STAINING OF YEAST CELLS WITH DIFFERENT FLUORESCENT DYES

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ABSTRACT

In order to optimize fermentation processes, it is important to characterize the physiological and metabolic activity of the yeast cells throughout the procedure. Four fluorescent dyes were compared: red fluorescent dyes propidium iodide (PI) and thiazole orange cyanide (TO3CN) and green fluorescent dyes thiazole orange (TO) and new cyanine dye Sofia Green. The absorbance spectra were observed and the emission spectra before and after binding to DNA were compared. The excitation and emission peak for each dye was determined. The rate of emission enhancement after binding to DNA was established. The dyes were combined and applied for live/dead staining of *Saccharomyces cerevisiae cells* and monitored by fluorescence microscopic image. Propidium iodide and Sofia Green dyes stained only dead cells. TO and TO3CN stained both dead and live cells. A double staining procedure was carried out. Propidium iodide was used in combination with TO dye and Sofia Green combined with TO3CN dye. The optimal concentration of the dyes for the cell staining was determined: 4 µg/ml for PI, 1 µg/ml for TO, 0.1 µg/ml for Sofia Green and 10 µg/ml for TO3CN. The optimal incubation time for cell staining was evaluated. A drop from the suspension was applied on a slide and was observed under a fluorescence microscope. The use of such combination of fluorochromes that selectively permeate in live or dead cells could lead to the development of rapid procedure for yeast viability investigation during fermentation in different industrial and food processes.

Key words*: fluorescent dyes, emission spectra, cell staining, fluorescence microscope*

INTRODUCTION

The brewing and wine industries rely predominantly on the yeast *Saccharomyces cerevisiae*. In order to optimize fermentation processes, it is important to characterize the physiological and metabolic (the viability and vitality, respectively) of the yeast cells throughout the procedure. Yeast are viable when the cells have intact membranes. Vitality, on the other hand, is defined by quantifiable metabolic activity as well as the ability to proliferate. It is possible to have yeast that are viable but not actively proliferating, an event which can adversely affect fermentation.

Cell counting method using fluorescent microscope can monitor yeast concentration and viability throughout fermentation to ascertain cell health and the amount of yeast to be pitched or repitched, all of which contributes to the quality and flavor consistency of the final product [2]. Analyzing physiological and metabolic characteristics of the yeast cells permits operators to efficiently monitor yeast viability and vitality for quality control purposes, which impacts long-term storage and other physiological stresses [8]. Traditionally, methylene blue has been implemented to measure yeast viability. The metachromatic stain can distinguish between live and dead cells using basic bright field microscopy [9]. Vital yeast cells convert methylene blue to a colorless solution via cellular dehydrogenase, while non-vital cells remain blue. Consequently, methylene blue is a tool for both viability and vitality analysis as it measures enzymatic activity. Even though this dye is considered the gold standard for the brewing industry, this method is time-consuming and susceptible to human error that results in user-dependent variations [3]. New fluorescent dyes that assess principles such as membrane integrity, metabolic activity (carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM)) have recently become available to analyze yeast viability and vitality [7]. Other stains such as propidium iodide (PI), ethidium bromide (EB), 4′,6-diamidino-2-phenylindole (DAPI), and 7-aminoactinomycin D (7-AAD), are membrane impermeable dyes that penetrate only membrane-compromised yeast, thereby identifying nonviable cells [6]. Dual staining methods acridine orange (AO) and PI can determine yeast viability, while CFDA-AM and PI can determine yeast vitality [4].

The aim of this study was comparison of optic characteristics of new fluorescent dye Sofia Green with other fluorescence dyes - red fluorescent dyes Propidium iodide and TO3CN and green fluorescent dyes TO. The possibility of staining cells *Saccharomyces cerevisiae* by combination of

Sofia Green with TO3CN and Propidium iodide with TO were investigated by fluorescence microscopy.

MATERIALS AND METHODS

Materials

All the used reagents were purchased from the following companies: Propidium iodide (PI) from Thermofischer scientific, TO3CN, TO and Sofia Green from Milkotronic Ltd, Deoxyribonucleic acid from fish sperm (DNA) from Sigma Aldrich. All solutions were prepared using deionized water from a Purelab ultra-system (Elga, Buckinghamshire, UK). Cell culture ISP-GO. Strain V30 *(Sacch. cerevisiae)* was purchased from the collection of the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC). Yeast extract, yeast bacto-peptone and glucose were purchased from Sigma Aldrich.

Apparatus

The measurements were performed using UV/Vis spectrophotometer Jenway 6900 and fluorescent spectrophotometer Perkin Elmer LS 45. The fluorescent microscopic images were captured using fluorescent microscope Olympus BX 51.

Studying of dye absorption spectra

Stock solutions of the dyes were prepared at concentration of 1 mg/ ml in 10 mM phosphate buffered saline (PBS) with pH 7.4. The stock solution of the dye was diluted to final concentration of 50 μg/ml and the absorption spectra of the fluorescent dyes were measured.

Studying of dye fluorescent spectra without and with DNA binding

Stock solutions of the dyes were prepared at concentration of 1 mg/ ml in 10 mM phosphate buffered saline (PBS) with pH 7.4. Stock solution of the DNA was prepared at concentration of 1 mg/ ml in deionized water.

For PI the stock solution of the dye was diluted to final concentration of 50 μg/ml and the DNA solution was diluted to reach concentration of 500 μg/ml. After that the solutions were mixed in a ratio dye: DNA = 1:1.

For TO3CN, TO and Sofia Green the stock solution of the dye and the DNA solution was diluted to reach concentration of 50 μg/ml. After that the solutions were mixed in a ratio dye: DNA = 1:12.5. The spectra before and after binding to DNA were studied.

Yeast growth conditions

Yeast ISP-GO. Strain V30 *(Sacch. cerevisiae)* was grown in a standard liquid YPD medium (1% yeast extract, 1% yeast bacto-peptone, 2% glucose) on a rotary shaker at 150 r.p.m. at a temperature of 28 °C for 48h.

Cell staining

The cell culture was inoculated in YPD medium and grown for 48 hours. The cell sample was taken at 24h. Cell suspensions in concentration of 5.0×10^6 in phosphate buffer, pH 7.4, was prepared and washed twice with dH_2O by centrifugation at 220 x g. A part from the cell suspension was treated at 70 ° C for 15 min. After that it was mixed with the untreated culture to obtain 50 % dead and 50 % live cells. From each live/dead yeast mixture, aliquots of 1 mL yeast suspension was stained with combination of two dyes – TO3CN and Sofia Green, PI and TO. The dye concentrations were varied (from 0,1 - 10 μg mL^{-1}) and the optimal concentration of each dye for cell staining was determined. After the addition of the fluorescent dyes the samples were incubated at room temperature. The incubation period varied from 5 to 60 minutes, and the optimal incubation time for each dye was determined. A drop from the stained suspension was applied on a slide and was observed under a fluorescence microscope Olympus BX 51. At least three repetitions were performed at every experimental stage.

RESULTS AND DISCUSSION

The optical properties of the fluorescent dyes TO, TO3CN, Sofia Green and PI have been investigated and compared. TO and TO3CN dyes were obtained from Milcotronic Ltd. by the

methodologies described in the following publications [5]. The PI dye was purchased from Sigma Aldrch. Sofia Green is a newly synthesized dye from Milkotronic Ltd. Sofia Green is green fluorescence nuclear staining dye from asymmetric monomethyncyanine group. This polycationic asymmetric monomethine cyanine dye has three positive charges [1]. The absorption spectra of the four dyes were sequentially studied. A comparison of the obtained results was made. TO and TO3CN stains live and dead cells, but PI and Sofia Green stains only dead cells.

First the absorption spectrum of each dye was observed using UV-Vis spectrophotometer, to decide the excitation maximum. On figure 1a and 1b are presented the absorption spectra of TO3CN, PI and Sofia Green, TO respectively. The excitation maximums for TO3CN and PI were 550 and 530 nm, for Sofia Green and TO were 476 and 500 nm. The final concentration of each dye for these measurements is 50 µg/ml. It was found that the absorption intensity of TO3CN is lower than intensity of other three dyes. Beside that TO3CN have a two absorption maximums corresponding to TO and CN groups.

After the establishment of the excitation maximum of each dye, the emission maximums before and after binding to DNA were observed. On figure 2 fluorescence spectra of PI and TO3CN are presented. It was observed that the emission maximum of PI is at 617nm and for TO3CN is at 603 nm.

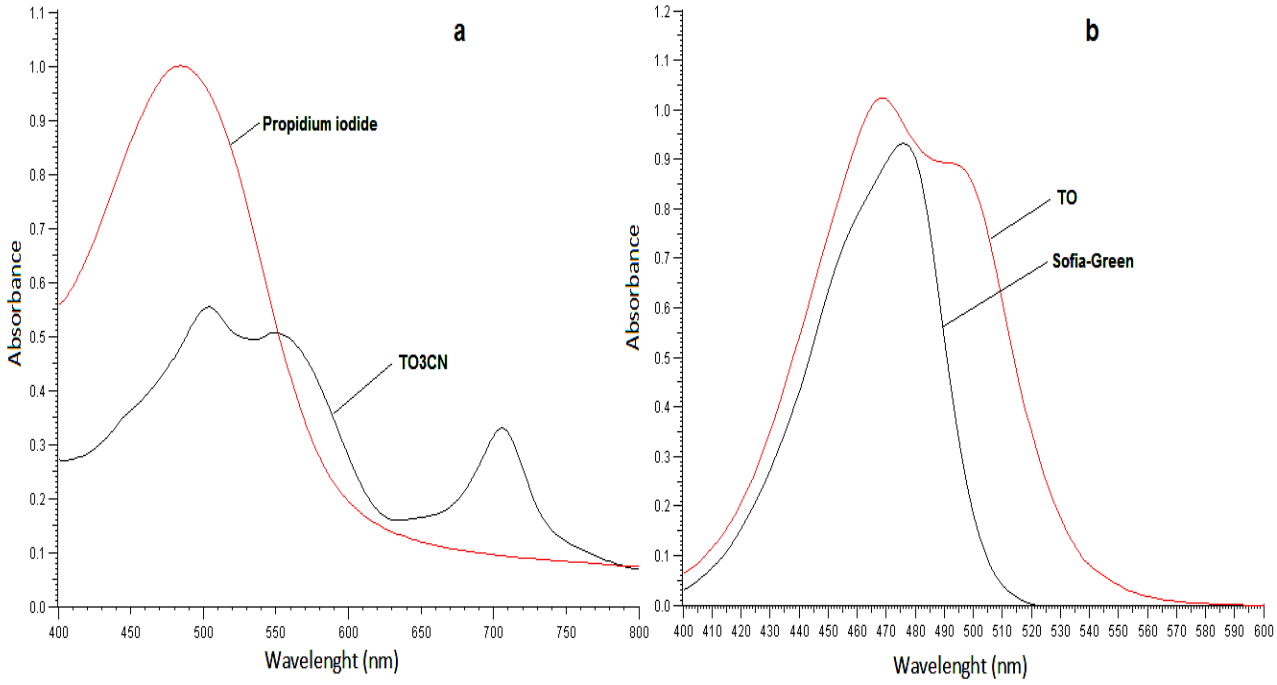

Figure 1. Absorption spectrum of TO3CN and PI (a) and of Sofia Green and TO (b)

The emission intensity of TO3CN before binding to DNA was much lower than the intensity of PI, which ensures minimal background. The increase of the fluorescence of TO3CN after binding to DNA was 50 times compared to PI which was only 23 times.

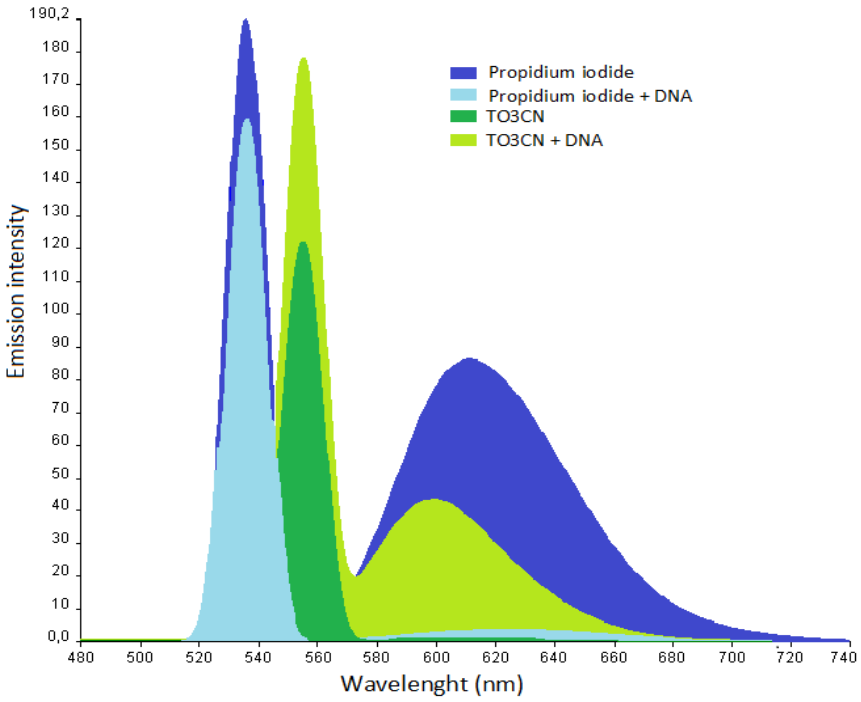

Figure 2. Fluorescence spectra of PI and TO3CN before and after binding to DNA

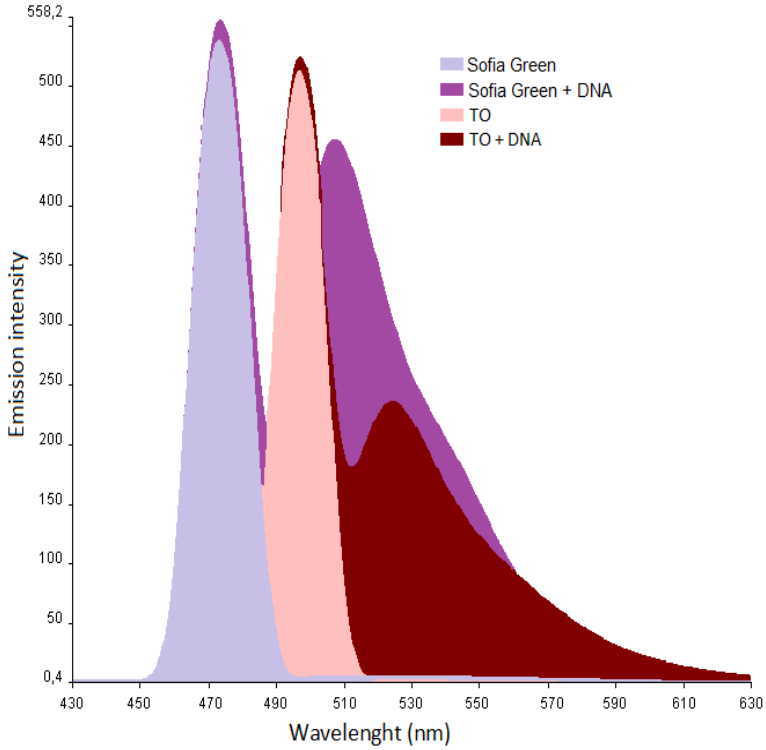

Figure 3. Fluorescence spectra of TO and Sofia Green before and after binding to DNA.

The same observation was carried out for Sofia Green and TO (Fig.3). It was found that both the dyes had low emission intensity before binding to DNA and a great increase of the fluorescence signal after binding to DNA. The increase was 65 and 85 times for TO and Sofia Green respectively. The basic optical characteristics of the four dyes were summarized in table 1.

Table 1. Basic optical characteristics of the investigated dyes

Dye	Excitation (nm)	Emission (nm)	Emission amplification after binding to DNA
Propidium iodide	530	617	23 times
TO3CN	550	603	50 times
Sofia Green	476	512	85 times
TO	500	530	65 times

Combination of dyes was applied for live/dead staining of *Saccharomyces cerevisiea* cells. First, the cell culture was grown for 24h. Then the cell suspension was treated at high temperature (70 °C for 15 min) and mixed with untreated culture at a ratio 50:50. A double staining procedure was carried out. Propidium iodide (staining only dead cells) was used in combination with TO (staining both dead and live cells) and Sofia Green (staining only dead cells) was used in combination with TO3CN (staining both dead and live cells). The optimal concentration of the dyes for the cell staining was determined: 4 µg/ml for PI, 1 µg/ml for TO, 0.1 µg/ml for Sofia Green and 10 µg/ml for TO3CN. The cells were incubated with the dyes from 5 to 60 min. It was found that the optimal time for all dyes was 15 min.

After that the samples were observed under a fluorescence microscope. On figure 4 the fluorescence microscopic image of *Saccharomyces cerevisiae* stained with TO and Propidium iodide are presented.

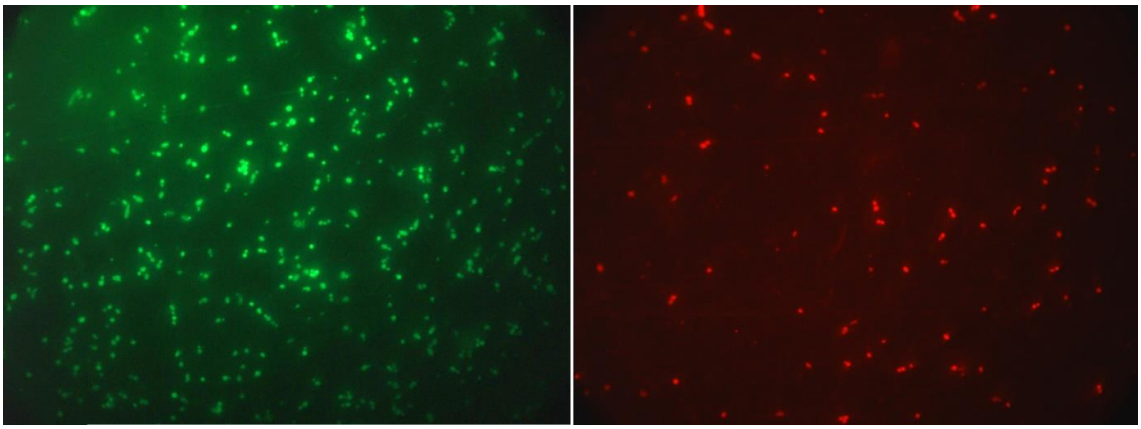

Figure 4. Fluorescent microscopic images of *Saccharomyces cerevisiae* stained with TO and Propidium iodide (x40)

On figure 5 the fluorescent microscopic images of *Saccharomyces cerevisiae* stained with TO3CN and Sofia Green are presented.

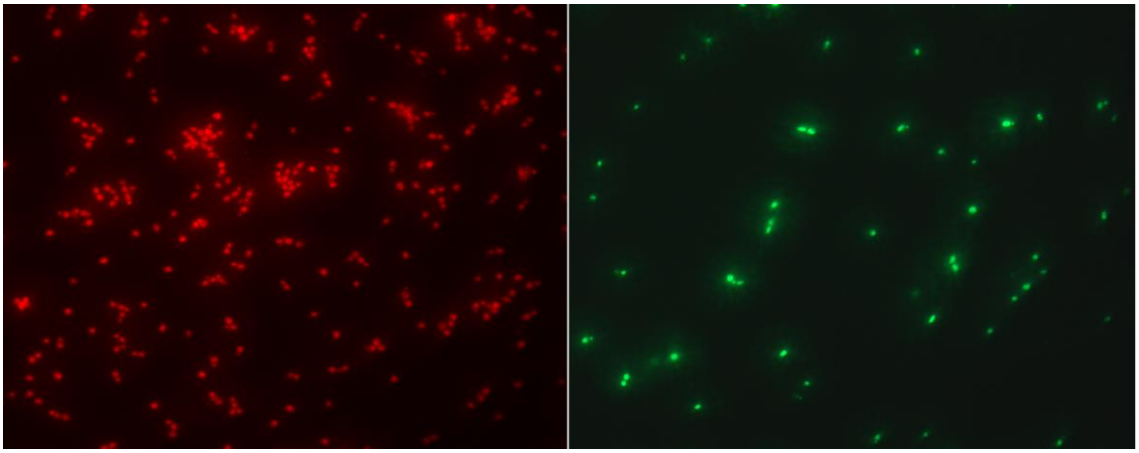

Figure 5. Fluorescent microscopic images of *Saccharomyces cerevisiae* stained with TO3CN and Sofia Green (x40)

The obtained results presented possibility of investigated dyes for determination of live and dead yeast cell count. Yeasts are an economically important organism used for ethanol production, in the beverage and alternative fuels industries as well as a leavening agent in the baking industry. Concentration and viability determinations are routinely performed for quality control purposes in yeast production, fermentation processes, and fungicides research to monitor proliferation of pathogenic yeasts. The most common method for determining yeast cell number and viability is manual counting on a fluorescent microscope. The four dyes can be useful for determination of cell viability and continuous control of processes.

CONCLUSION

The optic characteristics of new fluorescent dye Sofia Green was determined and compared with other fluorescence dyes - red fluorescent dyes Propidium iodide and TO3CN and green fluorescent dyes TO. The combination of Sofia Green with TO3CN and Propidium iodide with TO can be use for staining of cells *Saccharomyces cerevisiae* and determined the viability of yeast by fluorescence microscopy.

REFERENCES:

1. Atanasova, M., Ivanov, Y., "Studing of characteristic of fluorescent dye YO-Dam-1". Proceedings of University of Ruse, Biotechnology and food biotechnology, 54, 10.2: 70-74, 2015
2. Capece, A., Romaniello, R., Siesto, G., "Conventional and Non-Conventional Yeasts in Beer Production". Fermentation, 4(38):1-11, 2018
3. Chan, L. L., Kuksin, D., Laverty, D. J., "Morphological observation and analysis using automated image cytometry for the comparison of trypan blue and fluorescence-based viability detection method. Cytotechnology, 67(3):461-473, 2015
4. Chan, L. L., Wilkinson, R. A., Paradis, D. B., Lai, N., "Rapid Image-based Cytometry for Comparison of Fluorescent Viability Staining Methods". J Fluorescence, 22:1301–1311, 2012
5. Gu, Y., Fei, X., Lan, Y., Shib, B., Zhang, B., Jia, G., "Synthesis, crystal structure and spectral properties of thiazole orange derivative". J Chalcogenide Letters7 (5): 305-312, 2010
6. Kwolek-Mirek, M., Zadrag-Tecza, R., "Comparison of methods used for assessing the viability and vitality of yeast cells". FEMS Yeast Res, 14:1068–1079, 2014
7. Mascotti, K., McCullough, J., Burger, S. R.," HPC viability measurement: trypan blue versus acridine orange and propidium iodide". Transfusion, 40:693–696, 2000
8. Novak, J., Basarova, G., Teixeira, J.A.,"Monitoring of brewing yeast propagation under aerobic and anaerobic conditions employing flow cytometry". J Inst Brew, 113:249–255, 2007
9. Stoddart, M., "Cell viability assays: introduction". Methods Mol Biol.,740:1–6, 2011