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# The Effect of Exogenous Aromatic Alcohols on Growth and Flocculation of Commercial Beer Brewing Strains of *Saccharomyces cerevisiae* in Beer Wort

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## ABSTRACT

Flocculation is a phenomenon where yeast cells aggregate into dense masses which settle to the bottom of a growth vessel. In commercial brewing, flocculation of *Saccharomyces cerevisiae* is beneficial because finished fermentation products can be easily separated from the settled yeasts, and then new substrates can be added to reactivate the yeast for new fermentation. Aromatic alcohols such as 2-phenylethanol, tyrosol and tryptophol, natural biproducts of the fermentation process, have been shown to stimulate flocculation in lab strains of yeast through activation of a group of genes identified as *FLO* genes which include genes for lectin-like mannose receptors that bind to cell wall carbohydrates on adjacent cells to cause flocculation. Most research on flocculation, however, has been performed with conventional lab strains of *S. cerevisiae* in culture media that do not resemble the brewer's fermentation mix. We were interested studying the effect of exogenous aromatic alcohols on growth and flocculation in conditions more authentic to the brewer's fermentation chamber. We acquired 10 industrial beer brewing and two laboratory strains of yeast and studied the effects of 2-phenylethanol (2-PE), a presumptive quorum-signaling molecule, on flocculation of these strains in beer wort. 2-PE had no effect on the growth rate of the yeasts, although the growth rates of the different strains varied dramatically. Since growth and flocculation are influenced by carbon and nitrogen sources, we evaluated growth in three different types of media: SLAD, YB and YEPD, which differ in N and C sources. Most strains grew equally well in all three media types, though two of them exhibited 3-4 times higher growth in YEPD. None of the 12 strains grown in beer wort showed any difference in flocculation rates between controls and cultures containing 200 µM 2-PE. Different flocculation rates were observed between the different strains, with YMD4529 showing significant sedimentation rates as early as 15 min in the assay. This strain, another moderately flocculant strain and 2 non-flocculant strains were incubated in wort containing 200 µM of either 2-PE, tyrosol or tryptophol. None of the 3 aromatic alcohols affected flocculation compared to controls as determined by digital image analysis of sedimentation in cuvettes. We conclude that in a more authentic fermentation medium, flocculation of commercial yeast strains is unaffected by exogenous aromatic alcohols, but we are continuing these studies to evaluate their impact on expression of *FLO* genes in these strains.

## INTRODUCTION

### Flocculation in Yeast

- Aggregation of yeast cells into "flocs" during the fermentation process
- Flocs sink to the bottom of the fermentation chamber
  - Easier separation of alcohol product from yeast cells and collection of yeast cells for reuse
- **Lectin Hypothesis:** flocculation occurs when lectin-like receptors bind to cell surface carbohydrates like mannose

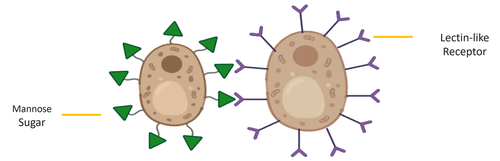



Image created in BioRender.com

- Influenced by:
  - Low pH – optimum range is approximately 3-5
  - Nitrogen source – $NH_4^+$ induces flocculation best
  - $Ca_2^+$ at concentrations as low as 8-10 mM
  - High cell density
  - Expression of FLO genes

### Aromatic Alcohols

- Byproducts of fermentation
- Shown to regulate expression of FLO genes and induce non-flocculant strains of yeast to flocculate
  - 2-Phenylethanol (2-PE) especially influential in expression of FLO genes
- Presumed quorum signaling molecule (QSM)
- Derived from amino acids

Phenylalanine → **2-Phenylethanol**

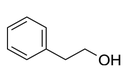


Image from MedchemExpress.com

Tryptophan → **Tryptophol**

Image from MedchemExpress.com

Tyrosine → **Tyrosol**

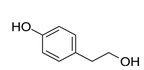


Image from MedchemExpress.com

## OBJECTIVES

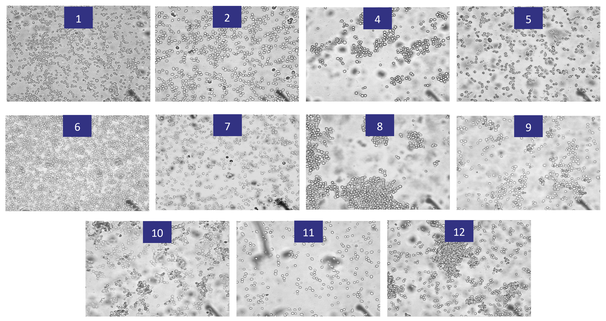
### Problem

- Nearly all studies of flocculation were performed using domesticated lab strains of *S. cerevisiae*
  - These strains are genetically and metabolically different from yeast strains used in commercial beer brewing
- Most studies employed synthetic, chemically-defined growth medium supplemented with $NH_4^+$ as a nitrogen source
  - In beer brewing, yeast grow and ferment in "beer wort," which has complex nitrogen sources

### Aims of this Study

- Compare differing nutrient requirements between different strains
- Determine the effects of 2-PE on growth
- Analyze the effects of 2-PE, tryptophol, and tyrosol on flocculating ability
- Explore the validity of the lectin hypothesis
- Examine differences in flocculation between different strains and their potential causes

## METHODS & RESULTS



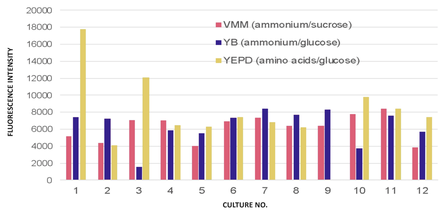

| Culture No. | Strain | Culture No. | Strain | Culture No. | Strain |
|---|---|---|---|---|---|
| 1 | YMD4518 (LACH) | 5 | YMD4537 (62 Duvel Moortgat Brewery Strain) | 9 | YMD4548 (WLP013) |
| 2 | YMD4520 (Belgian Strong Ale Yeast) | 6 | YMD4538 (F2) | 10 | YMD4553 (WY1725) |
| 3 | YMD4524 (US-05) | 7 | YMD4544 (LACH) | 11 | YMD4243 (Lab Strain 5288c) |
| 4 | YMD4529 (WLP006) | 8 | YMD4545 (Wmal) | 12 | YMD4260 (Lab Strain Sigma) |

Gift from Scott Britton, Director of Research at Duvel Moortgat Brewing Co., Belgium

- Stock cultures were maintained by weekly transfer on plates of Yeast Extract-Peptone-Dextrose broth (YEPD) and stored at 4˚C
- Seed cultures were grown in Yeast Extract-Peptone-Dextrose broth (YEPD) overnight for 24 hours at 30˚C, shaking at 225 rpm in a rotary water bath
  - Experimental cultures were started by diluting the cells to an inoculating density of $1x10^6$ cells per ml in beer wort and grown overnight as previously described
- Aromatic alcohols for experimental groups and ethanol for control groups were added to control groups at concentrations of 200 µM (500 mg/ml)

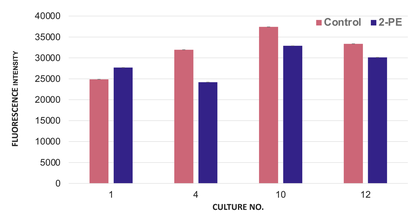
### Growth

#### Effect of Carbon and Nitrogen Source on Growth




- Cultures were grown in Vogel's Minimal Medium (VMM), Yeast Nitrogen Base (YB), or YEPD, centrifuged, and mixed with Calcofluor White
- Fluorescence emission of bound Calcofluor White was measured at 460 nm with an excitation wavelength of 350 nm

#### Effect of 2-PE on Growth




- Cultures were grown in beer wort, centrifuged, and mixed with Calcofluor White
- Fluorescence emission of bound Calcofluor White was measured at 460 nm with an excitation wavelength of 350 nm

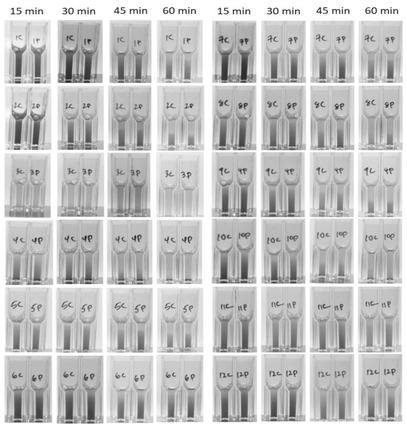
#### pH Change

| Source | pH |
|---|---|
| Initial Beer Wort | 4.3 |
| Supernatant YMD4518 | 3.3 |
| Supernatant YMD4529 | 3.5 |

pH of initial beer wort and the beer wort that had been acidified by the strains after 24 hours of growth was measured using a pH probe

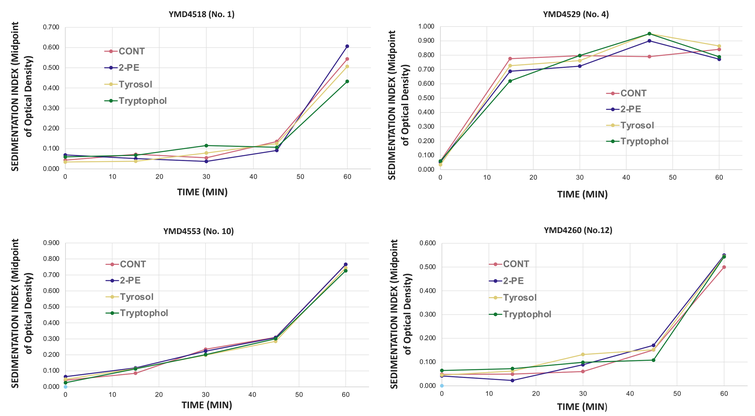
### Flocculation

#### Effect of 2-PE on Flocculation in Sedimentation Assays




- Overnight cultures were mixed thoroughly before 1 ml samples were transferred to plastic microcuvettes
- The cells were mixed again before being photographed over 15-minute intervals

#### Effect of 2-PE, Tryptophol, and Tyrosol on Flocculation in Select Strains

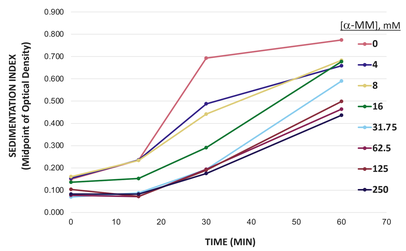



Four strains were selected based on their flocculating abilities:
- Strong: YMD4529
- Moderate: YMD4553
- Non-flocculating: YMD4518 & YMD4260

Sedimentation was quantified using ImageJ digital analysis on photos taken over 15-minute intervals.

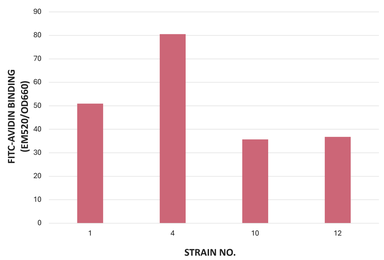
### Lectin Binding Properties

#### Effect of α-Methyl-Mannoside on Flocculation in YMD4529



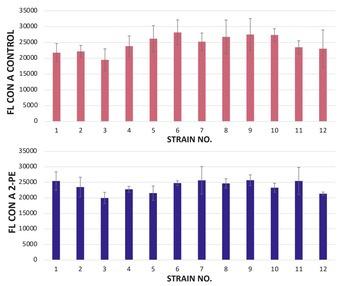

- Cells were grown in beer wort and centrifuged
- Pellets were resuspended in beer wort and added to dilutions of α-methyl-Mannoside in microcuvettes
- The cells were mixed again before being photographed over 15-minute intervals, and the photos were digitally analyzed

#### Lectin-Like Receptor Content Between Selected Strains




- Overnight cultures were centrifuged, washed twice with $dH_2O$, and resuspended in acetate buffer, and incubated with FITC-Avidin
- Fluorescence will be measured at 520 nm emission with an excitation wavelength of 495 nm.

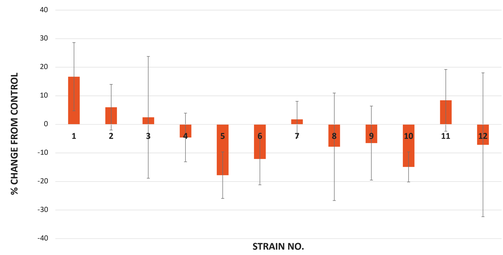
#### Mannose Content Between Strains




- Overnight cultures were centrifuged and washed with phosphate-buffered saline (PBS) and incubated with PSA-FITC and then Concanavalin A-Alexa Fluor®-350 (Con A)
- Fluorescence emission was measured at 442 nm with an excitation wavelength of 346 nm

## RESULTS

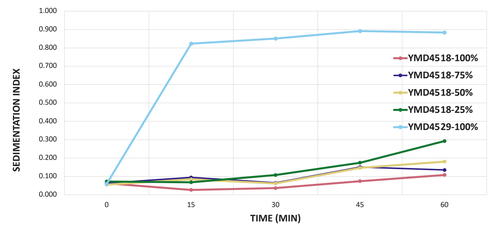
#### Effect of 2-PE on Mannose Content




- The percent change between controls and cultures treated with 2-PE was calculated.

### Co-Flocculation

#### Effect of Mixing YMD4518 & YMD4529 on Flocculation




- YMD4518 and YMD4529 were chosen because they are non-flocculant and strongly flocculant, respectively
- Overnight cultures of the strains were mixed in 1:3, 1:1, and 3:1 ratios, and 1 ml of each ratio was transferred to a microcuvette
- The cuvettes were mixed again before being photographed over 15-minute intervals, and the photos were digitally analyzed
- The percent change of the flocculation values from analysis versus the theoretical values based on the ratios of the cells was calculated.

## CONCLUSIONS

### Interpretation of Results

- Some strains grow similarly with different sources of carbon and nitrogen, but others have **different nutrient requirements**
- **2-PE can inhibit growth** of certain strains of *S. cerevisiae*
- YMD4529's superior flocculation ability is not because of its ability to better acidify its media
- The aromatic alcohols **2-PE, tryptophol, and tyrosol have no effect on flocculation or mannose content** in any of the 12 strains when grown in beer wort
- **The lectin hypothesis is confirmed**: observed flocculation is the result of lectin-like receptors binding to mannose residues on adjacent cells
- **Flocculation ability is influenced by the amount of lectin-like receptors**
- Differences in **mannose content on the cell surface do not influence flocculation**
- **Co-Flocculation interestingly resulted in lower flocculation values** compared to the theoretical flocculation values of mixed cultures
- Strains' genetic differences likely account for their perceived variations

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