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Automated Quantification of Budding *Saccharomyces cerevisiae* using a Novel Image Cytometry Method

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75th ASBC Annual Meeting
May 19-22, 2013
Hilton Tucson El Conquistador
Tucson, Arizona

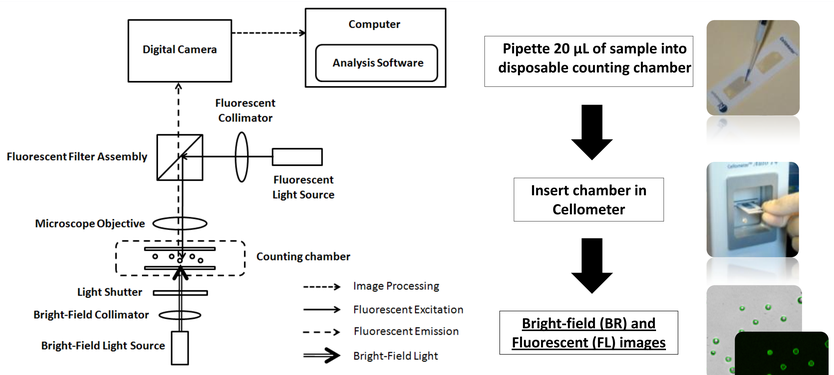
1. ABSTRACT

The measurements of concentration, viability, and budding percentages of Saccharomyces cerevisiae are performed on a routine basis in the biofuel and brewing industries. Generation of these parameters is of great importance in a manufacturing setting, where they can aid in the estimation of product quality, quantity, and fermentation time of the manufacturing process. Specifically, budding percentages can be used to estimate the reproduction rate of yeast populations, which directly correlates with metabolism of polysaccharides and bioethanol production, and can be monitored to maximize production of bioethanol during fermentation. The traditional method involves manual counting using a hemacytometer, but this is time-consuming and prone to human error. In this study, we developed a novel automated method for the quantification of yeast budding percentages using Cellometer image cytometry. The automated method utilizes a dual-fluorescent nucleic acid dye to specifically stain live cells for imaging analysis of unique morphological characteristics of budding yeast. In addition, cell cycle analysis is performed as an alternative method for budding analysis. We were able to show comparable yeast budding percentages between manual and automated counting, as well as cell cycle analysis. The automated image cytometry method is used to analyze and characterize corn mash samples directly from fermenters during standard fermentation. Since concentration, viability, and budding percentages can be obtained simultaneously, the automated method can be integrated into the fermentation quality assurance protocol, which may improve the quality and efficiency of the bioethanol production process.

2. CURRENT METHODS FOR MEASURING BUDDING YEAST CELLS

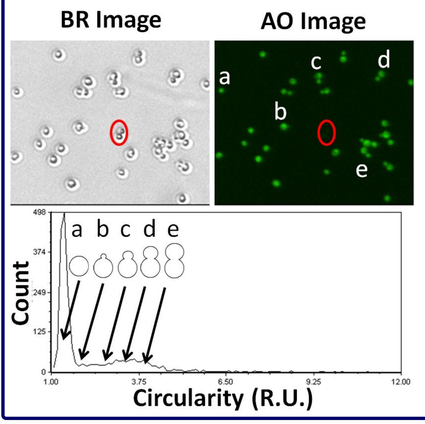
Methods	Description	Known Issues
Hemacytometer	Manually counting budding cells	•Time-consuming and tedious process •Requires experienced user for accurate counting
Fluorescence Microscopy	Visualization of Calcofluor-stained "budding scars"	•Qualitative observe instead of quantitative analysis •Not automated,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

3. CELLOMETER IMAGE CYTOMETRY INSTRUMENTATION



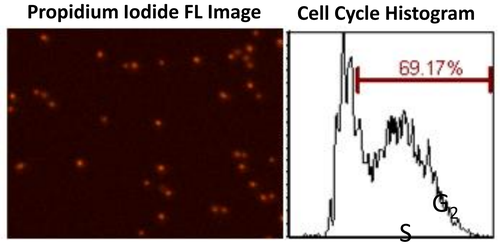
- Cellometer image cytometer utilizes an epi-fluorescence setup for fluorescent image analysis

4. BUDDING MEASUREMENT VIA MORPHOLOGY USING IMAGE CYTOMETRY



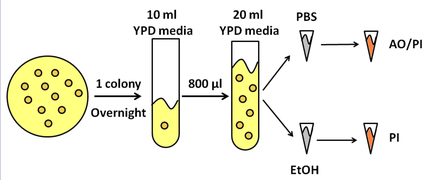
- Target yeast samples are stained with acridine orange (AO) and propidium iodide (PI)
- AO and PI are fluorescent nuclear stains that allow the live/dead cells to fluoresce in green/red
- Using Cellometer to capture only green fluorescence, only live cells are analyzed
- AO fluorescence can show distinct and apparent morphology as budding occurs
- Cellometer software counts all the AO positive yeast cells and outlines the shape of the yeast
- Advanced software algorithm then measures the circularity relative units for each yeast cell
- As the bud size increases, the circularity value increases, and plotted in a histogram
- Circularity histogram shows 2 populations, non-budding yeasts and budding yeasts with high circularity values

5. BUDDING MEASUREMENT VIA CELL CYCLE USING IMAGE CYTOMETRY



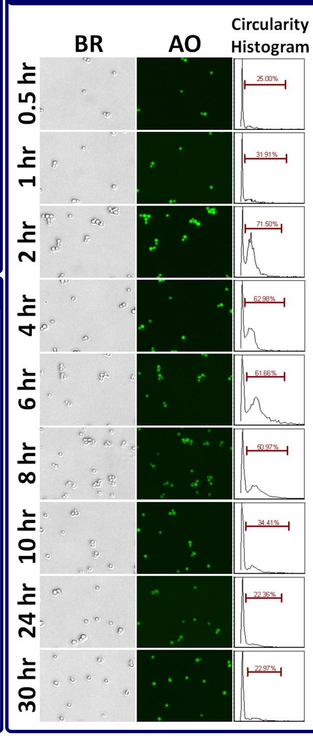
- Target yeast samples are stained with standard protocol of propidium iodide for cell cycle analysis
- DNA content is stained with propidium iodide in each yeast cell and measured using Cellometer
- Fluorescence intensity of each cell is then plotted in a cell cycle histogram showing the phases of yeast cell cycle
- Budding yeasts are measured from the beginning of S phase to G2 phase

6. EXPERIMENTAL PROTOCOL FOR BUDDING MEASUREMENT



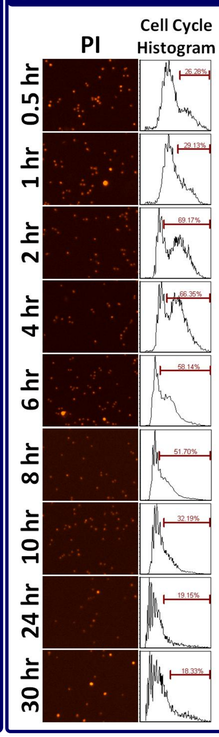
a) Yeast colonies are streaked out on an agar plate
b) A 10 ml YPD test tube is inoculated and allowed to culture overnight
c) A stationary phase sample of 800 µl is transferred to a tube of 20 ml YPD media
   - Samples are collected at 0.5, 1, 2, 4, 6, 8, 10, 24, and 30 hours
d) Collected yeast samples are resuspended in PBS or EtOH
e) Cells in PBS are stained with AOPI to measure circularity, and cells in EtOH are fixed and stained with PI to measure DNA content for cell cycle analysis

7. CIRCULARITY ANALYSIS RESULTS



%
25.00
31.91
71.50
62.98
61.66
50.97
34.41
22.36
22.97

8. CELL CYCLE ANALYSIS RESULTS

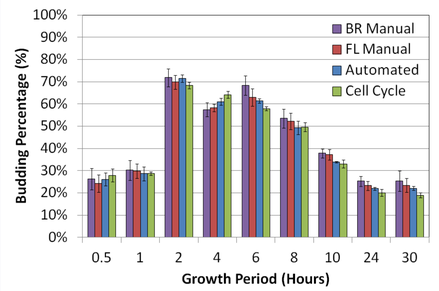


%
26.28
29.31
69.17
66.35
58.14
51.70
32.19
19.15
18.33

9. COMPARISON OF BUDDING MEASUREMENT METHODS

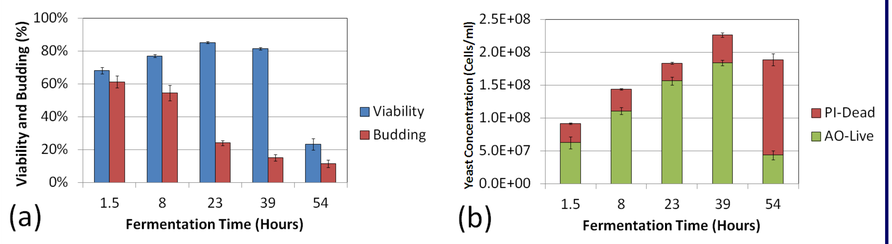
- **Bright-field image manual counting**
  - Bright-field images are captured by Cellometer image cytometer and the number of budding yeasts and total yeasts are manually counted
  - Percent budding yeasts are then calculated
- **Fluorescence image manual counting**
  - Fluorescent AO images are captured by Cellometer image cytometer and the number of budding and total yeasts are manually counted
  - Percent budding yeast are then calculated
- **Fluorescence automated counting via morphology analysis**
  - Fluorescent AO images are automatically counted and the circularity data is exported to FCS Express (De Novo Software)
  - Yeast population with large circularity values are gated to measure the budding percentages
- **Cell cycle analysis**
  - Fluorescent PI images are automatically counted and the fluorescence intensity data is exported to FCS Express (De Novo Software)
  - Yeast population with fluorescence intensity values that indicates S phase to G2 phase are gated to measure the budding percentages

10. COMPARISON OF TIME-COURSE BUDDING MEASUREMENT RESULTS



- Measured budding percentages using bright-field and fluorescent image manual counting, fluorescent automated counting, and cell cycle analysis are compared
- Results showed comparable measured budding percentages between all methods
- It showed that initially, budding percentages remained low at ~20%, after 2 hours, budding percentages increased dramatically to ~70%
- Over the 30 hour period, the budding percentages decreased from ~60% back down to ~20%

11. BUDDING MEASUREMENT OF BIOETHANOL FERMENTATION SAMPLES



- Bioethanol fermentation samples were collected at different time points during the fermentation
- Percent viability was measured at each time point using AOPI viability staining method **(a)**
- Percent budding was measured using the automated counting of circularity values, which showed high budding at time 1.5 hours and decreased to 10% at the end of fermentation **(a)**
- Yeast concentration for live and dead cells were also measured **(b)**

12. CONCLUSION

The development of a fast, accurate, and simple yeast analysis method can improve the current industry standard method, which relies mainly on manual counting using a hemacytometer. With the combination of these three parameters (concentration, viability, budding percentage), the fluorescence-based image cytometry method can be used to easily monitor yeast population characteristics during fermentation, which can allow researchers in the biofuel or brewing industry to improve their fermentation process, as well as improve the efficiency of quality assurance protocols. Future work may also involve supplementing the detection process with a yeast vitality parameter to complete the characterization of yeast during fermentation. We have demonstrated the capability of the image cytometry method for quantifying yeast budding via morphology and DNA content. This automated method can reduce the time required to obtain yeast characteristics in an industry setting, which is of great importance for the optimization of the fermentation process.