Enology-Grape Chemistry Group

Virginia Tech

Practical Monitoring and Management of Brettanomyces

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Presentation Outline

- Overview of Brett research from my lab
- Practical conclusions for today’s winemaking
- Review of others research
- HACCP-like Plans
- Review of practical Brett management issues

The faster the scientific advances, the greater the risk of widening the gap between what we know and what we do.

-Emile Peynand, 1984

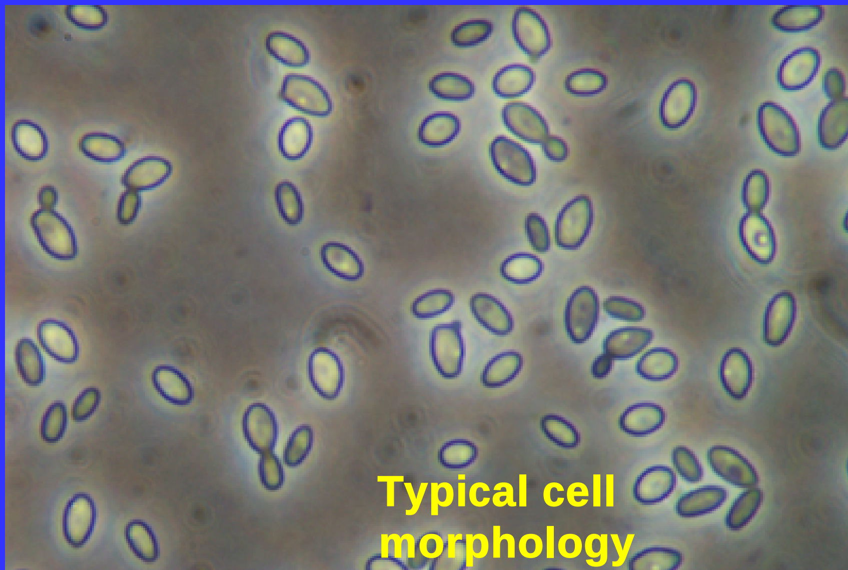
Misconceptions about Brett

- Brett usually comes in on grapes
- Some Brett is in all red wines
- Brett is not found in white wines
- Brett comes into wineries in new barrels
- Brett can only occur in barreled wines
- Brett is found only in dirty cellars
- All Brett is the same

Misconceptions about Brett

- Brett only develops in in dry wines
- Brett won’t grow over 13.5% alcohol
- Controlling oxygen can control Brett
- Brett growth always results in high VA
- Brett is a characteristic of all ‘French style’ wines

The many faces of *Dekkera/Brettanomyces*…



Source: Lisa Van de Water

Brett Descriptors

- Positive
  - Complex
  - Mature
  - Spicy
- Negative (partial list)
  - Animals
    - Sweaty horse/saddle
    - Wet dog
    - Manure
    - Barnyard
    - Mousy aftertaste
  - Plastic
    - Bandaids
    - Burnt plastic
  - Other
    - Burnt beans
    - Rancid
    - Metallic

Brettanomyces bruxellensis

- What is relationship between descriptors, cell growth and population densities?
- What are the specific chemical compounds responsible for these descriptors?
- What concentrations and ratios are need give a certain set of descriptors?
- What is the matrix/cultivar effect?
- What is the impact of strain variation?

Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir wines

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For overview see www.vtwines.info
***Enology Notes* #92, Published in Am. J. Enol. Vitic. 54:294-300**

Brettanomyces bruxellensis: Comparison of Growth Profiles and Metabolites among Ten Strains in Pinot Noir Wine

- *Question: Can differences in winemaker's experiences with* **Brettanomyces** *be attributed to strain, populations and/or metabolite differences?*

Experimental Design:

Ten genetically-characterized strains of *B. bruxellensis*

- **Pinot noir: 30 mg/L sulfur dioxide at crush. Ferment to dryness, press, clarify at 5°C (6 weeks).**
- **Rack to sterile containers, DMDC @ 700 mg/L.**
- **Bottled**
- **Initial inoculum: 50 CFU/mL (10 strains x 4 replications) + controls.**

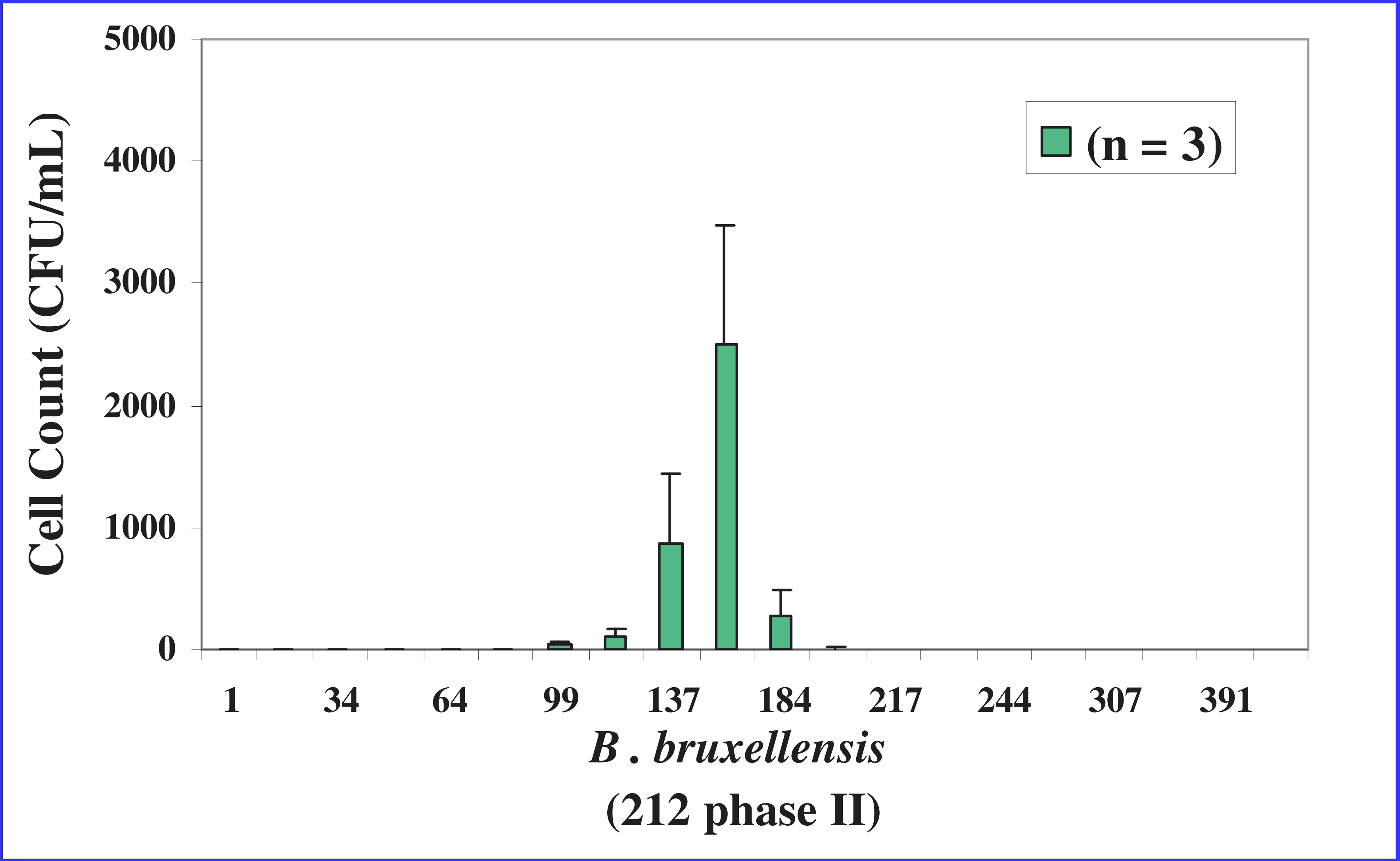
Sampling

Weekly plating for growth and chemical analysis for up to 712 days or until population declined to <30 CFU/mL.

Analyte quantification by HE-SPME, GC/MS:

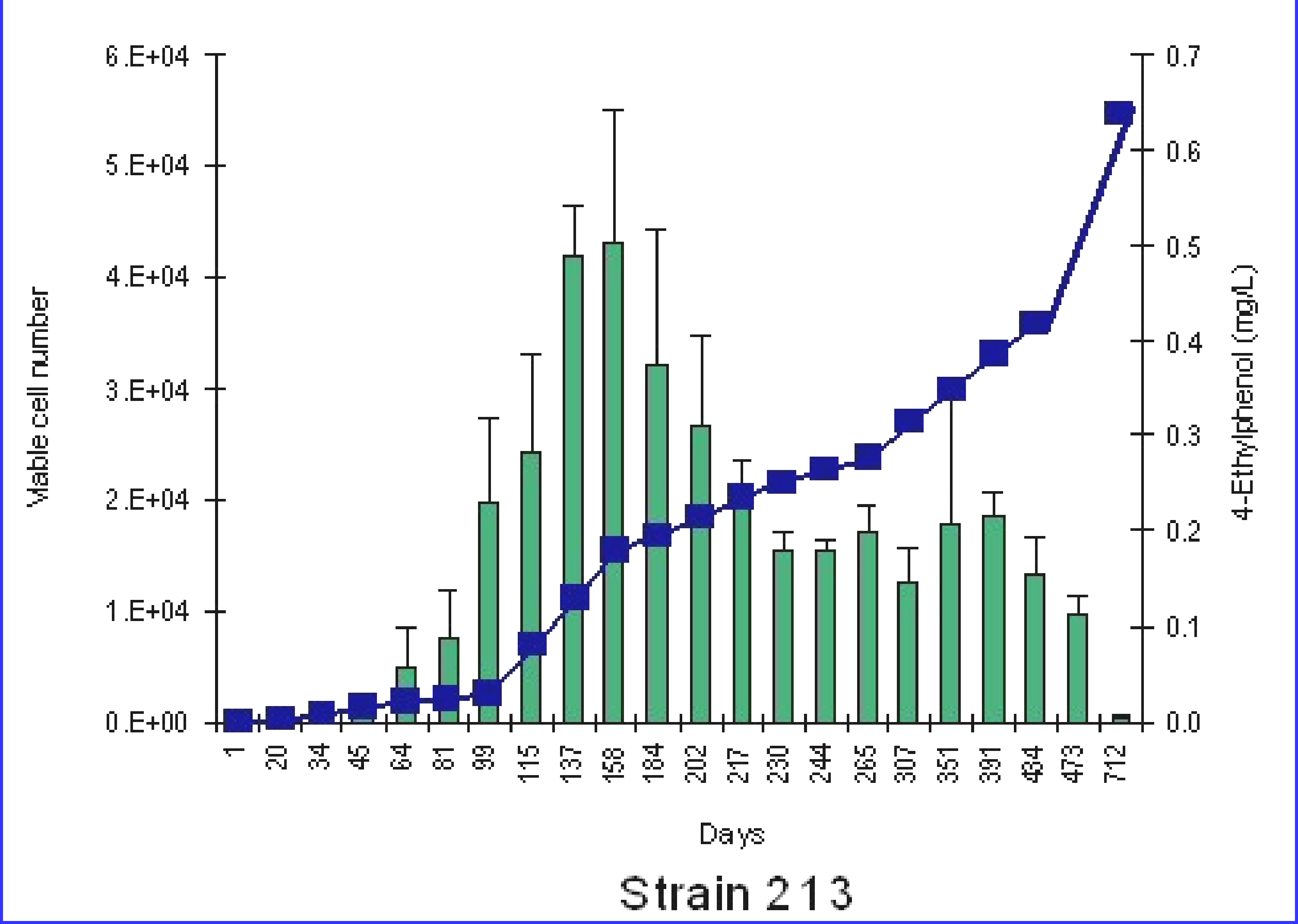
- 4-Ethylphenol (4-EP)
- 4-Ethylguaiacol (4-EG)
- 2-phenylethanol
- Guaiacol
- Isovaleric acid
- Ethyldecanoate
- trans-2-Nonenal
- Isoamyl alcohol
- Ethyl-2-methylbutyrate

Results

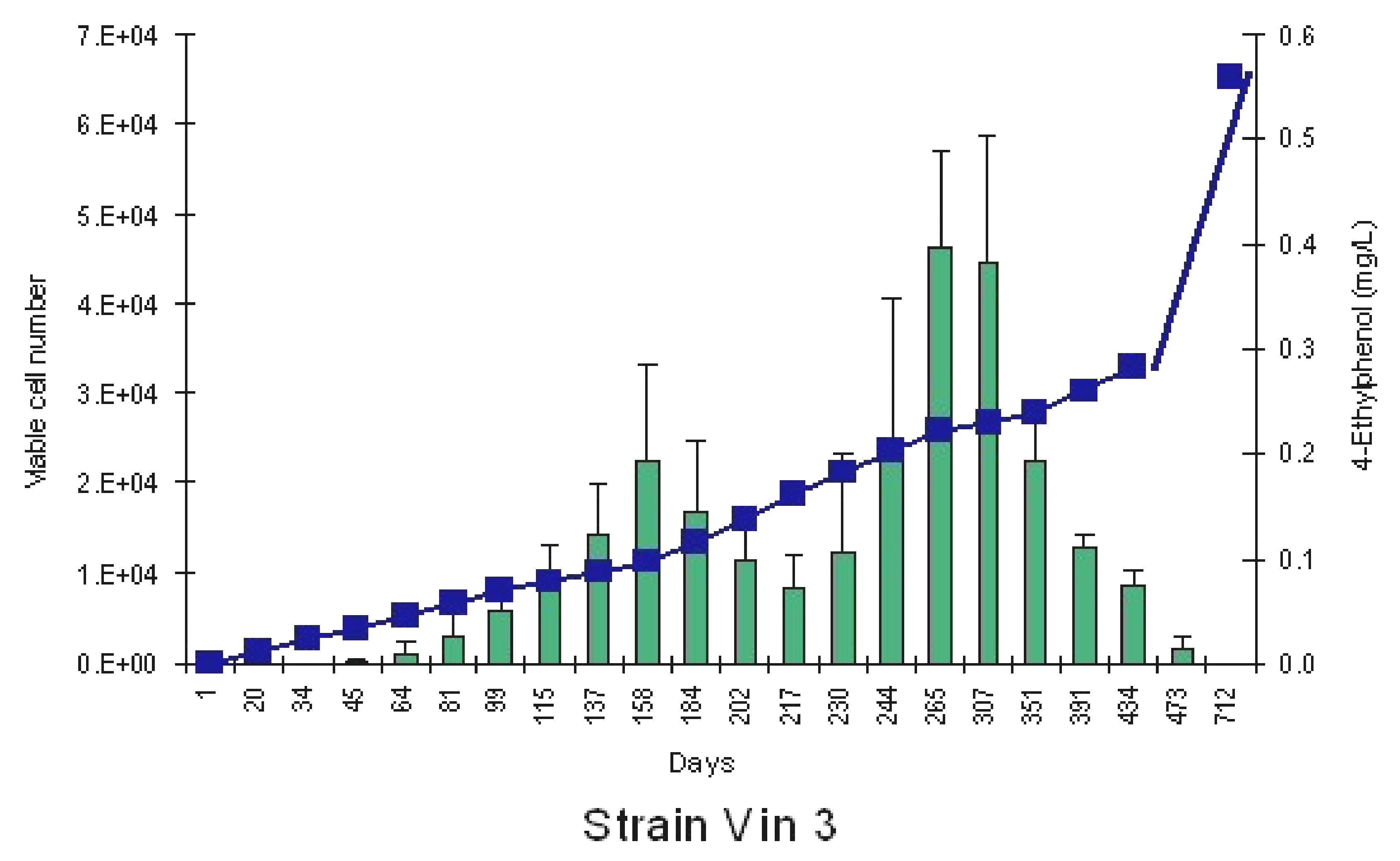


Cell Count (CFU/mL)
5000
4000
3000
2000
1000
0
(n = 3)
1
34
64
99
137
184
217
244
307
391
B . bruxellensis
(212 phase II)

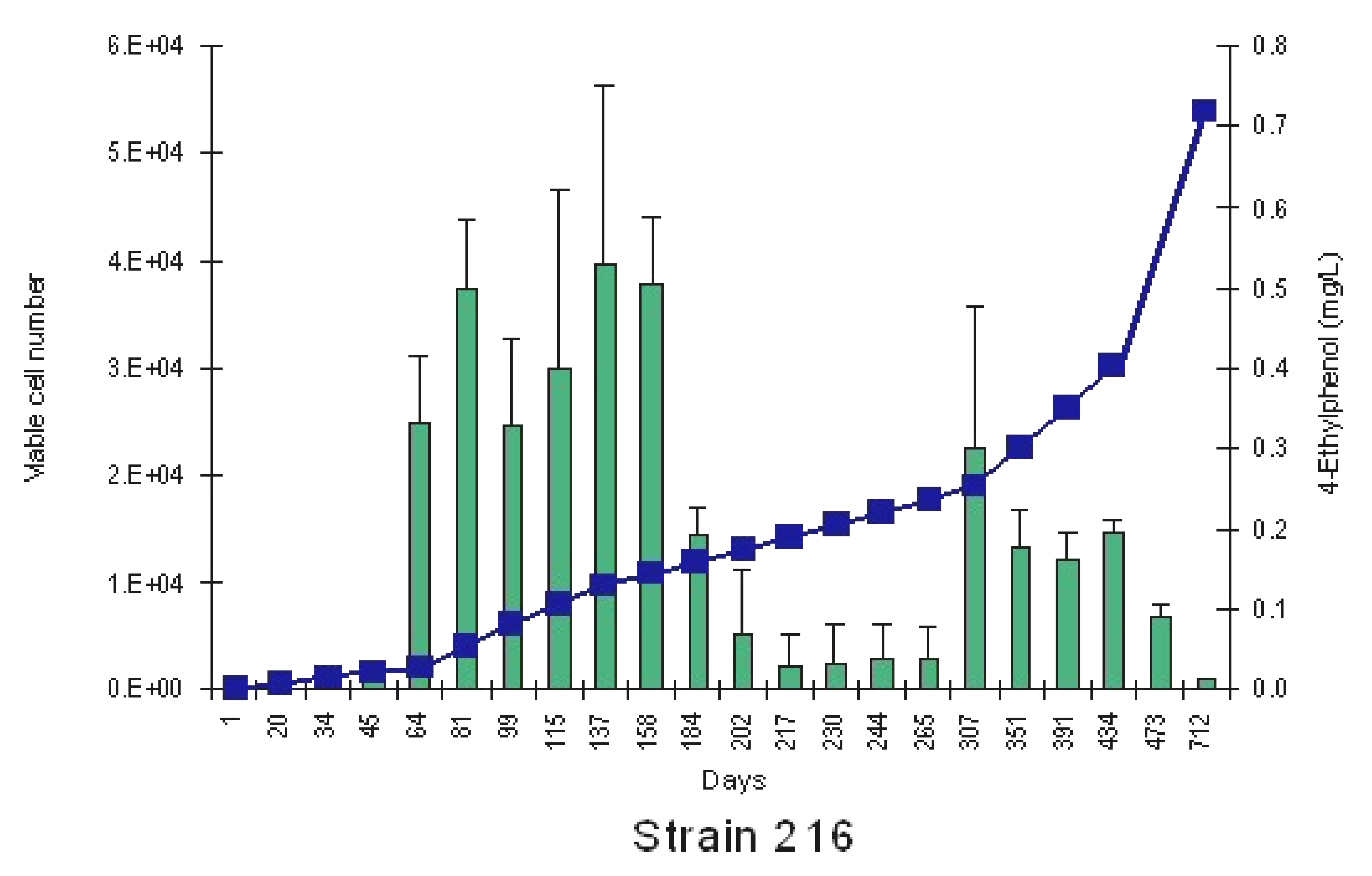
Results (cont.)



Results (cont.)



Results (cont.)



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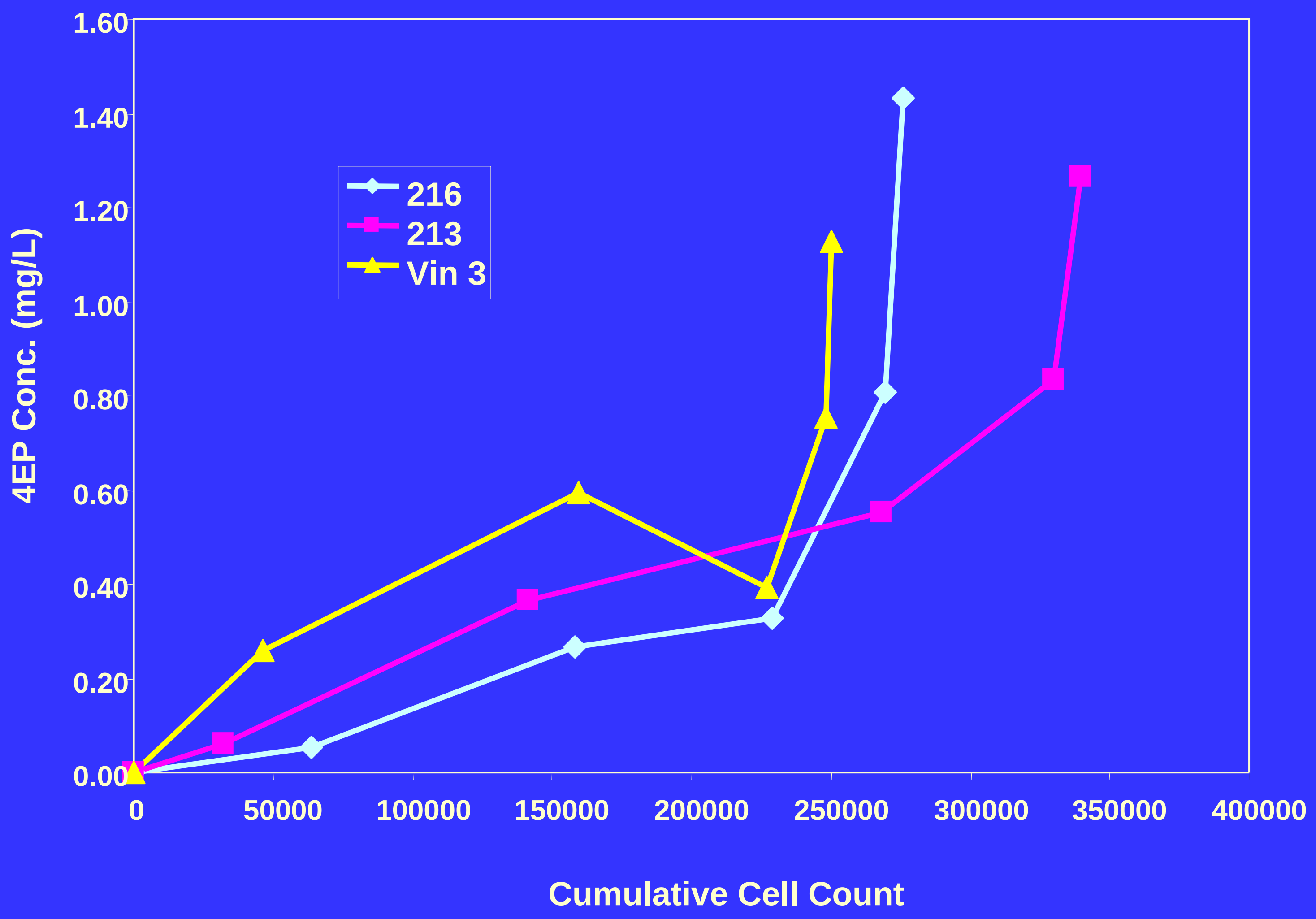
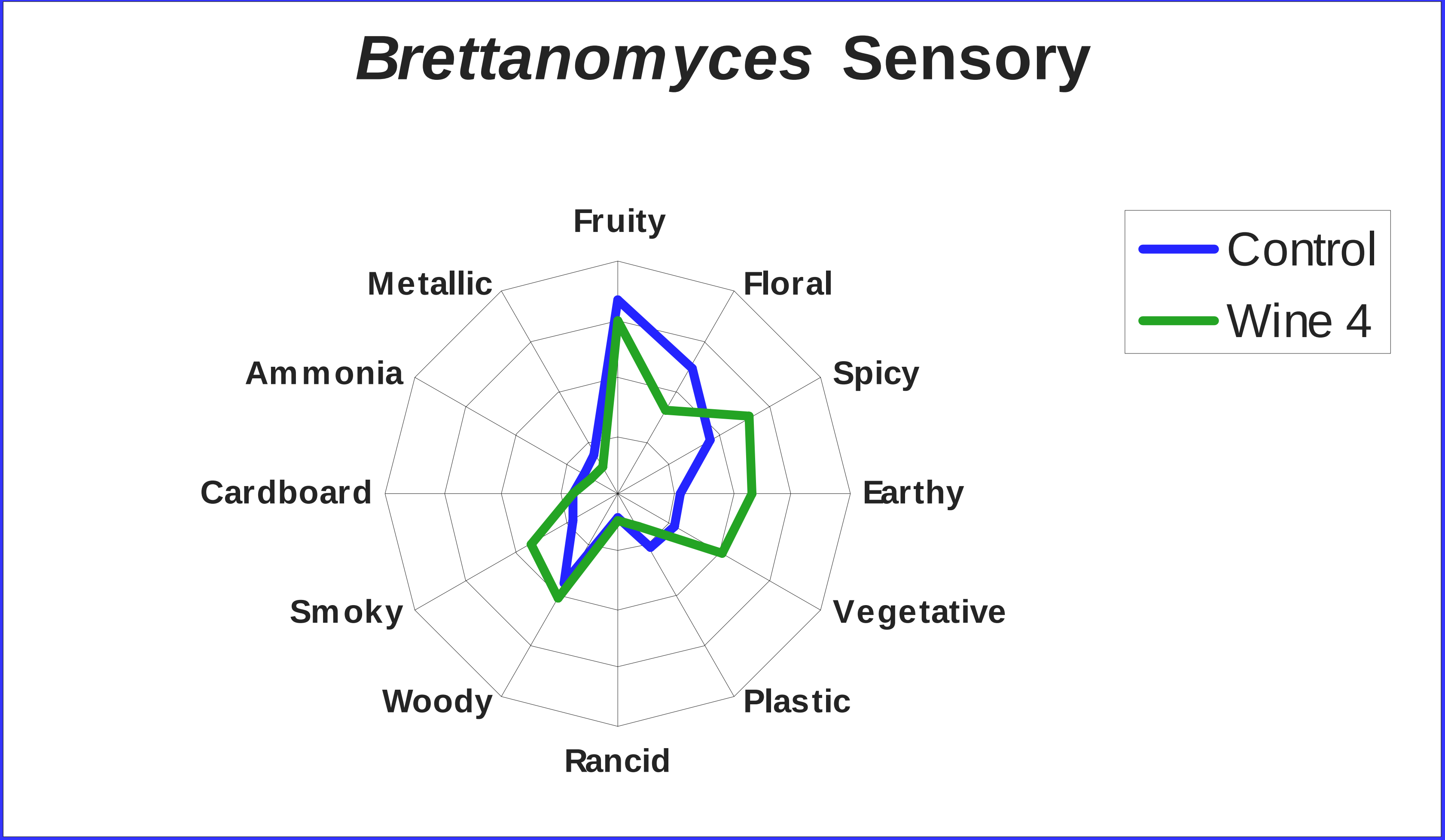
Brettanomyces

4-EP vs Cum. Cell Count, averaged



Pinot noir Sensory Evaluation



Conclusions

- Significant strain differences in length of growth cycle and peak population densities
- Blooms explained by VNC
- Large range of 4-ethylphenol (4-EP)
- Large range of 4-ethylguaicol (4-EG)
- 4-EP and 4-EG correlated
- 4-EP and 4-EG not correlated to isovaleric acid (IVA)

Conclusions

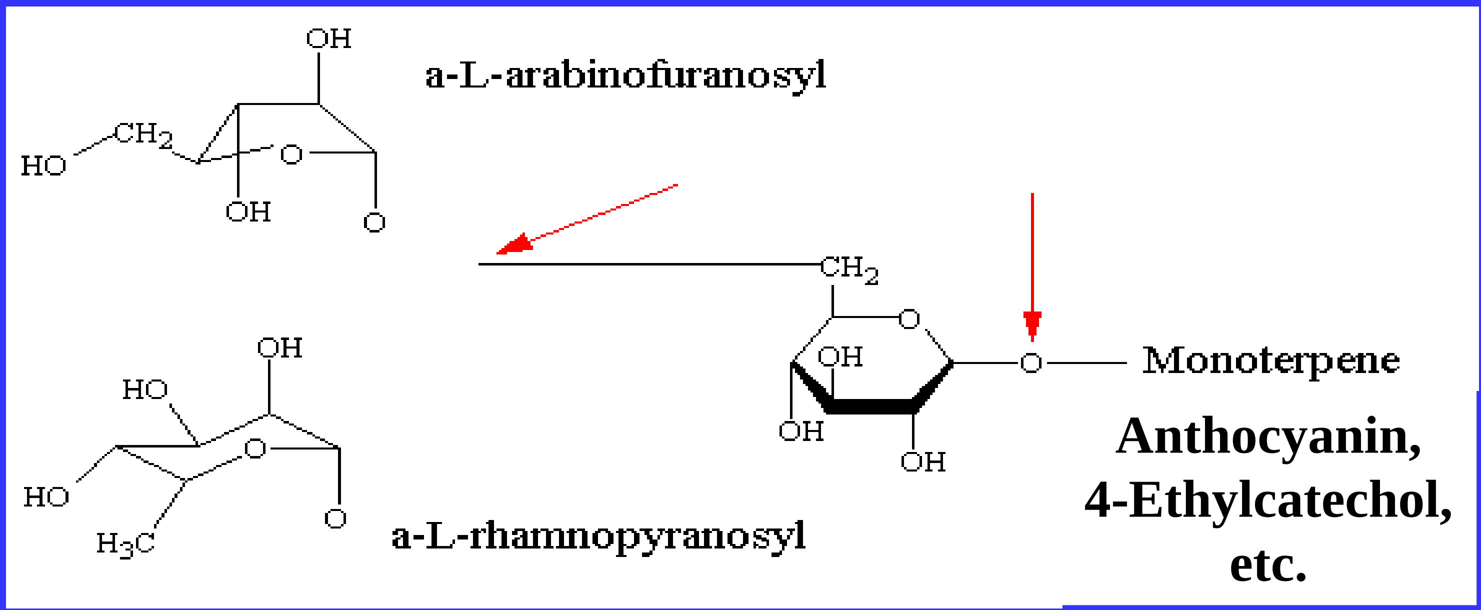
- With the exception of one strain, most 4-EP was produced <u>after</u> the population reached maximum cell density.
- The correlation between 4-EP and viable cell density was not as strong as the correlation with cumulative cell density.
- There were significant sensory differences among strains.
- 4-EP correlated to low glucose/fructose.

Viable But Not Culturable (VNC)

- Sublethally injured
  - Injury may be from any stress
    - Ethanol, pH, temperature, sulfite
  - May recover and still ferment and grow
- VNC
  - May still produce enzymes and metabolites
  - Associated with bacteria
  - Not studied extensively in yeasts

Important Enzymes: Esterases, Glucosidases

- Glycosidases
- Glucosidases



Glycosidase Activity in *Brettanomyces bruxellensis* strains

H.M. McMahon and B.W. Zoecklein.
***J. Ind. Micro. Biotech.* 23:198-203.**

A.K. Mansfield and B.W. Zoecklein.
***Am. J. Enol. Vitic.* 53:303-307.**

B. bruxellensis strain	Whole Cell	Permeabilized	Supernatant
211	27 c	142 e	11 bcd
212	5 d	341 a	9 bcd
213	34 c	105 f	14 bcd
214	19 c	110 f	6 cd
215	< LOD	74 g	11 bcd
216	59 b	321 b	24 a
Brux	26 c	182 d	11 bcd
Souche ‘Ave’	14 c	138 e	7 cd
Souche ‘O’	< LOD	< LOD	4 d
Souche ‘M’	82 a	179 d	9 bcd
Vin 1	32 c	14 g	< LOD
Vin 3	22 c	232 c	9 bcd
Vin 4	65 b	25 g	4 d
Vin 5	4 d	21 g	11 bc

Conclusions

- Large variation among strains in total enzyme activity.
- Eight strains of *Brettanomyces bruxellensis* had high *beta*-glucosidase activity (670-2,650 nM/mL/g dry cells).
- Large variation in supernatant and permeabilized activity.
- Glycosidase activity of Brett is likely how the organism can survive in oak and perhaps some wines for very long periods

Results of Physiological Tests

L. Joseph, T. Henick-Kling, L. Conterno

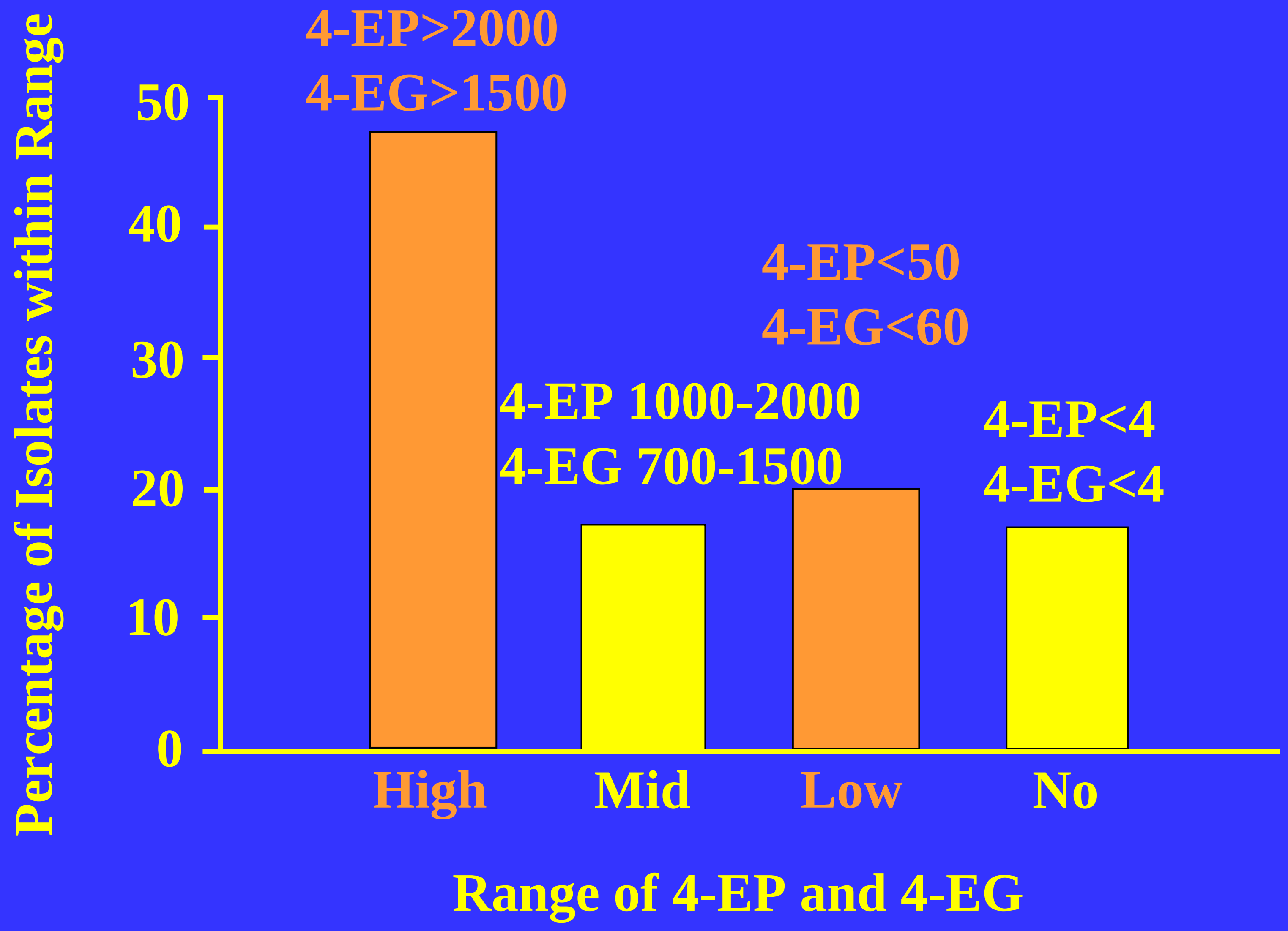
Regional differences in metabolism

- 75% of European strains used malic acid, 12% CA strains did
- All CA strains used nitrate, < 30% of European strains did
- 63% of European strains used ethanol, 18% CA strains did
- Most CA strains grew at 37 C, no European strains did

Physical Characteristics

- All isolates tolerant to 10% ethanol or higher.
- 33 isolates grew well at pH 2.
- More than 30% of isolates grew at 10^{o} C.
- More than 35% of isolates grew at 37^{o} C.
- 3 isolates (about 10%) grew at both temperature extremes.
- Almost 50% showed tolerance to 30 mg/L or greater free SO_2 at pH 3.4.

4-EP and 4-EG Production



Climate Impact on *Brett* Metabolites

Henschke (2004)

- 4-EP / 4-EG decrease in cool regions
- Malvidin-3-*p*-coumaryl glucoside may be precursor to 4-EP
- Malvidin-3-*p*-coumaryl glucoside in lower concentration in cool region, shaded fruit

Brett Growth

Physical effects

- Usually grows slowly, over many months
- Can grow within weeks if conditions are favorable
- Grows in the wine, almost never as a surface film
- Growth is stimulated by oxygen, but very little is required
- Slight CO_2 gas
- Sediment in bottle

Monitoring Brett

Methods:

- Metabolite analysis
- Sensory analysis
- Culturing
- Antibody methods
- Genetic markers: PCR, Scorpions

The key to monitoring and management is to have a good HACCP-like plan in place

HACCP: What is it?

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Hazard

Analysis &

Critical

Control

Point system

A means of assuring quality through the identification and monitoring of critical points during the production process.

HACCP-like plans

- Analysis of the dangers to product quality
- Identification and control of the critical steps in the production system
- Chemical, physical, microbiological, and/or sensory monitoring
- Verification

HACCP-like plans help to answer the following

- Why each analysis is performed
- Where the analysis fits into the scheme of quality wine production
- When results are needed
- The specific range for each result
- If the results are not within specification what to do.

7 Steps in Establishment of a HACCP-Like Plan

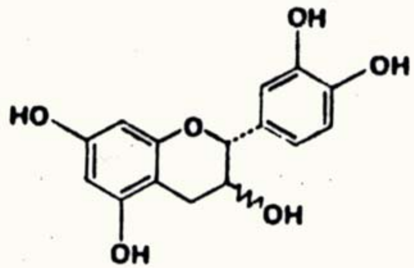
1. Create a production flow diagram
2. Identify the critical control point at each process step
3. Establish critical limits for each analysis to be conducted
4. Develop a monitoring procedure for each critical control point
5. Establish a plan for corrective action whenever critical limits are exceeded
6. Establish a record system to document action steps taken
7. Develop a verification plan for all analyses utilized

HACCP-like Plan
Step 1. Create a flow diagram

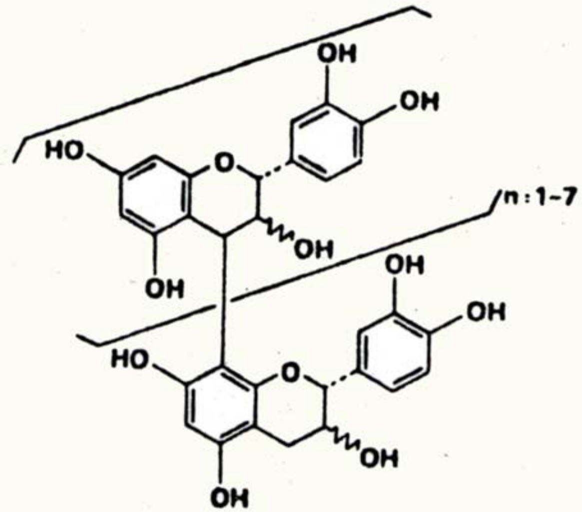
HACCP-Like Plan

Critical Control Point for Brett

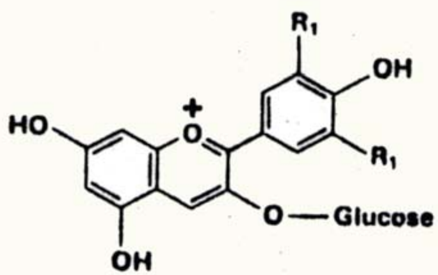
VINEYARD SAMPLING	Sanitation Monitoring
↓	
CRUSHING/PRESSING	Rot culling, temperature, SO_2, enzymes
↓	
JUICE/MUST	Sensory, ºBrix, pH, NSS, fermentable N, TA, SO_2, temp, oxygen, enzymes
↓	
FERMENTATION	Adjustment, ºBrix, pH, TA, fermentable N, Sensory, temp
↙ ↘	Yeast (strain, inoc. vol., % budding, % viability, purity)



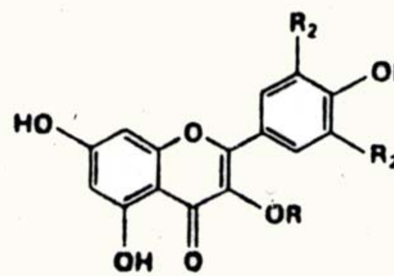
Catechins



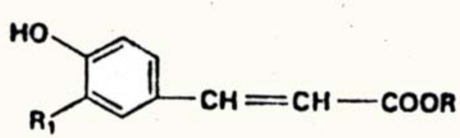
Procyanidins



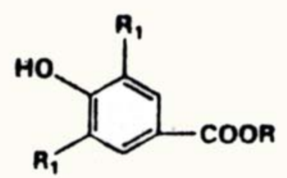
Anthocyanins



Flavonols



Hydroxy cinnamates



Hydroxy benzoates

Minimize Substrates for Growth

Measure Fermentable N (ammonia and alpha amino acids)

(Formol titration, www.vtwines.info or *Am. J. Enol. Vitic.* 53:325-329.)

Excess fermentable N:

- Lowers the production of esters
- Increases the production of aldehydes
- Increases the likely hood of volatile sulfur compound production
- Increases the fermentation rate and lose of volatiles
- Increases substrates for Brett

All Brett strains require biotin and thiamin

All can use Arginine as an N source

Excess DAP may serve as ‘food’ for Brett

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HACCP-Like Plan

Critical Control Point for Brett

VINEYARD SAMPLING	**Sanitation and monitoring**
CRUSHING/PRESSING	**Rot culling**, temperature, SO_2, **enzymes**
JUICE/MUST	Sensory, **ºBrix, pH**, NSS, **fermentable N**, TA, SO_2, temp, oxygen, **enzymes**
FERMENTATION	Adjustment, ºBrix, pH, TA, **fermentable N**, Sensory, **temp** **Yeast** (strain, inoc. vol., % budding, % viability, purity)

HACCP *Critical Control Points for Brett*

	Sanitation and monitoring
FREE RUN PRESSINGS	Sensory, tannin, and color MLF (strain, inoc. vol., purity)
COMPLETED FERMENTATION	Sensory, ºBrix, reducing sugar, pH, TA, alcohol, MLF status, oxygen, SO_2, protein stability, bitartrate stability
CLARIFICATION	Sensory, oxygen, protein stability, bitartrate stability
BBL MATURATION	Sampling, sensory, temp., sur lie, oxygen, SO_2, MLF status
BLENDING	Sensory, physical, chemical, biological stability
Pre-Bottling	Sensory, pH, micro check
BOTTLING	Sensory, fill level, SO_2, oxygen, membrane filtration micro check, DMDC, materials QC

Elements of Sensory Evaluation

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- Fully understand the objective (s)
- Evaluate representative samples
- Evaluate under proper conditions (temperature, TNSS, environment)
- Use trained evaluators with reference standards
- Minimize prejudice and bias
- Employ desirable and consistent tasting format
- Interpret results appropriately

Sensory effects of Brett

- Reduced varietal character
- Makes fruit ‘heavier’
  - Esterase activity degrades some fruity aromas
  - Floral aromas are also reduced
- Aromatic compounds
  - Masks varietal aroma and intensity
- Bitter/metallic finish
- Sometimes: mousy taint (ACPY/ACTPY)

- Lots of strain variation
- Some aroma/flavor compounds dependant on precursors produced by other yeast and bacteria

Brett Standards

- Components of FlavorSense, San Rafael, CA, *Brett* standard
  - 4-Ethylphenol
  - 4-Ethylguaiacol
  - Furfural
  - 3-methyl-2-buten–1-ol
  - Guaiacol
  - Isobutyl alcohol
  - Isobutryic acid
  - Isovaleric acid
  - Propionic acid

Brett Aromas

Sensory threshold levels depend on the matrix

- 4 EP 120-1200 ng/L
  Bandaids, Plastic
- 4 EG 70-150 ng/L
  Smokey, Spice, Burnt Beans, Medicinal
- Isovaleric Acid 1200 ng/L
  Rancid, vomit, barnyard
- Combination of these and other metabolites
  Provide the typical sweety horse, leather, horse blanket-type odors

Brettanomyces Sensory Detection

- Train to recognize danger signals using standards
  - When sensory effects are noticeable, it may be too late

- Matrix effect: cultivar, phenol composition Q and Q, metabolites:

  Tempranillo 4-EP 125, Cabernet Sauvignon 420 ug/L

- Synergistic effect on detection level:
  4-EP + 4-EG=426 ug/L
  4-EP alone=620 ug/L

❖ High 4-EP can mean High Brett character
❖ Low 4-EP can mean High Bret Character

HACCP *Critical Control Points for Brett*

	Sanitation and Monitoring
FREE RUN → COMPLETED FERMENTATION; PRESSINGS → COMPLETED FERMENTATION	Sensory, tannin, and color MLF (strain, inoc. vol., purity)
COMPLETED FERMENTATION	Sensory, ºBrix, reducing sugar, pH, TA, alcohol, MLF status, oxygen, SO_2, protein stability, bitartrate stability
CLARIFICATION	Sensory, oxygen, protein stability, bitartrate stability
BBL MATURATION	Sampling, sensory, temp., sur lie, oxygen, SO_2, MLF status
BLENDING	Sensory, physical, chemical, biological stability
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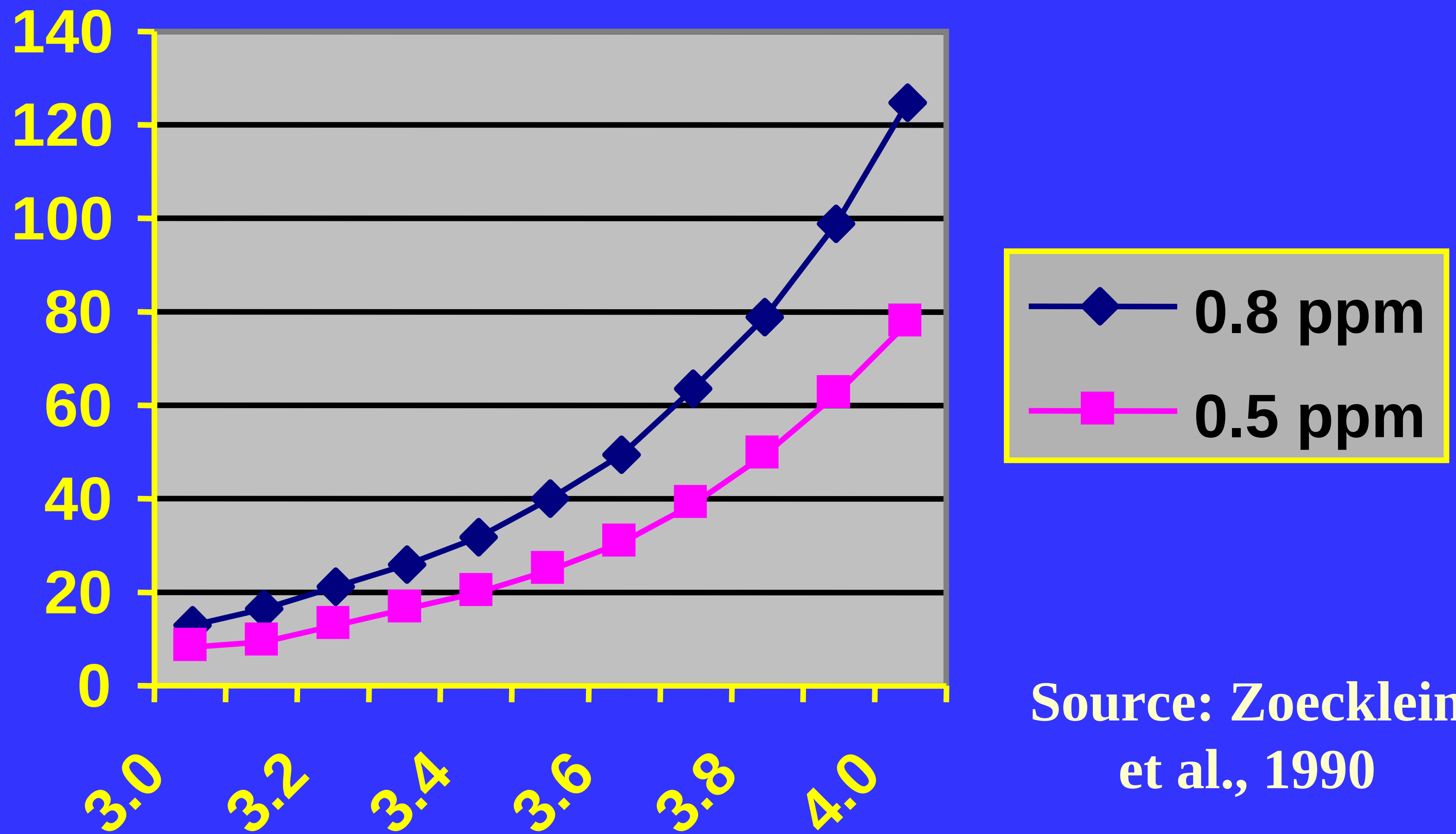
Wine Chemistry and Brett

- Alcohol synergistic but will not control
- Glucose and Fructose: 0.275 g/L = 1000 ug/L 4-EP
- VA concentration not correlated with 4-EP
- pH effects molecular FSO2
  - Biofilm formation
- Sulfur dioxide and pH synergistic
  - Fewer additions but larger concentration

Free SO_2 Needed to Achieve 0.5 and 0.8 ppm Molecular SO_2, at Different pHs



Source: Zoecklein et al., 1990

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HACCP *Critical Control Points for Brett*

Sanitation and Monitoring

FREE RUN / PRESSINGS	Sensory, tannin, and color MLF (strain, inoc. vol., purity)
COMPLETED FERMENTATION	Sensory, ºBrix, reducing sugar, pH, TA, alcohol, MLF status, oxygen, SO_2, protein stability, bitartrate stability
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BBL Maturation

- Old wood vs. new wood
  - cellobiose
  - 0.275 g/L can produce 1000 ug/L 4-EP
- Sampling
  - representative
  - avoid cross contamination
  - use disposable plastic pipetts
  - top with ‘clean’ wine (DMDC-**Velcorin** treated or filtered)

Brett and Sanitation

- Monitoring is key
- Understand differences between cleaning and sanitation
- Sanitation methods

Effect of Barrique Sanitation Procedures -

Manuel Malfeito-Ferreira, 2004

- Barrel sanitation experiment
  - Cold rinse, then hot water rinse 3x 70 C
  - Same as above plus SO2 1 month (200 ppm pH3)
  - Cold rinse, fill with 90 C water 15 min
  - Cold rinse, 70 C rinse, steam low pressure 10 min
    - Most effective treatment

- *Brett / Dekkera* was found 8 mm deep in staves.

Barrels cannot be "sterilized" with SO_2, rinsing, or ozone.

Isolate *Brett*+ barrels.

Ozone Treatment

- High-pressure water wash barrel
  - Thorough blast with sharp stream of hot water
  - Rinse for 2-3 minutes
  - Must remove all organics
  - Cool down completely
- Treat with ozonated water
  - Filter and deionize water before ozonating
  - At least 2-2.5 mg/L ozone in barrel, 0.1 mg/L out
  - Time x Concentration

Ozone Summary

- Strong oxidizing agent
- No chemical residue
- Half-life at ambient conditions 10-20 minutes
- Degrades microbial bio-films
- Degrades natural rubber
- Is a surface active agent-does not penetrate

Brett and Biofilms

- Liquid / solid interface
- 17 / 35 strains form biofilms (Joseph, 2004)
- pH effect
- Impact of cleaning compounds on biofilms

HACCP *Critical Control Points for Brett*

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Sanitation and Monitoring

FREE RUN, PRESSINGS	Sensory, tannin, and color MLF (strain, inoc. vol., purity)
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BOTTLING	Sensory, fill level, SO_2, oxygen, membrane filtration micro check, DMDC, materials QC

Wine Bottling and Brett

- Sanitation
- Monitoring
- Filtration and filtration monitoring
- DMDC can be effective
- Synergistic with pH, sulfur dioxide, and alcohol
- Oxygen pick up

Monitoring *Brett*

- Have a HACCP-like plan (www.vtwine.info)
- Isolate contaminated barrels
- Sample barrels with disposable plastic pipets
- Top with *Brett*-free wine (filtered, pasteurized and/or Velcorin-DMDC)
- Keep barrels topped-up or not opened
- Monitor carefully before bottling

HACCP Summary

- Define the production process, quality/style indicators, and their recommended values.
- Identify critical control points in the process where specific chemical methods can monitor quality indicators.
- Establish and carry out analysis methods that will give measures of quality/style indicators at each control point.
- Compare measured values with recommended values.
- Decide on action to modify any quality deficiencies.
- Carry out that action.
- Assess the result of that action by further analysis.

Encourage/Discourage *Brett*

- To ENCOURAGE

  Wine composition
  - Red wine
  - pH > 3.6
  - Molecular SO_2 < 0.2 mg/L
  - Alcohol 13% or below
  - Residual hexose sugars
  - Biotin, thiamine
  - Amino acids
  - Yeast lees present

- To DISCOURAGE

  Wine composition
  - White wine
  - pH < 3.6
  - Molecular SO_2 0.4 mg/L or greater
  - Alcohol > 13%
  - RS < 0.2 g/L
  - Vitamins depleted

    "Nutrient desert"
  - Clarified

Encourage/Discourage *Brett*

- To ENCOURAGE
  <u>Winemaking operations</u>
  - Temperature 25-30 C
  - Oxidative conditions
  - New barrels
  - Poor sanitation
  - Cross-contamination
  - Barrels washed in cold water
  - No aggressive barrel sanitation

- To DISCOURAGE
  <u>Winemaking operations</u>
  - Temperature < 16 C
  - Keep containers topped / closed
  - Older but uninfected barrels
  - Good hygiene
  - Keep infected wine separate
  - High-pressure hot water wash
  - Ozone/burn sulfur wick in barrel

Brettanomyces Detection

- Direct Microscopic Examination
  - Difficult when < 1000 cells/ml
  - Requires skill in identifying cells
- Culturing
  - Sampling method is very important
  - Detects only microbes that are present and alive
  - Disadvantages:
    - Must select and prepare media properly
    - False negatives (VNC)
    - Takes time for growth (3-7+ days)
    - Requires skill in identifying colonies