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 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 µL 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 throughout of the beer products.

2. CURRENT METHODS FOR MEASURING YEAST VIABILITY AND VITALITY

<table>
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<tr>
<th>Methods</th>
<th>Description</th>
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<td>Hemocytometer</td>
<td>Manually counting budding cells</td>
<td>Time-consuming and tedious process</td>
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<td>Fluorescence Microscopy</td>
<td>Visualization of fluorescently labeled yeast cells</td>
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<td>Flow-Based Analysis</td>
<td>Quantitative analysis, Automated analysis</td>
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<td>(communications)</td>
<td>Requires experienced user for proper operation</td>
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<td>(concentration)</td>
<td>Cannot visually yeasts cells</td>
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3. CELLMETER IMAGE CYTOMETRY INSTRUMENTATION AND PROTOCOL

- **Pipette 20 µl of sample into Acridine Orange and Propidium iodide fluorescent stains**
- **Insert chamber in Cellometer**

4. DEVELOPMENT OF VIABILITY Detection METHOD FOR 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

- **Healthy and Dead yeast indicated by AOPI stainings**
- **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 signal**
- **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 resting time would further reduce the AO signals of the dead cells**
- **Therefore, in order to optimize the AOPI staining of yeast, 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

- **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 were obtained and stained with AOPI**
- **AOPI showed bright AO and dim PI signals, which was opposite to what was expected**
- **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 hypothesized that some molecular components in the media were disrupting AOPI staining of yeast**

8. FINAL SHANDY BEER YEAST PREPARATION PROTOCOL FOR AOPI STAINING

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

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

**Step 3** Gently decent supernatant & transfer pellet into microcentrifuge tube. Suspend pellet in 1.5 ml fresh water. Centrifuge until pellet re-forms.

**Step 4** Prepare working solution for the staining protocol as described in instrument handbook.

**Step 5** Prepare yeast slurry in 1:4 ratio to Working yeast solution (1:2) and AOPI stain (1:4).

**Step 6** Prepare 2 counting slides for each of the 8 counting chambers. Collect a total of approx 800 yeast cells each of the 8 counting chambers, to be counted individually.

**Step 7** Collect & homogenize shandy sample. Place 50 ml into microcentrifuge tube and spin to concentrate yeast cells.

**Step 8** Gently decent supernatant and replace with water. Homogenize contents of tube. Centrifuge again. Repeat.

**Step 9** Gently decent supernatant & transfer pellet into microcentrifuge tube. Suspend pellet in 1.5 ml fresh water. Centrifuge until pellet re-forms.

**Step 10** Prepare working solution for the staining protocol as described in instrument handbook.

**Step 11** Collect & homogenize shandy sample. Place 50 ml into microcentrifuge tube and spin to concentrate yeast cells.

**Step 12** Gently decent supernatant and replace with water. Homogenize contents of tube. Centrifuge again. Repeat.

**Step 13** Gently decent supernatant & transfer pellet into microcentrifuge tube. Suspend pellet in 1.5 ml fresh water. Centrifuge until pellet re-forms.

**Step 14** Collect yeast slurry in 1:4 ratio to Working yeast solution (1:2) and AOPI stain (1:4).

**Step 15** Prepare 2 counting slides for each of the 8 counting chambers. Collect a total of approx 800 yeast cells each of the 8 counting chambers, to be counted individually.

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?