The Identification and Characterization of Indigenous Yeast in a Chardonnay Fermentation of an Ohio Winery

By

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The Identification and Characterization of Indigenous Yeast in a Chardonnay Fermentation of an Ohio Winery

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Abstract

The Identification and Characterization of Indigenous Yeast in a Chardonnay Fermentation of an Ohio Winery

The identification and characterization of indigenous yeast populations during the initial eight days of spontaneous fermentation of an Ohio Chardonnay must were examined and compared to a controlled inoculated fermentation in two consecutive harvest seasons, 2000 and 2001. Identification of the indigenous yeasts were determined by physiological tests, whereas strain succession was partly assessed by electrophoretic karyotyping. It was hypothesized that there is a difference in the yeast species present based on the type of fermentation. Based on the results of the physiological tests, three yeast species were identified in the spontaneous fermentation during consecutive harvest seasons. During both 2000 and 2001 seasons, *Hanseniaspora uvarum* was the predominant yeast species with few *Pichia membranaefaciens* present as well, in fermentation days one through five. As expected, with an increase in alcohol content, the indigenous yeasts did not survive, instead the more alcohol tolerant yeast, *Saccharomyces,* dominated and completed the fermentation process. Colony Forming Units (CFUs) were determined for both harvest seasons by performing serial dilutions and plating in triplicate on Sabouraud agar plates. Significant differences existed in yeast populations between the 2000 and 2001 fermentations regardless of type ($p=0.0001$, Multiple Regression Analysis). The yeast populations in the 2000 inoculated fermentation ranged from 10^6 to 10^8 per ml in the initial week followed by a decline in numbers to 10^3 per ml by the end of weeks two and three. By comparison, the 2001 inoculated fermentation ranged from 10^4 to 10^8 per ml in week one, followed by a gradual decrease to 10^3 per ml upon completion of fermentation. The spontaneous 2000 fermentation indicates an increase from 10^6 to 10^8 CFU per ml in the first week and remained in that range through the second followed by a gradual decline to approximately 10^6 CFU per ml upon completion of fermentation. In contrast, yeast populations during the 2001spontaneous fermentation increased from 10^4 to 10^8 CFU per ml in the first week. A subsequent decrease in numbers occurred through weeks two through four with a final CFU number of approximately 10^5 CFU per ml. Electrophoretic karyotyping of select samples of both harvest season indicates variability within the fermentation types and year to year. Although the same three yeasts were present in the spontaneous fermentation of both harvest seasons, these data suggest there is year-to-year variability in both spontaneous and inoculated fermentation.

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I. Past and present research of yeast in wine production

Wine production using spontaneous fermentation is believed to date as far back as 6000 BC (Pretorius 2000). Fermentation was not fully understood until 1863 when Louis Pasteur discovered that yeasts play a key role in the fermentation of wine by converting the grape sugars into alcohol and carbon dioxide. Basing his work on that of Antonie van Leeuwenhoek, Pasteur was able to determine that yeast was the necessary catalyst in wine fermentation. By 1890, Müller-Thurgau discovered that the flavor of the wine could be controlled by inoculating grape-must with yeast (Pretorius 2000). Despite improvements in wine production by this discovery, there remain two schools of thought in winemaking; those that continue to use traditional method of spontaneous fermentation versus those that inoculate with commercially produced yeast.

What continues to be examined today is not only the role yeasts play in winemaking, but also the identification of the indigenous yeasts present during various stages of wine fermentation. Many studies have been conducted in wine producing countries throughout the world to identify the indigenous yeasts present and the effect they have on the aroma and flavor of the wine. Studies conducted in New South Wales, Australia (Heard and Fleet 1985), Spain (Fernandez et al 1999; Guillamón et al 1998; Torija et al 2001), Wädenswil, Switzerland (Schütz and Gafner 1994), France (Frezier and Dubourdieu 1992) and the United States (Egli et al 1998; Cocolin et al 2000) have identified various indigenous yeasts present in spontaneous fermentation. These yeasts include *Hanseniaspora uvarum, Kloeckera apiculata, Pichia membranaefaciens, Candida stellata, Hansenula anomala, Cryptococcus* spp.*, Torulaspora delbrueckii* and *Saccharomyces* spp., with the prevailing yeast during the latter stages of fermentation

being *Saccharomyces cerevisiae*. The prevalence of *S. cerevisiae* is due to its ability to tolerate high levels of alcohol (Soden et al 2000). However, the diverse population of indigenous yeasts present in the initial stages of fermentation, not able to survive the increase in alcohol content, are believed to play a crucial role in the aroma and flavor of the wine (Fraile et al 2000; Fugelsang 1997; Guillamón et al 2000; Heard and Fleet 1985; Pretorius 2000).

Physiological tests have been used over the course of many years to identify organisms present in fermentation. As an aid in identification, physiological tests can distinguish the organism's ability to utilize different carbon and nitrogen compounds. However, many of these tests are time consuming and the results may be ambiguous. Therefore molecular techniques have been developed to further aid in the identification process of organisms. One such method, electrophoretic karyotyping, separates yeast chromosomes by Pulse Field Gel Electrophoresis (PFGE) (Birren and Lai 1993). In this method, the orientation of the electric field changes periodically and allows separation of the larger yeast chromosomes (Birren and Lai 1993). Conventional gel electrophoresis permits separation of small DNA up to 20 kilobases (Kb) in size to move through the gel matrix in a snake-like movement because of the differences in size and charge. In PFGE, separation of the larger coiled DNA fragments of up to 12 megabases (Mb), is established by alternating angles of electric pulses thus permitting the large DNA to move though the gel matrix (Birren and Lai 1993). This allows comparisons of yeasts chromosomes to determine if variability exists.

Several studies have examined the chromosome DNA patterns of typical yeasts found in fermentation. PFGE results obtained from Naumov et al (2000 and 2001),

examined the chromosome DNA patterns of the *S. bayanus* and *S. cerevisiae* while Cadez et al (2002), used various molecular techniques, including PFGE in the assessment of genetic diversity within species of the genera *Hanseniaspora* and *Kloeckera*.

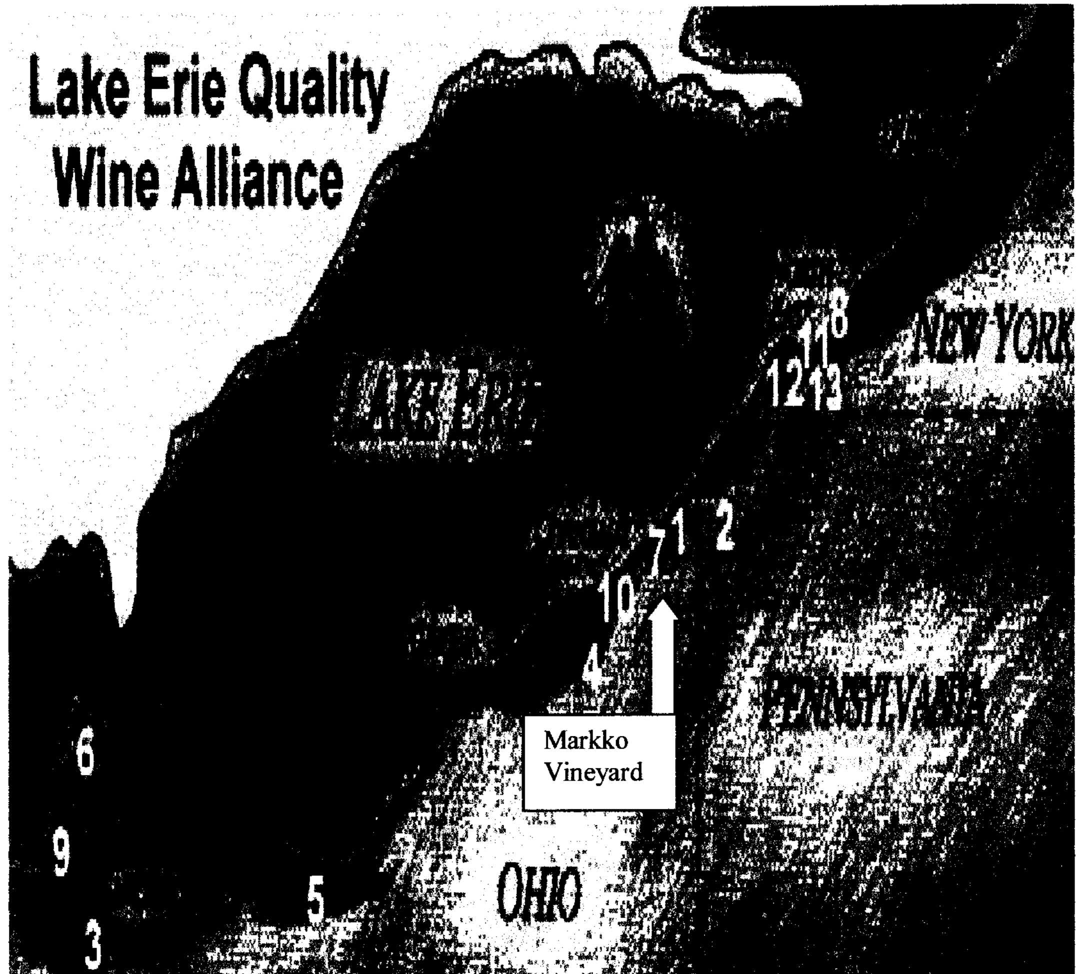
III. Ohio Wine Region

According to Arnie Esterer, owner of Markko Vineyard, initially there was much controversy when winemakers chose Ohio as a winemaking region. Harsh winters were thought to be detrimental to the survival of the vine and soil composition was believed to be less than ideal (personal communication, 2000). In the late 1800's, the rainy conditions of Ohio paired with humidity was thought to be the cause of "rot and mildew" (Riesen 1994) which enhanced the negativity surrounding Ohio as a winemaking region. However, today there are over 60 Ohio wineries producing quality wines. Thirteen wineries, located along the shores of Lake Erie (Fig. 1) benefit from lake effects. The lake's heat retention not only extends the fall growing season but offers protection to the vines located near the shore line from the low temperatures of harsh winters. Fungal disease of the vines, once thought to be a result of the climate, are in fact prevented as a result of a continuous breeze caused by the temperature difference between lake and land (Lake Erie Quality Wine Alliance 2002).

Fig. 1: Winery locations of the Lake Erie Wine Region. Site of this study, Markko Vineyard (#7), shown with arrow. Map provided by the Lake Erie Wine Alliance website, 2002.

Legend:

1. Buccia Vineyards
2. Conneaut Cellars Winery
3. Firelands Winery
4. Harpersfield Vineyard
5. Klingshirn Winery
6. Lonz Winery
7. Markko Vineyard
8. Mazza Vineyards
9. Mon Ami Restaurant & Historic Winery
10. Old Firehouse Winery
11. Penn Shore Vineyards
12. Presque Isle Wine Cellars
13. Arrowhead Wine Cellars


Lake Erie Quality
Wine Alliance
NEW YORK
PENNSYLVANIA
OHIO
Markko
Vineyard
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13

IV. Project Introduction

This project was among the first to examine the indigenous yeasts present during the fermentation process in Ohio wines. In conjunction with Cleveland State University (CSU), wines from Harpersfield Vineyard and Markko Vineyard in the Lake Erie region were studied.

The Markko Vineyard was the site of collection for the Youngstown State University (YSU) research team. Markko Vineyard is located in Conneaut, Ohio approximately 60 miles north of YSU (Fig. 1). This winery was chosen for its locality and the reputation of its wines. In addition, the vintner, Arnie Esterer, was enthusiastic about the project. The results of this study may help him produce even better quality wines.

The YSU research team has identified the indigenous yeasts present during the initial 8 days of a spontaneous fermentation of Chardonnay must of Markko Winery during two consecutive harvest seasons by physiological methods. Morphological characteristics and physiological properties were used to identify the yeasts present during a spontaneous fermentation, and strain typed by PFGE.

Based on previous studies conducted throughout the world, common species of indigenous yeasts identified during the initial stages of fermentation are *M. pulcherrima, C.stellata, H. uvarum, T. delbrueckii* and *P. membranaefaciens* with *Saccharomyces sp.* present during the latter stages of fermentation (Guillamón et al 1998; Schütz and Gafner 1994; Frezier and Dubourdieu 1992; Egli et al 1998; Cocolin et al 2000).

It was hypothesized there was a difference in the yeasts present depending on the type of fermentation.

Goal: The goal of this project was to study the indigenous yeasts during the initial stages of a spontaneous fermentation of a Chardonnay grape must and to isolate and identify those yeasts present.

Objectives:

1. Isolate, identify and quantify indigenous yeasts present during both a spontaneous fermentation and an inoculated fermentation.
2. Obtain representative isolates of the yeasts, identify by morphological and physiological characteristics and strain type by using PFGE.
3. Determine the variation between different types of fermentation.

Methods

I. Study design

Several Ohio wineries were chosen by CSU and YSU to examine indigenous yeasts in wines based on their locality and high quality products. The YSU research team identified the indigenous yeasts present during spontaneous fermentation of Chardonnay must at Markko Vineyard during two consecutive harvest seasons and compared to those in an inoculated fermentation.

Fermentation samples were collected daily for 10 days from a spontaneous fermentation of Chardonnay grape must at Markko Vineyard, then once every four days until the sugar content was 0.2 %. An additional spontaneous and inoculated fermentation from the same grape must was conducted in triplicate at YSU. The must for the inoculated fermentation was treated with 25 ppm metabisulfite and allowed to settle overnight then inoculated with 1g/gal commercial Premier Cuvée® *Saccharomyces bayanus*. The fermentations conducted at YSU were sampled in the same manner as the fermentation at Markko Vineyard. Each fermentation sample was plated in triplicate from 4 dilutions to determine the abundance of yeasts. Colony counts from each of the spread plates with numbers less than 200 yeast colonies were performed. Plates with numbers greater than 200 may lead to inaccuracies and were therefore not counted. Five representative isolates were selected based on phenotypic differences observed from each day and type of fermentation then were plated on Sabouraud agar plates. The isolated yeasts were identified based on physiological methods and strain typed using PFGE.

II. Sampling methodology

The fermentation at Markko Vineyard was carried out in 55 gallon oak barrels, whereas paralleled experiments were conducted in 9-liter Pyrex ® glass containers at the YSU laboratory. Sampling required removing 1 ml of grape must from the fermentation carboys using a sterile pipette and mixing it with 99 ml of sterile water. Fermentation samples collected from Markko Vineyard were cooled to 0°C during transportation to the lab. Fermentation temperature and soluble solid levels were recorded at the time of sample collection. Soluble solids, also referred to by winemakers as °Brix, are measurements that determine the level of residual sugars during the fermentation which also allow the winemaker to predict the percent alcohol content of the final product. As yeast numbers increase and utilize grape sugars as a source of energy, alcohol is produced and a decrease in °Brix level is expected.

III. Yeast Colony Isolation

A series of 4 dilutions (10^{-2} through 10^{-6}) of the fermentation samples were plated in triplicate on Sabouraud agar plates to determine colony-forming units (CFU). Spread plates of each diluent were incubated at 25 °C until sufficient growth was observed. Colony forming units were determined by counting the number of yeast colonies present on the agar plate using the Bantex 900A colony counter and the Eagle Eye ™ II Imaging System (Stratagene). Representative isolates from each sample were chosen according to phenotypic characteristics such as texture, size and color then plated individually on Sabouraud agar plates. These cultures were incubated at 25 °C until sufficient growth was observed, at which time the cultures were maintained at 5 °C.

Samples were identified by the date the sample was collected, fermentation carboy (S= YSU spontaneous, C= YSU control, F7= Markko spontaneous), replicate number (1-3) and assigned an alphabetical identity (A-D). For example, a sample identified as 10-15-00 S1-A, was collected from the spontaneous fermentation replicate 1 (S1) on 10-15-00 and was the first isolate chosen (S1-A). A sub sample of each culture was preserved at -20°C (Appendix A). Growth curves were generated and compared from colony counts of each fermentation treatment for two consecutive harvest seasons.

IV. Physiological Tests

A series of physiological tests (Fugelsang 1997; Kurtzman and Fell 1998; Barnett and Pankhurst 1974) were performed to identify the yeasts present in the initial eight days of fermentation. Isolated colonies were washed twice (Fugelsang 1997) to remove bacteria that may interfere with biochemical testing and incubated at 25 ° C until growth was observed.

Carbon and nitrogen assimilation tests were used to determine the yeast's ability to utilize the compounds. The results were compared to a taxonomic key (Kurtzman and Fell 1998). The carbon assimilation method (Fugelsang 1997) required Yeast Nitrogen Base (YNB) prepared following the manufacturer's instruction and combined with agar at 2% w/v, then autoclaved. The YNB agar preparation was equilibrated in a water bath of 44°C. Once the temperature was within 5 °C of the target temperature, the unknown yeast was suspended in 3-ml distilled water. The yeast suspension was poured into the center of a sterile petri dish followed by the molten YNB medium in a swirling motion to disperse the suspension and agar. Upon solidification, 5 mg of various carbon

compounds were placed on the center of individual agar plates, using sterile practices and incubated at 25 °C. The plates were examined after 3-7 days for carbon utilization, indicated by turbidity around the carbon source.

The nitrogen assimilation method required the use of Yeast Carbon Base (YCB) and agar prepared in the same manner as the carbon assimilation method previously described. Various nitrogen sources were placed on individual agar plates and incubated at 25 °C. Plates were examined after 3-7 days for nitrogen utilization, indicated by turbidity around the nitrogen source.

V. Electrophoretic Karyotyping

Samples from the 2000 and 2001 harvest seasons were selected based on identification through physiological tests, then strain typed by Pulse Field Gel Electrophoresis (PFGE). Comparisons of known yeasts were made to select samples.

Agarose yeast seeded plugs were made using the BioRad CHEF Genomic DNA plug kit (BioRad)®. Specifically, one colony of selected yeast was grown in 50 ml of YPD (Yeast Peptone Dextrose) broth overnight with agitation at 30 °C. The cells were collected in 50 ml conical tubes by centrifugation at 5K for 10 minutes at 4°C. The supernatant was removed and the cells re-suspended in 10 ml cold 50mM EDTA. Cell concentration was determined by counting a 1:100 dilution of cells in a hemocytometer (See Appendix B for hemocytometer usage) at 400x power. The concentrations of cell suspension, Cell Suspension Buffer and Cleancut Agarose were based on 1.0 ml of plugs to be made (See Appendix B for calculations*). The Cleancut Agarose was microwaved and equilibrated in a waterbath at 50 ° C. Based on calculations for cell concentration,

$6x10^8$ cells were removed and centrifuged at 5k for 10 minutes at 4°C. The cells were re-suspended in Cell Suspension Buffer and equilibrated to 50 °C. Lyticase stock was added to Cell Suspension Buffer followed by an addition of Cleancut Agarose maintaining the mixture at 50°C. The mixture was transferred to plug molds using sterile transfer pipettes and allowed to solidify. The solidified plugs were then pushed into a 50 ml conical centrifuge tube containing Lyticase solution and incubated for 2 hours at 37 °C. The Lyticase solution was then removed and the plugs rinsed with sterile water. Proteinase K Reaction Buffer was then added followed by an addition of Proteinase K stock. The plugs were incubated overnight at 50 °C without agitation. After overnight incubation, the plugs were washed four times in 10 ml of 1X Wash Buffer for 1 hour each at room temperature with gentle agitation. A dilution of 1:1000 of 1mM PMSF to Wash Buffer was added during the third wash to inactivate the residual Proteinase K in the event the plugs are to be used in subsequent enzymatic reactions. A 1.0 % gel for Pulse Field Gel Electrophoresis (PFGE) was prepared by placing 100 ml of 0.5x TBE in an Erlenmeyer flask with 1g/100ml agar (PFGE grade agar III, Amresco®) and covered to prevent loss of water from evaporation. An initial weight of flask with lid and solution was determined and heated until the agar dissolved completely. A final weight of the flask with lid and solution was determined and enough sterile water added until the initial weight and final weight were equal. The agar solution was then equilibrated at 50 °C. The plugs were placed on gel comb then placed in the gel mold. The equilibrated agar was gently poured into mold and allowed to solidify. A volume of 2000ml of 0.5X TBE (Tris-borate-EDTA) Buffer was poured into CHEF Mapper™ system and allowed to cool to 14 °C. The solidified gel was equilibrated in the cooled

buffer in the CHEF Mapper™ system for approximately 10 minutes prior to beginning the run. The gel was subjected to PFGE for 24 hours at 6V/cm with an initial switch-time of 60 seconds, a final switch-time of 120 seconds and an angle of 120°. Upon completion, the gel was stained in ethidium bromide and 0.5X TBE for 30 minutes, then examined under ultraviolet light in the Eagle Eye ™ II Imaging System (Stratagene). Ethidium bromide inserts between the DNA bands allowing for the chromosomal bands of the known yeasts to be viewed under ultraviolet light and compared to the fermentation yeasts sample.

VI. Statistical Analysis

To perform Multiple Regression Analysis using the SAS statistical program, it was necessary to log (ln) transform the CFUs [ln (CFU number)] to account for non-normal distribution of the data (Kleinbaum et al, 1998). The median number of CFUs per day of each fermentation (triplicate spontaneous and triplicate control of each harvest season) type was used to statistically analyze these data.

Four separate Multiple Regression Analyses were performed to determine if differences existed in CFUs of each fermentation type within a harvest season and differences from year to year of each fermentation type. For each analysis the dependent variable is (ln) CFU. The first regression was to determine a difference between spontaneous and controlled fermentation in the 2000 harvest season. The second analysis was to determine a difference between spontaneous and controlled fermentation in the 2001 harvest season. The third analysis was to determine the differences in the spontaneous 2000 versus spontaneous 2001 fermentations. The fourth and final analysis

Results

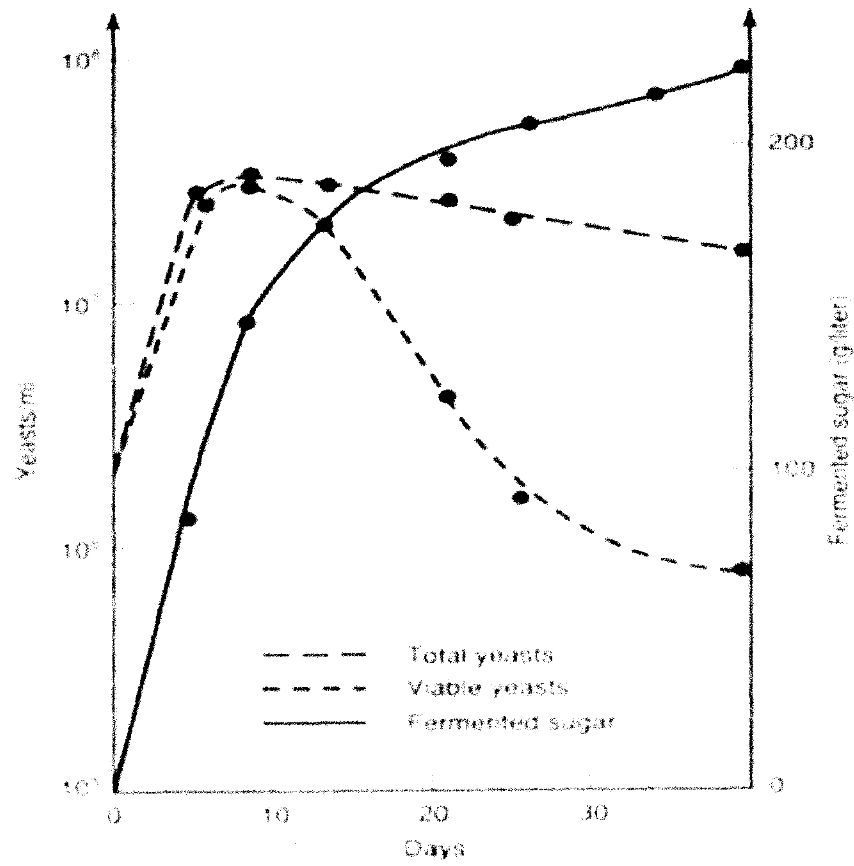
I. Yeast abundance

Growth curves were generated based on the information obtained from the colony counts (CFU) for each fermentation type of both harvest seasons. In a typical viable yeast growth curve (Fig. 2) low numbers are expected within the first few days of fermentation, followed by a substantial increase, signaling yeast growth. After approximately five to six days, a leveling of CFUs is expected, followed by a decrease towards the latter stages of fermentation indicating the loss or death of viable yeasts (Jackson 1994).

Growth curves generated from the inoculated fermentations of the 2000 harvest season (Fig. 3) indicates yeast populations ranged from 10^6 to 10^8 per ml in the initial week followed by a decline in numbers to 10^3 per ml by the end of weeks two and three. A final colony count of approximately 10^3 CFU per ml was determined when the fermentation was complete (22 days). In comparison, the results from 2001 inoculated fermentations ranged from 10^4 to 10^8 per ml in week one, followed by a gradual decrease to 10^3 per ml upon completion of fermentation (40 days).

Yeast populations for the 2000 spontaneous fermentations (Fig. 4) increase from 10^6 to 10^8 CFU per ml in week one and remained in that range through the second week. A gradual decline in numbers to approximately 10^6 CFU per ml was seen upon completion of fermentation (22 days). In comparison, yeast populations during the 2001spontaneous fermentations, increased from 10^4 to 10^8 CFU per ml in the first week. A subsequent decrease in numbers occurred through weeks two through four with a final CFU number of approximately 10^5 CFU per ml (40 days).

Fig. 2: Typical yeast growth curve of viable yeasts (----) numbers in fermentation compared to total yeasts numbers and fermented sugar levels (Jackson 1994)



Total yeasts
Viable yeasts
Fermented sugar
Days
Fermented sugar (g/liter)
200
100
0
10
20
30

Fig. 3: Comparison of average number of yeast colonies per ml in inoculated Chardonnay fermentation during the 2000 and 2001 harvest season conducted in triplicate at YSU laboratory.

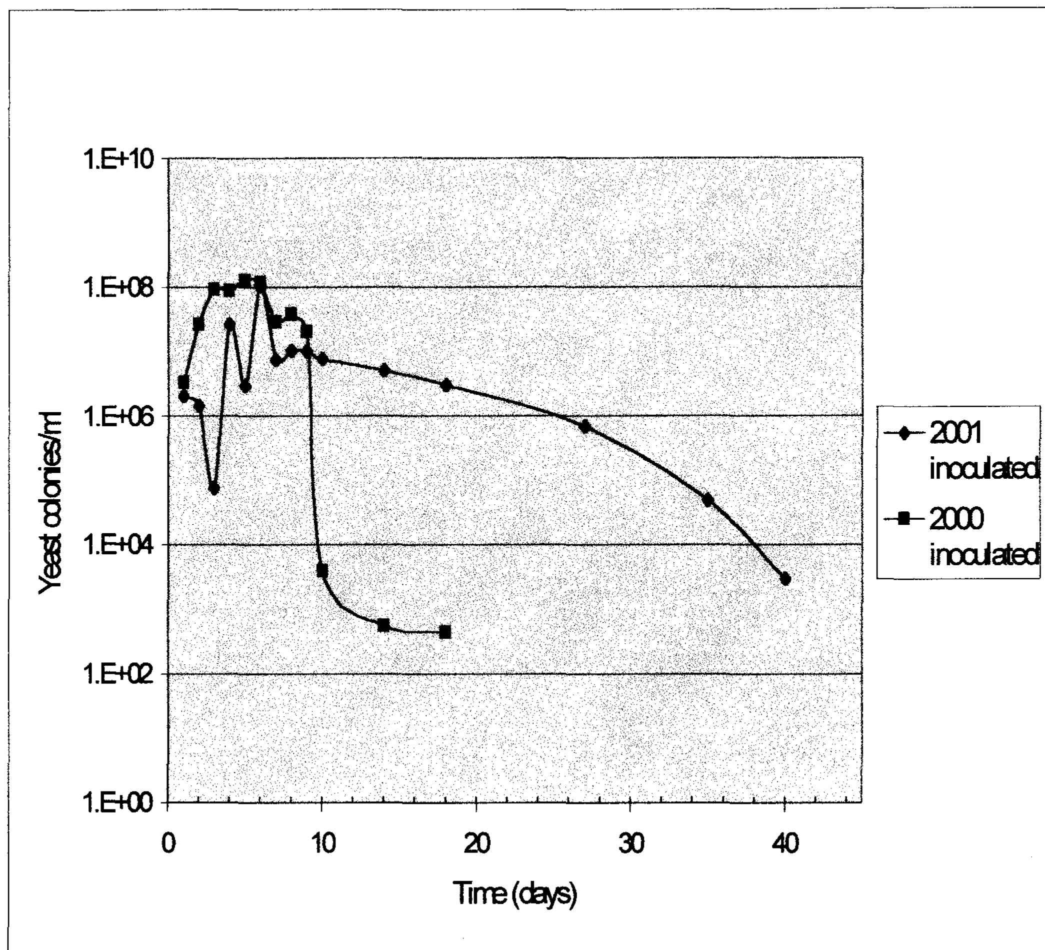
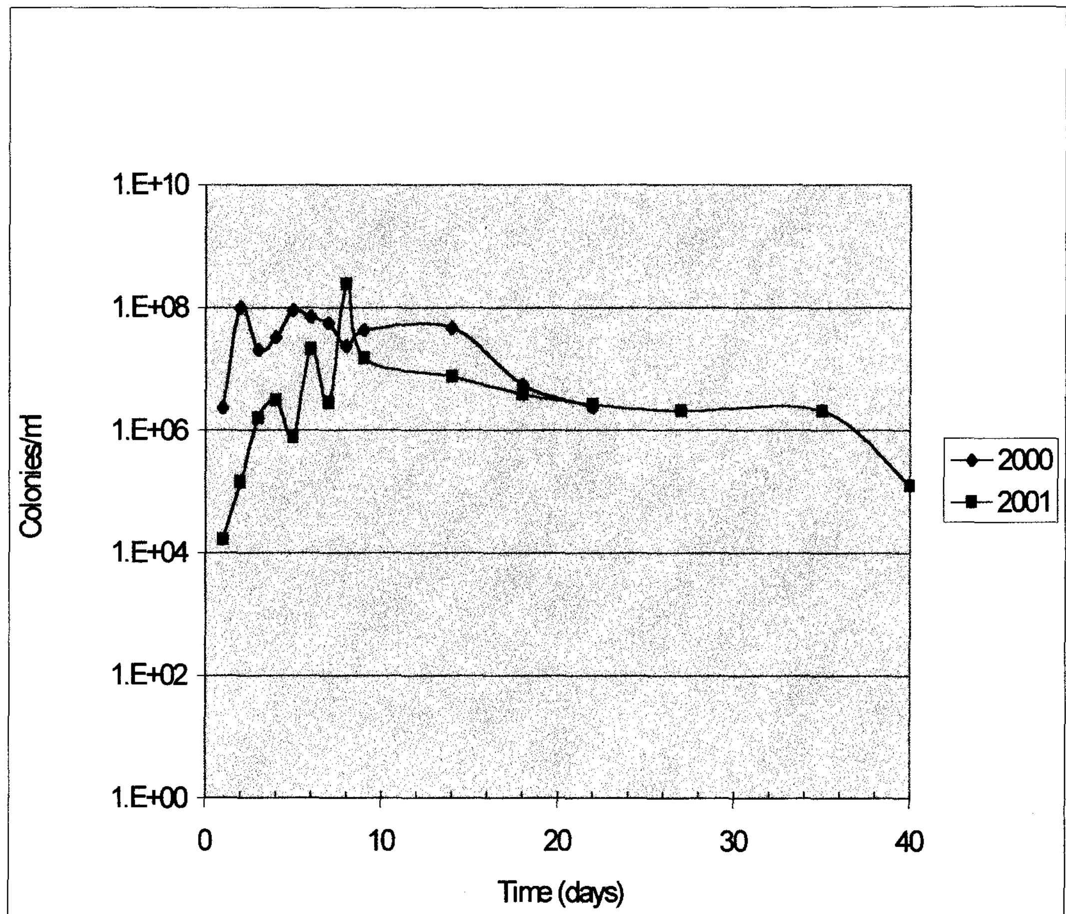

1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
Yeast colonies/ml
0
10
20
30
40
Time (days)
2001 inoculated
2000 inoculated

Fig. 4: Comparison of average number of yeast colonies per ml in spontaneous Chardonnay fermentation during the 2000 and 2001 harvest season conducted in triplicate at YSU laboratory.


1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
Colonies/ml
0
10
20
30
40
Time (days)
2000
2001

Based on the Multiple Regression Analyses for the 2000 harvest season: $\ln(\log)\ count = 15.13 - 2.38\ (treat) + 1.41\ (days) + 0.522\ (treat)(days) + (-.0195\ days^2) + 0.0051(days^3)$. Where treatment = 0 is control and treatment =1 is spontaneous. Based on this calculation, there is a significant difference (p=<.0001) between the CFUs in the 2000 spontaneous and 2000 control fermentations (Fig.5).

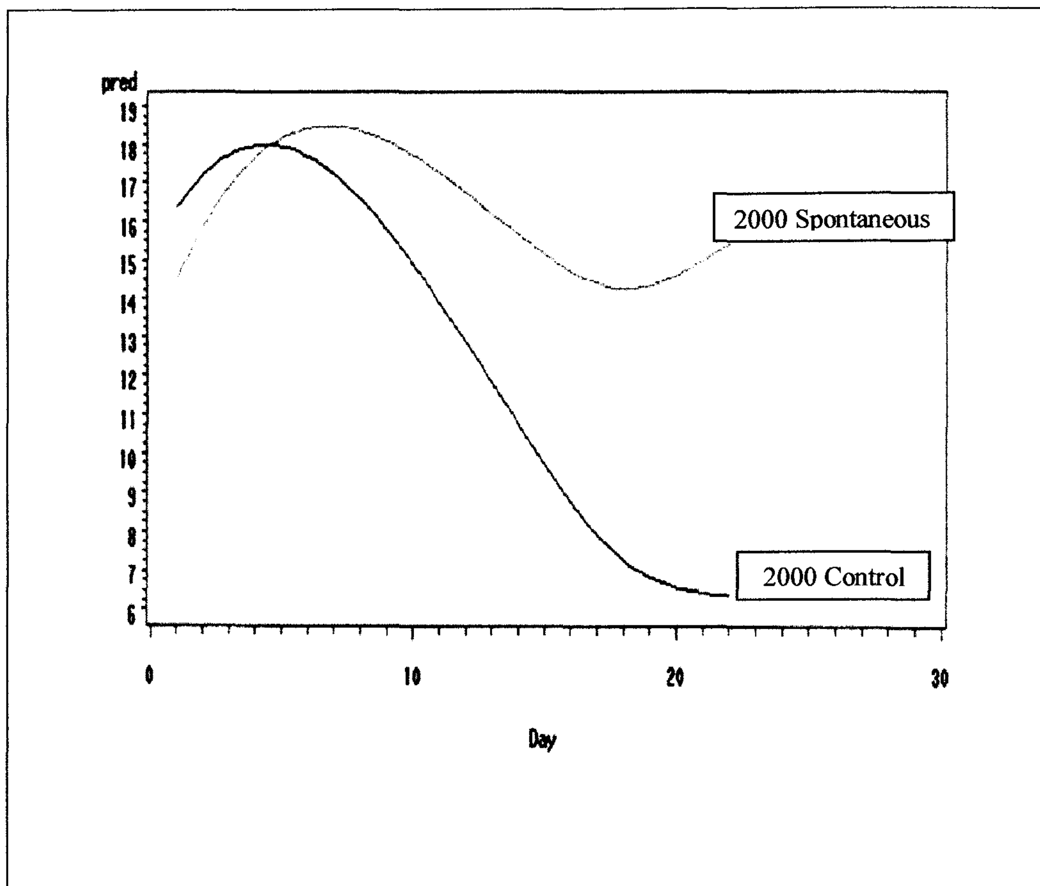
For the 2001 harvest season: $\ln(\log)\ count = 13.45 - 1.59\ (treat) + 0.741\ (days) + 0.106\ (treat)(days) + (-0.039\ days^2) + 0.0005(days^3)$. Where treatment = 0 is control and treatment =1 is spontaneous. Accordingly, there is a significant difference (p=<.0001) between the CFUs in the 2001spontaneous and 2001 control fermentations (Fig. 6).

The following equations were used to determine significance between 2000 spontaneous versus 2001 spontaneous (Fig.7) and 2000 control versus 2001 control (Fig. 8) respectively:

Spontaneous year to year analysis: $\text{Ln count} = 15.14 - 3.54(year) + 0.68(days) + 0.238(year)(days) + (-0.048)(days^2) + (0.0006)(day^3)$. Where year = 0 implies 2000 and year =1 implies 2001. Based on the calculation, there is a significant difference between the CFUs in the spontaneous fermentations from year to year (p value = <0.0001).

Control year to year analysis: $\text{Ln count} = 17.64 - 4.48(year) + 0.12(days) + 0.676(year)(days) + (-0.044)(days^2) + (0.0006)(day^3)$. Where year = 0 implies 2000 and year =1 implies 2001. Based on the calculation, there is a significant difference between the CFUs in the control fermentations from year to year (p value = <0.0001).

Fig.5: Predicted values of yeasts growth curve for spontaneous and control Chardonnay fermentation of the 2000 harvest season using Multiple Regression Analysis (SAS). P=0.0001


pred
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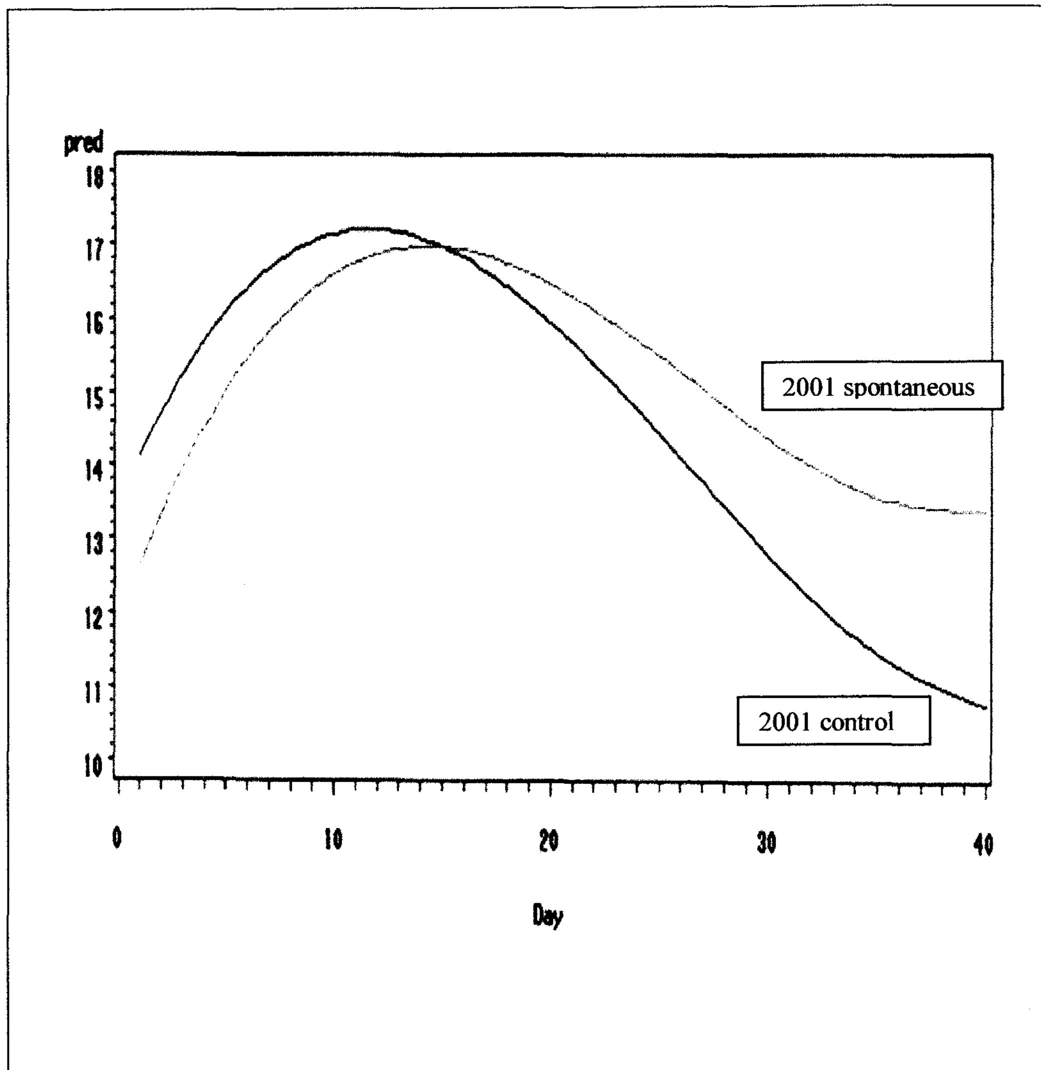
16
15
14
13
12
11
10
9
8
7
6
0
10
20
30
Day
2000 Spontaneous
2000 Control

Fig. 6: Predicted values of yeasts growth curve for spontaneous and control Chardonnay fermentation of the 2001 harvest season using Multiple Regression Analysis (SAS). P=0.0001



pred
18
17
16
15
14
13
12
11
10
2001 spontaneous
2001 control
0
10
20
30
40
Day

Fig. 7: Predicted values of yeasts growth curve for spontaneous Chardonnay fermentation of 2000 and 2001 harvest season using Multiple Regression Analysis (SAS). P=0.001

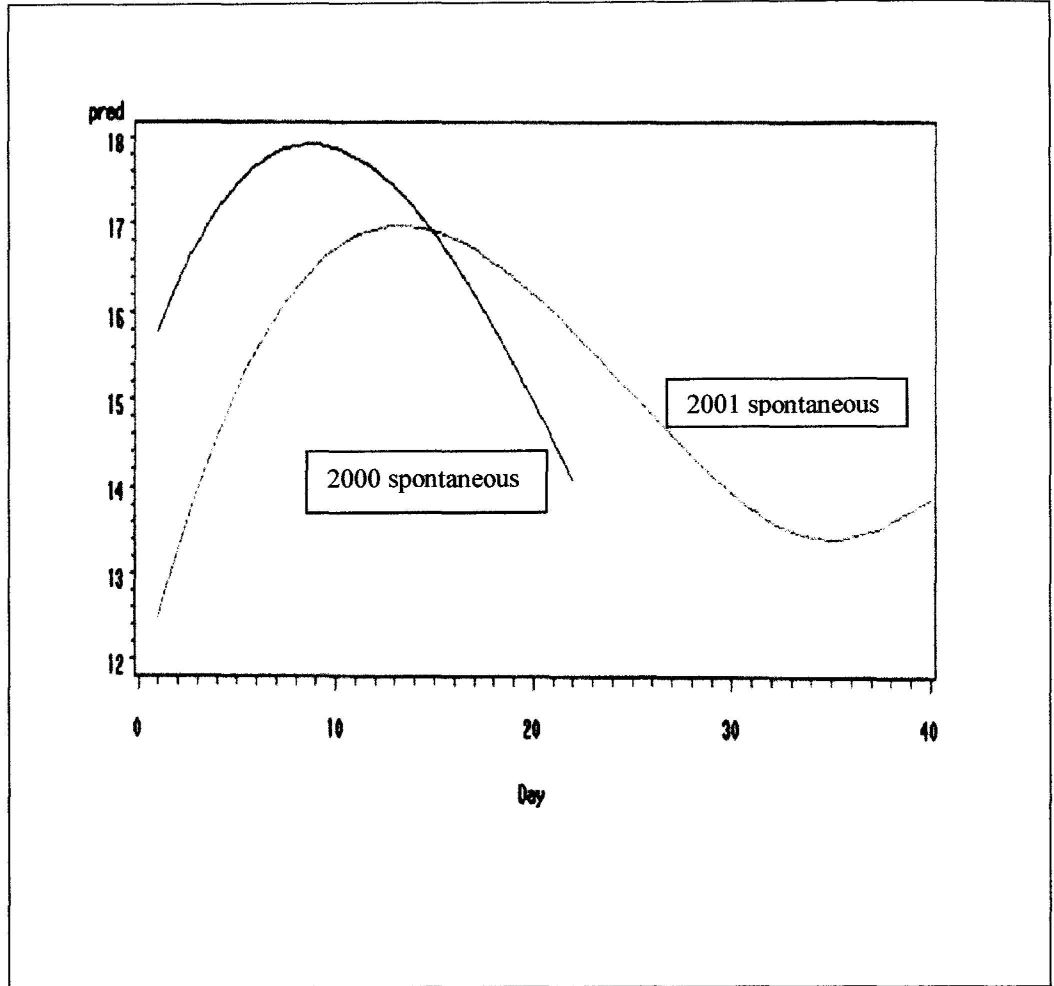
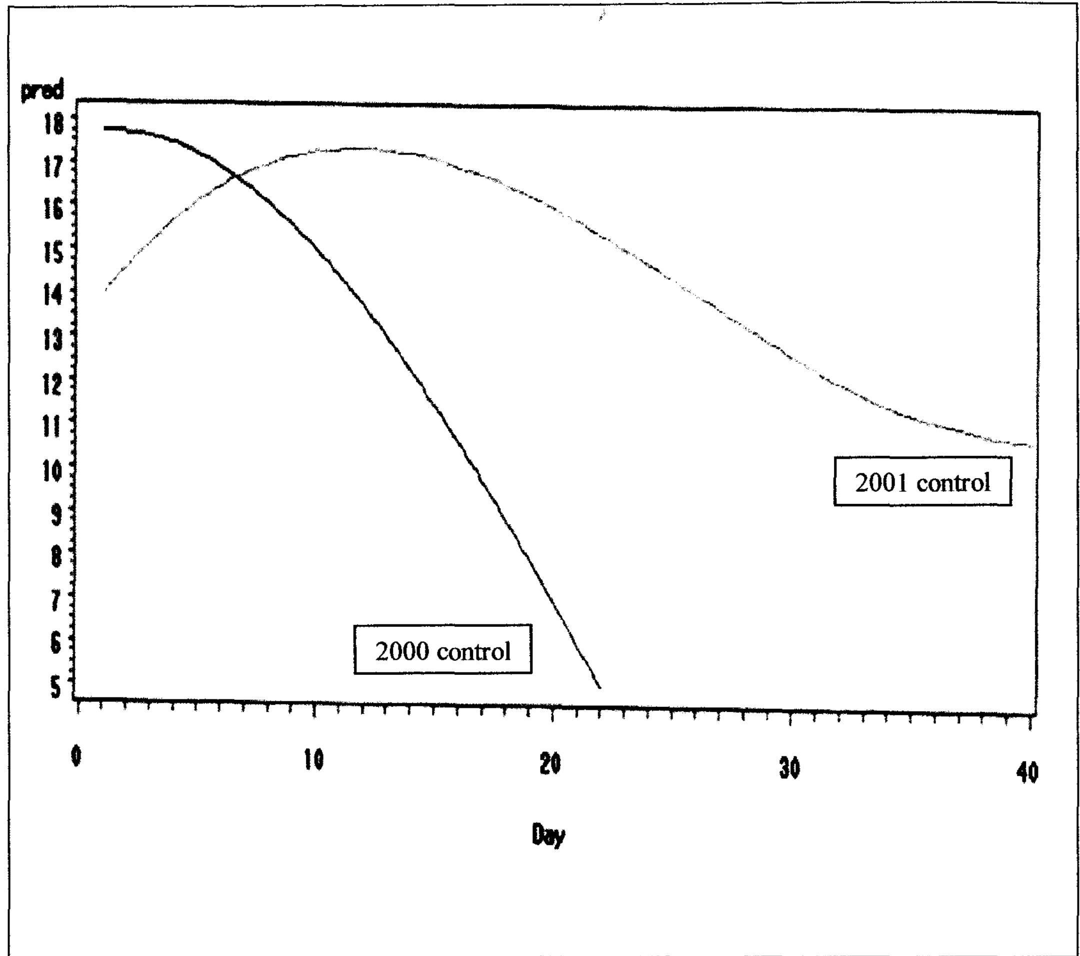

pred
18
17
16
15
14
13
12
0
10
20
30
40
Day
2001 spontaneous
2000 spontaneous

Fig.8: Predicted values of yeasts growth curves for control Chardonnay fermentation of the 2000 and 2001 harvest season using Multiple Regression Analysis (SAS). P=0.0001


pred
18
17
16
15
14
13
12
11
10
9
8
7
6
5
2001 control
2000 control
0
10
20
30
40
Day

The average yeast numbers per ml compared to °Brix measurements during the sampling period of the two consecutive harvest seasons are depicted in Figs. 9 through 12. As expected, as yeast numbers increased, °Brix levels for the spontaneous and inoculated fermentation's decreased.

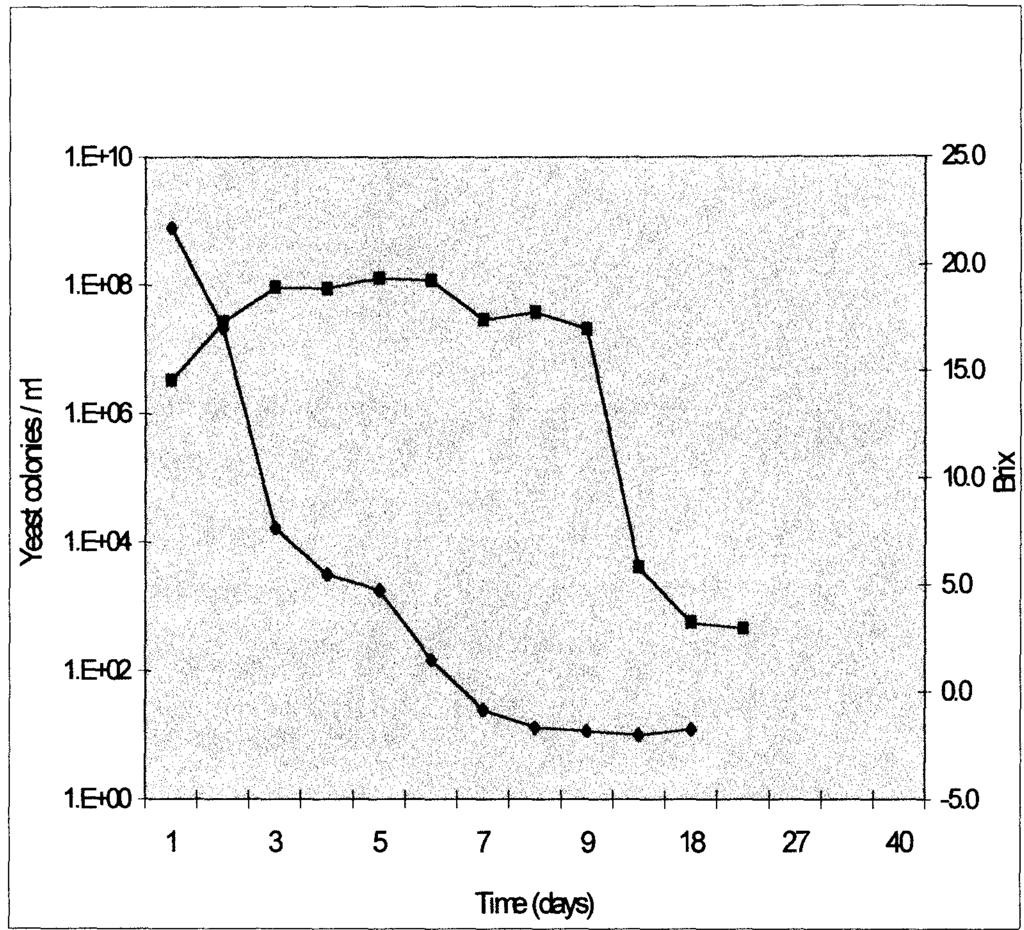
II. Physiological Tests

Based on the CFU results, physiological tests and phenotypical differences, three yeasts were identified in the 2001 spontaneous fermentations. In fermentation days one through five, *H. uvarum* was predominant with few *P. membranaefaciens* present, whereas after day five of fermentation, *Saccharomyces* began to dominate (Fig. 13) and completed fermentation. In comparison, the data in Fig.14 depicts the percent distribution of yeast present in the 2001 control fermentation. Tables 1-7 provide sample physiological test results of 2000 and 2001 harvest season for *H. uvarum* (Tables 1-3), *Saccharomyces* (Tables 4- 6) and *P.membranaefaciens* (Table 7) samples, respectively.

III. Electrophoretic karyotyping

Selected samples identified through physiological tests as *Saccharomyces* spp. in the 2000 and 2001 harvest seasons were run simultaneously on Pulse Field Gel Electrophoresis (PFGE) as indicated in Fig. 15. A size marker of *S. cerevisiae* (Lane 1) and commercial *S. bayanus* (Lane 7) were used for comparative purposes. As shown, the spontaneous samples in lanes 2-6 show variability whereas, the controlled fermentation samples in Lanes 8-10 appear similar.

Fig.9: Comparison of average number of yeasts per ml to Brix levels during the control Chardonnay fermentation of the 2000 harvest season conducted in triplicate at YSU laboratory.



1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
Yeast colonies / ml
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
Brix
1
3
5
7
9
18
27
40
Time (days)

Fig. 10: Comparison of average number of yeasts per ml to Brix levels during the spontaneous Chardonnay fermentation of the 2000 harvest season conducted in triplicate at YSU laboratory.

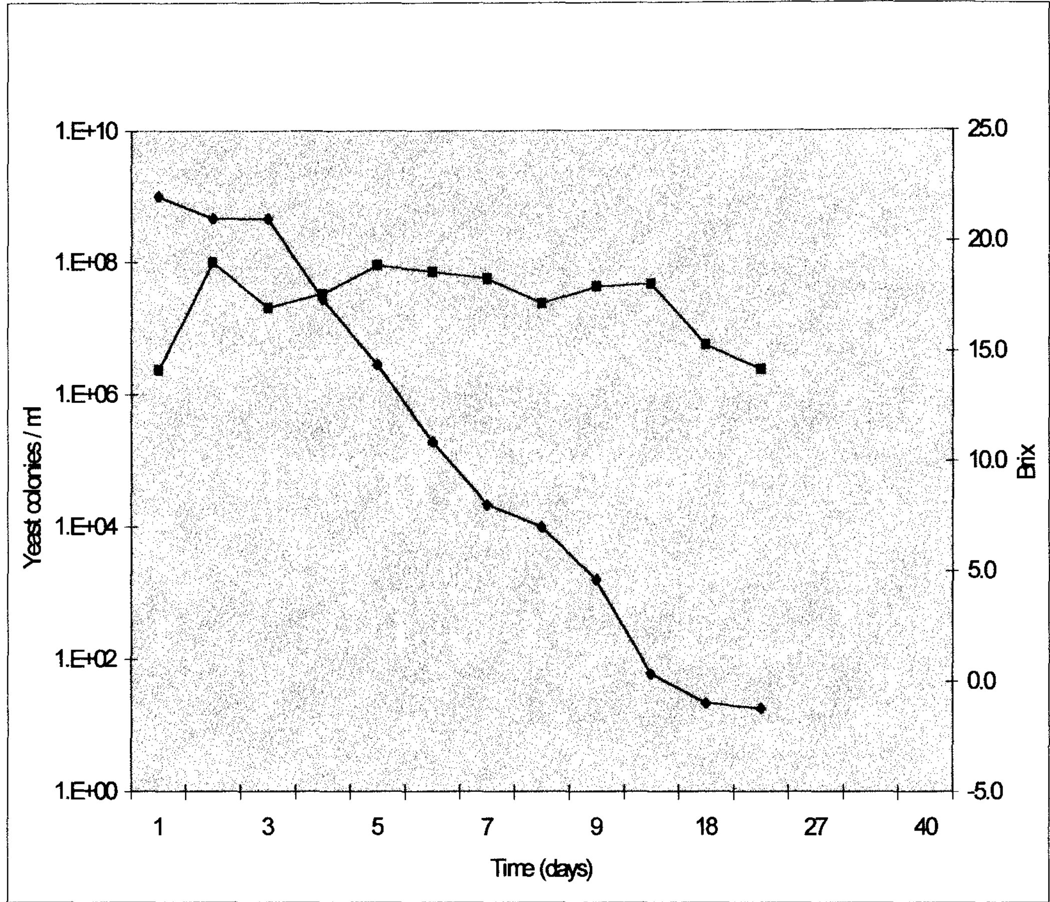

1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
Yeast colonies / ml
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
Brix
1
3
5
7
9
18
27
40
Time (days)

Fig. 11: Comparison of average number of yeasts per ml to Brix levels during the control Chardonnay fermentation of the 2001 harvest season conducted in triplicate at YSU laboratory.

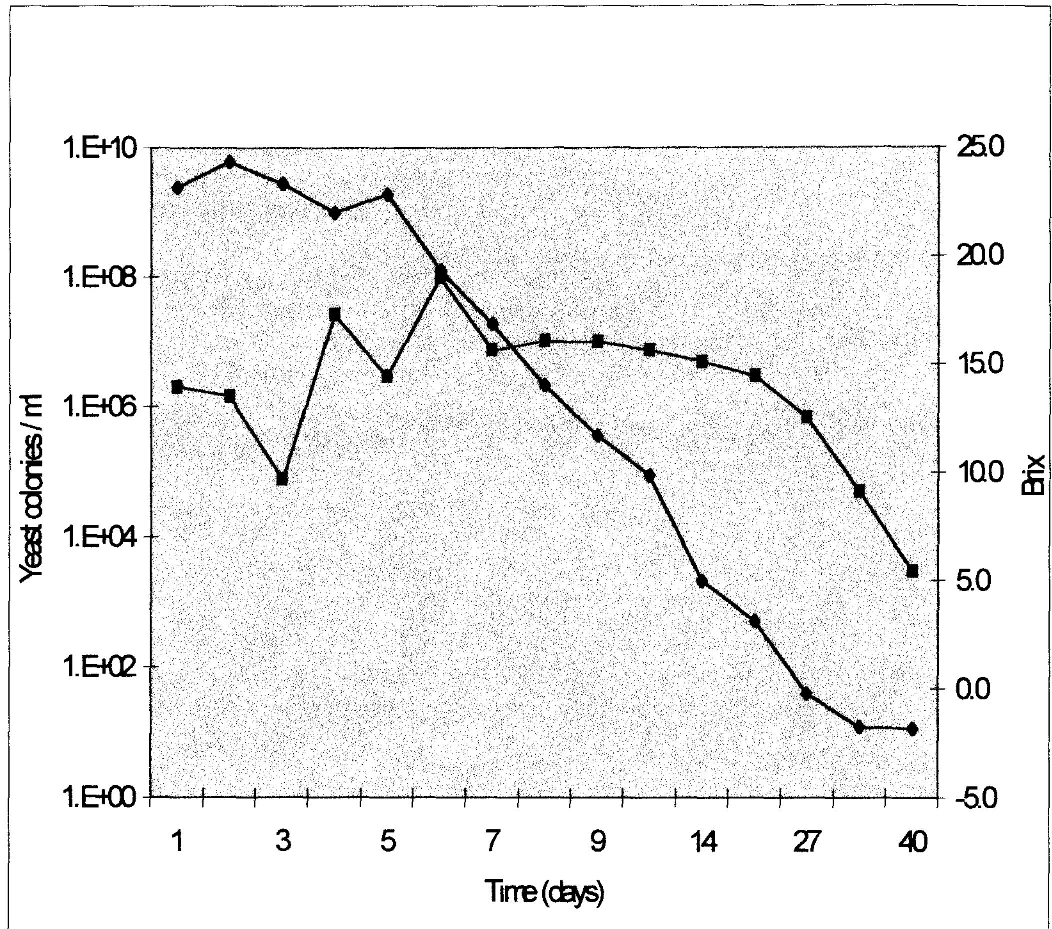

1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
Yeast colonies / ml
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
Brix
1
3
5
7
9
14
27
40
Time (days)

Fig.12: Comparison of average number of yeasts per ml to Brix levels during the spontaneous Chardonnay fermentation of the 2001 harvest season conducted in triplicate at YSU laboratory.

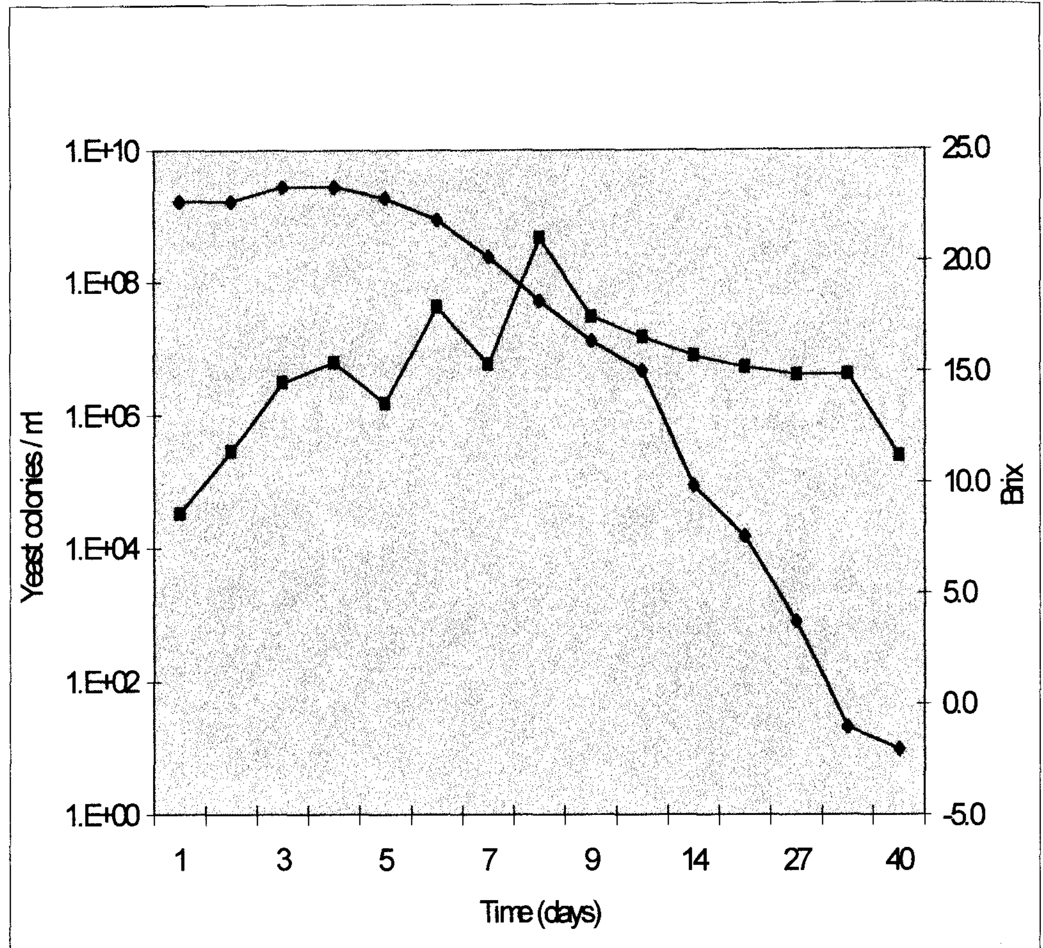
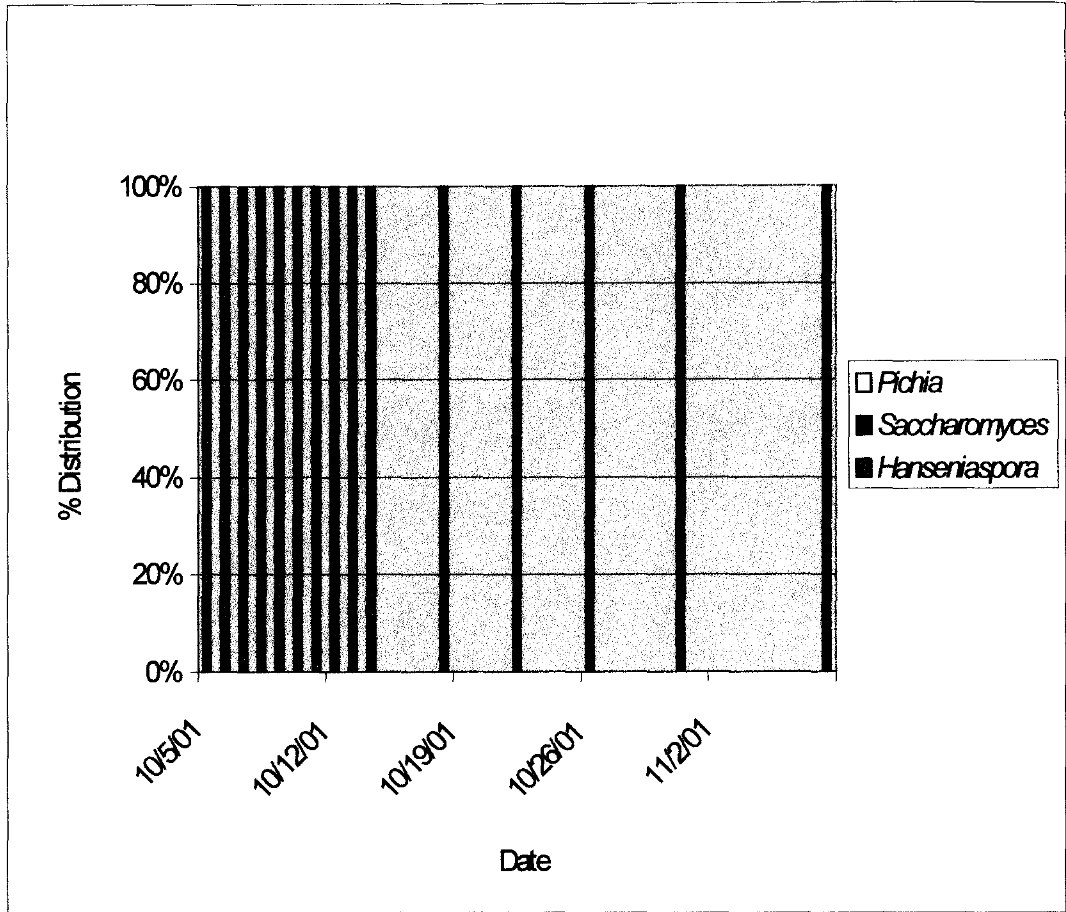

1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
Yeast colonies / ml
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
Brix
1
3
5
7
9
14
27
40
Time (days)

Fig.13: Distribution of yeasts present based on physiological tests and phenotypical differences in spontaneous Chardonnay fermentation during the 2001 harvest season conducted in YSU laboratory. *Pichia* present <1% therefore cannot be seen on chart. *Hanseniaspora uvarum* present (>60%) during initial five days, *Saccharomyces* sp. present (>60 %) after day five through completion of fermentation.


100%
80%
60%
40%
20%
0%
% Distribution
10/5/01
10/12/01
10/19/01
10/26/01
11/2/01
Date
Pichia
Saccharomyces
Hanseniaspora

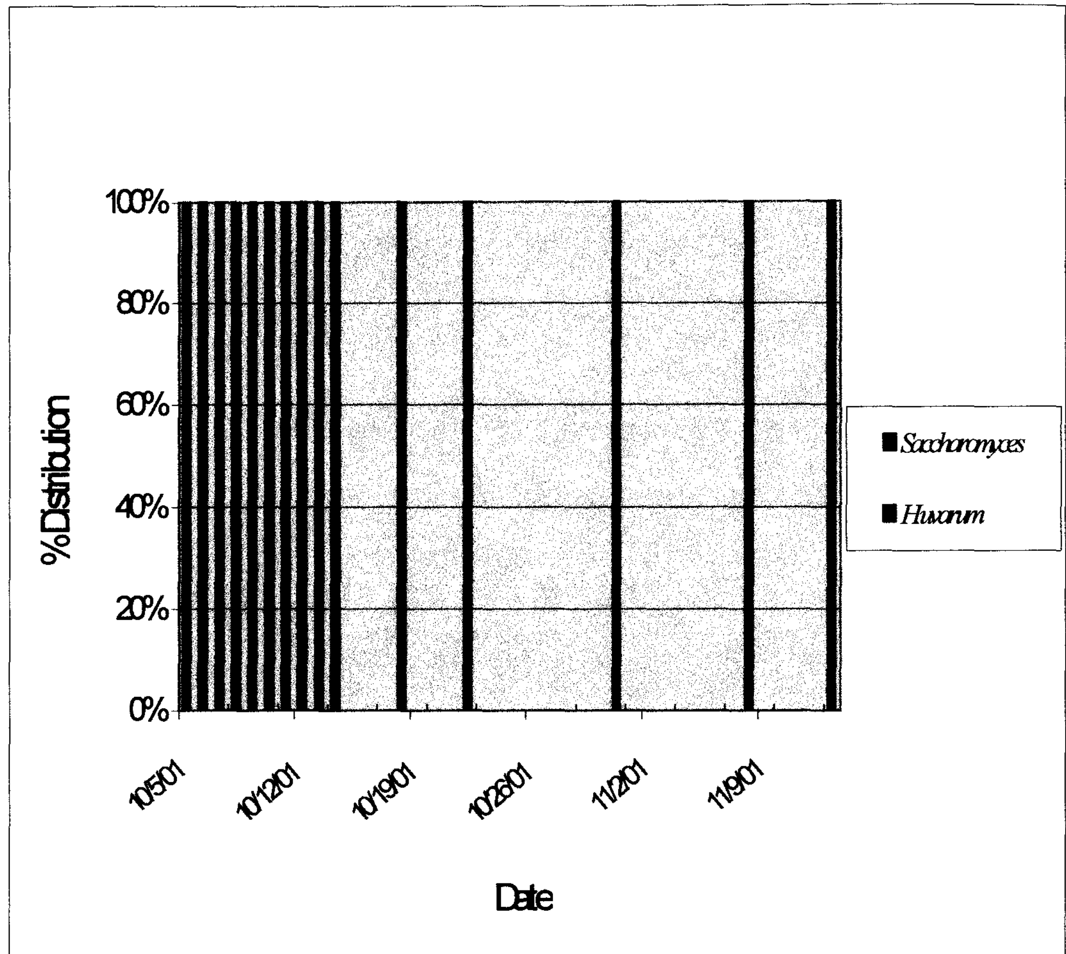

100%
80%
60%
40%
20%
0%
% Distribution
10/5/01
10/12/01
10/19/01
10/26/01
11/2/01
11/9/01
Date
Saccharomyces
H. uvarum

Table 1: Results of physiological tests for yeasts identified as *H. uvarum* from the Chardonnay fermentation of the 2000 harvest season on sample days 10-15-00 through 10-17-00. Sample numbers and test results in red indicate phenotype appearance is that of *H. uvarum* with test results differing.

Description: Colonies are off white to tan in color, appear flat with a creamy, glossy appearance on Sabouraud agar plates. At 40 X magnification cells appear small, oblong in shape with bipolar budding and appearing in chains.

	Nitrogen Assimilation				Carbon Assimilation					
Sample Id	Glucosamine	Cadavarine	L- lysine	Nitrate	D-Glucose	Maltose	Myo-inositol	Glucuronate	Lactic acid	Identification
10-13-00 S3-C	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-13-00 S2-B	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-13-00 S3-C	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-13-00 F7 -C	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-13-00 F7-E	-	+	+	-	+	-	-	-	-	*H.uvarum*
10-13-00 S2-B	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-13-00 S2-A	-	-	+	-	+	-	-	-	-	
10-15-00 S2-E	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 S1-G	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 F7	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 F7-C	-	+	+	-	+	-	-	-	-	*H.uvarum*
10-15-00 F7-D	-	+	+	-	+	-	-	-	-	*H.uvarum*
10-15-00 F7-BI	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 S1-E1	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 S1-F	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 S1-H	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-15-00 S1-J	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-17-00 S1-I	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-17-00 S3-G	-	+	+	-	+	-	-	-	-	*H. uvarum*

Table 2: Results of physiological tests for yeasts identified as *H. uvarum* from the Chardonnay fermentation of the 2000 harvest season on sample days 10-17-00 through 10-18-00, CSU samples, and 10-05-01 through 10-12-01. Sample numbers and test results in red indicate phenotype appearance is that of *H. uvarum* with test results differing.

Description: Colonies are off white to tan in color, appear flat with a creamy, glossy appearance on Sabouraud agar plates. At 40 X magnification cells appear small, oblong in shape with bipolar budding and appearing in chains.

Sample Id	Glucosamine	Cadavarine	L- lysine	Nitrate	D-Glucose	Maltose	Myo-inositol	Glucuronate	Lactic acid	Identification
10-17-00 S3-GI	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-17-00 S2-F	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-18-00 S1-A	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-18-00 S1-A	-	+	+	-	+	-	-	-	-	*H. uvarum*
CSU #1	-	+	+	-	+	-	-	-	-	*H. uvarum*
CSU #39	-	+	+	-	+	-	-	-	-	*H. uvarum*
CSU #52	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-5-01 C3-A	-	+	+	+	+	-	-	-	-	
10-5-01 C1-E	-	+	+	-	+	-	-	-	-	*H.uvarum*
10-5-01 S2-D	-	+	+	-	+	-	-	-	-	*H.uvarum*
10-5-01 S3-D	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-5-01 S1-B	-	+	+	-	+	-	-	-	-	*H.uvarum*
10-09-01 S1-A	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-09-01 S3-B	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-09-01 S3-D	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-12-01 S1-E	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-12-01 S3-E	-	+	+	-	+	-	-	-	-	*H. uvarum*
10-12-01 S2-E	-	+	+	-	+	-	-	-	-	*H. uvarum*

Table 3: Results of physiological tests for yeast identified as *Saccharomyces* from the Chardonnay fermentation of the 2000 harvest season on sample days 10-12-00 through 10-15-00. Sample numbers and test results in red indicate phenotype appearance is that of *Saccharomyces* with test results differing.

Description: Colonies are raised, off-white with a slightly glossy appearance on Sabouraud agar plates with a characteristic "bread" odor.

	Nitrogen Assimilation				Carbon Assimilation					
Sample Id	Glucosamine	Cadavarine	L- lysine	Nitrate	D-Glucose	Maltose	*Myo*-inositol	Glucuronate	DL-lactic acid	Identification
10-12-00 F7-C										
10-13-00 S3-B	-	+	+	-	+	-	-	-	+	
10-13-00 C2-A	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-13-00 C3-A	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-14-00 S1-B	-	+	-	-	+	+	-	-	-	
10-14-00 S1-D	-	-	-	-	+	-	-	-	-	
10-14-00 S2-D	-	-	+	-	+	+	-	-	+	
10-15-00 C1-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-15-00 C2-B	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-15-00 C3-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-15-00 F7-B	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-15-00 F7-A	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-15-00 S1-E	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-15-00 S2-A	-	+	-	-	+	+	-	-	+	
10-15-00 S2-B	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-15-00 S2-D	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-15-00 S2-G	-	-	-	-	+	-	-	-	-	

Table 4: Results of physiological tests for yeast identified as *Saccharomyces* from the Chardonnay fermentation of the 2000 harvest season on sample days 10-15-00 through 10-17-00. Sample numbers and test results in red indicate phenotype appearance is that of *Saccharomyces* with test results differing.

Description: Colonies are raised, off-white with a slightly glossy appearance on Sabouraud agar plates with a characteristic "bread" odor.

	Nitrogen Assimilation				Carbon Assimilation					
Sample Id	Glucosamine	Cadavarine	L- lysine	Nitrate	D-Glucose	Maltose	*Myo*-inositol	Glucuronate	DL-lactic acid	Identification
10-15-00 S2-I	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-15-00 S2-J	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-16-00 S1-D	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-16-00 S1-G	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-16-00 S2-B	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-16-00 S2-D	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-16-00 S3-A	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-17-00 S1-B	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-17-00 S1-D	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-F	-	-	-	-	+	-	-	-	+	
10-17-00 F7-B	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S2-E	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-E	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S1-A	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-B	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-D	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S1-F	-	-	-	-	+	+	-	-	+	*Saccharomyces*

Table 5: Results of physiological tests for yeast identified as *Saccharomyces* from the Chardonnay fermentation of the 2000 harvest season on sample days 10-17-00 through 10-18-00.

Description: Colonies are raised, off-white with a slightly glossy appearance on Sabouraud agar plates with a characteristic "bread" odor.

	Nitrogen Assimilation				Carbon Assimilation					
10-17-00 S2-d	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-H-	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S3-I	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S2-A	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-17-00 S2-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S3-E	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S1	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S2-E	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S3-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S2-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S3-A	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S3-B	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S1-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S2	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 C1-A	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-18-00 C3-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-18-00 C2-B	-	-	-	-	+	+	-	-	-	*Saccharomyces*
S. bayanus & S. cerevisiae	-	-	-	-	+	+	-	-	-	*Saccharomyces*

Table 6: Results of physiological tests for yeast identified as *Saccharomyces* from the Chardonnay fermentation of the 2000 and 2001 harvest season on sample days 10-18-00, 10-5-01 through 10-12-01.

Description: Colonies are raised, off-white with a slightly glossy appearance on Sabouraud agar plates with a characteristic "bread" odor.

10-18-00 S1	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-18-00 S2-E	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-18-00 S3-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-18-00 S2-C	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-5-01 C3-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-5-01 C2-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-5-01 C1-A	-	-	-	-	+	+	-	-	-	*Saccharomyces*
10-9-01 S1-E	-	-	-	-	+	+	-	+	-	*Saccharomyces*
10-09-01 S3-E	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-09-01 C1-A	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-09-01 C1-B	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-09-01 C1-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-09-01 S2-D	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-12-01 C2-A	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-12-01 C3-D	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-12-01 C1-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-12-01 S1-B	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-12-01 S2-A	-	-	-	-	+	+	-	-	+	*Saccharomyces*
10-12-01 S3-C	-	-	-	-	+	+	-	-	+	*Saccharomyces*

Table 7: Results of physiological tests for yeast identified as *Pichia* from the Chardonnay fermentation of the 2000 and 2001 harvest season. Sample numbers and test results in red indicate phenotype appearance is that of *Pichia* with test results differing.

Description: Colonies are off white to yellow in color, flat with matte texture or appearance on Sabouraud agar plates. At 40 X magnification cells appear small oblong in shape with budding noted and appearing in chains of cells. Characteristic acetone-like odor.

Sample Id	Glucosamine	Cadavarine	L- lysine	Nitrate	D-Glucose	Maltose	*Myo*-inositol	Glucuronate	DL-lactic acid	Identification
10-12-00 F7-A	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-12-00 F7-B	+	+	+	-	+	-	-	-	+	*P .membranaefaciens*
10-13-00 F7-A	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-13-00 F7-D	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-14-00 F7-C	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-14-00 F7-D	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-15-00 F7-F	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-15-00 F7-E	+	+	+	-	+	+	-	-	+	
CSU #6	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*
10-05-01 S1-E	+	+	+	-	+	-	-	-	+	*P. membranaefaciens*

PFGE results for samples identified as *P. membranaefaciens* and *H. uvarum* present in the 2000 and 2001 harvest seasons are depicted in Fig.16. A size marker of *S. cerevisiae* (Lane 1) was used for comparative purposes. As depicted in Fig. 16, differences exist between the CSU *Pichia* sample (lane 2) and the YSU *Pichia* sample (lane 3). The PFGE results of the *H. uvarum* samples indicates similarity exists with all but two of the samples in which a doublet chromosome can be seen at approximately 600 Kb (lanes 5 and 8).

Fig. 15: PFGE results of 2000 and 2001 harvest season *Saccharomyces* yeasts present in days one, three and eight of fermentation. A 1% Pulse Field gel subjected to 24 hrs. at 6 v/cm with an initial switch time of 60 seconds and a final switch time of 120 seconds with an angle of 120°.

Legend: **Lane 1:** Cleveland State University *S. cerevisiae* identified through various micro and molecular biological techniques. **Lanes 2:** Markko 2000 spontaneous fermentation (10-15-00 F7-B), **Lane 3:** YSU 2000 spontaneous (10-15-00 S2-B), **Lane 4:** YSU 2000 spontaneous (10-18-00 S1), **Lanes 5:** YSU 2001spontaneous (10-9-01 S2-D), **Lane 6:** YSU 2001 spontaneous (10-12-01 S2-A), **Lane 7:** commercial *S. bayanus* Premier Cuvee used in inoculating control fermentation. **Lane 8:** YSU 2000 control fermentation (10-13-00 C2-A), **Lane 9:** YSU 2000 control (10-18-00 C1-A), **Lane 10:** YSU 2001 control (10-5-01 C3-C), **Lane 11:** YSU 2001 control (10-9-01 C1-A), **Lane 12:**YSU 2001 control (10-12-01 C3-D).

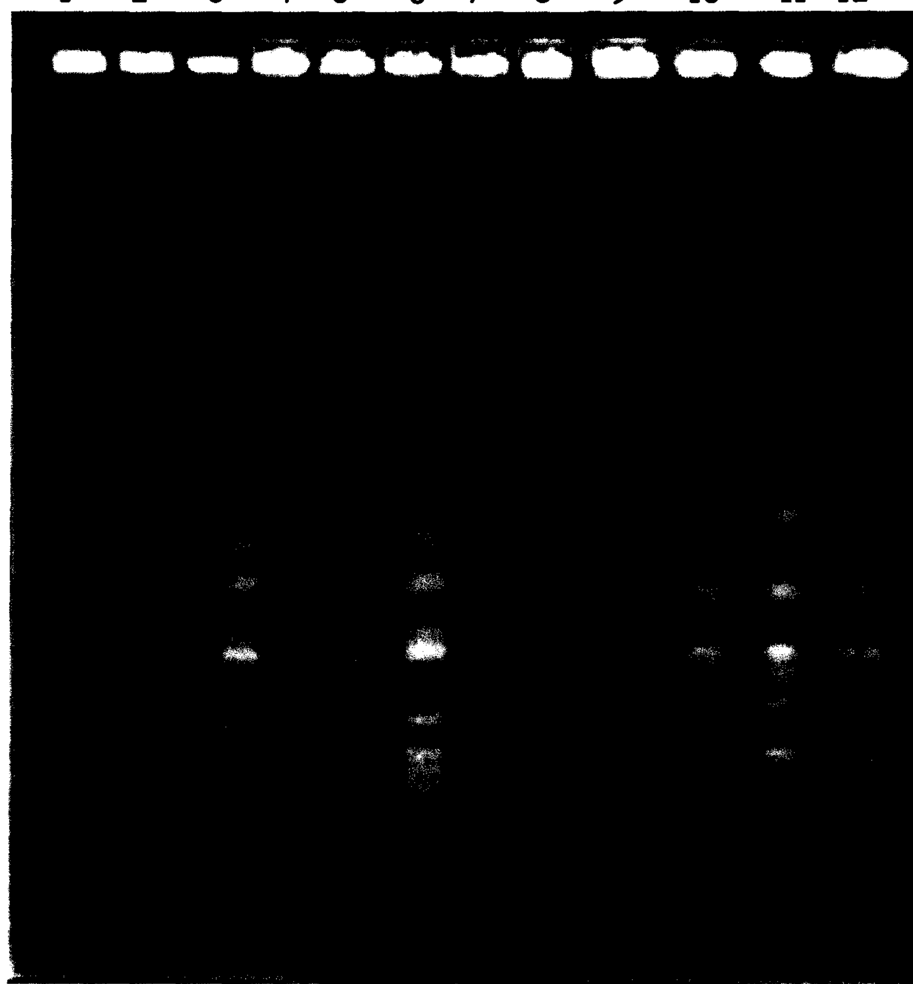
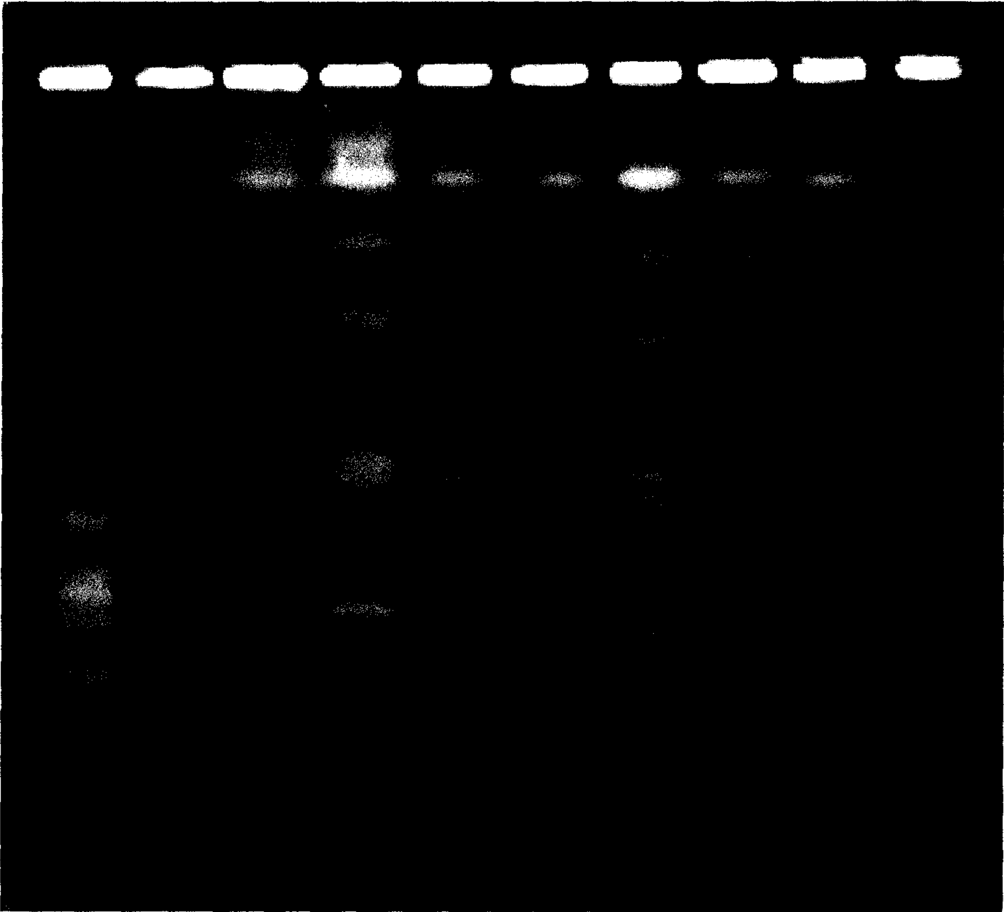

Lanes
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 16: PFGE results of 2000 and 2001 harvest season yeast *Pichia* and *Hanseniaspora*. A 1% Pulse Field gel subjected to 24 hrs. at 6 v/cm with an initial switch time of 60 seconds and a final switch time of 120 seconds with an angle of 120°.

Legend: **Lane 1**: CSU S. *cerevisiae* size marker. **Lane 2:** CSU *Pichia* #6. **Lane 3:** YSU 2001 spontaneous (10-5-01 S1-E). **Lane 4:** CSU #52 *Hanseniaspora*. **Lane 5:** YSU 2000 spontaneous10-13-00 S2-B. **Lane 6:** Markko 2000 spontaneous 10-15-00 F7-D. **Lane 7:** YSU 2000 spontaneous 10-18-00 S1-A. **Lane 8:** YSU 2001 spontaneous 10-5-01 C1-E. **Lane 9:** YSU 2001 spontaneous 10-9-01 S3-B.**Lane 10:** YSU 2001 spontaneous 10-12-01 S2-E.

Lanes 1 2 3 4 5 6 7 8 9 10



Discussion

Based on the results of physiological tests and electrophoretic karyotyping, we fail to reject the hypothesis, "There is a difference in the yeast present depending on the type of fermentation".

Physiological test results for the initial eight days of fermentation were completed for the 2000 and 2001 harvest seasons. Each physiological test was examined for utilization of the carbon or nitrogen source at day four after inoculation. As shown in Tables 1 – 7, a plus (+) sign designates utilization of the particular carbon or nitrogen source when administered to the yeast-seeded agar, whereas a negative (-) indicates the carbon or nitrogen sources were not utilized. Turbidity or cloudiness of the yeast-seeded agar was indicative of the use (+) of the chemical compound as a nutrient source. The results were then compared to a taxonomic key to identify the yeast (Kurtzman and Fell 1998). Based on the evidence supported by the physiological tests (Tables 1-7) there are three predominant yeasts, *H. uvarum, Pichia* and *Saccharomyces* present in the spontaneous fermentations in the two consecutive harvest seasons. In comparison, the control data indicates that on day one of the 2001 fermentations, *H. uvarum* (10-5-01 C1-E), is present, whereas in the 2000 control fermentation, no isolates were identified as *H. uvarum.* In subsequent days, *Saccharomyces* dominated and completed the fermentations.

Colony counts based on phenotypes of the 2001 season during the initial five days of spontaneous fermentation, indicate that the number of *H. uvarum* is predominant (>60%) with few *P. membranaefaciens* present. After day five, *Saccharomyces* dominates (>60 %) and completes the fermentation (Fig.13). By comparison, *H.*

uvarum is present only in few numbers on day one and day three of sampling, followed by *Saccharomyces.* Subsequently, the *Saccharomyces* dominated and completed the control fermentation (Fig.14). Colony counts based on phenotypes of the 2000 harvest season were not determined.

However, based on the results of the physiological tests (Tables 1-7) and electrophoretic karyotyping, it can be concluded that the three yeasts present in the 2001 harvest season were also present in the 2000 harvest season. Additionally, it can be concluded that *H. uvarum* was predominant during the initial stages of fermentation, with very few *P. membranaefaciens* present, and the fermentation was completed by *Saccharomyces*. According to results of the physiological tests and the method of isolation, *P. membranaefaciens* appeared only in the fermentation conducted at Markko Vineyard only during the first several days of the 2000 fermentations. Conversely, *P. membranaefaciens* appeared in the YSU laboratory and at Markko Vineyard during the initial three days of the 2001 fermentations. These results are similar with results obtained in a study conducted by Gafner and Shütz (1995) where *H. uvarum* was found in higher numbers with few other non-*Saccharomyces* present. Although the authors show that additional yeasts such as *Pichia anomala, P. guilliermondi, P. kluyveri, Candida castellii, C.glabrata, Cryptococcus* spp. and *Torulaspora delbrueckii* are present in the different vineyards studied, it is acknowledged that additional species and variability may exist in other regions of study. Additional studies conducted over consecutive harvest seasons indicate variations in strains from year to year in the same winery as well as the identification of yeasts not found in this study (PovheJemec et al 2001 and Izquierdo Canas 1997). It appears that the variation in yeast species present in fermentation is not

only dependent on the type of fermentation, spontaneous versus control, but also on the region and year in which the grapes are grown.

Slightly higher numbers of yeasts are present in the initial day of fermentation in the 2000 and 2001 harvest season (Figs. 3 and 4) in comparison to a study conducted by Egli et al (1998). According to Egli et al, initial colony counts were calculated at approximately 10^4 CFU/ml in an untreated fermentation (0 mgl^{-1} SO_2) compared to 10^2 CFU/ml in a treated fermentation (50 mgl^{-1} SO_2). Comparatively, data collected from this study indicates the initial colony counts are approximately 10^6 CFU/ml in both 2000 and 2001 inoculated fermentation (25 mgl^{-1} SO_2) and the 2000 spontaneous fermentation (0 mgl^{-1} SO_2), whereas 10^4 CFU/ml were observed in the 2001 spontaneous fermentation. The latter corresponds with the results obtained from Egli et al.

According to Multiple Regression Analysis of these data (Holcomb, 2002) there are significant differences ($p = < 0.0001$) between the spontaneous and control CFUs of the 2000 harvest season (Fig.5) the spontaneous and control of the 2001 harvest season (Fig. 6) and year to year comparisons of spontaneous and control (Figs. 7 and 8). The R square values (multiple correlation coefficient) generated from the Multiple Regression Analyses interpret the proportion of variability in the response variable (CFUs) and the explanatory variables (treatment, days or years). That is, the R square value indicates the variation seen in the ln CFU counts is explained by variation within the treatments and the days of fermentation (spontaneous versus control) and variation within the treatments and the year of fermentation. The R-values for the spontaneous versus control of the 2000 and the 2001 harvest season have a value of 88.4 % (R-square value = 0.884) and 62.8 % (R-square value = 0.628), respectively. Therefore, 88.4 % and 62.8 percent of the

variation seen in the ln CFU counts are explained by variation within the treatments and the days of fermentation. Whereas, year to year CFU Multiple Regression Analyses completed on the spontaneous and control fermentation indicates that 67.8% (0.678) and 76.9 % (0.769) of the variation can be explained by variation in the years and days.

Although the yeast growth curve of the 2000 control fermentation has an unexpectedly high initial CFU count, it shows an expected increase in yeast numbers, followed by a leveling off period where the yeasts consume the grape sugars as a source of energy. As the yeasts deplete their energy source, ethanol becomes toxic and the yeasts begin to decrease in number (Fig. 9). In comparison, the 2000 spontaneous and the 2001 control and spontaneous fermentations show an increase in numbers followed by a gradual decline in numbers corresponding to the gradual decline in °Brix levels (Figs. 10-12).

The °Brix levels and CFU comparisons of each type of fermentation and from year to year (Figs. 9-12) appear to have similar trends. As the yeast numbers increase, a decrease in the °Brix levels is observed, indicating the yeasts are utilizing the grape sugars.

These data correspond with numerous studies conducted in which variability is shown to exist in spontaneous fermentation within a season and from year to year. Due to many environmental factors, spontaneous fermentations are not predictable. Therefore variability is expected; thus the controversy regarding the use of spontaneous fermentation for consistent wine products. On account of many wine makers preferring control fermentation because of its predictability, this author would have expected less significant differences in the control fermentation. However, noting the uniqueness of

the environmental conditions it stands to reason that variability may exist in control fermentation as well.

According to the results of PFGE shown in Fig.15, there is some variability in the karyotypes among the spontaneous fermentation *Saccharomyces* yeasts. Similar results have been found in Valdepenas (Spain), in which four different karyotypes were found (Briones et al 1996). In this study, variability appears dependent upon the season. In the 2000 harvest season, similar karyotypes are present from three separate samples collected on day 3 (10-15-00, F7-B and 10-15-00, S2-B) and day 6 (10-18-00, S1) of fermentation. Although the karyotypes of the 2001 harvest season differ from the two separate samples collected on day 5 (10-9-01, S2-D) and day 8 (10-12-01, S2-A), additional similarity does exist between sample day 8 (10-12-01, S2-A) and the samples in the 2000 harvest season. Furthermore, the karyotype of sample 10-9-01 S2-D, collected on day 5 of the 2001 harvest season, is similar to that of the karyotypes in the control samples for both harvest seasons. The yeasts karyotypes found in the control fermentation are similar to those of the inoculum, Premier Cuvee® *S. bayanus,* in both harvest seasons.

Although both *S. bayanus* and *S. cerevisiae* are haploid yeasts (chromosome number, n=16) their respective karyotypes are quite different. This facilitates differentiation between the two. According to Naumov et al (2001), *S. cerevisiae* contain three or more chromosomes in the range of 245-370 Kb compared to *S. bayanus* in which two bands are present in the same range. Additionally, a large chromosome at 1600 Kb is seen in *S. cerevisiae* while the large chromosome in *S. bayanus* is seen at approximately 1300 Kb. In addition to the differentiation between *S. cerevisiae* and *S. bayanus,* Naumov examined the karyotypes of two varieties of *S. bayanus, S. bayanus var. uvarum*

and *S. bayanus var. bayanus*. Slight differences exist between the varieties in which two chromosome bands are seen at 245 and 370 Kb in *S. bayanus var. uvarum* while three chromosome bands are present in *S. bayanus var. bayanus.*

In this study, differences exist between samples of the *S. bayanus* yeast (Fig. 16). One sample from the spontaneous fermentation (10-9-01 S2-D, Lane 5) corresponds with the patterns of *S. bayanus var. bayanus* where three chromosomal DNA bands are seen in the 245 – 370 Kb region. In comparison, results from the control fermentation show that all but one sample (10-9-01 C1-A, Lane 12) corresponds to the banding patterns of *S. bayanus var. bayanus* described by Naumov et al. Sample 10-9-01 C1-A appears with two chromosomal DNA bands within the 245 – 370 Kb region which suggests the presence of *S. bayanus var. uvarum*. Therefore, it is believed that two varieties of *S. bayanus* exist in the control and spontaneous fermentations conducted in the YSU laboratory as well as various strains of *Saccharomyces*. Further testing is necessary to conclusively state the strain identity of the *Saccharomyces* present in the fermentations.

PFGE results of the *H. uvarum* yeasts (Fig. 16) identified slight variability in the karyotypes. Two samples from separate collection dates of the 2000 spontaneous (10-13-00 S2-B, Lane 5) and 2001 control fermentation (10-5-01 C1-E, Lane 8) show slight differences in the 600 Kb region. A doublet chromosome is seen in that region, whereas there is a single band appearing in the remaining fermentation samples. According to Cadez et al (2002), the karyotype pattern of *H. uvarum* ranged in size from 2200 to 600 Kb. This is consistent with the PFGE results of this study. Additionally Cadez et al observed doublet chromosomes found in the smallest chromosome fragments. This is also consistent with the present study. Several explanations could account for the doublet

chromosome. According to Cadez et al, *H. uvarum* has a species specific pattern and chromosomal-length polymorphisms occurring in the largest and smallest chromosomal DNA fragments. Another possible explanation of the doublet chromosome is that *H. uvarum*, like *S. bayanus* and *S. cerevisiae,* is a haploid ascomycete. However, during various stages of the yeasts life cycle and certain environmental conditions, the yeasts have the ability to become diploid. During meiosis, the cell duplicates its chromosomes and becomes, temporarily, a quadriploid cell. The two sets of chromosomes migrate and divide forming four haploid cells which are eventually released from the mother cell. Under environmental conditions where food availability is abundant, yeast cells can be diploid and undergo mitosis. It may be possible that the two samples chosen for karyotyping were in a diploid state. Each sample was collected at the beginning stage of fermentation when the food source is readily available. To ascertain a definitive answer, Southern Blot probing would be necessary to determine if these are homologous chromosomes containing the same genes or displaying different genes.

PFGE results for the *P. membranaefaciens* yeasts identified in the 2001 harvest season (Fig. 16) show some variability compared to that of the CSU sample. Each sample (CSU #6 and 10-5-01 S1-E) shows two chromosomal DNA fragments in the 2200 Kb region (based on size marker of *S. cerevisiae)*. However, the band seen at approximately 1300 Kb in the YSU sample is absent in the CSU sample. Conversely, while the band at approximately 800 Kb is present in the CSU sample, it is absent in the YSU sample. Although both yeasts are of the genera *Pichia*, there may be strain differences between the two samples explaining the differences in banding patterns. Both CSU and YSU samples were obtained from different vineyards. As previously observed

in other studies, strain differences may exist within a region and also from region to region. No comparison could be made for any yeasts in the 2000 harvest season despite repeated attempts to isolate this species.

Various biotic and abiotic factors may have influenced the fermentation of this study therefore may present differences in the fermentation types and contribute to season to season variability. The time of harvesting affects the grape sugar (°Brix) levels, with the sugar levels increasing later in the season. Consequently, the levels of sugars at the time of harvest may influence the length of time it takes for the fermentation to be complete. The earlier harvest on 10-4-01 showed a °Brix level at pressing of 20.8. Whereas the later harvest date on 10-11-00 had a °Brix level at the time of pressing of 23.5. The length of fermentation was considerably longer in the 2001 harvest season (40 days) compared to the length of fermentation in 2000 (20 days).

Temperature is another factor that greatly influences fermentation. Typically, a colder outside temperature increases the amount of time for fermentation to start. The differences in temperature in each season were slightly different. Although the temperature of the 2000 season (13°C) was colder, the fermentation process lasted 20 days in comparison to 40 days in the 2001 harvest season (17°C). By comparison, the temperature within the laboratory was cooler (17°C) in the 2001 fermentation compared to 22-25°C during the 2000 fermentation. During the 2001 fermentation it was necessary to move the wine carboys to a different room so that the fermentation would not be compromised. This lower temperature may have possibly affected the rate of fermentation explaining the extended length of days compared to that of the 2000 fermentation.

An additional factor influencing fermentation includes the number of grape pressing prior to the Chardonnay grape pressing of this study. Although the winery equipment is washed, residual yeast colonies remain and may influence the fermentation by introducing the winery microflora to the composition of the pressed grapes. In the 2000 harvest season, the Chardonnay grapes were the first grapes of the season to be harvested whereas in the 2001 harvest season the Chardonnay grapes were fourth to be pressed. Though the Chardonnay grapes were the fourth grapes to be pressed in the 2001 harvest, there appeared to be no additional yeast genera present that were not present in the 2000 fermentation.

Future experiments should be conducted as a continuation of this study. A more extensive examination of consecutive harvest seasons using molecular techniques such as PFGE, Southern blotting and Denaturing Gradient Gel Electrophoresis to allow further and more extensive examination of yeasts not previously identified by the physiological tests.

Phenotypical colony counts for each fermentation type and year could be beneficial in enumerating the presence and absence of certain yeasts throughout the fermentation. Unfortunately, the first harvest season (2000) is lacking phenotype colony counts. However, the experience of examining the differences in colony morphology within the first season aided the phenotype colony count the following harvest season.

In future studies, valuable information could be obtained through replicated spontaneous and controlled winery fermentation opposed to or in addition to laboratory fermentation. Laboratory fermentation was thought to be better able to control factors such as temperature fluctuations. However, as this study indicated, temperature

fluctuations may have influenced the length of fermentation. In addition, laboratory fermentation does not replicate wine making in its natural environment of the winery. Therefore, conducting replicated winery fermentation will give a better indication of the yeasts that are present throughout the process. The identification of the winery yeasts is important for the winemaker in order to produce better quality wines.

In conclusion, the results of this study correspond in part with other studies conducted throughout the world on spontaneous and control fermentation. These results show that variability exists in fermentation, regardless of type, within a region and over consecutive harvest seasons. These results also show that despite the variability, some species of yeasts are present from year to year in the same winery. In addition, there are also differences in the types of yeasts present depending on the type of fermentation.

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Appendix A

Method of preservation of cultures in 30 % glycerol at -20°C.

Aseptically dispense 3 ml of YEPD broth into sterile culture tubes. Inoculate YEPD broth with one colony of yeast. Vortex well. Place tube in rotating wheel at room temperature for 24 hours. Pipette 1 ml of culture in Eppendorf tube. Centrifuge for 10 seconds at 13K. Remove supernatant. Re-suspend cell pellet in 30 % sterile glycerol. Vortex. Pipette yeast and glycerol suspension into cryogenic vial. Place in -20°C storage.

If a sample of preserved culture is needed, remove one loopful of preserved culture with a sterile loop and swab onto agar plate. Allow culture to incubate at room temperature until growth is observed. Replace preserved culture immediately in cold storage once obtaining sample.

Appendix B

Hemocytometer usage as described in CHEF Genomic DNA Plug Kits Instruction Manual (Bio- Rad)

A hemocytometer is usually divided into nine large squares with each square being 1 x 10^{-4} cm^2 or 0.1 mm^3. Count five to ten of the 25 center squares at 400x power, to get a representative sample of the yeast cell suspension. You should have approximately 25 to 75 cells per square and should be relatively free of clumps.

Use the equation below to determine the cell concentration:

Cells counted / Number of squares = Average cells per square

Average cells per square x 25 squares x Dilution factor x 10^4 = cells per ml

Desired cell concentration / Actual cell concentration x ml of plugs to be made = ml of cell suspension to use.

Appendix C

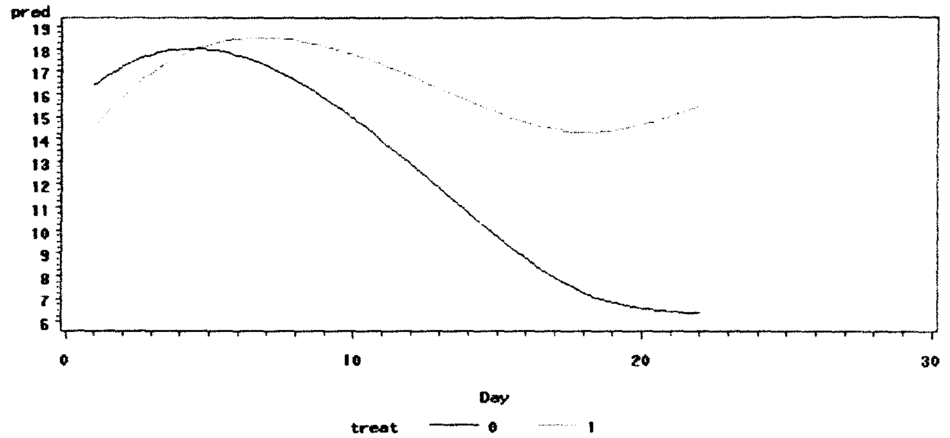
Four separate Multiple Regression Analyses were performed to determine if differences existed in CFUs of each fermentation type within a harvest season and differences from year to year of each fermentation type (SAS 2000) For each analysis the dependent variable is (ln) CFU. The first regression was to determine a difference between spontaneous and controlled fermentation in the 2000 harvest season. The second analysis was to determine a difference between spontaneous and controlled fermentation in the 2001 harvest season. The third analysis was to determine the differences in the spontaneous 2000 versus spontaneous 2001 fermentations. The fourth and final analysis was to determine if a difference existed between the controlled 2000 versus the controlled 2001 fermentations. This tool was used as a mechanism to determine how well the set of the four independent variables (spontaneous versus control 2000 harvest season, spontaneous versus control 2001 harvest season, spontaneous 2000 versus spontaneous 2001 and control 2000 versus control 2001) explained the dependent variable ln(CFUs).

2000 treat=0 is control, treat=1 is spontaneous

Yes, there is a difference between the control and spontaneous

R-Square	Coeff Var	Root MSE	Median Mean
0.884296	7.673268	1.217241	15.86339

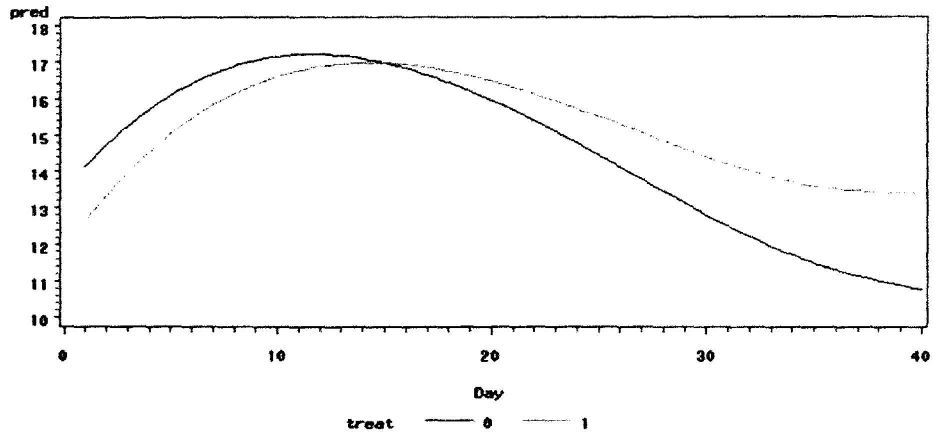
Parameter	Estimate	Error	t Value	Pr > |t|
Intercept	15.12939849	0.58775197	25.74	<.0001
treat	-2.38138355	0.47400253	-5.02	<.0001
Day	1.40624942	0.21399206	6.57	<.0001
treatday	0.52203661	0.04573459	11.41	<.0001
day2	-0.19418737	0.02286285	-8.49	<.0001
Day*Day*Day	0.00509879	0.00068139	7.48	<.0001



2001
treat=0 is control, treat=1 is spontaneous
Yes, there is a difference between control and spontaneous

R-Square	Coeff Var	Root MSE	Median Mean
0.627591	8.847737	1.343341	15.18287

Parameter	Estimate	Error	t Value	Pr > |t|
Intercept	13.45365613	0.48236146	27.89	<.0001
treat	-1.59606868	0.41488969	-3.85	0.0002
Day	0.71424942	0.11064972	6.46	<.0001
treatday	0.10609663	0.02406354	4.41	<.0001
day2	-0.03942999	0.00687909	-5.73	<.0001
Day*Day*Day	0.00049731	0.00011627	4.28	<.0001

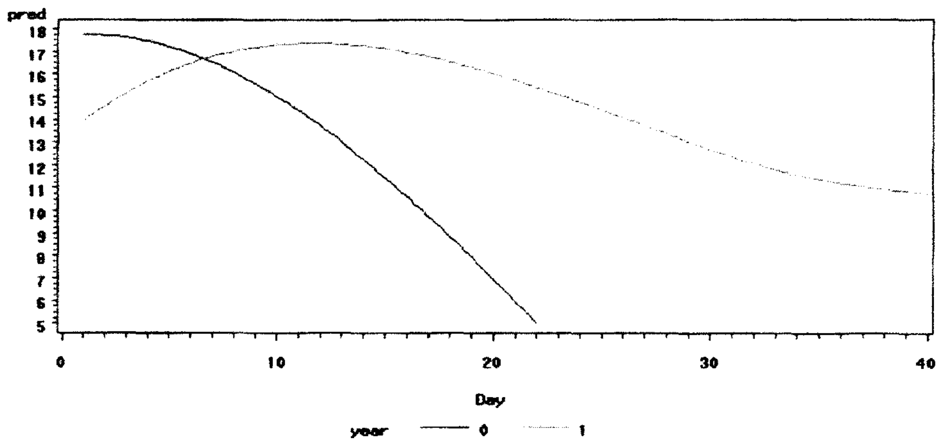


Control

Yes, there is a difference in **year=0 implies 2000 and year=1 implies 2001**

R-Square	Coeff Var	Root MSE	Median Mean
0.769069	11.38833	1.722994	15.12947

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	17.64258585	0.65213674	27.05	<.0001
year	-4.47635485	0.66012170	-6.78	<.0001
Day	0.11750710	0.15625331	0.75	0.4544
yearday	0.67571668	0.06266617	10.78	<.0001
day2	-0.04381585	0.01012077	-4.33	<.0001
Day*Day*Day	0.00056203	0.00017549	3.20	0.0020



Spontaneous

year=0 implies 2000 and year=1 implies 2001

Yes, there is a difference in the spontaneous group.

R-Square	Coeff Var	Root MSE	Median Mean
0.678439	6.991157	1.107482	15.84118

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	15.14552026	0.41917133	36.13	<.0001
year	-3.54195594	0.42430379	-8.35	<.0001
Day	0.68340077	0.10043431	6.80	<.0001
yearday	0.23839262	0.04027969	5.92	<.0001
day2	-0.04750177	0.00650529	-7.30	<.0001
Day*Day*Day	0.00064662	0.00011280	5.73	<.0001