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Application of Flow Cytometry to *Saccharomyces cerevisiae* Population Analysis

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Abstract: This study was focused on the development and the application of a rapid and reliable staining method for the characterisation of *Saccharomyces cerevisiae* cells. The experiments were carried out during the shaken flask batch cultivation of yeasts on YEPD medium under aerobic conditions at 27 °C. Stained samples were analysed with an epifluorescence microscope or by employing a flow cytometer. Three different fluorescent probes such as propidium iodide (PI), fluorescein diacetate (FDA), and fluorescein isothiocyanate (FITC) were used for staining. PI was used to determine cell viability in a native sample and DNA content in a sample fixed by ethanol. To assess protein distribution in the yeast population the FITC amine-reactive probe was used. Instantaneous cell enzyme activity was measured as the amount of fluorescein liberated from FDA by intracellular esterase activity.

Keywords: Cell physiology · Esterase activity · Flow cytometry · Fluorescence microscopy · Nucleic acid content · Protein content · *Saccharomyces cerevisiae* · Viability

Introduction

Direct optical methods such as flow cytometry and fluorescent microscopy have increased their application potential in the field of biotechnology over the last few years. The main advantage, which opens the possibility for flow cytometry to become an efficient tool in microbial analysis, is its ability to even quantitatively analyse particles at rates of thousands in a very short time period at single cell level. In connection with fluorescent staining it represents an extremely powerful tool for monitoring various cellular attributes within microbial populations.

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Nowadays, many studies have already been carried out using flow cytometry. The most common application in yeast analysis is a determination of viability [1–3] that is based on the determination of cell functions such as membrane integrity, existence of membrane potential, enzyme activity, respiration ability and intracellular pH that can be taken as a reliable viability marker.

Flow cytometry and fluorescent staining are also able to provide a detailed measurement of many physiological attributes. Enzyme activities can be measured as a fluorescence intensity of the fluorescent product of enzyme hydrolysis [4] characterised by an uptake and the hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in *Saccharomyces cerevisiae*. Fluorescent dyes with pH-dependent absorption or emission spectra enable the determination of the intracellular pH of yeast [5].

Potentiometric probes offer a method for the detection of changes in membrane potential referred to as membrane hyperpolarisation and depolarisation and can be also used for visualising mitochondria exhibiting transmembrane potentials of approximately 150 mV. This property is employed in the measurement of respiratory activity of yeasts [6] and assessment of mitochondrial membrane potential [7][8].

The flow cytometric determination of intracellular components such as ions, proteins, nucleic acids [9] or reserve carbohydrates [10] is also not exceptional. A formation of fluorescent patches on the cell wall surface originated from budding can be observed after primulin staining. Studies of cell organelles, cytoskeleton organisation [11], cell morphology and fluid flow are more commonly carried out employing epifluorescence or confocal scanning microscopy, however even in this field flow cytometry is being used more often. Up-to-date flow cytometry is used in connection with modern molecular biology methods such as fluorescent *in situ* hybridisation [12] or an analysis of fluorescence originated from green fluorescent protein [13].

Material and Methods

Strain

All experiments were performed with *Saccharomyces cerevisiae* 15-1-410 RIVE from the Yeast Collection of the Research Institute for Viticulture and Enology, Slovak Republic.

Inoculum

Preculture was prepared in 100 ml media in 250 ml Erlenmeyer flasks, 150 rpm, 27 °C, 48 h, under aerobic conditions.

Culture Conditions

Yeasts were proliferated in liquid YEPD medium (yeast extract 1%, peptone 2%, glucose 2%, pH ~6.5). 250 ml YEPD in 700 ml Erlenmeyer flasks were inoculated with 2 ml of inoculum. The cultivations were carried out for 30 h at 27 °C, 150 rpm and under aerobic conditions.

Staining Protocols

For the viability determination, yeasts were stained with propidium iodide (PI), applying a concentration of 15 μg/ml, the incubation time was 10 min at 25 °C. Fluorescein diacetate (FDA) (10 μg/ml, 30 min, 25 °C) was used for a study of esterase activity. Nucleic acid and DNA content were stained with PI (15 μg/ml, 45 min, 25 °C) after fixation with 70% EtOH (30 min, 4 °C), without or after RNase treatment (2.5 μg/ml, 1 h, 37 °C), respectively. Protein content was assessed after the fixation of the sample (70% EtOH, 30 min, 4 °C) using FITC (1 μg/ml, 30 min, 0 °C). In all cases phosphate buffer saline (PBS) pH 7.4 was employed as a reaction buffer, except for protein determination, where a bicarbonate buffer with pH 9.0 was used.

Instrumentation

Stained samples were analysed using a BX-51 Olympus microscope equipped for epifluorescence connected with a C5050Z Olympus digital camera (Japan). Digital image data were evaluated using the LUCIA imaging system (LIM, CZ) and by flow cytometry employing the PAS III Partec flow cytometer (Partec, Germany), software: Partecflow and WinMDI 2.8.

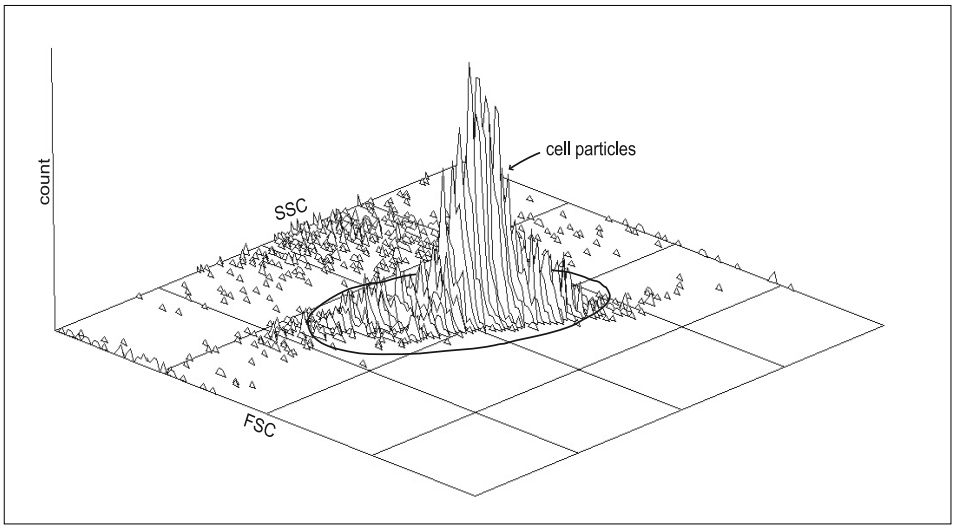

Fig. 1. 3D projection of FSC *versus* SSC dot-plot diagram obtained by flow cytometry after 17 h cultivation

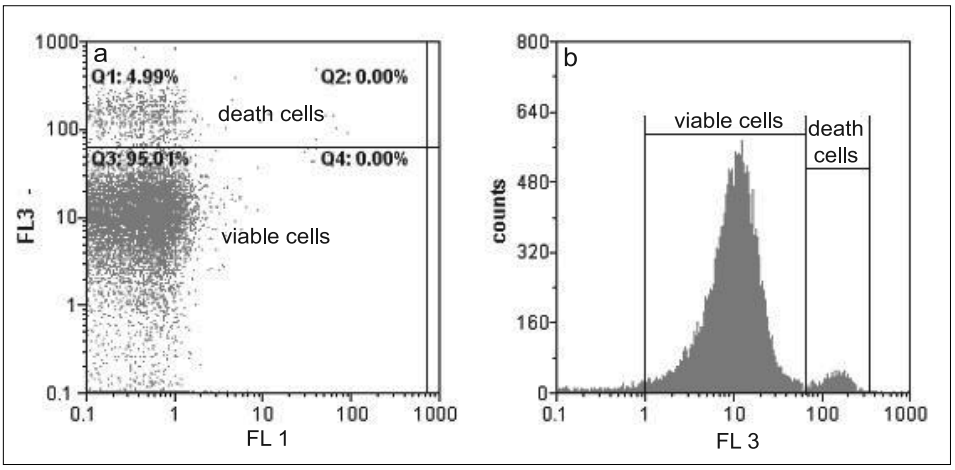

Fig. 2. Two different possibilities of expressing viability as a) dot-plot diagram or b) histogram after flow cytometry analysis

Results and Discussion

Rapid detection and characterisation of the physiological state of microorganisms is very important in many fields of applied microbiology and biotechnological processes. Therefore, in this study we tried to apply some optical methods for analyses of *Saccharomyces cerevisiae* culture as a model microorganism. The viability, esterase activity, and content of protein and nucleic acid were measured. All the experiments were carried out on a flow cytometer and obtained results were subsequently confirmed by fluorescent microscopy.

In flow cytometry, cells are carried within a fast flowing fluid stream; as they pass through the light beam, forward scatter (FSC), side scatter (SSC) and fluorescence with different wavelengths are measured. FSC and SSC are parameters giving information about cell morphology and volume; therefore, they can be used to distinguish cells from impurities based on a different morphological characteristic. An example of the discrimination of yeast cells and impurities applying gating in a dot-plot diagram is shown in Fig. 1.

Viability, *i.e.* the percentage of viable cells was calculated as non-stained particles (with defined morphological characteristics) after the incubation of samples with propidium iodide (Fig. 2). This dye is not able to penetrate intact membranes of microorganisms; on the other hand it can freely enter the cells with damaged or interrupted membranes. During the 30 h cultivation period the viability did not vary a lot, the meaningful decrease in viability was achieved in a late stationary phase (Fig. 3).

Instantaneous cellular hydrolytic activity can be measured as the amount of fluorescent product liberated from non-fluorescent substrates by intracellular esterase activity. There is a whole range of different fluorescent substrates designed for various kinds of enzymes. One of them is fluorescein diacetate, a non-fluorescent, cell permeable substrate that is intracellulary converted by a non-specific esterases to a bright green fluorescein. The changes in esterase activity, measured as a mean intensity of green fluorescence assayed in single cells are recorded in Fig. 4. The maximum hydrolytic activity was achieved at the end of the exponential and at the beginning of the stationary phase, where the C-source is depleted and yeast probably starts to utilize reserve carbohydrates.

The amount of intracellular proteins, nucleic acids, and DNA changes during the cell cycle, therefore the information of their content can give us information about the state in which they occur. Employing the conventional methods for the assessment of these macromolecules, it is nearly impossible to obtain information for particular cells. Even in the case of the whole sample analysis, the isolation step of analysed matter must precede terminal analytical methods. All these disadvantages are eliminated when using flow cytometry and selective staining of macromolecules. To quantify protein content, the amine-reactive probe fluorescein isothiocyanate was used. For nucleic acid and DNA, the above-mentioned propidium iodide was applied. It binds to a nucleic acid with little or no sequence preference and after binding fluorescence increases by about ten times.

Most flow cytometers are equipped with the software that utilise mathematical models enabling cell cycle analysis from

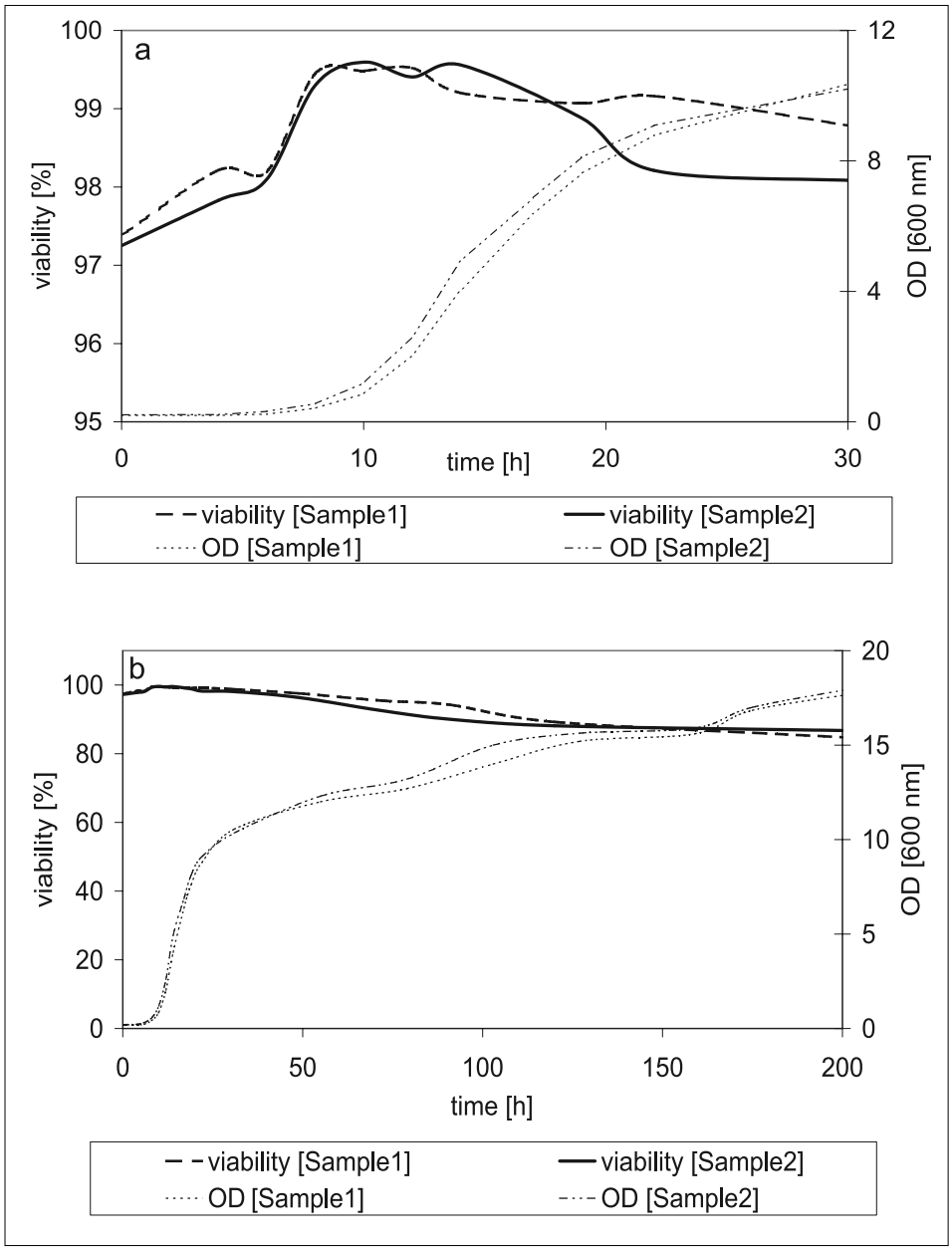

Fig. 3. Changes of *Saccharomyces cerevisiae* viability during a) 30 h, b) 200 h aerobic cultivation

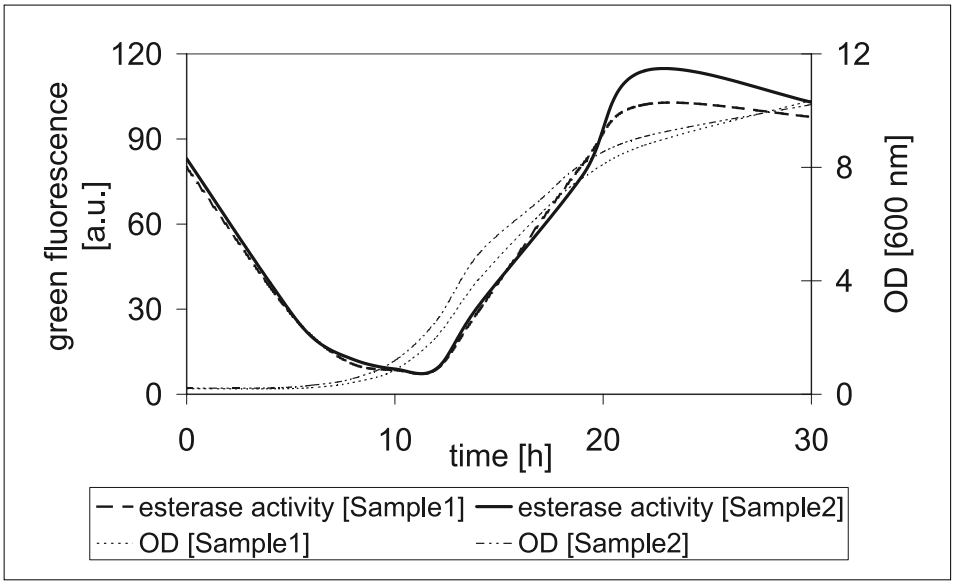

Fig. 4. The changes of esterase activity during the yeast growth, expressed as a fluorescence intensity of fluorescein liberated from fluorescein diacetate

an intensity histogram obtained from cells stained with PI (Fig. 5). These software packages are very often developed for analysis of mammalian cells, and in some cases are not applicable in yeast analysis to distinguish different phases of cell cycle. This phenomenon is due to a much larger ratio of mitochondrial to chromosomal DNA that disturbs an assay, therefore the possibility to calculate the percentage of cells in particular states of the cell cycle depends strongly on the yeast strain and the software. Likewise, manual gating can be used, but with a lower accuracy.

Nevertheless, the study of changes in the overall protein and nucleic acid content can provide valuable information about the cell or population dynamics. The fluorescence of cells induced from the stain can be viewed as a histogram, from which the parameters are calculated. The simplest and also the most common method is to calculate with the mean values, and of course a detailed peak and histogram analysis is possible too. The changes in the mean fluorescence of stained protein and nucleic acid during the growth and aging process of *S. cerevisiae* cells are shown in Fig. 6.

Conclusion

The usefulness of flow cytometry is based on its ability to simply and rapidly analyse a very high number of particles. In this study, some of the examples of the practical application of fluorescent staining in connection with flow cytometry have been shown. *Saccharomyces cerevisiae*, the yeast important to many industrial processes, was chosen as a model microorganism. The basic characteristics of the cell physiology and its changes were measured. Firstly, the possibility of viability assays was evaluated; flow cytometry provides a method for almost online analysis and it is better than conventional extremely time-demanding plate count methods. The existence of a wide variety of fluorescent stains offers much more specific and accurate viability determination than staining with methylene blue.

Non-specific enzyme activity and intracellular macromolecule content were measured during yeast growth. Significant changes in fluorescence were determined for all parameters reflecting the vitality and physiological state of yeast, such as a typical accumulation of proteins at the beginning of the exponential phase or an increase of intracellular hydrolytic activity after the depletion of the elementary carbohydrate source. It was evaluated that the above-mentioned parameters can be effectively measured using flow cytometry and there is no need to use demanding isolation and treatment steps that can, more-

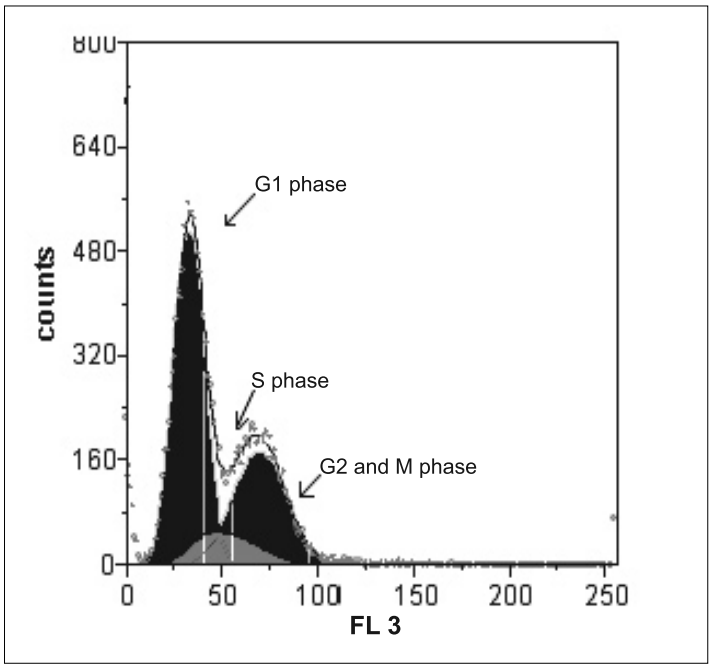

Fig. 5. Example of cell cycle analysis from a linear-scale histogram obtained after propidium iodide staining of ethanol fixed and RNase treated cells

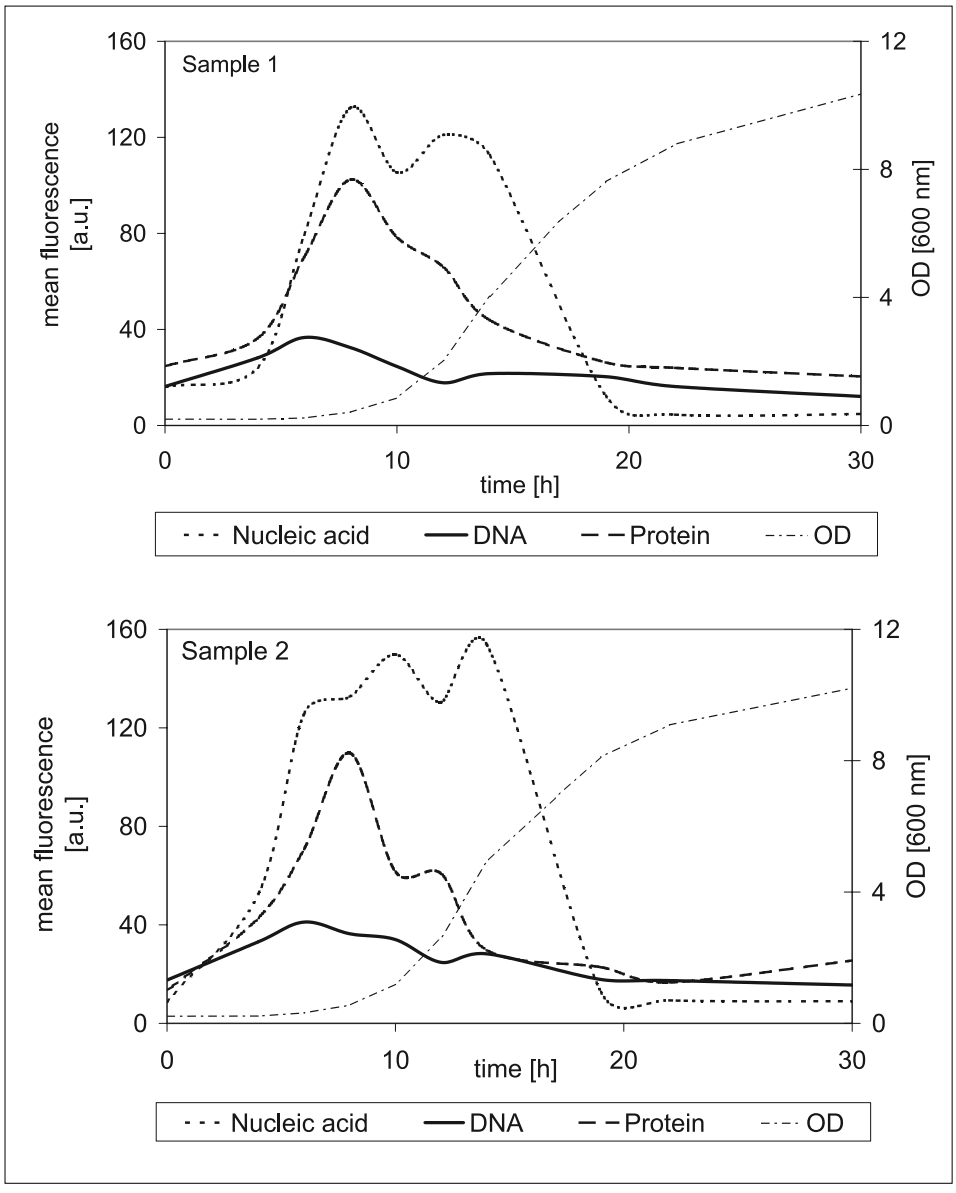

Fig 6. The changes of nucleic acid, DNA, and protein content during the yeast growth for two parallel experiments, obtained as a fluorescence response of stained macromolecules

over, influence the cell physiology and also the results. This study, of course, does not cover all the possibilities for the application of flow cytometry as a tool for yeast analysis; the purpose was to optimise the staining conditions for yeasts and display the possibilities for the application of flow cytometry to *Saccharomyces cerevisiae* population analysis.

Acknowledgements

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