191

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A rapid viability validation method of flash pasteurized yeast using Cellometer image cytometer

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1. ABSTRACT

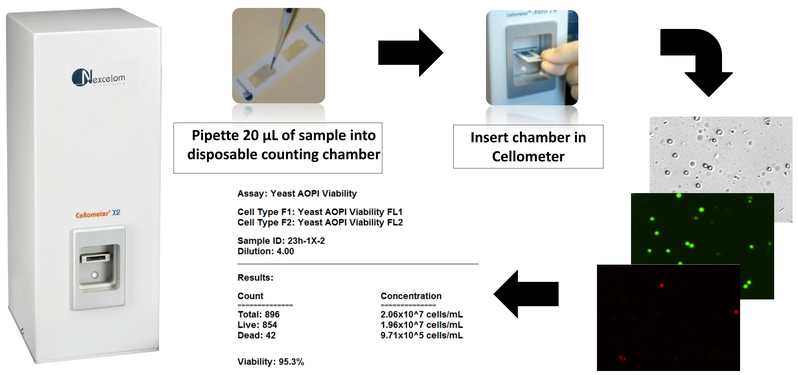
Flash pasteurization has traditionally been deployed in the brewing industry to stabilize beer by reducing the number of beer spoilage organisms per ml, ensuring long and stable shelf life free from defective flavors and aromas created by the likes of *L. brevis* and *P. damnosus*. As innovative new beers from the craft segment arrive, new challenges to beer stability sometimes manifest. In the case of beers containing high levels of residual fermentable sugar, a new contaminant microorganism must be accounted for in the form of the brewer's own yeast that conducted the initial fermentation of the beer. Some craft brewers are turning to pasteurization to reduce viable yeast counts. A novel method of verifying successful reduction of viable yeast cells in final packages of beer was developed that provides the brewer with real-time validation of the effectiveness of both pasteurization operations as well as sanitary bottle, can, or keg filling operations.

In order to determine if the beer product is ready for bottling after flash pasteurization, yeast viability is quickly measured using the Cellometer image cytometer. First, 50 ml of the product is collected and the sample is concentrated to approximately 250 µL. The yeast cells are then stained using the yeast dilution buffer and acridine orange (AO)/propidium iodide (PI) fluorescent stains to determine live/dead cell count and viability. Since the yeast cell concentration is low, multiple samples are analyzed to ensure the results are statistically accurate. The ability to quickly measure yeast viability enables a more efficient process for QC and manufacturing, which can further improve the quality and throughput of the beer products.

2. CURRENT METHODS FOR MEASURING YEAST VIABILITY AND VITALITY

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

3. CELLOMETER IMAGE CYTOMETRY INSTRUMENTATION AND PROTOCOL



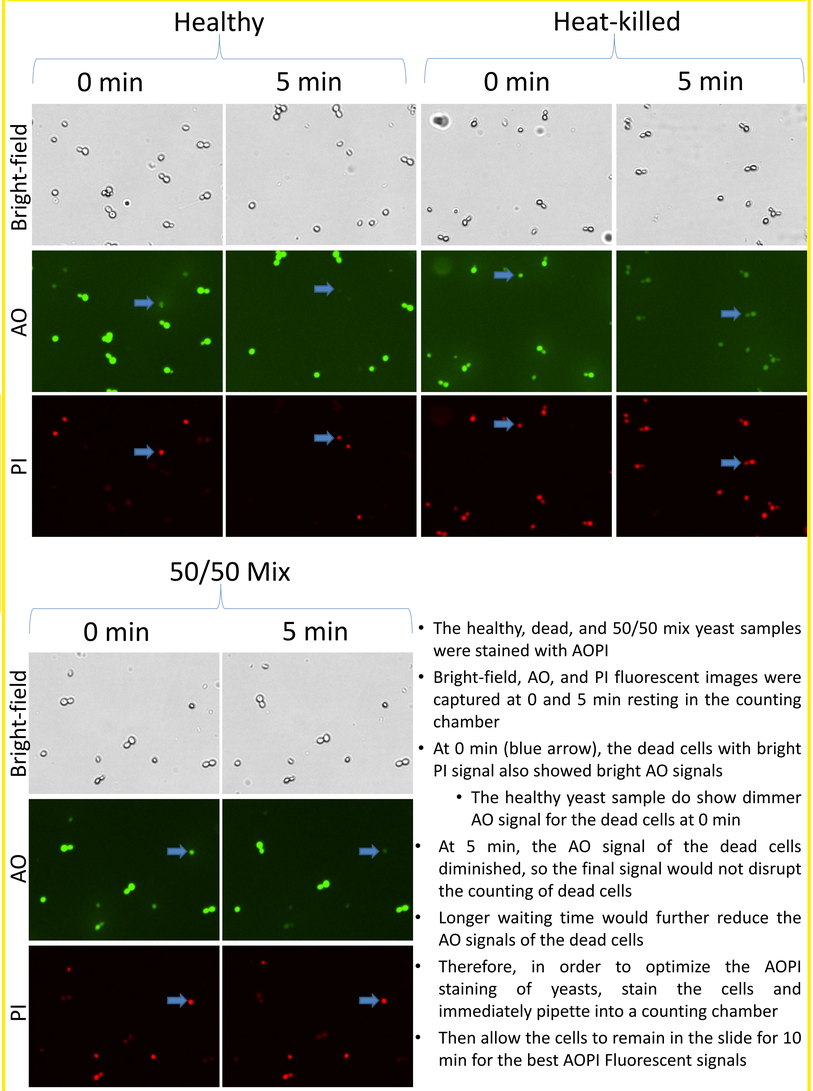
- Cellometer image cytometer automatically counts live and dead yeasts in the sample using Acridine Orange and Propidium Iodide fluorescent stains

4. DEVELOPMENT OF VIABILITY DETECTION METHOD FOR HEAT-KILLED YEAST

Cultured Munton yeast in YPD media overnight → Collect 20 mL of yeast sample and split into 2 tubes → Heat-kill one tube in boiling water for 15 min → Mix 50/50 of the healthy and heat-killed yeast

- The heat-killed yeast sample often time would show double fluorescent signals of AO and PI for the dead cells
- It was discovered previously that allowing AOPI stained cells to remain in the slide for 5 – 10 min, the non-specific double AOPI signal would fade, leaving only PI signals for the dead cells
- The purpose of this experiment is to determine the best AOPI staining procedure for heat-killed yeast, which should be similar to flash pasteurization
- The expected results should be clear visual identification of AO – Live and PI – Dead yeast cells after staining and measured on the Cellometer X2

5. OPTIMIZATION OF AOPI STAINING OF HEAT-KILLED YEAST



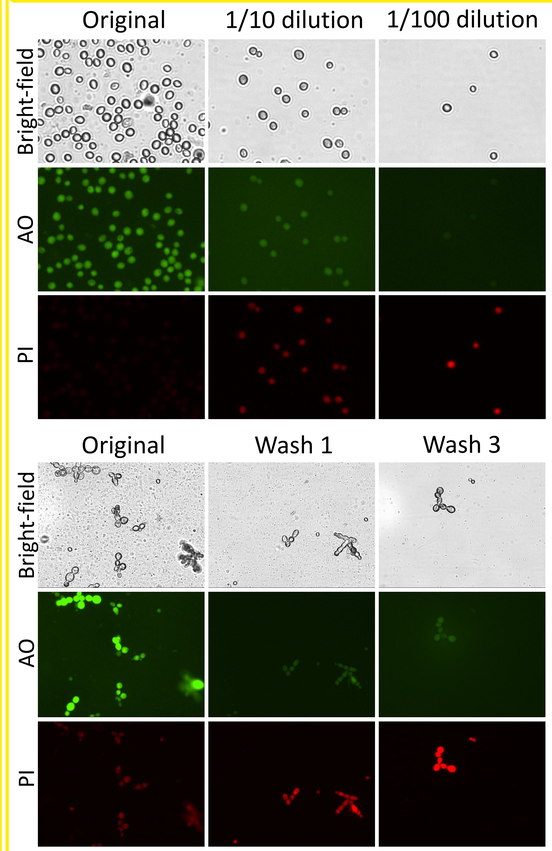
- The healthy, dead, and 50/50 mix yeast samples were stained with AOPI
- Bright-field, AO, and PI fluorescent images were captured at 0 and 5 min resting in the counting chamber
- At 0 min (blue arrow), the dead cells with bright PI signal also showed bright AO signals
  - The healthy yeast sample do show dimmer AO signal for the dead cells at 0 min
- At 5 min, the AO signal of the dead cells diminished, so the final signal would not disrupt the counting of dead cells
- Longer waiting time would further reduce the AO signals of the dead cells
- Therefore, in order to optimize the AOPI staining of yeasts, stain the cells and immediately pipette into a counting chamber
- Then allow the cells to remain in the slide for 10 min for the best AOPI Fluorescent signals

6. AOPI VIABILITY DETECTION OF FLASH PASTEURIZED YEAST

Collect flash pasteurized yeast sample (50 mL) → Centrifuge to concentrate the yeast sample → Stain the sample with AOPI → Pipette into counting chamber and wait 10 min

- The optimized AOPI viability detection method was tested on flash pasteurized yeast sample
- The sample was tested without dilution, at 1/10, and at 1/100 dilution factor
- The purpose is to observe and measure any AO – Live yeasts in the sample
  - The viability of the sample should be ~0% because all the cells should be dead
- Previously, it has been shown that flash pasteurized yeasts in Shandy beer did not show clear AO and PI fluorescent signals
- By testing different dilutions, we hypothesized that some component in the medium may be inhibiting the AOPI staining correctly

7. OPTIMIZATION OF AOPI STAINING OF FLASH PASTEURIZED YEAST IN SHANDY BEER



- Initially, the flash pasteurized yeast samples when stained with AOPI showed bright AO and dim PI signals, which was opposite to what was expected (Left)
- After 1/10 times dilution in water, the AO fluorescent intensity decreased, while the PI intensity slightly increased
- Finally, after 1/100 times dilution in water, the AO intensity reduced to background level, while the PI intensity increased to a more definitive dead cell fluorescence
- By dilution, we hypothesize that some molecular components in the media were disrupting AOPI staining of yeast

- A bottle of old shandy beer sample was obtained and concentrated
- Notice in the original sample on the left, the bright-field image showed numerous particles in the background
- As expected , the PI signal remained low similar to previous experiments
- The yeast sample was then washed by centrifuging, removing supernatant, and resuspended in water
- It appears that the particles concentration was reduced significantly, and the PI intensity increased
- A final wash was performed, which showed a dramatic increase in PI intensity

8. FINAL SHANDY BEER YEAST PREPARATION PROTOCOL FOR AOPI STAINING

Step 1 — Collect & homogenize shandy sample. Place 50 ml into centrifuge tube and spin to concentrate yeast cells

Step 2 — Gently decant supernatant and replace with water. Homogenize contents of tube. Centrifuge again. Repeat.

Step 3 — Gently decant supernatant & transfer pellet to a 1.5ml microcentrifuge tube. Suspend pellet in 1.5ml fresh water. Centrifuge until pellet re-forms.

Step 4 — Decant the supernatant water from the microcentrifuge tube, leaving the pellet intact. Replace with 100 µL clean water and vortex to homogenize

Step 5 — Prepare yeast slurry in 1:4 ratio with Revvity Yeast Buffer solution (1:2) and AOPI stain (1:4)

Step 6 — Prepare 4 counting slides for evaluation, dosing 20µL into each of the 8 counting chambers. Run thru Cellometer X2 as detailed in instrument handbook.

9. CONCLUSION

In conclusion, we have demonstrated the capability of using image cytometry to perform fluorescence-based evaluation of effectiveness of flash pasteurization of yeast cells. Some obstacles that were overcome:

- Extremely low concentration of cells; repeated bench centrifuging of the sample concentrated cells enough to draw valid conclusions based on observation of 400+ cells per sample.
- Extremely high turbidity of the sample medium; multiple washes of the sample between centrifugation treatments encouraged stratification and removal of grapefruit pulp and other fruit solids which initially masked the AO and PI signals.

Some questions remain to be answered:

- Is there a way to quantitatively determine the effectiveness of flash pasteurization of yeast cells at low concentrations in a challenging substrate like shandy?
- What is the maximum tolerable threshold of live cells observed that will not present instability in the final packaged beer?

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