of Brewing and Malting, Plc., Prague, Czech Republic). Both CM and wort were sterilized by autoclaving at 121°C, 100 kPa for 30 min. Phosphate buffered saline (PBS) contained (in g L^{-1}): 8.0, NaCl; 1.14, Na_2PO_4; 0.2, KCl; 0.8, KH_2PO_4 in distilled water.

Isolation of cells of different age

Biotinylation. The brewing yeast was allowed to grow in an Erlenmeyer flask with 50 mL CM on a rotary shaker for 48 h at 25°C. Before biotinylation, the cells were centrifuged (2,000 g, 3 min) and washed with ice cold PBS (pH 8.0) five times. The cells were re-suspended in PBS and the cell concentration was adjusted to 1×10^8 cells mL^{-1} (measured by microscopic counting) by dilution with PBS. This cell suspension (3 mL) was biotinylated with 12 mg EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, USA) in a test tube at 25°C for 30 min.

Cell aging. The biotinylated cells were centrifuged (2,000 g, 3 min), re-suspended in 10 mL of CM and allowed to grow in a test tube placed on a rotary shaker at 25°C for 8–48 h depending at what age it was desired to separate the biotinylated cells or their offspring. For instance, the biotinylated cells allowed to grow for 48 h enter into several cell cycles thus accumulating numerous bud scars on their surface. Conversely the biotinylated cells, allowed to grow for a short time (8 h) entered only into one cell cycle after the lag phase, thus giving birth to a population of young cells. After cultivation, the cell suspension was centrifuged (2,000 g, 3 min) and washed three times with ice cold buffer (PBS with 0.1% (w/v) bovine serum albumin and 2 mmol L^{-1} EDTA). Biotin binder magnetic particles (Dynabeads, Dynal Biotech ASA, Oslo, Norway) were also washed three times with ice cold buffer (PBS with 0.1% w/v bovine serum albumin and 2 mmol L^{-1} EDTA).

Separation of old cells. The washed magnetic particles (3 mL, 4×10^8 beads mL^{-1}) were added to the cell suspension (10 mL), previously grown on CM for 24 to 48 h, and the mixture was kept at 8°C under gentle agitation for 20 min. Subsequently, the test tube was placed into a magnet for 2 min and the supernatant was discarded. Conjugates of magnetic particles with old cells were washed and centrifuged (2,000 g, 3 min) with 10 mL of PBS in order to remove the non-biotinylated cells. This procedure was repeated 10 times. The old cells retained by biotin-avidin interaction between the surfaces of cells (biotinylated) and magnetic particles (avidin coated) were released from the magnetic particles by keeping them at 60°C for 12 h in deionized water[8].

Separation of young cells. Biotin binder magnetic particles (3 mL, 4×10^8 beads mL^{-1}) were added to the cell suspension (10 mL), previously grown for 8 to 18 h, and the mixture was kept at 8°C under gentle agitation for 20 min. Subsequently, the test tube was placed in a magnet for 2 min and the supernatant containing mostly virgin cells was collected.

Fixation and storage of cell samples

Prior to bud scar staining the samples of cell suspensions from batch and continuous cultures were centrifuged (2,000 g, 3 min), re-suspended in 70% (v/v) ethanol and kept at 4°C.

Bud scar staining

An aliquot of 0.5 mL (OD = 0.8 at 600 nm) cell suspension was washed twice in PBS and then it was re-suspended in 0.5 mL of Alexa Fluor 488-labelled wheat-germ agglutinin (lectin from *Triticum vulgaris*; Sigma-Aldrich, UK) solution (1 mg mL^{-1}) in PBS. Cells were gently agitated in darkness at room temperature for 15 min, harvested by centrifugation (2,000 g, 3 min) and washed three times in PBS. The stained cell culture was re-suspended in 0.5 mL PBS and examined using flow cytometry.

Flow cytometry measurement

The flow cytometry measurements of stained bud scar fluorescence intensities were carried out using a Partec Pas III (Partec GmbH, Münster, Germany) analyzer equipped with an argon ion laser (15 mW laser power with excitation wavelength 488 nm). The bud scar fluorescence was detected on FL1 channel (530 nm) with at least 5,000 cells in each analysis. Each sample was analyzed in triplicate.

Bud scar counting

The age (number of completed cell cycles) of isolated cells was determined by staining (Alexa Fluor 488-labelled wheat-germ agglutinin) and counting the number of bud scars with a fluorescence microscope (Olympus BX51, Olympus Optical Ltd., Japan). Bud scars were counted on at least 50 cells from entire random fields. By shifting the plane of focus, it was possible to count bud scars on the undersides of cells.

Methylene blue staining

The yeast suspension was diluted in a test tube to a suspension of approximately 5–10 cells in a microscopic field. Then 10 μL of a well-mixed suspension of cells was placed on a microscopic slide and 5 μL of methylene blue were added. The counting was carried out using a magnification of 400× during outmost five min. Cells stained dark blue were considered to be dead, along with broken, shriveled and plasmolyzed cells. Cells stained light blue were considered alive and budding yeast cells were counted as one cell, if the bud was less than one half the size of the mother cell. Viability was calculated from the ratio between total and dead cells.

Cell size determination

The average diameter, width, length and circularity of the yeast cell were analyzed by an automatic image analyzer program LUCIA (Laboratory Imaging s.r.o., Czech Republic).

Batch fermentation

Fermentation cylinders (53 cm high, 5.5 cm diameter) with a conical bottom section (2.2 cm diameter) and a working volume of 650 mL were used for the batch beer fermentations. Prior to fermentation, sterile wort (10% w/w) was prepared from a powdered wort concentrate, cooled to 8°C and filled into the fermentation cylinders. This wort was aerated for 15 min (250 mL min^{-1}) and subsequently inoculated with yeast to the desired concentration. Yeasts were previously grown on wort (8°C, 10% w/w) and were collected by centrifugation (2,000 g, 5°C, 10 min). After the 10th day of fermentation at 8°C, cell samples were taken from three different heights of the yeast sediment (Fig. 1) by means of a sterile needle introduced through a rubber plug (Fig. 1). The collected cell fractions were fixed as described earlier.

Continuous fermentation

A gas-lift reactor (GLR) of the concentric draught tube type, with an enlarged top section for degassing and a total working volume of 2.9 L, was used for continuous fermentation experiments focused on the aging of immobilized biomass. The GLR was placed into a thermostated cold room (8°C). The gas flow (100 mL min^{-1} + 150 mL min^{-1} CO_2) was adjusted with a mass flow controller (Aalborg GFC17, Aalborg Instruments, Orangeburg, US). Prior to inoculation, the reactor was filled with a sterilized slurry consisting of dried spent grains (40 g dry weight) in distilled water (1.5 L). Subsequently, the GLR was loaded with CM and then inoculated with 500 mL of yeast cell suspension grown on a rotary shaker at 8°C for 48 h. After 24 h of batch growth, the start-up period of the reactor was initiated. The CM was fed at a total residence time of 7 h and the temperature inside the GLR was maintained at 8°C. Within 13 days a fully developed yeast biofilm was formed around the spent grain particles[4].

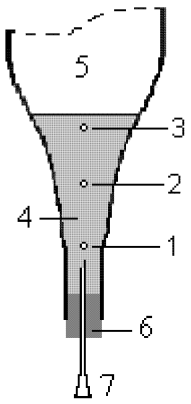

Fig. 1. Sedimentation zone of a fermentation cylinder: 1, bottom zone sampling position, 1.0 cm above the rubber plug; 2, core zone sampling position, 2.6 cm; 3, top zone sampling position, 3.8 cm; 4, yeast sediment; 5, young beer; 6, rubber plug; 7, needle.

Immobilized cell collection

Approximately 1 g of dry biocatalyst (carrier + immobilized cells) was taken from the reactor through the sampling port and washed with 2 × 100 mL of distilled water on a sintered glass filter in order to remove the free and loosely bound cells. Then the biocatalyst was suspended in 50 mL of CM without glucose and yeast extract and the mixture was agitated with a magnetic stirrer (2 cm bar, 200 min^{-1}) for 20 min. The shear stress created by mechanical agitation caused the liberation of the immobilized yeast biofilm. Subsequently, the carrier (spent grains) was allowed to sediment for 4 min and the released immobilized biomass from the supernatant was used for viability staining (methylene blue) or underwent a fixation process prior to bud scar staining.

RESULTS

Chitin is localized in a ring around the bud scar and its increasing overall level in the yeast cell wall throughout the yeast's lifespan is one of the indicators of ageing[5,14,16]. Prior to flow cytometry analysis, the bud scars of a yeast population were counted using fluorescence microscopy

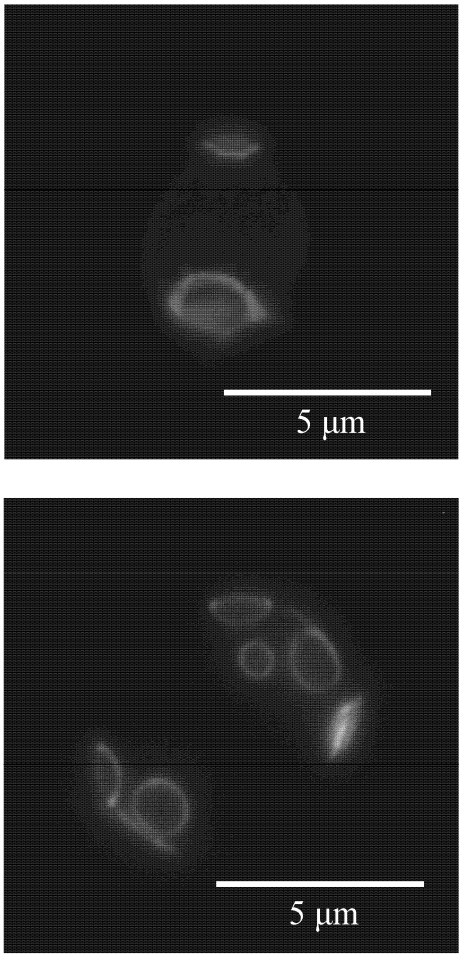

Fig. 2. Yeast cells with bud scars stained with a chitin selective fluorescent dye (Alexa Fluor 488-labelled wheat-germ agglutinin, a lectin from *Triticum vulgaris*) and observed with fluorescence microscopy.

Table I. Cell size as determined by image analysis.

Parameter	Value
Equivalent diameter (μm)	7.00 ± 1.04
Length (μm)	7.73 ± 1.25
Width (μm)	4.98 ± 0.71
Circularity	0.95 ± 0.023

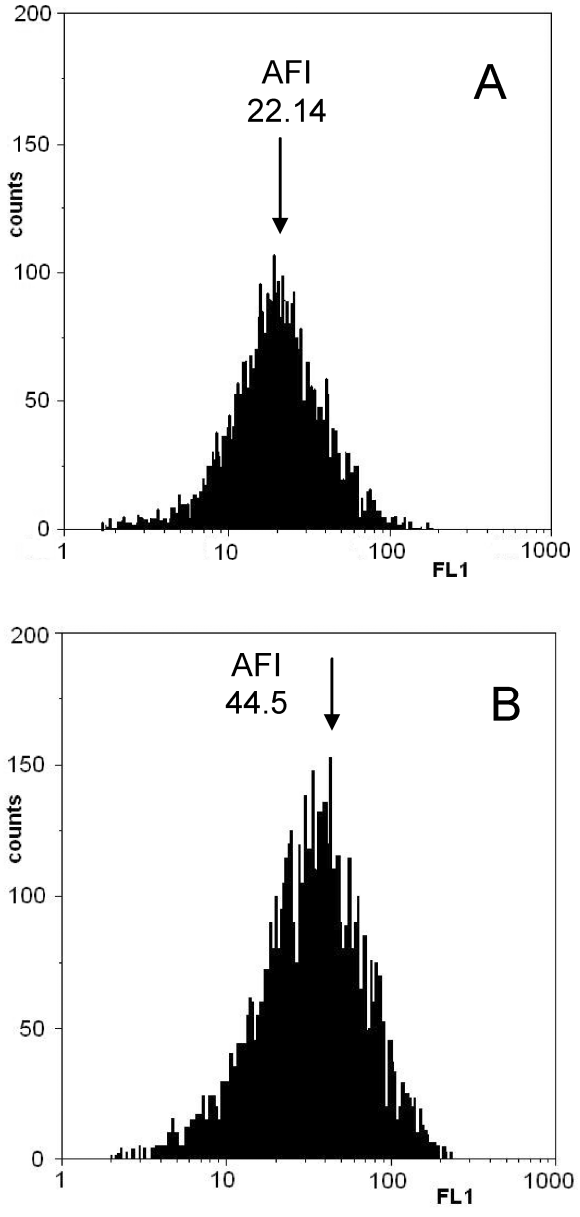

Fig. 3. Histograms of cells with stained bud scars (channel FL1). The arrows indicate the average fluorescence intensities (AFI, relative units) of two yeast populations: A, cell population with 1.4 bud scars per cell on average as determined by microscopic counting. B, cell population with 5.9 bud scars per cell on average as determined by microscopic counting.

in conjunction with Alexa Fluor 488-labelled wheat-germ agglutinin (WGA), which is highly specific for N-acetyl-glucosamine, the major component of chitin (Fig. 2).

The same yeast population, with microscopically counted bud scars, was then stained with Alexa Fluor 488-WGA and analyzed in a flow cytometer. The cell clusters

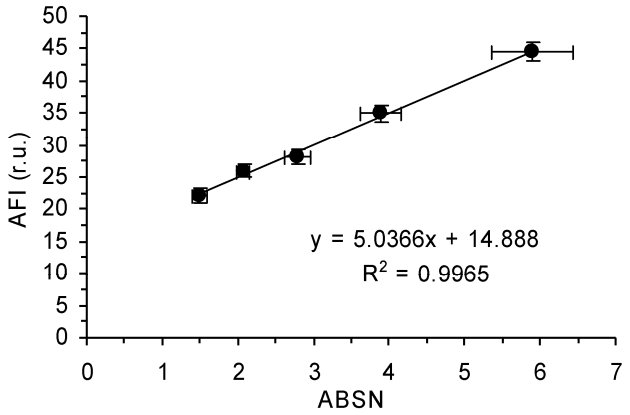

Fig. 4 Calibration curve: average fluorescence intensity (AFI) of different yeast populations with stained bud scars detected by flow cytometry (relative units) vs. average bud scar number per cell (ABSN) counted with fluorescence microscopy.

were eliminated from the obtained histograms based on the information on cell size (Table I). The fluorescence signal of cell clusters from the primary histograms was removed as undesirable by setting the size gates on the forward scatter channel from 3 to 12 μm. By setting these size gates, the cytometric analysis of the cell population with stained bud scars allowed the evaluation of the bud scar fluorescence intensities of single and budding cells. The resulting histograms showed a clear shift towards higher average fluorescence intensities (AFI) with increasing average bud scar numbers per cell (Fig. 3).

By matching the average bud scar fluorescence intensities (AFI) of stained cell populations obtained from flow cytometer, with different average bud scar numbers per cell (ABSN) determined by counting using a fluorescence microscope, a calibration curve was produced (Fig. 4). The calibration curve gave a satisfactory correlation coefficient which allowed the use of this method for the rapid average age determination of a yeast population.

Subsequently, the flow cytometric average age determination of yeast populations was tested in different model situations. For instance, traditional batch beer fermentation was mimicked in lab-scale conical fermenters (Fig. 1) with different initial cell concentrations (pitching rate). The results showed, that except with an extremely high pitching rate (60×10^6 cells mL^{-1}), the average age of the cells in the cone increased from the top to the bottom zone (Fig. 5). Simultaneously, it was observed that with increasing pitching rate both diminished the age differences between zones and increased the average age of cells in the industrially most important core zone (Fig. 5).

The flow cytometric bud scar measurement was also applied for the prediction of immobilized yeast ageing in a continuous bioreactor. It was observed that the initial gradual growth of flow cytometrically determined average bud scar number (ABSN) of immobilized cells was followed by a 60% increase in the fifth week of reactor operation. The same parameter for free cells remained almost constant during the entire continuous experiment (Fig. 6). The method most commonly used in the industry, to identify and discard pitching yeast of inferior quality, is vital staining with methylene blue. The dead immobilized cell

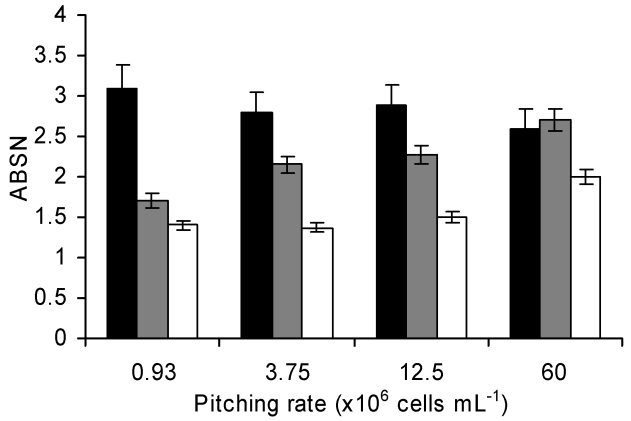

Fig. 5 Relationship between average bud scar number (ABSN) per cell of yeast populations in different cone zones (bottom, ■; core, ■; top, □) as determined by flow cytometric measurement vs. pitching rate of batch beer fermentation.

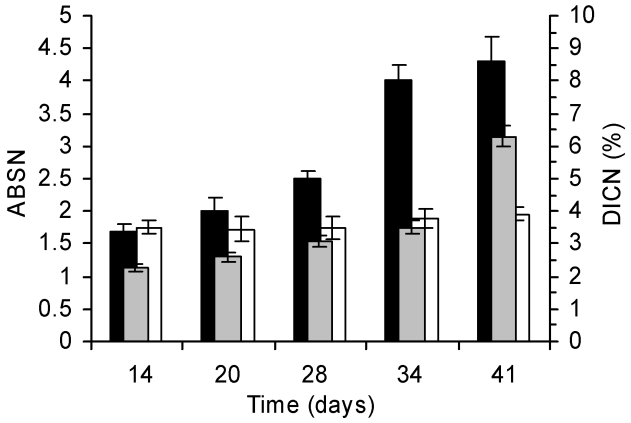

Fig. 6 Dead immobilized cell number (DICN, ■) and average bud scar number (ABSN) of free (□) and immobilized (■) cells during a continuous fermentation experiment.

number (DICN), obtained by vital staining, increased the most significantly in the last phase of the continuous beer fermentation (6th week) to values above 6%.

DISCUSSION

The number of divisions an individual cell has undertaken is the most widely accepted indicator of replicative cell age and can be directly calculated by analysis of the cell wall for bud scars using confocal microscopy[15]. However, this method is rather labour demanding and does not represent the most convenient method for real-time analysis of a large number of cells. A reproducible flow cytometric determination of the average bud scar number per cell of a yeast population would therefore be useful. For this purpose a chitin specific fluorescent dye (wheat germ agglutinin labelled with Alexa Fluor 488) was used to stain the yeast bud scars (Fig. 2). Compared to previously used dyes, such as calcafluor[20] or fluorescein isothiocyanate[17], the photo bleaching profile of the fluorescent dye applied in this work (Alexa Fluor 488) showed a prolonged stability allowing its reproducible detection in a flow cytometer.

The reproducibility of the stained bud scar measurements led to a calibration curve characterized by a good correlation coefficient between the average microscopically counted scars per cell (ABSN) and the average fluorescence intensity (AFI) of the whole population after staining. The lower end of the calibration curve is delimited by the presence of at least one bud scar on the cell surface, while the upper limit is given by the intrinsic replicative lifespan of the studied yeast strain. Based on this correlation it was possible to use flow cytometry for the rapid determination of the average age of a yeast population. Nevertheless, it has to be stated that the chitin content of bud scars, its distribution and position on the cell surface may be strain and condition specific[16] and therefore the presented calibration curve (Fig. 4) is not automatically applicable for all yeast strains or cultivation conditions.

Fermentation performance and metabolic by-product formation (flavour and aroma compounds) are strongly influenced by the metabolic state of the yeast during beer production. Consequently some of the technologically important processes such as yeast flocculation and efficiency of fermentation[17] are directly influenced by the age of the yeast population. For instance flocculation is mainly influenced by the properties of the cell wall which gradually change throughout the yeast's lifespan[1].

In beer fermentation technology, the yeast culture is used between 3 and 20 times, and the risk of degeneration increases with each serial re-pitching[11,19]. Therefore it is essential to collect, wash, store and reuse the potentially most productive fraction of the settled yeast biomass. Although in beer manufacturing practice, it is typically the central portion of the yeast crop (middle aged and virgin cells) which is collected, the age distribution within the yeast sediment is a matter of some controversy. According to some authors, the age of the yeast increases gradually from the top to bottom fraction of the yeast sediment[7]. Others located the oldest yeast fraction closer to the centre of the cone and suggested that the precise location may vary according to the characteristics of the yeast strain employed and the dimensions of the fermentation vessel[18]. The data obtained by using our flow cytometry age determination method supports the former finding (at the pitching rates corresponding to those used in industry of 5–20 $\times 10^6$ cells mL^{-1}) and locates the highest and lowest average bud scar number per cell (ABSN) in the bottom and top zone of the fermenter cone, respectively. The reduced age gradient at the highest pitching rate can be explained by the lower number of cell cycles that the extremely dense initial cell population (ABSN = 1.85) undergoes until depletion of fermentable sugars, compared to experiments with a lower pitching rates (Fig. 5).

Brewing yeast attached onto cellulose based spent grain particles (brewing by-product) have been studied in continuous beer fermentation[2]. Since the immobilized yeast in a continuous reactor show a gradual loss of viability due to aging[4], the monitoring of this yeast deterioration and scheduling of yeast replacement is a task of high relevance[3]. In traditional batch fermentations, yeast are considered of poor quality when dead cell counts are above 5%, therefore the deteriorating immobilized yeast viability in a continuous reactor creates an elevated risk of

impaired product quality. As regards continuous fermentation, an increase in dead immobilized cell numbers (DICN) above 6% was seen on the 6th week of reactor operation (Fig. 6). The fact that the ABSN increase occurred approximately 7 days before the increase of DICN (Fig. 6) shows that the presented method of yeast age determination could be used as an indicator of the immobilized biomass aging with practical implications. For instance, the ABSN value could be used for scheduling the replacement of aged biocatalyst during long-term continuous beer fermentations. The time gap, between the ABSN increase and the deterioration of immobilized biomass viability, allows the withdrawal of the "old" biocatalyst from the reactor and its replacement by clean carrier or fresh biocatalyst before the loss of immobilized cell viability impairs the process performance or product quality.

ACKNOWLEDGEMENTS

The study was financially supported by the Grant Agency of the Czech Republic (GAČR 104/06/1418) and MŠMT (MSM 6046137305, Czech Republic).

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(Manuscript accepted for publication October 2009)