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

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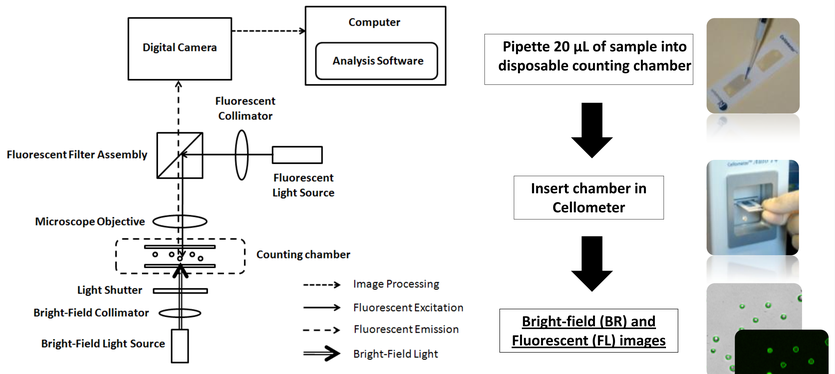
1. ABSTRACT

The study 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.

2. CURRENT METHODS FOR MEASURING METABOLIC CHANGES IN YEAST

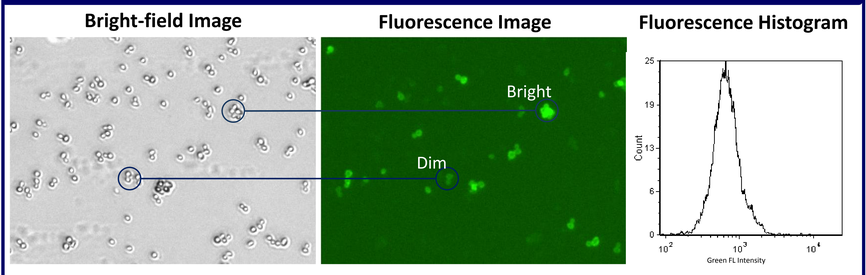
Methods	Description	Known Issues
Hemacytometer	•Manually counting viable cells	•Time-consuming and tedious process •Requires experienced user for accurate counting
Fluorescence Microscopy	•Visualization of fluorescently-stained yeast cells	•Qualitative observe instead of quantitative analysis •Not automated, low throughput
Flow Cytometry	•Quantitative fluorescent analysis •Automated analysis	•Relatively expensive and high maintenance •Requires experienced user for proper operation •Cannot visually observe budding yeasts

3. CELLOMETER IMAGE CYTOMETRY INSTRUMENTATION



- Cellometer image cytometer utilizes an epi-fluorescence setup for fluorescent image analysis

4. FLUORESCENCE INTENSITY ANALYSIS USING IMAGE CYTOMETRY



- Target yeast samples are stained with the appropriate fluorescent labels
- Using Cellometer image cytometer to capture bright-field and fluorescent images
- The Cellometer software counts all the yeast cells and outlines the shape of the yeast in bright-field
- The mask that is generated is used in fluorescent images to measure intensity from each yeast particle
- The fluorescence intensity data is then exported to FCS Express 4 for data analysis and presentation

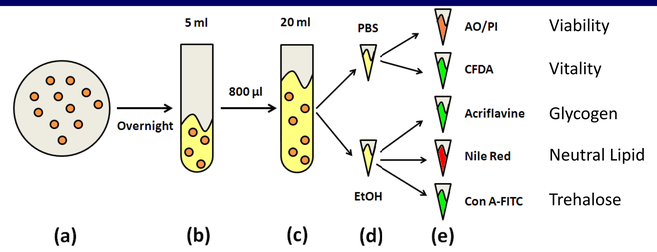
5. DESCRIPTION OF PHYSIOLOGICAL AND METABOLIC PARAMETERS

Parameters	Description
Viability	• Measurement of percent live cells in the sample • Determine amount of viable yeast used for fermentation • Improve consistency by utilizing consistent amount of live cells
Vitality	• Measurement of percent of cells able to perform enzymatic reactions • Metabolically active cells are fermenting during production • Vitality and viability do not usually correlate
Trehalose	• Sugar disaccharide that supplies energy during cell cycle • Protects against stress, high EtOH, heat, dehydration, oxidation, pH • Mobilized quickly, related to survival capacity
Neutral Lipid	• Energy-rich molecules that are stored in yeasts • Cannot be metabolized • Protect from toxic substance like EtOH, prolong survival capacity
Glycogen	• Macromolecule that provides the energy and carbohydrates required for yeast sterols and lipid synthesis • Mobilized quickly to provide energy during and after fermentation • It has been shown to correlate to vitality

6. DETECTION METHOD OF PHYSIOLOGICAL AND METABOLIC PARAMETERS

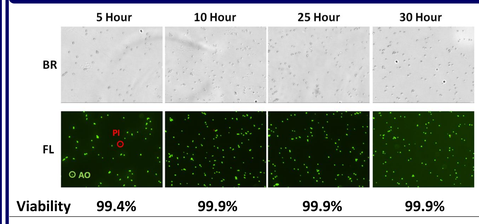
Parameters	Detection Method
Viability	Measured by Acridine Orange (AO, EX: 475 nm, EM: 535 nm) and Propidium Iodide (PI, EX: 525 nm, EM: 595 nm) fluorescent nucleic acid staining method
Vitality	Measured by Carboxyfluorescein Diacetate (CFDA) fluorescent enzymatic staining method (EX: 475 nm, EM: 535 nm)
Trehalose	Measured by staining with Concanavalin A-FITC (EX: 475 nm, EM: 535 nm)
Neutral Lipid	Measured by staining with Nile Red (EX: 525 nm, EM: 600 nm)
Glycogen	Measured by staining with Acriflavine (EX: 475 nm, EM: 535 nm)

7. EXPERIMENTAL PROTOCOL FOR BUDDING MEASUREMENT



a) Yeast colonies are streaked out on an agar plate
b) A 5 ml YPD test tube is inoculated and allowed to culture overnight
c) A stationary phase sample of 800 µl is transferred to a tube of 20 ml YPD media
   - Samples are collected at 5, 10, 25, and 30 hours
d) Collected yeast samples are resuspended in PBS or EtOH
e) For PBS: stained with AOPI, CFDA; for EtOH: stained with Acriflavine, Nile Red, Con A-FITC

8. PHYSIOLOGICAL AND METABOLIC PARAMETER TIME-COURSE MEASUREMENT

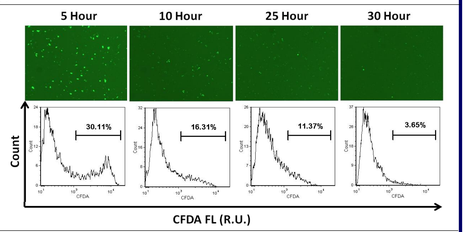


Viability

1. Bright-field and fluorescent images of AO/PI-stained yeasts
2. AO+ and PI+ yeast cells are automatically counted at each time point
3. Viability was then measured at each time point

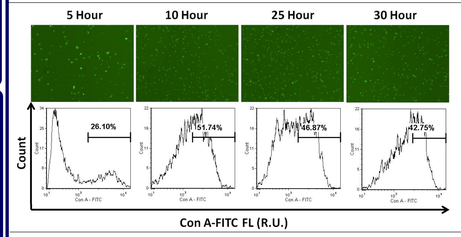
Vitality

1. Fluorescent images and histograms of CFDA-stained yeasts
2. Reduced number of high fluorescence intensity cells as culture progress to 30 hours
3. Histograms at each time point indicated reduced vitality of cells



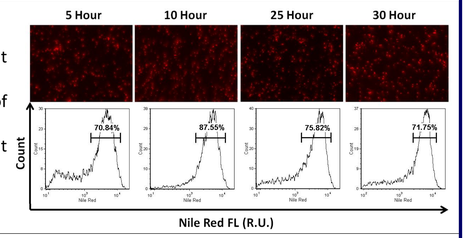
Trehalose

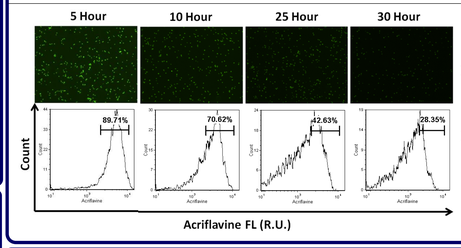
1. Fluorescent images and histograms of Con A-FITC-stained yeasts.
2. Trehalose positive yeasts were initially low
3. Increased in the first 10 hours, and then decreased at 30 hours
4. Indicated trehalose content was quickly absorbed at the start of the culture.



Neutral Lipid

1. Images show high level of lipid content in fluorescent intensity, which slowly decreased at each time point
2. At 10 hour time point, there is an initial increase of Nile Red positive cells
3. Neutral lipid content seemed to be mostly consistent during the culture period.

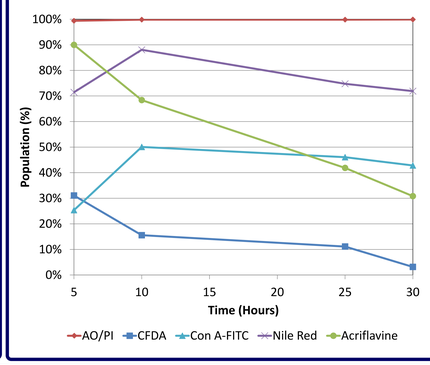




Glycogen

1. All of the cells contained glycogen, which decreased in fluorescence intensity as the culture progressed to 30 hours.
2. Histograms for each time point show this decrease, which indicated glycogen reduction in the yeasts.

9. TIME-COURSE MEASUREMENT RESULTS AND CONCLUSION



- The measured physiological and metabolic data corresponded to previous publications
- Brewing fermentation process requires the essential ingredient of yeast to perform properly
- Physiological and metabolic characteristics of yeast can affect the product quality and consistency
- Image cytometry systems can be used to monitoring physiological parameters
- Understanding changes in viability, vitality, glycogen, neutral lipid, and trehalose content
  - Can potentially ensure the quality and consistency of the products produced

Chan et al., JIMB, 39:1615-1623, 2012