Aging of immobilized brewing yeast in a continuous bubble-column reactor

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Abstract

The aim of this work was to study the physiological changes of immobilized brewing yeast Saccharomyces cerevisiae in a continuous reactor. Continuous cultivation was carried out at three different temperatures (15, 20 and 25°C) with an industrial brewing yeast strain (UNICER, Bebidas de Portugal, S.A.). During the continuous experiment the viability of free and immobilized cells was followed using vital staining and flow cytometry. The results of viability determination were used to calculate the specific death rate of the immobilized cells (K_d). Further, the flow cytometry was used to follow the glycogen content of cells and the number of budding cells. The experiments showed that the viability of free cells stayed constant in the course of the whole experiment. Conversely, the viability of immobilized cells decreased during the whole time of the cultivation as well as the viability decreased in the direction from the surface to the depth of the biofilm. The study of the immobilized biomass showed that specific immobilized cell death rate (K_d) was a function of cultivation temperature and that the values of K_d increased with temperature. Higher content of glycogen and a lower count of budding cells was found in immobilized cell fraction.

Introduction

The yeast *Saccharomyces cerevisiae* has a limited replicative lifespan. Each cell is only capable of a finite number of divisions, usually within the range 10-30 divisions, before entering a non-replicative state termed senescence, leading to death and autolysis (Powell et al. 2000). As a consequence of aging and senescence, the polyploid brewing yeast cells are subject to modifications in terms of physiology, morphology and gene expression. Such modifications include decrease of viability (Barker and Smart 1996), increase in size, wrinkling of the cell surface, increase of generation time, increasing bud scar number and decrease in metabolic activity (Jazwinski 1990).

The study of the aging process of brewing yeast strains has also a practical significance. The aged brewing yeasts show changed flocculation characteristics and fermentation performance (Powell et al. 2003). It is believed that the performance of lager strain begins to degenerate after 10 serial repitchings. When comparing the maximum age that brewing yeast can reach in traditional technology with the long periods of time that immobilized cells are spending in a continuous reactor, e.g. primary beer fermentation operating for several months (Tata et al. 1999), the question of the immobilized cell age and physiology turns to be even more relevant. Although the viability and fermentation capacity (vitality) of immobilized brewing yeast in continuous fermentation systems have already been reported to decrease (Branyik et al 2004a), there is little known on the senescence and aging process of immobilized yeast in continuous beer fermentation systems and on their impact on product quality. Hence, elucidating the influence of immobilization methods and process parameters on aging of

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brewing yeast and understanding the effect of senescence on cell vitality and fermentation performance would be of a great practical importance. As a consequence, proper measures to increase the operational lifetime and fermentation performance of the bioreactor could be taken.

Materials and Methods

Yeast strain and culture conditions

The brewing yeast *Saccharomyces uvarum (carsbergensis)* was supplied by the brewing company UNICER, SA. The yeast for inoculation of the continuous bubble-column reactor were cultivated in 100 mL of synthetic medium under aerobic conditions on a rotary shaker (120 rpm) at 30 ºC for 30 h. The composition of the synthetic medium was as follows (g/L): KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.4; yeast extract, 1.0; glucose, 10. The phosphate buffer (PBS) solution consists of: KCl, 200.0 mg/ L; KH_2PO_4, 200.0 mg/ L; NaCl, 8.0 g/ L; $Na_2HPO_4.12H_2O$, 2.89 g/ L.

Continuous culture experiments

The continuous culture experiments were conducted in a 440 mL aerated bubble-column reactor at room temperature. The inlet and outlet were situated 1 and 11.5 cm above the bottom of the reactor, respectively. The sedimentation barrier (semicircle plan, 1.5 cm radius) reached 1 cm above and 5 cm below the outlet. After placing the carrier (6 – 7 g dry weight) into the reactor, 100 ml of pre-cultured brewing yeast suspension was added and subsequently the reactor was filled with synthetic medium. The continuous feed was started after 16 h of batch growth always at a dilution rate of $D = 0.7\times\mu_{max}$ (T), and it was kept constant during the whole experiment as well as the temperature was kept constant by means of a cooling jacket. The medium was supplied at the bottom of the reactor by means of a peristaltic pump (Watson Marlow 101 U/R, Falmouth, England). Sterile air was passed into the bubble column at 0.6 L min^{-1}, through a pipe with 4 holes (1 mm diameter each). Under these conditions the carrier in the reactor was in continuous motion and did not tend to settle. In the course of the continuous culture experiments the immobilized biomass concentration was measured and samples for vital staining and fixation were taken at regular intervals.

Flow cytometry

The flow cytometric measurements were taken using a Partec Pas III (Partec GmbH, Germany) analyzer equipped with an argon ion laser (15 mW laser power with excitation wavelength 488 nm) and a HBO lamp. The final volume submitted to analysis was 1 mL of in phosphate buffer solution (PBS), to which 10 - 6 µL of cells suspension, either vital cells or stained fixed cells, was added so that the final cell concentration during analysis did not exceed 1000 cells per second. Typically 10 000 cells were analyzed for each sample. Every assay was performed in triplicate. All samples were vortexed for 10 s immediately prior to analysis. Samples of free cells were collected at the outflow of the reactor and either directly used for vital staining measurement or underwent a fixation process prior to further analyses. To make possible to analyze the immobilized cells of the continuous experiment, ca. 0.2 g dry weight of the biocatalyst (carrier + immobilized cells) taken from the reactor through the outflow were washed with 2 × 100 mL of distilled water, then 50 mL of synthetic medium without glucose and yeast extract were added and agitated with a magnetic stirrer (2 cm bar, 200 rpm) for 20 min. After the agitation the biocatalyst was allowed to sediment (4 min) and the released biomass from the supernatant was either used for vital staining or underwent a fixation process prior to further analyses. This immobilized biomass liberated from the carrier by agitation is referred to as the 1st biofilm fraction. Then the liquid with suspended biomass was removed with a syringe and the whole process of washing with distilled water, agitation and sampling was repeated two more times. The corresponding immobilized biomass samples are referred to as the 2nd and 3rd biofilm fraction.

Fixation

For the determination of glycogen, samples were collected by centrifugation (5 min, 7000 rpm) and fixed in ethanol 70% (v/v) for at least 24h at 4 ºC. Prior to analysis the fixed cells were centrifuged for

5 min at 7000 rpm, washed with 1mL of PBS and then centrifuged again. The recovered yeast cells were before staining resuspended in 2 mL of PBS and vortexed for 1 min.

Viability staining

Propidium Iodide fluorescently stains nucleic acid but does not diffuse appreciably into intact cells. Therefore, this dye preferentially stains dead cells that have porous membranes. In order to determine the amount of dead cells, fresh samples taken from the continuous and batch culture (free and immobilized cells) were diluted in PBS and stained with 6μL of Propidium Iodide (PI; Sigma, Germany) stock solution (1 mg/ mL in PBS, stored at 4°C). Measurements were carried out within 5 min after the addition of PI.

Glycogen staining

The relative amount of glycogen in cells can be determined by flow cytometry using Acriflavine. It is a fluorescent dye able to covalently bind to polysaccharide glycogen only if the cell membrane was permeabilized by ethanol (fixation) prior to staining. An aliquot of 0.5 mL of the diluted sample was removed and added 10 μL of Acriflavine (Sigma, Germany) solution (1 mg/ mL in PBS, stored at 4°C). Incubation occurred at room temperature in darkness for 30 min. The sample was then analyzed by flow cytometry and the Acriflavine fluorescence signal intensity acquired in FL1 channel.

Results and Discussion

The yeast attachment to the surface of the carrier made of spent grains can be hypothesized to be a result of continuous culture induced physiological changes (selection pressure) of the yeast cell surface (Brányik et al. 2004b). The net yeast biofilm accumulation to non-porous surfaces is mainly a result of the dynamic character of the biofilm where opposite processes such as cell growth/dead, adhesion/release, adsorption/desorption and attachment/detachment take place between carrier and the surrounding environment. Therefore, the rate of the immobilized yeast biofilm development can be expressed as a result of a balance between cell deposition rate (R_{dep}), immobilized biomass growth rate (R_{grw}), immobilized biomass dead rate (R_{dead}) and immobilized biomass detachment rate (R_{det}). The overall mass balance for immobilized biomass can thus be expressed as:

$$\frac{dX_{im}}{dt} = R_{dep} + R_{grw} - R_{dead} - R_{det} = R_{dep} + \mu_{im} X_{im} - K_d X_{im} - k_{det} X_{im} \qquad [1]$$

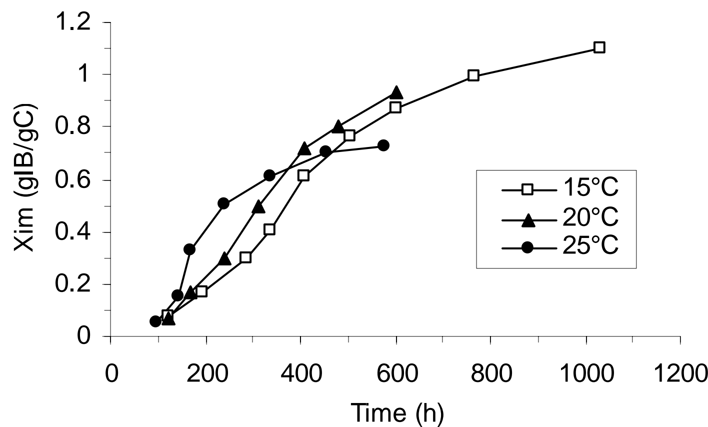

Figure 1. Development of immobilized biomass X_{im} (gIB/gC, IB – immobilized biomass, C – dry carrier) during continuous culture experiment at different temperatures (15, 20 and 25 °C) and corresponding dilution rates ($D_{15} = 0.07\ h^{-1}$, $D_{20} = 0.14\ h^{-1}$, $D_{25} = 0.2\ h^{-1}$).

The immobilized cell growth, dead and the yeast biofilm detachment can be further characterized by the specific growth rate of the immobilized biomass μ_{im} (h^{-1}), specific immobilized biomass dead rate K_d (h^{-1}) and the specific immobilized biomass detachment rate coefficient k_{det} (h^{-1}), respectively. Consequently, the biomass accumulation rate is temperature dependent, which was proved by continuous cultivations at different temperatures (Figure 1). Higher temperature caused an increased initial immobilized biomass accumulation rate. However, the results concerning the maximum immobilized biomass load, higher at lower temperatures (Figure 1), are rather unclear. It can be speculated that either the disturbed material balance due to samples of biocatalyst taken for analysis or alterations in yeast biofilm structure at lower temperatures can influence the maximum biofilm load.

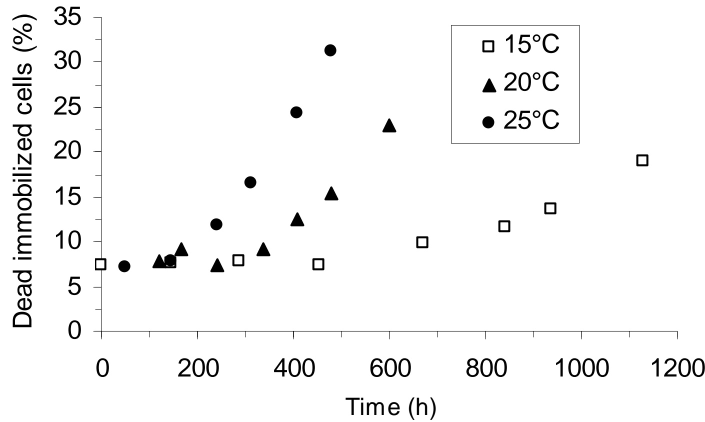

Figure 2. Percentage of dead cells among 1st fraction of immobilized cells (IC) measured by vital staining (PI) and flow cytometry during continuous culture experiment with ai industrial brewing yeast strain. The applied dilution rates at different temperatures (15, 20 and 25 ºC) were $D_{15} = 0.07\ h^{-1}$, $D_{20} = 0.14\ h^{-1}$ and $D_{25} = 0.2\ h^{-1}$.

Selective fluorochrome such as Propidium iodide were applied into the suspension of yeast cells and then analyzed by flow cytometry in order to obtain information about the viability of the free and immobilized cells of the reactor. The obtained results suggest that the immobilized biomass is exposed to a gradual aging process. It has been found that the immobilized cells from biofilm contain higher number of dead cells and generally can be considered metabolically less active. Further it was observed that the number of dead cells in the immobilized biofilm increased with time (Figure 2), while in free biomass it remained almost constant in the range from 5 to 10 % (data not shown). The higher percentage of dead immobilized cells was caused by the longer period spent in the bioreactor comparing to free cells which exposes them to a gradual aging process leading to cell dead. The effect of temperature on death rate was studied in a continuous system and it was found that the onset of the immobilized yeast biofilm was postponed for lower cultivation temperatures (Figure 2). This effect can be ascribed to the slower exhaustion of the Hayflick limit (maximum lifespan potential) at lower temperatures caused longer generation times.

The specific immobilized cell death rate coefficient (K_d) was calculated from the experimental values obtained after the percentage of the immobilized dead cells exceeded the free dead cells by least-square fitting of plot of $\ln(N_{live\ cells}/N_{total\ cells})$ vs. time obtained from the equation:

$$\frac{N_{live}}{N_{total}} = e^{-Kd(T)\cdot t} \qquad [2]$$

Subsequently, a plot of $\ln K_d$ vs. reciprocal temperatures yields a straight line with a slope E/R and y-intercept of lnA according to the Arrhenius equation (Figure 3):

$$K_d(T) = A \cdot e^{\frac{-E}{R \cdot T}} \qquad [3]$$

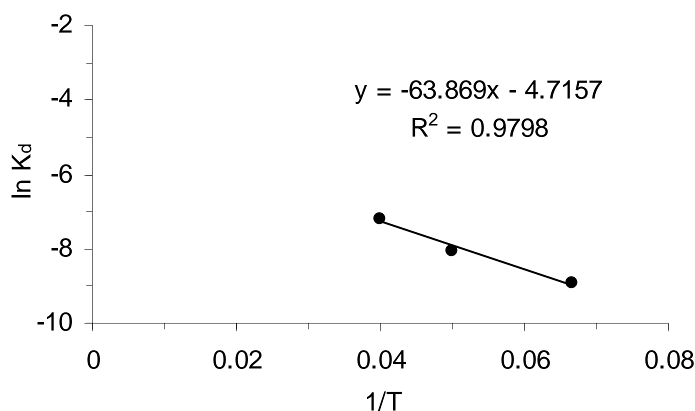

Figure 3. Plot of $\ln K_d$ vs. reciprocal temperatures (see eq. 3).

The kinetic parameter of immobilized yeast aging (K_d - specific immobilized biomass dead rate coefficient) and its temperature dependence can be used in practical brewing applications for predicting the regular time schedule of the replacement of the "old" biocatalyst by clean spent grains carrier in the continuously operating reactor. Kinetic parameters of immobilized and free yeast, including specific dead rate coefficient, and their substrate and temperature dependence will be applied in a kinetic model in order to predict the immobilization rate of brewing yeast on spent grain particles and their long-term fermentation performance.

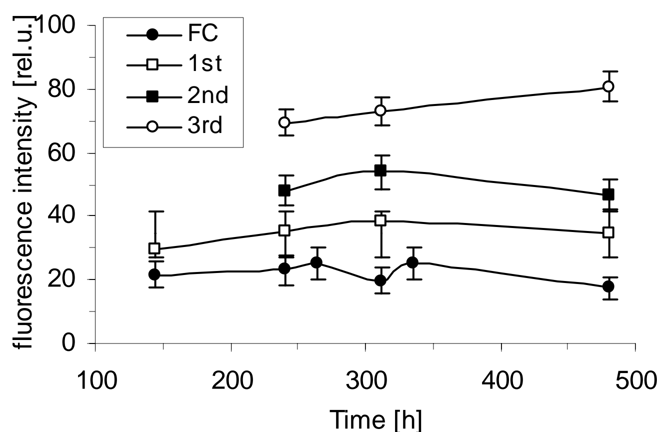

Figure 4. Variation of the cell glycogen content during the time of the experiment for free cells (FC), 1st, 2nd and 3rd immobilized cells fraction at 25 ºC. Cell glycogen concentration was expressed as an average fluorescence intensity (relative units) emitted by cells stained with Acriflavine.

The immobilized biomass was removed from the spent grain particles by repeated mechanical agitation. The biomass liberated after the three subsequent agitations was called 1st, 2nd and 3rd immobilized biomass fraction. Although somewhat simplified, the 1st, 2nd and 3rd biofilm fractions can be ragarded as biofilm layers from outer, through middle and until deeper regions, respectively.

Complementary information on the yeast physiological conditions (free and immobilized cells) was obtained with flow cytometric analysis of intracellular glycogen content and budding cell number in different cell fractions. The budding cell/single cell ratio was during the whole experiment decreasing in the following order i.e. free cells > 1st > 2nd > 3rd immobilized cell fraction, which is in accordance with increasing cell age and higher dead cell count found in the same order (data not shown). Glycogen is referred to as intracellular storage carbohydrate which can provide both carbon source and metabolic energy necessary for stress responses (Majara et al. 1996). High accumulation of glycerol, a

common osmolyte produced by yeast cells to counteract osmotic stress, has been reported for immobilized yeast (Galazzo and Bailey 1990). In the case of the studied immobilized cell system, the cell glycogen content increased in the order free cells < 1st < 2nd < 3rd immobilized cell fraction (Figure 4) thus supporting the hypothesis that the solid immobilzation matrix induces osmotic stress and subsequent physiological responses resulting at the level of cellular composition (Doran and Bailey 1986; Shen et al. 2003). In addition, mass transfer limitation inside yeast biofilm may result in stressful conditions for immobilized cells, such as nutrient starvation and ethanol stress, leading to alteration of immobilized cell behaviour. Since these alterations are often undesirable for the beer quality, the goal of the further research will be to both understand and avoid factors inducing these changes.

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