### VWT 272
#### Class 16

#### Quiz 14 15

<table>
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<th>Number of quizzes taken</th>
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<td>Median</td>
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Kilgore Trout once wrote a short story which was a dialogue between two pieces of yeast. They were discussing the possible purposes of life as they ate sugar and suffocated in their own excrement. Because of their limited intelligence, they never came close to guessing that they were making champagne.

Kurt Vonnegut (1922-2007)
Plan of Study

• Yeast Ecology
  – Biofilms
• Yeast Nutrition
• Yeast Inoculation
• LAB Ecology
• LAB Use
• LAB Nutrition
  – Diacetyl
• LAB Monitoring
Yeast Refresher

- Wine Yeast
  - Eukaryotic single celled microorganism (has nucleus)
  - Fungus Kingdom (<1% of all known fungi)
  - Can Respire
    - Use O$_2$ to reproduce and “recharge” ADP to ATP
  - Can Ferment
    - Does NOT use O$_2$ to reproduce and “recharge” ADP to ATP
  - Reproduce asexually by budding
  - ~5 μm in length
Welcome to the (Grape) Jungle!

• Grape surfaces have between $10^2$ and $10^5$ yeast per cm$^2$

• Over 50 species of yeast have been identified
  – *Saccharomyces cerevisiae* is very rare!

• Yeast type changes by ripeness, and location on the berry

• Insects are vectors of yeast dispersal in vineyards
Epicuticular Wax Surface
of Mature Grape Berries

Fig. 1. Scanning electron micrographs of the epicuticular wax surface of mature grape berries. 9500X. (A) Cabernet Sauvignon; (B) Pinot noir; (C) Grenache; (D) Zinfandel.
Welcome to the (Grape) Jungle!

• Pre-verasion
  – Cryptococcus species
  – Rhodotorula species
  – Aureobasidium species

• At harvest
  – $10^3$ more yeast toward the peduncle
  – Hanseniaspora uvarum/Kloeckera apiculate
  – Metschnikowia pulcherrima/Candida pulcherrima
  – Candida stellate/Candida zemplinina
Welcome to the (Winery) Jungle!

• Following grape processing *Saccharomyces* populations can increase < 3 orders of magnitude
  – 10 to 10,000 cells/mL

• *Saccharomyces* can produce biofilms
  – Common with *Saccharomyces*, *Brettanomyces*, and *Candida* yeast
    • Behavior increased with presence of polyphenols
Welcome to the (Winery) Jungle!

• Yeast species found in wineries
  – Saccharomyces
  – Candida
  – Cryptococcus
  – Brettanomyces

• Many winery surfaces are very difficult to clean
  – Stainless steel
  – Wood
  – Concrete
Biofilms

• A group of microorganisms where cells adhere to a surface and to each other
Biofilms

• Different phenotype than free floating “planktonic cells”
  • Phenotype – an organism’s observable traits
Biofilms

• Different phenotype than free floating “planktonic cells”
  • Phenotype – an organism’s observable traits
Biofilms

- Microbes are embedded in self produced Extracellular Polymeric Substance (EPS)
  - Slime
  - Polysaccharides, proteins, extracellular DNA
Welcome to the (Fermentation) Jungle!

- In uninoculated fermentations rely on grape and winery based yeast
- Early fermentation are dominated by non-\textit{Saccharomyces} yeast
  - Usually by the yeast present on the berry
    - \textit{Hanseniaspora uvarum}/\textit{Kloeckera apiculate}
    - \textit{Metschnikowia pulcherrima}/\textit{Candida pulcherrima}
    - \textit{Candida stellate}/\textit{Candida zemplinina}
  - Temperature of fermentation determines which non-\textit{Saccharomyces} yeast will dominate
    - \textit{Hanseniaspora} and \textit{Candida} species are more dominant in cold conditions
    - \textit{Hansenula}, \textit{Issatchenkia} and \textit{Saccharomyces} are more dominant in warm conditions
Welcome to the (Fermentation) Jungle!

- Mid fermentation is characterized by the rapid increase of population of *Saccharomyces* yeast
  - *Pichia* species may emerge after cold soaked musts
- Late fermentation are totally dominated by *Saccharomyces* yeast
  - Often no other viable yeast can be found
- In *inoculated fermentations* *Saccharomyces* yeast quickly dominate population
Welcome to the (Aging) Jungle!

• Type of organisms present
  – Winery practices
  – Sanitation
  – Storage vessels
    • Wood & Cement – hard to clean
    • Stainless Steel – easier but not perfect
  – Yeast found in non-biologically active state
    • *Candida*, *Pichia*, *Brettanomyces* & *Zygosaccharomyces*
Yeast Growth Curve

Number of cells vs Time

- Lag Phase
- Exponential Growth Phase
- Stationary Phase
- Death Phase
Yeast Nutrition

• Energy
  – From Glucose/Fructose

\[
\text{C}_6\text{H}_{12}\text{O}_6(aq) \rightarrow 2\text{CH}_3\text{CH}_2\text{OH}(aq) + 2\text{CO}_2(g)
\]

  Glucose          Ethanol          Carbon dioxide

• Micronutrients
  – From grape juice or supplement
  – No commercial analysis for quantification
  – Biotin, pantothenate, potassium, sterols, fatty acids
Yeast Nutrition

• Nitrogen source
  – Free Amino Nitrogen (FAN)
    • α-amino acids
    • Not peptides or proteins
    • Not Proline
  – Ammonium salts (NH₄⁺)

• $\text{FAN} + (\text{NH}_4^+) = \text{Yeast Assimilable Nitrogen (YAN})$
Yeast Nutrition

- YAN highly variable
  - Dependant on
    - vineyard nutrition
    - Rootstock

FAN + (NH\(_4^+\)) = Yeast Assimilable Nitrogen (YAN)

<table>
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<th>Free Amino N</th>
<th>Ammonium</th>
<th>YAN</th>
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<tbody>
<tr>
<td>n=1524</td>
<td>mg N/L</td>
<td>mg N/L</td>
<td>mg N/L</td>
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<tr>
<td>Mean</td>
<td>135</td>
<td>79</td>
<td>213</td>
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<tr>
<td>Min</td>
<td>29</td>
<td>5</td>
<td>40</td>
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<tr>
<td>Max</td>
<td>370</td>
<td>325</td>
<td>559</td>
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</table>

- NO correlation between FAN and NH\(_4^+\) concentrations in juices
Yeast Nutrition

- **FAN**
  - Determined in a lab
  - NOPA
  - Difficult to do
  - Report as mg N/L

- **NH_{4}^{+}**
  - Determined in a lab
  - Ion selective electrode
  - Easy to do
  - Report as mg N/L
YAN Requirements

• How Much?

<table>
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<tr>
<th>Total YAN (in grape + additive)</th>
<th></th>
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<tbody>
<tr>
<td>≤ 23 °Brix</td>
<td>250 mg N/L</td>
</tr>
<tr>
<td>&gt; 23 but &lt; 25 °Brix</td>
<td>300 mg N/L</td>
</tr>
<tr>
<td>≥ 25 °Brix</td>
<td>350 mg N/L (or more)</td>
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</table>

• Can there be too much N?
  – Excess N leads to
    • High Fermentation Rate
      – High temperature fermentations
      – Fast/Hot fermentations = less aroma complexity & amount
    • Leftover N for spoilage organisms
YAN Requirements

• When to add?
  – Professor Linda Bisson (UC Davis)
    • After *Saccharomyces* have become dominant
      – “No reason to feed the yeast you don’t want!”
    • Ethanol is inhibitory to N transport into the yeast cell
    • Some nutrients needed for alcohol tolerance need to be available when new cells are being made

  **Feed 24 to 48 hours after inoculation**

  – Lisa van der Water (BSG Wine Division)
    • At yeast rehydration
      – Gives yeast a boost for a fast start
    • At yeast inoculation
    • During the first half of fermentation
      – Multiple additions
      – After yeast have stopped growing but before they are incapable of uptake

  **Feed early and often**
YAN Requirements

• What to add?
  • Diammonium phosphate DAP
    – Provides NH$_4^+$
    – And phosphate (also used by the yeast)
    – Cheap
  • Yeast autolysate
    – Provides amino acids
    – Provides micronutrients
    – Vary widely in purity and quality
    – Expensive
  • “Blends” of DAP and Autolyzed yeast
    – Invented by Lisa van der Water (Superfood)
YAN Requirements

• How Much Nitrogen?
  • Diammonium phosphate DAP
    – 21 mg N/100 mg DAP
  • Yeast autolysate
    – Varies by manufacturer
    – Usually not provided
  • Superfood
    – ~ 10 mg N/100 mg Superfood
  • Fermaid
    – ???
  • Yeast Hulls/Yeast Ghosts
    – Trace amounts of N
    – Sterols & Lipids useful in building up cell membrane
    – Adsorb “toxins”???
    – Solids act as “surfboard” for yeast to get them up/into the ferment
Yeast Inoculation

5/10 Method

1. Prepare a chlorine-free, 5:1 water to juice mixture of approximately 5 times the yeast weight at 35 - 40°C (95 - 104°F).

2. Gently sprinkle the yeast into the water-juice mixture and, without stirring, allow 10 minutes for hydration.

3. Stir the yeast into the water-juice mixture allowing it an additional 10 minutes to fully hydrate.

4. Add enough juice to the yeast suspension to lower the temperature by 5°C. Let mixture acclimate for 5 minutes.

5. Repeat above until the temperature of the yeast suspension is less than 10°C warmer than the must or juice to be inoculated.

6. Slowly add the yeast mixture into one area of the must or juice. Do not mix or pump over for at least 10 hours.
Bacteria Refresher

• Bacteria
  – Prokaryotic microorganism (no nucleus)
  – DNA in rings - plasmids
    • not rod like structures - Chromosomes
  – Tiny
    • often < 1μm in length
  – Incredibly numerous
  – Can reproduce quickly by fission
• Classification
  • Gram +/Gram –
  • Aerobic/Aerobic
Malo/Lactic Fermentation

• First recorded in 1837 by Freiherr von Babo
  – Occurred in spring
  – Released CO₂

• Louis Pasteur isolated bacteria from wine in 1866
  – Considered all bacteria “spoilage organisms”

• Hermann Müller-Thurgau suggested in 1891 that bacteria could be responsible for the acid reduction

• Brad Web & John Ingraham produce the first M/L starter culture (ML34) in 1959
  – *Luconostoc oenos* bacteria collected from a redwood tank of Barbera at the Louis Martini Winery, St Helena
  – First successful LAB inoculation at Hanzel Vineyards, Sonoma

• **Viniflora Oenos** bacteria introduced in 1993 by Danish company Chr. Hansen
  – freeze dried, direct inoculation
Malo/Lactic Fermentation
Why Malo/Lactic Fermentation?

• Decrease acidity
  – TA decreases by 0.01 to 0.03 g/L as tartaric
  – pH increases 0.1 to 0.3

\[\text{HO-} \quad \text{OH} \quad \text{HO} \quad \text{HO}\]
\[\text{O} \quad \text{O} \quad \text{O} \quad \text{O}\]

Malic Acid

\[\text{HO} \quad \text{OH} \quad \text{OH}\]
\[\text{O} \quad \text{O}\]

Lactic Acid
Why Malo/Lactic Fermentation?

• Bacteria consume nutrients needed by other (spoilage?) organisms
  – “Scorched Earth Policy”
Why Malo/Lactic Fermentation?

• Sensory changes
  – Acetic Acid
  • Produced from sugar by bacteria
    – if $O_2$ is available – otherwise Ethanol
    – $O_2$ can stimulate growth of M/L (and other) bacteria

Acetic Acid

Ethanol
Why Malo/Lactic Fermentation?

• Sensory changes
  – Diacetyl
    • Produced from Pyruvic Acid
      – Multiple/Complex formation pathways
      – From sugars
      – From Citric Acid
  • Not just butter
    – When combined with S containing amino acids
      » Nutty, chocolate, cheese, popcorn

Pyruvic Acid

Diacetyl

Citric Acid
Inoculate with LAB or not?

• Inoculated
  – Decide which bacteria
  – Decide when to inoculate
  – Starter cultures are hard to maintain
    • Can inoculate entire vintage with “bad” bacteria
    • Require large inoculation volume
  – Direct add LAB are Expensive
  – Fast

• Uninoculated/Spontaneous
  – No choice of which bacteria
    • Risk of unpleasant aromas/flavors
  – No choice of time
  – Free
    • Until you have to clean up/declassify/dump the wine
  – Slow
When to inoculate with LAB?

• Prior to yeast
  – Good
    • No Ethanol, low SO₂, available nutrients, warm temperatures?
  – Bad
    • Produce Acetic Acid (VA)
    • Can produce other compounds inhibitory to yeast growth (toxins???)
    • Usually wont complete MLF prior to beginning of yeast fermentation
When to inoculate with LAB?

• With yeast
  – Good
    • No initial Ethanol, low SO₂, warm temperatures?
  – Bad
    • Produce Acetic Acid (VA)
    • Both fermentations are slow with low viable cell populations
    • Yeast tend to outcompete bacteria
    • Yeast consume Diacetyl
    • Yeast “hog” nutrients
      – Stuck MLF
    • Yeast can produce SO₂
When to inoculate with LAB?

• During Yeast Fermentation
  – Good
    • Low Ethanol (?), low $\text{SO}_2$ (?), warm temperatures(?)
  – Bad
    • No nutrients available to LAB
    • Produce Acetic Acid (VA)
    • Yeast fermentations tend to slow with decreased viable cell populations
      – Can stick yeast fermentation
    • Yeast consume Diacetyl
    • Yeast can produce $\text{SO}_2$
When to inoculate with LAB?

• After Yeast Fermentation
  – Good
    • No issue with stuck yeast fermentation
    • Lower Acetic Acid Production
    • Can add nutrients
    • Can provide warm temperatures
    • Low SO₂
    • Produce Diacetyl
  – Bad
    • High Ethanol
    • Yeast consume Diacetyl
    • Bacteria can produce off characters
# Oenococcus oeni Growth Factors

<table>
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<tr>
<th>Condition</th>
<th>EtOH [%]</th>
<th>pH</th>
<th>Free SO2 [mg/L]</th>
<th>Total SO2 [mg/L]</th>
<th>Temperature [°C]</th>
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<td>&lt; 13</td>
<td>&gt; 3.4</td>
<td>&lt; 8</td>
<td>&lt; 30</td>
<td>18 to 22</td>
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<td>difficult</td>
<td>13 to 15</td>
<td>3.1 to 3.4</td>
<td>8 to 12</td>
<td>30 to 40</td>
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<td>harsh</td>
<td>15 to 16.5</td>
<td>2.9 to 3.1</td>
<td>12 to 20</td>
<td>40 to 60</td>
<td>10 to 14</td>
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<tr>
<td>very harsh</td>
<td>&gt; 16.5</td>
<td>&lt; 2.9</td>
<td>&gt; 20</td>
<td>&gt; 60</td>
<td>&lt; 10</td>
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LAB Nutrition

• Most fermentations have enough left over
  – Available Nitrogen sources
    • α-amino acids
    • Peptides
    • NOT $\text{NH}_4^+$
  – Energy source
    • Even in “dry wines” some glucose remains
    • Pentoses (5 Carbon sugars)
      – Arabinose, Xylose, ribose
    • Malic Acid
  – Micronutrients
    • B vitamins
    • pantothenic acid
    • $\text{O}_2$ ???
• Can be supplemented by commercial LAB food
LAB Nutrition
Diacetyl (2,3 butanedione)

- **Diacetyl**
  - Produced by LAB from Citric Acid
  - Detection threshold
    - White wine 0.2 mg/L
    - Red wine 2.8 mg/L
  - Movie Theater Popcorn Smell
  - Positive factors for production
    - Species of bacteria
      » *Lactobacillus* & *Pediococcus*
    - High concentration (> 0.5 g/L) of Citric Acid
    - Small amounts of O₂ (un-topped barrel?)
    - Temperature 18 °C (65 °F) to 25 °C (78 °F)
    - No yeast (post alcoholic fermentation)
    - Low SO₂
    - No cysteine (S containing Amino Acid)
      » Makes Thiazole – hazelnut/toast/popcorn smell
Monitoring MLF

• Paper Chromatography
  – Cheap & Easy
  – Disappearance of Malic “spot”

• Enzymatic Testing
  – Expensive & Precise
  – Done when malic acid < 50 mg/L?

• Listen to the barrel

\[
\begin{align*}
\text{HO} & \quad \text{OH} & \quad \text{OH} \\
\text{O=C=O} \\
\text{HO} & \quad \text{C=O} & \quad \text{OC}
\end{align*}
\]
Next Week

• Question Time

![Keep Calm](image)

![I Mustache You a Question](image)

![But I Will Give You Time to Mullet Over](image)