Evaluating Genes of Interest for their Impact on Ethanol Tolerance in Commercial Wine Strains of *Saccharomyces cerevisiae*

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Viticulture and Enology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2021

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Abstract

In order to better understand their impact on ethanol tolerance in *Saccharomyces cerevisiae*, six individual genes were overexpressed in two different commercial wine yeast strains, Montrachet and Elixir, and fermented in a high brix, defined, wine-like medium. These genes mainly consisted of those involved in the production of unsaturated fatty acids, although genes dealing with glycerol efflux and reactive oxygen species sequestration were also evaluated. The fermentations were monitored for brix, biomass, and nitrogen utilization. HPLC analysis of hexose sugars, glycerol, and ethanol was reserved for the most interesting of these genes, *FPS1* and *FAS1*, in order to further understand the metabolism associated with these genetic changes. A final fermentation was then run using mutants lacking the *FPS1* gene, and mutants overexpressing both components of the FAS complex, *FAS1* and *FAS2*. The results showed a clear trend of greater ethanol tolerance, measured as the amount of ethanol produced before the cessation of fermentation, with less expression of *FPS1* in Montrachet, but little result in Elixir. The *FPS1* deletion mutant for the Montrachet strain developed higher biomass, utilized more sugar, and produced more ethanol compared to the control. Likewise, overexpression of the FAS complex showed a deleterious effect on the same ethanol tolerance proxies of Elixir, with more muted results in Montrachet. FAS overexpression caused reduced biomass and more sluggish fermentation. These responses demonstrate not only the potential of leveraging these genes for the production of novel commercial strains of *S. cerevisiae*, but also the interesting strain dependent responses associated with their differential expression.

Chapter 1: Research Objectives

Fermentation-derived alcohol production is amongst the world's most significant biotechnologies. Alcoholic fermentations in the biofuel and alcoholic beverage industries amounted to about 100 billion liters of ethanol worldwide each year as of 2018 (Walker and Walker 2018). The economic impact of these industries is great, with the fuel ethanol market worth approximately $60 billion in 2017, and a beer and wine market worth approximately $570 billion and $100 billion, respectively, in 2018 (Eliodório et al. 2019). The vast majority of this alcohol is produced by yeast of the *Saccharomyces* genus, most specifically by budding yeast, *Saccharomyces cerevisiae*.

Saccharomyces cerevisiae ferments simple hexose sugars glucose and fructose into ethanol, allowing for alcohol production from a diverse set of organic inputs such as fruits, grains, and starches. The myriad of inputs to industrial fermentation spawns an even greater number of products, from biofuels to wine. All are equally reliant on one species of microbe. As a result, the problems associated with fermentation by *Saccharomyces cerevisiae* are significant across all industries in which alcohol production is critical. Because the use of *Saccharomyces cerevisiae* as the driver of inoculated fermentations is virtually ubiquitous, improvements to the organism can be potentially cost saving, quality improving, and risk reducing to a variety of industries. For the purposes here the focus will be directed toward the wine industry.

The past half century has produced significant advances in genome sequencing and editing. The ability to identify genes within the *Saccharomyces* genome as well as to elucidate their function has allowed scientists to make predictions of phenotypic changes based on differential gene expression, and then to observe these changes in living mutants. Should these

genetic annotations lead to useful phenotypes, these advances provide an avenue for the creation of new yeast strains that are better suited to their fermentative tasks. It is therefore critical that genes of interest be identified that might potentially alleviate industry problems, and that these genes are tested in order to determine their capacity to tailor the species to industry needs.

Stuck fermentations, or fermentations that stop prematurely, are a universal problem in alcohol production. Stuck fermentations can be a result of sudden or drastic temperature changes, nutrient depletion, interactions between microbe species, and, most notably for the purposes here, rising concentrations of ethanol. The primary product of fermentation, ethyl alcohol damages the yeast cells which produce it (Bisson 1999).

Saccharomyces cerevisiae is more ethanol tolerant than most microbes, a fact that allows it to wrest control of its environment, but there are limits to this tolerance. The alcohol tolerance of the fermenter creates an upper limit to the amount of ethanol that can be produced from a single fermentation. It would therefore be advantageous to control the ethanol tolerance of the fermenter in order to, for example, maximize ethanol produced from grain inputs in a single biofuel fermentor, or to limit stuck fermentations from high sugar musts in the wine industry. Decreasing ethanol tolerance can also be potentially desirable. Strains of *Saccharomyces cerevisiae* more susceptible to ethanol toxicity could be used to ensure a wine beverage with low alcohol and residual sugar without expending the energy necessary to prematurely arrest fermentation by chilling. While high ethanol concentrations have long been known to cause stuck fermentations, the methods by which *Saccharomyces* tolerates increasing concentrations is likely complex and remains largely unknown.

The primary determinant of alcohol tolerance in *Saccharomyces cerevisiae* is thought to be membrane structure (Tesniere 2019)(Henderson and Block 2014). The cell membrane is composed of a lipid bilayer containing both saturated and unsaturated fatty acids with attached glycerophosphate heads, sterols, and proteins. In the presence of excessive amounts of alcohol these fatty acids can interdigitate, causing a dramatic thinning of the cell membrane. This thinning of the cell membrane often exposes hydrophobic portions of integrated proteins which can result in conformational changes and lack of function. The interdigitation of the membrane eventually allows alcohol to enter the cell, causing the production of radical oxygen species and significant changes in redox state. This mode of action suggests that changes in membrane composition to limit membrane interdigitation, as well as increased ability to quench oxidation reactions and maintain redox state might improve a yeast strain's ability to survive growing ethanol concentrations.

The purpose of this research was to investigate the overexpression of a selected set of genes on the ethanol tolerance of two different commercial wine strains of *Saccharomyces cerevisiae*. Ethanol tolerance was measured here by proxy, with the direct measurements being cell mass, sugar utilization, and ethanol production. Mutants overexpressing genes coding for fatty acid, membrane protein, and superoxide dismutase production were grown in a defined medium emulating a high sugar content grape must. Their biomass, fermentative capacity, and metabolism were monitored in order to asses changes in their ability to ferment high levels of sugar to high concentrations of ethanol. A total of six genes were individually over expressed and tested for their effects on alcohol tolerance in an attempt to identify genes useful for the creation of novel *Saccharomyces cerevisiae* strains. The most interesting of these was then subject to

further testing by fermentation at a sugar content more typical for the wine industry, and null mutants were tested in like fashion to the initial high brix fermentation.

Chapter 2: Literature Review

This literature review will seek to explain the current state of research regarding ethanol toxicity of the yeast cell, as well as to provide rationale as to why the individual genes used in the following experiments were chosen. This information is useful to most commercial applications of *Saccharomyces cerevisiae*, but the scope of this writing has been limited mainly to that which pertains directly to the wine industry.

2.1 Ethanol Toxicity and the Cell Membrane of Yeast

There is mounting evidence that the main target of the deleterious effects of rising ethanol concentrations is the yeast cell membrane (Tesniere 2019)(Henderson and Block 2014). It is known that high concentrations of alcohol increase passive proton movement into the cell, and that higher concentrations of ethanol yield greater increases in proton movement (Casey and Ingledew 1986)(Madeira et al. 2010). Further exacerbating this problem, high concentrations of ethanol can interfere with Pma1, an H+ ATPase integral membrane protein charged with pumping protons out of the cell (Aguilera et al. 2006). Increased H+ ATPase activity in *Saccharomyces cerevisiae* strains has been correlated with increased ethanol tolerance (Tesniere 2019). The combination of these effects results in a reduction in the cell's ability to maintain internal pH and membrane potential, reducing the cell's capacity for nutrient uptake and eventually causing depolarization. In addition to altering permeability and protein function, the fluidity of the membrane increases with greater concentrations of ethanol, and the ability of the yeast strain to resist such changes has been correlated with increased tolerance (Jones et al. 1987, Alexandra et al. 1994, Huffer et al. 2011). These observable effects on the membrane in the

presence of ethanol, as well as the membrane's position dividing the cell from its environment, indicate it as being critical to the cell's ability to survive in such environments. Because the membrane seems to be the primary target, efforts to create strains of *S. cerevisiae* with increased ethanol tolerance have largely been concerned with understanding exactly how ethanol affects the membrane, and in what ways these can be combated.

In order to better understand the effects of ethanol on the membrane, an overview of the components and function of the membrane is necessary. The basic structure of the yeast cell membrane is made up of a bilayer of phospholipids embedded with membrane proteins and sterols. The major phospholipid classes present in the *S. cerevisiae* membrane are phosphotidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) (Henderson and Block 2014)(Renne and de Kroon 2017). These classes differ in which molecule is bonded onto the sn3 position of the glycerol-3-phosphate backbone of the phospholipid, and with this backbone, make up the hydrophilic portion of the membrane. Esterified to the sn1 and sn2 positions, and forming the hydrophobic portion of the membrane, are two fatty acid chains. The majority of those found in yeast membranes are palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), and oleic acid ($C_{18:1}$) (Henderson and Block 2014)(Renne and de Kroon 2017). These glycerophospholipids make up the barrier between the cell and its environment, and a variety of different integral proteins span it in order to sense the external environment and facilitate transport of select molecules across the membrane.

It is primarily through interactions with the glycerophospholipids that ethanol damages the membrane. When it comes into contact with the membrane, ethanol forms hydrogen bonds

with both the phosphate head and the glycerol backbone. In studies of contact probabilities via nuclear magnetic resonance spectroscopy, it has been observed that the greatest probabilities, and therefore the majority of these bonds, occur between the ethanol molecules and the glycerol backbone (Feller et al. 2002)(Holte et al. 1997). When these bonds form, the ethanol molecules arrange themselves such that their more hydrophilic hydroxyl reaches toward the outside of the membrane and the water solvent, while the remaining hydrocarbons plunge down into the fatty acid chains of the membrane's interior (Tierney et al. 2005)(Ly et al. 2002)(Vanegas et al. 2012). As more ethanol molecules make similar bonds and arrange themselves in between the phospholipids, the space between the phospholipids increases due to the steric interactions of the bonded ethanol molecules, causing an increase in membrane fluidity (Feller et al. 2002)(Holte et al. 1997). Eventually the ethanol drives the phospholipids far enough apart that they interdigitate, meaning that the fatty acid tails of the phospholipids slide past each other to be in parallel (Vanessa et al. 2012)(Rowe and Cutrera 1990). This causes a dramatic thinning of the cellular membrane. It is this thinning of the membrane and disruption of the phospholipids which are believed to be the direct effects on the membrane proteins under ethanol stress.

The phospholipids surrounding membrane bound or integrated proteins are critical to protein function, and changes in these can cause reduced or loss of function for the embedded proteins. The surrounding phospholipids can be classified as either annular or non annular. The bulk of these lipids are annular, sometimes called boundary lipids, meaning that their primary function is to anchor the protein in place and to surround it with an annular shell (Lee 2004). The class of lipid serving as an annular lipid is not thought to be important, as they need only hold and shelter the protein. The turnover rate for these lipids moving in and out of their annular

position is quite high. Non-annular lipids, however, are much more specific and have a much lower turnover rate. Non-annular lipids act as cofactors for the proteins, and are often found attached to transmembrane alpha helices or at the connections between protein subunits. Both their fatty acid tails and head groups are highly specific, and as a result they do not frequently shift or trade positions with other lipids. Non-annular lipids are considered essential to the function of the protein much like any other cofactor (Lee 2004). As there are two types of protein associated lipids, it follows that there are two ways in which changes to these by membrane thinning can affect proteins.

The process of membrane thinning by interdigitation could disrupt protein function by altering the position at which non-annular lipids are situated in the membrane, eliminating them as an effective cofactor, and subsequently rendering the proteins unable to perform their functions (Lee 2004). The second way in which this reduction in membrane thickness causes a loss of protein function is through a phenomenon known as hydrophobic mismatch. Hydrophobic mismatch occurs when the membrane thins to such a degree that the hydrophobic portions of the protein are exposed to the polar solvent, rather than their usual position inside their annular shell of acyl tails (Lee 2004). This exposure of hydrophobic residues will cause the protein to aggregate in order to shield itself from the polar solvent more effectively. This conformational change will, in all likelihood, render the protein non-functional. It is likely by one or both of these effects that the aforementioned Pma1 H+ ATPase interference occurs. Additionally, it is possible that the cessation of sugar utilization is caused by a change in conformation of the sugar transport proteins.

2.2 The Impact of Sterols and Acyl Chains on Strain Ethanol Tolerance

There have been a variety of studies investigating the membrane composition and differences of more ethanol tolerant strains in attempts to develop correlations between membrane components and increased tolerance (Kim et al. 2011)(Chi and Arneborg 1999) (Henderson et al. 2013)(Dong et al. 2015). Others have attempted to alter the components of model membrane systems and then subject them to different levels of ethanol (Vanegas et al. 2012). While these results can potentially lead to greater understanding of which membrane components and what types may be best suited to combatting ethanol effects, it is important to note that the frequently used method of "ethanol shock" does not well mimic a realistic fermentation scenario. A large proportion of those studies looking into the ethanol tolerance of yeast do so by adding ethanol to the media during the growth phase of the culture, thereby subjecting it to ethanol shock. This may test how the membrane handles ethanol, but not in a way typical of that observed in a commercial fermentation. In a typical fermentation the ethanol is developed more slowly, and the vast majority of it is produced only once the culture population has reached its stationary phase (Cramer et al. 2001). Additionally, in a commercial setting, it is not enough that a yeast cell survive a high concentration of ethanol. It must also remain metabolically active in order to avoid a stuck fermentation. A yeast cell that survives a high degree of ethanol stress, but no longer ferments sugar to alcohol is of limited utility.

In one such study, where ethanol shock was used to determine the effects of different membrane components on ethanol tolerance, Tierney et al. exposed model membrane systems of dipalmitoylphosphatidylcholine (DPPC), cholesterol, and ergosterol to increasingly more concentrated solutions of ethanol (Tierney et al. 2005). They found that ergosterol concentration

within these model membranes increased the concentration of ethanol necessary for the membranes to enter the interdigitated phase (Tierney et al. 2005). Venegas et al. performed a similar experiment, with vesicles of differing concentrations of DPPC and dioleoylphosphatidylcholine (DOPC) (Vanessa et al 2012). Similar model systems were observed by the same group via atomic force microscopy and fluorescence imaging to form a phase diagram of the systems at 20% ethanol (Vanegas et al 2010). The results of both trials indicated that increasing concentrations of ergosterol and unsaturated fatty acids (DOPC) reduced the membrane's readiness to interdigitate. These trials were in agreement with del Castillo (1992) which reported ergosterol and unsaturated fatty acids to be helpful in maintaining membrane fluidity when *Saccharomyces* is exposed to ethanol (del Castillo 1992). Ergosterol is the main sterol in *Saccharomyces cerevisiae* and has been the subject of a variety of inquiries into ethanol tolerance.

Ergosterol interspersed throughout the membrane is thought to help prevent interdigitation by protecting the acyl chains from their loss of favorable Van Der Waals forces as the phosphate heads of the lipids are pulled apart by the steric effects of the ethanol (Vanegas et al. 2012)(Henderson and Block 2014). This is theorized to occur because the ethanol pushes ergosterol embedded in the membrane down toward the terminal ends of the acyl chains, preventing them from sliding past each other (Henderson and Block 2014).

But despite studies finding ergosterol to be beneficial in this regard, there are some that find no correlation between ergosterol concentrations and a strain's ability to survive at high concentrations of ethanol (Henderson et al. 2013)(Mannazzu et al. 2008). In a study of 22 different *Saccharomyces* strains, Henderson et al found a correlation between ergosterol content

and maximum cell mass achieved, but no correlation between ergosterol and final ethanol concentration (Henderson et al. 2013). In a study looking at 3 different strains in a lipid nutrient deficient fermentation media, Mannazu et al observed that higher ratios of C16 fatty acid chains:total fatty acids and unsaturated fatty acids:total fatty acids were critical to ethanol tolerance, while ergosterol content and total lipid amount were not (Mannazzu et al. 2008). This supports the idea that longer and unsaturated fatty acids hinder bilayer interdigitation. Longer fatty acid chains increase the thickness of the membrane, increasing the number of carbons between head groups (Renne and de Kroon 2017). Unsaturated fatty acid chains are thought to work much like ergosterol in the sense that steric interactions prevent interdigitation. Saturated fatty acids may be more likely to interdigitate because the acyl chains are much more straight, whereas the double bond in an unsaturated chain causes the chain to splay outward, increasing the head group spacing needed for the opposite acyl chain to slide by (Vanegas et al. 2012)(Ding et al. 2009). This could potentially be why yeast that survive greater levels of ethanol tend to have higher concentrations of unsaturated fatty acids, and greater percentages of longer saturated fatty acids.

If greater amounts of long chain fatty acids and greater relative amounts of unsaturated fatty acids have been observed to prevent membrane interdigitation, and membrane interdigitation presents perhaps the most deleterious effect of ethanol on the cell, then it would make sense to attempt alterations to the *Saccharomyces* membrane in order to capitalize on these potential benefits. This first requires some understanding of how these fatty acids are produced and desaturated. Fatty acids can be acquired in three ways by the yeast cell. They can either be taken up from the environment, salvaged from the degradation of more complex lipids, or

synthesized de novo (Tehlivets et al. 2007). De novo synthesis of fatty acids (acyl chains) is extremely dependent on a variety of factors including strain, temperature, media, and growth phase (Renne and de Kroon 2017). It begins with acetyl-CoA (C2). Acetyl-CoA is carboxylated using CO_2 by acetyl-CoA carboxylase (Acc) into malonyl-CoA (C3) (Renne and de Kroon 2017). This is the rate limiting step in the de novo synthesis of acyl chains in yeast, and requires biotin as a cofactor. Cytosolic Acc is coded for by the gene *ACC1*, and the mitochondrial variant is coded for by *HFA1* (Tehlivets et al. 2007). The acetyl-CoA then acts as a very important two carbon donor to a following series of cyclical reactions ending in the finished chain. These reactions begin by the attachment of malonyl-CoA to a protein complex known as fatty acid synthase (FAS) (Tehlivets et al. 2007). The FAS complex is composed of an alpha and a beta subunit, coded for by the genes *FAS2* and *FAS1*, respectively. This protein complex is extremely complex, with the alpha subunit containing acyl carrier protein, 3-ketoreductase, 3-ketosynthase, and phosphopantheteine transferase, and the beta subunit containing acetyl transferase, enoyl reductase, dehydratase, and malonyl-palmitoyl transferase (Tehlivets et al. 2007). The FAS complex and its associated reactions will then elongate the acyl chain to be largely C16:0 or C18:0, but with minor amounts of C10:0, C12:0, and C14:0 (Renne and de Kroon 2017) (Tehlivets et al. 2007). FAS is capable of synthesizing chains of up to C20:0, but only very rarely does so. Another set of proteins: Elo1p, Elo2p, and Elo3p, of which Elo1p is the most important, have a partially overlapping function with the FAS complex. They will elongate acyl chains mainly from C14:0 to C16:0 or from C16:0 to C18:0 but will in rare cases elongate fatty acid chains up to C26:0 (Tehlivets et al. 2007). Interestingly, once a fatty acid has been desaturated, the FAS complex will no longer elongate it. However, Elo1p (coded for by the gene *ELO1*) has

been shown to elongate monounsaturated acids C14:1 and C16:1 to C:16:1 and C18:1 (Schneiter et al. 2000)(Sec et al. 2015).

There are no polyunsaturated fatty acids in *Saccharomyces cerevisiae*, and only two species of monounsaturated fatty acids. Both, palmitoleic (C16:1) and oleic (C18:1), are produced by the desaturation of palmitic (C16:0) and stearic (C18:0), respectively. This occurs by the formation of a double bond between carbons 9 and 10, catalyzed by delta 9 fatty acid desaturase. This protein is produced by the gene *OLE1* (Kim et al. 2011)(Nasutian et al. 2017) (You et al. 2003).

Kim et al. 2011 overexpressed delta 9 fatty acid desaturase gene *OLE1* as well as expressing two fatty acid desaturases not normally found in *Saccharomyces cerevisiae* in a laboratory strain of *Saccharomyces* (Kim et al. 2011). They found that when *OLE1* was overexpressed, the yeast contained a higher concentration of unsaturated fatty acids, and had enhanced viability when exposed to ethanol shock (Kim et al. 2011). Interestingly, while the strains constructed with the other two desaturases indeed created several polyunsaturated fatty acids, the overall ratio of unsaturated to total fatty acids did not change, and there was no enhanced viability when exposed to ethanol shock (Kim et al. 2011). This suggests that it is indeed the relative amount of unsaturated fatty acids to the total fatty acids that is conferring the ethanol tolerance benefits, and not the rate of unsaturation. In 2003, You et al. performed an experiment in which they deleted *OLE1* in a laboratory strain, supplemented it with known amounts and species of unsaturated fatty acids, and then exposed those mutants to ethanol shock. They determined that ethanol tolerance differences were largely attributable to oleic acid (C18:1), and that palmitoleic acid (C16:1) seemed to have very little ability to increase tolerance

(You et al 2003). Further generating interest in the *OLE1* gene, a study in 2017 which overexpressed *OLE1* in laboratory yeast grown on synthetic complete media observed an increase in membrane oleic acid (C18:1), enhanced proton efflux, reduced membrane permeability, lower intracellular hydrogen peroxide concentration, and enhanced growth in the presence of low concentrations of alcohol (Nasutian et al. 2017). In the same study, overexpression of *OLE1* was also found to constitutively activate Hog1, a mitogen-activated protein kinase, which was then able to activate expression of much of the high osmolarity glycerol (HOG) pathway, normally responding to osmostress, heat, and reactive oxygen species (Nasutian et al. 2017). One would think, based on the results of these two studies, that *OLE1* would likely see a higher level of expression when levels of ethanol are higher later in fermentation. Dong et al. 2015 did an experiment in which a laboratory yeast strain was grown in YPD media, and at different phases of growth, cells were removed and tested for ethanol shock tolerance, membrane composition and permeability, cell swelling rate, and gene expression by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). They looked at the expression of 3 different genes: *ACC1*, *HFA1*, and *OLE1*. What they found was that *OLE1* was quite active in the exponential phase of the culture, but was far less active at stationary phase (Dong et al. 2015). This could potentially be because the desaturation reaction catalyzed by Ole1p requires oxygen (You et al. 2003). But the samples taken during stationary phase had higher survivability when exposed to an ethanol challenge than the exponential phase, and it is during stationary phase of cell growth in which the majority of ethanol is produced in a wine fermentation (Dong et al. 2015)(Bisson 1999). *ACC1* and *HFA1*, the two genes coding for malonyl-CoA production in the cytosol and mitochondria and the rate limiting step in fatty acid

synthesis, were upregulated during both exponential and stationary phases as compared to the lag phase (Dong et al. 2015). This suggests that fatty acids are being produced by the cell from inoculation to lag phase, but that desaturation may decrease in proportion as the culture matures. None of the above experiments have observed the effects of self produced ethanol on a mutant overexpressing *OLE1* in a high brix defined media.

ACC1, coding for the cytosolic acetyl-CoA carboxylase, was expressed at higher levels during the stationary phase than *OLE1*, when the samples had a higher rate of survivability from ethanol (Dong et al. 2015). It has been repeatedly observed that acetyl-CoA carboxylase activity can have an effect on average acyl chain length. In an experiment in which the concentration of malonyl-CoA was changed to observe its effect on fatty acid synthesis, Hori et al observed that higher concentrations of malonyl-CoA, produced by acetyl-CoA carboxylase, led to higher average acyl chain lengths produced (Hori et al. 1987). In a much more recent study, it would seem that these results were corroborated and expanded upon. Hofbauer et al. demonstrated that hyperactivity in the *ACC1* gene by phosphorylation of a regulatory protein (Snf1 kinase) led to both greater overall fatty acid production and a greater ratio of C18:C16 fatty acids (Hofbauer et al. 2014). It has also been observed that increased temperatures have increased the activity of *ACC1*, and therefore the average chain length (Hori et al. 1987)(Schneiter et al. 2000). However as the temperature increases, the percentage of unsaturated fatty acids as a proportion of total fatty acids decreases (Renne and de Kroon 2017). Because higher oleic acid (C18:1) concentrations increase the ethanol tolerance of *Saccharomyces cerevisiae* it could be advantageous to increase the C18:C16 fatty acid ratio within the cell membrane but without the accompanying reduction in unsaturation (You et al. 2003).

As previously stated, the next step following acetyl-CoA carboxylase in de novo fatty acid synthesis is the fatty acid synthase complex, the two distinct subunits of which are coded for by the *FAS1* and *FAS2* genes. *FAS1*, which codes for the beta subunit, has some bearing on the level of expression of *FAS2*, the alpha subunit. Excesses of beta subunits have been observed to cause an increase in alpha subunits, possibly by the deactivation of some sort of repressor, while a reduction in beta subunits has been shown to cause a down regulation in *FAS2* (Wenz et al. 2001). Similarly, when multiple copies of *FAS2* have been inserted into a laboratory strain via a plasmid, *FAS1* has been shown to compensate with increased expression (Chirala 1992). In this way, an increase in expression in either gene should increase expression of the complex as a whole. In an experiment with sake mashes, Furukawa et al. observed that overexpression of the *FAS1* gene caused considerable increases in medium chain fatty acids (C6 to C12), at the expense of long chain fatty acids (Furukawa et al. 2003). Unusually, given the results of Chirala's experiments in 1992, Furukawa et al. did not observe a similar increase when *FAS2* was overexpressed (Furukawa et al. 2003)(Chirala 1992). However, when both *FAS1* and *FAS2* were overexpressed together, the proportion of medium chain fatty acids was highest (Furukawa et al. 2003). While the experiment did not address the alcohol tolerances of the yeast mutants, it can be theorized that overexpression of *FAS1*, and the accompanying rise in medium chain fatty acids, could result in lower alcohol tolerance by causing shorter, medium chain fatty acids to take the place of longer, long chain fatty acids in the membrane phospholipids. Significant increases in fatty acid production could also cause a reduction in mol percentage of unsaturated fatty acids incorporated into the membrane if there is not also a corresponding increase in delta 9 fatty acid desaturase activity coded for by *OLE1*. The overexpression of both *FAS1* and *FAS2* in a single

mutant would likely exacerbate these problems, assuming that they indeed occur. In an unpublished experiment, Simmons et al fermented a series of mutants over expressing various genes and collected lipidomic and metabolic data. Amongst these mutants was one overexpressing only the *FAS1* gene. This particular mutant displayed a lower maximum optical density, a lower concentration of C18 fatty acids, and a higher concentration of fatty acids of C16 or fewer, as compared to a control. It can be theorized that such a mutant would succumb to self produced alcohol earlier in a high brix fermentation, and that the effects observed would be more pronounced in a mutant over expressing both genes in the FAS complex, but neither of these were tested.

2.3 Reactive Oxygen Species

The presence of moderate to high concentrations of ethanol cause an increased formation of reactive oxygen species mainly through a combination of osmotic and chaotrope stresses (Eardley and Timson 2020). The cell also responds to ethanol stress through a set of highly conserved stress response proteins known as heat shock proteins (Costa et al. 1993). Heat shock proteins are a group of proteins which respond to various stressors such as heat stress, ethanol stress, and a release from anoxia (Costa et al. 1993). However, the activation of the associated genes and the stimulation of synthesis can cause an increase in oxygen consumption which generally causes an increase in reactive oxygen intermediates, adding to the reactive oxygen species already created when the cell is under ethanol stress (Costa et al. 1993). These reactive oxygen species (ROS) then must either be sequestered, or the cell risks protein misfolding, a loss of redox balance, and DNA damage (Jiménez and Benítez 1988)(Costa et al. 1997).

Yeast confront ROS through enzymes known as superoxide dismutases, which dismutate superoxide radicals to hydrogen peroxide, which is then decomposed by catalase or a peroxidase (Costa et al. 1993). The superoxide dismutases in yeast are coded by *SOD1* and *SOD2*, with *SOD1* coding for the cytosolic (CuZnSOD) and *SOD2* coding for the mitochondrial (MnSOD) (Costa et al. 1997). In a 1997 experiment by Costa et al, it was observed that levels of superoxide dismutase expression increased in both the cytosolic and mitochondrial forms throughout exponential phase, but that MnSOD continued to increase into stationary phase while CuZnSOD did not (Costa et al. 1997). Mutants deficient in MnSOD also displayed a lower viability when exposed to ethanol shock during stationary phase (Costa et al. 1997). With stationary phase being where the most ethanol is produced by the cell, this indicates that *SOD2*, and not *SOD1* is the primary superoxide dismutase determining ethanol tolerance in yeast. This is in line with the results of Jiménez and Benítez, who determined that mitochondrial genome presence and integrity is critical for viability under ethanol shock in wine yeasts (Jiménez and Benítez 1988). In a study looking at portions of the genome of wine yeast EC1118 not shared by laboratory strains, a copy of a similar gene to *SOD2* was found (Novo et al. 2009). This genome portion was associated with genes acquired in transfer events conferring beneficial phenotypes for a wine environment (Novo et al. 2009). Yeast mutants lacking the *SOD2* gene have been found to reduce ethanol tolerance in some genomic studies, while in others there appears to be little difference from controls in deletion or overexpression mutants (Fujita et al. 2006)(Lewis et al. 2010). However, it should be noted that these experiments were performed with different yeast strains and with different conditions. In an attempt to better understand *SOD2*'s effect on ethanol tolerance, Lee et al expressed *Saccharomyces cerevisiae SOD2* on a plasmid in *Ethanolic*

Escherichia coli and observed the bacteria to display greater viability when exposed to ethanol (Lee et al. 2010). There is therefore evidence both that *SOD2* may confer some degree of ethanol tolerance, and that it may not. These conflicting results are likely due to strain and condition differences across experiments. Observations in a scenario more closely mimicking a commercial wine fermentation could potentially shed light on the usefulness of *SOD2* in breeding wine strains for high brix musts.

2.4 The Effects of Glycerol Efflux

The high brix musts which produce high alcohol wines are associated with high osmotic pressure due to the concentration of soluble solids in solution. The yeast cell responds to these stresses with the production and exportation of glycerol via the high osmolarity glycerol (HOG) signaling pathway (Hohmann et al. 2007). There is evidence that this response mechanism may not only allow a culture to grow in a high brix environment, but also have an effect on the ability of that culture to survive the resulting ethanol produced.

Glycerol is produced in the cell as a competing part of the carbon metabolism; that is to say that the production of glycerol comes from the same sugar substrate as ethanol (Goold et al. 2017)(Hohmann et al. 2007)(Cordier et al. 2007). It is produced in the cell for two primary purposes, the first being in response to high osmolarity, and the second being as a way to reoxidize NAD+ back to NADH after it has been reduced to form biomass or organic acids during anaerobic fermentation (Zhang et al. 2006)(Goold et al. 2017). The process which produces glycerol consists of two enzymes that branch off of the glycolytic pathway. The first is NAD+ dependent glycerol-3-phosphate dehydrogenase, coded for by the genes *GPD1* and

GPD2, and the second is glycerol-3-phospate phosphatase, coded for by *GPP1* and *GPP2* (Hohmann et al. 2007)(Zhang et al. 2006)(Cordier et al. 2007). The latter catalyzes the rate limiting step (Zhang et al. 2006). When exposed to a high osmolarity environment, the HOG pathway is activated by a series of MAP kinases, activating *HOG1*. *HOG1* then in turn produces a protein which regulates transcription of a variety of genes in response to the stress, amongst them the expression of *GPD1* and *GPP2* (Hohmann et al. 2007). While these two genes are expressed during high osmostress, their isogenes *GPD2* and *GPP1* are stimulated by anaerobic conditions (Zhang et al. 2006). In this way, it would appear that *Saccharomyces cerevisiae* has a gene for each enzyme in the process for both conditions that require glycerol production. The glycerol produced serves as an osmolyte to prevent cell lysis (Cordier et al. 2007)(Hohmann et al. 2007). As the sugar begins to deplete as a result of alcoholic fermentation, the osmolarity of the extracellular environment lowers and the cell needs to remove the glycerol. Glycerol does not diffuse well through the cell membrane, so the aquaglyceroporin Fps1 is opened and glycerol is allowed to pass through out of the cell (Hohmann et al. 2007)(Luyten et al. 1995). The HOG signaling pathway is not necessary for the inactivation of *FPS1* (Luyten et al. 1995). It is the *FPS1* gene, and the aquaglyceropourin that it codes for, that are the most interesting part of glycerol production with regard to ethanol tolerance.

The *FPS1* gene was identified in 2009 in a large scale genomic study looking into ethanol tolerance in *Saccharomyces cerevisiae*. The authors screened yeast differential tolerance to high ethanol stress. The stress was by ethanol shock on a minimal growth media. The authors then grouped the approximately 250 observed determinants into categories based on functions to get a better understanding of what aspects of yeast cellular processes are more or less important to

surviving ethanol shock. Of the large pool of gene mutations observed, the authors picked *FPS1* for further tests (Teixeira et al. 2009). The original null mutants of *FPS1* displayed a lag phase immediately after the addition of alcohol up to 6% (vol/vol) which was not seen with the control, and in the subsequent continuation of exponential phase displayed a lower rate of growth. Overexpression mutants were then subjected to 6% (vol/vol) of radio labeled ethanol, and it was observed that these mutants had lower intracellular concentrations of the radio labeled ethanol as compared to the control (Teixeira et al. 2009). This suggests that the glycerol export channel may actually help remove ethanol from the intracellular environment. Finally, mutants over expressing *FPS1* by both one copy and two copies were allowed to ferment in a high brix YPD media supplemented with amino acids in order to measure various metabolites. The mutants showed a higher final concentration of alcohol and higher glucose consumption, but very similar growth curves. The two over expression mutants produced similar levels of alcohol up to approximately 12% (vol/vol), at which point the mutant expressing only one extra copy was unable to proceed, while the other mutant eventually fermented to 14% (vol/vol) alcohol (Teixeira et al. 2009). A similar study, again using deletion mutants to screen for alcohol tolerance also found the deletion of *FPS1* to hinder a culture's ability to tolerate ethanol shock (Fujita et al. 2006). These results would seem to point toward *FPS1* as a potential asset in making modified yeast strains for high levels of alcohol production, were they not inconsistent with much of the available literature.

Zhang et al found an increase in ethanol production at the expense of glycerol in *FPS1* deletion mutants, however this was in an 8% glucose media so ethanol only ever reached 5% (Zhang et al. 2006). Similar results to these were obtained in null mutants for *FPS1* with *GLT1*

(coding for glutamate synthase) overexpressed, which yielded higher amounts of ethanol and lower amounts of glycerol (Kong et al. 2006). These contrasts to Teixeira et al. could be indicative of strain or environmental conditions causing different results, and none truly tested tolerance to high concentrations of ethanol. It is, however, vital to understand these shifts in glycerol and ethanol production because in a high sugar/ethanol fermentation NADH requiring processes will be upregulated such as fatty acid synthesis (Ma and Liu 2010). By upregulating glycerol production, *FPS1* overexpression could help to regenerate NAD+ back to NADH and maintain the redox potential of the cell. Even in low sugar/ethanol systems this is important, as altering the redox balance of the cell can cause synthesis of products with a sensory off-character (Goold et al. 2017). There appears to be little work regarding *FPS1* specifically with regard to ethanol tolerance, and none as of yet fermenting mutants in a high brix, defined, wine-like medium.

Based on the previous observations above identifying genes with potentially advantageous attributes, this experiment sought to differentially express *FAS1*, *FAS2*, *ACC1*, *OLE1*, *FPS1*, and *SOD2* in two commercial wine yeast strains. Data were taken for biomass, sugar utilization, and ethanol and glycerol production in an attempt to observe any differences in ethanol tolerance, as we have defined it here, with changes in these specific genes.

Chapter 3: Methods

3.1 Strains and Mutants

The yeast strains used in these experiments were commercial wine yeasts Montrachet (Red Star) and Elixir (Vitilevure). Both base strains were supplied by the University of California, Davis culture collection. These were chosen due to previously observed differences in biomass and maximum alcohol produced during similar fermentations to those here.

3.1.1 Yeast overexpression plasmid construction. The hygromycin yeast CEN6 vector, pRS41H, was used to construct a yeast expression plasmid containing the TDH1 promoter, PGK1terminator, and restriction sites allowing for the insertion of genes of interested that would be C-terminally HA-tagged. Briefly, the TDH1 promoter was PCR amplified using Q5 polymerase (NEB; M0491S), run on a 1.2% agarose gel, and the PCR fragment gel extracted (Bioneer PCR/Gel Extraction kit; K3037). The PCR fragment was then digested with *HindIII/ XmaI* restriction sites, cloned into the pRS41H vector and verified by sequencing. The PGK2 terminator was then PCR amplified using primers containing the *NotI/SacI* restriction sites, with an HA tag and stop codon in the *NotI* primer to be in-frame with the gene of interest at the C-terminal end. The PCR fragment was extracted as previous described, cloned into the pRS41H-pTDH1 plasmid, and sequence verified. Yeast target genes (*ACC1, FPS1, FAS1, FAS2, OLE1, SOD2)* were PCR amplified from genomic DNA using primers containing the restriction sites *XmaI/NotI* and were cloned into the pRS41H-pTDH1-HA-tPGK1 plasmid and sequence verified. Each construct was transformed into *S.cerevisiae* wine strains Elixir and Montrachet using the lithium acetate method described in Gietz et al. (2002), and positive clones were selected using YPD plates containing 300 ug/ml hygromycin. Each yeast strain containing the ethanol gene

plasmids were tested for protein expression via western blot using an anti-HA antibody (Sigma; H3663) at a 1:5000 dilution.

3.1.2 FPS1 double yeast deletion strain. Deletion of the *FPS1* gene in Montrachet and Elixir wine yeast, which are diploid strains, required two rounds of gene deletion, one at each locus. For the first *FPS1* locus deletion, F1 and R1 PCR primers containing homology to the *FPS1* gene and to a kanamycin marker cassette were created:

FPS1 F1: AAAGCTCTAAACGACTTTCTGTCCAGTGAATCTGTTCATACGGATCCCCGGGTTAATTAA
FPS1 R1: ACGCAAAACATTCCCGCAACACTTTCATTCATCCACCAATGAATTCGAGCTCGTTTAAAC

PCR amplification of a kanamycin cassette from the plasmid pFA6a-3HA-KanMX containing homology ends to *FPS1* was performed using Verifi Taq (PCR biosystems; PB10.42), and the DNA fragment size checked by agarose gel electrophoresis. The PCR product was transformed into *S.cerevisiae* wine strains Elixir and Montrachet using the lithium acetate method described in Gietz et al. (2002) and positive clones were selected using YPD plates containing 300 ug/ml G418. These clones were verified to be heterozygous for *FPS1* gene and the kanamycin cassette via PCR amplification.

3.2 Media

Two medias were used for the fermentations, differing only in sugar content, and one media was used for all experiments in order to grow suitable cell masses prior to inoculation with the required antibiotics to ensure plasmid stability in the inoculum.

3.2.1 Yeast Extract Peptone Dextrose (YPD) Media This media was used in order to build up cell mass prior to inoculation into the fermentation medium. The media was made in 1 L

batches. To 950 ml of distilled water, 20 g glucose, 20 g peptone, and 10 g yeast extract were added. This was allowed to dissolve and then more distilled water was added up to a total final volume of 1 L. This media was then sterile filtered. Immediately prior to use the media was supplemented with hygromycin to a concentration of 300 ug/ml and homogenized. For inoculum containing FPS1 deletion mutants, G418 was also added to a concentration of 300 ug/ml. This media was used due to an incompatibility between the MMM fermentation media and these antibiotics.

3.2.2 Minimal Must Media (MMM) This was the fermentation media used for all three experiments, differing across them only in sugar concentration. For the purposes of this writing the two iterations of MMM used will be referred to as Regular Brix MMM and High Brix MMM. The methods used to create both follow, and are the same except where specifically noted as differing. An ergosterol stock was first prepared by adding 62.5 mg 90% ergosterol to 95% ethanol to a volume of 25 ml. This was vortexed in order to dissolve ergosterol and stored in a refrigerator. 450 ml of DI water was added into a 1 L flask and placed onto a magnetic stir plate. 110 g D-fructose and 110 g D-glucose (140 g of each of these for the high brix media) were added slowly until dissolved. 4 ml of the previously prepared ergosterol stock was added. Following the completed dissolving of the sugar, 6 g L(+)tartaric acid, 3 g L(-)malic acid, and 0.5 g citric acid, anhydrous were added and allowed to dissolve. 200 ml more of DI water was then added. After this second water addition, the following were added: 1.7 g YNB (w/out amino acids and ammonium sulfate), 2 g vitamin-free casamino acids, 6 mg myo-inositol, 0.26 g $CaCl_2*2H_2O$, 0.2 g L-arginine*HCl, 1 g L-proline, 0.1 g L-tryptophan, and 0.1 g ammonium phosphate. DI water was then added to a final volume of 1 L. For the high brix media, a mixture

of equal parts fructose and glucose was slowly added up to a DMA reading of 28 brix. The media was then sterile filtered and the pH was adjusted to 3.25 using 3N KOH.

3.3 Fermentations

3.3.1 Biomass formation and inoculation Each mutant strain was inoculated into a 50mL centrifuge tube of 25mL sterile YPD containing 300ug/mL hygromycin (and 300ug/mL G418 in the case of *fps1* mutants). These tubes were left open and placed into a temperature controlled shaker at 30 degrees Celsius and 175rpm for 24 hours. Following this period of growth the tubes were centrifuged for 10 minutes at 14,000 rpm in an Eppendorf 5804 R centrifuge. The supernatant was poured off and the cell pellets were resuspended in sterile water. The tubes were again centrifuged for 10 minutes at max rpm. The supernatant was poured off again and the resulting washed cell pellets were resuspended in 50 ml of MMM (high brix for experiments 1 and 3, regular brix for experiment 2). These suspensions were then measured for OD600 in a spectrophotometer, and the appropriate amount was added to a 500 ml flask, along with an appropriate amount of sterile MMM to make 400 ml with an OD600 of approximately 0.05. These flasks were sealed with stoppers equipped with a one way CO_2 release and placed inside of a temperature controlled shaker at 30 degrees Celsius and 175rpm.

3.3.2 Fermentation sampling The fermentations were sampled approximately every 12 hours for the first 48 hours to monitor brix and OD600. Samples for nitrogen and HPLC analysis were taken every 24 hours beginning at inoculation. Dry cell weight measurements were taken every 48 hours beginning at around hour 36 of fermentation. Brix was monitored by Anton-Paar

DMA. Optical density (OD600) was measured by spectrophotometer without dilution for the first and second samplings, at a 1:25 dilution for the third, and then a 1:50 dilution for the remaining measurements. Dry cell weight measurements were taken by weighing an unused 0.45 micron cellulose acetate filter on a small piece of aluminum foil. 15 ml at the first dry cell weight sampling and then 5 ml in each successive sampling were then run through the filter, followed by twice that volume of DI water. The filter was then placed inside of an incubator on top of its aluminum foil and allowed to dry for 24 hours at 95 degrees Celsius. The dried filter/foil was then measured and the dry cell weight was taken as the difference between this and the initial weight of the filter/foil per volume filtered. Nitrogen measurements were taken by the University of California, Davis Winery via Gallery Machine as NOPA and NH_3. Samples given to the winery had been removed as a 1mL aliquot, sterile filtered, and then frozen at -20 degrees Celsius until completion of the fermentation. Samples for the HPLC analysis were taken and stored in this same fashion.

3.3.3 HPLC analysis HPLC analysis was performed on thawed samples diluted 1:20. Calibration curves were made at concentrations of 0.5, 1, 10, and 20 g/L for glucose, fructose, glycerol, and ethanol. Samples were run through two BioRad Aminex HPX-87H 300 x 7.8mm columns in series on an Agilent Infinity II HPLC with refractive index detector (RID). The mobile phase used was 5 mM sulfuric acid. The method used an injection volume of 20 ul, a pump speed of 0.5 ml/min, and a temperature of 50C. Pressure throughout the columns was approximately 85 bar. Total retention time was approximately 1 hour per sample.

3.4 Statistical Analysis

Data was analyzed for statistically significant differences using analysis of variance in ExcelStat software with an alpha of 0.05. Fisher's least significant difference values were calculated for all differences found to be significant.

Chapter 4: Results

The results are divided into four parts. These consist of the initial screening of the chosen genes, overexpressed in both base strains, the overexpression of *FPS1* in both base strains in a lower brix media, the *FPS1* deletions, and the FAS complex overexpressions.

4.1 Initial Screening

The initial screening of the overexpression strains was split into two fermentations for reasons of space and practicality. The division was made by starting strain, so each gene was overexpressed in triplicate in both fermentations, with the first fermentation being for mutants made from Montrachet (Red Star) and the second fermentation being for mutants made from Elixir (Vitilevure). These fermentations, like the rest, were monitored for brix, optical density, dry cell weight, and yeast assimilable nitrogen. These specific parameters were chosen because they are metrics for both how far the fermentation has progressed (brix), and how vigorous the growth of the yeast cells are in the face of first high osmolarity and then increasing ethanol concentrations (optical density, dry cell weight, and yeast assimilable nitrogen). Changes in these could potentially be indicative of changes in the strain's ability to tolerate high concentrations of ethanol.

The brix curve for the initial screening of Montrachet can be seen below in Figure 1. The density of the initial media at inoculation was read by the DMA to be approximately 28 brix, and none of the yeasts were able to ferment to dryness. This was intentional, as the point at which the fermentation stuck was to be taken as an indicator of the ethanol tolerance of that particular mutant/control. In the case of Montrachet, the most significantly different mutant was the *FPS1* overexpression, which ended its fermentation about one brix earlier than the rest.

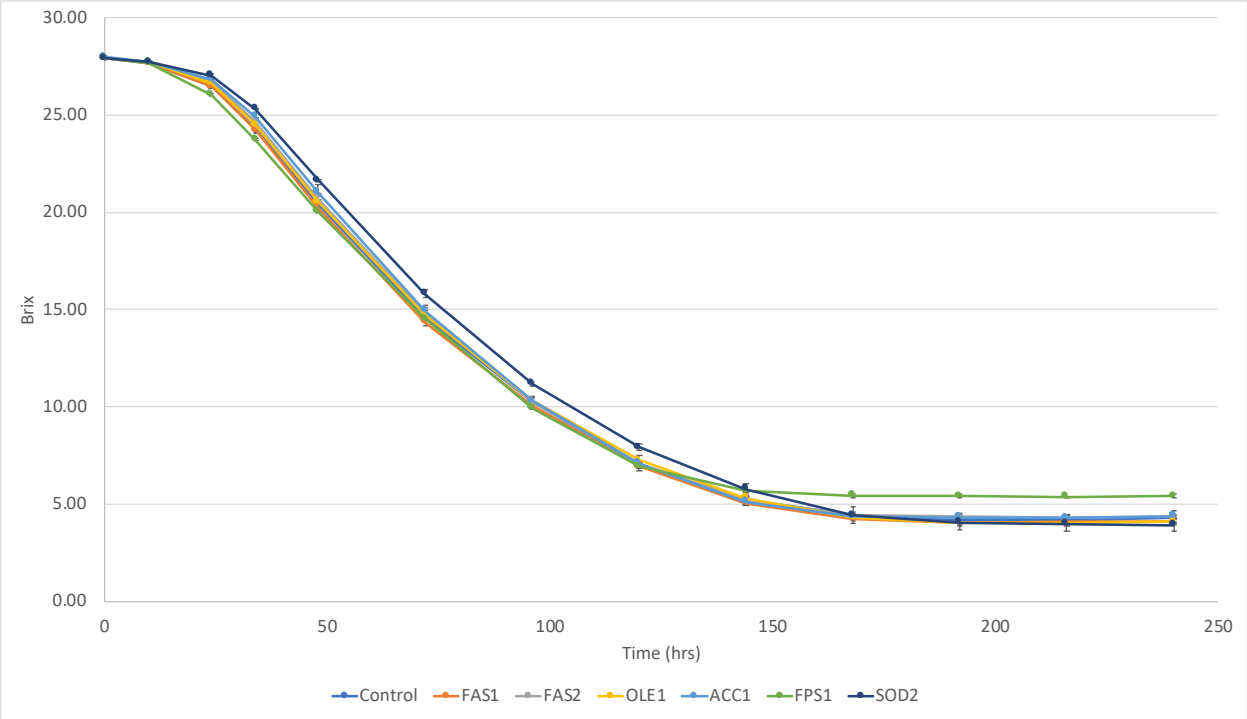


Figure 1. Brix curve of mean values of the initial screening of overexpression mutants made from the commercial strain Montrachet n=3

The overexpression of *SOD2* yielded an extended lag phase, but eventually reached similar fermentation rate and ultimately reached a slightly lower density than that of the empty plasmid control. A summary of the differences between the mean values of each mutant throughout fermentation can be seen below in Table 1. While significant differences around the middle of fermentation can be interesting, the differences that are ultimately the most impactful are those toward the end of fermentation when there is a higher concentration of ethanol.

Table 1. Statistically significant differences from controls in mean value (n=3) brix measurements for Montrachet mutants at different time points

Sample \Time (hrs)	0	10	24	34	48	72	96	120	144	168	192	216	240
Control	27.900	27.667	26.467	24.300	20.400	14.600	10.233	7.033	5.200	4.433	4.200	4.200	4.300
ACC1	27.967	27.733	26.833	24.900	21.033	14.933	10.300	7.100	5.100	4.367	4.333	4.300	4.400
FAS1	27.933	27.667	26.467	24.233	20.233	14.367	10.033	6.933	5.033	4.267	4.067	4.100	4.133
FAS2	27.900	27.733	26.733	24.600	20.700	14.800	10.233	7.067	5.200	4.433	4.367	4.300	4.400
FPS1	27.900	27.700	26.033	23.733	20.033	14.533	9.933	6.933	5.667	5.433	5.400	5.367	5.400
OLE1	27.900	27.733	26.633	24.467	20.533	14.700	10.300	7.267	5.300	4.333	4.067	4.033	4.100
SOD2	27.900	27.733	27.033	25.300	21.633	15.800	11.200	7.933	5.767	4.433	4.067	4.000	3.933
Least Significant Difference: 0.295													
Yellow denotes significance at alpha = 0.05													

The optical density measurements were taken as a way of indirectly measuring the biomass of the cultures. The optical density measurements, taken at 600nm, have been plotted below in Figure 2 for the Montrachet fermentation. As expected based upon the brix curve, the *FPS1* mutant had a reduced biomass compared to the control. The *SOD2* mutant displayed a delayed increase in biomass formation, but eventually reached levels comparable to the control. This was also consistent with the brix curve in Figure 1. The accompanying statistical summary can be found in Table 2. The *FPS1* mutant showed the only statistically significant optical density at the final time point, 240 hours.

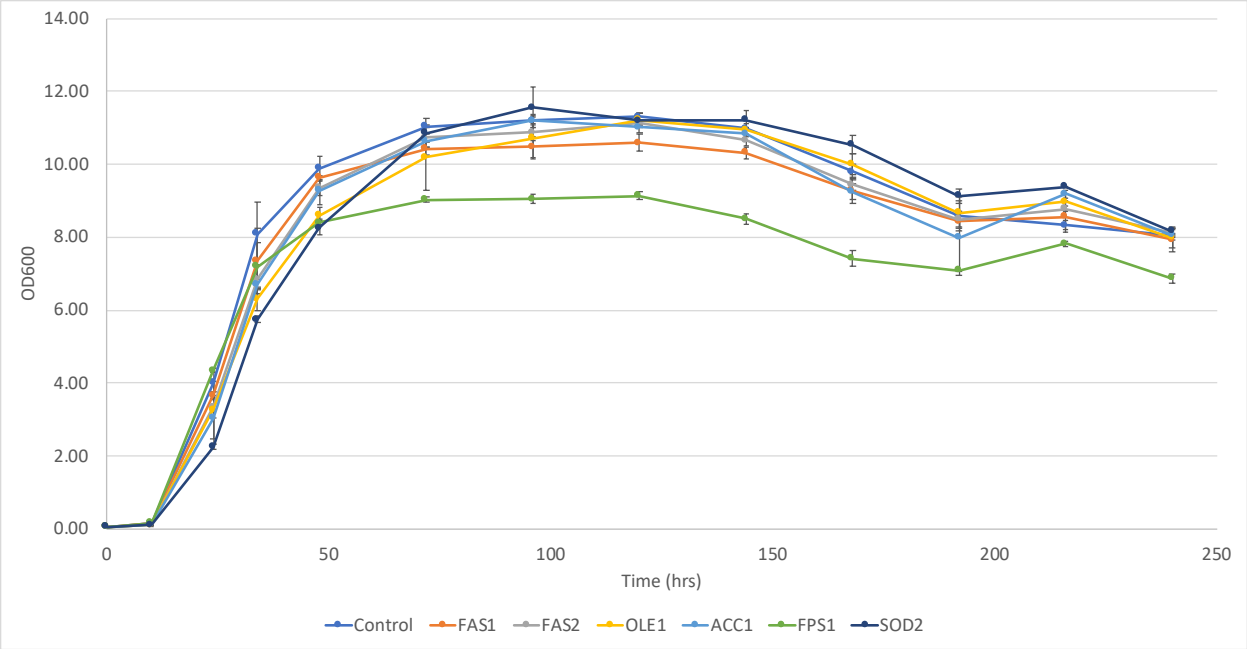


Figure 2. Optical density at 600nm mean values of the initial screening of overexpression mutants made from the commercial strain Montrachet n=3

Table 2. Statistically significant differences from controls in mean value (n=3) OD_{600nm} measurements for Montrachet mutants at different time points

Sample \Time (hrs)	0	10	24	34	48	72	96	120	144	168	192	216	240
Control	0.052	0.139	4.007	8.100	9.883	11.033	11.200	11.300	10.983	9.800	8.583	8.333	8.067
ACC1	0.050	0.125	3.033	6.717	9.283	10.650	11.200	11.017	10.833	9.250	7.983	9.183	8.067
FAS1	0.048	0.145	3.660	7.350	9.633	10.433	10.483	10.583	10.317	9.283	8.433	8.567	7.933
FAS2	0.048	0.118	3.340	6.850	9.350	10.733	10.883	11.150	10.667	9.433	8.483	8.783	8.133
FPS1	0.048	0.167	4.340	7.200	8.400	9.033	9.050	9.133	8.500	7.417	7.083	7.817	6.867
OLE1	0.051	0.108	3.267	6.317	8.600	10.200	10.717	11.217	10.950	10.000	8.667	8.967	7.983
SOD2	0.046	0.107	2.240	5.750	8.250	10.867	11.567	11.200	11.217	10.533	9.133	9.383	8.167
Least Significant Difference: 0.491													
Yellow denotes significance at alpha = 0.05													

The brix curve for the initial screening of the Elixir mutants is below in Figure 3. The *FPS1* overexpression mutant again ceased fermenting at a higher brix than the control, and this time was joined by *FAS1*. The other portion of the FAS complex, *FAS2*, also showed some significant differences, but not as strongly as the *FAS1* mutant. The differences observed in *SOD2* in the Montrachet did not appear with the comparable Elixir mutant.

A summary of the statistically significant differences in samples per time point is below in Table 3. The largest and most consistent of the significant differences are attributed to the *FPS1* mutant.

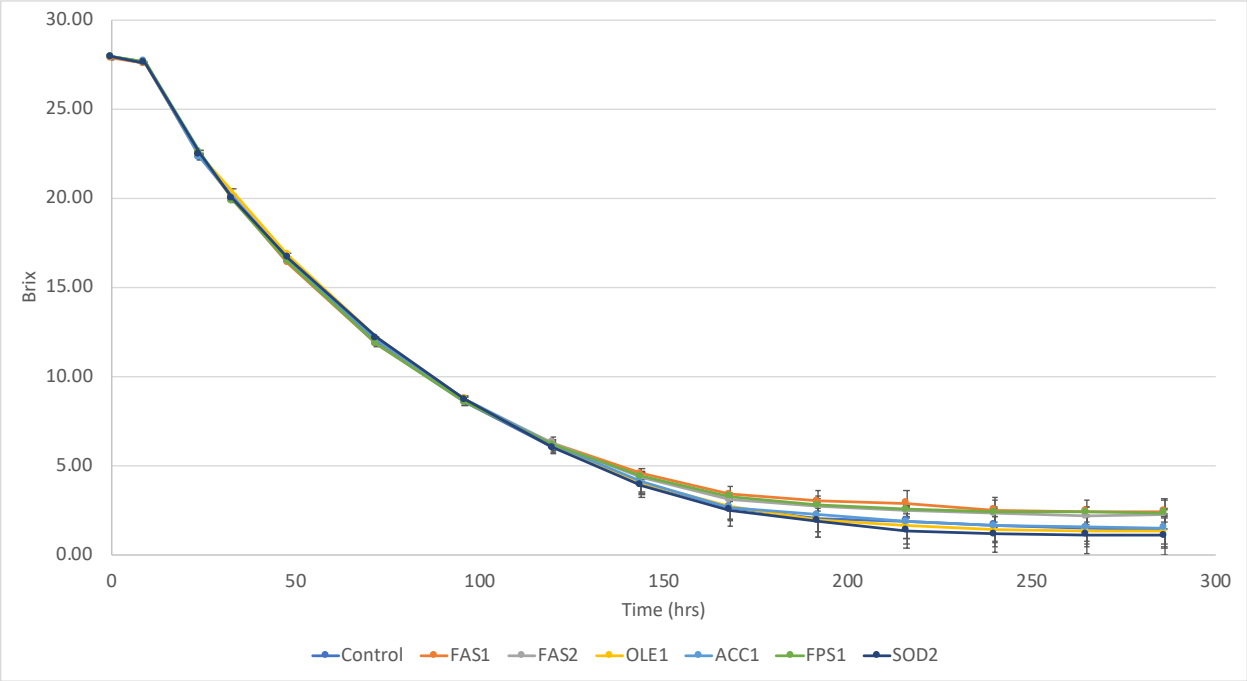


Figure 3. Brix curve of mean values of the initial screening of overexpression mutants made from the commercial strain Elixir n=3

The differences between the mutants and the control are summarized below in Table 3. The differences do not appear until 168 hours post inoculation, when the yeast has produced almost all of the ethanol of which it is capable.

Table 3. Statistically significant differences from controls in mean value (n=3) brix measurements for Elixir mutants at different time points

Sample \Time (hrs)	0	9	24	33	48	72	96	120	144	168	192	216	240	265	286
Control	27.900	27.533	22.300	20.067	16.467	11.833	8.533	6.033	4.000	2.633	2.033	1.867	1.667	1.467	1.400
ACC1	27.933	27.633	22.267	20.000	16.533	12.000	8.700	6.133	4.067	2.667	2.233	1.833	1.600	1.533	1.500
FAS1	27.833	27.533	22.333	19.900	16.333	11.800	8.600	6.267	4.533	3.400	3.000	2.867	2.500	2.433	2.433
FAS2	27.900	27.567	22.367	20.067	16.600	12.033	8.700	6.267	4.300	3.067	2.733	2.467	2.300	2.167	2.267
FPS1	27.900	27.600	22.567	19.833	16.400	11.800	8.533	6.133	4.367	3.267	2.767	2.533	2.400	2.400	2.333
OLE1	27.900	27.600	22.367	20.300	16.800	12.133	8.733	6.100	4.000	2.700	1.967	1.633	1.400	1.333	1.300
SOD2	27.900	27.567	22.467	19.967	16.633	12.133	8.700	6.000	3.900	2.500	1.867	1.333	1.167	1.100	1.067
Least Significant Difference: 0.695															
Yellow denotes significance at alpha = 0.05															

In the case of Elixir, there was more variation between the replicates. This caused both the higher LSD value in the above table, and for replicate to be a significant source of variation.

The optical density data for the Elixir mutants is below in Figure 4. The data show a far less exaggerated reduction in biomass for the *FPS1* mutant than seen in Montrachet, but both *FPS1* and *FAS1* showed reductions in biomass, as was expected based upon the corresponding

brix curve in Figure 2. The summary of the differences of the least squares means and their significance for each time point is below in Table 4.

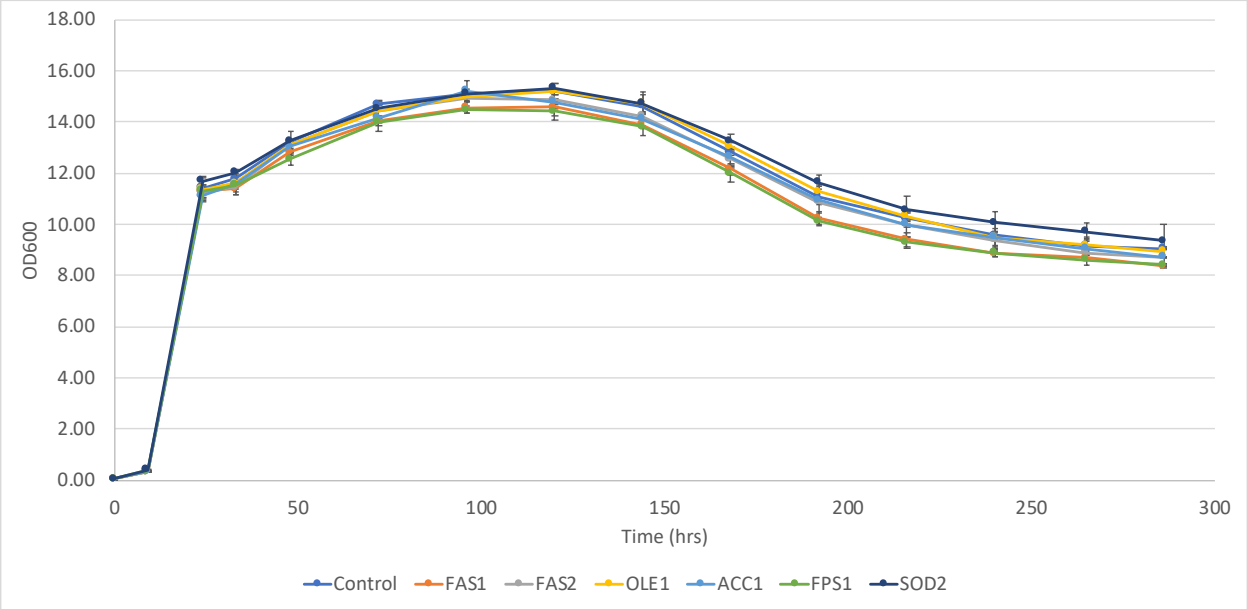


Figure 4. Optical density at 600nm mean values of the initial screening of overexpression mutants made from the commercial strain Elixir n=3

The differences in the data are easier to see in the table below than in Figure 4. There were small but significant reductions in OD_{600nm} for the majority of the duration of the fermentation for both the *FPS1* and the *FAS1* mutants, with a few significant differences in other mutants. Most notably, the *SOD2* mutant displayed a slightly higher biomass formation toward the end of fermentation, but was not significantly different upon the completion of fermentation.

Table 4. Statistically significant differences from controls in mean value (n=3) OD_{600nm} measurements for Elixir mutants at different time points

Sample \Time (hrs)	0	9	24	33	48	72	96	120	144	168	192	216	240	265	286
Control	0.048	0.368	11.417	11.783	13.217	14.700	15.083	15.200	14.600	12.817	11.083	10.233	9.567	9.167	9.050
ACC1	0.051	0.327	11.117	11.550	13.033	14.167	15.217	14.783	14.117	12.633	10.950	9.983	9.483	9.050	8.717
FAS1	0.045	0.353	11.283	11.400	12.833	14.033	14.567	14.583	13.900	12.150	10.233	9.400	8.867	8.700	8.367
FAS2	0.054	0.328	11.267	11.433	13.133	14.433	14.933	14.867	14.233	12.550	10.867	10.000	9.383	8.900	8.733
FPS1	0.052	0.395	11.300	11.517	12.550	14.000	14.483	14.417	13.800	11.983	10.133	9.300	8.900	8.600	8.417
OLE1	0.055	0.333	11.350	11.600	13.117	14.433	15.000	15.200	14.717	13.050	11.283	10.317	9.500	9.183	8.933
SOD2	0.047	0.414	11.683	12.017	13.250	14.533	15.117	15.333	14.717	13.250	11.650	10.567	10.083	9.717	9.367
Least Significant Difference: 0.399															
Yellow denotes significance at alpha = 0.05															

Dry cell weight was used as a direct way to measure biomass. Briefly, small volumes of each sample were filtered and the filter was dried out in an incubator overnight. The weight difference of the filter before and after it was used and dried was taken as the dry cell weight per volume filtered. The expectation was that the biomass observations seen by optical density would be observed again with the dry cell weight measurements. The dry cell weights of the Montrachet fermentation are below in Figure 5.

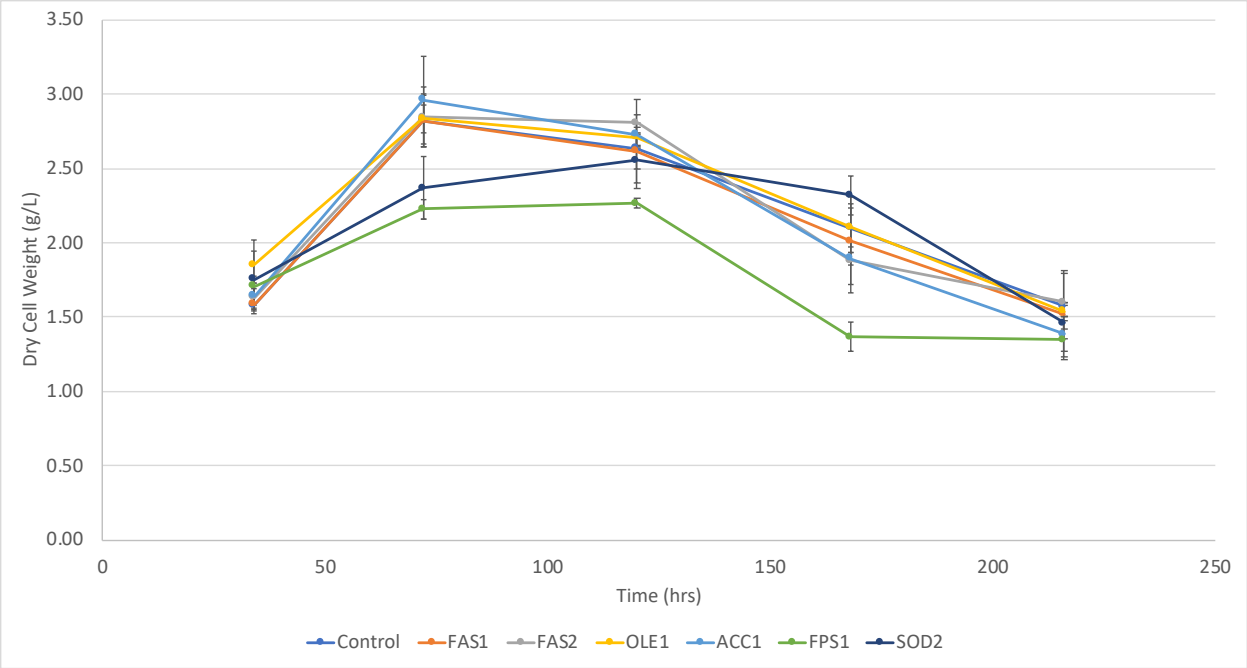


Figure 5. Dry cell weight mean values of the initial screening of overexpression mutants made from the commercial strain Montrachet n=3

The dry cell weight data indeed showed similar trends to those seen in Figure 3, albeit with a higher degree of variability. The *FPS1* mutant reaches much lower maximum biomass than the control, and the *SOD2* mutant has a slower growth. Despite the variability shown by the standard deviation error bars above, replicate was not found to be a statistically significant factor upon analysis of variance. The primary concern, sample by time interactions, were however significant. A summary of the differences and statistical significance is in Table 5.

Differences were found mainly for the *FPS1* mutant toward the middle of fermentation. A difference near the end of fermentation at the final time point was narrowly avoided at 95% confidence. Significant differences in optical density between *FPS1* and the control began around 34 hours, which was not observed with the dry cell weight, but began at the next time point.

Table 5. Statistically significant differences from controls in mean value g/L (n=3) dry cell weight measurements for Montrachet mutants at different time points

Sample\Time (hrs)	34	72	120	168	216
Control	1.580	2.820	2.633	2.100	1.573
ACC1	1.640	2.960	2.727	1.893	1.387
FAS1	1.587	2.820	2.613	2.013	1.520
FAS2	1.633	2.847	2.807	1.880	1.600
FPS1	1.707	2.227	2.267	1.367	1.347
OLE1	1.853	2.833	2.707	2.107	1.540
SOD2	1.753	2.367	2.553	2.320	1.460
Least Significant Difference: 0.250					
Yellow denotes significance at alpha = 0.05					

The dry cell weight data for the Elixir fermentation can be seen in Figure 6. Similar to the optical density data in Figure 4, any difference existing was far more muted than those found in Montrachet. The slight difference in biomass detected by the spectrophotometer in the form of OD_{600nm} was not found and the only statistically significant factor found by analysis of variance was time. Differences between replicates and samples were not significant. The variability in the data is probably caused by the large amount of error introduced by the process of dry cell weight collection, and could cause small differences to be missed if they indeed exist.

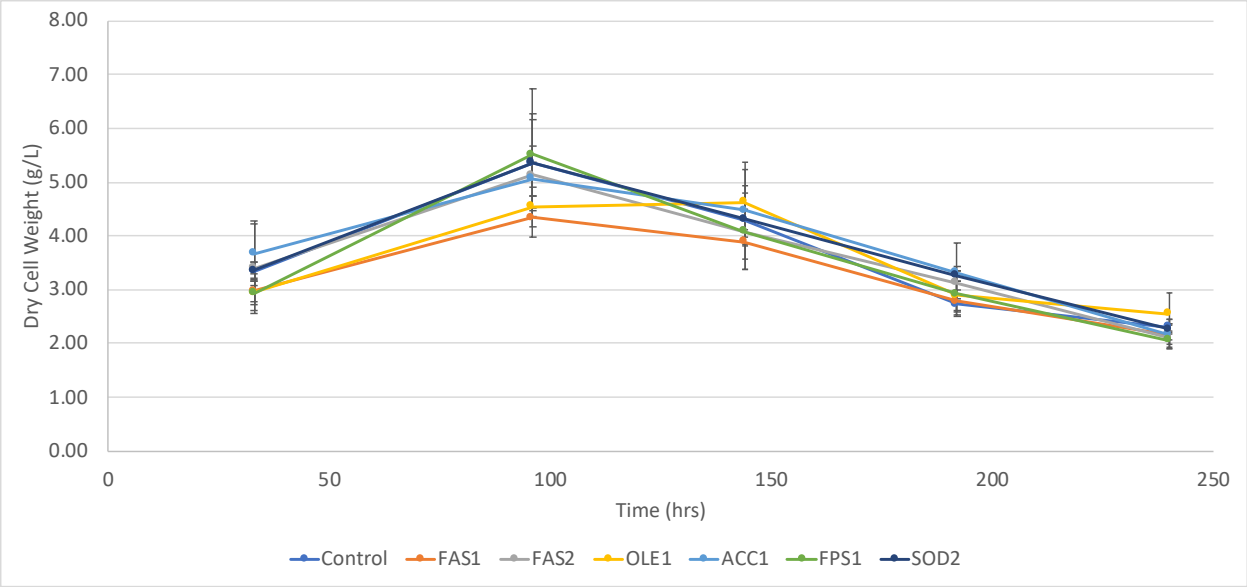


Figure 6. Dry cell weight mean values of the initial screening of overexpression mutants made from the commercial strain Elixir n=3

The final data taken for all mutants in the initial screening were for yeast assimilable nitrogen. Yeast assimilable nitrogen in the media over time for the Montrachet fermentation is in Figure 7 below. There was considerable variation in nitrogen uptake at 24 hours, with only the *FAS1* mutants not showing a statistically significant difference from the controls. At 24 hours all of the mutants that showed differences in nitrogen uptake from the control utilized the nitrogen more slowly with the exception of the *FPS1* mutants. The cells likely began to lyse around 120 hours as the nitrogen concentration in the media began to rise. The graph shows a slightly higher amount of nitrogen returned to the media by the *FPS1* mutants, but these differences are not significant except at 96 hours. A summary of the differences in means at each time point and their statistical significance is in Table 6 below.

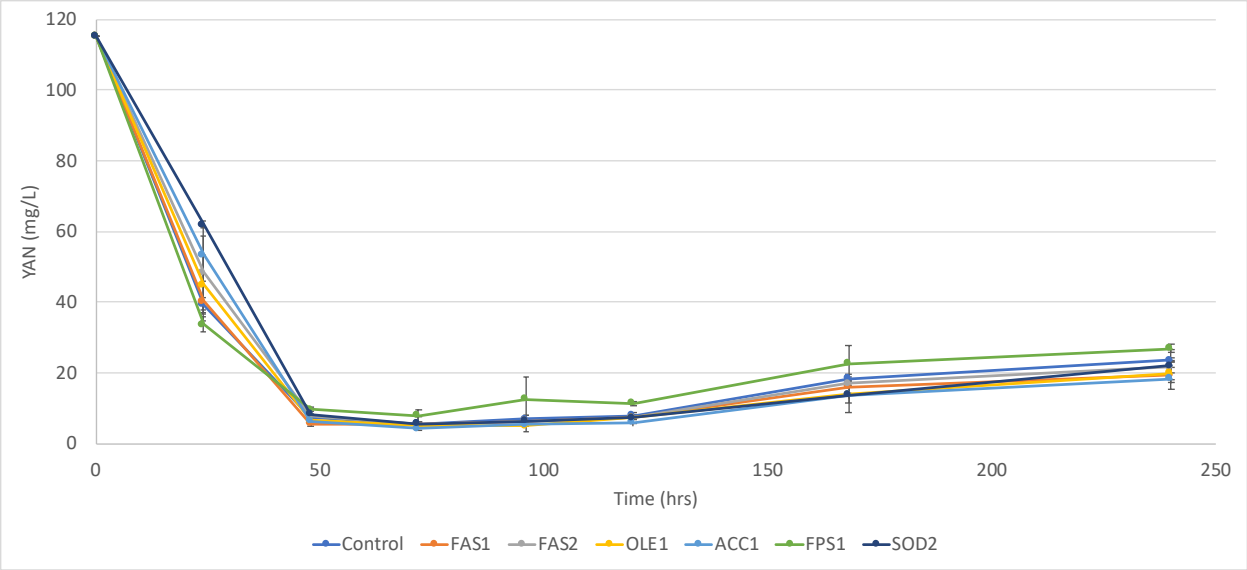


Figure 7. Yeast assimilable nitrogen mean values of the initial screening of overexpression mutants made from the commercial strain Montrachet n=3

Table 6. Statistically significant differences from controls in mean value mg/L (n=3) yeast assimilable nitrogen measurements for Montrachet mutants at different time points

Sample\Time (hrs)	0	24	48	72	96	120	168	240
Control	115.320	39.333	7.333	5.667	7.000	8.000	18.333	23.667
ACC1	115.320	53.333	6.333	4.333	5.333	6.000	13.667	18.333
FAS1	115.320	40.333	5.667	5.333	6.333	7.333	16.000	19.333
FAS2	115.320	48.333	7.667	5.333	6.333	7.333	17.000	21.667
FPS1	115.320	33.667	9.667	8.000	12.667	11.333	22.667	27.000
OLE1	115.320	45.000	6.667	5.000	5.000	7.333	14.000	20.000
SOD2	115.320	62.000	8.333	5.667	6.333	7.333	13.667	22.000
Least Significant Difference: 4.591								
Yellow denotes significance at alpha = 0.05								

The yeast assimilable nitrogen utilization data for the Elixir fermentation is below in Figure 8. There were no significant differences in nitrogen across the mutants and controls.

Analysis of variance showed only time to be a significant factor. Again there is nitrogen reentering the media, but at the same rates for all mutants and controls.

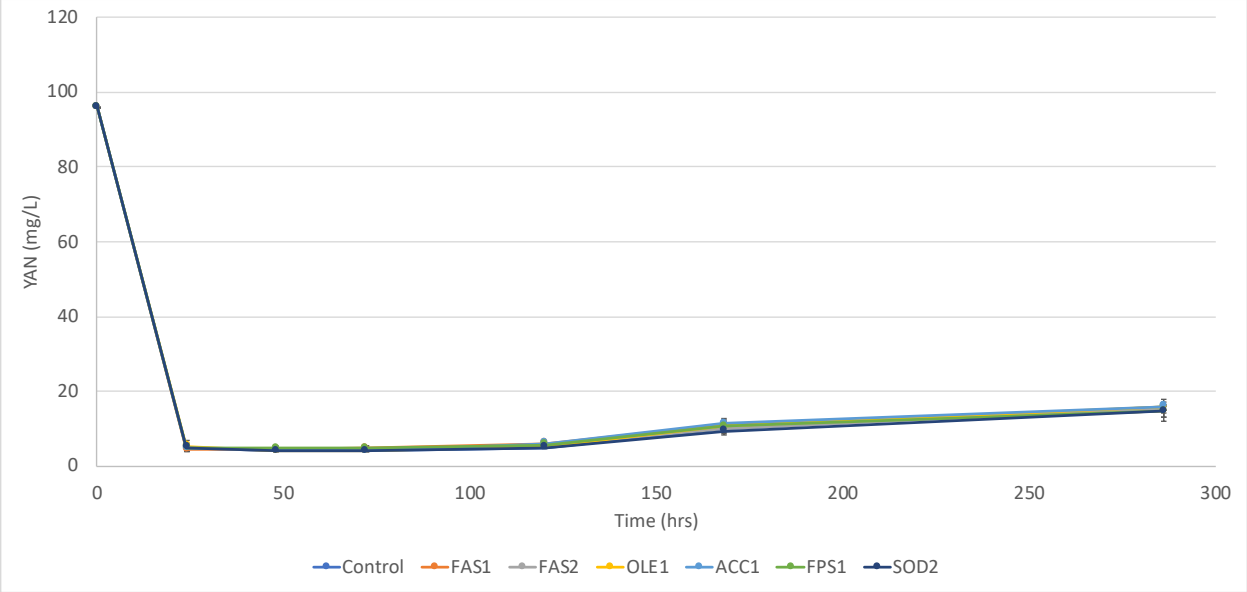


Figure 8. Yeast assimilable nitrogen mean values of the initial screening of overexpression mutants made from the commercial strain Elixir n=3

Based on all of the preceding data, *FPS1* and *FAS1* were determined to cause the most significant changes in the original strains. Therefore, *FPS1*, *FAS1*, and *FAS2* were the only genes chosen to continue the experiments after the initial screening. From the initial screening, samples were taken for glucose, fructose, glycerol and ethanol by HPLC from the controls and the *FPS1* and *FAS1* mutants for each base strain. The sugar utilization curves for the Montrachet fermentation are below as Figure 9 and Figure 10. The second time point in both figures has what is likely a dilution error in one replicate in both of two treatments.

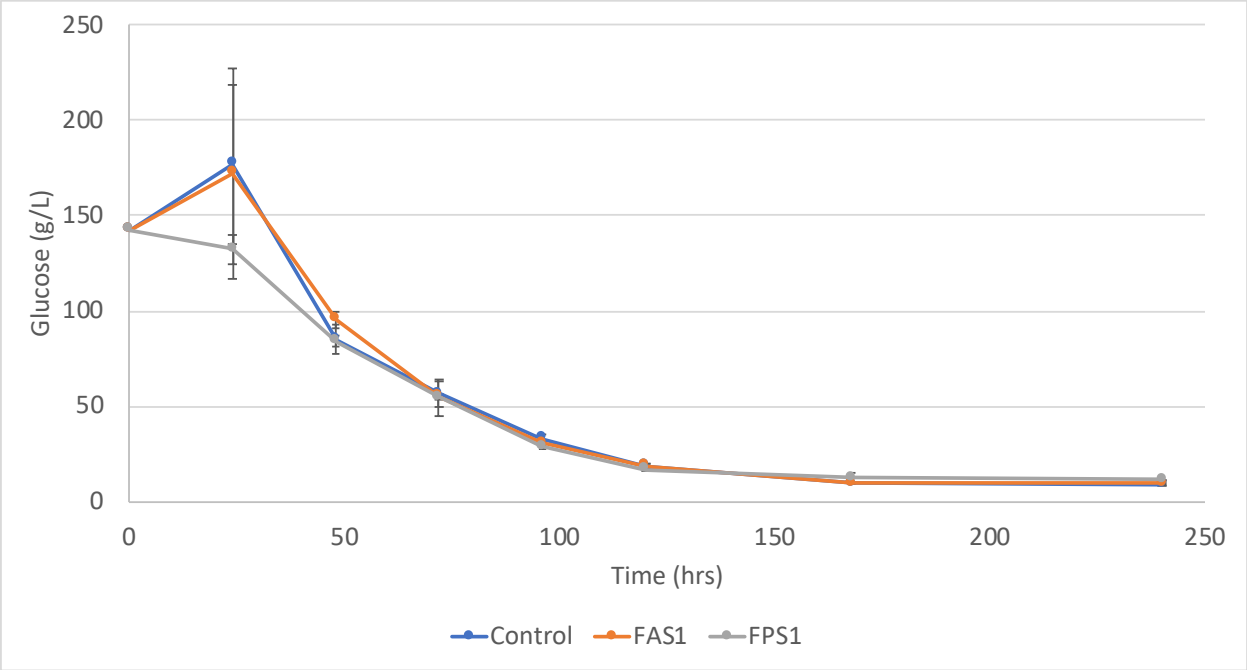


Figure 9. Glucose mean values of the initial screening of selected overexpression mutants made from the commercial strain Montrachet n=3

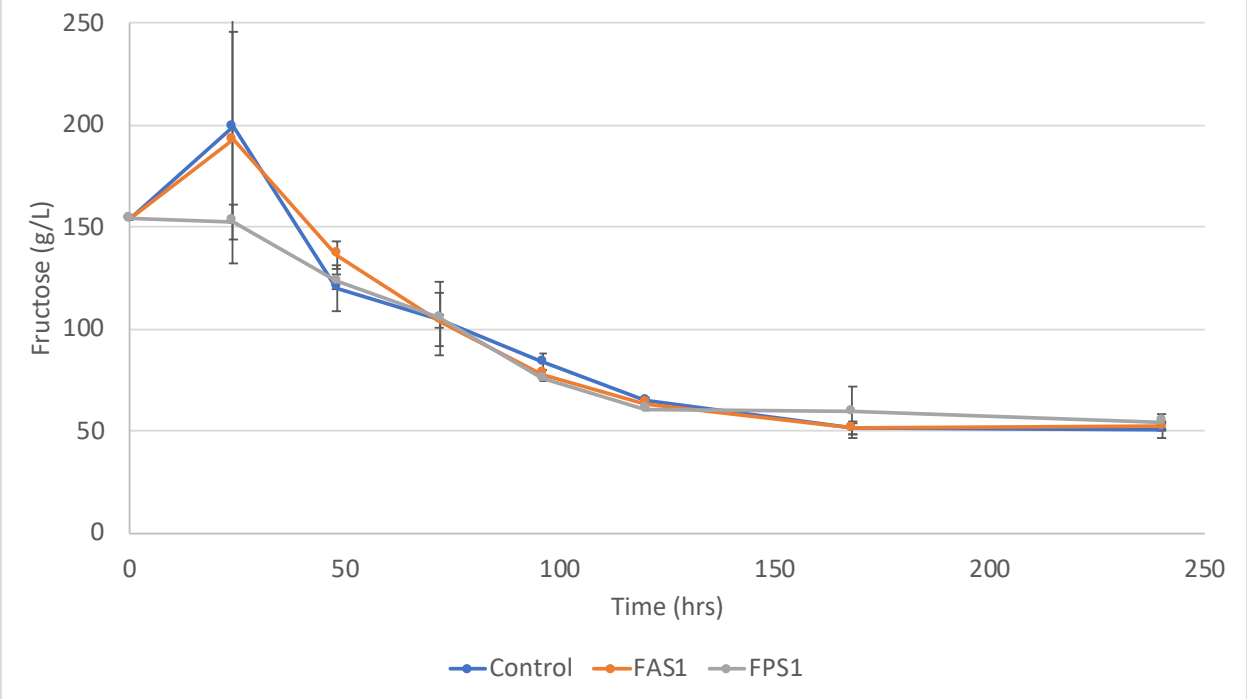


Figure 10. Fructose mean values of the initial screening of selected overexpression mutants made from the commercial strain Montrachet n=3

The utilization of both sugars by the control and mutants did not differ. Analysis of variance showed only time to be a significant factor for both sugars. This is in contrast to the initial brix measurements taken throughout fermentation, which showed the *FPS1* mutants ceasing fermentation about one brix earlier than the controls (Figure 1). This could potentially be because the brix measurements were performed by DMA, which measures density as a way to determine total soluble solids. The difference seen by the DMA may actually have been caused by the increase in glycerol in the media for the *FPS1* mutants. This difference can be seen in Figure 11.

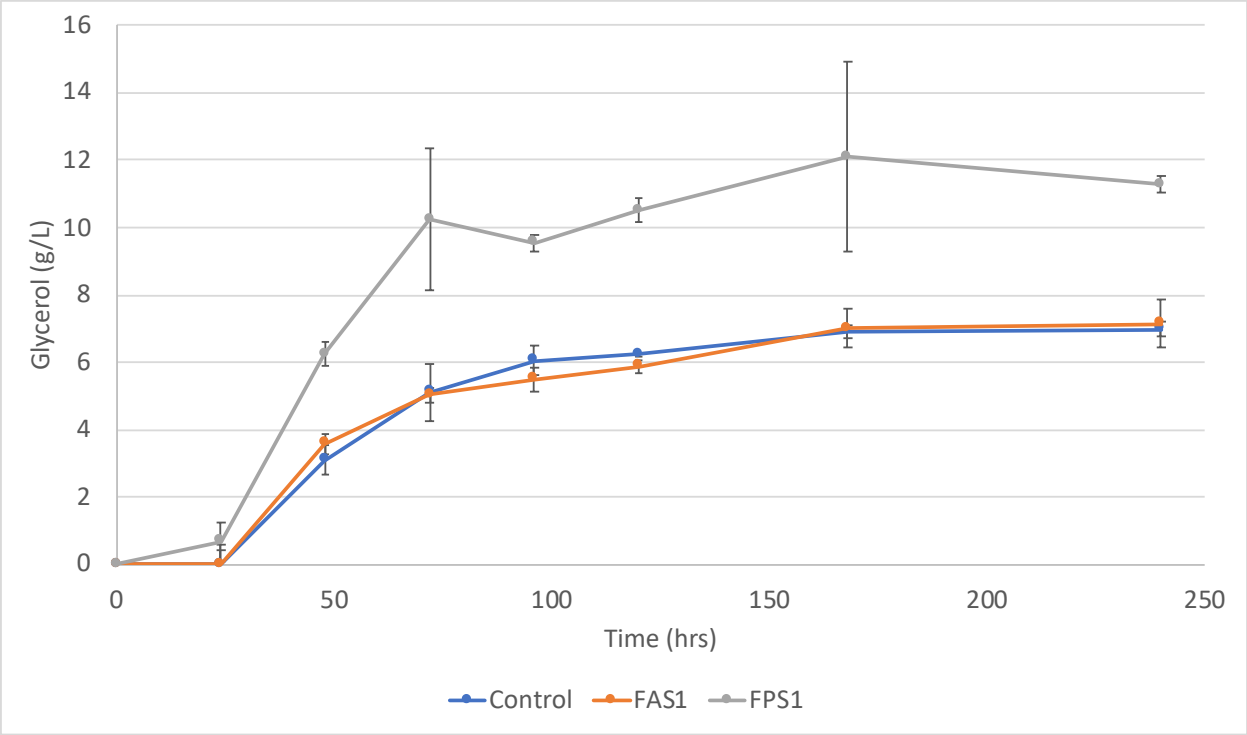


Figure 11. Glycerol mean values of the initial screening of selected overexpression mutants made from the commercial strain Montrachet n=3

There was no significant difference between the *FAS1* mutants and the controls, but a considerable increase from the controls to the *FPS1* mutants. This is to be expected, as the *FPS1* gene codes for a glycerol export channel in the membrane. Glycerol is a dense substance, and could potentially account for the initial DMA readings. A summary of the differences and their statistical significance is below in Table 7.

Table 7. Statistically significant differences from controls in mean value (n=3) glycerol for Montrachet mutants at different time points

Sample\Time (hrs)	0	24	48	72	96	120	168	240
Control	0.000	0.047	3.089	5.102	6.059	6.227	6.912	6.989
FAS1	0.000	0.000	3.577	5.026	5.490	5.889	7.003	7.151
FPS1	0.000	0.691	6.249	10.239	9.540	10.513	12.105	11.271
Least Significant Difference: 1.338								
Yellow denotes significance at alpha = 0.05								

Based on this increase in glycerol, it would be expected that the *FPS1* mutant would produce less ethanol, as ethanol and glycerol are competing products. The ethanol curve for the selected mutants of the initial Montrachet fermentation is below in Figure 12. The *FPS1* mutants did indeed appear to have a slightly lower final ethanol concentration when looking at the graph, but these differences were not significant. Analysis of variance showed only time to be a statistically significant factor.

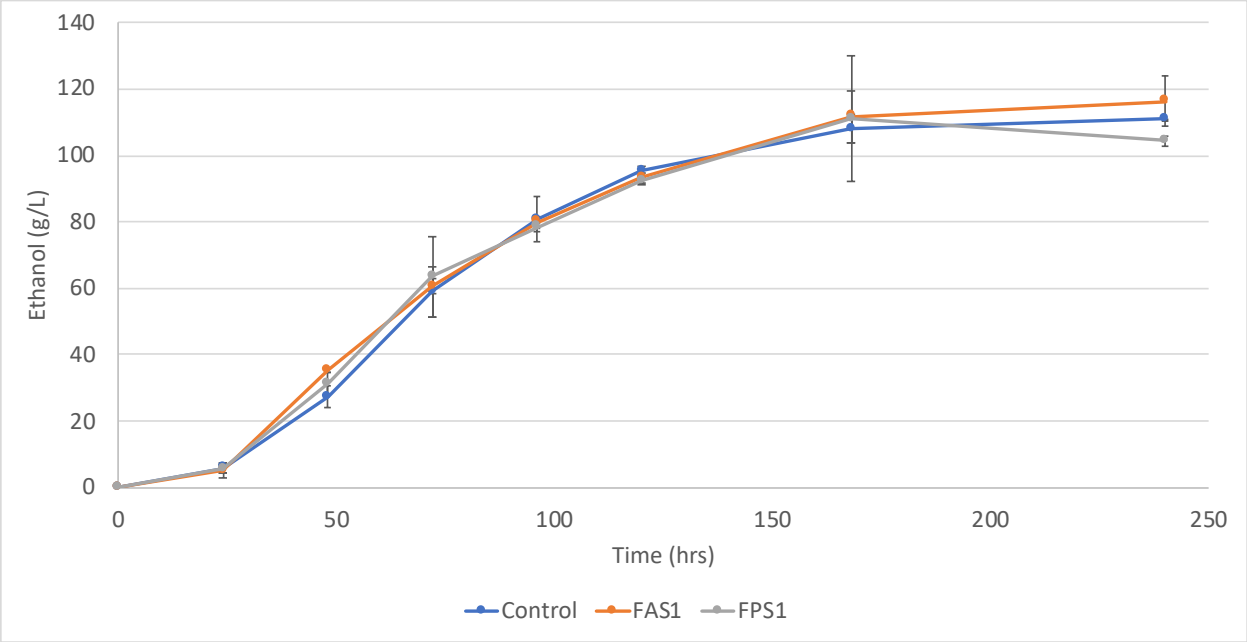


Figure 12. Ethanol mean values of the initial screening of selected overexpression mutants made from the commercial strain Montrachet n=3

The Montrachet *FPS1* mutants showed considerable increases in glycerol over the control, but in all other cases there were not statistically significant differences between mutants and controls for the HPLC data taken for the initial Montrachet screening. The increase in glycerol and decrease in biomass formation in the Montrachet *FPS1* mutant suggests glycerol production at the expense of biomass. It is possible that the increase in glycerol export channels increases the amount of glycerol leaving the cell, and therefore the amount of glycerol available to the cell for use in lipid synthesis.

The results for the *FPS1* and *FAS1* mutants in the Elixir fermentations were similar with regard to sugar utilization. Below are the curves in Figures 13 and 14. Just as with Montrachet, there were no significant differences between any of the mutants or controls in sugar uptake. For both glucose and fructose the only significant factor found by ANOVA was time.

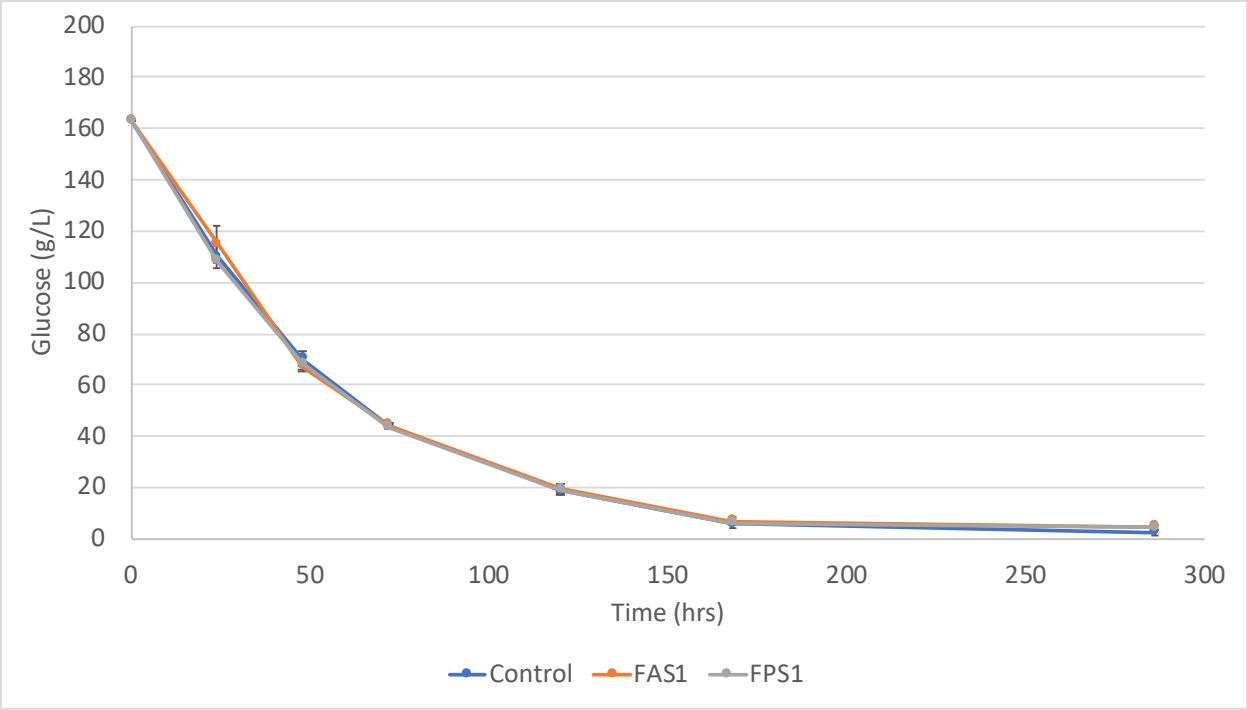


Figure 13. Glucose mean values of the initial screening of selected overexpression mutants made from the commercial strain Elixir n=3

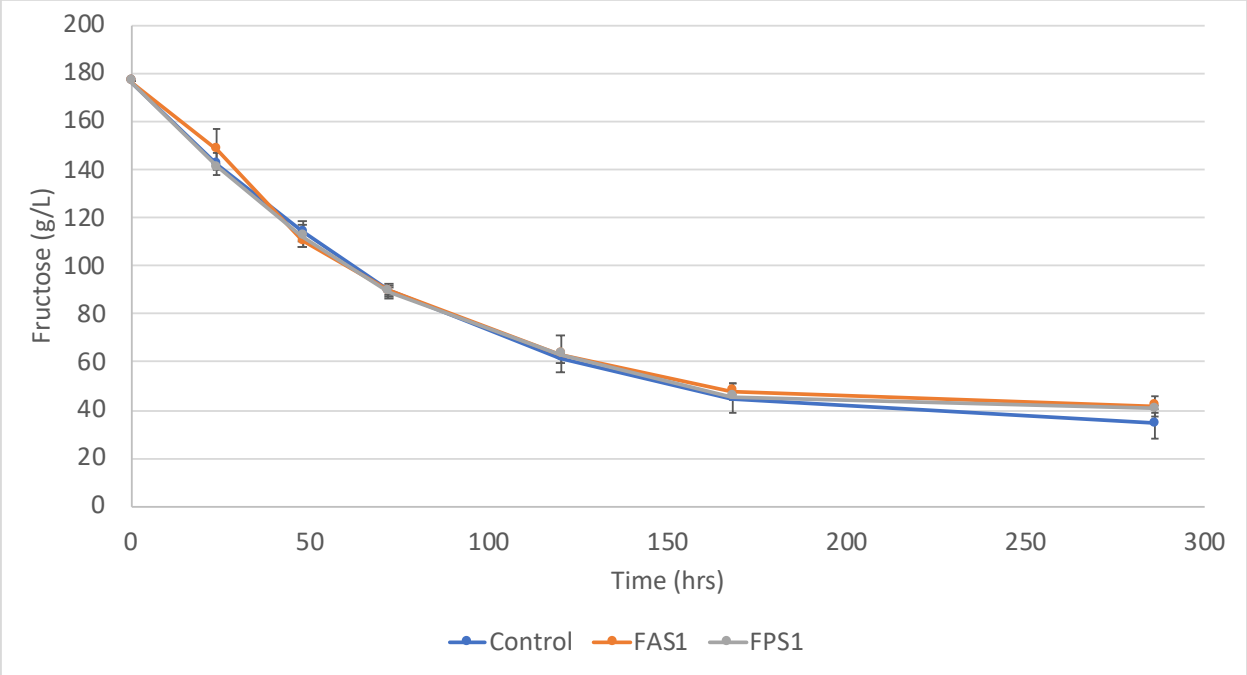


Figure 14. Fructose mean values of the initial screening of selected overexpression mutants made from the commercial strain Elixir n=3

Just like with the Montrachet mutants, this leaves the disparity in brix by DMA seen in Figure 2 likely attributable to soluble compounds other than hexose sugars. In the case of Montrachet, the dramatic increase in glycerol could potentially have caused the DMA reading, but referring to Figure 2, both the *FPS1* and the *FAS1* Elixir mutants ceased fermentation earlier than the control. The glycerol data for these can be seen in Figure 15 below.

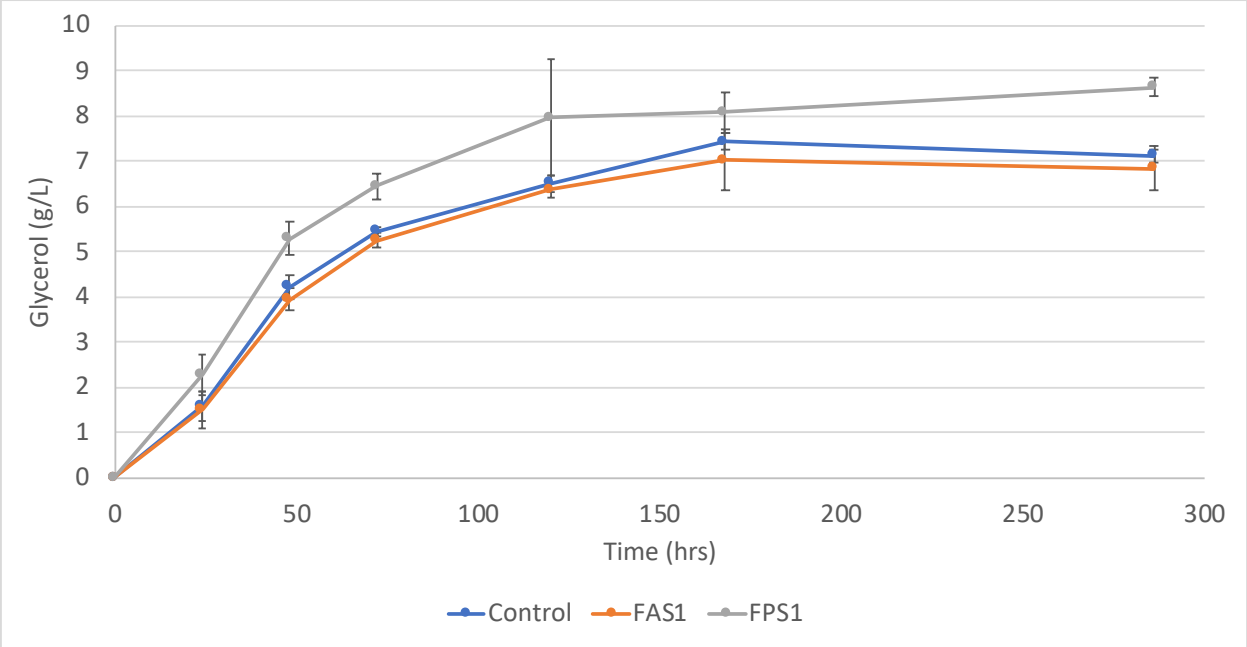


Figure 15. Glycerol mean values of the initial screening of selected overexpression mutants made from the commercial strain Elixir n=3

There is an increase in glycerol for the *FPS1* mutant in Elixir as well, but not to as dramatic of an extent as seen with Montrachet. What is particularly interesting is that there is no increase for the *FAS1* mutants, however these used the same amount of sugars as the controls and stopped fermentation at a higher density. The means and statistical significance of the differences between the mutants and controls are below in Table 8.

Table 8. Statistically significant differences from controls in mean value (n=3) glycerol for Elixir mutants at different time points

Sample\Time (hrs)	0	24	48	72	120	168	286
Control	0.000	1.583	4.221	5.452	6.500	7.428	7.110
FAS1	0.000	1.500	3.945	5.234	6.357	7.016	6.838
FPS1	0.000	2.278	5.291	6.439	7.958	8.070	8.623
Least Significant Difference: 0.680							
Yellow denotes significance at alpha = 0.05							

As there was a statistically significant increase in glycerol production for the *FPS1* mutants, it would be expected that there would be a corresponding decrease in ethanol production. The Ethanol curve for the selected Elixir mutants and controls is below in Figure 16.

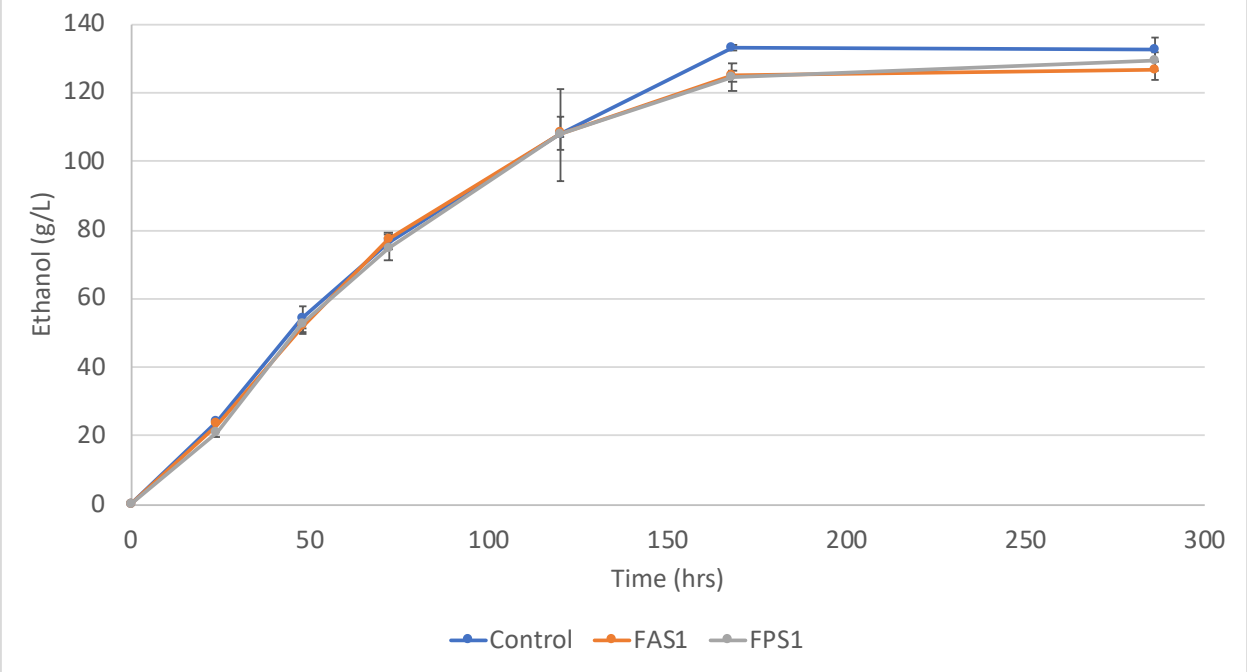


Figure 16. Ethanol mean values of the initial screening of selected overexpression mutants made from the commercial strain Elixir n=3

The sample by time interactions was not found to be a statistically significant factor by ANOVA. The control strain produced the most ethanol at 168 hours, but upon the completion of fermentation both mutants had reached a comparable concentration. This presents some very interesting questions regarding where the excess glycerol comes from in the *FPS1* mutants if it is utilizing the same amount of sugar and producing the same amount of ethanol as the control. The answer probably lies in the fact that *FPS1* codes for a glycerol membrane channel and these numbers are concentrations in the media. The *FPS1* mutants may produce the same amount of glycerol as the control, but allow more efflux out of the cell.

4.2 FPS1 Regular Brix Fermentation

As *FPS1* overexpression had the greatest impact of all of the genes observed in the initial screenings, it was decided that the *FPS1* overexpressions for both strains, as well as their controls would be fermented again in a lower brix media in order to determine if the same effect could be observed at a sugar concentration more typical for the wine industry. The fermentation conditions and samplings were the same, with the only difference being the sugar content of the initial media being reduced from ~28 brix to ~22 brix. Because space and practicality were no longer issues given the reduced number of flasks needed, both Montrachet and Elixir were run in the same fermentation. The brix curve by DMA for the regular brix fermentation is below as Figure 17. All flasks finished fermentation, reaching a density below that of water because of the ethanol produced. As can be seen easier in Table 9, both *FPS1* mutants fermented slightly quicker during the middle of fermentation than their respective controls. This increase in rate was

not seen in the high brix experiment, possibly because the large amount of sugar led to substrate inhibition, eliminating any difference in rate that might otherwise exist.

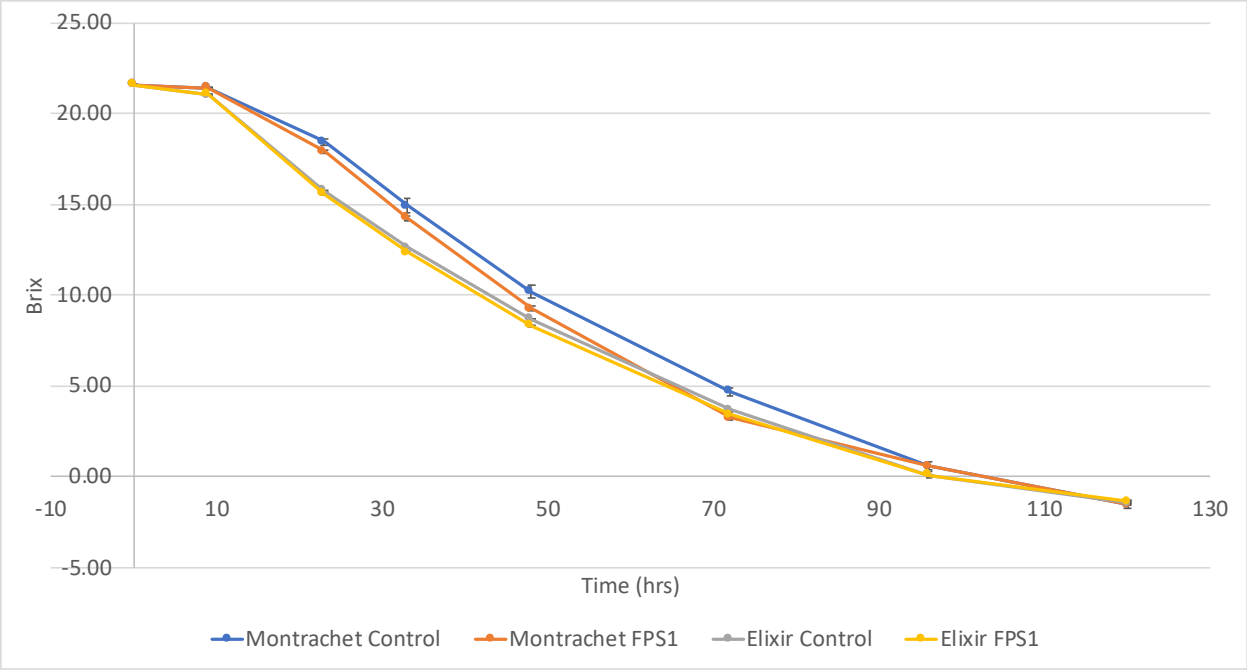


Figure 17. Brix curve of mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 9. Statistically significant differences from controls in mean value (n=3) brix measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	9	23	33	48	72	96	120
E Control	21.600	21.000	15.733	12.600	8.700	3.700	0.100	-1.433
E FPS1	21.600	21.067	15.567	12.367	8.333	3.433	0.100	-1.333
M Control	21.600	21.400	18.433	14.900	10.200	4.700	0.600	-1.533
M FPS1	21.600	21.400	17.900	14.233	9.267	3.233	0.600	-1.533
Least Significant Difference: 0.214								
Yellow denotes significance at alpha = 0.05								

The optical density of the flasks was taken as before in the initial screening, and plotted in Figure 18 below. While there was a considerable reduction in OD_{600nm} for the Montrachet *FPS1* mutant in the high brix media (Figure 3), in the regular brix media there was far less effect. The difference rather than being near immediate as in the high brix, started small and widened as fermentation continued. Elixir showed a similar small difference between mutant and control throughout much of stationary phase. The statistical significance is shown in Table 10. Interestingly, the Montrachet *FPS1* mutant showed both a faster rate of fermentation and a lower biomass, indicating an increase in the sugars converted to ethanol per cell.

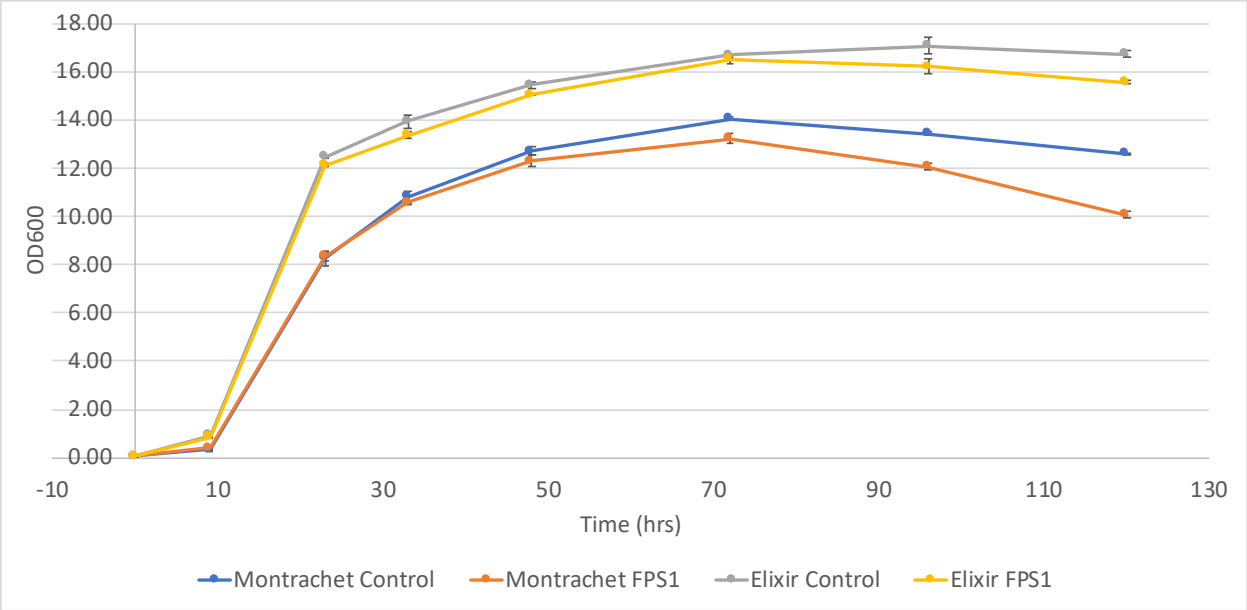


Figure 19. Optical density mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 10. Statistically significant differences from controls in mean value (n=3) OD_{600nm} measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	9	23	33	48	72	96	120
E Control	0.067	0.942	12.450	13.950	15.450	16.683	17.083	16.733
E FPS1	0.066	0.863	12.117	13.350	15.050	16.533	16.200	15.567
M Control	0.066	0.329	8.267	10.817	12.717	14.050	13.417	12.600
M FPS1	0.065	0.400	8.350	10.567	12.283	13.217	12.067	10.067
Least Significant Difference: 0.245								
Yellow denotes significance at alpha = 0.05								

The dry cell weight data, although only three data points due to the brevity of the fermentation, agrees with this reduction in Montrachet *FPS1* mutant biomass toward the end of fermentation. See Figure 20 below, with the statistical information following in Table 11.

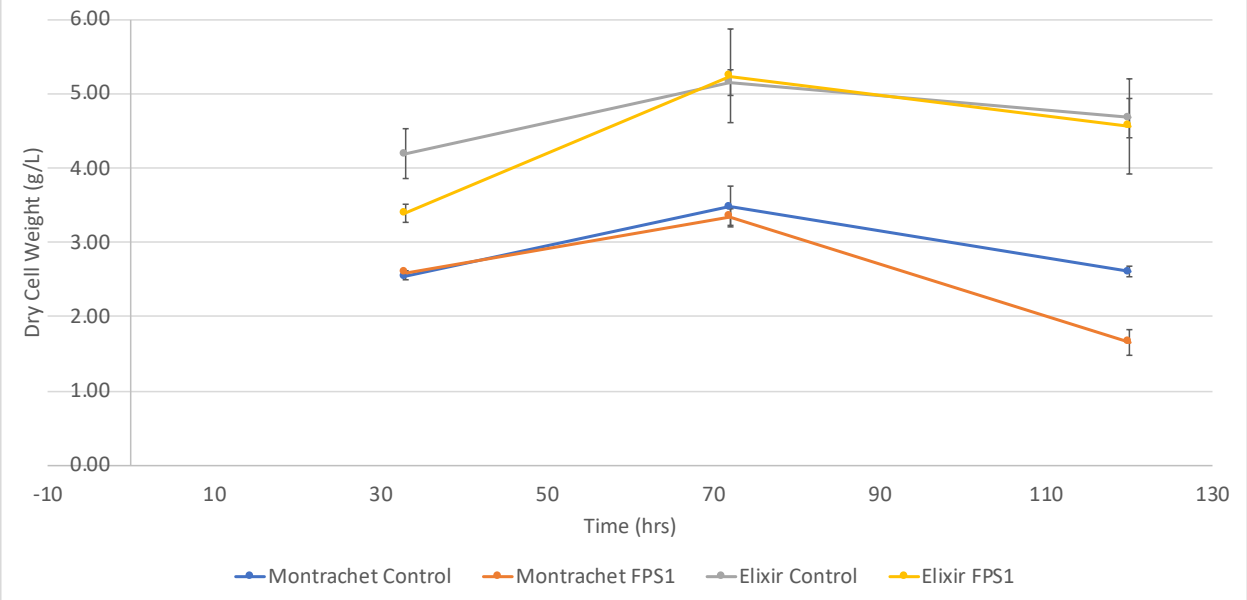


Figure 20. Dry cell weight mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 11. Statistically significant differences from controls in mean value g/L (n=3) dry cell weight measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	33	72	120
E Control	4.193	5.153	4.673
E FPS1	3.400	5.240	4.560
M Control	2.540	3.480	2.607
M FPS1	2.593	3.347	1.660
Least Significant Difference: 0.530			
Yellow denotes significance at alpha = 0.05			

Nitrogen utilization again showed the Montrachet *FPS1* mutant taking up more nitrogen by the 24 hour mark, and there was a statistically significant increase in the nitrogen returned to the media at the end of fermentation. There were no significant differences in the nitrogen concentrations of the media between the Elixir control and mutant. A graphical representation of the mean values over time is in Figure 21, and the statistical significance of the mutant means relative to their respective controls is in Table 12.

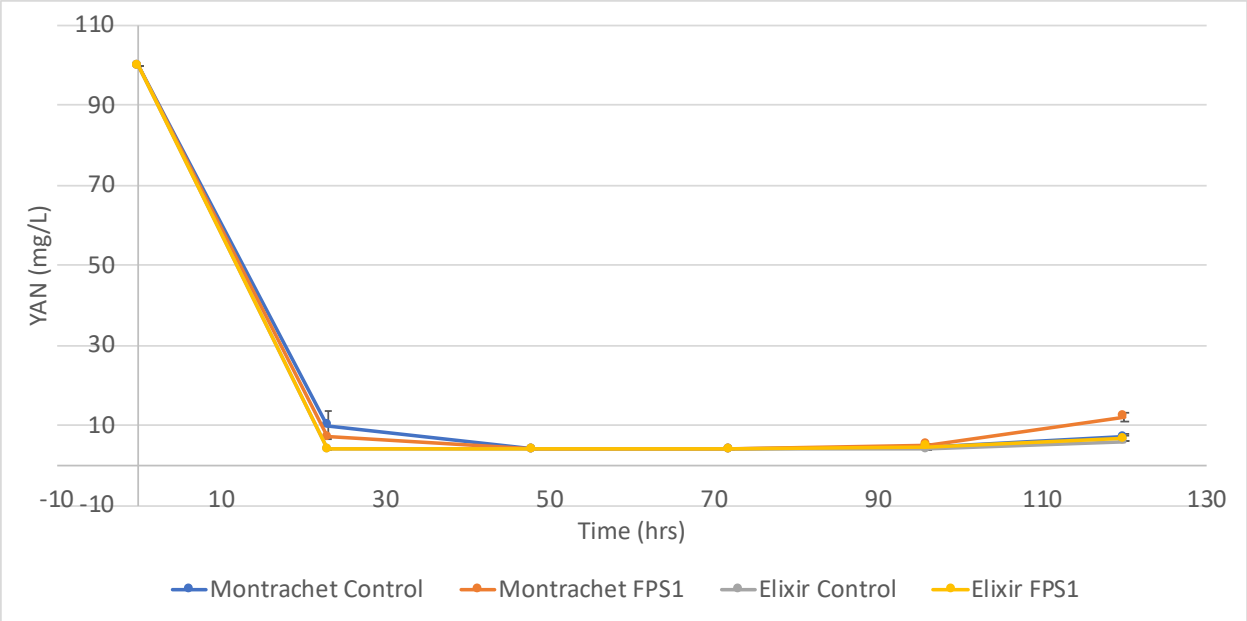


Figure 21. Yeast assimilable nitrogen mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 12. Statistically significant differences from controls in mean value mg/L (n=3) yeast assimilable nitrogen measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	23	48	72	96	120
E Control	99.667	4.000	4.000	4.000	4.000	6.000
E FPS1	99.667	4.000	4.000	4.000	4.667	6.667
M Control	99.667	10.000	4.000	4.000	4.667	7.000
M FPS1	99.667	7.000	4.000	4.000	5.000	12.000
Least Significant Difference: 1.333						
Yellow denotes significance at alpha = 0.05						

HPLC analysis for glucose, fructose, glycerol, and ethanol were performed on samples taken throughout fermentation in the same fashion as in the initial screenings. The sugar

utilization curves for both glucose and fructose are below as Figures 22 and 23. With both sugars, the only statistically significant differences were between the Montrachet control and mutant. The *FPS1* overexpression mutant utilized sugar more quickly in the middle of fermentation. The statistically significant values can be found in Tables 13 and 14 below.

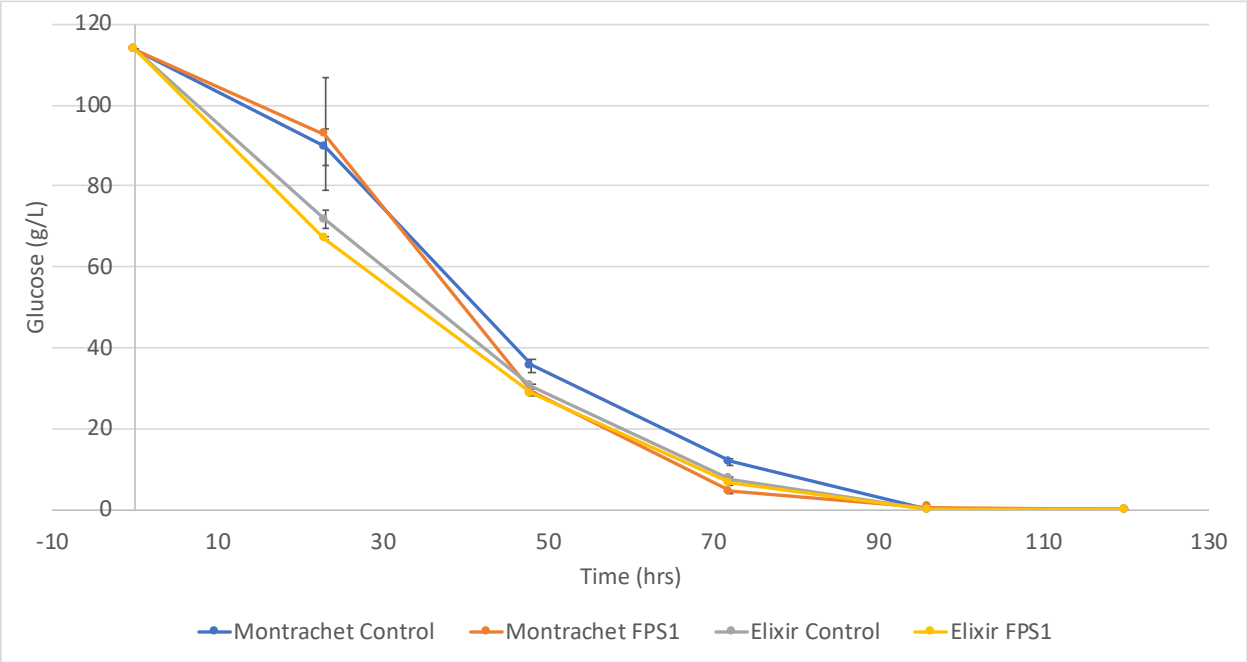


Figure 22. Glucose mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

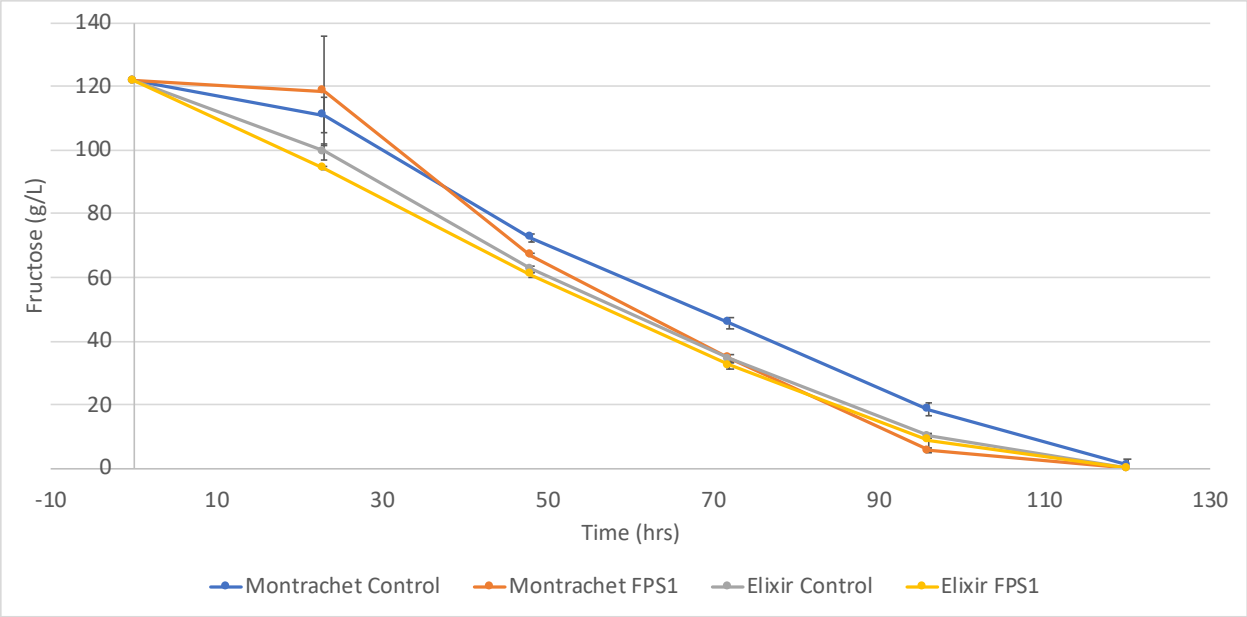


Figure 23. Fructose mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 13. Statistically significant differences from controls in mean value g/L (n=3) glucose measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	23	48	72	96	120
E Control	113.592	71.684	30.459	7.573	-0.808	-1.105
E FPS1	113.592	66.976	28.599	6.511	-0.879	-1.105
M Control	113.592	89.572	35.598	11.818	-0.402	-1.105
M FPS1	113.592	92.709	29.282	4.396	0.609	-1.105
Least Significant Difference: 4.966						
Yellow denotes significance at alpha = 0.05						

Table 14. Statistically significant differences from controls in mean value g/L (n=3) fructose measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	23	48	72	96	120
E Control	121.860	99.413	62.542	34.274	10.063	-0.669
E FPS1	121.860	94.317	60.897	32.340	8.874	-0.669
M Control	121.860	110.968	72.424	45.745	18.514	1.006
M FPS1	121.860	118.558	66.978	34.582	5.480	-0.521
Least Significant Difference: 6.306						
Yellow denotes significance at alpha = 0.05						

Glycerol measurements were again where the greatest differences between the controls and mutants were observed. The difference between the Montrachet control and its *FPS1* overexpression mutant were greater in the high brix media than here in the regular brix media. This can be seen in Figure 24 below, and the accompanying statistical information in Table 15.

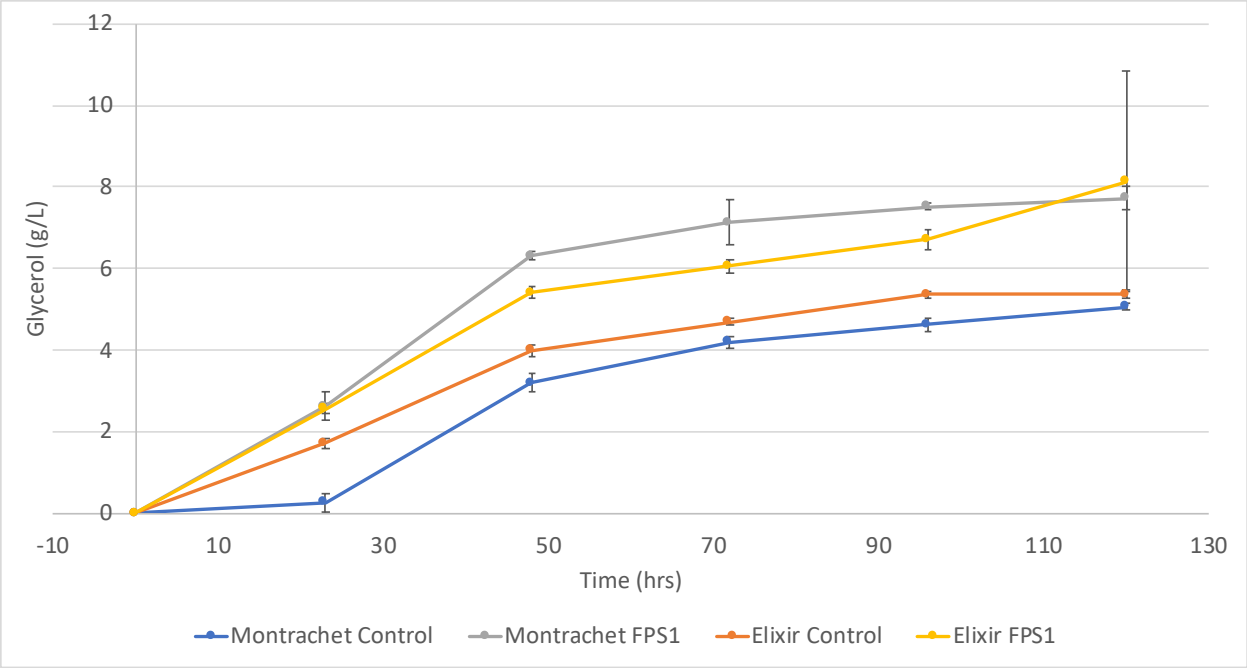


Figure 24. Glycerol mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 15. Statistically significant differences from controls in mean value g/L (n=3) glycerol measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	23	48	72	96	120
E Control	0.000	1.710	3.981	4.695	5.357	5.372
E FPS1	0.000	2.564	5.411	6.053	6.704	8.123
M Control	0.000	0.260	3.203	4.193	4.633	5.062
M FPS1	0.000	2.614	6.308	7.133	7.507	7.721
Least Significant Difference: 0.946						
Yellow denotes significance at alpha = 0.05						

The final metabolite measured by HPLC for this fermentation was ethanol. The ethanol curve is below in Figure 25. None of the time points had significant differences between the controls and their mutants except for at two points highlighted in Table 16 where the Montrachet

FPS1 mutant has a higher ethanol concentration. This is likely due to the faster sugar/nitrogen usage of the mutant. The overexpression of *FPS1* in Montrachet led to a mutant with more fermentative vigor, but reduced biomass.

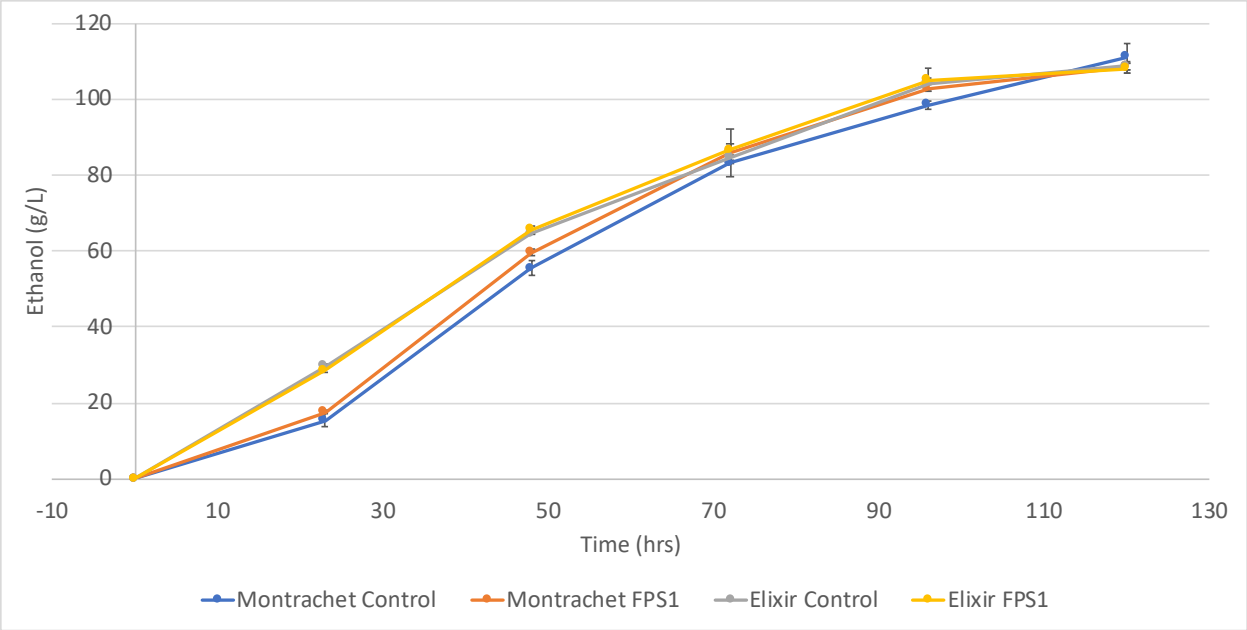


Figure 25. Ethanol mean values of the regular brix fermentation of *FPS1* overexpression mutants made from both commercial strains n=3

Table 16. Statistically significant differences from controls in mean value g/L (n=3) ethanol measurements for *FPS1* overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	23	48	72	96	120
E Control	0.000	29.668	64.572	84.710	104.175	108.703
E FPS1	0.000	28.541	65.639	86.645	104.996	108.143
M Control	0.000	15.468	55.452	83.190	98.458	111.123
M FPS1	0.000	17.610	59.524	85.877	102.926	108.383
Least Significant Difference: 3.033						
Yellow denotes significance at alpha = 0.05						

4.3 *FPS1* Deletion

In the final experiment, strains lacking the *FPS1* gene and strains overexpressing both *FAS1* and *FAS2* together were made for both Montrachet and Elixir and fermented in the high brix media. This was done in an attempt to establish a relationship between the level of expression of *FPS1* and ethanol tolerance, and to see if a whole FAS overexpression would exacerbate the deleterious effects of the *FAS1* overexpression. The sampling was the same as for the previous two experiments. In this case all mutants and controls were run at the same time, but in the interest of clarity the data has been divided into two sections, one for the *fps1*Δ, and one for the FAS complex overexpression.

The *fps1*Δ caused the Montrachet mutant to ferment more quickly and to a lower brix than the control (Figure 26). The Montrachet mutant acted much more similarly to the Elixir mutant and control, which also differed over much of the fermentation but by a smaller margin. Below are the curves for the controls and mutations, as well as a table showing the statistical significance of the differences between the mutant and control means per time point (Table 17).

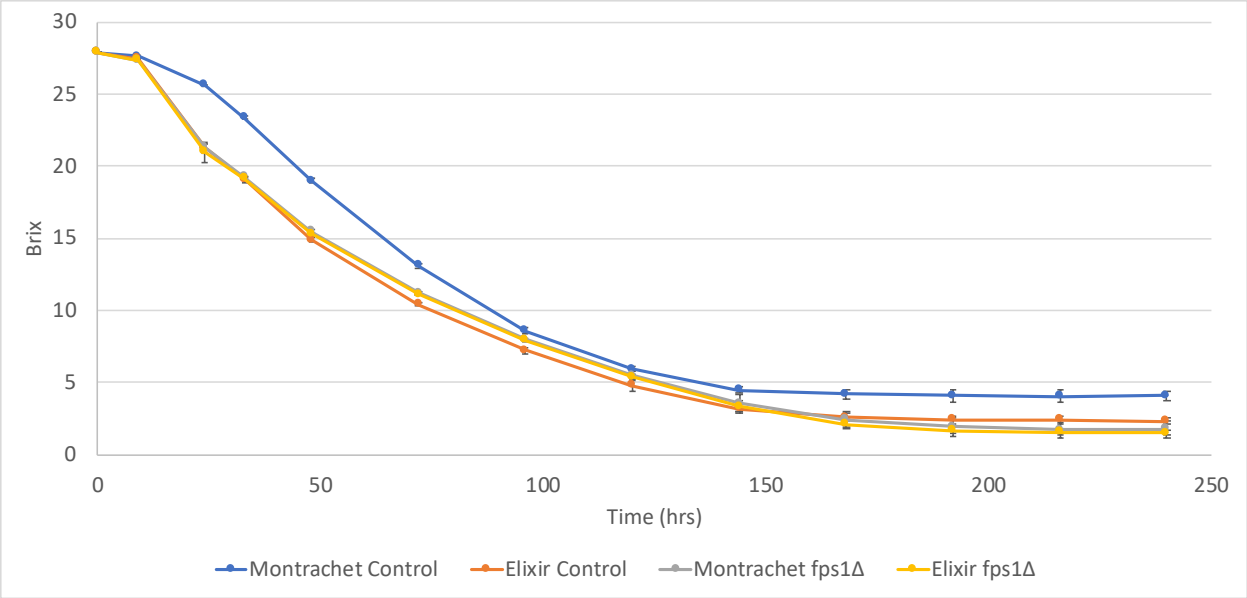


Figure 26. Brix curve of mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 17. Statistically significant differences from controls in mean value (n=3) brix measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample \Time (hrs)	0	9	24	33	48	72	96	120	144	168	192	216	240
E Control	27.900	27.433	21.233	19.100	14.900	10.433	7.200	4.800	3.200	2.600	2.400	2.400	2.333
E -FPS1	27.900	27.333	21.000	19.167	15.300	11.100	7.933	5.400	3.367	2.100	1.667	1.567	1.533
M Control	27.900	27.633	25.600	23.367	19.000	13.100	8.600	5.933	4.500	4.200	4.100	4.067	4.100
M FAS	27.900	27.700	25.167	22.667	18.500	12.533	8.233	5.667	4.567	4.267	4.267	4.267	4.267
Least Significant Difference: 0.441													
Yellow denotes significance at alpha = 0.05													

The optical density data also indicated that these mutations were affecting the base strains differently. In Figure 27, there is a large increase in optical density between the Montrachet control and the Montrachet mutant, while there is a much smaller analogous increase for Elixir.

Interestingly, the two *fps1*Δ mutants, although made from very different base strains, behaved the same. The graph of OD_{600nm} and a table denoting statistically significant changes from the controls (Table 18) follow.

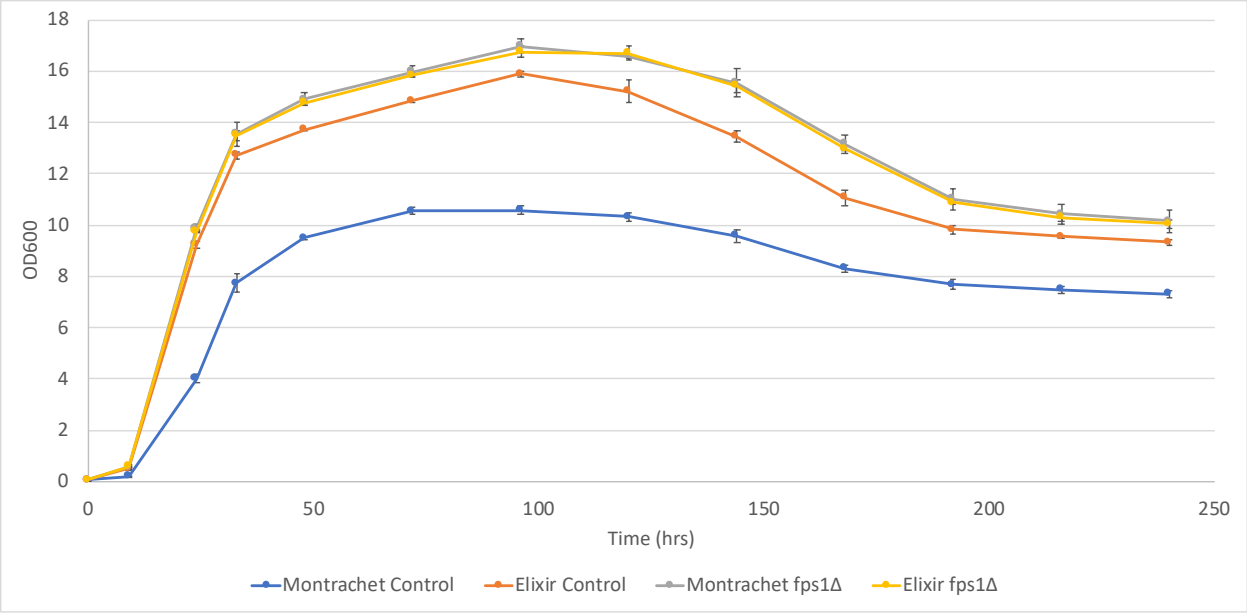


Figure 27. Optical density mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 18. Statistically significant differences from controls in mean value (n=3) OD_{600nm} measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample \Time (hrs)	0	9	24	33	48	72	96	120	144	168	192	216	240
E Control	0.058	0.487	9.253	12.717	13.717	14.850	15.883	15.217	13.450	11.067	9.817	9.550	9.333
E -FPS1	0.061	0.585	9.753	13.483	14.767	15.833	16.750	16.700	15.433	12.983	10.883	10.300	10.033
M Control	0.048	0.188	4.010	7.733	9.483	10.550	10.583	10.317	9.567	8.300	7.683	7.467	7.317
M -FPS1	0.057	0.553	9.893	13.550	14.917	15.983	16.983	16.583	15.550	13.167	11.000	10.433	10.150
Least Significant Difference: 0.329													
Yellow denotes significance at alpha = 0.05													

The dry cell weight data largely agree with the optical density. In Figure 31, the *fps1*Δ in Montrachet groups much more closely with the two Elixir based strains than with the Montrachet control. The graphical representation as well as the statistical table (Table 19) are below.

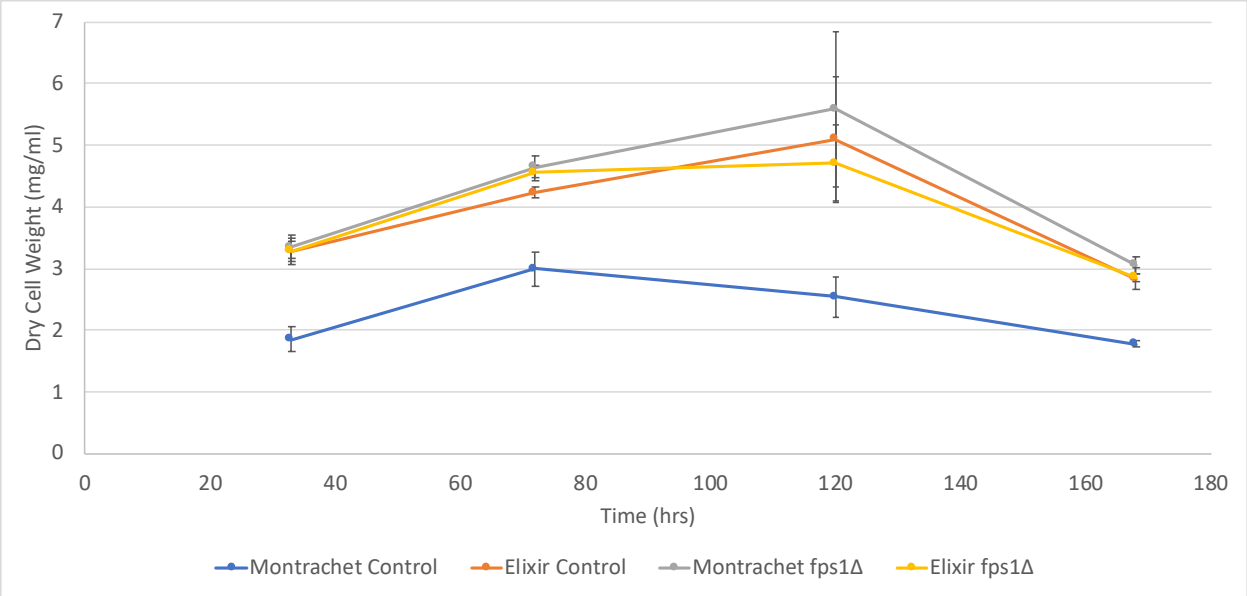


Figure 28. Dry cell weight mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 19. Statistically significant differences from controls in mean value g/L (n=3) dry cell weight measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	33	72	120	168
E Control	3.280	4.233	5.100	2.833
E -FPS1	3.287	4.553	4.707	2.853
M Control	1.853	2.993	2.540	1.773
M -FPS1	3.353	4.640	5.587	3.053
Least Significant Difference: 0.645				
Yellow denotes significance at alpha = 0.05				

Nitrogen uptake between the Montrachet *fps1*Δ and the Elixir control/*fps1*Δ strain were extremely similar (Figure 29). The *fps1*Δ in Montrachet caused greater maximum nitrogen uptake, and enhanced ability to retain that nitrogen as environmental conditions became increasingly hostile. The statistical significance of the mutant values at each time point are below in Table 20.

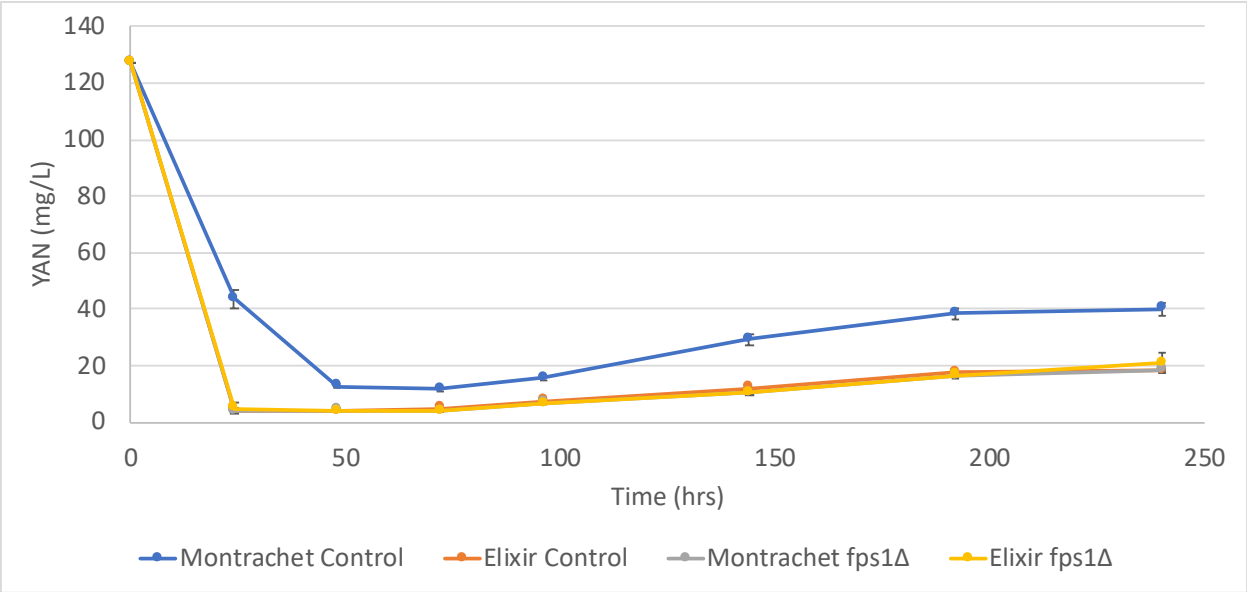


Figure 29. Yeast assimilable nitrogen mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 20. Statistically significant differences from controls in mean value mg/L (n=3) yeast assimilable nitrogen measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	127.000	4.333	4.000	5.000	7.333	12.000	17.667	18.333
E -FPS1	127.000	5.000	4.000	4.000	6.667	10.333	16.667	21.000
M Control	127.000	43.667	12.667	11.667	15.667	29.333	38.333	40.000
M -FPS1	127.000	4.000	4.333	4.000	7.000	10.333	16.333	18.667
Least Significant Difference: 1.994								
Yellow denotes significance at alpha = 0.05								

The *fps1*Δ mutants for both strains again behaved the same. The utilization of both sugars by the two mutants were not significantly different. The Montrachet again showed a greater reaction to the *FPS1* deletion, being grouped more closely with the two Elixirs rather than with the Montrachet control. The Montrachet *fps1*Δ mutant ultimately utilized more of both glucose (Figure 30) and fructose (Figure 31) than the Montrachet control. The statistical tables for glucose and fructose are found below as Table 21 and Table 22.

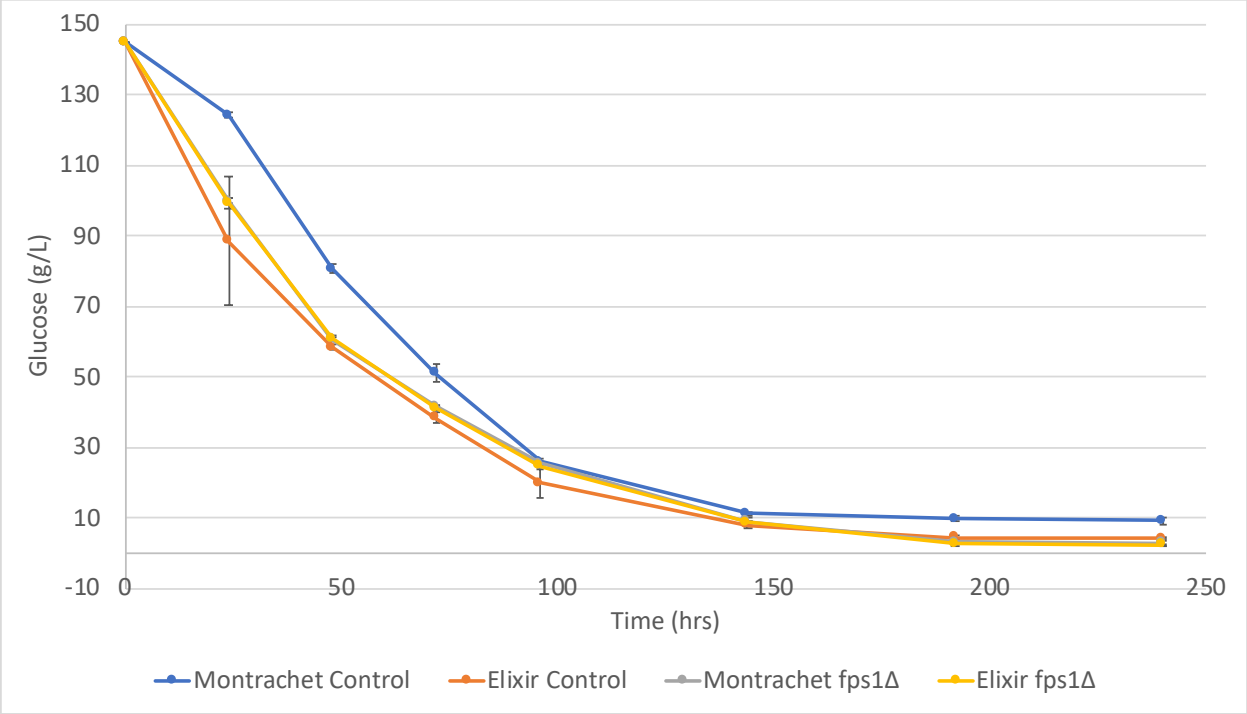


Figure 30. Glucose mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

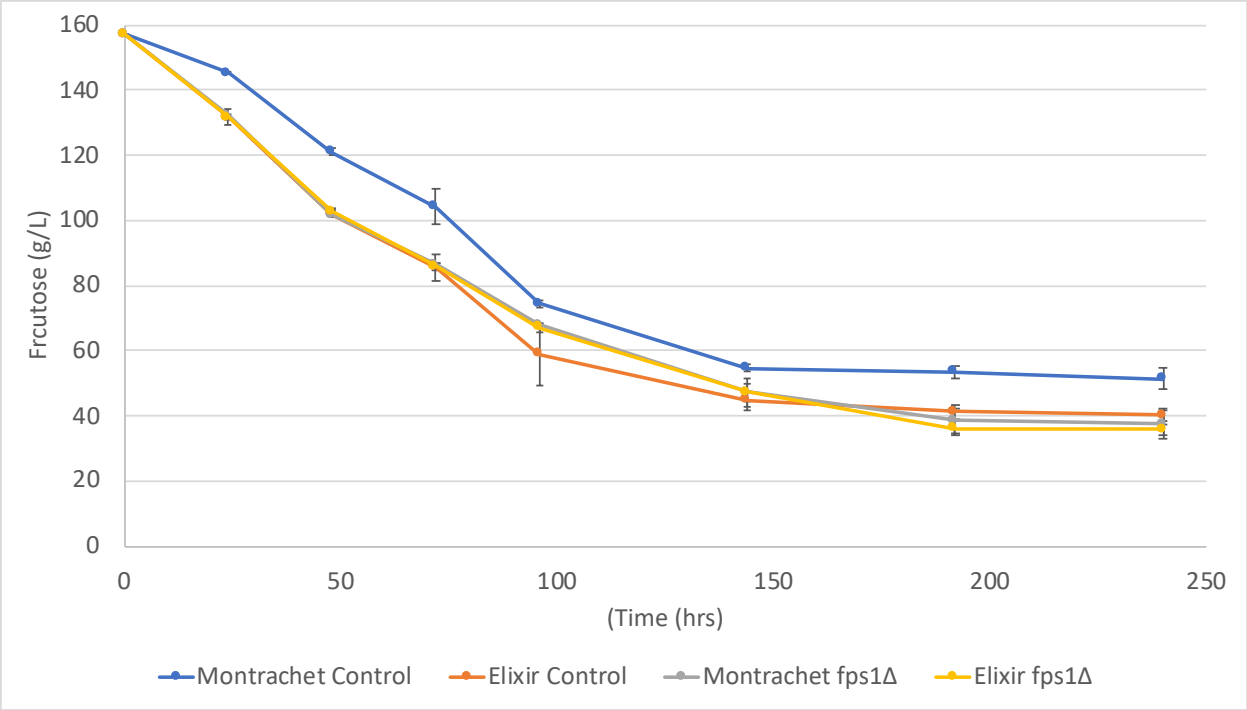


Figure 31. Fructose mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 21. Statistically significant differences from controls in mean value g/L (n=3) glucose measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	144.869	88.562	58.236	38.342	19.838	7.969	4.476	4.243
E -FPS1	144.869	99.367	60.957	41.120	24.736	8.623	2.682	2.514
M Control	144.869	124.115	80.620	51.030	25.868	11.195	9.839	9.123
M -FPS1	144.869	99.893	60.687	41.704	25.419	8.876	3.070	2.920
Least Significant Difference: 4.604								
Yellow denotes significance at alpha = 0.05								

Table 22. Statistically significant differences from controls in mean value g/L (n=3) fructose measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	157.352	131.644	102.103	85.618	59.001	44.791	41.284	40.170
E -FPS1	157.352	131.617	102.904	85.982	67.236	47.230	36.307	35.849
M Control	157.352	145.251	121.129	104.288	74.434	54.621	53.477	51.399
M -FPS1	157.352	132.105	101.852	86.559	68.063	47.217	38.523	37.654
Least Signifcant Difference: 4.226								
Yellow denotes significance at alpha = 0.05								

Glycerol measurements for both *fps1*Δ mutants were, as expected, lower than the controls. Glycerol levels should be lower because *FPS1* codes for the glycerol export channel in the membrane and has a regulatory role in glycerol synthesis. With no export channel and lowered synthesis the resulting drop in glycerol in the media makes sense. These differences can be seen below in Figure 32 and the accompanying statistically significant differences can be found in Table 23.

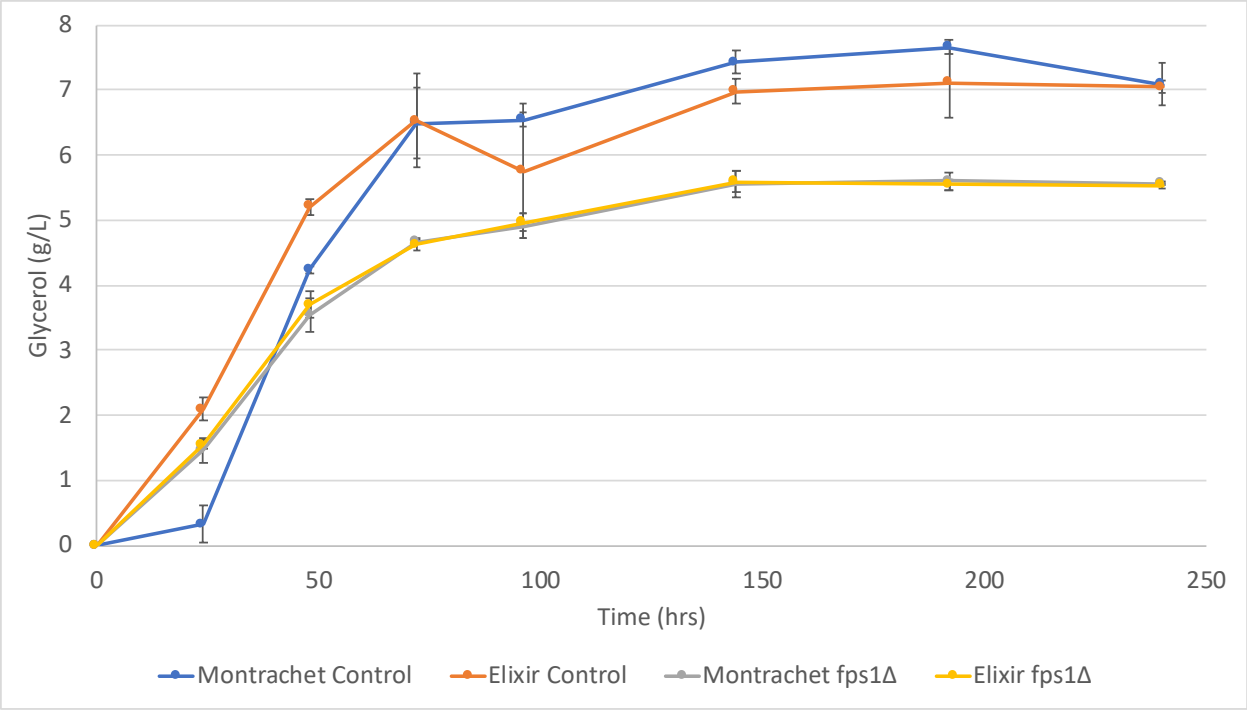


Figure 32. Glycerol mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 23. Statistically significant differences from controls in mean value g/L (n=3) glycerol measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	0.000	2.091	5.203	6.526	5.751	6.976	7.112	7.052
E -FPS1	0.000	1.551	3.706	4.630	4.963	5.596	5.544	5.536
M Control	0.000	0.327	4.247	6.494	6.547	7.433	7.662	7.091
M -FPS1	0.000	1.468	3.551	4.668	4.907	5.556	5.600	5.559
Least Significant Difference: 0.448								
Yellow denotes significance at alpha = 0.05								

The final ethanol concentrations are potentially the best indicator of ethanol tolerance given the definition applied. The concentration of self produced ethanol at which the yeast was

no longer able to continue fermentation is, for the purposes of commercial fermentations, the upper limit to the ethanol tolerance of the inoculum. The Montrachet *fps1*Δ mutant produced more ethanol than the control (Figure 33). The final ethanol concentration of the mutant was approximately 1.6% ABV higher than that of the control. The Elixir *fps1*Δ mutant did not differ significantly from the control, and neither of these from the Montrachet mutant. This increase in ethanol from mutant to control shows the deletion of the *FPS1* gene in the base strain Montrachet to increase its ethanol tolerance, but without similar effect in the Elixir base strain.

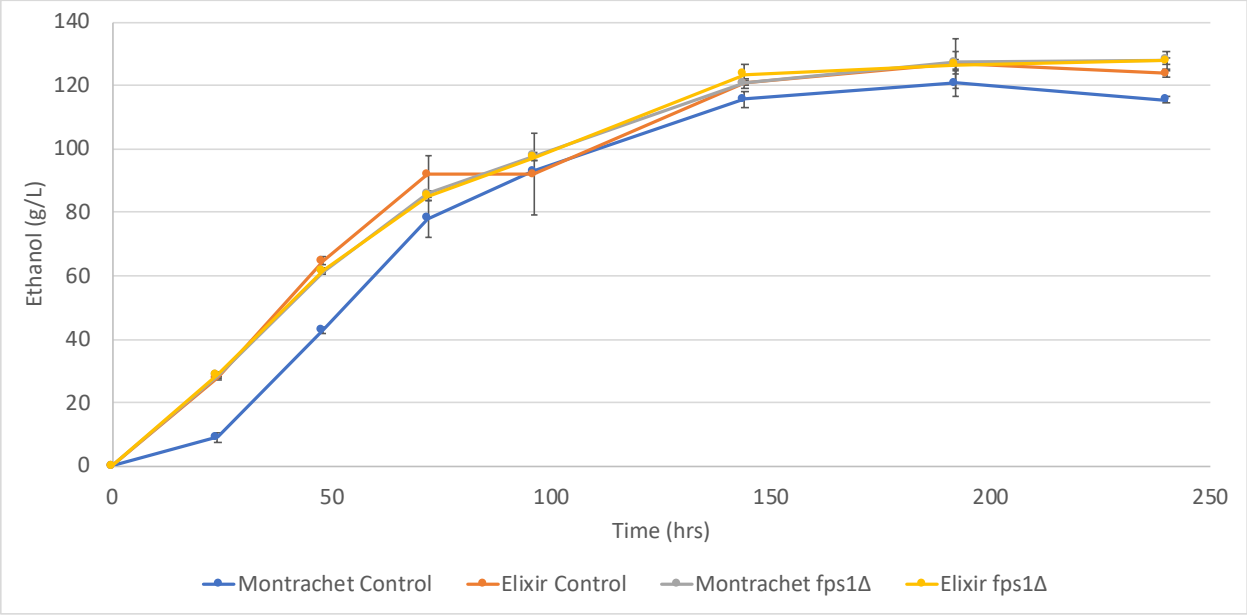


Figure 33. Ethanol mean values of the high brix fermentation of *fps1*Δ mutants made from both commercial strains n=3

Table 24. Statistically significant differences from controls in mean value g/L (n=3) ethanol measurements for *fps1*Δ mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	0.000	27.666	64.618	92.069	92.075	120.785	126.911	123.768
E -FPS1	0.000	28.883	61.681	85.119	97.314	123.614	126.609	127.771
M Control	0.000	8.966	42.923	78.054	92.836	115.741	120.746	115.473
M -FPS1	0.000	28.436	61.277	85.853	97.738	120.650	127.359	128.009
Least Sigificant Difference: 5.114								
Yellow denotes significance at alpha = 0.05								

The deletion of *FPS1* in Montrachet caused an appreciative increase in ethanol produced, which could indicate an increase in ethanol tolerance. These mechanisms are likely strain dependent, as a similar increase was not observed with Elixir. Overexpression of *FPS1* in Montrachet caused a significant increase in glycerol production and a reduction in biomass, but no differences in sugar utilization or ethanol production. When overexpressed in a media containing a more industry standard concentration of sugar (~22 brix) what differences in glycerol and biomass production were initially observed in Montrachet were somewhat muted.

4.4 FAS Complex Overexpression

The other half of the final fermentation was the overexpression of both *FAS1* and *FAS2* in the same strains. The whole FAS complex overexpression led to the Elixir mutant displaying a slower fermentation and finishing at a higher brix (Figure 34). The same overexpression in

Montrachet appeared to have very little effect. The statistically significant differences are outlines in Table 25 below.

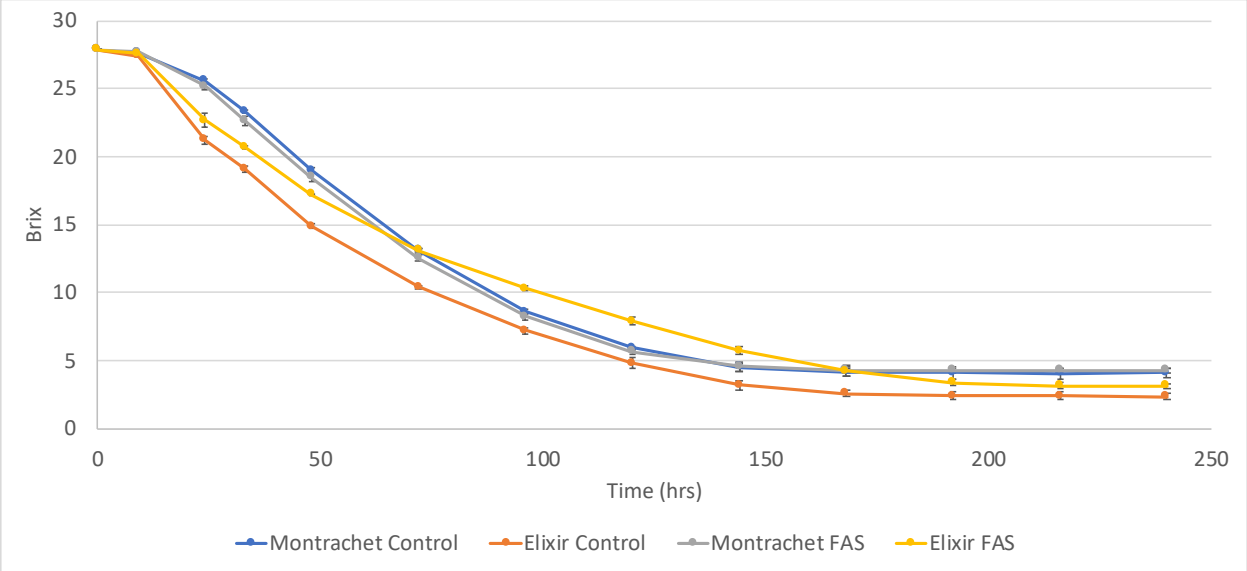


Figure 34. Brix curve of mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 25. Statistically significant differences from controls in mean value (n=3) brix measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample \Time (hrs)	0	9	24	33	48	72	96	120	144	168	192	216	240
E Control	27.900	27.433	21.233	19.100	14.900	10.433	7.200	4.800	3.200	2.600	2.400	2.400	2.333
E -FPS1	27.900	27.333	21.000	19.167	15.300	11.100	7.933	5.400	3.367	2.100	1.667	1.567	1.533
M Control	27.900	27.633	25.600	23.367	19.000	13.100	8.600	5.933	4.500	4.200	4.100	4.067	4.100
M FAS	27.900	27.700	25.167	22.667	18.500	12.533	8.233	5.667	4.567	4.267	4.267	4.267	4.267
Least Significant Difference: 0.441													
Yellow denotes significance at alpha = 0.05													

As can be seen in Figure 35, there was a large disparity between the biomass of the Elixir control and that of the Elixir overexpressing the FAS complex. There was also a similar reduction for the Montrachet strain, but to a far lesser degree. Figure 35 is accompanied below by Table 26, which shows the significant differences.

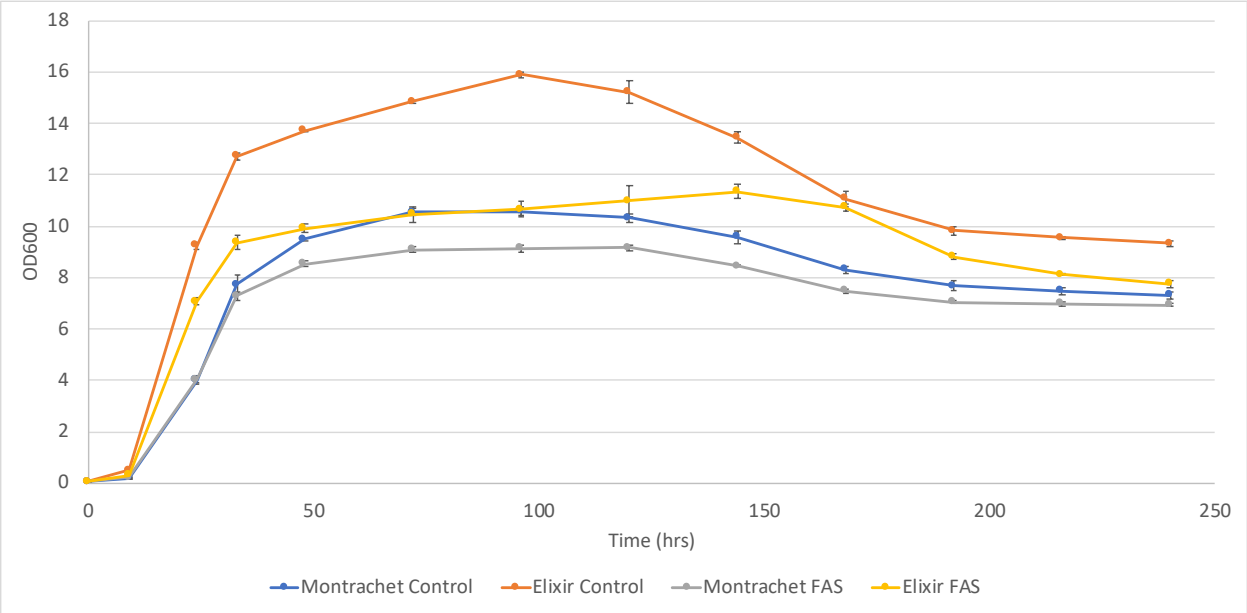


Figure 35. Optical density mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 26. Statistically significant differences from controls in mean value (n=3) OD_{600nm} measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample \Time (hrs)	0	9	24	33	48	72	96	120	144	168	192	216	240
E Control	0.058	0.487	9.253	12.717	13.717	14.850	15.883	15.217	13.450	11.067	9.817	9.550	9.333
E FAS	0.056	0.312	7.073	9.367	9.917	10.450	10.667	10.983	11.350	10.733	8.817	8.117	7.750
M Control	0.048	0.188	4.010	7.733	9.483	10.550	10.583	10.317	9.567	8.300	7.683	7.467	7.317
M FAS	0.055	0.220	4.033	7.283	8.533	9.083	9.133	9.150	8.433	7.467	7.050	6.983	6.917
Least Significant Difference: 0.329													
Yellow denotes significance at alpha = 0.05													

In Figure 36, The FAS complex mutant shows reduced biomass as compared to the control, with the same overexpression having little effect on the Montrachet. This is in agreement with the optical density measurements as expected. The statistical information follows in Table 27.

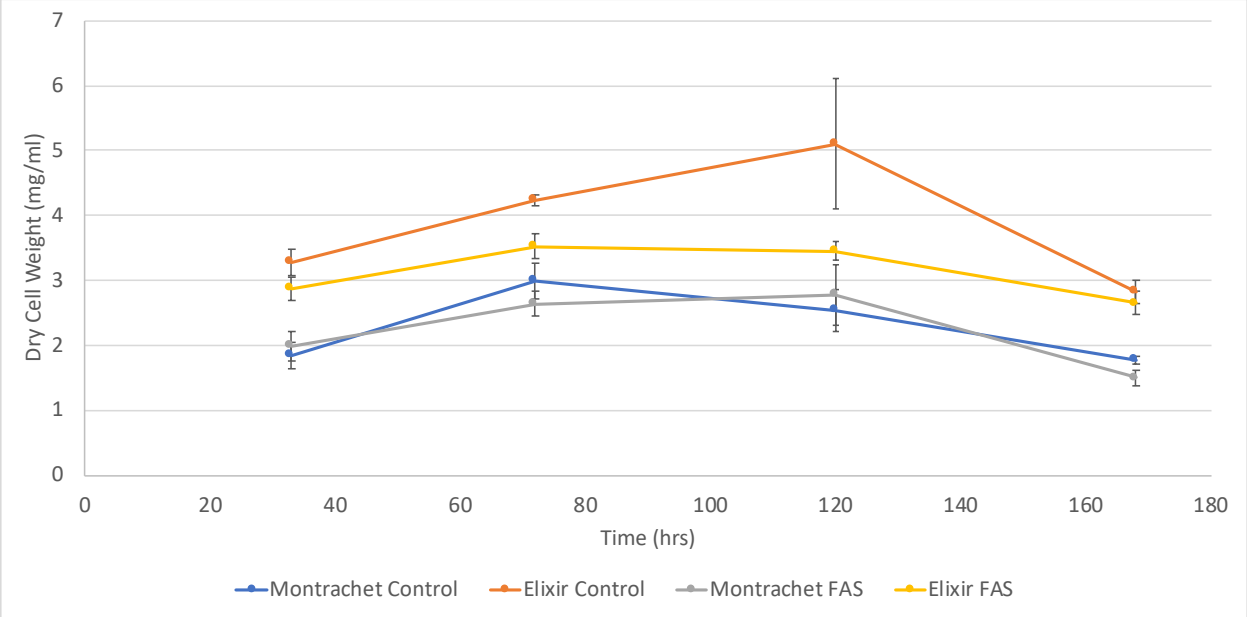


Figure 36. Dry cell weight mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 27. Statistically significant differences from controls in mean value g/L (n=3) dry cell weight measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	33	72	120	168
E Control	3.280	4.233	5.100	2.833
E FAS	2.873	3.527	3.453	2.653
M Control	1.853	2.993	2.540	1.773
M FAS	1.993	2.640	2.787	1.500
Least Significant Difference: 0.645				
Yellow denotes significance at alpha = 0.05				

Nitrogen uptake did not differ considerably between the controls and FAS complex mutants. The most significant difference was the Montrachet overexpression mutant's increased ability to retain nitrogen as the fermentation reached the ending stages (Figure 37). Table 28 highlights the significance.

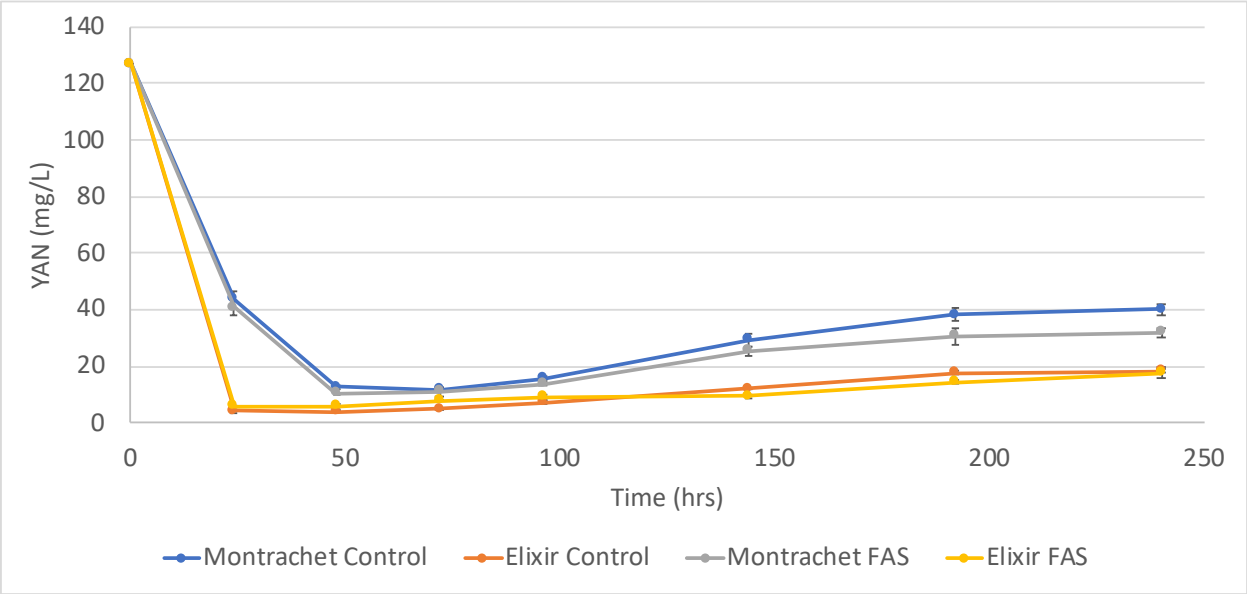


Figure 37. Yeast assimilable nitrogen mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 28. Statistically significant differences from controls in mean value mg/L (n=3) yeast assimilable nitrogen measurements for FAS complex mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	127.000	4.333	4.000	5.000	7.333	12.000	17.667	18.333
E FAS	127.000	6.000	6.000	8.000	9.000	9.333	14.333	17.667
M Control	127.000	43.667	12.667	11.667	15.667	29.333	38.333	40.000
M FAS	127.000	40.667	10.333	11.000	13.667	25.333	30.667	32.000
Least Significant Difference: 1.994								
Yellow denotes significance at alpha = 0.05								

The sugar utilization curves for the FAS complex mutants show a slower sugar uptake for both glucose (Figure 38) and fructose (Figure 39) for the Elixir mutant as compared to the control. The Elixir mutant utilized less glucose throughout the fermentation, and finished with more residual glucose as compared to the control. There was no difference in final sugar utilized for the Montrachet mutant from the control for either sugar, and only small differences in the rate of usage. Tables 29 and 30 show the significance of these curves.

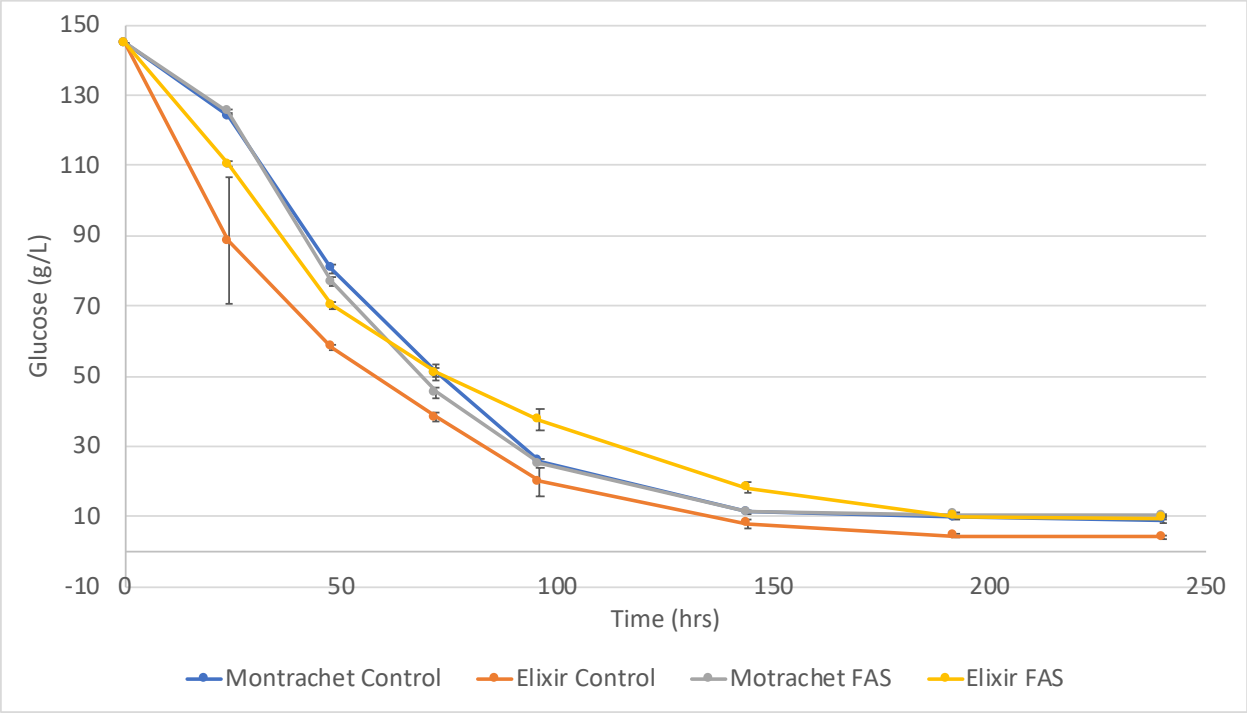


Figure 38. Glucose mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

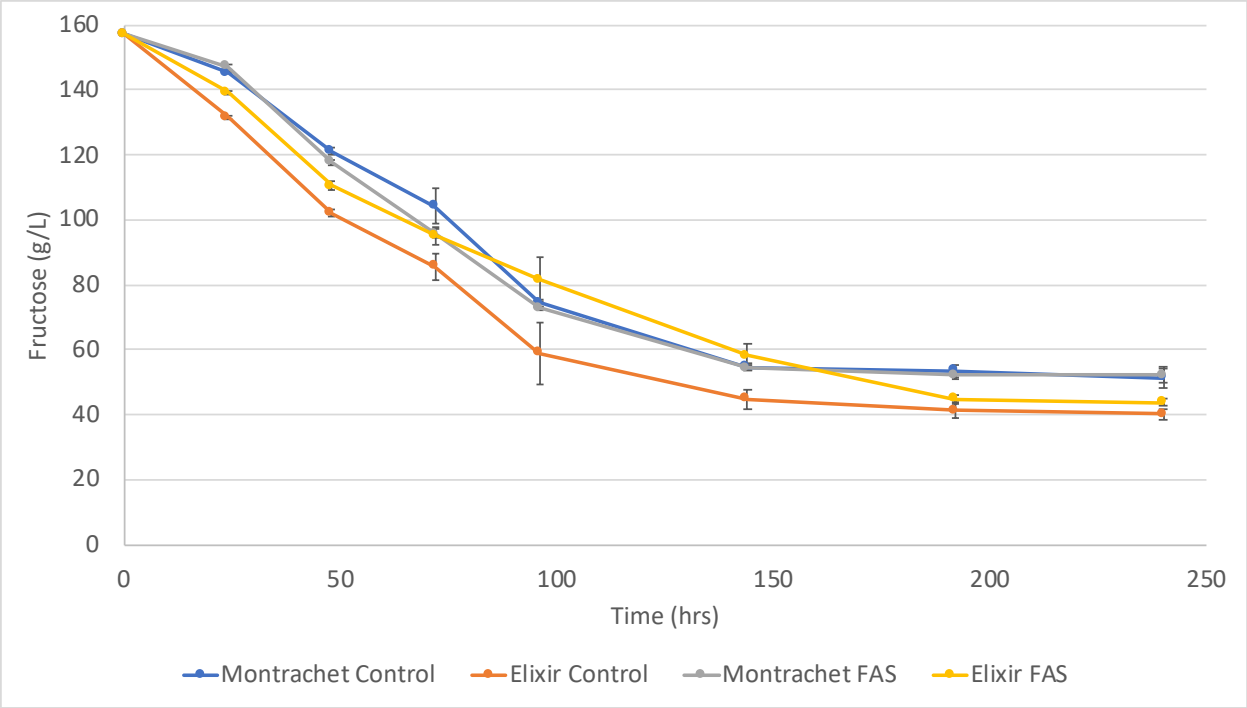


Figure 39. Fructose mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 29. Statistically significant differences from controls in mean value g/L (n=3) glucose measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	144.869	88.562	58.236	38.342	19.838	7.969	4.476	4.243
E FAS	144.869	110.438	70.166	50.915	37.445	18.210	10.081	9.541
M Control	144.869	124.115	80.620	51.030	25.868	11.195	9.839	9.123
M FAS	144.869	125.205	76.808	45.333	24.972	11.369	10.473	10.219
Least Significant Difference: 4.604								
Yellow denotes significance at alpha = 0.05								

Table 30. Statistically significant differences from controls in mean value g/L (n=3) fructose measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	157.352	131.644	102.103	85.618	59.001	44.791	41.284	40.170
E FAS	157.352	139.169	110.470	95.083	81.656	58.198	44.904	43.777
M Control	157.352	145.251	121.129	104.288	74.434	54.621	53.477	51.399
M FAS	157.352	147.148	117.852	95.733	72.765	54.487	52.153	52.240
Least Signifcant Difference: 4.226								
Yellow denotes significance at alpha = 0.05								

Glycerol measurements were particularly interesting for the last round of mutants, as the FAS complex mutants saw a change in glycerol production. As the FAS genes deal with competing metabolic pathways, it is likely that these changes are caused by a redirection of cellular efforts toward fatty acid synthesis. The Montrachet FAS mutant allowed less glycerol into the media than the control by certain time points, but ended at approximately the same amount. The Elixir FAS mutant, however, ended up producing significantly less glycerol overall (Figure 40). This is particularly interesting because, as expected, both *fps1*Δ mutants allowed less glycerol into the media as compared to the controls, and did not differ between each other (Figure 32). The *FPS1* deletion is causing reduced glycerol efflux in Montrachet and improving its ability to ferment lower, while FAS complex overexpression in Elixir is reducing glycerol production, but hindering its ability to ferment lower. This could potentially be because increased intracellular glycerol lends a benefit to the cells, or because of strain specific adaptations, or could indicate that the benefit Montrachet is getting out of the *FPS1* deletion may be

independent of glycerol directly. The latter is unlikely as *FPS1* is a glycerol export channel with a small role in the regulation of glycerol production. The significance is shown in Table 31.

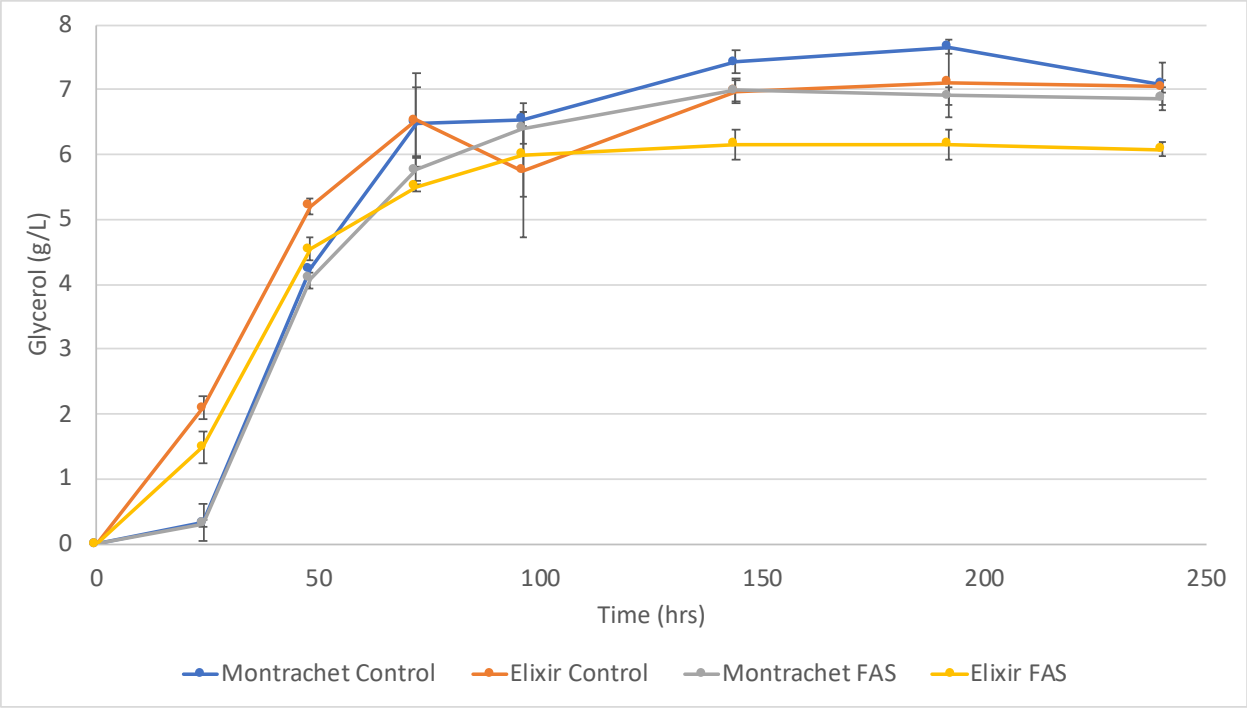


Figure 40. Glycerol mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 31. Statistically significant differences from controls in mean value g/L (n=3) glycerol measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	0.000	2.091	5.203	6.526	5.751	6.976	7.112	7.052
E FAS	0.000	1.497	4.540	5.507	5.997	6.160	6.158	6.078
M Control	0.000	0.327	4.247	6.494	6.547	7.433	7.662	7.091
M FAS	0.000	0.310	4.100	5.765	6.409	6.987	6.906	6.864
Least Significant Difference: 0.448								
Yellow denotes significance at alpha = 0.05								

The Elixir FAS complex mutant produced ethanol more slowly than the control, with many time points toward the middle of fermentation having less ethanol in the media, but ultimately produced approximately the same amount (Figure 41). While the Elixir FAS complex overexpression mutant produced less biomass and utilized slightly less glucose, it reached the same final ethanol. In a practical sense, reaching the same final ethanol would mean that the control and mutant have the same upper limit to ethanol tolerance. The Montrachet FAS mutant was nearly the same as the control, only differing at one point near the end of fermentation. The significance of these results can be seen in Table 32.

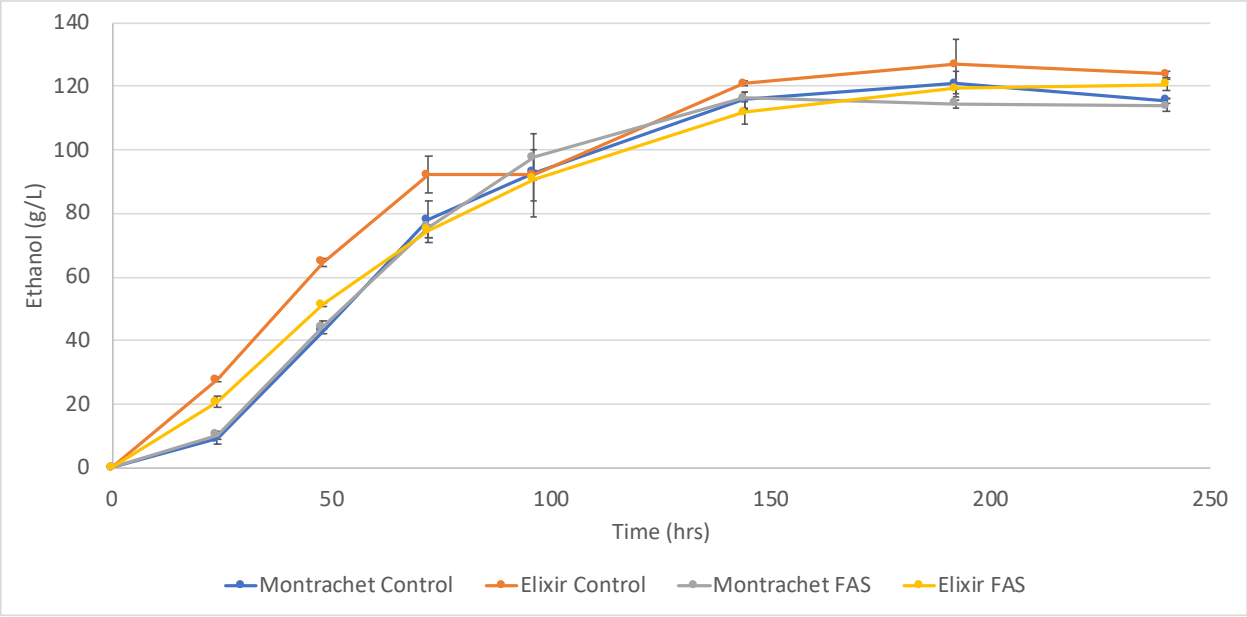


Figure 41. Ethanol mean values of the high brix fermentation of FAS complex overexpression mutants made from both commercial strains n=3

Table 32. Statistically significant differences from controls in mean value g/L (n=3) ethanol measurements for FAS complex overexpression mutants at different time points during the regular brix fermentation

Sample\Time (hrs)	0	24	48	72	96	144	192	240
E Control	0.000	27.666	64.618	92.069	92.075	120.785	126.911	123.768
E FAS	0.000	20.684	51.398	74.647	90.880	111.725	119.301	120.610
M Control	0.000	8.966	42.923	78.054	92.836	115.741	120.746	115.473
M FAS	0.000	10.437	44.386	75.854	97.634	116.195	114.464	113.817
Least Sigificant Difference: 5.114								
Yellow denotes significance at alpha = 0.05								

The overexpression of the FAS complex had more effect than either *FAS1* or *FAS2* individually. Overexpression of the complex in Elixir led to a slower fermentation, lower glucose consumption, and reduced biomass, but the same final ethanol concentration. This would appear to be strain dependent as the effects were far more obvious in the Elixir mutant than in the Montrachet mutant.

Chapter 5: Discussion

These experiments were the first in which these genes were overexpressed in commercial wine yeast strains in a high brix, well defined, wine-like medium. Many of the genes observed here had been altered before in *Saccharomyces cerevisiae* in order to assess their impact on ethanol tolerance, but never in a way as analogous to a true commercial fermentation as this.

The most exciting finding, a possible increase in ethanol tolerance, came in the form of a deletion of the *FPS1* gene in the base strain Montrachet. The *FPS1* gene codes for a glycerol transport channel in the membrane, responsible for glycerol efflux out into the extracellular environment. This glycerol production and subsequent efflux is used by the cell to resist changing osmotic pressures in the high sugar environment, as well as a way to maintain cellular redox potential and regenerate NADH. It is important to note that the specific gene deleted does not control glycerol production, but its export out of the cell, and as seen in the deletion mutants, a lack of *FPS1* does not completely prevent glycerol from moving into the medium. Given that glycerol is still being produced, the cell should still be gaining the benefits of resisting early osmotic pressure brought on by the high sugar medium, as well as the regeneration of NADH and redox maintenance. This is not to say that the production of glycerol remains at the same magnitude, however, and these benefits are likely reduced. *FPS1* plays a regulatory role in glycerol production, so a deletion of the gene would likely reduce glycerol production, and this carbon may be redirected toward ethanol production. This is the most likely explanation for the increase in ethanol observed, and has been observed in other studies (Zhang et al 2006). It is entirely possible that the cells were not more ethanol tolerant, but simply produced more ethanol from the same level of sugar taken up.

Another place where the cell could be losing is in the removal of glycerol once extracellular sugar concentrations become low enough that the osmotic pressure the intracellular glycerol was originally needed for is reduced. However, glycerol is still leaving the cell, and in similar amounts regardless of base strain. Montrachet may therefore be gaining a benefit from the lack of extra membrane spanning proteins. Having more membrane spanning proteins may prove harmful to the cell once the concentration of ethanol has grown high enough to cause portions of the cell to interdigitate. It could be that the reaction of the fps1 protein to the resulting hydrophobic mismatch could be particularly damaging to the membrane as the protein aggregates and further disturbs membrane stability.

This explanation fails to account for a few factors. The first being the near immediate reduction in biomass accumulated in the *FPS1* overexpression mutant in Montrachet. This reduction occurs well before the concentration of ethanol in the cell reaches a point that should significantly inhibit the yeast's ability to grow. The opposite effect is observable in the *FPS1* deletion mutant, which caused a near immediate increase in biomass. This was accompanied by a more robust fermentation, meaning faster sugar utilization and nitrogen uptake. Perhaps the most peculiar part is that these results are in stark contrast to those of Texeira et al. (2009) in which the overexpression of *FPS1* resulted in more biomass and a higher final ethanol concentration, while the deletion resulted in a lower biomass. The main differences between this study and Texeira et al. (2009) are the base strain and the media. The base strain used in the Texeira et al. (2009) experiment was a laboratory strain (BY4741), and the media was YPD. In this experiment, commercial wine strains served as the base strains and the media was a grape juice-like solution.

The most likely explanation for this discrepancy has to do with strain dependent responses to the differential expression of the gene. In this experiment, the differences between the response of Montrachet and that of Elixir were quite considerable. If added to the conclusions of Texeira et al. (2009), one ends up with 3 different strains all demonstrating different responses to levels of FPS1 expression. BY4741, according to Texeira et al. (2009), benefitted from more *FPS1* expression, Elixir, in this experiment, did not show dramatic differences in either direction, and Montrachet, also in this experiment, benefitted from less *FPS1* expression. Because of the clear and convincing results of the Texeira study, the results gathered here were not as expected, but several other studies have concluded that deletion of *FPS1* in their different laboratory strains has increased the production of ethanol, suggesting that this may be the more common result (Zhang et al. 2006)(Kong et al. 2006). Unfortunately, these studies were performed in far simpler lab media, and with lab strains of yeast. Zhang et al. (2006) also overexpressed a second gene in the mutants. The goals of both of these studies were directed toward increasing ethanol production at the expense of glycerol, and not at improving the survivability of the yeast in high ethanol conditions. While beyond the scope of this study, it would be interesting to perform a transcriptomics analysis of a variety of *S. cerevisiae* strains differentially expressing the *FPS1* gene to try and elucidate what differences in the rest of the genome this may be causing. Information such as this may shed light on the strain specific interactions that appear to be causing such conflicting results in mutants from different genetic backgrounds.

This leads to a question of how Montrachet and Elixir differ. While both are commercial wines strains, they were both isolated at different times and in different places. Their genetic

differences could be relatively significant, and these differences could potentially inform why the two are seeing differences in response to the level of *FPS1* expression.

Similar in terms of strain dependency, the FAS complex over expression had a greater effect on the Elixir strain than on the Montrachet strain. The FAS complex expression was greater in magnitude than the *FAS1* expression alone, and far greater than the *FAS2* which had no significant effect. There has been some disagreement in the literature as to the effectiveness of overexpressing these genes individually in order to increase total activity of the complex (Furukawa et al. 2003)(Chirala et al. 1992)(Wenz et al. 2001). The results here agreed with Furukawa et al. (2003) which showed no difference in recorded response with *FAS2* overexpression alone, a modest response with *FAS1* alone, and the greatest response with both *FAS1* and *FAS2* in a single strain. In this experiment the Elixir FAS mutant grew a lower biomass, fermented more slowly, and utilized slightly less glucose ultimately. It did, however, end with no significant difference in ethanol concentration. Because there is less biomass, it would suggest that more sugar is being consumed, and more ethanol produced, per cell. More sugar being consumed per cell could potentially be expected, as the upregulated fatty acid synthesis of the cell requires carbon, but the expectation would be to have a lower level of produced ethanol as the cell would likely be producing higher levels of acetate instead to feed the increased level of fatty acid synthesis. It would be useful to examine acetate levels in both strains overexpressing the FAS complex, as well as to collect lipidomic data in order to determine the differences in lipid metabolism and fermentation between the two strains. This could potentially provide valuable information about why Elixir was so strongly affected by the overexpression, and why Montrachet had a muted response by comparison. Both *FAS1* and *FAS2* are essential

genes for survival, and as a result can not be completely removed from the genome to leave a viable mutant, but it would also be interesting to reduce expression and determine if a beneficial effect on the fermentation could be achieved. The reduced expression of the FAS complex was not tested in mutants in this experiment due primarily to time constraints.

Taken together, the increase in ethanol tolerance by Montrachet with an *FPS1* deletion, and a decrease in ethanol tolerance by Elixir with FAS overexpression could indicate that the two strains overcome in different ways concentrations of ethanol that prove lethal to other microbes. Glycerol production and efflux may be a more important component to the complex phenotype of ethanol resistance in Montrachet, while for Elixir the composition of the membrane may be more significant.

The *SOD2* gene may also merit further study, although only a limited amount of data was collected with regard to it here. The Montrachet *SOD2* mutant displayed a lengthened lag phase, which caused it to take longer to complete fermentation, but it did ferment to a slight but statistically significant lower brix level. It also displayed a higher biomass than the control at several points along fermentation in both strains. These results were not as striking as those observed with the *FPS1* gene and the FAS complex, but as differences in strain response have been shown here to sometimes be significant, it would be advantageous to test the effect of differential expression of *SOD2* in several different *S. cerevisiae* strains. In this experiment alone, Montrachet appeared to be affected to a greater degree by the *SOD2* overexpression than Elixir. Owing to the fact that *SOD2* codes for a superoxide dismutase and is involved in maintaining redox balance, the increased response to both this and *FPS1* could indicate that

maintaining redox balance is a more important component to ethanol tolerance in Montrachet than in Elixir.

The final remaining genes tested were *OLE1* and *ACC1*, both involved in fatty acid synthesis. Neither caused considerable changes from controls in either case. One potential reason for this is due to the anaerobic conditions of the fermentations. The hypothesis that the overexpression of these genes would increase the yeast's ability to ferment to a higher concentration of ethanol was predicated on the assumption that oleic acid (C18:1) concentrations in the membrane would increase. That increased concentrations of oleic acid can aid in increasing yeast ethanol tolerance is a fairly well researched phenomena, and is a relatively safe assumption (You et al. 2003)(Renne and de Kroon 2017). However, the desaturation step that forms oleic acid (C18:1) from stearic acid (C18:0) requires both the fatty acid desaturase enzyme coded for by *OLE1* and oxygen. As these fermentations were run anaerobically in stoppered flasks, there may not have been sufficient oxygen to supplement the increase in ole1p and actually give rise to an appreciable increase in the oleic acid concentration of the cell. *ACC1*, having been shown to increase the C18:C16 chain length ratio in *S. cerevisiae* upon being made hyperactive, was expected to increase the stearic acid concentration in the pool out of which ole1p desaturated, thus increasing the oleic:palmitoleic acid ratio (Hofbauer et al. 2014). It is possible that the C18:C16 ratio was indeed increased, but that this change alone without a subsequent increase in desaturation was not enough to cause an appreciable change. As there were no lipidomic data were taken, it is impossible to say whether or not this occurred. Even assuming that these changes did occur and the mutants produced more oleic acid, there is a second reason why these genes may not have shown any significant results: integration.

Having a greater production of oleic acid, and having a greater amount of oleic acid integrated into the membrane are two different things. In order to incorporate these fatty acid chains into the membrane they must be attached to a phosphate head group via an acyl transferase enzyme. Two acyl transferases in particular, coded for by *ALE1* and *ICT1* have a high affinity for unsaturated fatty acid chains and may enhance integration of these into the membrane if overexpressed (Ghosh et al. 2008)(Riekhof et al. 2007). By not ensuring increases in these acytransferases as well as the increase in oleic acid, it is entirely possible that more oleic acid was produced, but was never actually incorporated into the membrane where it would presumably cause a difference.

It would have been extremely valuable to have collected lipidomic data on these mutants. By quantifying the different lipids in the membranes it would become possible to determine if the gene overexpression for many of these genes had the intended immediate effect. If there were evidence that these changes in membrane lipid composition occurred, it would be more clear why these genes did or did not have the desired effect.

The plasmids in these experiments were stabilized by including a hygromycin resistance gene and supplementing the media with hygromycin. Unfortunately, for reasons that are not fully understood, the MMM fermentation media dramatically reduced the effectiveness of the hygromycin against cells not containing the plasmid. For this reason the cell cultures were grown up in YPD media appropriately supplemented with hygromycin and then inoculated into the MMM. There is a limited number of cell divisions once this inoculation takes place, but it does provide a small chance for the cells to shed the plasmid, rendering some cells no different from the base strain. As statistically significant differences between control flasks and mutant flasks

were observed this certainly did not happen in all cases, but there is a chance that it may have reduced the magnitude of the differences observed, or there is the remote possibility that it caused real differences to remain undetected. However even were such a thing to occur, the proteins already produced by the cells before the shedding of the plasmid would remain even after the plasmid itself were lost.

The experiments would also have benefitted from HPLC analysis of organic acids. Due to the high sugar and ethanol concentrations in the samples 1:20 dilutions needed to be made in order to accommodate the resulting HPLC peaks. Because these dilutions were so small and the sugar peaks so large it was impossible to quantify the relatively small organic acid peaks. If a less dilute sample were used the sugar peaks become large enough to engulf the organic acids, but if a dilution is made to reduce the sugar peak size, it renders the organic acid peaks too small to accurately quantify by RID. Time and scheduling constraints prevented returning to the HPLC, but with a less dilute sample it could potentially be possible to quantify organic acids by DAD without the interference of the sugar peaks seen on the RID. The calibration curves used for the HPLC in these experiments can be found in the supplemental information.

It is important to note that while some of the candidate genes observed in this study returned little to no definitive difference from the controls, this by no means should disqualify them from future study. Strain differences observed in these experiments highlight the importance of utilizing a variety of base strains when making mutants in order to capture a range of possible responses to the differential expression. Differences in temperature, pH, and dissolved oxygen also could play considerable roles in the effectiveness of these genetic alterations.

Chapter 6: Conclusion

The success of increasing the ethanol tolerance of the commercial yeast Montrachet by the deletion of the *FPS1* gene is an important step toward understanding how the *Saccharomyces cerevisiae* genome can be leveraged for greater ethanol production. However, the obvious strain dependency of the results from both *FPS1* and the FAS complex mutants tested here emphasizes the importance of future study into not only different candidate genes, but also different base strains and the interactions between the two.

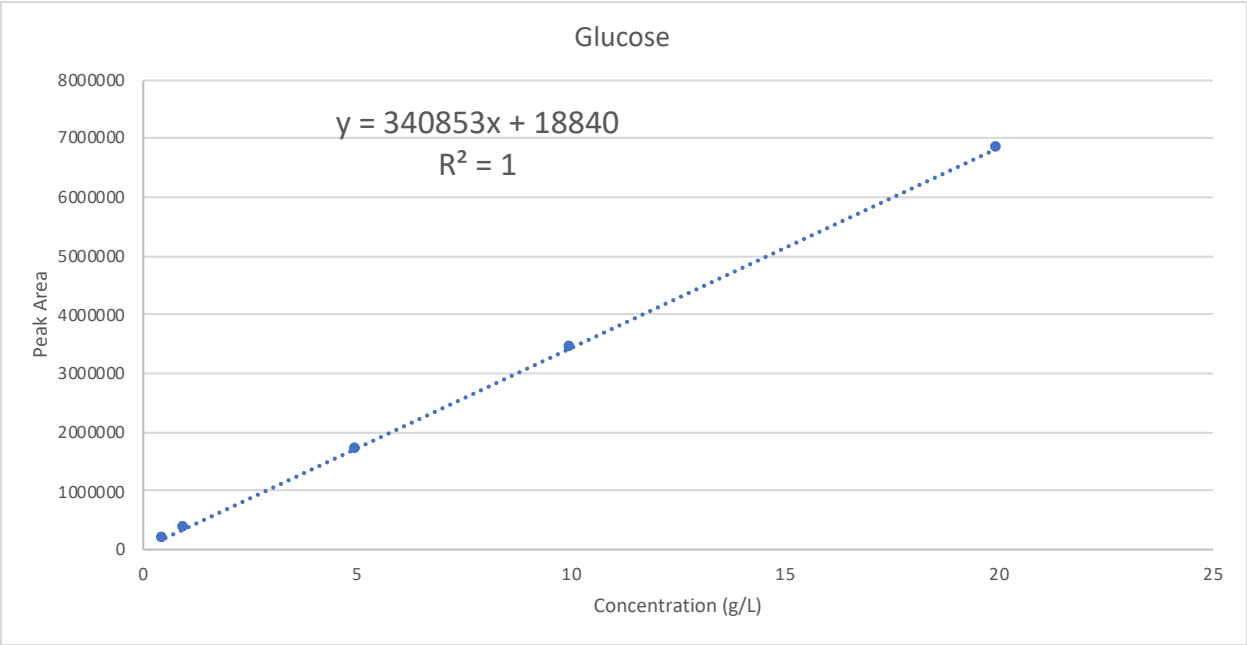
FPS1 and the FAS complex should be the focus of further study into both their effects on a strain's ability to tolerate growing ethanol concentrations, but also the differences in response that strains have to differential expression of these genes. *FPS1* has been demonstrated here in the Montrachet strain to provide significant differences in the maximum amount of ethanol produced in a high brix fermentation. Because Elixir did not show the same significant response, a variety of different commercial strains should be tested in order to determine how common the beneficial response seen in Montrachet is. It could be that most strains do not at all benefit from *FPS1* deletion, and that Montrachet is a rare exception. This would make the mechanism by which Montrachet benefits all the more interesting. It could also be that *FPS1* deletion provides these benefits to most strains, in which case deletion of *FPS1* could be an important tool in the development of new, more ethanol tolerant commercial strains. The other effects of the deletion of *FPS1* on wine sensory characteristics should also be weighed. While *FPS1* deletion in Montrachet allowed for the yeast to produce more alcohol, it also significantly reduced the amount of glycerol excreted into the media. Glycerol can be a desirable component to wine mouthfeel. A yeast producer or a winemaker may have to weigh the relative merits and

drawbacks to increasing ethanol tolerance while simultaneously decreasing glycerol in the finished product.

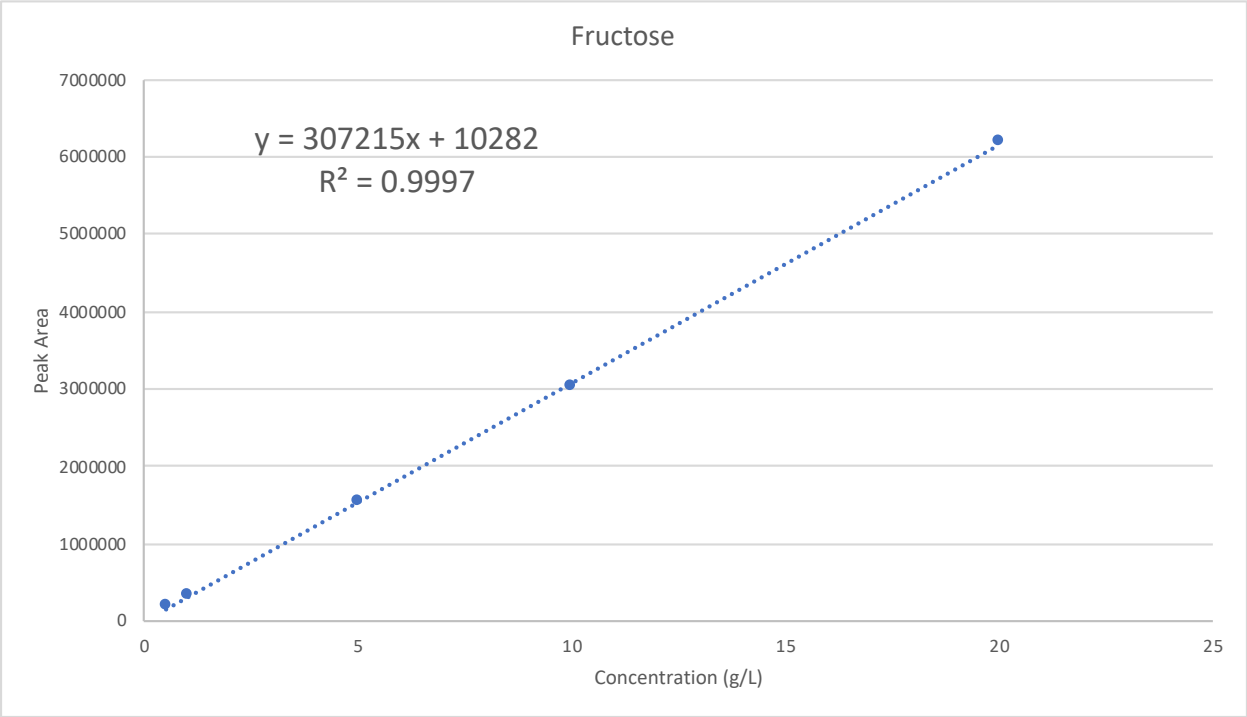
Likewise, the FAS complex should receive further attention. Similar attempts should be made in order to determine how common more drastic responses like the one seen here in Elixir are across commercial strains. Because only overexpressions of these particular genes were tested here, it would also be of great utility to observe mutants with reduced FAS activity in order to possibly achieve an ethanol tolerance benefit. While these experiments show decreases in ethanol tolerance with overexpression, that is only half of a potentially useful trend.

This study highlights the useful potential of the *FPS1* gene and the FAS complex in developing new commercial strains of *Saccharomyces cerevisiae*. It also emphasizes the importance of strain differences in their response to differential gene expressions. In order to maximize the effectiveness of new commercial strain development it is crucial to continue to identify genes such as these which may alter ethanol tolerance in a predictable way, and to refine these predictions by observing reactions to differential expression in a variety of existing strains.

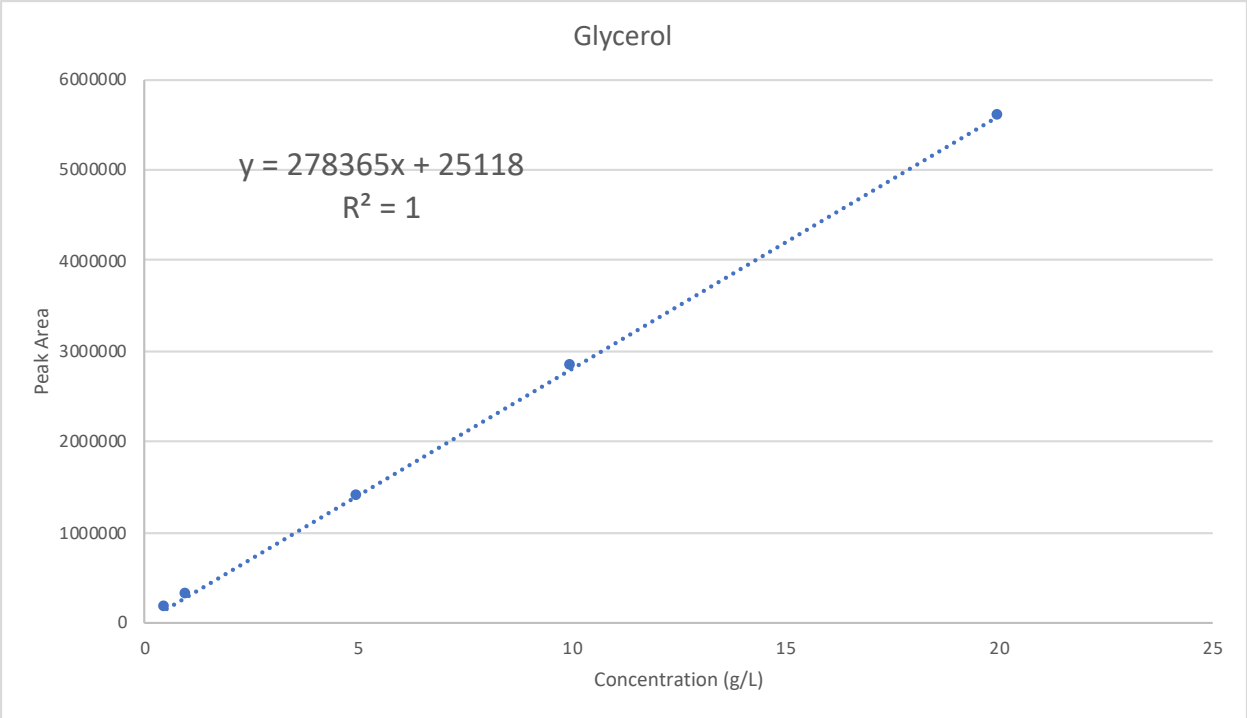
Supplemental Information



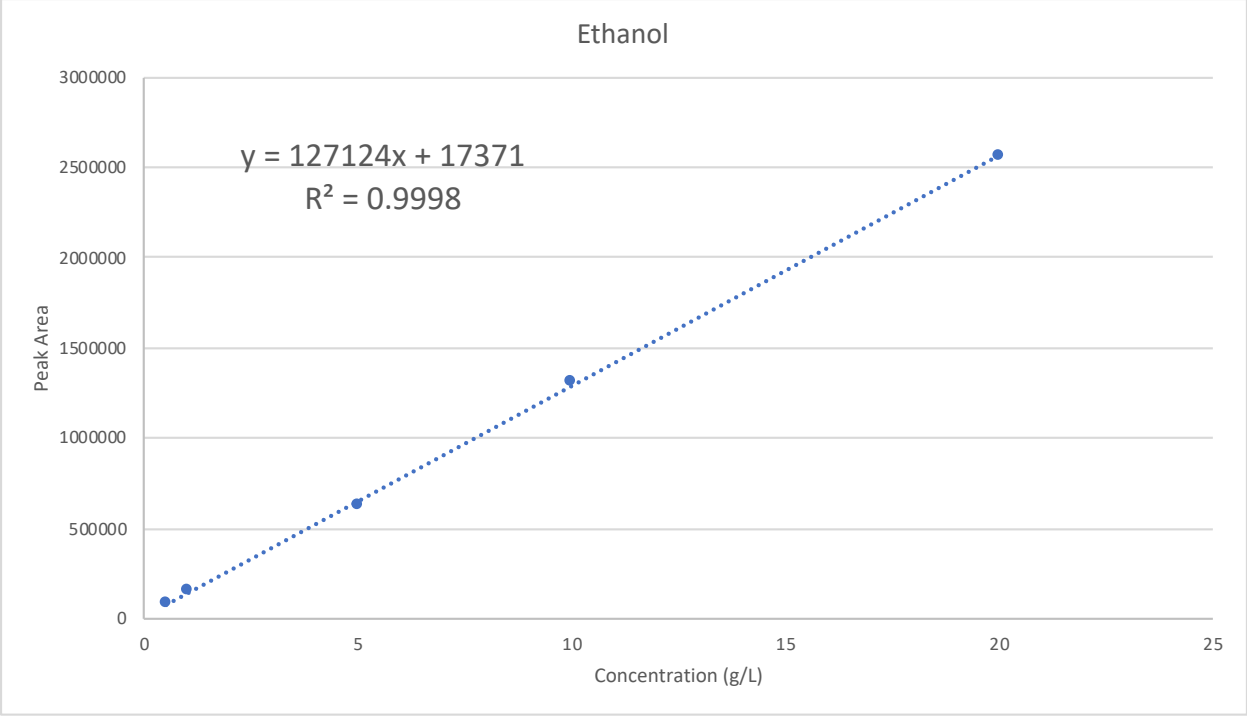
Supplemental 1. HPLC calibration curve for glucose



Supplemental 2. HPLC calibration curve for fructose



Supplemental 3. HPLC calibration curve for glycerol



Supplemental 4. HPLC calibration curve for ethanol

Supplemental 5. Analysis of variance for the Montrachet initial screening brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	92	25230.124	274.240	8203.775	**<0.0001**
Error	180	6.017	0.033		
Corrected Total	272	25236.141			

Supplemental 6. Type III sum of squares for the Montrachet initial screening brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.030	0.015	0.442	0.644
Sample	6.000	7.813	1.302	38.955	0.000
Time	12.000	25192.530	2099.378	62801.892	0.000
Sample*Time	72.000	29.751	0.413	12.361	0.000

Supplemental 7. Analysis of variance for the Montrachet initial screening optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	92	3760.055	40.870	439.645	**<0.0001**
Error	180	16.733	0.093		
Corrected Total	272	3776.788			

Supplemental 8. Type III sum of squares for the Montrachet initial screening optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.828	0.414	4.456	0.013
Sample	6.000	37.569	6.261	67.355	0.000
Time	12.000	3652.983	304.415	3274.629	0.000
Sample*Time	72.000	68.674	0.954	10.260	0.000

Supplemental 9. Analysis of variance for the Montrachet initial screening dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	36	29.007	0.806	34.092	**<0.0001**
Error	68	1.607	0.024		
Corrected Total	104	30.614			

Supplemental 10. Type III sum of squares for the Montrachet initial screening dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.083	0.041	1.756	0.181
Sample	6.000	1.745	0.291	12.309	0.000
Time	4.000	25.014	6.254	264.598	0.000
Sample*Time	24.000	2.164	0.090	3.815	0.000

Supplemental 11. Analysis of variance for the Montrachet initial screening yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	57	210020.163	3684.564	457.796	**<0.0001**
Error	110	885.333	8.048		
Corrected Total	167	210905.497			

Supplemental 12. Type III sum of squares for the Montrachet initial screening yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	28.000	14.000	1.739	0.180
Sample	6.000	223.333	37.222	4.625	0.000
Time	7.000	207782.830	29683.261	3688.056	0.000
Sample*Time	42.000	1986.000	47.286	5.875	0.000

Supplemental 13. Analysis of variance for the Montrachet initial screening glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	229965.197	9198.608	42.215	**<0.0001**
Error	46	10023.290	217.898		
Corrected Total	71	239988.487			

Supplemental 14. Type III sum of squares for the Montrachet initial screening glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	223.755	111.877	0.513	0.602
Sample	2.000	629.482	314.741	1.444	0.246
Time	7.000	225850.451	32264.350	148.071	0.000
Sample*Time	14.000	3261.509	232.965	1.069	0.408

Supplemental 15. Analysis of variance for the Montrachet initial screening fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	153003.329	6120.133	20.937	**<0.0001**
Error	46	13446.591	292.317		
Corrected Total	71	166449.919			

Supplemental 16. Type III sum of squares for the Montrachet initial screening fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	359.499	179.749	0.615	0.545
Sample	2.000	495.381	247.690	0.847	0.435
Time	7.000	148091.351	21155.907	72.373	0.000
Sample*Time	14.000	4057.098	289.793	0.991	0.477

Supplemental 17. Analysis of variance for the Montrachet initial screening glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	1005.070	40.203	60.689	**<0.0001**
Error	46	30.472	0.662		
Corrected Total	71	1035.542			

Supplemental 18. Type III sum of squares for the Montrachet initial screening glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.035	0.017	**0.026**	0.974
Sample	2.000	173.296	86.648	130.803	0.000
Time	7.000	776.154	110.879	167.381	0.000
Sample*Time	14.000	55.584	3.970	5.994	0.000

Supplemental 19. Analysis of variance for the Montrachet initial screening ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	126265.404	5050.616	146.756	**<0.0001**
Error	46	1583.096	34.415		
Corrected Total	71	127848.500			

Supplemental 20. Type III sum of squares for the Montrachet initial screening ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	4.991	2.495	0.073	0.930
Sample	2.000	59.344	29.672	0.862	0.429
Time	7.000	125861.855	17980.265	522.452	0.000
Sample*Time	14.000	339.214	24.230	0.704	0.759

Supplemental 21. Analysis of variance for the Elixir initial screening brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	106	28381.069	267.746	1435.299	**<0.0001**
Error	208	38.801	0.187		
Corrected Total	314	28419.870			

Supplemental 22. Type III sum of squares for the Elixir initial screening brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	16.146	8.073	43.276	0.000
Sample	6.000	10.748	1.791	9.603	0.000
Time	14.000	28334.496	2023.893	10849.432	0.000
Sample*Time	84.000	19.680	0.234	1.256	0.099

Supplemental 23. Analysis of variance for the Elixir initial screening optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	106	6317.223	59.596	967.975	**<0.0001**
Error	208	12.806	0.062		
Corrected Total	314	6330.029			

Supplemental 24. Type III sum of squares for the Elixir initial screening optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.615	0.308	4.996	0.008
Sample	6.000	21.736	3.623	58.840	0.000
Time	14.000	6285.684	448.977	7292.366	0.000
Sample*Time	84.000	9.188	0.109	1.777	0.001

Supplemental 25. Analysis of variance for the Elixir initial screening dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	36	109.589	3.044	12.426	**<0.0001**
Error	68	16.659	0.245		
Corrected Total	104	126.248			

Supplemental 26. Type III sum of squares for the Elixir initial screening dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.246	0.123	0.501	0.608
Sample	6.000	2.532	0.422	1.723	0.129
Time	4.000	101.856	25.464	103.942	0.000
Sample*Time	24.000	4.955	0.206	0.843	0.672

Supplemental 27. Analysis of variance for the Elixir initial screening yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	50	143433.782	2868.676	4539.443	**<0.0001**
Error	96	60.667	0.632		
Corrected Total	146	143494.449			

Supplemental 28. Type III sum of squares for the Elixir initial screening yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	2.000	1.000	1.582	0.211
Sample	6.000	5.306	0.884	1.399	0.223
Time	6.000	143409.211	23901.535	37822.209	0.000
Sample*Time	36.000	17.265	0.480	0.759	0.824

Supplemental 29. Analysis of variance for the Elixir initial screening glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	22	192355.368	8743.426	1654.419	**<0.0001**
Error	40	211.396	5.285		
Corrected Total	62	192566.764			

Supplemental 30. Type III sum of squares for the Elixir initial screening glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	1.943	0.971	0.184	0.833
Sample	2.000	12.885	6.442	1.219	0.306
Time	6.000	192258.986	32043.164	6063.165	0.000
Sample*Time	12.000	81.554	6.796	1.286	0.264

Supplemental 31. Analysis of variance for the Elixir initial screening fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	22	143764.324	6534.742	394.947	**<0.0001**
Error	40	661.835	16.546		
Corrected Total	62	144426.159			

Supplemental 32. Type III sum of squares for the Elixir initial screening fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	3.510	1.755	0.106	0.900
Sample	2.000	42.570	21.285	1.286	0.287
Time	6.000	143536.952	23922.825	1445.849	0.000
Sample*Time	12.000	181.292	15.108	0.913	0.543

Supplemental 33. Analysis of variance for the Elixir initial screening glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	22	489.827	22.265	131.266	**<0.0001**
Error	40	6.785	0.170		
Corrected Total	62	496.612			

Supplemental 33. Type III sum of squares for the Elixir initial screening glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.286	0.143	0.843	0.438
Sample	2.000	14.690	7.345	43.304	0.000
Time	6.000	470.990	78.498	462.799	0.000
Sample*Time	12.000	3.860	0.322	1.897	0.065

Supplemental 34. Analysis of variance for the Elixir initial screening ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	22	140868.729	6403.124	457.947	**<0.0001**
Error	40	559.290	13.982		
Corrected Total	62	141428.018			

Supplemental 35. Type III sum of squares for the Elixir initial screening ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	25.541	12.771	0.913	0.409
Sample	2.000	87.791	43.896	3.139	0.054
Time	6.000	140613.520	23435.587	1676.096	0.000
Sample*Time	12.000	141.876	11.823	0.846	0.605

Supplemental 36. Analysis of variance for the regular brix fermentation brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	33	7021.034	212.759	12393.220	**<0.0001**
Error	62	1.064	0.017		
Corrected Total	95	7022.098			

Supplemental 37. Type III sum of squares for the regular brix fermentation brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.069	0.034	2.008	0.143
Sample	3.000	20.598	6.866	399.936	0.000
Time	7.000	6976.722	996.675	58056.437	0.000
Sample*Time	21.000	23.646	1.126	65.589	0.000

Supplemental 38. Analysis of variance for the regular brix fermentation optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	33	3478.269	105.402	4664.965	**<0.0001**
Error	62	1.401	0.023		
Corrected Total	95	3479.670			

Supplemental 39. Type III sum of squares for the regular brix fermentation optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.216	0.108	4.789	0.012
Sample	3.000	187.687	62.562	2768.925	0.000
Time	7.000	3221.060	460.151	20365.729	0.000
Sample*Time	21.000	69.306	3.300	146.067	0.000

Supplemental 40. Analysis of variance for the regular brix fermentation dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	13	43.730	3.364	34.301	**<0.0001**
Error	22	2.158	0.098		
Corrected Total	35	45.888			

Supplemental 41. Type III sum of squares for the regular brix fermentation dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.143	0.072	0.731	0.493
Sample	3.000	31.077	10.359	105.629	0.000
Time	2.000	8.657	4.328	44.136	0.000
Sample*Time	6.000	3.854	0.642	6.549	0.000

Supplemental 42. Analysis of variance for the regular brix fermentation yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	89241.736	3569.669	5428.258	**<0.0001**
Error	46	30.250	0.658		
Corrected Total	71	89271.986			

Supplemental 43. Type III sum of squares for the regular brix fermentation yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	1.750	0.875	1.331	0.274
Sample	3.000	35.153	11.718	17.818	0.000
Time	5.000	89095.903	17819.181	27096.936	0.000
Sample*Time	15.000	108.931	7.262	11.043	0.000

Supplemental 44. Analysis of variance for the regular brix fermentation glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	139359.762	5574.390	610.614	**<0.0001**
Error	46	419.941	9.129		
Corrected Total	71	139779.703			

Supplemental 45. Type III sum of squares for the regular brix fermentation glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	26.546	13.273	1.454	0.244
Sample	3.000	395.127	131.709	14.427	0.000
Time	5.000	137675.805	27535.161	3016.177	0.000
Sample*Time	15.000	1262.284	84.152	9.218	0.000

Supplemental 46. Analysis of variance for the regular brix fermentation fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	151860.052	6074.402	412.559	**<0.0001**
Error	46	677.291	14.724		
Corrected Total	71	152537.343			

Supplemental 47. Type III sum of squares for the regular brix fermentation fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	39.570	19.785	1.344	0.271
Sample	3.000	817.685	272.562	18.512	0.000
Time	5.000	149878.755	29975.751	2035.883	0.000
Sample*Time	15.000	1124.042	74.936	5.089	0.000

Supplemental 48. Analysis of variance for the regular brix fermentation glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	496.566	19.863	59.993	**<0.0001**
Error	46	15.230	0.331		
Corrected Total	71	511.796			

Supplemental 49. Type III sum of squares for the regular brix fermentation glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	1.130	0.565	1.706	0.193
Sample	3.000	63.733	21.244	64.166	0.000
Time	5.000	413.577	82.715	249.832	0.000
Sample*Time	15.000	18.126	1.208	3.650	0.000

Supplemental 50. Analysis of variance for the regular brix fermentation ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	118026.187	4721.047	1386.134	**<0.0001**
Error	46	156.672	3.406		
Corrected Total	71	118182.859			

Supplemental 51. Type III sum of squares for the regular brix fermentation ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	4.227	2.113	0.621	0.542
Sample	3.000	314.755	104.918	30.805	0.000
Time	5.000	117225.050	23445.010	6883.627	0.000
Sample*Time	15.000	482.156	32.144	9.438	0.000

Supplemental 52. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	79	20920.570	264.817	3548.016	**<0.0001**
Error	154	11.494	0.075		
Corrected Total	233	20932.064			

Supplemental 53. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation brix measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.326	0.163	2.182	0.116
Sample	5.000	194.441	38.888	521.023	0.000
Time	12.000	20612.711	1717.726	23014.050	0.000
Sample*Time	60.000	113.092	1.885	25.254	0.000

Supplemental 54. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	79	5370.873	67.986	1632.876	**<0.0001**
Error	154	6.412	0.042		
Corrected Total	233	5377.285			

Supplemental 55. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation optical density measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.350	0.175	4.204	0.017
Sample	5.000	927.292	185.458	4454.326	0.000
Time	12.000	4174.500	347.875	8355.235	0.000
Sample*Time	60.000	268.731	4.479	107.573	0.000

Supplemental 56. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	25	80.254	3.210	20.823	**<0.0001**
Error	46	7.092	0.154		
Corrected Total	71	87.346			

Supplemental 57. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation dry cell weight measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.664	0.332	2.155	0.128
Sample	5.000	42.436	8.487	55.053	0.000
Time	3.000	31.454	10.485	68.008	0.000
Sample*Time	15.000	5.700	0.380	2.465	0.010

Supplemental 58. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	49	213453.924	4356.203	2878.896	**<0.0001**
Error	94	142.236	1.513		
Corrected Total	143	213596.160			

Supplemental 59. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation yeast assimilable nitrogen measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	10.431	5.215	3.447	0.036
Sample	5.000	6163.285	1232.657	814.630	0.000
Time	7.000	203349.326	29049.904	19198.296	0.000
Sample*Time	35.000	3930.882	112.311	74.223	0.000

Supplemental 60. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	49	339409.722	6926.729	858.844	**<0.0001**
Error	94	758.127	8.065		
Corrected Total	143	340167.849			

Supplemental 61. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation glucose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	18.339	9.170	1.137	0.325
Sample	5.000	3098.350	619.670	76.833	0.000
Time	7.000	333280.437	47611.491	5903.341	0.000
Sample*Time	35.000	3012.596	86.074	10.672	0.000

Supplemental 62. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	49	245090.271	5001.842	735.960	**<0.0001**
Error	94	638.857	6.796		
Corrected Total	143	245729.128			

Supplemental 63. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation fructose measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	16.640	8.320	1.224	0.299
Sample	5.000	3995.972	799.194	117.592	0.000
Time	7.000	239584.455	34226.351	5035.993	0.000
Sample*Time	35.000	1493.204	42.663	6.277	0.000

Supplemental 64. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	49	874.137	17.840	233.532	**<0.0001**
Error	94	7.181	0.076		
Corrected Total	143	881.318			

Supplemental 65. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation glycerol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	0.156	0.078	1.023	0.364
Sample	5.000	30.146	6.029	78.927	0.000
Time	7.000	814.206	116.315	1522.648	0.000
Sample*Time	35.000	29.629	0.847	11.082	0.000

Supplemental 66. Analysis of variance for the FPS1 deletion/FAS complex overexpression fermentation ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	49	287053.656	5858.238	588.653	**<0.0001**
Error	94	935.482	9.952		
Corrected Total	143	287989.138			

Supplemental 67. Type III sum of squares for the FPS1 deletion/FAS complex overexpression fermentation ethanol measurements

Source	DF	Sum of squares	Mean squares	F	Pr > F
Replication	2.000	10.662	5.331	0.536	0.587
Sample	5.000	2856.459	571.292	57.405	0.000
Time	7.000	282358.135	40336.876	4053.167	0.000
Sample*Time	35.000	1828.400	52.240	5.249	0.000

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