

Isolation, Identification and Initial Fermentative Characterization of Fourteen Wild Yeast Strains from Pinot Noir Grapes Grown in the South Okanagan, British Columbia.

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Abstract

In this study, we characterized fourteen unique yeast isolates from spontaneous fermentations of Pinot Noir grapes from the southern Okanagan region of British Columbia. Spontaneous fermentations rely on the wild yeast present on grapes skins to convert glucose to ethanol. Yeast isolates were identified through amplification of the ITS2 region by a polymerase chain reaction in addition to their colony morphology traits using various media and microscopes. Because ethanol production is of interest to both biofuel and wine industries, we conducted two experiments to determine potential usefulness of our isolates. In the first experiment, we determined which of our late-fermenting yeast isolates (those isolated after day five) had the fastest fermentation kinetics. We found that the yeast isolate FSL 23 was the fastest fermenter. Secondly, we looked to determine if three of our yeast isolates could outcompete the industry standard *S. cerevisiae* (EC-1118), in either a high sugar condition (200 g/L) and a low sugar condition (20 g/L). Although the commercial *S. cerevisiae* outcompeted the three Okanagan strains after 20 days in each condition, the yeast isolate FSL 17 was the most competitive of the Okanagan strains. Our work has revealed two yeast isolates, FSL 23 and FSL 17, with strong fermentative abilities, which could make them useful in either the biofuel or wine industries.

Introduction

The majority of wine fermentation is carried out by commercial yeast species such as *Saccharomyces cerevisiae*¹; however, it has been reported that spontaneous fermentations, which are carried out by many different genera of yeast² can result in a more complex taste of wine³⁻⁵. The wine industry could benefit from the discovery of wild yeasts with desirable traits such as faster fermentations. In the province of British Columbia, the wine industry is a growing sector of the economy with the Okanagan Valley being the oldest, largest, and most important

wine region in this area⁶. This makes characterizing the performance of yeast from the South Okanagan of British Columbia an important objective of global wine industries.

Being able to continuously produce wines with a particular taste is important to the wine industry and for this reason, commercial yeast strains are often used. However, wine produced by the multiple yeasts in a spontaneous fermentation can greatly alter the flavours and aromas producing more complex wines⁵. It is the population present on the grape skin that is the source of yeast for spontaneous fermentation. Many factors affect the diversity of the yeast population. Some of these factors include production area⁷, climatic conditions, age of vineyard, grape variety, harvesting technique, and the type of wine produced⁸. In addition, as stated in Maro *et al.*, commercial yeasts can negatively influence the natural microflora in grape must to the extent that certain desired metabolic components, such as higher alcohols and other metabolic by-products, are no longer produced in adequate amounts^{9,10}. Some wine producers are readopting the traditional spontaneous fermentation method for wine production in order to produce unique attributes that differentiate their wine and improve wine quality¹¹.

In the present study, we isolated and characterized 14 wild yeast from the Black Sage Bench of British Columbia's south Okanagan region. Many studies have previously examined wild yeast populations in different geographic locations^{8,11}, but besides one study focusing on the northern Okanagan¹², the Okanagan Valley has been left uncharacterized. The Okanagan Valley stretches for 155 km¹³. Due to the vast area, there are a range of mesoclimates found here which influence the type of yeast strains that thrive in each individual vineyard and can result in yeast with unique adaptations. Therefore, we expect the north and south Okanagan to be home to differing yeast populations. Furthermore, examining the different yeast strains present in the British Columbia's south Okanagan region will reveal information about the biodiversity of this region.

Various industries from biofuel production to wine producers are interested in finding the most efficient yeast for the production of ethanol. Throughout a fermentation, the ethanol levels increase due to the yeast's metabolic activity. Therefore, yeast isolated later on in fermentation would be expected to have a higher ethanol threshold and may be more useful to industry. For these reasons, we looked at the three morphologies that were isolated on and after day five of the Pinot Noir fermentation when the diversity of yeast decreased and ethanol levels were thought to have debilitated the vast majority of yeast.

It is common practice to inoculate crushed grapes with commercial yeast, in anticipation that they will outcompete the wild yeast¹⁴. For this reason, we inoculated crushed grapes with several isolated wild strains and a commercial strain in a mixed population. Environmental factors such as the amount of available sugar can largely influence a strain's ability to thrive; therefore we tested this mixed population in both a low and high sugar environment¹⁵. Determining the growing habits of our identified yeast may reveal more insights into the potential use of these yeast in industry.

Materials and Methods

Grape selection and crushing

Whole Pinot Noir grape clusters were harvested randomly from Stoneboat Vineyards in Oliver, B.C., and transferred aseptically into four replicate bags. Samples were transported on ice for 6 hours to our laboratory at Science World in Vancouver before crushing. The grapes were aseptically crushed and stems removed so that they could be poured through a funnel into each of the four 2000 mL conical flasks.

Spontaneous fermentation

Time points were collected at T= 0, 0.5, 1, 1.5, 2, 3, 5, 7, 9, 12, 14, 16, 19, 21, 23 days following initial crushing. Fermentation flasks were swirled and any forming grape skin mass was broken up prior to each time point. Time points T=0 to T=3 were serially diluted to 10^{-2} , 10^{-3} , and 10^{-4} ; 100 μL of these dilutions were plated on yeast-peptone-dextrose (YPD) plates containing biphenyl and chloramphenicol. For the time points after T=3, 10^{-4} , 10^{-5} , 10^{-6} dilutions were plated. Plates were grown until colonies were clearly visible (~2 days) and subsequently stored at 4°C until the fermentation was complete. Unique colony morphologies were identified and, if possible, two replicates were chosen for each one to further characterize. Yeast were isolated from any of the quadruplicate fermentations. The following equation was used to calculate the amount (in grams) of ethanol produced by each fermentations:

$$\text{Grams of ethanol} = \text{mass loss (g CO}_2\text{)} \times \frac{1 \text{ mol}}{44.01 \text{ g}} \text{CO}_2 \times \frac{46.07 \text{ g}}{1 \text{ mole}} \text{C}_2\text{H}_5\text{OH}$$

Media

YPD agar mixture was prepared with 10 g of yeast extract (Fisher Scientific), 20 g of peptone (Fisher Scientific), 20 g of dextrose (BioShop), and 20 g agar (BioShop) and made up to 1 L with distilled water. YPD agar was autoclaved for 30 min prior to pouring. Selective plates for yeast growth were made by the addition of chloramphenicol (0.1 mg/mL) and biphenyl (0.15 mg/mL) to cooled YPD agar. Wallerstein (WL) agar plates were made according to the manufacturer's instructions (Becton, Dickinson and Company). Cycloheximide plates were made by the addition of cycloheximide (Sigma- Aldrich) to a final concentration of 20 mg/ L.

Microscopy

Yeast were grown in an overnight culture of 2 mL in liquid YPD. Cultures were diluted by the addition of 50 μL of overnight culture to 2 mL of fresh YPD. Cultures were allowed to grow for 8

hours and imaged using the 60X objective for a final magnification of 600X on a light microscope (Reichert). Colonies on plates were imaged using a dissecting scope (Nikon).

Molecular ID

DNA Extraction from Yeast Colony Morphology Isolates

Yeast DNA was extracted from each of the 24 isolates by a phenol chloroform extraction, DNA was precipitated with ethanol, dried, resuspended in 50 µL of distilled water and stored at 4°C.

Amplification of ITS2 regions in yeast isolates

A polymerase chain reaction mixture for 15 reactions was made with 144 µL dH20, 37.5 µL 10x Thermo Buffer (NEB), 7.5 µL dNTP (10mM), 11.25 µL MgCl2 (50mM), 11.25 µL DMSO (100%), and 1.875 µL polymerase (Taq-Thermo Pol). 15 µL of this mixture was added to 10 µL of template DNA (1000 µg) in PCR tubes. Amplification conditions were: 1X initial denaturation phase (95°C for 2 minutes), 35X [denaturation phase (95°C for 30 seconds), annealing phase (47°C for 30 seconds), extension phase (72°C for 1 minute)], and 1X final extension phase (72°C for 10 minute). 20 µL of PCR products were run on a 2% solution agarose gel through gel electrophoresis for 80 minutes at 90 V against a 100 bp DNA ladder (GeneDirex).

Fermentations

Fermentations for Figure 1 were performed as follows. Yeast cultures were grown on YPD agar plates, colonies were resuspended in liquid media and cell densities were calculated with a hemocytometer. Yeast were added to a final concentration of 2×10^6 cells/ mL in triplicate bottles (250 mL sterile mason jars), each with 200 mL of grape juice (Welch's); jars were fitted with lids and air locks. A negative control consisted of 200 mL of grape juice without yeast. The mass was measured 7 times over the course of 19 days. Specific gravity of the grape juice was

measured with a hydrometer prior to the addition of yeast and again at the end of the fermentation.

Fermentations for Figures 2, 3, and 4 were performed as follows. Three yeast species (FSL 17, FSL 19 and FSL 7) and a commercial *S. cerevisiae* (EC-1118) strain were grown on YPD plates. A master inoculum was created with an equal concentration of each of the individual yeast species (4×10^8 cells/mL), which was determined by use of a hemocytometer. Eight autoclaved bottles were filled with 200 mL of yeast-peptone (YP), four of them with 20 g/L dextrose and the other four with 200 g/L dextrose. The six experimental bottles were then filled with 501 μ L of the master inoculum, leaving one control for each sugar concentration. Every two days, for 20 days, flasks were weighed and serial dilutions were performed to 10^{-3} , 10^{-4} and 10^{-5} and 100 μ L were plated on YPD plates.

Results

Isolation of 14 yeast with unique morphologies from spontaneous Pinot Noir fermentation

Yeast were isolated from quadruplicate Pinot Noir fermentations over a span of 23 days and plated on YPD containing biphenyl and chloramphenicol to suppress the growth of mold and bacteria. Multiple yeast isolates with different colony morphologies were selected and further characterized on YPD, Wallerstein media, and cycloheximide (20mg/L)- which is known to suppress the growth of *Saccharomyces* species (Table 1). Colonies were examined for their colour, both from the top or agar side (bottom) of the specimen; margin; elevation; shine; and form (Table 1). To better distinguish the unique topology of colonies, individual yeast colonies were observed using a stereoscope (Figure 5). These terms originate from well established classification practices such as those in the Colony Morphology Protocol (ASM MicroLibrary;

Appendix 2)¹⁶. Light microscopy analysis (600X) of actively growing yeast was also performed (Figure 5). Following these procedures, we identified 14 unique morphologies amongst our Pinot Noir isolated yeast.

Molecular identification of isolated yeast

The ITS2 region is a part of ribosomal DNA that is conserved amongst many species because it is a region near the highly conserved nuclear 5.8S gene and the gene for large subunit ribosomal RNA. Even so, this region is still variable enough to distinguish between closely related genera and is commonly used to differentiate yeast¹⁷⁻¹⁸. Through analysis of ITS2 polymerase chain reaction (PCR) products, as seen in Figure 6, the ITS2 region in the 14 isolated yeast range from 250 base pairs to approximately 450 base pairs. The PCR verified most of the colony morphologies as being distinct yeast. Two pairs of yeast, FSL 14 and FSL 5 as well as FSL 7 and FSL 2, possessed similar ITS2 PCR product sizes; however, their morphologies distinguished them as distinct yeast (Figure 5).

To tentatively identify the isolated yeast (Table 2) we compared the following traits to yeast databases: cycloheximide resistance, morphological traits in colonies and single cells, and ITS2 region size (Enology Access and CBS-KNAW Fungal Biodiversity Centre).

Fermentation profile of yeast isolated in late stage fermentation

At room temperature, the fermentations (200mL) were set up in triplicate for FSL 19, FSL 22, and FSL 23 in grape juice (Ocean Spray). The controls were set up without yeast to account for evaporation and to monitor contamination. We recorded the loss in mass 7 times in 19 days. We carried out our fermentations until the loss in mass was negligible, which occurred at 19 days. The mass losses of the fermentation with FSL 19 and the fermentation with FSL 22 were not statistically different from the mass loss of the controls ($p= 0.629$ for FSL 19 and $p= 0.612$ for

FSL 22). FSL 23 lost significantly more mass compared to the control ($p= 0.002201$, using a two-tailed student t-test; Figure 1). FSL 23 is tentatively identified as *Saccharomyces cerevisiae*.

The hydrometer readings support the observation of mass loss in FSL 23. The initial specific gravity reading of Ocean Spray was 1.058, following fermentation with FSL 23 the specific gravity was 1.001, which indicates a final concentration of 7.74% ethanol. This concentration was calculated using the Standard SG Drop Method as seen in Ritchie Products Company¹⁹. There was 7.15% Ethanol produced in the 200 mL fermentations. There were no significant differences in the specific gravities of the FSL 19 fermentation, the FSL 22 fermentation, and the controls.

Analyses of community dynamics between isolate yeast and a commercial yeast strain

We examined the change in population number among three yeast isolates and one industrial yeast under two different sugar conditions, in an effort to determine if there were differences in competitiveness between yeast upon varying sugar concentrations (Figure 2 and Figure 3). This may be important as grapes appear to be increasing in sugar concentration due to climate change²⁰.

Equal amounts of yeast; FSL 7, FSL 17, FSL 19 and the commercial EC-1118, were added to triplicate cultures of high (200 g/L) and low (20 g/L) sugar, YP media. Yeast strains used were chosen for having easily distinguishable morphologies on YPD plates, and therefore their colony numbers could be counted easily.

Initially in the low sugar concentration FSL 7, FSL 17, and FSL 19's colony forming units (CFU) were greater than that of EC-1118 (*S. cerevisiae*) (Figure 2). However, FSL 7, FSL 17, and FSL 19 gradually decreased in number, while the EC-1118 did not.

In the high sugar concentration, both FSL 7 and FSL 19 decreased in number within the first four days in the fermentation (Figure 3). FSL 17 had the greatest number of CFU in the first 10 days of the fermentation and outcompeted all the species, yet after 20 days of the fermentation, EC-1118 showed the greatest number of colonies.

The yeast in the low sugar fermentations completely converted all 20g/L sugar to ethanol as each fermentation lost over the calculated 1.95 g of CO₂. However, the high sugar fermentation did not ferment to completion as the predicted 19.54 g of CO₂ was not produced (Figure 4).

Discussion

Isolation and characterization of yeasts from fermentation:

The Okanagan is a specific region of importance as it the largest wine producing region in British Columbia²¹ and its indigenous yeast potential has been largely unstudied. We have identified 14 unique yeast from Pinot Noir grape skins isolated from the Black Sage Bench region of the Okanagan. Secondly, we have identified one yeast (FSL 23) as a robust fermenter, demonstrating the ability to ferment quickly compared to the other yeast tested under our conditions. We have confirmed the common belief that by inoculating a fermentation with a commercial yeast strain (EC-1118), the growth of the wild yeast tested is suppressed. However, FSL 17 appears to have persisted during early stages of fermentations.

Although, the 14 yeast identified have unique colony morphologies under differential and selective growth conditions (Figure 5), it is difficult to determine precisely what species to which they belong. The base pair lengths of the ITS2 region was used to tentatively differentiate the yeast at the level of species. PCR amplification size were estimated based on the gel electrophoresis product and thus there is a level of uncertainty associated with the results due to the nature of this technique. Future work will involve the genetic sequencing of the yeast for more precise identification.

Most Effective Fermenter

FSL 23 was the fastest fermenter under our conditions, and so, it may be valuable to people who wish to produce ethanol quickly. We think that FSL 23 may be in the genus *Saccharomyces* (Table 2). In the future, we would like to compare FSL 23's ability to ferment to that of other *S. cerevisiae* strains. If FSL 23 is able to outcompete commonly used commercial *S. cerevisiae* strains, then one may be able to use FSL 23 to improve the efficiency of processes that require fermentation.

FSL 22 and FSL 19 did not ferment. As seen in Figure 1, their mass loss was similar to the control and therefore did not plateau. A longer fermentation period may provide different results as seen in the initial fermentation. It is possible these yeast did not ferment in the initial fermentation but were able to survive.

Competitive Advantage in Yeast

We chose to examine two different sugar concentrations to determine if these sugar concentrations would impact the dynamics of the yeast communities. The competitive advantage shown by *S. cerevisiae* (EC-1118), against our wild yeast (FSL 7, FSL 17, FSL 19) was mainly consistent with past research²². Initially, the wild species thrived, but were eventually

outcompeted by EC-1118. In the high sugar fermentation, EC-1118 completely outnumbered the other species (Figure 3), while in low sugar; FSL 17 was still present at the end of the fermentation, but in lower concentration than EC-1118 (Figure 2). The experiment's results suggest that commercial EC-1118 is able to out-compete the three selected wild yeasts: FSL 7, FSL 17, and FSL 19 in two sugar concentrations. However, it would be beneficial to keep testing FSL 17, as it demonstrated robust growth in the early stage of fermentation and may be useful to industry.

It would be interesting to more closely monitor the dynamics of the wild yeast species as it would more easily explain the trends we see. This could be established with greater biological replicates and more frequent colony counts.

We have started to characterize the indigenous yeast population of the Okanagan. Within the scope of this experiment, we have determined several characteristics of the isolated yeast. Future studies into these yeast, perhaps in different environmental conditions like sugar sources, could yield additional applications in the fields of biofuel production or microbial bioremediation.

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Figures and Tables

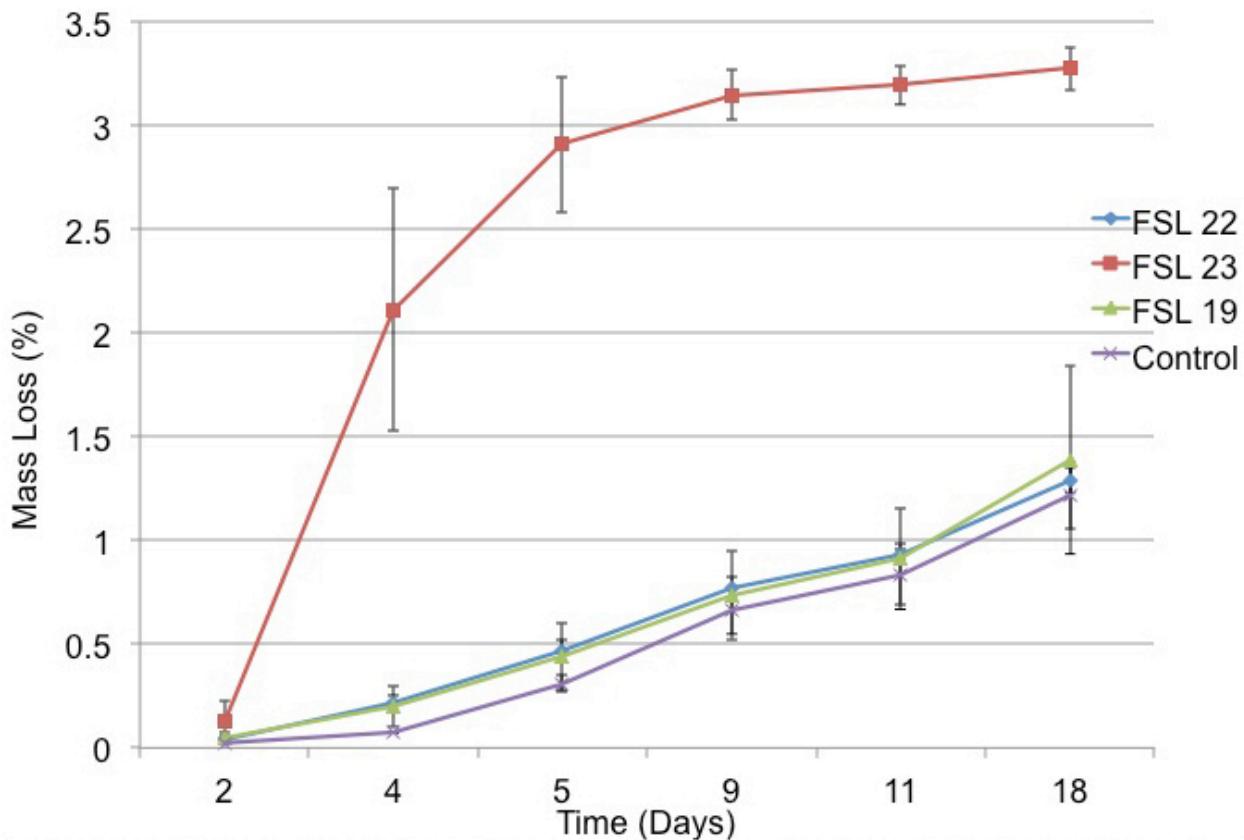


Figure 1: Average mass loss in respective fermentations of three FSL yeasts over 18 days in grape juice. Note that FSL 22 and FSL 24 are members of the same morphology group. Test done in triplicate with error bars representing stand deviation.

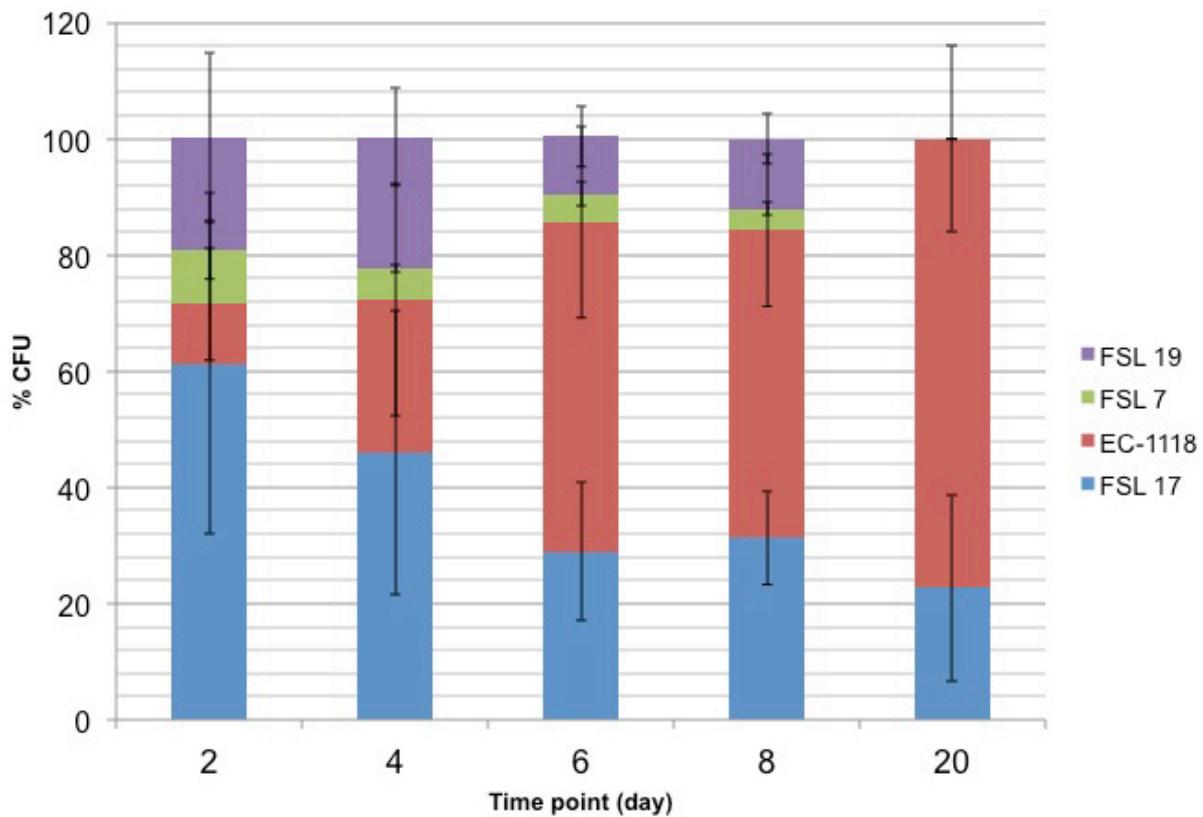


Figure 2: Average percentage of colony forming units (CFU) in low sugar fermentations.

Relative average colonies of each individual yeast species from total number of colonies per plate in triplicate samples of the low (20 g/L dextrose) sugar solutions were counted and plated at indicated time points over a period of 20 days. Error bars represent standard deviation.

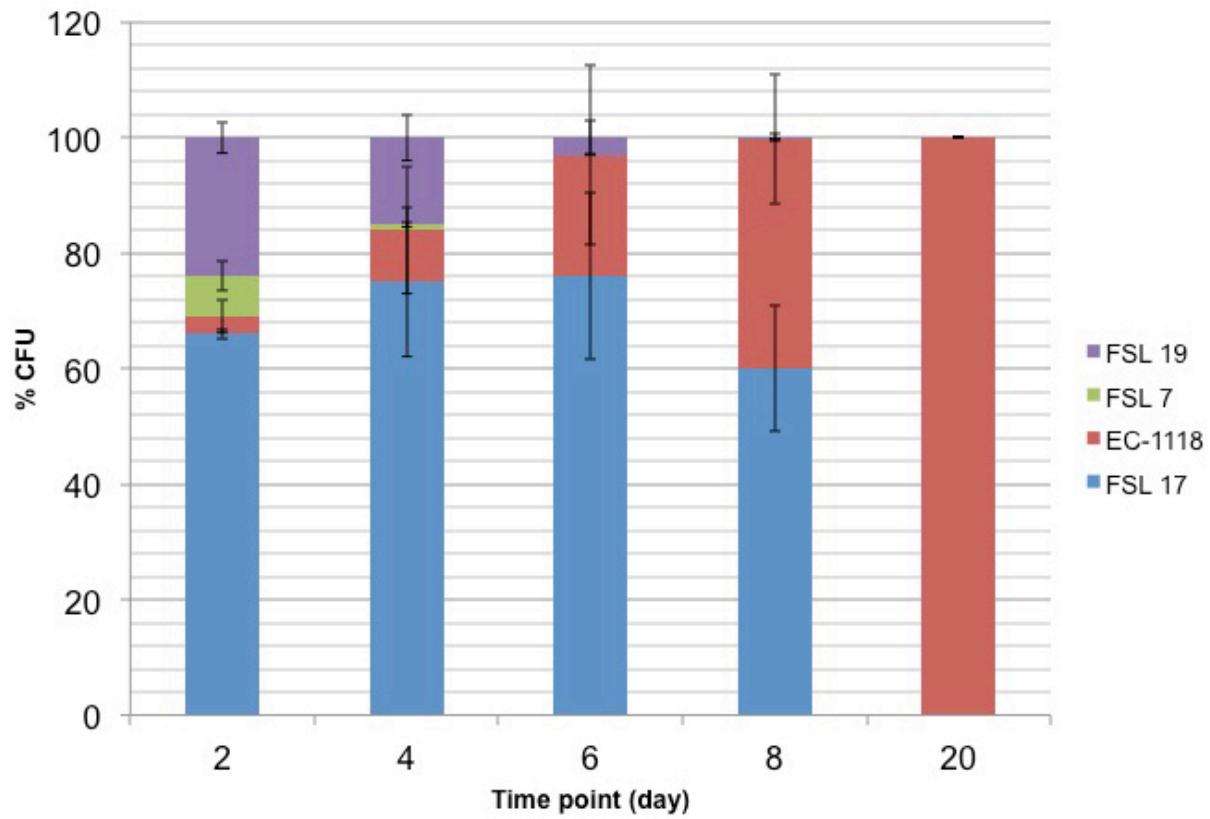


Figure 3: Average percentage of colony forming units (CFU) in high sugar fermentations.

Relative average colonies of each individual yeast species from total number of colonies per plate in triplicate samples of the high (200 g/L dextrose) sugar solutions were counted and plated at indicated time points over a period of 20 days. Error bars represent standard deviation.

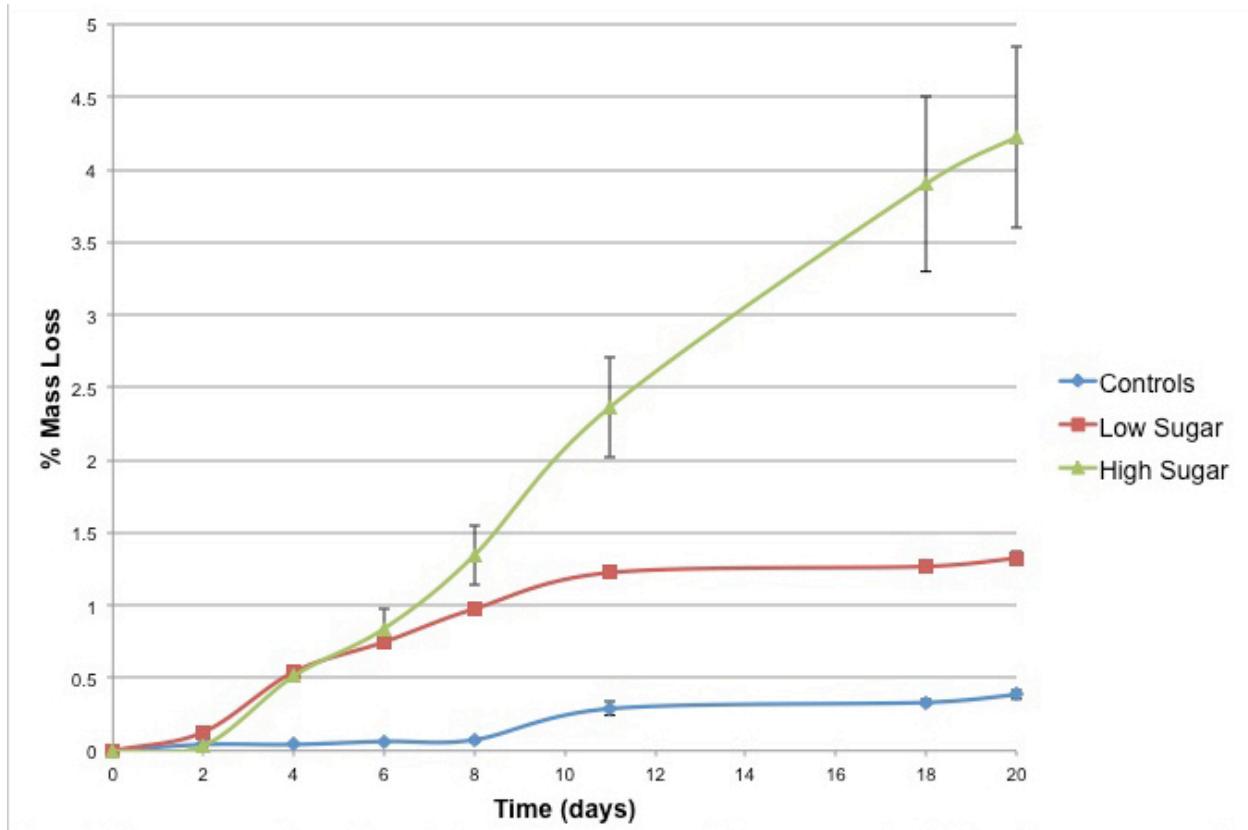
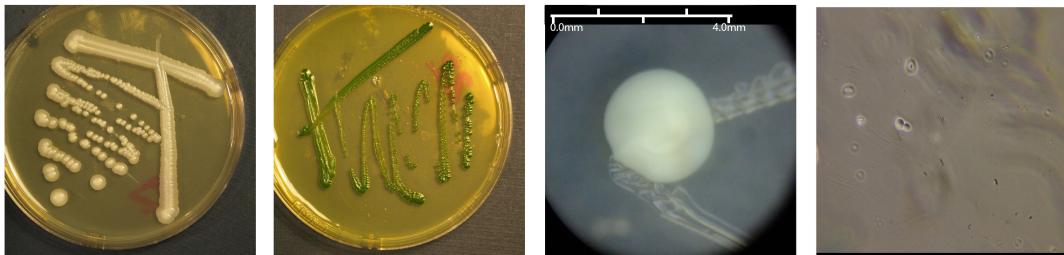
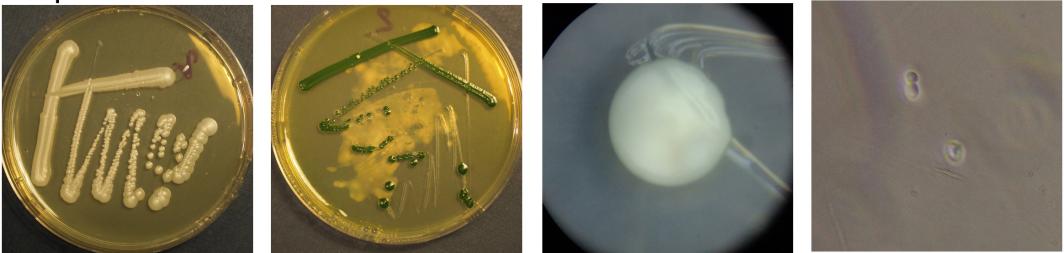


Figure 4: Average percent mass loss of mixed population fermentations over a period of 20 days. Mass was measured every 2 days for a period of 20 days of triplicate fermentations of high (200 g/L), low (20 g/L), and single controls (with no yeast added). Error bars represent standard deviation.

Group A- FSL 22



Group B- FSL 2



Group C- FSL 10



Group D- FSL 9

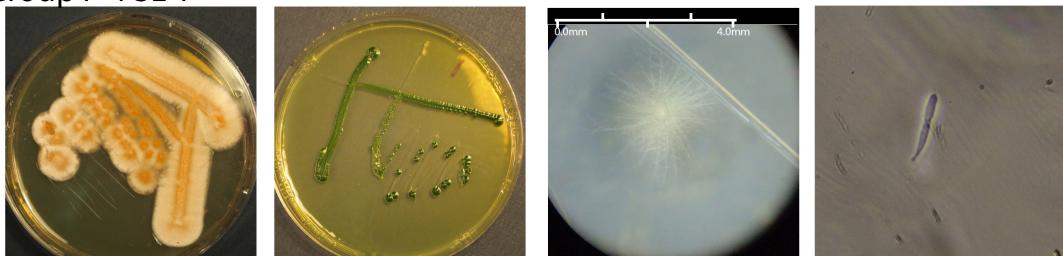


Group E- FSL 11



[Figure 5 continued on next page]

Group F- FSL 1



Group G- FSL 3



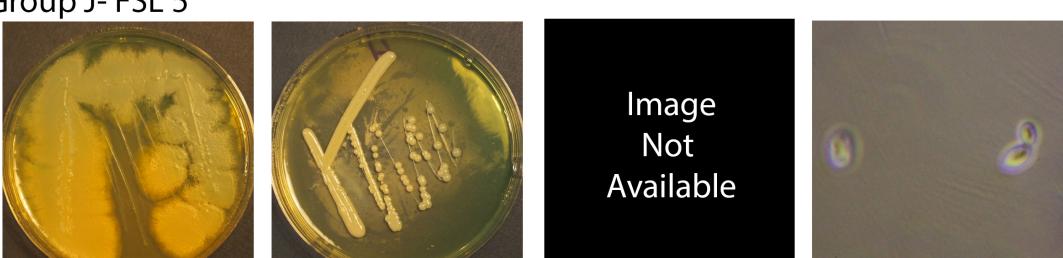
Group H- FSL 4



Group I- FSL 15

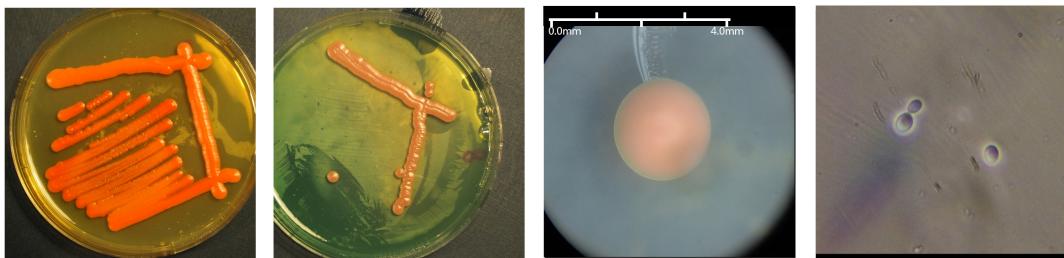


Group J- FSL 5



[Figure 5 continued on next page]

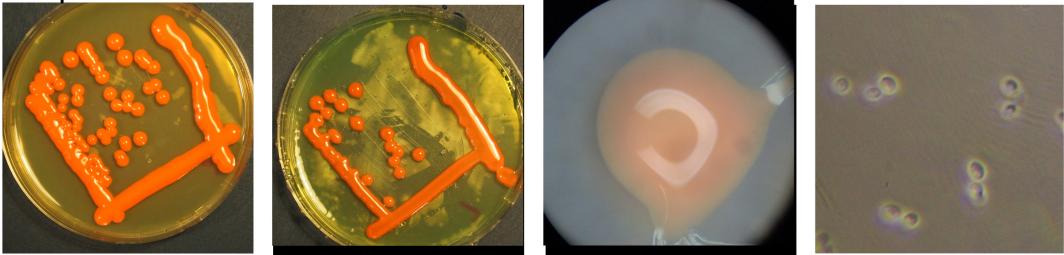
Group K- FSL 8



Group L- FSL 14



Group M- FSL 7



Group N- FSL 6



Figure 5: Morphology composite figure. From left to right, the images show: yeast on YPD media, yeast on Wallerstein media, yeast colony using dissecting microscope, and yeast imaged under a light microscope (600x).

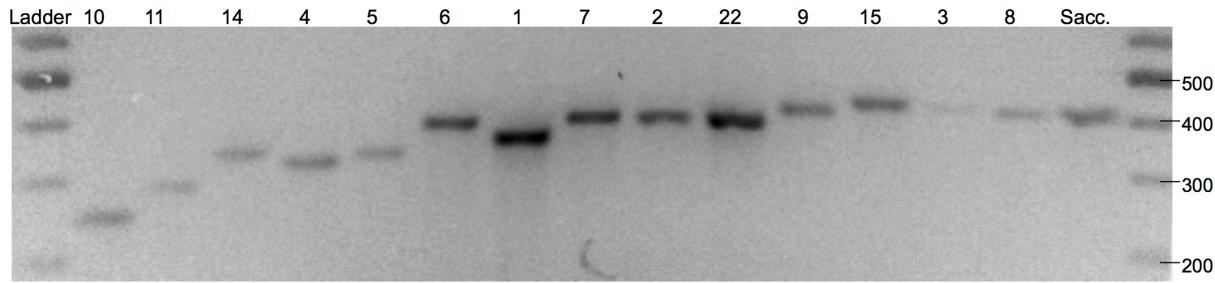


Figure 6: Polymerase chain reaction agarose gel image - gel run at 90V for 50 minutes.

Sacc. represents *S. cerevisiae* control. See control in Appendix 1.

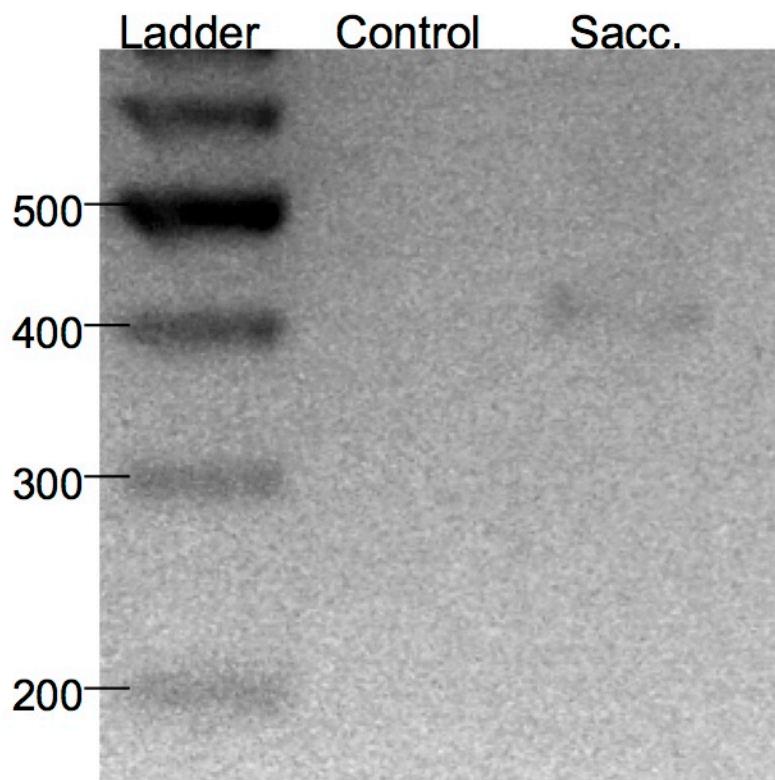
Group	Strain Number	YPD						Wallerstein						Cycloheximide (200mg/L) R=resistance; S=sensitive
		YPD-top-colour	YPD-bottom-colour	Shiny/Dull	Elevation	Form	Margin	WL-top-colour	WL-bottom-colour	Shiny/Dull	Elevation	Form	Margin	
A	FSL 22, 24	light grey, translucent	cream	dull	flat	circular	entire	very dark green	dark green	dull	flat	circular	entire	R
B	FSL 2, 13	cream	cream	dull	flat	circular	entire	very dark green (evenly pigmented)	dark green	dull	flat	circular	entire	R
C	FSL 10, 19	beige	beige	dull	raised	circular	entire	light green w/ white edges	dark green-brown aura	dull	raised	circular	entire	S
D	FSL 9, 18, 20, 21, 23*	white	white	dull	raised	circular	entire	light green w/ white tip	dark green w/ lighter margin	dull	convex	circular	entire	S
E	FSL 11, 17	light cream	light cream	dull	raised	irregular	undulate	light blue-green	light blue-green	dull	raised	irregular	undulate	S
F	FSL 1	orange w/ white edges	orange w/ thin white edges	shiny	umbonate	irregular	filiform	dark green	dark green	shiny	raised	circular	entire	R
G	FSL 3	peach	beige	dull	raised	circular	entire	green-grey	dark green w/ white margin	dull	raised	circular	entire	R
H	FSL 4	peach	beige	shiny	raised	circular	entire	greyish	dark green w/ white margin	dull	raised	round	entire	R
I	FSL 15	white	white	dull	raised	circular	entire	dark green w/ white margin	dark green w/ white margin	dull	raised	circular	entire	R
J	FSL 5	orange	orange	dull	umbonate	rhizoid	filiform	gold	yellow-green w/ yellow-pink margin	dull	flat	irregular	filiform	R
K	FSL 8	light pink (lighter than FSL 7)	light pink	dull	flat	circular	entire	bright pink	pink w/ lighter margins	dull	raised	circular	entire	R
L	FSL 14	pink	pink	dull	flat	filamentous	filiform	pink	pink w/ green middle	dull	flat	filamentous	filiform	R
M	FSL 7	light pink	light pink	shiny	raised	circular	entire	pink	dark green w/ pink margin	shiny	raised	circular	entire	R
N	FSL 6	pink-orange	pink-orange	shiny	convex	circular	entire	dark pink	shiny	raised	circular	entire	entire	R

Table 1: Morphological description of yeast on Yeast Peptone Dextrose plates and Wallerstein Nutrient plates. Yeast are denoted code names: FSL + number. Yeast are grouped into Group A-N based on morphological similarities.

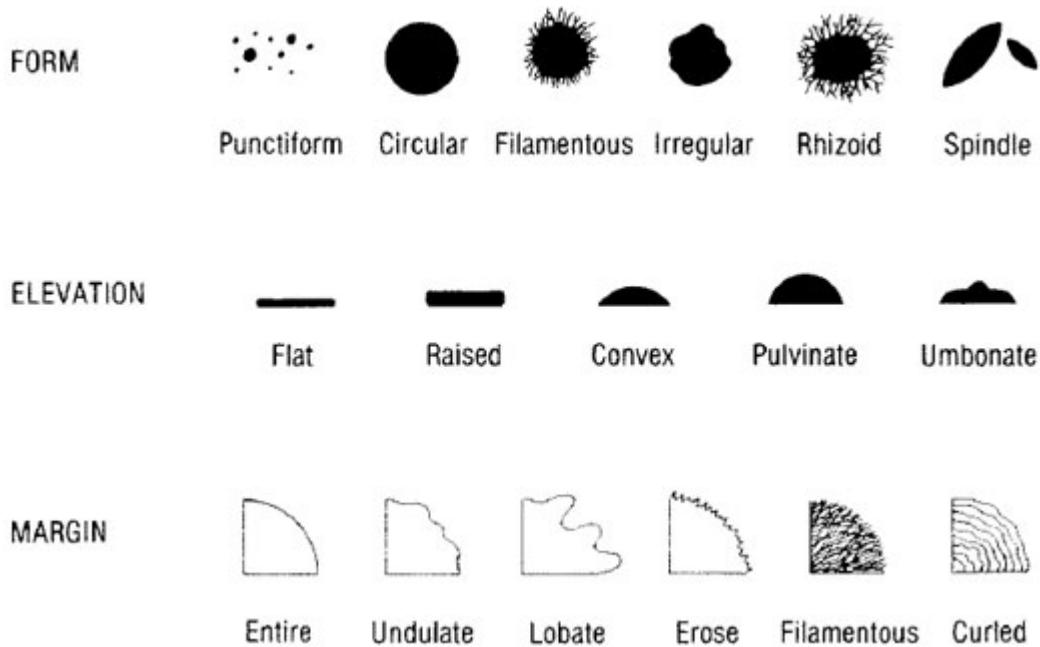
Morphology	FSL Yeast	Tentative Genus Species	Matching PCR
A	FSL 22, 24	Hanseniaspora	Yes ~400
B	FSL 2, 13	Hanseniaspora uvarum	Yes ~400
C	FSL 19	Metschnikowia pulcherrima	Yes ~250
D	FSL 9, 18, 20, 21, 23	saccharomyces cerevisiae or cryptococcus	Yes ~410
E	FSL 11, 17	Pichia fermentans or another species of Pichia	Yes ~300
J	FSL 5	Aureobasidium Pullans	Yes ~350
N	FSL 6	Rhodotula glutinis	Yes ~ 391

Table 2: Tentative identification of yeast isolated from the Okanagan grape fermentation.

Appendices



Appendix 1: The control gel run for the PCR amplification. The control lane contains no DNA and the Sacc. lane contains the DNA of *Saccharomyces cerevisiae* that was used for comparison.



Appendix 2: The Colony Morphology Protocol from American Society for Microbiology.

We used this image to describe the form, elevation, and margin of yeast for Table 1.