130

Assessment of brewing yeast age based on selective bud scar staining and subsequent fluorescence measurement by flow cytometry

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SUMMARY

To study the yeast cell aging in continuous beer fermentation processes has a considerable practical significance. Hence, an expeditious method of yeast age estimation was developed based on selective bud scar staining and subsequent fluorescence intensity measurement by flow cytometry. The calibration curve resulting from cytometric determination of average bud scar fluorescence intensities of cell populations with different average bud scar numbers showed a good correlation. The combination of flow cytometry with bud scar staining resulted in a method of rapid cell age estimation particularly suitable for continuous main fermentation systems with elevated risk of immobilized cell aging.

INTRODUCTION

The brewing yeast *Saccharomyces cerevisiae* has a limited replicative lifespan determined by its genes and influenced by environmental factors. Brewing yeast are capable of a finite number of divisions (10-30 divisions) before entering a non-replicative state termed senescence, leading to death and autolysis [5]. Yeast display an array of changes during their aging including decrease of viability, increase in size, cell surface wrinkling, increase of generation time, increasing bud scar number and decreased metabolic activity [1, 4, 6]. The study of the aging process of brewing yeast strains has also a practical significance. Serial fermentation can select for an undesirable sub-population enriched with elderly cells. The aged brewing yeasts show changed flocculation characteristics and fermentation performance [7]. It is believed that the performance of lager strain begins to degenerate after 10 serial repitchings.
The question of yeast aging is particularly important in continuous fermentation processes taking into consideration the long periods of time (several months) that immobilized cells are spending in a continuous reactor [9]. The viability and fermentation capacity (vitality) of immobilized brewing yeast in continuous

fermentations have already been reported to decrease [3]. However, there is little known on the impact of senescence and aging of immobilized yeast in continuous fermentation on product quality. Hence, elucidating the influence of aging process on cell viability and fermentation performance would be of a great practical importance. As a consequence, measures to ensure stable fermentation performance of bioreactors could be taken.

There are many methods of cell age and viability assessment; however, traditional culture-based techniques do not provide real-time information on microbial physiology. Conversely, flow cytometry offers the prospect of real-time analysis of a large number of cells. The number of bud scars present on the cell surface is directly related to the number of times a cell has divided and thus represents a biomarker for replicative cell age estimation. The feasibility of flow cytometric method of yeast bud scar determination was verified by calibration of the fluorescence intensity of bud scars vs. microscopic bud scar counting. The developed method was applied for immobilized biomass age estimation during continuous fermentation.

METHODOLOGY

Microorganisms and medium

A bottom fermenting brewing yeast strain (*Saccharomyces cerevisiae subsp. carlsbergensis*) no. 96 obtained from the culture collection of brewing yeast (Research Institute of Brewing and Malting, Prague, Czech republic) was used throughout the experiments. The yeast were cultivated in a complex medium (CM) with the following composition (in g/l): 5, KH_2PO_4; 2, $(NH_4)_2SO_4$; 0.04, $MgSO_4.7H_2O$; 2, yeast extract (Merck, Darmstadt, Germany); 20, glucose. CM was sterilized by autoclaving at 121 °C, 100 kPa for 30 min. Phosphate buffer saline (PBS) contains (in g/l): 8, NaCl; 1.14, Na_2PO_4; 0.2, KCl; 0.8, KH_2PO_4 in distilled water.

Isolation of cells of different age

Biotinylation: brewing yeast (strain 96) 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 (5 000 min^{-1} for 3 min) and washed with ice cold PBS (pH 8.0) 5 times. An aliquot of 3 ml was removed and the cell concentration was adjusted to 1×10^8 cells/ml. This cell suspension was biotinylated with 4 mg EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, USA) per ml of cell suspension in a test tube at 25 °C during 30 min.

Cell aging: the biotinylated cells were resuspended in 10 ml of CM and allowed to grow in a test tube placed on a rotary shaker at 25 °C for 14 to 48 h depending at what age it was desired to separate the biotinylated cells or their offspring.

Before separation step the cells suspension was centrifuged (5 000 min^{-1} for 3 min) and washed with ice cold buffer (PBS with 0.1 % bovine serum albumin and 2 mM EDTA) 3 times. Biotin binder magnetic particles (Dynabeads, Dynal Biotech ASA, Oslo, Norway) were also washed 3 times with ice cold buffer (PBS with 0.1 % BSA and 2 mM EDTA).

Separation of old cells: the washed magnetic particles (3 ml) were added to the cell suspension (10 ml), previously grown for 48 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 was discarded. Conjugates of magnetic particles with old cells were washed 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 magnetic particles by autoclaving at 120 °C for 10 min in deionized water.
Separation of young cells: biotin binder magnetic particles (3 ml) were added to the cell suspension (10 ml), previously grown for 14 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. The cultivation of the biotinylated cells and the harvest of young cells could be repeated if necessary.

Fixation and storage of cell samples
Prior to staining the analysed cell fractions were centrifuged (5000 rpm, 3 min), re-suspended in 70 % ethanol (fixation process) 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 1 mg/ml Alexa Fluor 488-labelled wheat-germ agglutinin (lectin from Triticum vulgaris; Sigma-Aldrich, UK) solution in PBS. Cells were gently agitated in darkness at room temperature for 15 min, harvested by centrifugation (5 000 min^{-1} for 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. The flow cytometric measurements 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). Bud scar fluorescence was detected in FL1 channel (530 nm) for at least 5 000 cell in each analysis.

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

Glycogen staining
The relative content of glycogen in yeast was determined using acriflavine (Sigma Aldrich, Germany), which is a fluorescent dye able to covalently bind to polysaccharide glycogen after permeabilisation of the cell membrane by ethanol (fixation). An aliquot of 0.5 ml (OD = 0.8 at 600 nm) of cell suspension was removed and 10 µl of acriflavine solution (1 mg/ml in PBS, stored at 4 ºC) were added. Incubation occurred in darkness at room temperature for 30 min. The sample was then analysed by flow cytometry in FL1 channel (530 nm) for at least 5 000 cell in each analysis.

Methylene blue staining
The yeast suspension was diluted in a test tube until a suspension with approximately 5-10 cells in a microscopic field. Then 10 µl of well-mixed suspension of cells was placed on a microscopic slide and 5 µl of methylene blue were added. The counting was made using a magnification of 400 during outmost 5 minutes. Cells stained dark

blue were considered to be dead along with broken, shriveled and plasmolyzed cells. Cells stained light blue should be considered alive and the 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.

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 experiments focused on aging of immobilized biomass. The GLR was placed into a thermostated cold room (8 °C). The gas flow (100 ml/min + 150 ml/min CO_2) was adjusted with a mass flow controller (Aalborg GFC17, Aalborg Instruments, Orangeburg, New York, USA). Prior to inoculation, the reactor was filled with sterilized slurry consisting of spent grains (40 g dry state) in distilled water (1.5 l). Subsequently, the GLR was charged with CM and then inoculated with 500 ml of yeast cell suspension grown on a rotary shaker at 8 °C for 48 hours. After 24 h of batch growth, the start-up period of the reactor initiated. The CM was fed at a total residence time of 7 h and the temperature inside GLR was maintained at 8 °C. Within 13 days a fully developed yeast biofilm was formed around the spent grain particles. For more details see [2].

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, then 50 ml of synthetic medium without glucose and yeast extract was added and agitated with a magnetic stirrer (2 cm bar, 200 rpm) for 20 min. Subsequently the biocatalyst was allowed to sediment for 4 min and the released biomass from the supernatant was either used for viability staining (methylene blue) or underwent a fixation process.

RESULTS AND DISCUSSION

The number of divisions an individual cell has undertaken is the most widely accepted indicator of cell age and can be directly calculated by analysis of the cell wall for bud scars using confocal microscopy [5]. 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. Therefore a chitin specific fluorescent dye (wheat germ agglutinin labelled with Alexa Fluor 488) was used in this work to stain the yeast bud scars (figure 1). Comparing to previously used dyes, such as calcofluor [8] or fluorescein isothiocyanate [7], the photobleaching profile of the fluorescent dye applied in this work (Alexa Fluor 488) shows prolonged stability allowing its reproducible detection in flow cytometer.

The cytometric analysis of the cell population with stained bud scars allowed the evaluation of the fluorescence intensities of only single or budding cells (figure 1). The flow cytometer enabled the removal of cell clusters from the statistical interpretation of the results based on cell size measurement and subsequent deletion of the undesirable data. The resulting histograms showed a clear shift towards higher average fluorescence intensities (AFI) with increasing average bud scars number (figure 2).

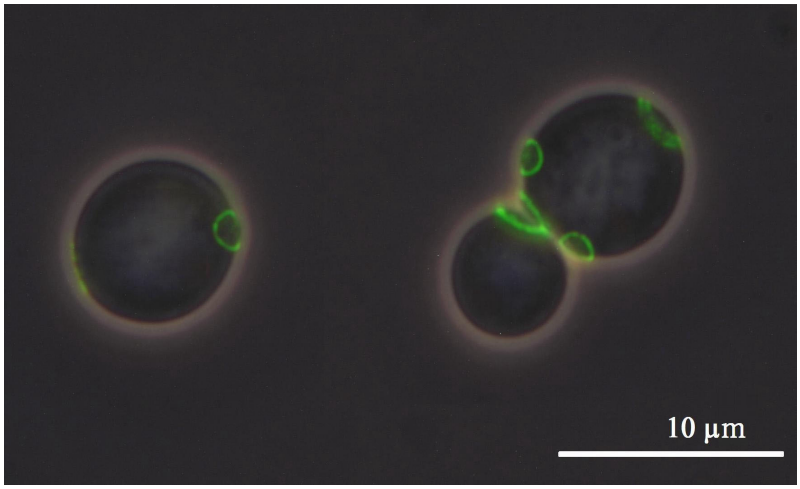

Figure 1: Single or budding yeast cells with bud scars stained with a chitin selective fluorescent dye (Alexa Fluor 488-labelled wheat germ agglutinin, a lectin from Triticum vulgaris) and observed in fluorescence microscope.

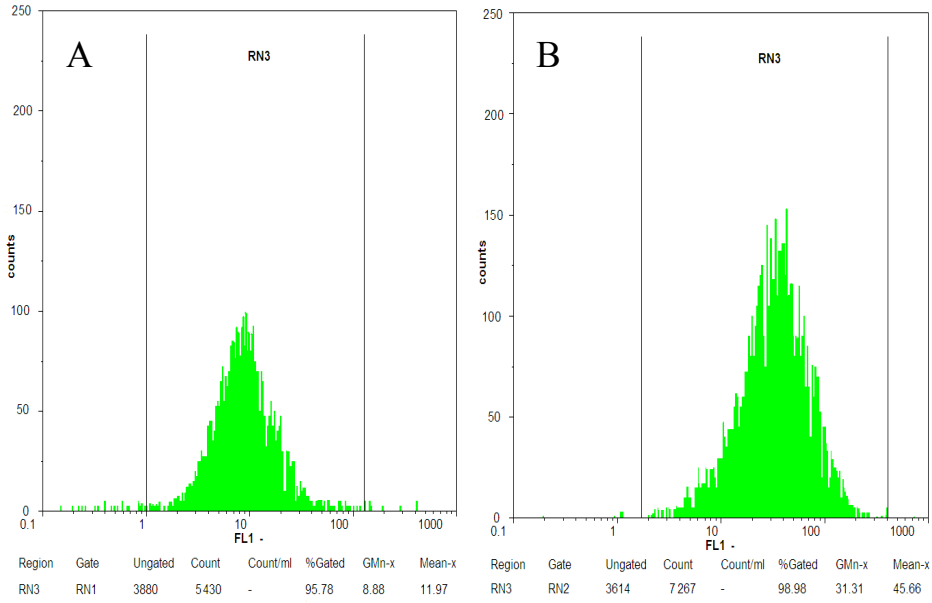

Figure 2: Histograms of stained bud scar fluorescence intensities (channel FL1): A-cell population with 1.4 bud scars in average, B-cell population with 5.9 bud scars in average.

The calibration curve resulting from cytometric determination of average bud scar fluorescence intensities (AFI) of cell populations with different average bud scar numbers, determined by counting in fluorescence microscope, is shown in figure 3. It is characterized by a good correlation which allows using this method for average age determination of brewing yeast. However, it has to be stated that the chitin content of bud scars, its distribution and position on cell surface may be strain and condition specific [6] and therefore the presented calibration curve (figure 3) is not automatically applicable for all yeast strains or culture conditions.

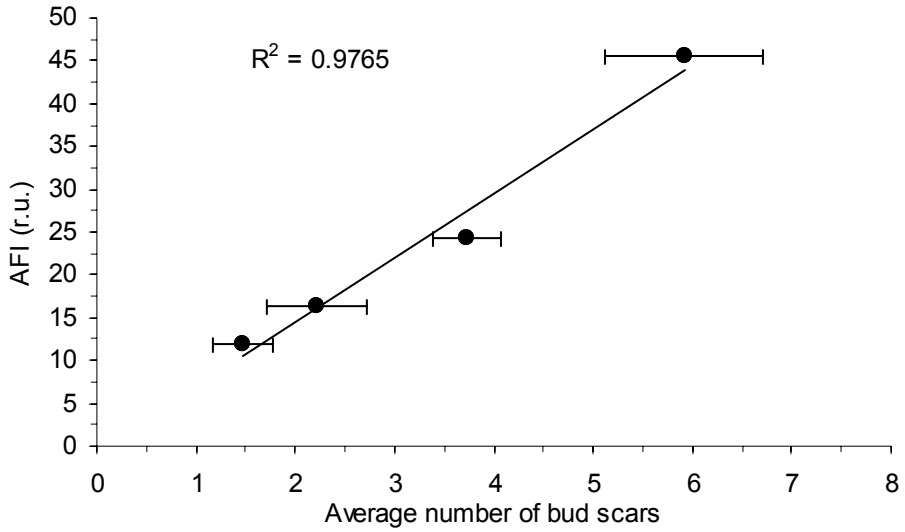

Figure 3: Calibration curve: average fluorescence intensity (AFI) of stained scars on single or budding cells detected by flow cytometer (relative units) vs. average bud scar number counted in fluorescence microscope.

This novel method of cell age determination was tested on a model situation when the immobilized yeast were allowed to age in a continuous fermentation reactor. The continuous fermentation experiment was divided in two main stages. In the first stage there was a regular replacement of biocatalyst (biomass + carrier) by clean non-colonized spent grain particles (figure 4A). When replacing the biocatalyst by clean carrier, a non-colonized carrier surface is introduced into reactor allowing the adhesion of free cell population with higher viability onto carrier. This keeps the immobilized cell viability similar to that of the free cells. The second stage without biocatalyst replacement can be considered as an aging period (figure 4B). These stages allowed the comparison of two situations where the first can be described in term of cell viability as a close steady-state, while the second is characterized by a gradual aging and loss of viability of the immobilized biocatalyst.

As regards immobilized dead cell numbers, it can be seen that the regular replacement of carrier during the first period of the continuous experiment resulted in relatively constant immobilized dead cell number around 14 % (figure 4A). However, as the carrier replacement stopped during the aging period, the percentage of dead immobilized cells increased and reached values close to 27 % (figure 4B).

Cellular components such as chitin of bud scars and intracellular glycogen were stained by fluorescent dyes and their alterations during the continuous fermentation experiment are depicted in figure 4. During the period with biocatalyst replacement the values of the average fluorescence intensity (AFI) of glycogen and bud scars seem to be constant (figure 4A). In the course of the aging period an increase in AFI of chitin rings (bud scars) was observed while the AFI of glycogen remained unchanged (figure 4B). The fact that the average bud scar fluorescence signal increase occurred approximately 7 days before the increase of dead immobilized cell number proved, that the bud scar AFI could be used as an indicator of the immobilized biomass aging with practical implications. For instance, the bud scar AFI could be used for scheduling the replacement of aged biocatalyst during long-term continuous beer fermentations while the intracellular glycogen content did not seem to be a suitable indicator of cell aging in this experiment. The time gap between the buds scar AFI increase and the deterioration of immobilized biomass viability allows the withdrawal

of “old” biocatalyst from reactor and its replacement by clean carrier or fresh biocatalyst, before the loss of immobilized cell viability could negatively influence the process performance or product quality.

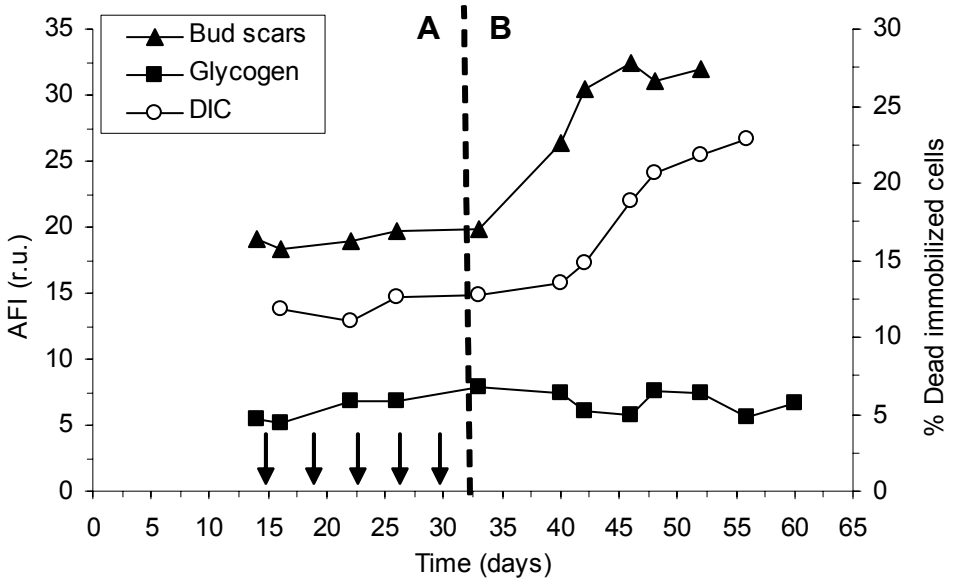

Figure 4: Relationship between dead immobilized cell count (DIC); average fluorescence intensity (AFI, in relative units) of bud scars and intracellular glycogen of immobilized cells during continuous fermentation experiment: **A -** period of regular biocatalyst replacement (arrows indicate the replacement of 4 g of biocatalyst by clean spent grains), **B** - aging period without biocatalyst replacement.

CONCLUSIONS

(i) A relationship between stained bud scar fluorescence and cell aging was observed and a novel method of rapid cell age determination was developed.
(ii) The combination of flow cytometry with bud scar staining resulted in a suitable method of rapid cell age estimation.
(iii) The calibration curve relating the stained bud scar fluorescence intensity to the average bud scar number of the studied cell population was satisfactory (figure 3).
(iv) The developed method was particularly suitable for immobilized biomass age estimation during long-term continuous fermentation. The gradual immobilized biomass aging was detected by increasing average bud scar fluorescence intensity preceding the decrease of immobilized cell viability by approximately 7 days (figure 4).

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