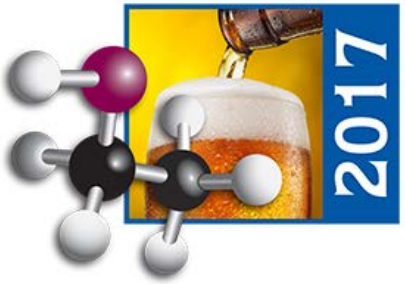

ASBC Annual Meeting

June 4–7 ■ Fort Myers, Florida

See what SCIENCE can brew for you

A novel concentration and viability detection method for Brettanomyces using image cytometry

Leo Chan, Ph.D.

Nexcelom Bioscience

Presentation outline

- What is Brettanomyces and why are they used in the brewing industry?
- What are the current methods to count and measure viability of Brettanomyces?
- What is the Cellometer X2 image cytometer and how is it operated?
- Measure Brettanomyces growth, viability, and elongation over 8 days growing in a flask
- Measure Brettanomyces growth, viability, and elongation over 40 days in fermentation

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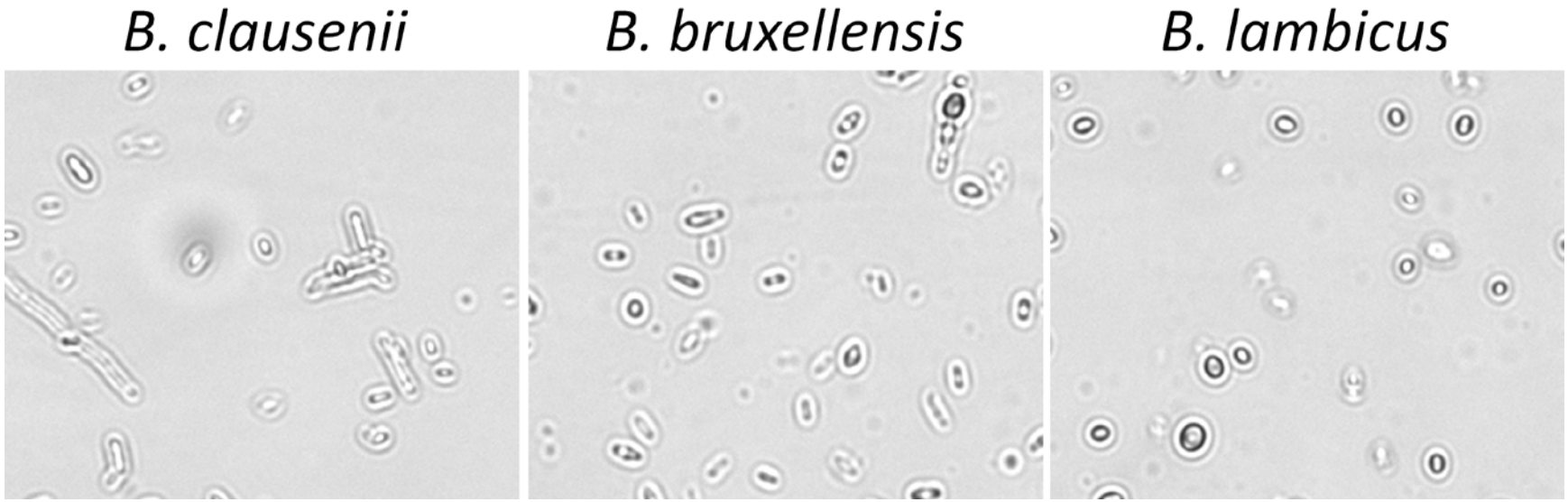
There has been increasing interests in recent years for using Brettanomyces for fermentation



- Can form pseudo-hyphae – elongation of yeast cell
- Produce novel flavors and aroma compounds
- Create complex flavors by Allagash or Crooked Stave Artisan Beer Project

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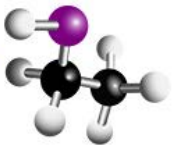
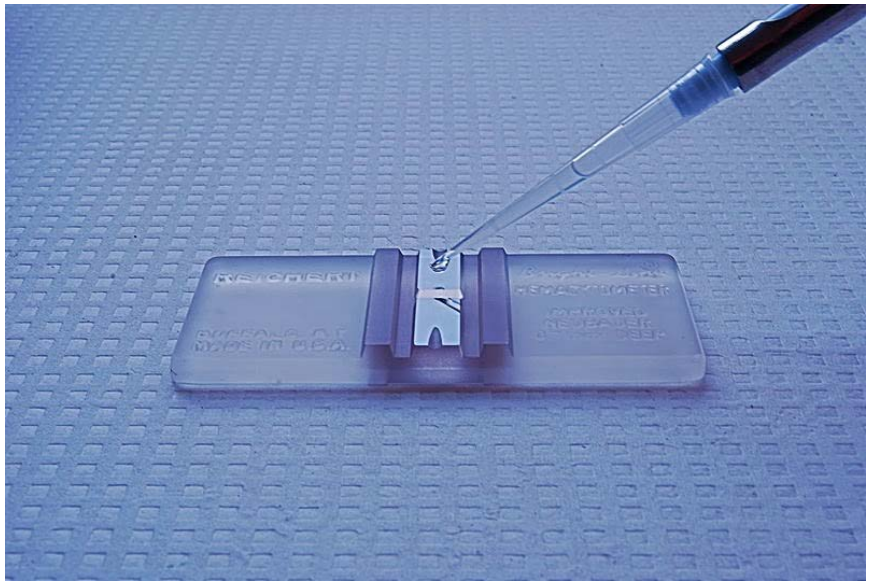


Hemacytometer is commonly used but has operator-dependent variation



- Hemacytometer is the most common method to count yeasts
- Counting is difficult for Brettanomyces due to the pseudo-hyphae
- Although common, but can be time-consuming and have operator-dependent inconsistency

Optical density is only measuring cell density based on cell mass not by individual cells

- Spectrophotometers are used to measure optical density of Brettanomyces
- By using a standardize curve, the optical density is corrected to a cell concentration
- There is an uncertainty of what is actually in the sample without looking at the cells
- Standard curves are required for different cell types
- This is not for measuring viability

Pouring yeasts directly by weight can be very inaccurate and highly inconsistent

- Measuring yeast by weight is does not produce viability
- Thus, putting in the same weight every time doesn't guarantee same amount of live yeasts
- Overall, this method is highly inconsistent and inaccurate

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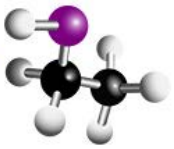
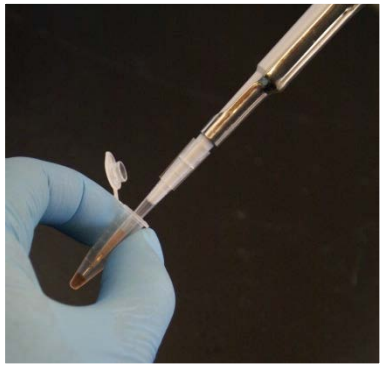


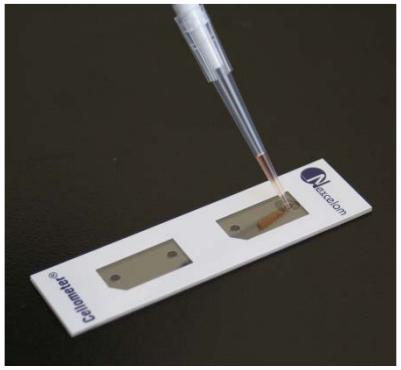
Cellometer X2 is an image cytometer that can automatically analyze yeast count and viability

- Cellometer X2 captures fluorescent images in green and red channels
- The images are automatically analyzed to produce yeast concentration and viability
- Takes ~60 secs per sample

Image cytometry yeast counting and viability detection method is simple

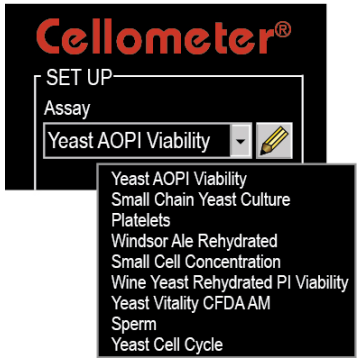


1. Stain sample

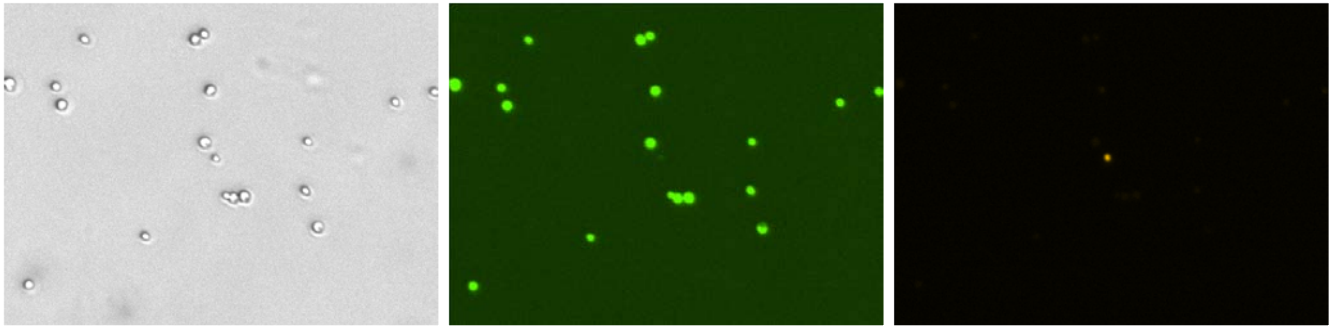


2. Pipette 20uL into counting chamber

3. Insert chamber into instrument



4. Select assay and click count



5. Bright field and fluorescent images are acquired and analyzed

Assay: Yeast AOPI Viability

Cell Type F1: Yeast AOPI Viability FL1
Cell Type F2: Yeast AOPI Viability FL2

Sample ID: Yeast AOPI Viability-2
Dilution: 4.00

Results:

Count	Concentration
Total: 1148	5.00x10^7 cells/mL
Live: 928	4.05x10^7 cells/mL
Dead: 220	9.50x10^6 cells/mL

Viability: 81.0%

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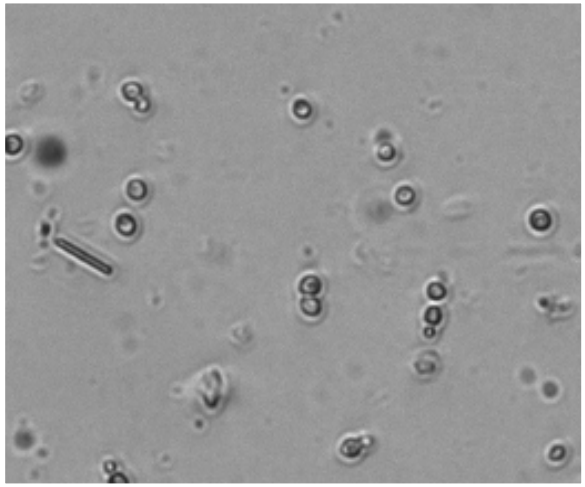
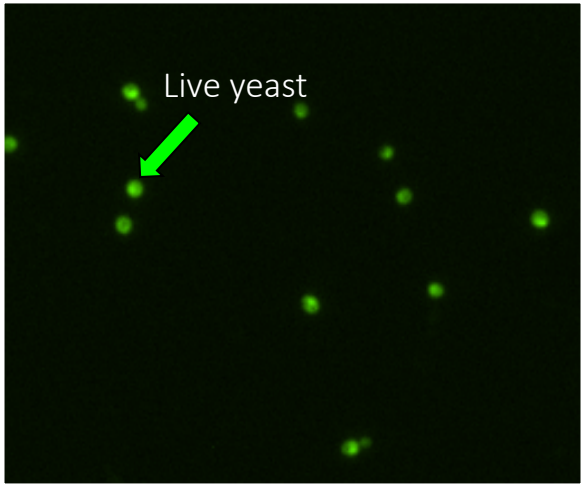
Yeasts are stained with AOPI to distinguish between live and dead cells

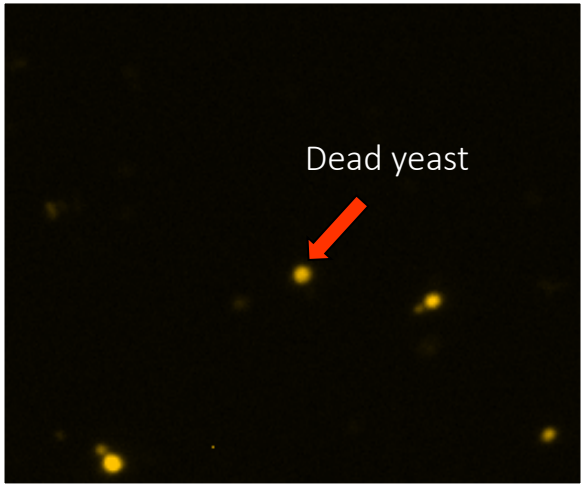
Bright field image



Live cell image



Dead cell image



- Acridine orange (AO) and propidium iodide (PI) stain live and dead yeasts, respectively
- Using fluorescence can eliminate the counting of nonspecific particles in bright field images
- AOPI fluorescence is clean and only stains the nuclei of the yeasts

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Bretts were directly analyzed daily from flasks cultured for 8 days

1. 3 flasks (clausenii, bruxellensis, and lambicus) were prepared and cultured in autoclaved solution of dried malt extracts
2. The flasks were stirred continuously over 8 days
3. Cellometer X2 was used to capture images daily and analyzed for concentration and viability

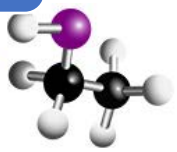
Brettanomyces propagation experimental work flow

3 separate flasks were filled with 200 mL of wort (DME)

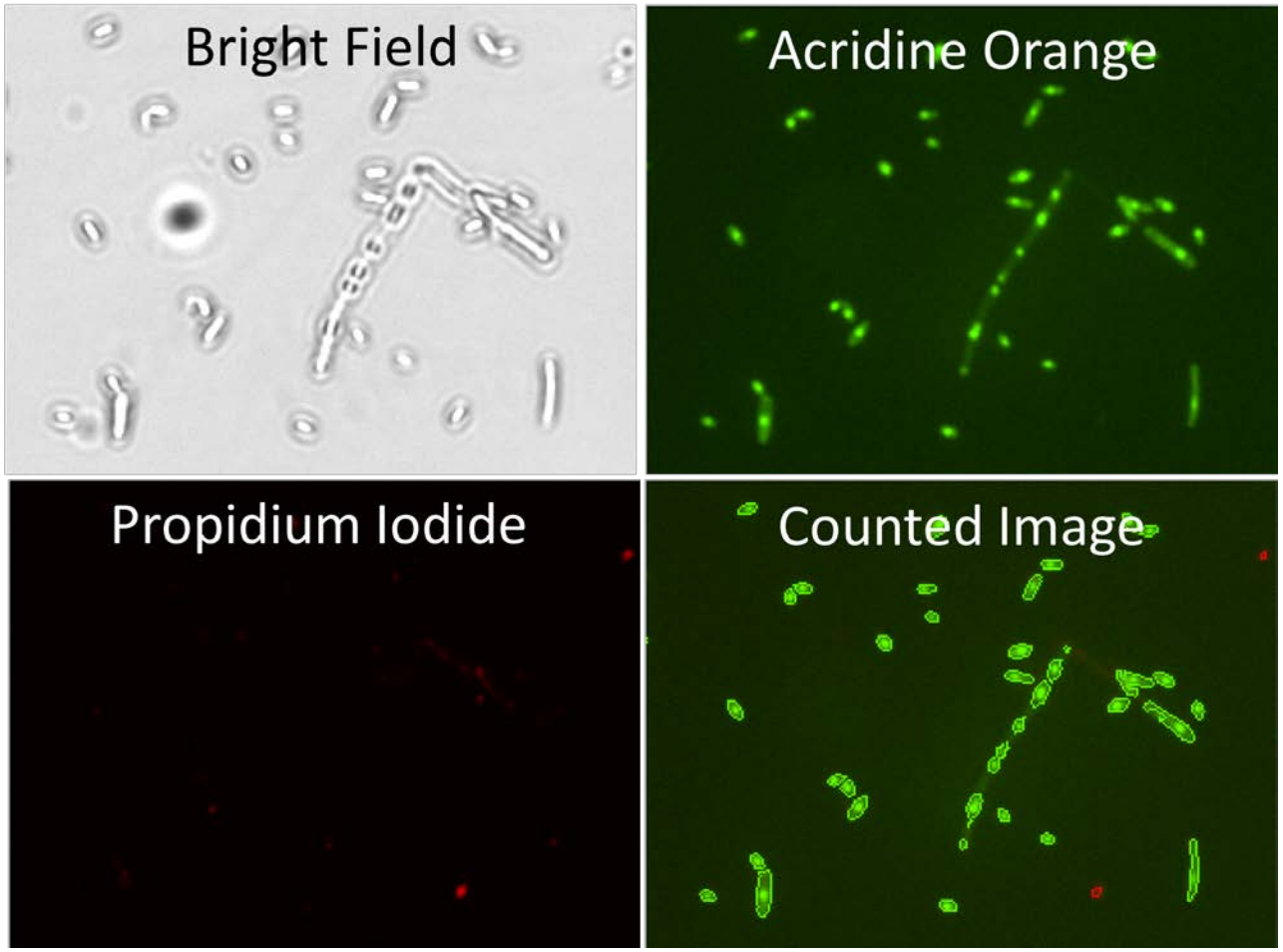
Clausenii, bruxellensis, and lambicus (White Labs) were added at 20, 20, and 14 mL

The flasks were put on a stir plate and aerated with constant stirring

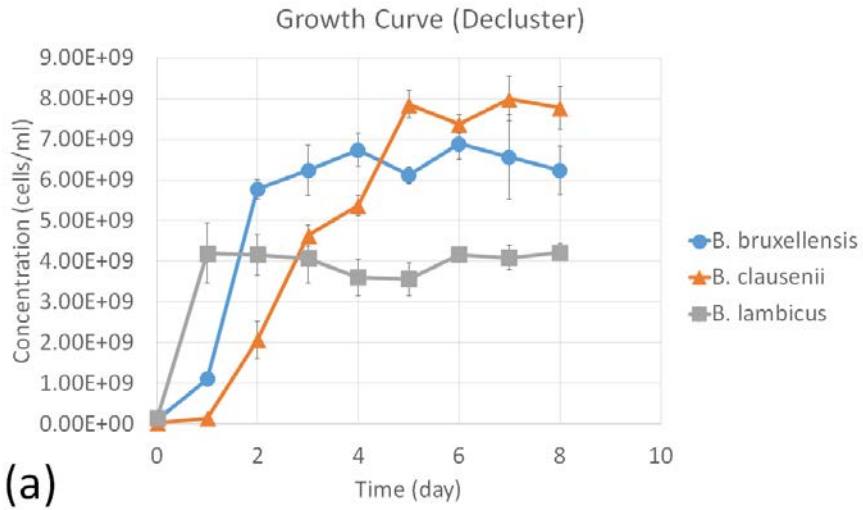
Cellometer X2 was used to monitor concentration, viability and % elongation over 8 days

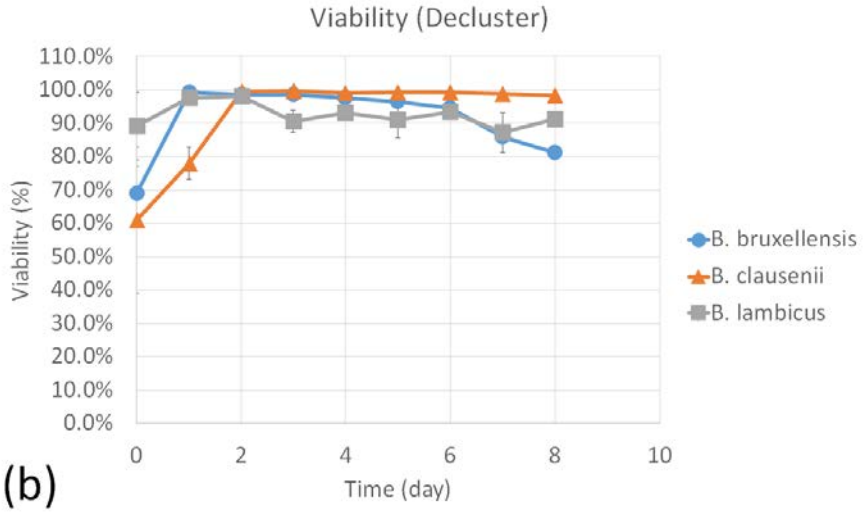


AOPI can be used to stain Brett and count single nuclei within the pseudo-hyphae



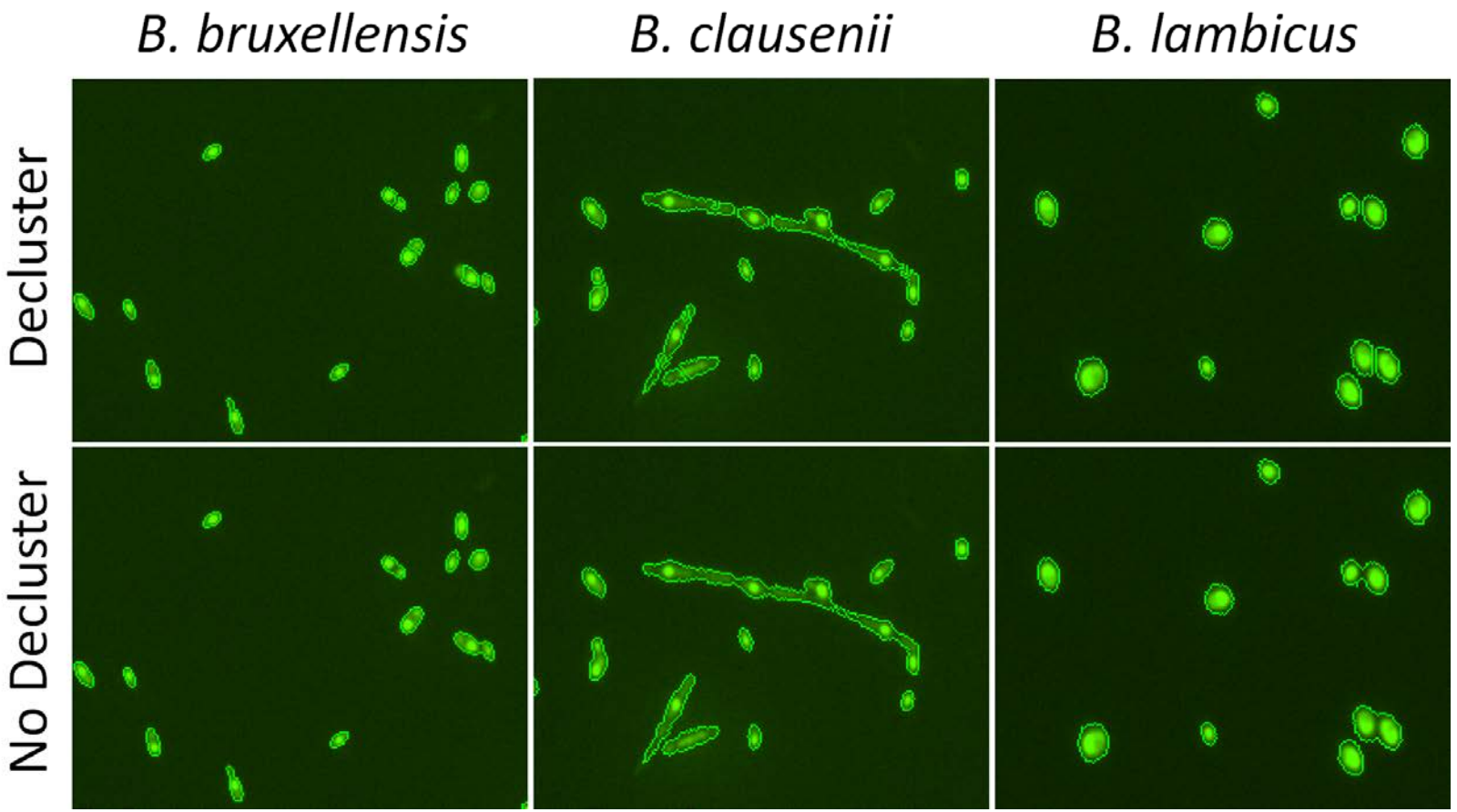
Each Brett strain seemed to plateau at a certain concentration





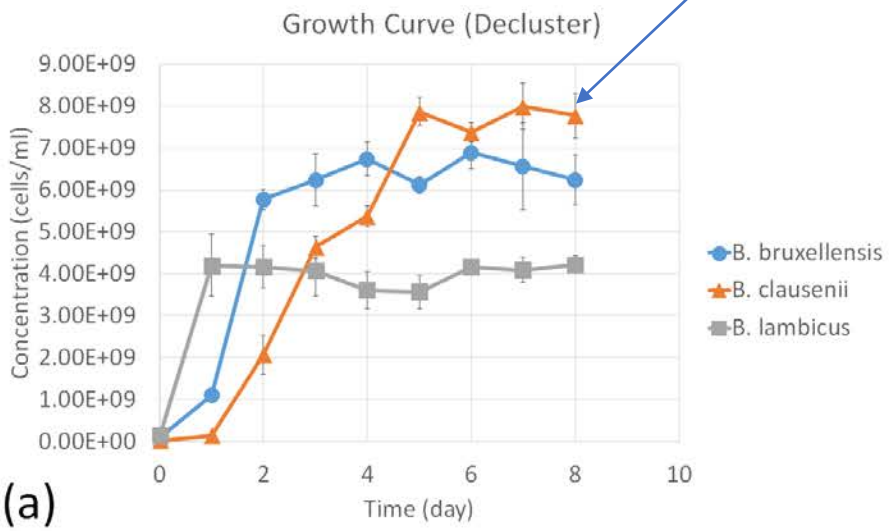
- Growth plateau ranked from clausenii > bruxellensis > lambicus
- The viability of clausenii was consistently high, while bruxellensis and lambicus seemed to decrease slight at the end of the culture

Using declustering algorithm can count Brett as a single multicellular organism or multiple cells



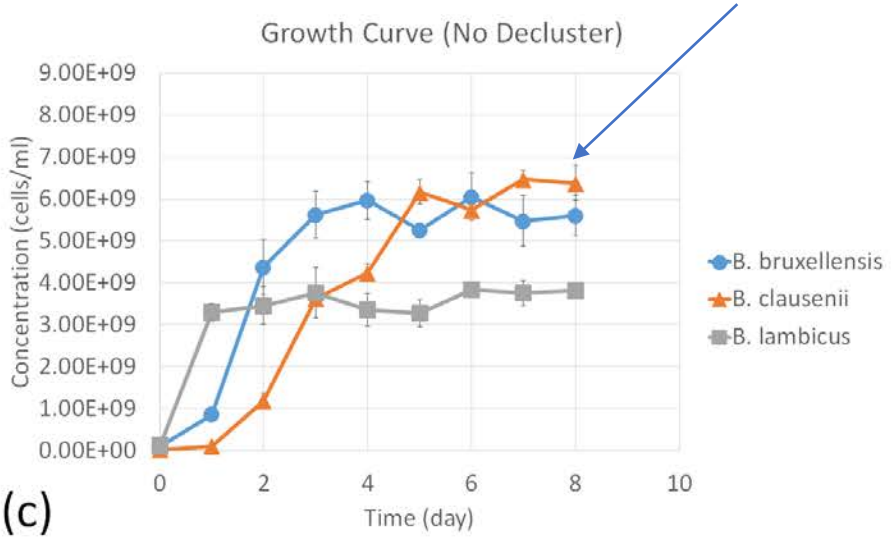
Only clausenii showed difference for counting w/ or w/o declustering

8 x 10^9 cells/ml



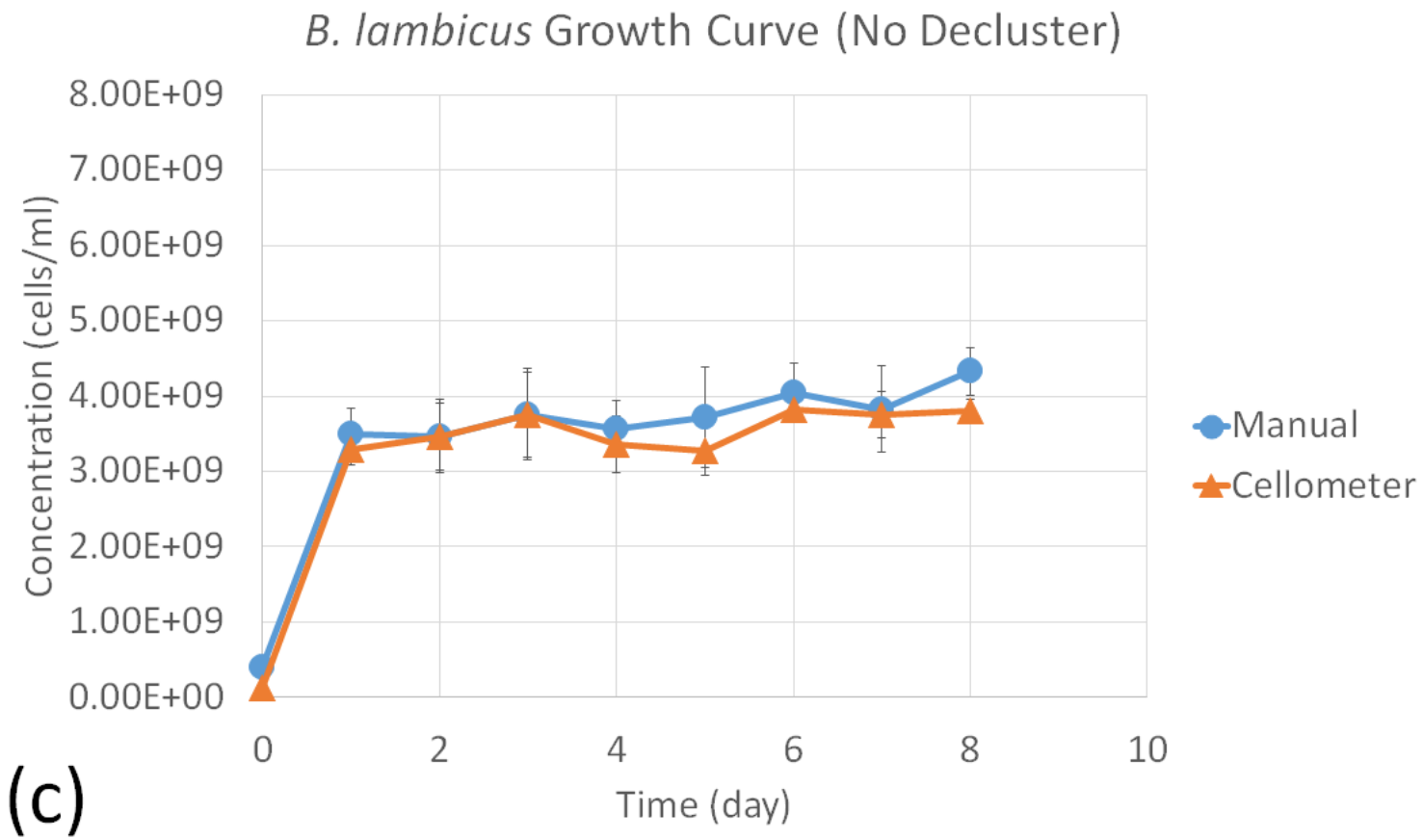
(a)

6 x 10^9 cells/ml



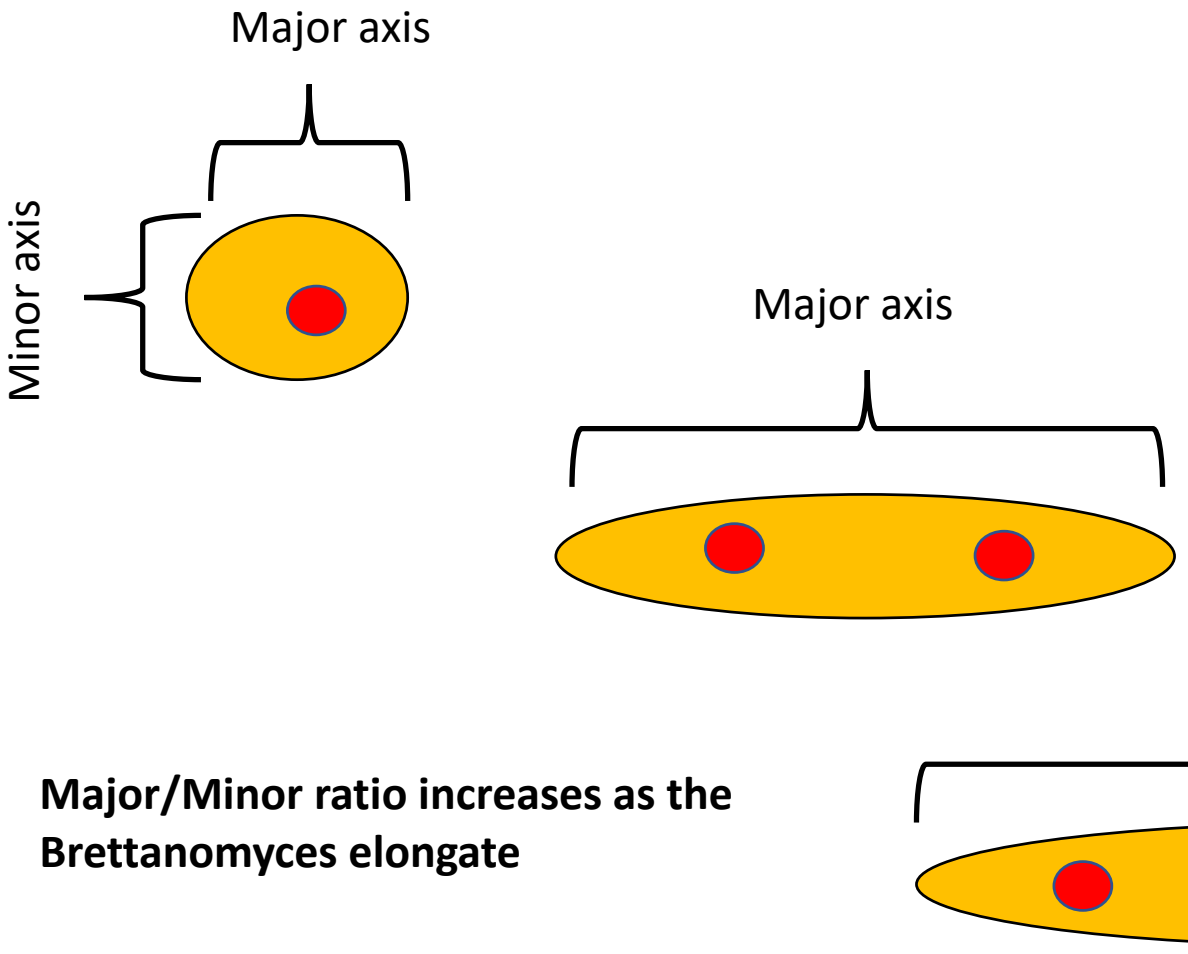
(c)

The Brettanomyces counting was validated by also performing a manual counting



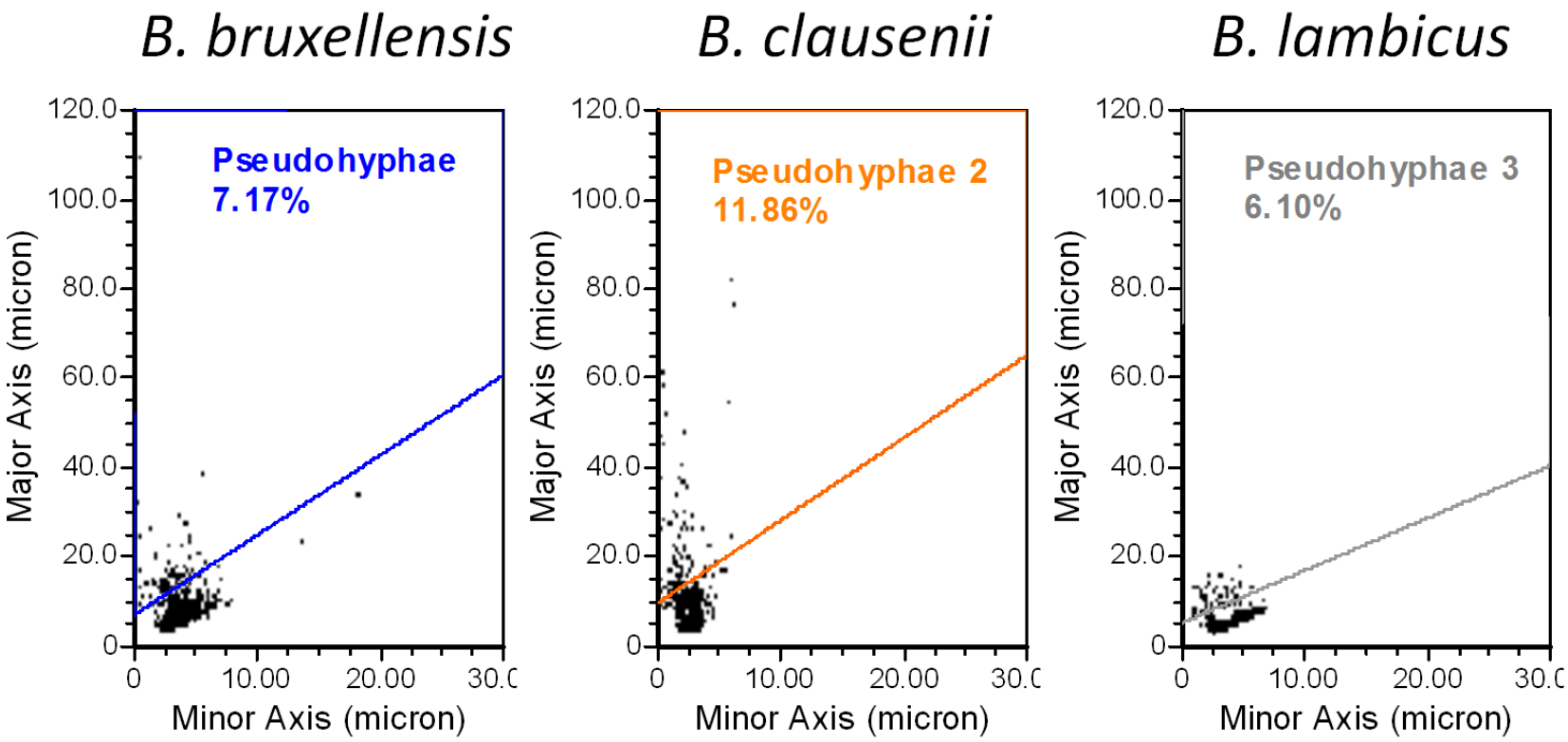
(c)

How to measure % elongation of the pseudo-hyphae

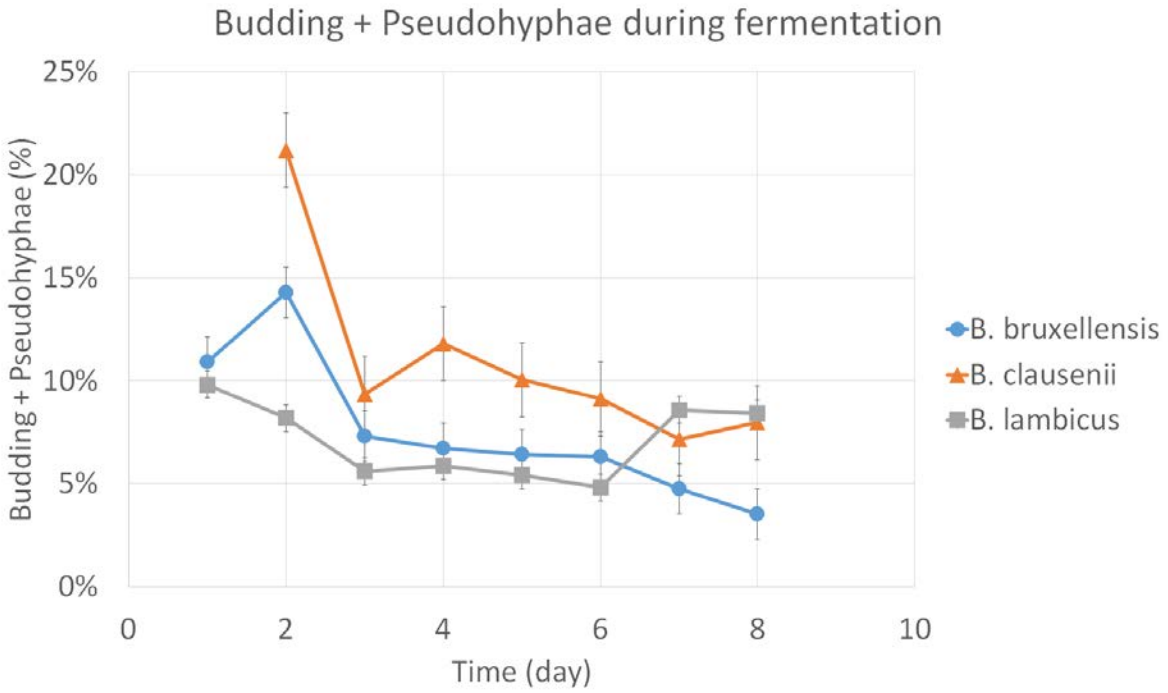


Major/Minor ratio increases as the Brettanomyces elongate

FCS express software was used to plot major/minor axis of Bretts to determine elongation



The %elongation of Bretts were monitored for 8 days



- The 3 strains of Bretts were analyzed to monitor changes in % elongation over 8 days
- It showed that clausenii generated the most pseudo-hyphae

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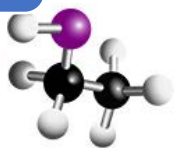
Brettanomyces fermentation experimental work flow

15 gallon stainless cylindroconical fermenters at 72°F

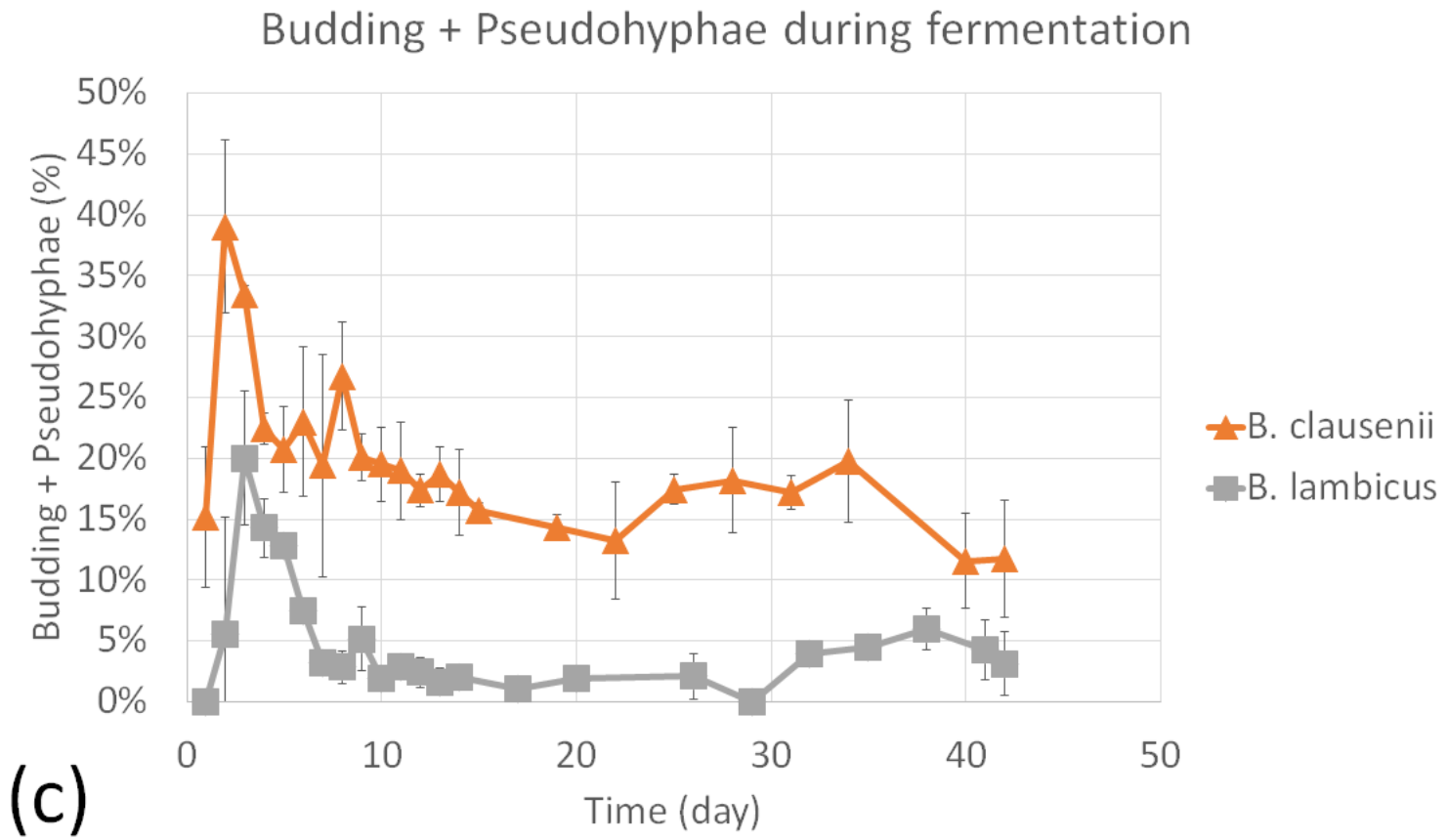
Clausenii and lambicus (Wyeast and White Labs) were added at 115 and 125 mL into 3 gallon of wort

Yeast samples were collected from sample porter in the middle of the fermenter. Sanitized before collection.

Cellometer X2 was used to monitor concentration, viability and % elongation over 42 days



Monitoring Brett concentration, viability, and pseudo-hyphae over 40 days of fermentation



(c)

Take home messages

- Brettanomyces have been increasingly used in beer products for novel flavors
- There is no standard method for analyzing Bretts consistently
- Using Cellometer X2 image cytometer can monitor Brett concentration, viability, pseudo-hyphae % during fermentation
- Breweries currently using or thinking about using Bretts can improve and standardize the cell count method to produce consistent products

Acknowledgment

- University of Maine
  - Jason Bolton
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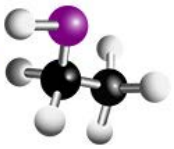
- Nexcelom Bioscience
  - Dmitry Kuksin
  - Suzanne Shahin



References

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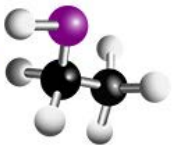
  - JIMB 39:11, 1615-1623
  - JIMB 44:1, 119-128
- Strain development
  - FEMS Microbio. Eco. 80:3, 578-590
  - Int. J Food Microbio. 157:1, 45-51
- Monitoring production quality
  - BioControl 57, 451-461
  - FEMS Microbio. Eco. 76:1, 145-155

More publications here
http://www.nexcelom.com/Support/References-Publications.html



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