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BIOTECHNOLOGY METHODS

Novel image cytometric method for detection of physiological and metabolic changes in *Saccharomyces cerevisiae*

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Abstract The studying and monitoring of physiological and metabolic changes in *Saccharomyces cerevisiae* (*S. cerevisiae*) has been a key research area for the brewing, baking, and biofuels industries, which rely on these economically important yeasts to produce their products. Specifically for breweries, physiological and metabolic parameters such as viability, vitality, glycogen, neutral lipid, and trehalose content can be measured to better understand the status of *S. cerevisiae* during fermentation. Traditionally, these physiological and metabolic changes can be qualitatively observed using fluorescence microscopy or flow cytometry for quantitative fluorescence analysis of fluorescently labeled cellular components associated with each parameter. However, both methods pose known challenges to the end-users. Specifically, conventional fluorescent microscopes lack automation and fluorescence analysis capabilities to quantitatively analyze large numbers of cells. Although flow cytometry is suitable for quantitative analysis of tens of thousands of fluorescently labeled cells, the instruments require a considerable amount of maintenance, highly trained technicians, and the system is relatively expensive to both purchase and maintain. In this work, we demonstrate the first use of Cellometer Vision for the kinetic detection and analysis of vitality, glycogen, neutral lipid, and trehalose content of *S. cerevisiae*. This method provides an important research tool for large and small breweries to study and monitor these physiological behaviors during production, which can improve fermentation conditions to produce consistent and higher-quality products.

Keywords Image cytometry · Cellometer Vision · *Saccharomyces cerevisiae* · Viability · Vitality · Glycogen · Neutral lipid · Trehalose

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Introduction

The studying and monitoring of physiological and metabolic changes in *Saccharomyces cerevisiae* (*S. cerevisiae*) has been a key research area for the brewing, baking, and biofuels industries, which rely on these economically important yeasts to produce their products [1–3]. Specifically for breweries, physiological characteristics such as viability, vitality, glycogen, neutral lipid, and trehalose content can be measured to better understand changes in physiological and metabolic status of *S. cerevisiae* during fermentation [4–9]. In addition, monitoring changes in these parameters during fermentation can help improve or optimize current production processes to yield more consistent and higher-quality products. Furthermore, glycogen, neutral lipid, and trehalose content have been shown to play an important role in predicting yeast viability during and post-fermentation, which is essential for the efficiency and time-duration of the production process [10, 11].

Monitoring relative viability and vitality of yeasts during fermentation is commonly used to determine the efficiency

of the ethanol production process. Traditionally, yeast viability is defined as the ability for cells to reproduce, and it is measured by counting the number of colony-forming units (CFUs) [12–14]. However, this method typically requires days in order to determine the viability of the target yeast sample. More commonly, viability can be determined using a hemacytometer and a metachromatic stain, such as methylene blue to distinguish between live and dead yeasts [14, 15]. Metabolically active live cells with dehydrogenase activity convert the methylene blue to a colorless substance, while the dead cells retain the blue color of the stain [15–19]. However, this counting method is prone to human-error and is also time-consuming. In recent years, fluorescence-based membrane integrity viability stains have also been used to determine the number of viable and membrane-compromised nonviable cells in a sample [5, 16, 20–22]. Here, we would like to make the distinction between "viability" and "vitality" of yeasts. Out of the three described viability detection methods, methylene blue can actually determine the metabolic activity or "vitality" of yeasts, while "viability" should be determined using membrane integrity stains. In order to measure the metabolic activity or vitality of yeasts in fermentation, specific fluorescent stains such as carboxyfluorescein diacetate and Calcein AM that become fluorescent when acetate residues are cleaved by active esterase are commonly required, which can be used to determine the fermenting capability of the yeast population [8]. Previous work has shown that metabolic activity decreases with longer periods of fermentation [5].

Glycogen is an important physiological macromolecule that controls the functionality of yeasts during fermentation [9, 23]. It provides the energy and carbohydrates required for sterols and lipids synthesis, which is necessary for proper functions of cytoplasmic membrane as well as the absorption of nutrients to ensure the efficiency of fermenting yeasts. Glycogen typically contains less energy storage than neutral lipids due to the energetic differences between glucose and lipids. However, since glycogen can be mobilized faster than neutral lipids, it can break down quickly to provide the necessary components and energy during and after fermentation. Glycogen is accumulated early in the logarithmic phase and decreases during fermentation. It has been shown that glycogen content is directly correlated with the vitality, but not the proliferation of yeasts [6, 9]. In addition, a previous publication has shown a noticeable decrease in glycogen content for fermentation periods of 4–38 h for various yeast strains [3].

Neutral lipids are energy-rich molecules stored in yeasts, which are synthesized early in the lag phase during fermentation due to stress, but not in the logarithmic phase. Fatty acids in neutral lipids cannot be metabolized, thus the level of neutral lipid remains consistent or increases during fermentation [9]. In a previous publication, the increase in yeast cell size can be attributed to the increase in neutral lipid content after a continuous growth period of 48 h [8]. It has been shown that neutral lipids can protect yeast cells from toxic substances such as ethanol and sugar. Therefore, the importance of neutral lipid content can be related to the survival capacity of yeasts, which may prolong ethanol production during fermentation as ethanol concentration increases [8, 9].

Trehalose is a disaccharide that supplies energy during the cell cycle. It can also protect yeasts against stress, high alcohol concentration, heat, dehydration, oxidation, pH, and osmotic stress. Furthermore, trehalose can enhance yeast cell membranes by forming a stable matrix and binding to polar groups of lipids and proteins, which can prevent denaturation and undesirable reactions. Intracellular trehalose is quickly mobilized and provides energy during initial fermentation [4, 9]. A previous publication has shown a rapid increase in trehalose content in the early logarithmic growth phase during fermentation [4, 24]. Similar to neutral lipids, the protective effect of trehalose may allow prolonged ethanol production during fermentation, which can potentially reduce overall production time.

Traditionally, these physiological changes can be qualitatively measured using fluorescence microscopy to examine the fluorescence intensities associated with each parameter [9, 21, 22, 25, 26]. However, conventional fluorescent microscopes lack automation and fluorescence analysis capabilities that are necessary to quantitatively analyze a large number of cells. To overcome these issues, flow cytometry has been utilized to automate fluorescence data collection and analysis for a large population of yeasts, which can improve efficiency and statistical accuracy of the experiments [16, 27–29]. However, the instruments require a considerable amount of maintenance, highly trained technicians, and the available systems are relatively expensive to both purchase and maintain.

In recent years, advances in imaging optics, high-powered light-emitting diodes (LEDs), and optical lens technologies have allowed the development of a new generation of affordable automated image-based cytometry systems. By making use of these new technologies, Nexcelom Bioscience has developed a fluorescence-based image cytometry system (Cellometer Vision), which has demonstrated automated viability detection of yeasts [20]. In addition, Cellometer Vision has the capability of performing fluorescence-based cell-population analysis on mammalian cells [30–32]. In this work, we demonstrate the first use of Cellometer Vision for the kinetic detection and analysis of vitality, glycogen, neutral lipid, and trehalose content of *S. cerevisiae*. Standard *S. cerevisiae* are cultured for 30 h in the appropriate yeast medium, where samples are collected at different time points to measure each physiological parameter including viability. The *S.*

cerevisiae are fluorescently stained with acridine orange/propidium iodide, carboxyfluorescein diacetate, acriflavine, Nile red, and Concanavalin A-fluorescein isothiocyanate to quantitatively determine viability, vitality, glycogen, neutral lipid, and trehalose content, respectively. Image cytometry provides an efficient, accurate, and inexpensive alternative method that would allow researchers in the brewing industry to conveniently study yeast metabolic activities during fermentation.

Methods

Image cytometry instrumentation and disposable counting chamber

The Cellometer Vision utilizes bright-field (BR) and dual fluorescence (FL) imaging modes to analyze and measure the physiological changes in cell populations at the individual cell level. The bright-field imaging uses a white light emitting diode (LED) while the fluorescence imaging uses two monochromatic LEDs at 470 and 525 nm. These LEDs are combined with specific filters based upon the excitation/emission wavelengths of the fluorescent probes used. Fluorescent Channel 1 (FL1) uses VB-535-402 (EX: 475 nm/EM: 535 nm) while fluorescent channel 2 (FL2) uses VB-595-502 (EX: 525 nm/EM: 595 nm) or VB-660-502 (EX: 540 nm/EM: 660 nm).

A target cell sample (20 µl) is pipetted into the Nexcelom disposable counting chamber, and then inserted into Cellometer Vision for image acquisition. The counting chamber is held in position via a motorized stage that allows images to be captured at four different locations. The Cellometer software then analyzes the images captured in the three channels (BR, FL1, and FL2) to determine the concentration and fluorescence intensities of the target cell population. Cells are identified and outlined by the software, and the fluorescent intensities are measured within each outlined cell under FL1 and FL2. The measurable cell concentration is between 1×10^5 and 7×10^7 cells/ml. Cell population results are analyzed using FCS Express Flow Cytometry (De Novo Software). The overall detection time is typically less than 2 min, but is dependent on the exposure time appropriate for the stains used and the concentration of the cells.

Preparation of *Saccharomyces cerevisiae*

Fresh cultures of *S. cerevisiae* EBY100 were maintained on YPD-agar (Difco). Prior to each experiment, a single colony was inoculated into 5 ml of liquid YPD (Difco) and cultured at 30 °C for 16 h (Fig. 1a). Overnight cultures were diluted into fresh YPD, 5 h prior to the start of each experiment at a ratio of 800 µl of stock culture to 20 ml of fresh media (Fig. 1b). Five milliliters of the diluted culture was collected at 5-, 10-, 25-, and 30-h time points to measure nutritional parameters during the lag, logarithmic, stationary, and nutrient-deprived phases of growth, respectively (Fig. 1c).

At each time point, the yeast cells were separated into two tubes: the first tube (2 ml) was centrifuged for 10 min at 6,000 rpm and resuspended in phosphate buffered saline (PBS, Sigma-Aldrich) for viability and vitality measurements; the second tube (3 ml) was fixed in 95 % ethanol (EtOH, Sigma-Aldrich) for 15 min on ice (Fig. 1d), and then resuspended in PBS prior to fluorescent staining (Fig. 1e).

Viability detection using acridine orange and propidium iodide

A solution of acridine orange and propidium iodide (AO/PI, CS-0106-5ML) was obtained from Nexcelom Bioscience and used to measure the viability of *S. cerevisiae* at each time point. The yeast sample was stained by mixing 20 µl of live cells and 20 µl of AO/PI stain. The stained sample was immediately analyzed by image cytometry in quadruplicate.

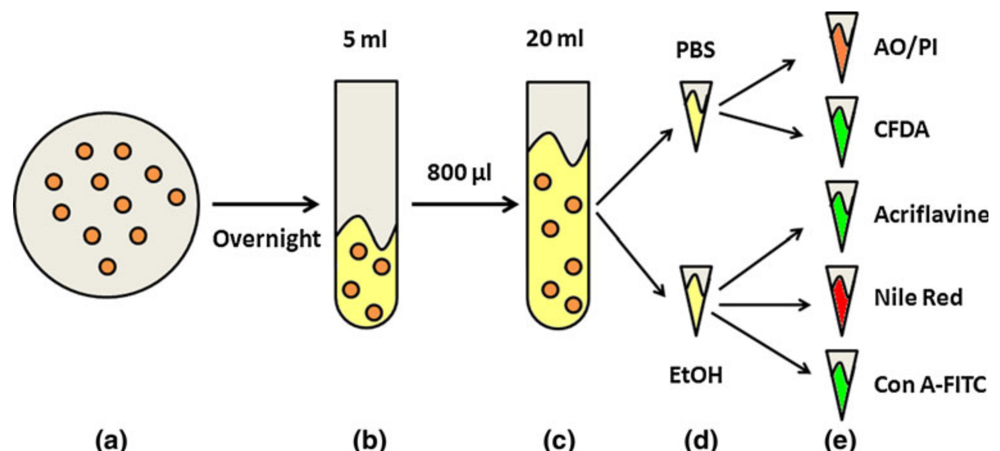

Fig. 1 Physiological characterization assay protocol flow chart. **a** *S. cerevisiae* was streaked out on YPD-agar and then **b** cultured in a 5-ml YPD glass test tube. **c** The sample was cultured overnight to stationary phase, where 800 µl was transferred to a 20-ml YPD test tube. **d** The sample was collected at 5-, 10-, 25-, and 30-h time points and resuspended in PBS or EtOH. **e** The samples were then stained with AO/PI, CFDA, acriflavine, Nile red, and Con A-FITC

Vitality detection using carboxyfluorescein diacetate

In order to measure the vitality of *S. cerevisiae*, carboxyfluorescein diacetate (CFDA) was purchased from Invitrogen to measure the metabolic activity of the yeasts. The CFDA was first dissolved in 100 % dimethyl sulfoxide (DMSO, Sigma-Aldrich) to prepare a stock concentration of 20 mM, and then diluted directly in PBS to 1 mM. For each time point sample, 20 μl of 1 mM CFDA was added to 1 ml of yeasts at a final staining concentration of 20 μM, which was then incubated for 20 min at 37 °C. Following the incubation, the sample was centrifuged and resuspended in 1 ml PBS, prior to fluorescence analysis via image cytometry in quadruplicate.

Glycogen staining using acriflavine

Acriflavine powder (Sigma-Aldrich) at 0.5 g was dissolved in 10 ml of 1 M hydrochloric acid (Sigma-Aldrich). A stock solution of 12.5 mg/ml potassium sulfate (K_2SO_4, Sigma-Aldrich) was prepared in deionized H_2O, where 1 ml of acriflavine solution was mixed in 9 ml of K_2SO_4 to a concentration of 5 mg/ml. In order to measure glycogen content, 2 μl of acriflavine was added to 1 ml of yeasts at a final staining concentration of 10 μg/ml and incubated in the dark for 1 h at room temperature. Following the incubation, each sample was centrifuged and resuspended in 1 ml of PBS. Washing was repeated a total of four times prior to fluorescence analysis via image cytometry in quadruplicate.

Neutral lipid staining using Nile red

In order to measure neutral lipid content, a stock solution of Nile red (Sigma-Aldrich) was prepared in DMSO at 5 mg/ml. The stock solution was then further diluted in acetone to 1 mg/ml. For each time point sample, 10 μl of Nile red solution was added to 1 ml of yeast at a final staining concentration of 10 μg/ml and incubated in the dark for 30 min. Both tubes were incubated in the dark for 30 min at room temperature. Following the incubation, each sample was centrifuged (6,000 rpm, 10 min) and resuspended in 1 ml of PBS for four times, prior to fluorescence analysis via image cytometry in quadruplicate.

Trehalose staining using Concanavalin A-fluorescein isothiocyanate

In order to measure the trehalose content, a stock solution of 1 mg/ml Concanavalin A-fluorescein isothiocyanate (Con A-FITC, Sigma-Aldrich) was prepared in PBS. For each time point sample, 20 μl of Con A-FITC was added to 1 ml of yeast at a final staining concentration of 20 μg/ml, which was then incubated for 20 min in the dark at room temperature. Following the incubation, each sample was centrifuged (6,000 rpm, 10 min) and resuspended in 1 ml PBS for a total of two times prior to fluorescence analysis. Image cytometric analysis was performed in quadruplicate.

Cellometer image cytometric detection method

Cellometer Vision was utilized to measure the physiological changes in *S. cerevisiae* via fluorescence-based image cytometric analysis. Three different user-changeable fluorescence optics modules (FOM) were used corresponding to the respective fluorescent stains. VB-535-402 was used for detection of AO, CFDA, acriflavine, and Con A-FITC at exposure times of 200, 2,000, 50, and 2,000 ms, respectively. VB-595-402 and VB-660-502 were used for the Nile red and PI stains at an exposure time of 1,000 and 2,000 ms, respectively.

To measure physiological changes in *S. cerevisiae*, bright-field imaging was used to locate and focus the yeasts for optimal image acquisition. After focusing, fluorescent images were captured and analyzed using the Cellometer software, where the fluorescence intensity values were measured for each cell. FCS Express Flow Cytometry software was used for interactive data analysis and presentation of target cell populations. Therefore, various physiological characteristics of each yeast sample including viability, vitality, glycogen, neutral lipid, and trehalose content were determined by quantification of cells stained with the appropriate fluorescent probes.

Results and discussion

Viability and vitality kinetic measurement

Viability was monitored by the measurement of AO and PI positive yeast in each sample, and calculated by using the mathematical formula, $\frac{\#AO^{+}Cells}{\#AO^{+}+\#PI^{+}Cells}$. The fluorescence imaging results are shown in Fig. 2 (pseudo-color green and red), which consistently showed a large number of AO-positive cells at each time point. The kinetic plot of viability is shown in Fig. 7, where the viability percentages were consistently at ~99 %. In contrast, vitality detection using CFDA showed a large reduction in metabolic activities of cell populations over the course of their growth cycle. The fluorescent images are shown in Fig. 3 (pseudo-color green), which showed decreasing fluorescence intensities as the yeast culture progressed to 30 h. In the kinetic plot (Fig. 7), active yeast percentages decreased from 31.1 to 3.2 % as expected, since yeasts would become inactive due to lack of nutrients. In general, although fermenting yeast display high "viability", it may not correspond to the

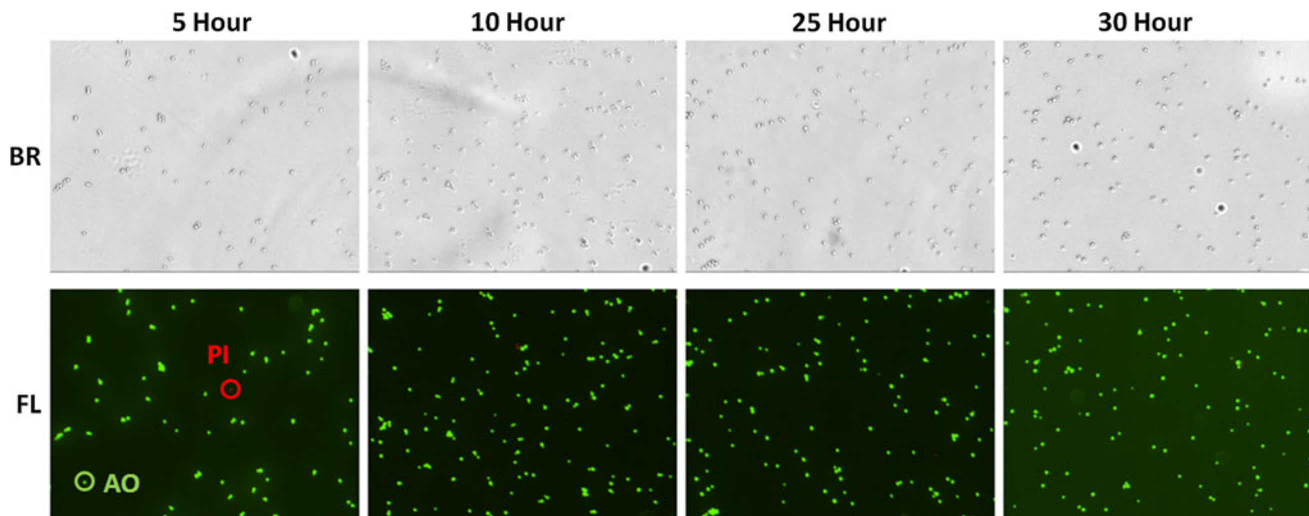

Fig. 2 Bright-field and fluorescent images of AO/PI-stained yeasts. The images are shown at each time point, where the numbers of AO- and PI-positive cells are used to calculate the viability of each sample. The viability remained high at ∼98 %, showing only a few PI-positive cells in the images

metabolic activity or "vitality" of these yeasts. The results indicated the measured viability did not correspond to the yeasts that are metabolically active as measured by CFDA during fermentation.

Physiological kinetic measurement

The glycogen content was monitored by measuring the fluorescence intensities of acriflavine-stained yeast. The fluorescent images are shown in Fig. 4 (pseudo-color green), where reduction in fluorescence intensities throughout the growth period is clearly visible, and consistent with previous findings for glycogen catabolism in yeast cells during growth [3, 9, 11]. In addition, the trend was directly correlated to vitality, as measured by CFDA. Statistically, cell populations with higher fluorescence intensities decreased from 90.0 to 30.8 % (Fig. 7a). The mean fluorescence intensity also decreased with respect to fermentation time from 3,500 to 1,200 FL intensity relative units (Fig. 7b), which indicated that glycogen was actively broken down during fermentation. Overall, the Cellometer image cytometer was able to quantitatively measure relative depletion of glycogen content within the yeast population.

The neutral lipid content was monitored by measuring the fluorescence intensities of Nile red-stained yeasts. The fluorescent images are shown in Fig. 5 (pseudo-color red). The percentages of yeast in the population with higher fluorescence intensities increased from 70.8 % in lag phase to 87.6 % during logarithmic phase before gradually decreasing to 71.9 % at the 30-h time point (Fig. 7a). Neutral lipid accumulation occurred when the yeasts were allowed to continue growth in a nutrient-deprived environment over 48 h [5, 8, 10], which was not shown in this experiment since the growth duration was only 30 h (Fig. 7). Our results indicated that neutral lipid content peaks during log phase growth, and decreasing slightly in population and fluorescence intensity over the growth period. The results were consistent with previous work showing that neutral lipids are only synthesized during the beginning of the growth phase, and do not break down as easily as glycogen [9]. According to previous work, neutral lipid content does increase under stress as well as prolong fermentation for storage of energy [8, 23], but the results showed no storage of neutral lipids up to the 30-h time point.

The trehalose content was monitored by measuring the fluorescence intensities of Con A-FITC-stained yeast. The fluorescent images are shown in Fig. 6 (pseudo-color green). The yeast population displaying higher trehalose content first increased from 26.10 to 51.74 %, and then decreased to 42.80 % at the 30-h time point (Fig. 7a). According to previous publications, trehalose is mobilized early during the growth period to provide energy for fermentation, and then decreases over time as nutrients in the media have been depleted [4, 9, 11]. It has also been shown that as the ethanol percentage in the environment increases, trehalose also accumulates to protect the cells from toxic agents [4, 24]. Our results showed a gradual decrease in average fluorescence intensity up to the 30-h time point, which indicated that no trehalose storage had occurred.

We have demonstrated a novel detection method for kinetic physiological measurements of *S. cerevisiae* using the Cellometer Vision image cytometer. The method can address the issues raised from current techniques such as the lack of automation, high maintenance, and affordability. Most importantly, it provides a convenient method for performing measurements on the physiological parameters of

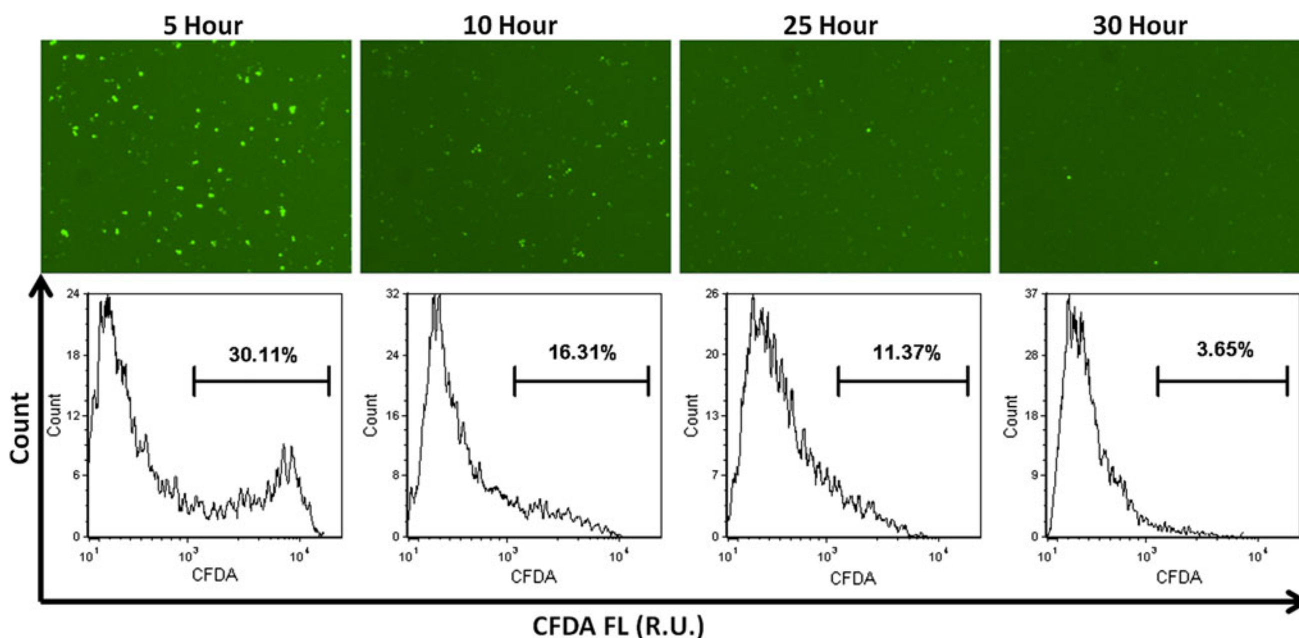

Fig. 3 Fluorescent images and histograms of CFDA-stained yeast. The images showed a decrease in the proportion of high fluorescence intensity cells as the culture progressed to 30 h. This decrease can be observed in the histograms at each time point, indicating a decrease in cell vitality

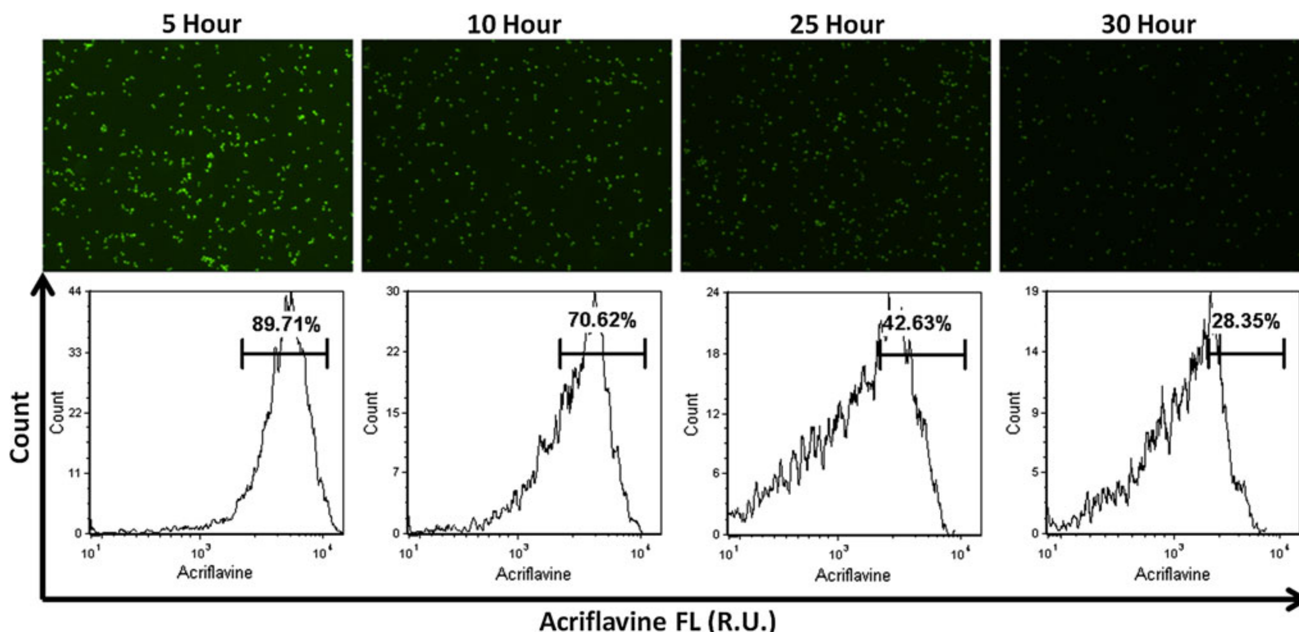

Fig. 4 Fluorescent images and histograms of acriflavine-stained yeasts. The images showed that all of the cells contained glycogen, which decreased in fluorescence intensities as the culture progressed to 30 h. The decrease in the highly fluorescent populations can be observed in the histograms from ~90 to 30 %, which indicated reduction in glycogen content in the yeasts

yeasts such as glycogen, neutral lipid, and trehalose content, allowing for fluorescence-based cellular analysis as well as monitoring metabolic changes during fermentation for quality control. The ability to optimize these parameters can improve product qualities, such as consistency, flavor, and shelf life. The ability to kinetically measure viability and vitality of yeasts during fermentation may also aid in improving consistency and quality of the end products. Future work may be focused on developing a novel automated method for determining budding percentages within a fermenting sample to further improve fermentation conditions and simplify quality control processes.

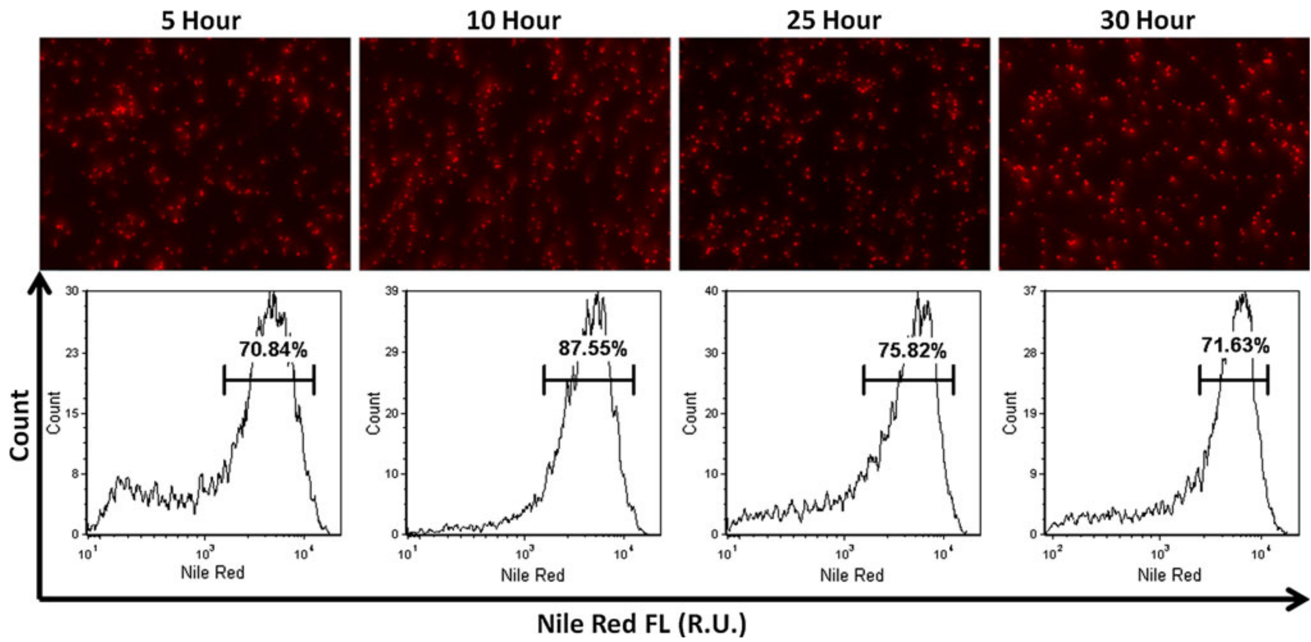

Fig. 5 Fluorescent images and histograms of Nile red-stained yeasts. The images showed a high level of lipid content, which decreased slightly as the culture progressed to 30 h. There was an initial increase in the Nile red-positive population, which can be observed in the histograms at the 10-h time point. However, the population percentages decreased to the basal level at the 30-h time point, which indicated neutral lipid content were relatively consistent in the yeasts during the entire culture period

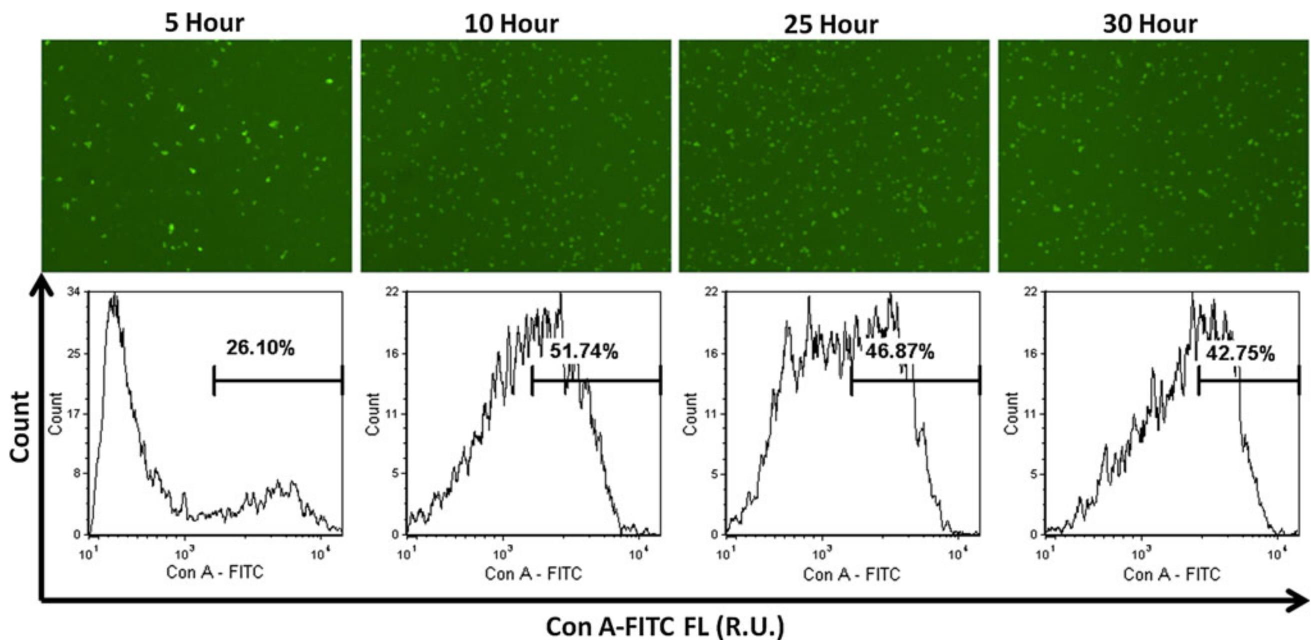

Fig. 6 Fluorescent images and histograms of Con A-FITC-stained yeast. The images showed the population of trehalose positive yeast was initially low, increasing ~25 % in the first 10 h, and then decreasing at 30 h. This indicates the trehalose was quickly absorbed at the commencement of culture

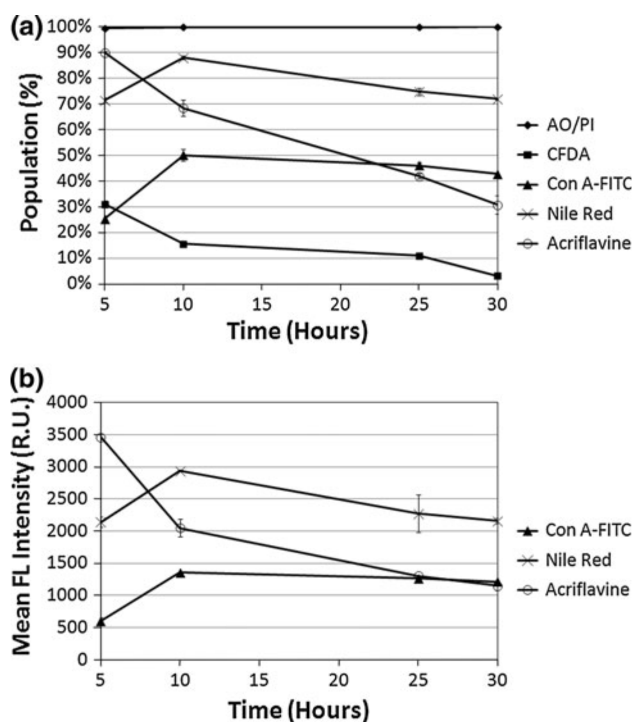

Fig. 7 Kinetic plot of physiological changes during a 30-h culture period. **a** Changes in highly fluorescent positive yeast populations for AO/PI, CFDA, acriflavine, Nile red, and Con A-FITC. AO/PI showed consistently high viability, while the CFDA showed reduction in vitality of the yeast. Acriflavine showed a significant decrease in glycogen content, while the neutral lipids, measured by Nile red, showed relatively consistent percentages with a gradual decrease at the 30-h time point. Trehalose was mobilized quickly in the initial stages of fermentation and decreased slightly over 30 h. Similar to neutral lipid, trehalose content may increase in a nutrient-deprived environment due to its role as a protective agent, but the results did not show storage of either lipids or trehalose during the 30-h incubation. **b** The mean fluorescence intensity of acriflavine, Nile red, and Con A-FITC-stained yeast. Similar to the trends exhibited in population analysis, acriflavine fluorescence decreased dramatically as glycogen broke down during fermentation; Nile red fluorescence fluctuated within $\sim$20 %, which was consistent with the fact that neutral lipids mobilize at a lower rate than glycogen; Con A-FITC fluorescence followed a similar pattern to Nile red, with gradual decrease at the 30-h time point

Conflict of interest The authors, LLC, and AP declare competing financial interests, and the work performed in this manuscript is for reporting on product performance for Nexcelom Bioscience, LLC. The performance of the instrumentation has been compared to standard approaches currently used in biomedical research institutions.

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