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# Yeast activity monitoring by fluorescent methods

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## SUMMARY

Yeast activity was monitored during pilot plant yeast propagation under aerobic and anaerobic conditions. The work was carried out with three widely used Czech lager yeast strains. Fluorescent intensity of DNA was analysed by flow cytometry and signal was used as a marker for cell cycle determination. During experiments were monitored changes in significant properties of yeast (glycogen, proteins, and DNA) by fluorescent methods. It seemed that the impact of aerobic conditions on preservation of flocculation abilities depends on the genetic properties of each strain. Only one strain did not show significant losses in flocculation abilities neither under aerobic nor strictly anaerobic conditions.

## INTRODUCTION

Fresh yeasts in optimal physiologic state are one of the most important ingredients for favourable, successful fermentation and for obtaining reproducible procedure duration as well [1]. During the last years a new focus has been set in yeast quality control and yeast management procedures. Traditionally, brewery yeasts are propagated through a series of poorly aerated batch fermentation vessels with low biomass yield and relatively long time of cultivation [2]. Many works [3,4,5] described that under aerobic condition we can obtain significantly higher biomass yield with better yeast physiological state from the point of view of fermenting power etc. Nevertheless there were occurred problems with optimal oxygen saturation, foam formation and some works described changes of gene expression under aerobic conditions. Harrison et al. [6] observed changes in yeast surface hydrophobicity, surface charge and in flocculation ability as well. Flocculation is generally causing profound changes in the settling properties of yeast cells. Flocks tend to settle individually from dilute suspensions, but at higher concentrations, zone settling occurs due to mutual interactions. This leads to boundary between the settling flocks and the clear supernatant [7]. Sedimentation remains the basis for the most of modern flocculation assays but the rate at which a flocculent yeast suspension sediments is a function of

the flock size, cell concentration, flocks density, and the extend of agitation imposed on the yeast suspension prior to the settling period [8].

During the propagation yeast produce storage carbohydrate polymer glycogen which is accumulate in the latter stages of the fermentation. There were also described changes in glycogen accumulation under aerobic compared to anaerobic conditions. Thanks Hutter work glycogen determination by flow cytometry nowadays is possible [9,10].

Flow cytometric analysis does an analysis of every single yeast cell. Measurement speeds up to 3000 cells per second are possible, but recommended measurement speeds are lower order. The flow cytometry mechanism involves passing individual cells through the path of a laser beam. Light is scattered into different directions and is detected by several detectors in accordance with required information (size, granularity, fluorescence). The resulting fluorescence from the cells can be recorded digitally and quantified. Stained cells should be examined microscopically [11].

The measurement of the DNA content of cells was one of the first major applications of flow cytometry and is still one of the biggest applications in this laboratory today. The DNA content of the cell can provide a great deal of information about the cell cycle, and consequently the effect on the cell cycle of propagation conditions. There are a number of dyes that can be used. In general, for quantitative DNA analysis, cells are fixed in ethanol. After this we can use a number of DNA-binding dyes, e.g. Hoeschst 33342, DAPI, Propidium Iodide, TO-PRO-3, etc. [11].


**MATERIAL AND METHODS**

Three proprietary bottom fermenting Czech yeast strains, denoted in accordance to RIBM 655 Culture Collection of Brewing Yeasts as strain No. 2, 7 and 95, were utilized in this study. Stock cultures were maintained on YPD slanting agars. All media were sterilized immediately following preparation by autoclaving for 20 minutes. Starting cultures for the bioreactor were grown aerobically at 25 °C by shaking in an Erlenmeyer flask (1000 ml) at 150 rpm. Industrial beer wort 13,8 °P was diluted to achieve final concentration 12 °P and used as a growth medium (table I). In order to prevent foam formation was used drop of the Antifoam 204 (Sigma Aldrich) and zinc sulphate was added in amount 0,1 mg/l. Temperature during the yeast propagation was carried out at 12 °C and in case of aerobic conditions was dissolved oxygen concentration carried out up to 5 % of maximal saturation. Aeration intensity was managed in accordance with increasing oxygen supply requirements. The other tests were carried out with various agitator speeds and with strain No. 2. Initially the medium was one time aerated and next conditions were anaerobic.

Experiments were carried out at a 5 litre laboratory bioreactor (Brown Company) with cooling jacket. System was controlled by automatic unite micro DCU Twin with a dissolved oxygen, agitation and temperature monitoring. Aeration was managed by Mass flow controller. The temperature hysteresis was ± 0,1 °C and the oxygen probe was accurate to ± 0,1 ppm.

Flocculation was examined by the modified Stratford flocculation test and with a controlled by micro sedimentation test as well [12].

Flow-cytometric analysis was carried out with a PAS III from Partec (Münster, Germany) and for the fluorescent dyes excitation was used blue light laser (488 nm) [13].

Cell cycle was monitored by procedure described bellow. Fixed cells were incubated in 0,1 % RNase for 40 minutes at 37 °C. After removal of the enzyme solution, the

pellet was resuspended in phosphate buffer (PBS), and 1 ml of propidium iodide staining solution (50 µg/ml) was added to cell pellet and mixed well. After next 40 minutes of incubation cells suspension in PBS were measured by flow-cytometric analysis (excitation: 488 nm and fluorescent detector FL3) [14, 15].

Glycogen content was measured in accordance with Hutter´s work. The yeast pellet was twice washed in PBS buffer and incubated in 1 ml 1 N HCl for 50 min. HCl was removed by centrifugation and stained with acriflavine solution for 1 h (excitation: 488 nm and fluorescent detector FL1) [16].

Protein content in cells was determined by fluorescent probe FITC Fluorescein Isothiocyanate, Isomer I (Sigma, F-1628). Centrifuged pellets were mixed with 100 µl PBS and 10 µl FITC solution (50 µg/ml of acetone). Sample was under 0 °C incubated for 30 minutes and immediately analysed by flow cytometry [17].

| Substance | Content |
|---|---|
| Zn | 0,15 mg/l |
| Ca | 50 mg/l |
| Mg | 100 mg/l |
| Bitterness | 37 B.U. |
| Extract | 13,8 ºP |

Table I: Industrial wort composition.

## RESULTS AND DISCUSSIONS

Not surprisingly, in the case of continuous oxygen supply, all examined strains reproduced significantly faster with higher spec. growth rates (table II).

| Anaerobic | | Aerobic | |
|---|---|---|---|
| Strain No. | Spec. growth rate (h-1) | Strain | Spec. growth rate (h-1) |
| 2 | 0,019 | 2 | 0,061 |
| 7 | 0,016 | 7 | 0,057 |
| 95 | 0,020 | 95 | 0,052 |

Table II: Comparison of strains growing parameters during aerobic and anaerobic propagation.

Changes of G2/M phase cells were determined by flow cytometry. G2/M phase cells were evaluated by pre-set mathematical function based on the fluorescence intensity evaluation (figure 1). Strain No.7 had the shortest lag phase with the fastest G2/M phase reaching and fastest loose in G2/M phase substitution as well. On the other hand strain No. 95 showed the slowest maximum of G2/M phase reaching with the longest maximum G2/M phase cells preservation. By the comparison one time aeration (figure 3) propagation with continuous oxygen supply (figure 2) should be generally noted that G2/M phase cells maximum was faster under aerobic conditions.

Above mentioned results are showing that optimal time for pitching propagated yeast is different not only for each strain but for aerobic conditions too.
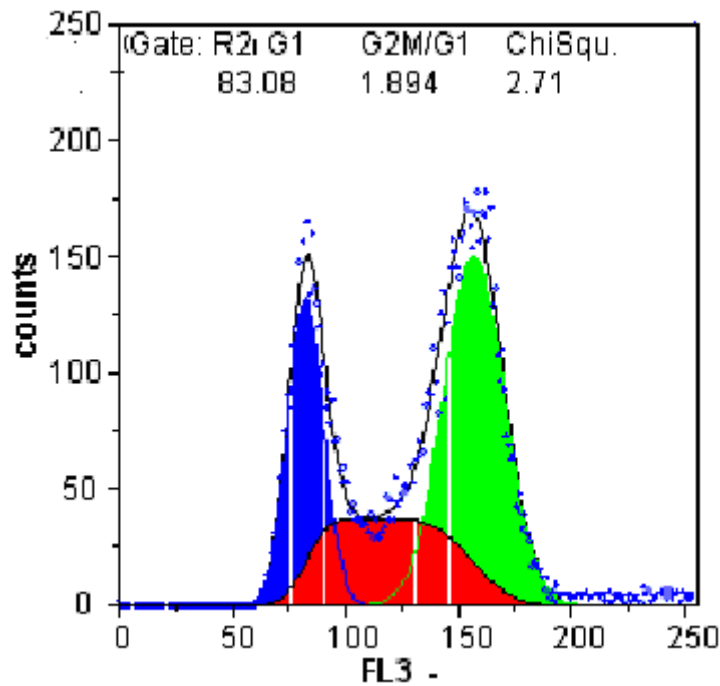


Figure 1: Cell cycle analysis based on the pre/set mathematical model.
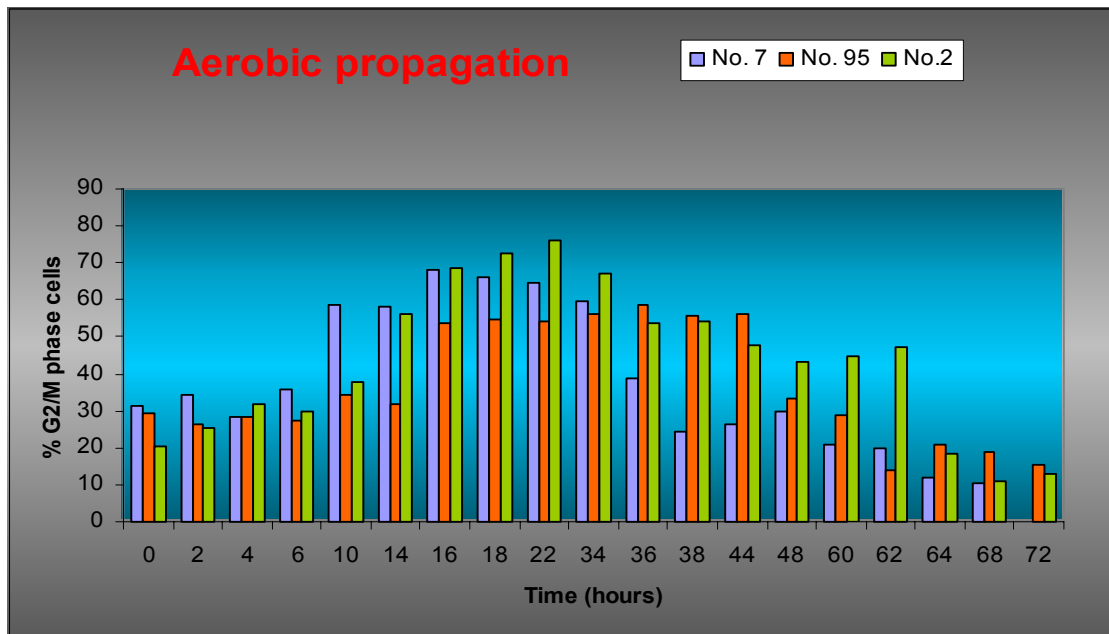


Figure 2: Changes in proportion of G2/M phase cells during aerobic propagation.

Strains had different tendencies to accumulate glycogen. Significantly higher accumulating potential showed strain No. 2. It appeared that the continuous oxygen supply generally caused higher tendencies for glycogen accumulation for all tested strains. Duration of protein amount had typical curves with maximum between 22 and

38 hours of propagation. No significant different of cells proteins was detected by comparison of tested strains under anaerobic and aerobic conditions as well (figure 4).
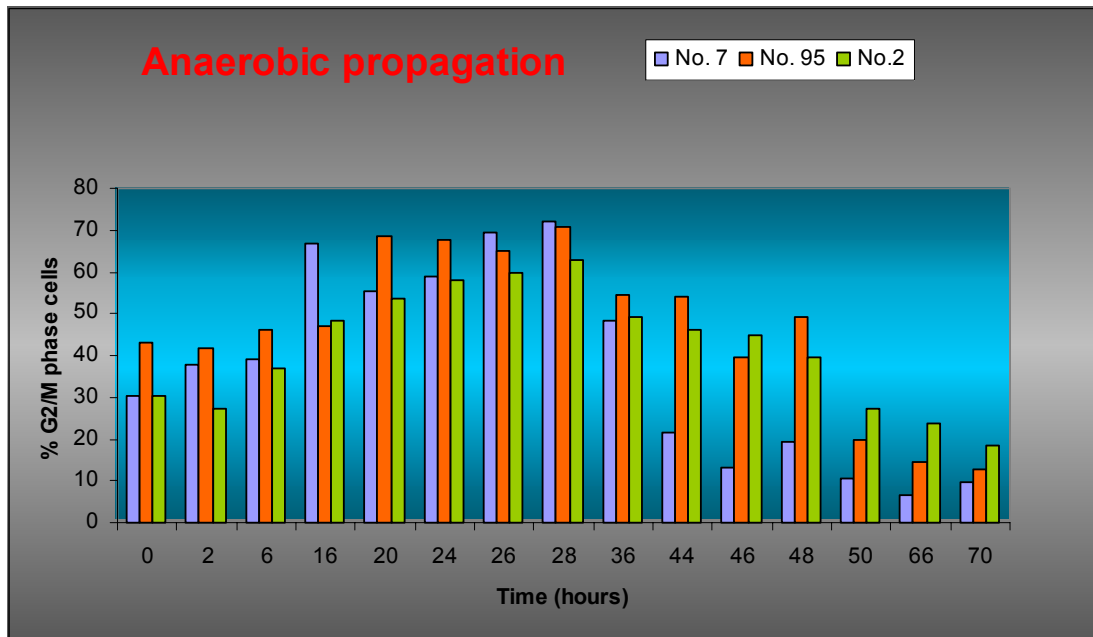


Figure 3: Changes in proportion of G2/M phase cells during anaerobic propagation.
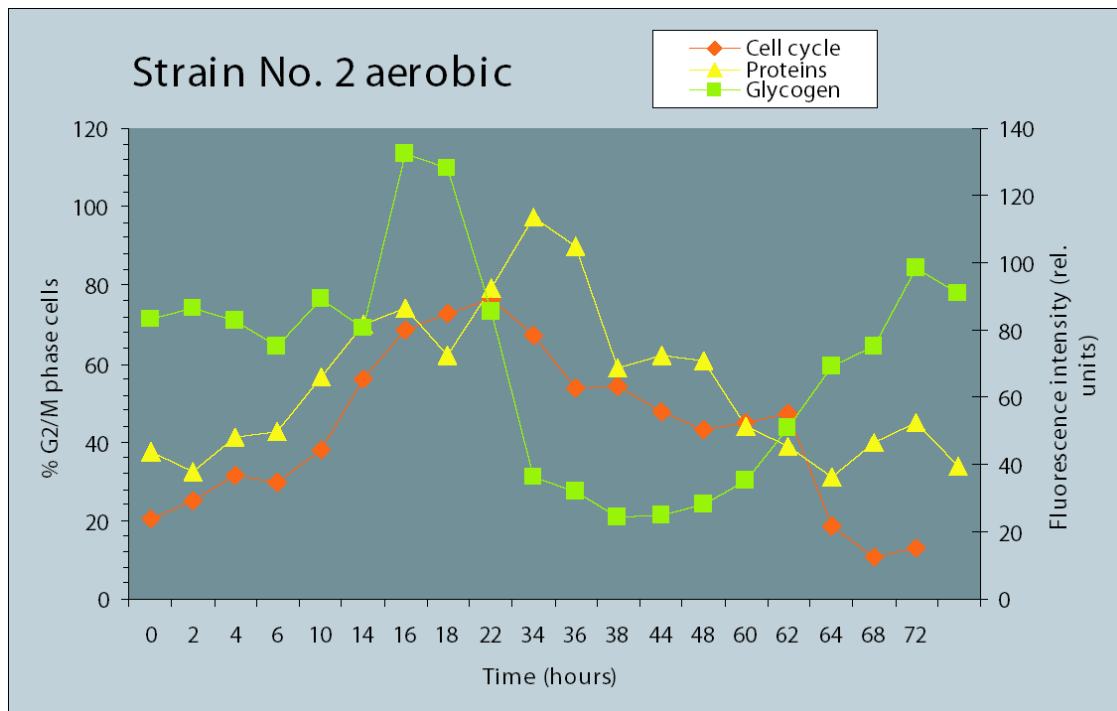


Figure 4: Changes in cells glycogen, proteins and DNA content during aerobic propagation.

Some particular influences of agitator speed on physiological parameters of strain No. 2 were observed. Maximum concentration of biomass was increased with rising

agitation speeds. This should be caused by some oxygen "contamination" of the process. The higher ratio of smaller yeast cells (controlled by FSC parameter) was observed by using maximum agitation speed. Surprisingly the markedly stronger flocculation was observed in test carried out with maximum agitation speed (table III). Flocculation potential of strains No. 7 and 95 was affected by continuous oxygen supply. It appeared that flocculation potential of these strains was dropping with continuous aeration but there also wasn't enough of experiments repetition for 100 % conclusions (figure 5).

| One time aeration (100 %), Strain No. 2 | | |
|---|---|---|
| Agitator speed (min -1) | % of relatively smaller cells | Flocculation (7 min) (%) |
| 150 | 18 | 86 |
| 300 | 22 | 90 |
| 600 | 34 | 99 |

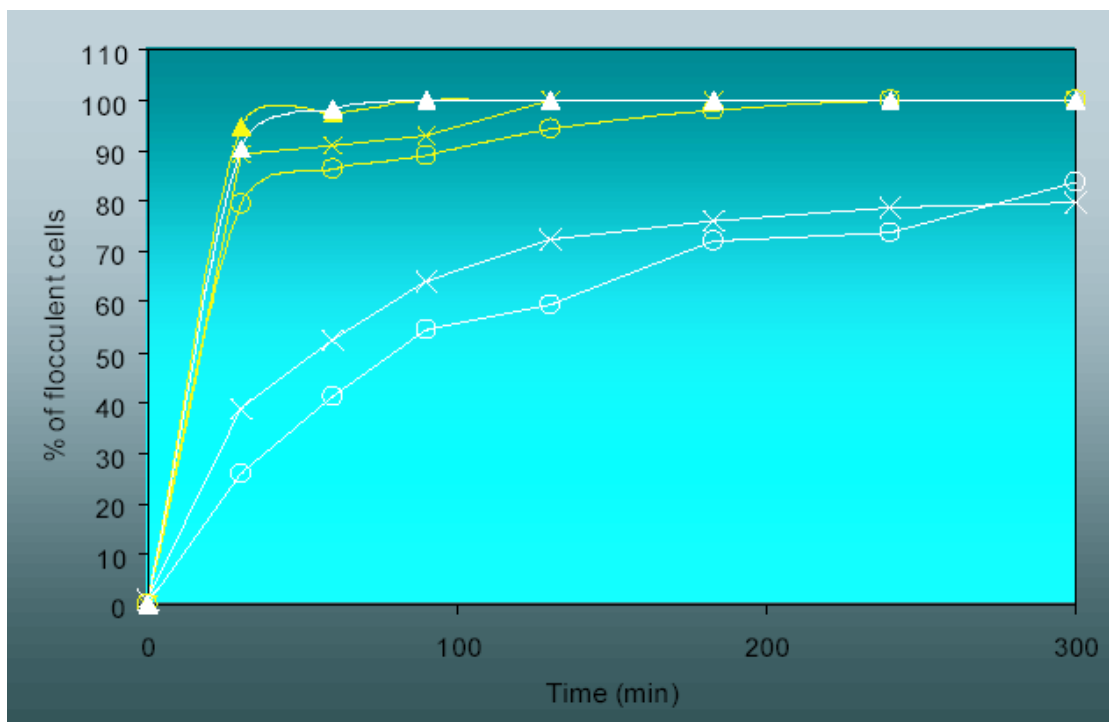Table III: Tests with strain No. 2 and various agitating speeds.



Figure 5: Continuous oxygen supply effects for the flocculation ability of examined strains (ΔNo2, ONo7, XNo95).

**CONCLUSION**

On the basis of these observations, it can be concluded that:
- strains have different tendencies to accumulate glycogen with highest accumulation potential for strain No. 2;
- flocculation potential of some strains seemed to be affected by continuous oxygen supply;

- in the case of continuous oxygen supply, all tested strains reproduced significantly faster and also faster reached the maximum of G2/M phase cells;
- strain No. 7 showed the shortest lag time and time for reaching maximum G2/M phase cells compared to the others;
- markedly stronger flocculation of strain No. 2 was observed when the agitation speed was 4 times faster than under initial conditions.

## ACKNOWLEDGEMENTS

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