

AN EXAMINATION OF CELL WALL PROPERTIES AFFECTING  
BREWING YEAST FLOCCULATION

by

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*This thesis is dedicated to my family, both near and far.*

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## Abstract

Flocculation, the process whereby yeast cells attach in groups and sediment to the top or bottom of a fermenter, is industrially important in many fermentation batch operations. These batch operations include wine, distilled spirits, cider, bio-ethanol and production of commercial yeast metabolites. In the case of brewing yeast, it has been determined that flocculation occurs due to three forces called hydrophobic interactions, zymolectin binding and to a lesser extent, surface charge neutralization. This project sought to more closely study hydrophobic interactions and zymolectin binding.

Earlier studies had shown that certain hydrophobic carboxylic acids, 3-OH oxylipins, formed in brewing yeast at flocculation onset. Therefore, these compounds showed potential as an indicator of overall cell surface hydrophobicity, and it was believed that flocculation level, cell surface hydrophobicity and oxylipin level would increase in unison in the yeast cell during brewing fermentations. During lab scale fermentations in shaker flasks and in a miniature fermentation assay setup, both flocculation level and cell surface hydrophobicity increased coincidentally. However, 3-OH oxylipins could not be isolated from whole cells or cell wall isolates grown in the shaker flasks or whole cells grown in the miniature fermentation assay at detection limits approximated as 50 ng/0.5 g wet yeast. Due to their minute levels in brewing yeast cells, it was proposed that 3-OH oxylipins may mediate flocculation and aggregation via a quorum sensing mechanism instead of by increasing cell surface hydrophobicity.

A disagreement exists in the literature where certain researchers believe zymolectin activity is induced, while others believe it is constitutive. The second part of this study attempted to address this by measuring zymolectin density during lab scale fermentations with a flow cytometer. Because of flow cytometry's capacity for multiparametric analysis, large amounts of data were produced which gave information on not only zymolectin density, but also cell size and cellular complexity. Upon statistical analysis of the data, it was not possible to either refute or confirm the claim that zymolectin activity is induced or constitutive. However, the results suggested there could have been a population of cells with fewer zymolectins, and this certainly warrants further investigation. During the lab scale fermentations, cell size measured by a flow cytometer appeared to be correlated with manual measures of cell size. Furthermore, cell size tended towards uniformity during the fermentation which has also been observed in similar studies employing flow cytometry. Conversely, the cellular complexity of the yeast in this study did not change as in other studies by this may have been due either to strain differences or the methods used herein.

## List Of Abbreviations And Symbols Used

ASBC	American Society of Brewing Chemists
BAME	Bacterial Acid Methyl Ester
BATH	Bacterial Adherence to Hydrocarbon
CSH	Cell Surface Hydrophobicity
DAG	Diacylglycerols
EDTA	Ethylenediaminetetraacetic Acid
FAME	Fatty Acid Methyl Ester
FSC	Forward Scatter
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HIP	Hexane Isopropanol
HSL	Homoserine Lactone
MAG	Monoacylglycerols
MATH	Microbial Adhesion to Hydrocarbon
MOA	Methods of Analysis
MS	Mass Spectrometry
NIST	National Institute for Standards and Technology
PBS	Phosphate Buffered Saline
pI	Isoelectric Point
SIM	Selective Ion Monitoring
SSC	Side Scatter
TAG	Triacylglycerols
TMS	Trimethylsilyl
WGA	Wheat Germ Agglutinin

YEPD	Yeast Extract Peptone and Dextrose
YIS	Yeast in Suspension
3-OH	Three Hydroxy
Ap	Surface Area of a Prolate Spheroid
a	Half Major Axis of a Prolate Spheroid
b	Half Minor Axis of a Prolate Spheroid
°P	Degrees Plato
Vp	Volume of a Prolate Spheroid
$\gamma_{LV}$	Interfacial Tension of the Vapour Phase
$\gamma_{SL}$	Interfacial Tension of the Liquid Phase
$\gamma_{SV}$	Interfacial Tension of the Solid Phase

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# Chapter 1: Introduction

There are three biological mechanisms by which *Saccharomyces cerevisiae* forms cell clusters, including mating aggregates, chain formation and flocculation (Strauss, 2005). All three of these mechanisms are of interest in basic biological research, however, flocculation is of particular interest in the brewing and fermentation sciences. Flocculation is a complex process which has been defined different ways, but it has been most aptly described as, “...the phenomenon wherein yeast cells adhere in clumps and either sediment rapidly from the medium in which they are suspended or rise to the medium’s surface. The process is reversible and should not be confused with chain formation that occurs in some yeasts” (Stewart & Russell, 1981).

In the brewing industry and other fermentation batch operations (e.g., wine, distilled spirits, cider, bio-ethanol and production of commercial yeast metabolites), a complete and timely flocculation is crucial (Nevoigt, 2008). In these batch operations (excluding wine and distilled spirits), the yeast sediment is harvested from the ferment upon completion, treated and often re-inoculated into to a new fermentation. The efficacy of the production process depends, in part, on the flocculation characteristics of the yeast strain used. If the yeast does not flocculate properly, this creates economic losses for the brewer or manufacturer and in the case of beer, can also impact the sensory quality of the final product (Huuskonen, Rautio and Londesborough, 2008).

While flocculation remains to be very important to the brewer, it and other aggregation processes are often not desirable in haploid yeast strains used in laboratories. Large-scale screening and genetic engineering are easier when cells are suspended in their environment or in

the reaction medium. This fact explains why flocculation has not been as highly studied in haploid research strains (Van Mulders et al., 2012). Nonetheless, it has been determined that flocculation occurs due to three phenomena termed hydrophobic interactions, zymolectin binding and to a lesser extent, surface charge neutralization (Speers, 2012). Zymolectin binding, a process where glycoproteins on the yeast cell surface bind to carbohydrate moieties on adjacent cells (Speers, 2012), is believed to be the predominant force driving yeast flocculation. However, it is often assumed that more than one of these three mechanisms are always operative during beer and other industrial fermentations (Speers, 2012).

Hydrophobic interactions in particular have been known to be involved in flocculation for some time and in fact, yeast cells were shown to reach maximum flocculation potential with a concurrent increase in cell wall hydrophobicity (Speers et al., 2006). Furthermore, during an investigation of oxylipin distribution in brewing yeasts, it was shown that 3-hydroxy oxylipins (i.e., 3-OH oxylipin), a particular species of hydrophobic carboxylic acids that can also be classified as oxygenated free fatty acids, localized to the yeast cell wall at flocculation onset (Kock et al., 2000). It was also shown that two 3-OH oxylipins, 3-OH 8:0 and 3-OH 10:0, could be isolated from brewing yeast strains (Kock et al., 2000; Strauss, 2005). Thus, the level of 3-OH oxylipins showed strong potential as a putative predictor of flocculation and overall yeast cell surface hydrophobicity (CSH).

In addition, while brewing scientists concede that the primary mechanism driving flocculation in brewing strains is zymolectin binding, there is a disagreement in the literature around whether this phenomenon is induced or always operative. Studies by Stratford and Carter (1993) showed that zymolectins are produced during the exponential growth phase and activated at flocculation onset. This observation was supported by experiments that demonstrated

flocculation was only inhibited by cyclohexamide when it was added in the early stages of cell growth and that flocculation could be induced by heat treatment. Likewise, Rhymes and Smart (2000) noted an increase in levels of the Flo 1 protein in two yeast strains at 15 and 48 hours growth time while employing immunofluorescence staining with fluorophore-labelled polyclonal anti-Flo 1p antibodies. On the contrary, Patelakis et al. (1998) found there was no significant change in zymolectin “density” (defined as zymolectins/unit surface area) over the course of a fermentation, suggesting the *FLO1* gene acts in a constitutive manner. Similarly, extensive work by Higgins and colleagues did not find any up-regulation of flocculation controlling *FLO* genes (personal communication, 2001) at 0, 1, 4, 7, 10 and 24 hours into a fermentation. Therefore, it was of interest to monitor zymolectin density over the course of a fermentation with a flow cytometer, and further investigate the zymolectin induction versus the constitutive activity dilemma.

To more closely examine how these two cell wall characteristics, (hydrophobic interactions and zymolectin binding), affect brewing yeast flocculation, two studies were devised. The first study was based at Dalhousie University and aimed to measure flocculation level, cell surface hydrophobicity and 3-OH oxylipin levels concurrently during lab scale fermentations. This work sought to demonstrate that during brewing fermentations 3-OH oxylipin levels in *Saccharomyces cerevisiae* increase and that each of flocculation level, cell surface hydrophobicity and 3-OH oxylipin levels increase coincidentally. The second study conducted at Heriot-Watt University hoped to show that there were two or more cell populations with respect to zymolectin density during lab scale fermentations, and thus show there was an up-regulation or induction of this flocculation mechanism.



# Chapter 2: Literature Review

## 2.1 Flocculation

Flocculation of brewer's yeast has been the concern of scientists as early as the 1800's (Speers, 2012). While early reviews by Stratford (1992) identified work by French scientist Louis Pasteur (1876) as the first to observe flocculation, neither Pasteur's text nor the 1876 English translation (Faulkner & Rob, 1879) mention the term "flocculation" specifically. It is now believed that Seyffert (1898) was in fact the first to study flocculation. However, it wasn't until the second half of the 20<sup>th</sup> century that brewing scientists truly began to grasp the forces that govern this important phenomenon. Through their work it has been determined that flocculation occurs due to three principle forces termed hydrophobic interactions, zymolectin binding and to a lesser extent, surface charge neutralizations (Speers, 2012).

### 2.1.1 *Hydrophobic Interactions*

Hydrophobic interactions have been known to be involved in flocculation for some time. Several early studies linked yeast flocculation and cell surface hydrophobicity. Iimura et al. (1980) argued that hydrophobicity could be an important force in wine yeast flocculence. Kamada and Murata (1984) demonstrated that yeast cells treated with a proteinase became less hydrophobic and less flocculent. Meanwhile, Amory and Rouxhet (1988) found a positive correlation between the flocculation of lager yeast and CSH. Several other researchers have implicated hydrophobicity as an important factor driving flocculation including Mozes et al. (1989) and Mestdagh et al. (1990).

More recently, and as previously alluded to, Kock et al. (2000) noticed that a particular species of oxygenated free fatty acids, 3-OH oxylipins, formed during cell growth and localized

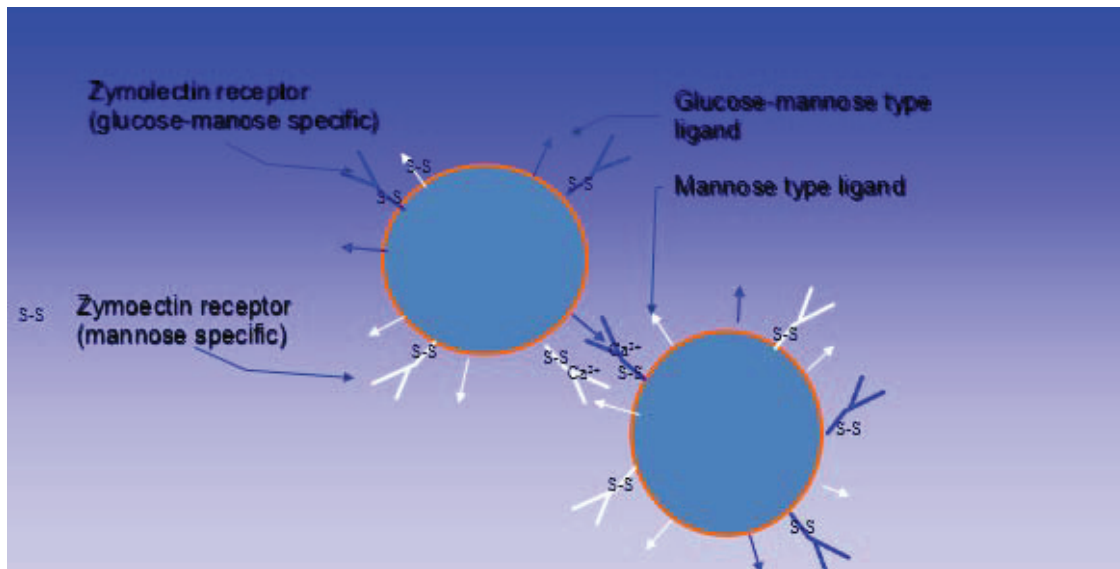
to the cell wall at flocculation onset. Furthermore, Speers et al. (2006) noted a pronounced increase in CSH that coincided with a maximal flocculation potential during fermentations in tall tube fermenters. Interestingly, brewing strains are not the only fungi that flocculate or aggregate after an increase in cell hydrophobicity. Studies have reported that hydrophobicity is an important determinant in either cell-to-cell or cell-to-surface adhesion in each of *Candida albicans* (Hodben et al., 1995), *Candida krusei* (Samaranayake et al., 1995) and *Schizophyllum commune* (Wosten et al., 1994), to name a few.

### **2.1.2 Zymolectin Theory**

Early studies of yeast flocculation implicated proteins as crucial for this process to occur. When cyclohexamide (a protein synthesis inhibitor) was added to fermenting wort, yeast lost their ability to flocculate (Baker & Kirsop, 1972). Later studies demonstrated that flocculating yeast had a cell surface protein that was not present on non-flocculating yeasts (Stewart et al., 1976). Knowing that proteins appeared important in the flocculation process, Taylor and Orton (1978) first proposed a model that a lectin similar to concanavalin-A must be involved in flocculation; concanavalin-A is known to bind cell wall mannan and is activated by calcium ions (Speers, 2012).

Ultimately, Speers et al. (1998) elegantly refined the “lectin hypothesis” of Taylor and Orton (1978) and later Miki et al. (1982a, 1982b) to its current definition as a “zymolectin hypothesis”. Speers et al. (1998) stated zymolectins are, “...any protein or glycoprotein structures associated with the yeast cell walls which contain specific carbohydrate binding domain(s) and whose presence causes or enhances cell flocculation”, as depicted in Figure 2.1. The presence and predicted function of zymolectins was strengthened when Jin and Speers

(2000) verified that non-flocculence caused by urea exposure, and thus zymolectin degradation, could be reversed by washing in a 100mM sodium acetate buffer. Washing in an acetate buffer is a common practice in molecular biology to neutralize alkaline denaturation (Birnboim & Doly, 1979).



**Figure 2.1.** The zymolectin theory of flocculation, as defined by Speers et al. (1998). In this model of flocculation either glucose-mannose specific or mannose specific zymolectin receptors bind their appropriate ligand, as shown.

### ***2.1.3 Surface Charge Neutralization***

One of the earliest models of flocculation was the colloid model based on the principles defined by Derjaguin, Landau, Verwey and Overbeek in the 1940's (Lake, 2008), and known as the "DLVO" theory. In this theory, yeast are presumed to be negatively charged particles in aqueous solutions that generally repelled each other (Stratford, 1992; Staver et al, 1993). It was noted that exposure to the divalent cations calcium, magnesium and manganese promoted flocculation and scientists presumed that these cations neutralized the repulsive negative charges allowing cell-to-cell adhesion (Stewart & Russell, 1981). Ultimately, it was demonstrated that

calcium was required for flocculation, whereas other divalent cations were not (Jin & Speers, 1998).

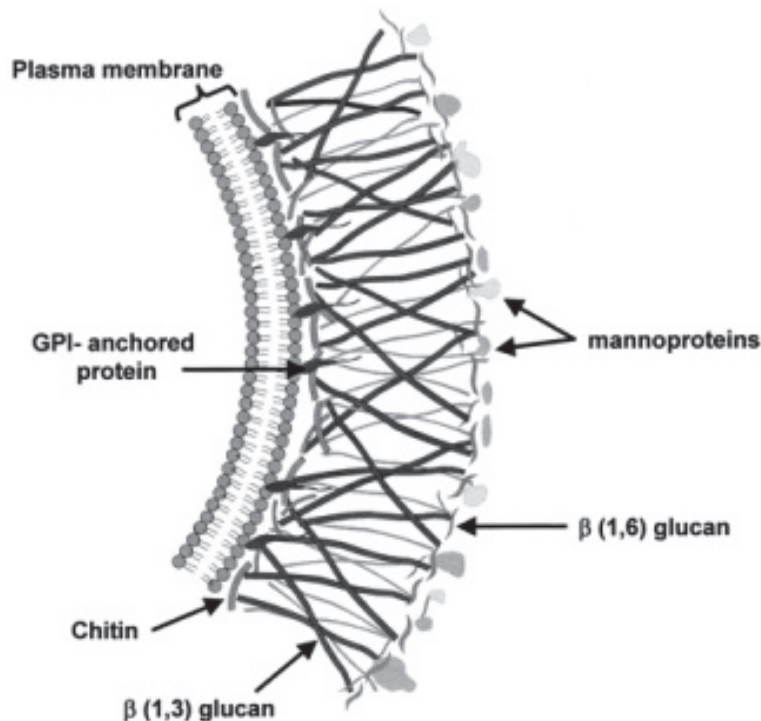
Nonetheless, neutralization of negatively charged repulsive cell surfaces is a factor often implicated in flocculation and can be exemplified in other works. The pH range correlated with maximum flocculation, pH 3.5-4.5 (Helm et al., 1953), has been shown to be close to the isoelectric point (pI) of yeast at  $\leq$  pH 3.5 (Beaven et al., 1979). Furthermore, Patel et al. (2011) reported a zeta potential decline (decrease in repulsive forces) over the course of a 120 hour fermentation, during which time yeast are known to increase in flocculence.

#### ***2.1.4 Yeast Cell Walls***

It has been assumed that at least part of the reason why some yeast cells are flocculent and some are not is due to the cell wall composition. While reports do vary, researchers generally agree that flocculent cells have higher levels of cell wall mannan than non-flocculent cells (Stewart and Russell, 1981; Amri et al., 1982). The fine architecture and composition of the yeast cell wall has been the subject of numerous reviews including those by Klis (1994) and Lesage and Bussey (2006). However, a particularly succinct review was written by Lipke and Ovalle (1998). First, it is important to note that the whole yeast cell is made up of two distinct portions: a plasma membrane which encases the cell cytoplasm and internal organelles and a rigid cell wall exterior to the plasma membrane, as in Figure 2.2. Studies show that the yeast plasma membrane is roughly 8 nm thick, while the cell wall is 100-200 nm thick (Porter, 2007).

In the review of Lipke and Ovalle (1998) they state that yeast cell walls are comprised mostly of  $\beta$ -1,3-glucan and mannoprotein, while chitin is a minor constituent. A  $\beta$ -1,3-glucan-chitin complex has been shown to be the majority of the inner cell wall, while  $\beta$ -1,6-glucan is

known to link the inner and the outer cell walls. The outer surface of the yeast cell wall consists primarily of mannoprotein attached to carbohydrate residues through N (N-acetylglucosamine) and O (via N-acetylgalactosamine) linkages. The  $\beta$ -1,3-glucan portion of the cell wall on average contains 1500 residues per polymer, has an approximate molecular mass of 240 000 and assumes a helical secondary structure. The  $\beta$ -1,6-glucan has been described as a highly branched polysaccharide. The mannoproteins meanwhile are polypeptides with a large amount of carbohydrate attached, so much so that they can be 50-95% carbohydrate by weight.



**Figure 2.2.** A schematic cross-section of a yeast plasma membrane and cell wall. The main constituents of the cell wall are shown (\*Used with permission from Selitrennikoff, 2001; see Appendix I).

### ***2.1.5 Flocculation Phenotypes and Genetic Aspects of Flocculation***

Categorization of yeast strains in terms of flocculence has divided them into two distinct phenotypes called NewFlo and Flo1. These two phenotypes differ based on which sugars inhibit

flocculation by competitively binding to the zymolectin mannan binding site. As well, Flo1 are generally haploid cells while NewFlo strains are often polyploidy brewing strains. Studies show flocculation of the NewFlo phenotypes is inhibited by mannose, glucose, sucrose, maltose, maltotriose and galactose in some strains. Meanwhile, flocculation in the Flo1 phenotype is inhibited by mannose only (Vidgren & Londesborough, 2011). Therefore, most brewer's strains are of the NewFlo phenotype because they will not tend to flocculate until the majority of fermentable sugars have been catabolized. A further distinction between the two phenotypes is that NewFlo strains have been shown to become flocculent when entering stationary phase, while studies show that Flo1 strains are flocculent during all stages of yeast cell growth (Stratford & Assinder, 1991; Masy et al., 1998).

Researchers have also concluded that there are at least nine genes that code for proteins involved in flocculation in both lager and brewing yeast strains. These genes are called FLO1, FLO5, FLO9, FLO10, FLO11, FLONL, FLONS and Lg-FLO1 (Vidgren & Londesborough, 2011). This is not to say though that all flocculent strains carry each of these genes in their genome. For example, researchers found that the completely sequenced genome of the haploid laboratory strain S288C only had the flocculin encoding genes FLO1, FLO5, FLO9, FLO10 and FLO11. S288C also had one additional FLO gene, FLO8, that codes for a transcription factor that regulated overall FLO gene expression (Vidgren & Londesborough, 2011). Furthermore, it has been demonstrated that the presence of the genes FLO1, FLO5, FLO9 and FLO10 together confer the Flo1 phenotype, while genes Lg-FLO1, FLONL and FLONS together result in the NewFlo phenotype (Vidgren & Londesborough, 2011).

### ***2.1.6 Environmental Conditions and Flocculation: Nutrients***

Generally, in conditions where there are limited nutrients cell division ceases and the flocculation potential of yeast cells increase dramatically (Porter, 2007). Nitrogen depletion in particular has been shown to induce flocculation, while addition of amino acids to starved cells was able to de-floc them (Smit et al., 1992). The amount of fermentable sugars have been shown to have a profound impact on the flocculation ability of brewer's yeast as well. With yeast of the NewFlo phenotype any of mannose, glucose, sucrose, maltose and maltotriose in consumable amounts are believed to inhibit flocculation by competitively binding to the mannan domain on the zymolectin (Speers et al., 2006). Similarly, addition of glucose to a growth medium has been shown to de-aggregate flocs of starved cells of the Flo1 phenotype (Soares & Duarte, 2002). Inorganic cations are also known to influence flocculation; calcium ions are essential for zymolectin-mediated flocculation (Stratford, 1989), but studies also show that manganese and zinc ions are required for flocculation (Smit et al., 1992; Rees and Stewart, 1998) too.

### ***2.1.7 Environmental Conditions and Flocculation: Oxygen***

As fermentation is an anaerobic process, there is generally an absence of oxygen during the brewing process. However, wort is typically oxygenated before it is pitched with yeast. Studies have shown that insufficient wort oxygenation leads to an early and incomplete flocculation, while a proper wort oxygenation leads to a later but eventually full flocculation (Vidgren & Londesborough, 2011). Early and incomplete flocculation due to poor oxygenation can be prevented with the addition of ergosterol and oleic acid (Vidgren & Londesborough, 2011). Thus, it is believed that oxygen concentration doesn't directly affect flocculation. Rather,

it affects synthesis of sterols and fatty acids which are components of the cell plasma membrane understood to anchor zymolectins in the cell (Straver et al., 1993).

### ***2.1.8 Environmental Condition and Flocculation: Ethanol***

There is some variation in the literature when it comes to how ethanol affects cell flocculation. A report by Jin and Speers (2000) suggested that increased ethanol concentrations decreased the repulsive forces between negatively charged cells by reducing the dielectric constant of the medium. The dielectric constant is an important determinant in an equation that can be used to describe electrostatic repulsion between brewing yeast cells based on DLVO theory (Speers et al., 1992). On the contrary, reports by D'Hautcourt and Smart (1999) suggested the effect of ethanol on flocculation is strain dependent. Meanwhile, Smukalla et al. (2008) found that increasing concentrations of ethanol promoted flocculation with the Flo1 strain EM93, and hypothesized that ethanol may control flocculation in brewing yeasts via quorum sensing.

### ***2.1.9 Environmental Conditions and Flocculation: Temperature and pH***

As with D'Hautcourt and Smarts (1999) understanding of how ethanol affects flocculation, the effect of temperature on flocculation seems strain-dependent. Jin and Speers (2000) showed that Flo1 strains flocculate over a wider temperature range than NewFlo strains. In particular they found that flocculation changed only slightly between 5 °C and 25 °C for Flo1 phenotypes, whereas NewFlo strains showed medium to high flocculation between 10 °C and 25 °C but low flocculation at 5 °C (Jin & Speers, 2000).

Helm et al. (1953) conducted one of the earliest studies of the pH-dependence of flocculation. In their study, they examined four strains, and while they found that the effect of



pH on flocculation were strain-dependent, they noted flocculation predominated at pH 2.5-4.5. Other research has found that the pI of yeast occurs at approximately  $\text{pH} \leq 3$  (Vidgren & Londesborough, 2011). During a beer fermentation the pH of the medium decreases due to consumption of buffering compounds and production of organic acids and it drops to a final pH of 4-5 (Priest & Stewart, 2006). It is believed that as the pH drops and gets closer to the pI of yeast, the repulsive forces between cells are reduced allowing flocculation to occur (Vidgren & Londesborough, 2011).

#### ***2.1.10 Yeast Flocculation Assays***

In studying the phenomenon of flocculation it is important to be able to quantify it. One of the first methods to quantify yeast flocculation was that of Helm et al. (1953) and it eventually came to be known as the Helm's assay. In this test, the researchers measured flocculation by the following means. A portion of yeast was first double washed in distilled water with 0.510 g/L of  $\text{CaSO}_4$ . Then a 1 gram sample of the wet yeast was transferred to 10 mL of pH 4.5 acetate buffer made up of 6.80 g/L sodium acetate, 4.05 g/L acetic acid and 150 mg/L  $\text{CaSO}_4$ . This mixture was agitated and the degree of sedimentation was determined after it had sat for 10 minutes.

While other researchers have experimented with modifying this method slightly, the American Society of Brewing Chemists (ASBC) adapted and adopted two versions of the Helm's assay as part of their standard Methods of Analysis (MOA). In these methods (ASBC, 2013, Yeast-11) one is based on flocculation in an acetate buffer while the second is based on differential absorbances between flocculation in a buffer of calcium acetate and flocculation in a buffer with the chelating agent disodium ethylenediaminetetraacetic acid (EDTA).

A second major flocculation assay technique was that developed by Gilliland (1951) who was dismissive of Helm's buffer technique. In the assay by Gilliland, flocculation was characterized by assessing the yeast in suspension (YIS) in wort not buffer, using colourimetric methods. Speers and coworkers (Macintosh et al., 2012) have also developed a powerful statistical technique to model YIS during a fermentation with a "tilted Gaussian" fit equation and this gives important information regarding the flocculation capacity of the yeast in question. This method (ASBC, 2013, Yeast-14) has been accepted as an official ASBC method. More recently, molecular biological techniques have been employed to estimate the flocculation ability and potential of different strains of yeast. In the studies by each of Carstens et al. (1998) and Huuskonen et al. (2012) molecular approaches were used to monitor expression of genes implicated in flocculation.

## **2.2 Cell Surface Hydrophobicity**

### ***2.2.1 Cell Surface Hydrophobicity Assays***

One of the first methods applied to measure CSH of brewer's yeast was by determining the contact angle of water droplets on a layer of yeast cells collected on a 0.45  $\mu\text{m}$  cellulose triacetate filter (Mozes & Rouxhet, 1987). In this test the contact angle between the liquid and solid interface is calculated using the Young-Dupré equation:

$$\cos\theta = (\gamma_{SV} - \gamma_{SL})/\gamma_{LV} \quad (2.1)$$

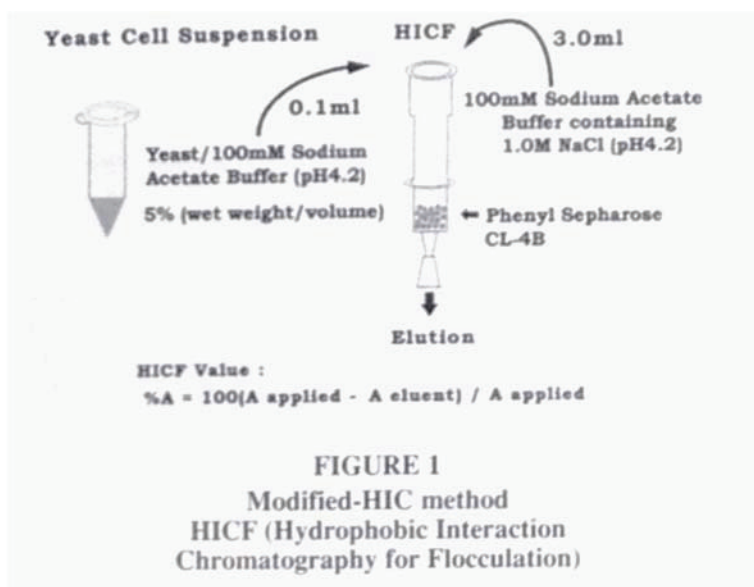
where  $\gamma$  is the interfacial tension between the solid (S), liquid (L) and vapour (V) phases (Menzies & Jones, 2010). In the equation the larger the contact angle the more hydrophobic the surface (Menzies & Jones, 2010). Another method frequently used to measure CSH, but one that has been reported to be inaccurate (Akiyama-Jibiki et al., 1997), is an assay based on the use of

paramagnetic, polystyrene-coated latex beads (Straver and Kijne, 1996). In this assay the percentage of cells that adhere to the latex beads can be determined by removing the beads from the medium with a magnet. The percentage adherence is indicative of the CSH (Straver & Kijne, 1996).

Yet another method for measuring cell hydrophobicity was developed by Rosenberg et al. (1980) and was originally known as bacterial adherence to hydrocarbon (BATH) and then later generalized as microbial adhesion to hydrocarbon (MATH). These authors devised this method because they felt other hydrophobicity assays were inferior for two primary reasons-1.) the experimental conditions with other assays influenced the observed hydrophobic interactions and 2.) they felt non-hydrophobic forces interfered with other assays (Rosenberg et al., 1980). The MATH assay in principle was a simple spectrophotometric and quantitative assay based on the degree of adherence of cells to various liquid hydrocarbons after mixing. Unfortunately, numerous researchers have found that small alterations in the protocol, such as the diameter of the test tubes or the pH of the medium, significantly affect the results (Pembrey et al., 1999).

A final method and one with reported good reproducibility was developed by Akiyama-Jibiki et al. (1997) and is shown in Figure 2.3. This method is an adaptation of hydrophobic interaction chromatography and is called hydrophobic interaction chromatography for flocculation. In this method miniature disposable chromatographic columns (1.5-3.0 mL) are packed with phenyl sepharose CL-4B. The columns are equilibrated with a salt-containing acetate buffer. A standardized (5 % w/v) sample of yeast cells is then applied to the column, a volume of the same salt-containing acetate buffer is added to the column and the eluent is collected. Many trials have demonstrated that those cells which are hydrophobic will adhere to the phenyl sepharose CL-4B (Speers, 2012). Therefore, the hydrophobicity of the sample is

determined based on the % differential absorbances between a (5 % w/v) sample of the yeast in the salt-containing sodium acetate buffer and the eluent collected after the same sample and buffer volume were applied to the column.



**Figure 2.3.** The hydrophobic interaction chromatography for flocculation method of Akiyama-Jibiki et al. (1997) used to determine cell surface hydrophobicity (\*Used with permission from Akiyama-Jibiki et al., 1997; see Appendix II).

## 2.3 Oxylipin Analysis

### 2.3.1 Definition and Structure of Oxylipins

Oxylipins in the broadest sense are saturated or unsaturated fatty acids possessing a hydroxyl group (Bhatt et al., 1998; Goldblatt, 2007). As with most fatty acids, oxylipins contain a polar carboxyl head, a hydrocarbon chain and the aforementioned hydroxyl group located on the carbon chain (Goldblatt, 2007). Oxylipins also have chiral centers and therefore produce both R and S enantiomers (Goldblatt, 2007). These compounds are ubiquitous in nature and can be extracted from plants, animals, fungi (*Sacchormyces cerevisiae*) and medically important pathogens (Sebolai et al, 2012).

### **2.3.2 Biological Functions of Oxylipins in Yeast and Other Fungi**

This vast class of lipids includes eicosanoids and hydroxyl oxylipins and they perform numerous biological functions (Sebolai et al, 2012). In all life forms, oxylipins act as signaling molecules in a hormone-like manner and in fungi they play a role in asexual and sexual development and in quorum sensing (Sebolai et al., 2012). It has been shown that oxylipins are produced via an incomplete  $\beta$ -oxidation pathway (Finnerty, 1989).

One of the earliest discoveries that proved oxylipins act as signalling molecules was when albumin-associated oxygenated lipids were shown to induce germ tube formation in *Candida albicans* (Aandleigh, 1964). Later work demonstrated that hydroxyl derivatives of oleic and linoleic acid were determinants in the life cycle of *Aspergillus nidulans* (Champe et al., 1989). Polyunsaturated lipids were further shown to be strong inducers of filamentation in the fungi, *Ustilago maydis* (Klose et al., 2004). Lastly, a 12-carbon polyunsaturated alcohol called farnesol was implicated in quorum sensing in the yeast *Candida albicans*, modulating conversion to and from hyphal and yeast forms (Hornby et al, 2001).

### **2.3.3 3-OH Oxylipins in *Saccharomyces cerevisiae***

Having found that the species of oxygenated free fatty acids, 3-OH oxylipins, were important in ascospore release and aggregation in the yeast strain *Dipodascopsis uninucleata* (Kock et al., 1999), Kock and co-workers researched 3-OH oxylipin distribution in *Saccharomyces cerevisiae*. The researchers observed oxylipin-containing osmiophilic layers that migrated through the cell and facilitated cell-to-cell adhesion and flocculation (Kock et al., 2000). Furthermore, Kock et al. (2000) were able to isolate the oxylipins 3-OH 8:0 and 3-OH 10:0 from the flocculent *Saccharomyces cerevisiae* strain ATCC 26602. The work of Strauss

(2005) revealed two more important points with respect to 3-OH oxylipins in brewing yeast strains. First, she was able to isolate naturally occurring 3-OH 8:0 from the *Saccharomyces cerevisiae* production brewing strains UOFS Y-2330, UOFS Y-1 and UOFS Y-3. She also showed that aspirin, an inhibitor of prostaglandin (an oxylipin) synthesis, inhibited both the production of 3-OH oxylipins and flocculation in the brewing strain *Saccharomyces cerevisiae* UOFS Y-2330 (Strauss, 2005).

#### ***2.3.4 Lipid Content and Lipid Distribution in Saccharomyces pastorianus (lager yeast) and Saccharomyces cerevisiae (ale yeast)***

When the lipid contents of the ale yeast *Saccharomyces cerevisiae* grown at 15 and 30 °C in a chemostat were compared, growth at 15 °C produced more total lipids, total fatty acids, triacylglycerols and phospholipids (mainly phosphatidylcholine) (Hunter & Rose, 1972). However, growth temperature differential did not affect the amount of diacylglycerols, free fatty acids, sterols or sterol esters. Similarly, the composition of fatty acids and the degree of unsaturation of the fatty acids was not effected by temperature (Hunter & Rose, 1972).

In a study of the lipid content of *Saccharomyces pastorianus* obtained from the end of an industrial beer fermentation, Blagovic et al. (2001) found that lipid constituted 4.4 % of dry cell biomass. Triacylglycerols, squalene, ergosterol and steryl esters made up of 51 % of total lipids. Fatty acid characterization revealed a wide array present in the cell; palmitic acid (16:0) was the most prevalent followed by palmitoleic acid (16:1). Of the phospholipids, phosphatidylcholine and phosphatidylinositol predominated at 33.1 and 21.6 %, respectively.

Later studies were carried out by Blagovic et al. (2005a) to compare the lipid contents of whole cells of *Saccharomyces cerevisiae* to the plasma membrane alone. The group found that

the lipid content of whole cells was 6.7 % dry biomass, while phospholipids were 36.8 % of total whole cell lipids. Phospholipids were measured on a mass fraction basis in the plasma membranes and were 0.086 mg/mg proteins. Steryl esters were the most prevalent component of whole cell neutral lipids, while ergosterol was the most abundant in the plasma membrane. Meanwhile, palmitoleic (16:1) and oleic (18:1) acid were the largest constituents of both the whole cells and the plasma membrane fatty acids, but were significantly less abundant in the plasma membranes (Blagovic et al, 2005a).

A final study was conducted by Blagovic et al. (2005b) to compare the lipid content of the *Saccharomyces pastorianus* plasma membrane to its mitochondria and in this study the yeast sample had been collected from the end of a beer fermentation. The group found the most prevalent phospholipid in the plasma membrane was phosphatidylinositol (37%) and in the mitochondria it was phosphatidylcholine (30%). Also, there was twice as much triacylglycerols and steryl esters in the plasma membrane compared to the mitochondria. Both membranes were found to have high contents of squalene and fatty acids and low amounts of ergosterol. These proportions were attributable to the fact that fermentation is an anaerobic process and that ergosterol synthesis requires oxygen (Blagovic et al., 2005b).

### ***2.3.5 Fatty Acid Extraction Methods***

One of the most commonly used lipid extraction techniques, particularly in biochemical work, was first proposed by Folch et al. (1957). In this extraction method a biological sample typically containing more than only lipid is immersed in a 2:1 by volume mixture of chloroform and methanol and shaken (Christie, 1993). A  $\frac{1}{4}$  volume of saline solution is added to the mixture such that two distinct layers form; a lower chloroform-methanol-water layer (86:14:1 by volume)

with almost all the lipids and an upper chloroform-methanol-water layer (3:48:47 by volume) with the non-lipid components (Christie, 1993). In this method total chloroform, methanol and water volume must be in an 8:4:3 ratio or selective lipid loss may occur (Folch et al., 1957).

A second commonly used lipid extraction technique was developed by Hara and Radin (1978) to mitigate the toxic effects of chloroform and methanol exposure. It was found that the hexane-isopropanol (HIP) extraction technique had several distinct advantages over the method of Folch et al. (1957). One is that the HIP extraction had accumulated less non-lipid material than the chloroform-methanol extraction. A second advantage is that the HIP lipid extract, after evaporation, can be applied directly to a chromatographic column without clogging the applicator syringe. Lastly, the HIP extract can be evaporated without washing, while unwashed chloroform-methanol extracts are known to foam during evaporation (Hara & Radin, 1978).

### ***2.3.6 Derivatization of Fatty Acid Extracts and Microbial Fingerprinting***

For fatty acids analysis to be conducted on any biological sample, the fatty acids must first be separated from the non-lipid portions of the sample and then liberated if they are part of diacylglycerols or triacylglycerols (Zhang, 2001). The liberation is typically a hydrolysis or a methanolysis that is acid or base-catalyzed. Free fatty acids are then converted to low molecular weight non-polar volatile derivatives that make for easier chromatographic analysis (Zhang, 2001). These volatile derivatives are called fatty acid methyl esters (FAME) and the principle ways they are prepared are acid-catalyzed esterification, acid-catalyzed transesterification, base-catalyzed transesterification and with diazomethane (Zhang, 2001). Recently, use of diazomethane has diminished though because of health and safety issues (Sparkman et al., 2011).



Many authors have written extensive reviews on FAME production, including Vyas et al. (2010) and Christie (1989).

A second common technique to render otherwise non-volatile compounds more volatile and thus better suited to chromatographic analysis is by adding a trimethylsilyl (TMS) group. In this technique a trimethylsilylating reagent can derivatize rather non-volatile compounds like certain alcohols, phenols, or carboxylic acids. The reagent works by substituting a TMS group for a hydrogen in the hydroxyl groups on the compounds (Sparkman et al., 2011). The TMS group also gives compounds characteristic peaks and spectra which aid in identification. Both Pierce (1968) and Knapp (1979) have written in depth reviews of TMS derivatization.

Different species of microbes, in particular bacteria, have unique fatty acid profiles. Because of this certain microbes can be typed by extracting their fatty acids, producing FAME and conducting chromatographic analysis. This approach has actually long been a diagnostic tool when detecting pathogens. In this identification technique, FAME of an unknown organism are run against standards with known bacterial acid methyl esters (Supelco Analytical, 2013).

### ***2.3.7 Fatty Acid Detection Methods: Gas Chromatography***

Pioneering work in basic chromatography by Day and Tsvet in the late 1800s paved the way for numerous modern day chromatographic techniques (Zhang, 2001). Gas chromatography (GC) in particular is a technology that is very useful for separating non-labile components of a mixture. With GC a minute sample volume (~1.0  $\mu$ l) is injected by a needle onto a heated port on the apparatus. The sample is vapourized in the injector port and carried by an inert gas (often Helium) mobile phase into a capillary column lined with a non-polar stationary phase (Graham & Fryhle, 2002). Columns can range in length and diameter depending on the application and the

columns are always housed in an oven. The temperature of the oven can be adjusted depending on the volatility of the samples analyzed. Components of a mixture will separate at different times in a gas chromatograph depending on their boiling points and interactions with the stationary phase to give a characteristic retention time (Graham & Fryhle, 2002). Many authors have written extensively on gas chromatography, including recently Sparkman et al. (2011).

### ***2.3.8 Fatty Acid Detection Methods: Gas Chromatography-Mass Spectrometry***

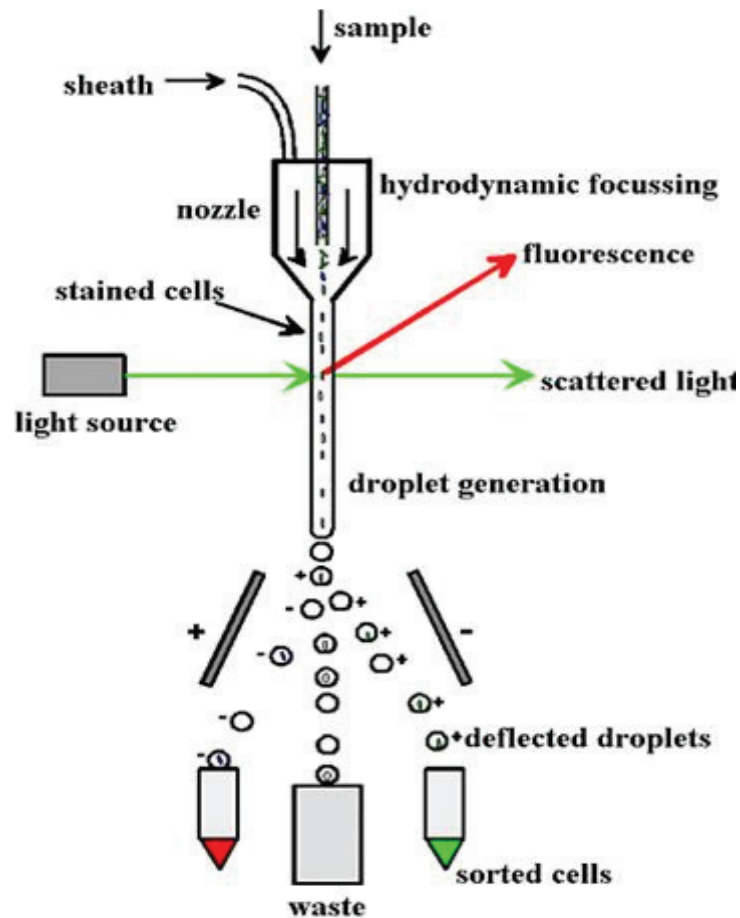
GC is often paired with a technology called mass spectrometry (MS). In MS gaseous molecules under very low pressures are subjected to high energy electrons. The high energy electrons remove an electron from the molecule in question to produce a radical cation called the molecular ion (Graham & Fryhle, 2002). The high energy electrons are in fact so strong that covalent bonds are broken and the molecule can further break into fragments smaller than the molecular ion, in what is known as fragmentation. In the detection step the fragments are accelerated then passed through a curved tube surrounded by a magnetic field. The magnetic field directs the fragments to a detector where they are sorted based on their mass/charge ( $m/z$ ) ratio (Graham & Fryhle, 2002). When coupled with GC this technology is very powerful; the gas chromatograph separates components of a mixture and the MS aids in identifying those components (Graham & Fryhle, 2002). Again, Sparkman et al. (2011) have written extensively on GC-MS.

## **2.4 Flow Cytometry**

### ***2.4.1 Flow Cytometry: How Technology Works***

Flow cytometry is a technology which allows particles or cells 0.2-150  $\mu\text{m}$  in size to be classified according to their physical characteristics (relative size, relative granularity, relative

fluorescent intensity) and chemical compositions, in what is known as multiparametric analysis (BD Biosciences, 2000). In this technology either a homogenous or heterogeneous population of cells or particles are transported by a high velocity fluid stream through a sample core, as in Figure 2.4. The sample core is irradiated by laser light and depending on the composition of the cells or particles unique patterns of scattered light are produced (BD Biosciences, 2000). The scattered light is collected by positioned lenses and sensed by computerized detectors. Forward scattered (FSC) light is proportional to cell size, and side scattered (SSC) light is proportional to cellular granularity (Allman et al., 1990), which can be understood as cellular complexity. Quite often the cells of interest are run through the sample core bound with fluorochromes that emit fluorescent light when subjected to the laser, and thus, further aid in characterization (BD Biosciences, 2000). With certain flow cytometry systems heterogeneous population of fluorochrome labelled cells can be separated into homogenous populations based on their level of fluorescence with a technique called fluorescence-activated cell sorting (BD Biosciences, 2000). Many authors have written detailed reviews of flow cytometry including Laerum and Farsund (1981) and Muirhead et al. (1985).



**Figure 2.4.** A schematic of how flow cytometry works (\*Used with permission from the University of California San Francisco School of Medicine, 2013; see Appendix III).

#### *2.4.2 Bi-Modal Distributions: What They Are, Relevance and Statistical Tests*

Many frequency distributions in the physical and life sciences, when conveyed in a histogram, are unimodal and the normal distribution is the best example of this. In these distributions the value which occurs most often is called, not surprisingly, the mode and it occurs at the apex of the histogram with the other values tailing off at the sides (National Institute of Standards and Technology, 2013). However, some frequency distributions are an amalgam of two or more distributions and are called bimodal or multimodal distributions. When a histogram

of the data shows a bimodal distribution, this can suggest two populations in the measurements (Murphy, 1964). Researchers are always encouraged to take caution though, and not immediately assume that a bi-modal distribution is indicative of two populations. Two of the more common causes of apparent bi-modality when the population is in fact unimodal are non-representativeness of the sample and sampling error (Murphy, 1964). Nevertheless, a host of statistical tests exist to assess for bi-modality or non-normality of the data.

Visual inspection is usually the first indicator that a data set may be bi-modal or non-normal, but there are several more rigorous tests employed in research. One principle test and one of the earliest described in the literature was that developed by Kolmogorov and Smirnov in 1933 (Kolmogorov, 1933). Kolmogorov's significant contribution was defining the empirical distribution function of the sample and comparing this to the cumulative distribution function of the reference distribution which in the case of normality testing is a normal distribution (Stephens, 1992). Another important normality test developed after the Kolmogorov-Smirnov test was that proposed by Anderson and Darling (Anderson & Darling, 1954) and, not surprisingly, called the Anderson-Darling test. A number of other tests can be utilized but a third and final powerful one was that developed by and given the name the Jarque-Bera test (Jarque & Bera, 1987). In an extensive simulation study by Yazici and Yolacan (2007), these three methods and eight others were compared. These researchers found that the Jarque-Bera test was the most powerful with a large sample size and when the distribution of the population was not known (Yazici & Yolacan, 2007).

### ***2.4.3 Fluorescent Microscopy***

Since Antonie van Leeuwenhoek's preeminent advancements in the 17<sup>th</sup> century, microscopy has been a fundamental technique in the biological and medical sciences. Unfortunately, live specimens or organisms are not particularly suited to microscopic visualization because of their light scattering properties and innate defence mechanisms to prevent light damage (Yuste, 2005). As a result, many specimens in the past have been chemically fixed to improve their light transparency. A series of images from fixed tissues isn't always ideal though as this forces the microscopist to guess how the living tissue functions (Yuste, 2005). The development of fluorescent microscopy, where specimens were stained with fluorophores, meant a technique that could effectively visualize molecules on live or fixed tissues even at low concentrations (Yuste, 2005). Phillip Ellinger, a pharmacology professor, was instrumental in developing the first intravital fluorescence microscope in 1929 (Masters, 2010).

More recently, technological improvements in fluorescent microscopy have greatly advanced the effectiveness of this technique including development of genetically encoded fluorophores (Chalfie et al., 1994). While many modern fluorophores are embedded in the organism via genetic engineering, many more still are conjugated to molecules which are known to bind to the specimen. Molecules commonly conjugated with fluorophores include proteins like avidin and streptavidin and lectins such as wheat germ agglutinin (Lodish et al., 2005). Wheat germ agglutinin (WGA) in particular selectively binds to N-acetylglucosamine (chitin monomer) and N-acetylneuraminic acid (sialic acid) residues (Wright, 1984), with N-acetylglucosamine linking mannoproteins to carbohydrates on the brewing yeast cell wall. Therefore, WGA-fluorophore conjugates can be used to visualize the brewing yeast cell wall, and track changes in the cell wall structure during a brewing fermentation.

## **Chapter 3: Thesis Objectives and Hypotheses**

The objectives of this thesis in the broadest sense were two-fold- 1.) to examine the role of cell surface hydrophobicity as a predominant force driving brewing yeast flocculation and 2.) to further assess zymolectin density and other cellular characteristics over the course of a fermentation. The experimental portion of the study was conducted in two distinct phases (Figure 4.1) and each phase tested different hypotheses and had unique objectives. The hypotheses and project objectives in Phase 1 and Phase 2 are indicated below.

### ***Phase I Hypotheses***

First, oxylipin levels in brewing yeast strains increase in the cell wall during a brewing fermentation. Second, an increase in yeast cell oxylipin concentration during a brewing fermentation is correlated with an increase in cell surface hydrophobicity and flocculation level.

### ***Phase I Objectives***

As neither oxylipin detection nor quantification had been conducted on the SMA strain (or LCC 125 or LCC 1240), the first objective was to develop a protocol to extract then derivatize yeast fatty acids. A second objective was to validate the extraction and derivatization protocols with yeast cell growth in Erlenmeyer flasks of yeast extract, peptone and dextrose broth. The third objective of this study was to monitor flocculation level, cell surface hydrophobicity and oxylipin level during a miniature fermentation (ASBC, 2013, Yeast-14) for the medium flocculent SMA yeast strain.

### ***Phase II Hypotheses***

Firstly, the distribution of zymolectin densities for a fermenting yeast cell population as determined by a flow cytometer should be bi-modal, suggesting two populations of cells-i.) a population of mother cells with a higher zymolectin density and ii.) a population of daughter cells with a lower zymolectin density. Secondly, the distributions of cell sizes and cell granularities, as determined by forward scatter and side scatter, respectively, should change in accordance with our knowledge of yeast cell populations during a fermentation.

### ***Phase II Objectives***

The first objective of Phase II was to organize the massive quantities of raw data (i.e., > 100,000 measurements/run) produced by the flow cytometer into statistically analyzable and organized spreadsheets. The second objective was to statistically analyze the data to better understand how fermentation time effected zymolectin level as well as cell size and granularity.



## Chapter 4: Experimental Design

Phase 1 of this project was conducted in the Dalhousie University Food Science laboratories, while Phase 2 was conducted at the International Center for Brewing and Distilling at Heriot-Watt University. For Phase 1 it was the initial plan that flocculation level, cell surface hydrophobicity and oxylipin level would be measured concurrently for 3 yeast strains; the highly flocculent LCC 125, the medium flocculent SMA and the non-flocculent LCC 1240 (Table 4.1) all grown in the miniature fermentation assay (ASBC, 2013, Yeast-14). Because no work like this had been done in the Dalhousie Food Sciences lab, there was no certainty that the oxylipin analysis in particular would work. Therefore, it was elected to first use the medium flocculent SMA strain. If the oxylipin work was successful, then it was planned to repeat the analyses with the highly flocculent (LCC 125) and non-flocculent (LCC 1240) strains.

The SMA strain was selected as the initial strain in Phase 1 because the Dalhousie University Food Science lab has experience with the growth and flocculation behaviour of this strain in the miniature fermentation assay (ASBC, 2013, Yeast-14). It was assumed that this strain would have medium levels of oxylipins relative to LCC 125 and LCC 1240 as it had a medium flocculent phenotype. Therefore, it would have been the best strain to develop extraction protocols with detection limits that encompassed presumed high (LCC 125), medium (SMA) and low levels (LCC 1240) of oxylipins.

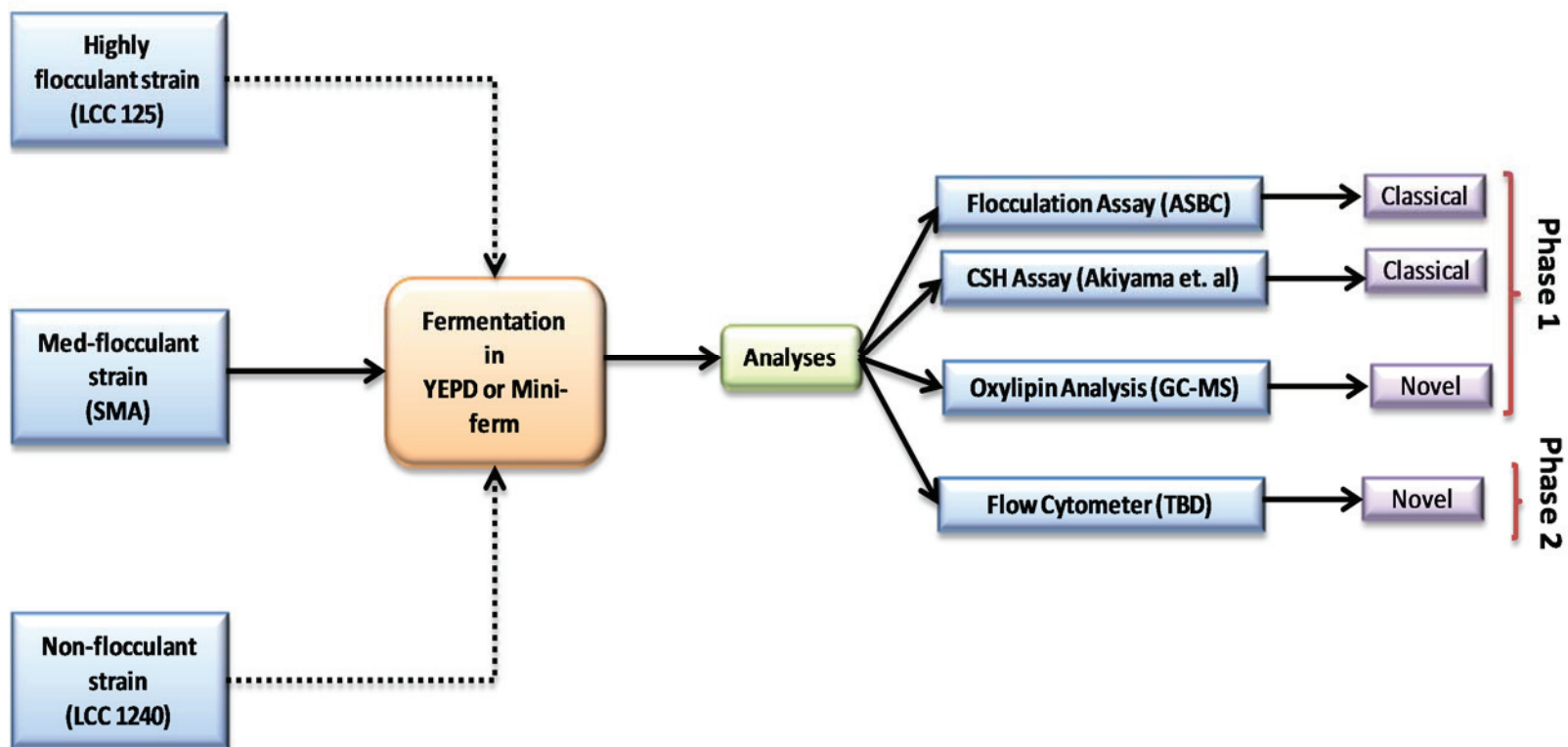
The overall approach of the work in Phase 1 was as appears in Figure 4.2, where the work was conducted in three parts. In the first part, the SMA yeast strain was initially grown in yeast extract, peptone and dextrose (YEPE) broth to validate the experimental techniques and to investigate the merits of whole-cell fatty acid analysis versus cell-wall associated fatty acid

analysis. It was hypothesized that cell rupture and subsequent isolation of the cell wall ghosts would concentrate the cell wall-associated lipids. In the second part the yeast was grown in a miniature fermentation assay (ASBC, 2013, Yeast-14) setup to more closely mimic an industrial beer fermentation. In the third part a mixed Bacterial Acid Methyl Ester (BAME) standard was used to determine chromatographic properties of the oxylipins, the characteristic fragments of the 3-OH structure in GC-MS and to ultimately aid in detecting 3-OH 8:0 and 3-OH 10:0 specifically.

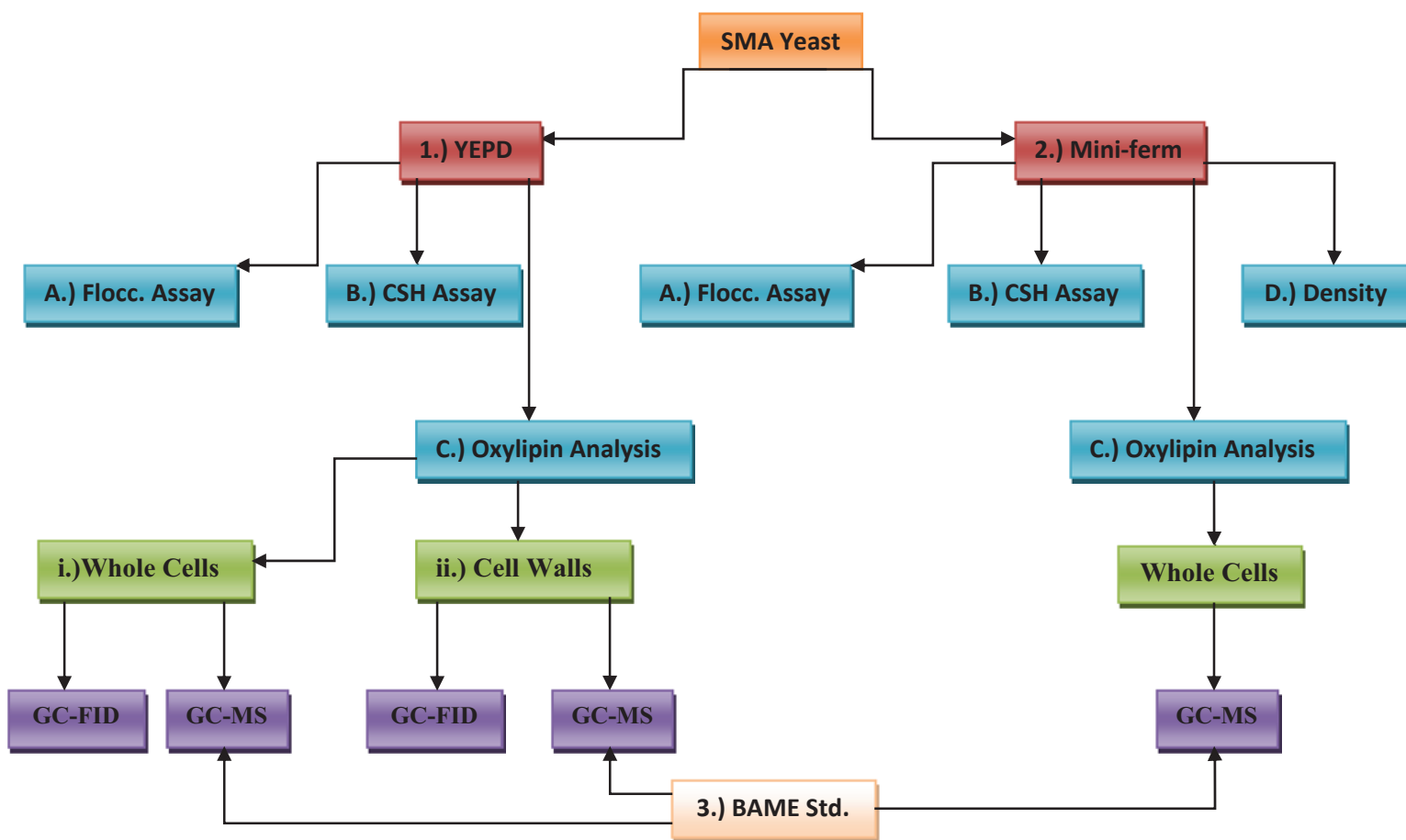
**Table 4.1.** Summary of information for the strains proposed to be used in this thesis project.

Strain	Ale/Lager	Level of Flocculence	Flocculation Phenotype	Source
LCC 125	Ale	Highly	NewFlo	Stewart et al., 1983
SMA	Lager	Medium	NewFlo	Wyeast Laboratoties, 2009; Yamauchi et al., 1995.
LCC 1240	Ale	Non-flocculent	Flo1	Sobczak, J., personal communication, July 29, 1998.

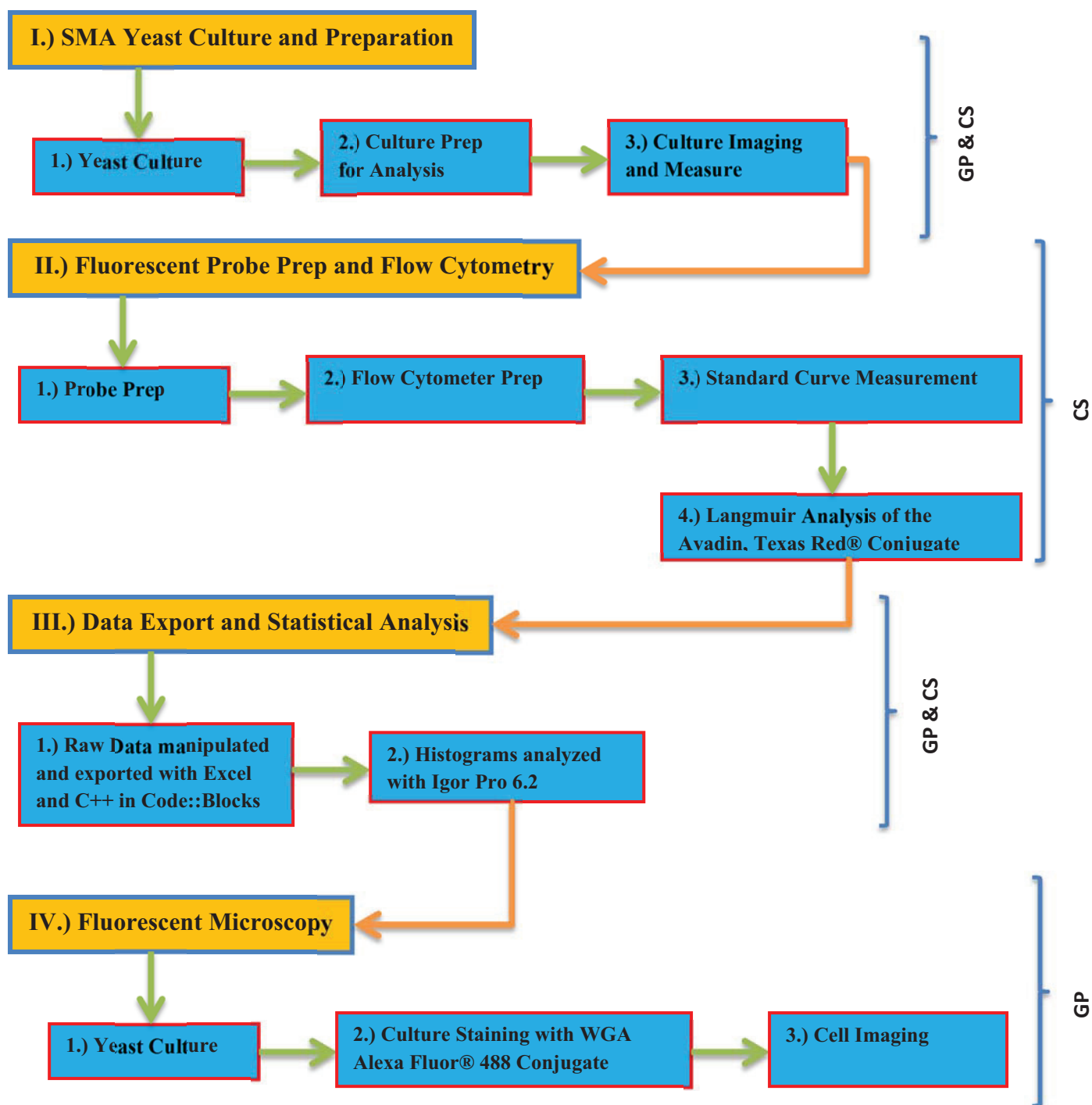
In Phase 2, the initial work conducted was part of a collaborative effort with another student who started flow cytometry experiments for his M.Sc. thesis project at Heriot-Watt University (Stanton, 2013). A large amount of data was produced from the flow cytometry work, and the challenge after the data was collected was to then organize and statistically analyze the information. Subsequent staining of the SMA yeast strain with a WGA, Alexa Fluor® Conjugate was conducted to complement the initial studies. A schematic of the preliminary lab work, later data analyses and complementary experiments conducted for Phase 2 are shown in Figure 4.3.



**Figure 4.1.** A schematic of the original overall experimental design. Phase 1 of this project was conducted in the Dalhousie University Food Science laboratories, while Phase 2 was conducted at the Institute of Brewing and Distilling at Heriot-Watt University.



**Figure 4.2.** A schematic of the overall experimental approach and analyses used in Phase 1. In part 1, the SMA yeast strain was initially grown YEPD broth to validate the experimental techniques and to compare whole-cell fatty acid analysis versus presumed cell-wall associated fatty acid analysis. In part 2, the yeast was grown in the miniature fermentation assay setup to more closely mimic an industrial beer fermentation. In part 3, a mixed BAME standard was used in an attempt to improve detectability of 3-OH oxylipins.



**Figure 4.3.** A schematic of the work done for Phase 2 of this thesis project at Heriot-Watt University. The Yeast Culture and Preparation work was done jointly by both the author (shown as GP) and Heriot-Watt M.Sc. student Chris Stanton, Jr. (shown as CS). Chris Stanton, Jr. then did Fluorescent Probe Prep and Flow Cytometry work, each did the Statistical Analyses of the raw data and the author did the Fluorescent Microscopy.

# Chapter 5: Materials and Methods

## 5.1 Phase 1

### 5.1.1 Part 1.) Growth in YEPD Broth

#### *Yeast Strain Used*

The SMA yeast strain used in this study came from Wyeast Laboratories, Inc. (Odell, OR). This strain originated at Versuchs-und-Lehranstalt für Brauerei (Berlin, DEU) and has been described by this organization as bottom fermenting and completely flocculent towards the end of a fermentation, with a constant high rate of fermentation.

#### *Culture in YEPD Broth*

In all cases a “double-grow up” approach was used to culture the SMA yeast strain, unless otherwise indicated (ASBC, 2013, Yeast-14). To do this with a sterile inoculating loop approximately one medium-sized colony was collected from either a YEPD agar slant or a YEPD agar plate that consisted of 20 g/L D-glucose (Bioshop, Burlington, ON), 20 g/L peptone (Bioshop, Burlington, ON), 10 g/L yeast extract (Bioshop, Burlington, ON) and 25-30 g/L agar (Bacto, Sparks, MD) . This growth was aseptically transferred to a 250 mL Erlenmeyer flask containing 100 mL sterile YEPD broth made from the same constituents previously described except for the agar. The flask was aerobically incubated on a New Brunswick Scientific Classic Series C24 Incubator Shaker (Edison, NJ) at 150 rpm for 24 hours at 25 °C. After 24 hours, the cell count of the 250 mL Erlenmeyer flask culture was determined with the ASBC standard method (ASBC, 2013, Yeast-4). Based on this amount a volume of culture from the first 250 mL Erlenmeyer flask was transferred to a second sterile 250 mL Erlenmeyer flask of YEPD broth such that the inoculation rate was  $1.5 \times 10^7$  cells/mL and the final volume was 100 mL. The

second flask was incubated on the same orbital shaker under the same conditions as the first flask.

#### *A.) Flocculation Assay*

The flocculation level was determined by the ASBC Flocculation Test (ASBC, 2013, Yeast-11-B). However, the preparation of the yeast sample was conducted as previously described, under Culture in YEPD Broth rather than in hopped wort. In this test, at each sampling time two 10 mL samples were aseptically withdrawn from the 250 mL Erlenmeyer flask and transferred to two sterile 15 mL centrifuge tubes marked “A” and “B”. Tube A was centrifuged at 630 xg for 2.5 minutes and the supernatant was discarded. 9.9 mL of sterile distilled water and 0.1 mL of 0.5 M EDTA was then added to tube A. The yeast pellet was re-suspended by withdrawing and expelling through a Pasteur pipette approximately 10 times and then vortexing the tube for 15 seconds. 1 mL of this vortexed suspension was then diluted in 9 mL of water and the absorbance of the diluted sample was determined at 600 nm.

Tube B was centrifuged at 680 xg for 2.5 min and the supernatant was discarded. The pellet was then re-suspended in a washing solution by the same method using a Pasteur pipet and vortexing as described earlier. The washing solution had been prepared by dissolving 0.51 g calcium sulfate in 1 L distilled water. Tube B was then centrifuged at 630 xg for 2.5 minutes, the supernatant was discarded and the pellet was re-suspended in 10 mL of a suspension solution prepared from 0.51 g calcium sulfate, 6.8 g sodium acetate and 4.05 g glacial acetic acid all dissolved in 1 L of distilled water. The suspension in Tube B was then re-suspended as described above, slowly inverted 5 times in 15 seconds and left to sit for exactly 6 minutes. After

6 minutes 1 mL of the suspension was added to 9 mL of sterile distilled water and the absorbance at 600 nm was determined. % Flocculence was calculated using the following equation (5.1):

$$\% \text{ Flocculence} = ((\text{Abs}_{600} \text{ Tube A} - \text{Abs}_{600} \text{ Tube B}) / \text{Abs}_{600} \text{ Tube A}) \times 100 \quad (5.1)$$

All absorbances were measured with a Thermo Scientific Genesys 20 spectrophotometer (Madison, WI) and an IEC Centra MP4R centrifuge (Needham Heights, MA) was used for all centrifugations.

### *B.) Cell Surface Hydrophobicity Assay*

The cell surface hydrophobicity was determined using the method of Akiyama-Jibiki et al. (Akiyama-Jibiki et al., 1997). In this method at each sampling time approximately 1.5 mL of yeast culture was aseptically withdrawn from the 250 mL Erlenmeyer flask and added to a pre-weighed 1.5 mL microcentrifuge tube. The tube was then centrifuged at  $1.32 \times 10^6$  xg for 1 minute and the supernatant was discarded. 1.0 mL of 100 mM sodium acetate buffer (pH 4.2) was added to the tube and the pellet was re-suspended by vortexing for approximately 10 seconds. The centrifuging and washing procedure was repeated once more and the pellet was finally re-suspended in 100 mM sodium acetate buffer (pH 4.2) at 5 % w/v based on the mass of the pellet. This suspension was stored at 4 °C until later use. A Hettich Mikro 20 centrifuge (Kirchlengern, DEU) was used for all centrifugations.

A volume of well-stirred Phenyl Sepharose CL-4B/ethanol storage solution corresponding to a sediment volume of 0.25 mL (500 µl) was then added to a 1.5 mL Bio-Rad disposable chromatography column. This column was centrifuged at 490 xg for 2.5 min to separate the resin and ethanol slurry and the ethanol was discarded. 1 mL of distilled water was added to the column to rinse it, the column was centrifuged at 490 xg rpm for 1.5 min and the



water was discarded. The column was then equilibrated by running 3 mL of 100 mM sodium acetate buffer (pH 4.2)/1.0 M NaCl through it. Then the absorbance at 660 nm of 100 µl of the 5% w/v yeast suspension with 3 mL of 100 mM sodium acetate buffer (pH 4.2)/1.0 M NaCl was determined. 100 µl of the 5% w/v yeast suspension was simultaneously applied to the equilibrated column along with 3 mL of 100 mM sodium acetate buffer (pH 4.2)/1.0 M NaCl. The eluent was collected and the cell surface hydrophobicity was calculated using the following equation (5.2):

$$\% \text{ HICF Value} = ((A_{660} \text{ applied} - A_{660} \text{ eluent}) / A_{660} \text{ applied}) \times 100 \quad (5.2)$$

All absorbances were measured with the Thermo Scientific Genesys 20 spectrophotometer and the IEC Centra MP4R centrifuge was used for all centrifugations.

### *C.) i.) Oxylipin Analysis of Whole Yeast Cells*

#### *I.) Yeast collection*

The SMA yeast strain was cultured as indicated under Culture in YEPD broth. Following incubation four 40 mL aliquots of culture were transferred to four sterile 50 mL centrifuge tubes. The tubes were centrifuged at 630 xg for 2.5 minutes and the supernatant was discarded. 3 mL CaSO<sub>4</sub> washing solution was added to each pellet and the pellets were re-suspended in the solution by vortexing. The four tubes were then centrifuged again at 630 xg for 2.5 minutes and the pellets were transferred with a solvent-cleaned scoopula to a Buchner funnel lined with Genuine Whatman No. 1 filter paper (W & R Balston Ltd., England). The yeast pellet was then dried by mounting the Buchner funnel on a 1000 mL side-arm flask under vacuum. An IEC Centra MP4R centrifuge (Needham Heights, MA) was used for all centrifugations.

## *II.) Hexane/isopropanol extraction*

To extract the fatty acids from the yeast pellets a modified version of a Goettingen Center for Molecular Biosciences HIP extraction (Albrecht-von-Haller, 2005) was employed. To do this a pre-weighed mass of dried yeast (~0.5 g) was added to a 40 mL durable, solvent-cleaned glass tube. 20 mL of n-hexane/2-propanol (3:2 v/v) with 0.0025 % (w/v) 2-butyl-6-hydroxytoluene was added to the glass tube. The tube was then sonicated for 4 minutes with a Branson 2510 sonicating bath (Danbury, CT), shaken for 15 seconds and centrifuged for 4 minutes at half-speed. Following centrifugation, 12.5 mL of 6.7 % (w/v) K<sub>2</sub>SO<sub>4</sub> that had been prepared with chloroform washed water was added to the glass tube and the tube was shaken vigorously by hand for 2 minutes. The resultant upper hexane layer was then transferred to a 10 mL durable, solvent-cleaned glass tube and the tube was dried under streaming nitrogen with an Organomation Associates N-EVAP 112 (Berlin, MA). The dried product was re-dissolved in 0.2 mL CH<sub>2</sub>Cl<sub>2</sub>. In between all aforementioned tube manipulations the tube atmospheres were evacuated with streaming nitrogen. An IEC HN-SII centrifuge (Needham Heights, MA) was used for all centrifugations.

## *III.) Production of fatty acid methyl esters (FAME) for GC*

FAME were prepared according to the standard transesterification protocol from the Dalhousie University Marine Lipids Lab (Marine Lipids Lab, 2013). In this method 1.5 mL of methylene chloride with 0.01 % butylhydroxytoluene (BHT) and 3.0 mL of Hilditch reagent were added to the dried product of the HIP extract dissolved in 0.2 mL CH<sub>2</sub>Cl<sub>2</sub>. The Hilditch reagent had been prepared by adding 1.5 mL of H<sub>2</sub>SO<sub>4</sub> to 100 mL of methanol that was dried by filtering it through a funnel and filter paper with 1 scoop (~1.3 g) of NaSO<sub>4</sub>. The 10 mL durable

glass tube was then placed in a VWR Analog Heat Block (Deptford, NJ) at 100 °C for 1 hour and cooled to room temperature at the duration of heating. A total of 3 mL of hexane and 1 mL of distilled water were then added and the tube was vortexed.

The top hexane layer was subsequently removed from the tube and added to a new solvent-cleaned 10 mL glass tube. The extraction procedure was then repeated twice more; each time 1 mL of hexane was added to the first tube, it was vortexed and the upper hexane layer was added to the new tube. Then 2 mL of distilled water was added to the second tube, it was vortexed and the bottom aqueous layer was removed. The hexane layer was dried by adding a scoop of NaSO<sub>4</sub> to the tube and shaking it. The solvent/FAME mixture was then added to a third pre-weighed, solvent-cleaned, durable 10 mL glass tube and the solvent was removed by drying under streaming nitrogen with the Organomation Associates N-EVAP 112. Based on weight differentials the FAME products were dissolved in hexane to give the desired concentration. In between all aforementioned tube manipulations the tube atmospheres were evacuated with streaming nitrogen.

#### IV.) *Analysis using GC-FID*

GC-FID analysis was performed on a Bruker 430-GC Ultra equipped with a Thermo Triplus AS autosampler and an Agilent 30 m x 0.25 mm 0.25 µm phase (DB-23, (50%-cyanopropyl)-methylpolysiloxane) thickness column. The injected volume was 1.0 µl and a splitless injection was used to maximize the amount of analyte on the column. Inlet temperature was 250 °C. Column temperature began at 69 °C and increased to 153 °C at 45 C°/min followed by a ramp at 2.3 C°/min to a second holding temperature of 174 °C. Column temperature then increased by a third ramp at 2.5 C°/min to a final temperature of 205 °C that was held for 8.4

minutes before run termination. Helium (Air Liquide, Dartmouth, NS) flowing at 1.0 mL/min was used as the carrier gas.

#### *V.) Analysis using GC-MS*

GC-MS analysis was performed on a Trace GC Ultra equipped with a Polaris Q mass spectrometer, a Thermo Triplus AS autosampler and a Phenomenex Zebron 30 m x 0.25 mm 0.25  $\mu$ m phase (FFAP, nitroterephthalic acid modified polyethylene glycol) thickness column. The injected volume was 1.0  $\mu$ l and a splitless injection was used to maximize the amount of analyte on the column. Inlet temperature was 200 °C and detector temperature was 250 °C. Column temperature began at 153 °C and increased to 174 °C at 2.3 C°/min, immediately followed by a ramp at 2.5 C°/min to a final temperature of 205 °C which was held for 8.27 minutes before run termination. Helium (Air Liquide, Dartmouth, NS) flowing at 0.25 mL/min was used as the carrier gas. For certain analyses, the GC-MS was run in selective ion monitoring mode to detect fragments with a mass-to-charge ratio of 103 specifically, and during these runs the same conditions described above were employed.

#### *C.) ii.) Oxylipin Analysis of Yeast Cell Wall Isolates*

The SMA yeast strain was cultured as indicated under Culture in YEPD broth, and following incubation eight 45 mL aliquots of culture were transferred to eight sterile 50 mL centrifuge tubes. The tubes were centrifuged at 630 xg for 2.5 minutes, the supernatant was discarded and each of the eight pellets were re-suspended in 5 mL of sterile YEPD broth. The contents of each of two 50 mL tubes were then pooled into a sterile 10 mL centrifuge tube. Each of the four pooled tubes were compressed twice at 8000 psi in a 40 mL French pressure cell using an Aminco French pressure cell press (Tavenol) to rupture the yeast cells and to prepare

ghosts. The “pressed” cultures were then stored on ice and subsequently centrifuged at 3870 xg for 10 minutes to isolate the cell wall fractions, as suggested by Campbell & Duffus (1988). A hexane/isopropanol extraction, production of FAME for GC, analysis using GC-FID and analysis using GC-MS were conducted as previously described. All centrifugations were conducted with the IEC Centra MP4R centrifuge.

### **5.1.2 Part 2.) Growth in the Miniature Fermentation Assay**

#### *Culture in Miniature Fermentation Assay*

The SMA yeast inoculum was prepared according to instructions in the ASBC miniature fermentation assay protocol (ASBC, 2013, Yeast-14). However, a pre-made Pale Ale wort (Festa Brew, Vaughn, ON) with an OG of 12.39-12.58 °P (measured as 12.5 °P) was used in place of the congress wort. The pre-made wort had been previously portioned into 473 mL cups that were frozen in a -30 °C blast freezer. Prior to use, the wort was defrosted in a microwave and prepared using an abridged version of the miniature fermentation assay (ASBC, 2013, Yeast-14) mashing and wort treatment protocol. That is, the contents of two 473 mL cups of defrosted wort were autoclaved for 20 min at 121 °C, and the sterile wort was cooled overnight at 4 °C for no more than 12 hours.

Following the cold break, the wort was centrifuged at 1950 xg for 15 minutes, and the supernatant was saved. Then 18 g of D-glucose was added to 410 mL of centrifuged wort to yield a density of 16.1 °P, and the wort was aerated by bubbling it with medical-grade, compressed oxygen for 5 minutes. The wort was then pitched with the SMA yeast at a rate of  $1.5 \times 10^7$  cells/mL to final volume of 450 mL. When the pitched wort had been thoroughly mixed, 15.0 mL aliquots were transferred to each of 30 sterile 20 mL test tubes that contained a sterile

PTFE boiling stone. Each tube was plugged with a sterile sponge bung and the rack was placed in a 21 °C bath until sampling. All centrifugations were conducted with the IEC Centra MP4R centrifuge.

#### *A.) Flocculation Assay*

The flocculation level was determined by the ASBC Flocculation (ASBC, 2013, Yeast-11-B) as previously described. However, the preparation of the yeast sample was conducted as indicated under Culture in Miniature Fermentation Assay. Also, two 10 mL aliquots (sample A and B) were taken from two 15 mL test tubes that had been vortexed to re-suspend any yeast mass from the bottom of the tube.

#### *B.) Cell Surface Hydrophobicity Assay*

The cell surface hydrophobicity assay was determined using the method of Akiyama-Jibiki et al. (Akiyama-Jibiki et al., 1997), as previously described. However, the approximately 1.5 mL of yeast culture was withdrawn from one of the test tubes that had been vortexed to re-suspend any yeast mass at the bottom of the tube.

#### *C.) Oxylipin Analysis of Whole Yeast Cells*

To collect yeast pellets at each sampling point four test tubes containing 15 mL each of fermenting beer were transferred evenly to two sterile 50 mL centrifuge tubes. The 50 mL tubes were centrifuged for 2.5 minutes at 630 xg and the supernatant was discarded. 10 mL of sterile distilled water was then added to each tube and the pellets were re-suspended by vortexing. The tubes were then pooled into one 50 mL centrifuge tube and again this tube was centrifuged for 2.5 minutes at 630 xg. The supernatant was discarded and the yeast pellet was stored at -30 °C

until later use. A hexane/isopropanol extraction, production of FAME for GC and analysis using GC-MS were conducted as previously described. All centrifugations were conducted with the IEC Centra MP4R centrifuge.

#### D.) *Gravity Determination*

Density was determined on a Plato scale (°P) using a handheld densitometer (Anton Parr DMA 35, Graz, Austria) and before each reading the sample was filtered through Whatman 4 filter paper (Buckinghamshire, UK).

#### **5.1.3 Part 3.) Use of Mixed Bacterial Acid Methyl Ester (BAME) Standard**

In the part 3 samples from part 1 and part 2 were re-run on the GC-MS against a BAME standard (Sigma Aldrich, Supelco; Oakville, ON) known to contain 3-OH 12:0 and 3-OH 14:0. The BAME standard came stored in methyl caproate at 10.0 mg/mL, and prior to use, the standard was diluted 1:10 in hexane to a working concentration of 1.0 mg/mL.

## 5.2 Phase 2

**\*Note:** What follows is a description of the Materials and Methods used in Phase 2 of this thesis project. As outlined in Figure 4.3, this study was a collaborative effort between the author and Heriot-Watt University M.Sc. student Chris Stanton, Jr. Therefore, particular emphasis and detail will be given to those parts of the study the author did jointly with Chris or those parts the author did on his own. Sufficient information will be provided to the reader for the section Chris Stanton, Jr. did by himself, but if further details are desired the reader is directed to a copy of Chris Stanton, Jr.'s thesis (Available upon request from School of Life Sciences at Heriot-Watt University).

### 5.2.1 I.) SMA Yeast Culture and Preparation

#### 1.) *Yeast Culture*

Stock cultures of the SMA yeast strain (Wyeast Laboratories Inc., Odell, OR) were stored on YEPD agar slants, prepared as described in Phase 1, and kept at 4 °C until used. To initiate growth, a loopful of an isolated colony was aseptically transferred to a 250 mL Erlenmeyer flask containing 100 mL sterile YEPD broth and the flask was aerobically incubated on a New Brunswick Scientific Excella E25 Incubator Shaker (Enfield, CT) at 100 rpm for 24 hours at 30 °C. After 24 hours approximately 50 g of the culture was transferred to a sterile 50 mL centrifuge tube. The tube was centrifuged at 1600 rpm for 3 minutes with a Denley BS400 centrifuge (Buckinghamshire, UK), and the supernatant was discarded. The remaining yeast mass was aseptically transferred to a new 250 mL Erlenmeyer flask containing 100 mL of YEPD broth and the flask was incubated for a further 24 hours under the same conditions as the first flask. Following this one more round of centrifugation, yeast transfer and incubation was completed to produce a culture that was 72 hours old.



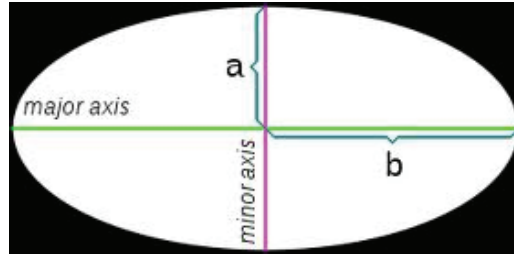
## *2.) Culture Prep for Analysis*

At 24, 48 and 72 hours, yeast were prepared for analysis by withdrawing 1.0 mL of yeast slurry and transferring it to a sterile 1.5 mL microcentrifuge tube. This was done in triplicate. The 1.5 mL microcentrifuge tubes were then centrifuged at 550 xg for 3 minutes with a Thermo Scientific Heraeus Pico 21 microcentrifuge (Marietta, OH) and the supernatant was discarded. The pellet was re-suspended in a pH 4 acetate buffer that had been supplemented with ethanol to a final concentration of 5.0 g/L and calcium ( $\text{Ca}^{2+}$ ) (from  $\text{CaCl}_2$ ) to a concentration of 1.0 mmol/L as described by Patelakis et al. (1998). Calcium and ethanol were added to the acetate buffer to encourage the yeast to flocculate and to simulate a beer fermentation. The microcentrifuge tubes were centrifuged again as previously described, and the washing procedure was completed twice more to insure adequate removal of YEPD broth.

## *3.) Culture Imaging and Measure*

To capture pictures and estimate cell sizes at each sampling time, the prepared cultures, as described above, were first diluted 1:10 in the acetate buffer supplemented with  $\text{Ca}^{2+}$  and ethanol. The samples were then placed on a microscope slide and viewed at 40 X magnification with a Zeiss Axioplot microscope (Thornwood, NY) equipped with an AxioCam ERc 5s (Toronto, ON) camera such that there were 20 to 30 cells per picture. Duplicate samples were prepared at each sampling time. Relative sizes of the cells were determined using the Zeiss Zen2011 (Thornwood, NY) software. The dimensions of the major and minor axes (as shown in Figure 5.2.1) of each cell were determined by calibrating the output of Zeiss Zen2011 to the scale of a micrometer (0.01 mm). Those cells selected for counting exhibited a prolate spheroid

shape (Figure 5.2.1). The cell volume and cell surface area were determined using Equations 5.2.1 or 5.2.2.



**Figure 5.2.1** A schematic of a prolate spheroid showing the minor and major axes, and their respective radii, given as “b” for the major axis and “a” for in the minor axis (\*Used with permission from the University of California Davis, 2013; see Appendix IV).

$$V_p = \frac{4}{3}\pi a^2 b \quad (5.2.1)$$

Where  $V_p$ ,  $a$  and  $b$  are the volume, the radius of the minor axis and radius of the major axis, respectively.

$$A_p = \sqrt[3]{V_p^2} \quad (5.2.2)$$

And where,  $A_p$  and  $V_p$  are the surface area and the volume of the prolate spheroid, respectively.

## 5.2.2 II.) Fluorescent Probe Prep and Flow Cytometry

### 1.) Probe Prep

The fluorescing molecular probe used in this study, an Avidin, Texas Red® conjugate (Life Technologies, Burlington, ON), had an excitation wavelength at approximately 595 nm and an emission wavelength at about 615 nm. Stock solutions of the probe were prepared by rehydrating in the pH 4 acetate buffer supplemented with ethanol and  $\text{Ca}^{2+}$  to a concentration of 2.0 mg/mL. All stock solutions were stored at -20 °C and were protected from light damage by

wrapping in tin foil. From the stock solution, nine concentrations of probe were prepared for the experimental work at 1.5, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/mL.

### 2.) *Flow Cytometer Prep*

The flow cytometry experiments were done with a Partec Cyflow® SL flow cytometer (Partec GmbH, GER) equipped with a 488-nm air cooled argon ion laser, and all preparations and machine use were done according to the manufacturer's instructions. The instrument settings for each parameter measured are indicated in Table 5.2.1. Manipulations of the data produced were prepared with the Partec software suite, Flowmax version 2.4e.

**Table 5.2.1.** The instrument settings for the Partec Cyflow® SL flow cytometer used in the experimental work.

Parameters	Gain	Log/linear	Lower limit	Upper limit
FSC	150	Log 4	0	999.9
SSC	200	Log 4	0	999.9
FL1 520 nm	550	Log 4	0	999.9
FL2 590 nm	550	Log 4	0	999.9
FL3 630 nm	550	Log 4	0	999.9

### 3.) *Standard Curve Measurement*

A standard curve was generated by mixing 10 µl of each probe concentration with 1390 µl of the pH 4 acetate buffer supplemented with ethanol and Ca<sup>2+</sup>. The samples had been prepared in triplicate and were used to produce an average emission at 630 nm for each concentration. During standard curve measurement, the samples were corrected for the autofluorescence of the buffer. The nine dilutions of the Avidin, Texas Red® conjugate probe at 1500, 1400, 1200, 1000, 800, 600, 400, 200 and 100 µg/mL were determined to have a final

probe concentration in the sample of 10.71, 10, 8.57, 7.14, 5.71, 4.29, 2.86, 1.43 and 0.71  $\mu\text{g/mL}$ , respectively.

#### *4.) Langmuir Analysis of the Avidin, Texas Red® conjugate*

To construct Langmuir plots as recommended by Patelakis et al. (1998), the amount of free probe in the supernatant and that bound to the cell had to be determined for the different concentrations of Avidin, Texas Red® conjugate previously described (1.5, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1  $\text{mg/mL}$ ). To do this, at each sampling time the prepared culture was placed in 9 microcentrifuge tubes at a concentration of approximately  $1 \times 10^6$  cells/mL and to a total volume of 1390  $\mu\text{l}$ . The samples were prepared in duplicate and 10  $\mu\text{l}$  of each probe concentration was added to each microcentrifuge tube, while more buffer was added to a tenth tube as a control. Each sample was vortexed for 10 seconds and centrifuged at 550  $\text{xg}$  for 4 minutes with the Thermo Scientific Heraeus Pico 21 microcentrifuge. The supernatant was carefully extracted from each tube, and the yeast pellet was re-suspended in fresh pH 4 acetate buffer. Each sample was then processed through the flow cytometer, and all samples that were not being immediately processed were covered to prevent light degradation to the fluorescent dye.

### **5.2.3 III.) Data Export and Statistical Analysis**

#### *1.) Raw Data Gated, Manipulated and Exported with Excel and C++ in Code::Blocks*

The raw data was manipulated and exported from the flow cytometer computer with Microsoft Excel and tools generated using C++ in Code::Blocks (Stanton, 2013). All measures exported from the flow cytometer were converted to a relative scale ranging from 0 to 16 380. The gate ranges for each parameter for each measure are summarized in Table 5.2.2.

**Table 5.2.2.** The gating ranges used for the flow cytometry data for each set of measurements, forward scatter (FSC), side scatter (SSC) and emission at 630 nm (630 nm).

Type and Time (hrs)	FSC Gate Range	SSC Gate Range	630 nm Gate Range
Yeast, 24	6 500-10 500	>0	>0
Supernatant, 24	<2 500	<100	>0
Yeast, 48	5 500-11 500	>0	>0
Supernatant, 48	<3 000	<100	>0
Yeast, 72	5 500-11 500	>0	>0
Supernatant, 72	<2 500	<100	>0

2.) *Histograms analyzed with Igor Pro 6.2*

In an effort to detect potential sub-populations, histograms of each parameter at each sampling time for the yeast samples incubated with the 1.5 mg/mL probe solution were analyzed with Igor Pro 6.2 (Wavemetrics, Inc., Portland, OR). Among its many functionalities, Igor Pro 6.2 is capable of fitting multiple curves to graphs and data sets using an automatic peak-finding algorithm that searches for peaks by finding maxima in the smoothed second derivative of the data. **Note:** In processing the 9 yeast samples with different concentrations of the Avidin, Texas Red® conjugate probe, the sample with a probe concentration of 1.5 mg/mL was always processed first. The remaining 8 samples were processed in the flow cytometer after, but this may have been up to several hours after removal from the main culture and the cells were not fixed at extraction. Therefore, the aliquot with the 1.5 mg/mL probe concentration was the most representative of the culture at the sampling time, and it was always used to produce the histograms of each measured parameter.

## 5.2.4 IV.) Fluorescent Microscopy

### 1.) Yeast Culture

The SMA yeast strain was cultured almost exactly as done for the flow cytometry experiments. That is, a loopful of culture was transferred from a YEPD agar slant stored at 4 °C to a sterile 250 mL Erlenmeyer flask with 100 mL of YEPD broth. The flask was incubated for 24 hours at 100 rpm and 30 °C. After 24 hours, 50 mL (~50 g) of culture was collected in a 50 mL sterile centrifuge tube, and centrifuged at 1600 rpm for 6 minutes with the Denley BS400 centrifuge. The resultant yeast pellet was transferred to 100 mL of fresh YEPD broth by pipetting approximately 5 mL of YEPD broth into the decanted 50 mL tube containing the yeast pellet and transferring the slurry to the 250 mL Erlenmeyer flask. The flask was incubated as described previously and the centrifugation and yeast transfer procedure was repeated once more such that cultures at 24, 48 and 72 hours age were produced.

### 2.) Culture Staining with Wheat Germ Agglutinin Alexa Fluor® 488 Conjugate

At each time interval, 24, 48 and 72 hours, a sample was stained using a protocol modified slightly from that first described by Lehnhart et al. (2007). In summary, at each time 3.0 mL was removed from the culture and mixed with 3.0 mL of 70 % ethanol. This mixture was stored at 4 °C for at least 30 minutes to fix the cells. Once at least 30 minutes had passed, the cells/ethanol mixture were transferred to 4 sterile 1.5 mL microcentrifuge tubes and centrifuged at 21 000 xg for 3 mins with the Thermo Scientific Heraeus Pico 21 microcentrifuge. The supernatant was discarded and each pellet re-suspended in 1.5 mL of pH 7.4 phosphate buffered saline (PBS). The absorbance at 600 nm ( $A_{600}$ ) was determined with a Genesys 20 Thermo

Spectronic spectrophotometer (Madison, WI) and dilutions were made if necessary so that the culture volume was 0.5 mL and had an  $A_{600}$  of approximately 0.800.

The pellet was then washed a further two times as done previously, and was re-suspended in a 0.5 mL aliquot of 1 mg/mL WGA Alexa Fluor® 488, conjugate (Life Technologies, Burlington, ON) in PBS. The probe/PBS solutions had been prepared earlier and were stored covered in tin foil at -20 °C to prevent light damage. The pellet and probe mixture, wrapped in tin foil, was then incubated for 15 minutes with frequent agitation. Following incubation, the cells were harvested by centrifugation at 2400 xg for 3 minutes in the same microcentrifuge, washed twice in 0.5 mL of PBS as described previously and finally re-suspended in 0.5 mL of PBS.

### *3.) Cell Imaging*

All stained cells at each sampling time were visualized at 100X magnification under oil immersion with the Zeiss Axioplan microscope equipped with the AxioCam ERc 5s camera. The microscope had been fitted with a single excitation filter set Zeiss No.09 (excitation: BP 450-490; beamsplitter: FT 510; emission: LP 520). Images were processed with the Carl Zeiss Microscopy software Zen2011.

# Chapter 6: Results and Discussion

## 6.1 Phase 1

### 6.1.1 Part 1.) Growth in YEPD Broth

#### *Flocculation and Cell Surface Hydrophobicity Measurements*

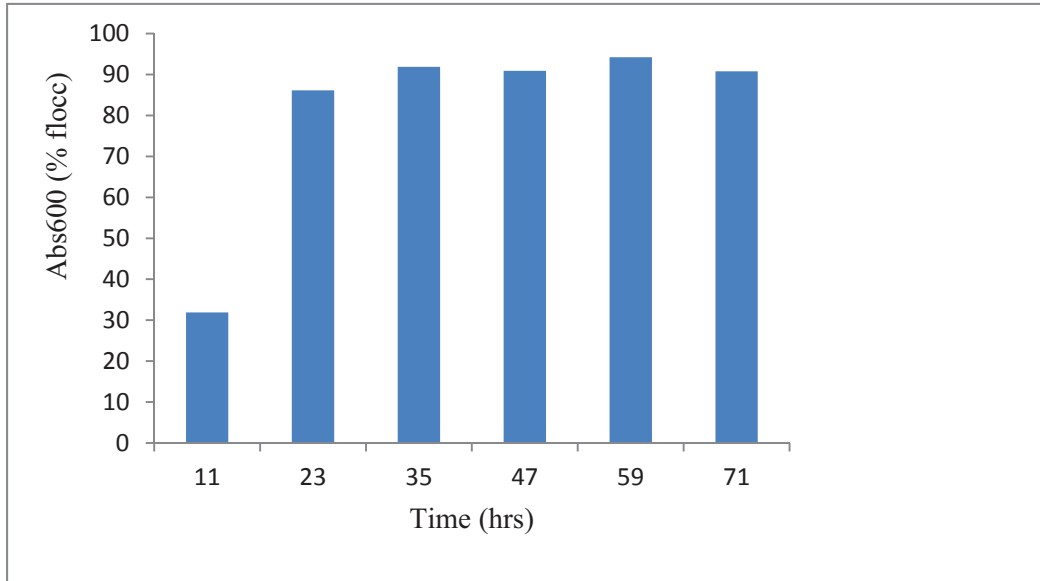
When the flocculation level was measured for the SMA yeast strain as per the ASBC Yeast-11-B method (Figure 6.1.1), the level increased sharply within the first 24 hours. It is noteworthy that this increase reflects the flocculation tendency of the yeast in a buffer. The cell surface hydrophobicity for the SMA yeast strain, meanwhile, did not show a pronounced increase until after 48 hours of growth (Figure 6.1.2). However, as with earlier work which observed a maximum flocculation potential with a concurrent increase in cell wall hydrophobicity (Speers et al., 2006), there was a coincident maximum level in flocculation capacity and cell surface hydrophobicity (Figures 6.1.1 and 6.1.2). Furthermore, the SMA yeast strain is known to flocculate towards the end of a fermentation (Wyeast Laboratories, 2009) and possesses the NewFlo phenotype (Yamauchi et al., 1995). Together these facts likely explain the delayed onset of flocculation and cell surface hydrophobicity.

#### *Oxylipin Analysis of Whole Yeast Cells*

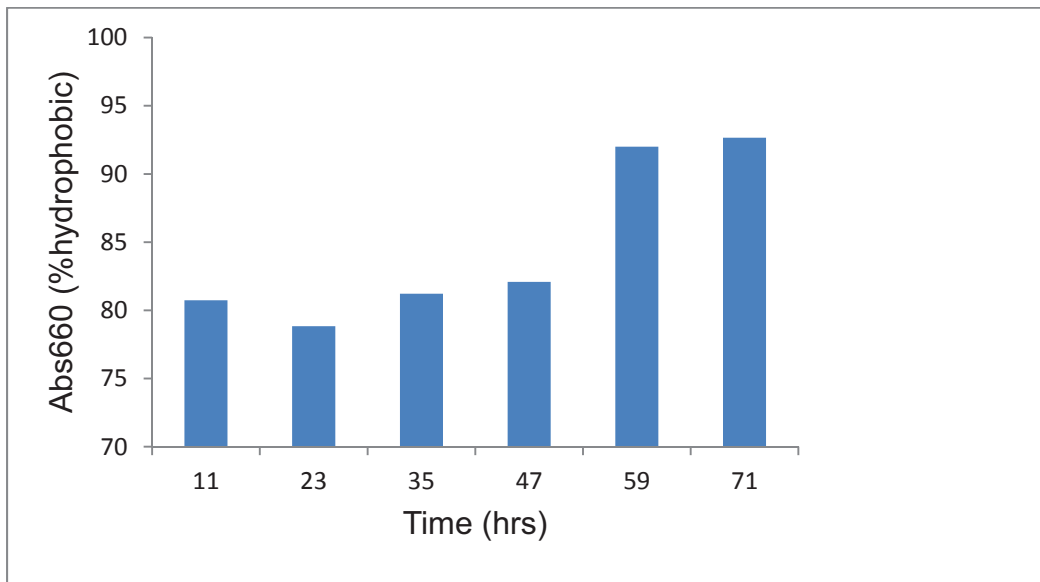
The initial fatty acid analysis was conducted with the SMA yeast grown in YEPD broth using a GC-FID apparatus. In this analysis the five most abundant fatty acids were C16:0, C16:1n-7, C18:0, C18:1n-9 and C18:1n-7, with 16:1n-7 the most prevalent fatty acid overall (Figure 6.1.3). Notably, neither C8:0 or C10:0 were included in the 6 most abundant fatty acid structures isolated. These results are in accordance with a previous study (Blagovic et al., 2001)



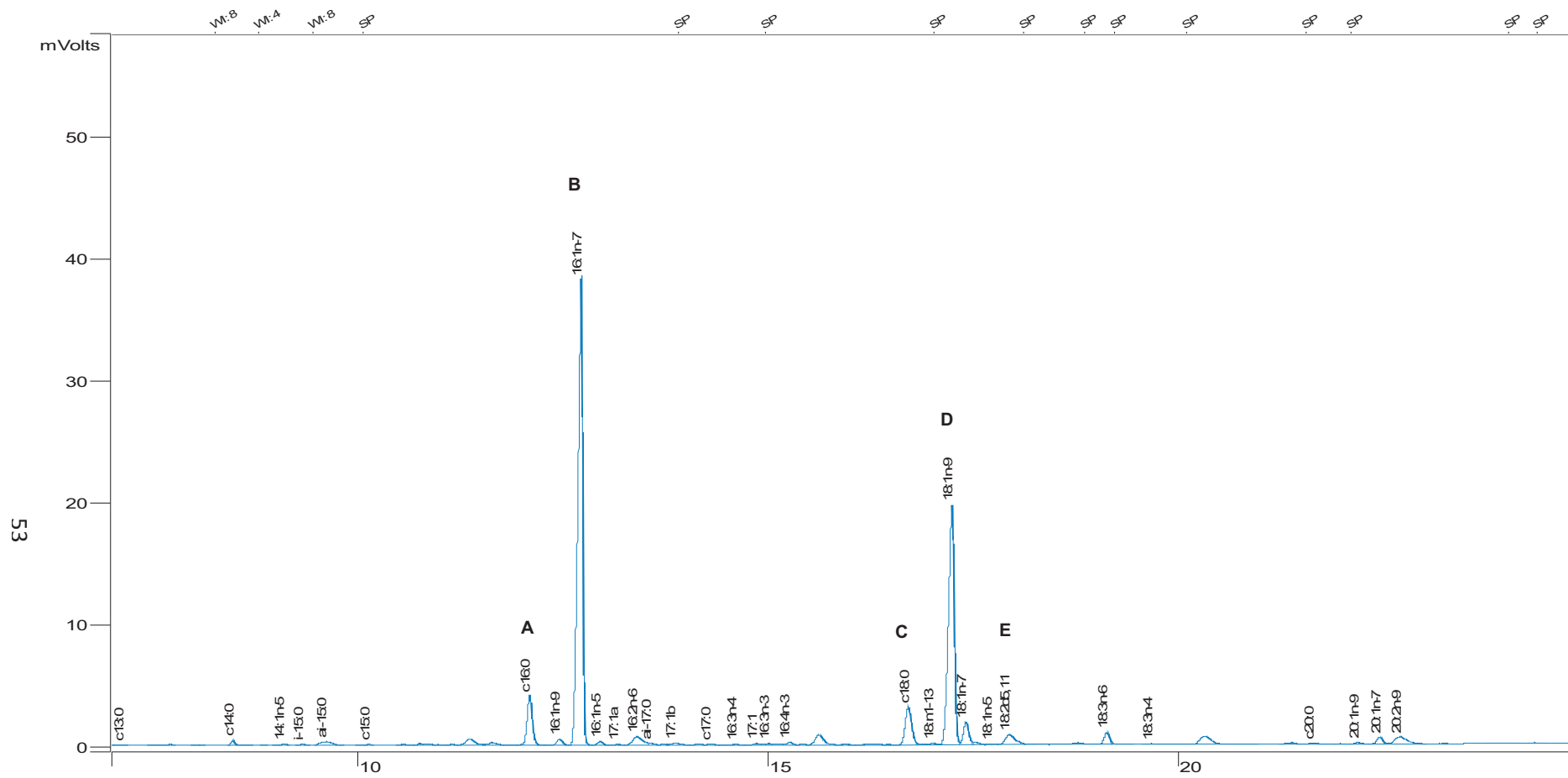
that found palmitic (C16:0) and palmitoleic (C16:1) acids in the highest concentration of those fatty acids extracted from brewer's yeast.



**Figure 6.1.1.** The flocculation level of the SMA yeast strain (a lager strain) grown in YEPD broth as determined by the ASBC Yeast-11-B method, with absorbance measured at 600 nm.

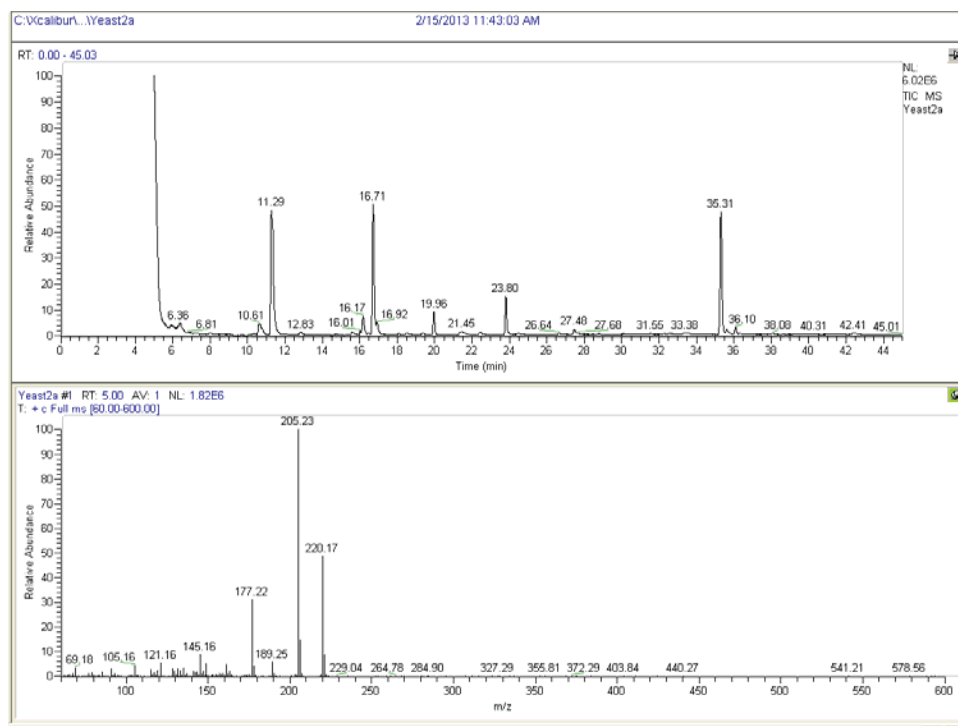


**Figure 6.1.2.** The cell surface hydrophobicity of the SMA yeast strain (a lager strain) grown in YEPD broth, as determined by the method of Akiyama-Jibiki et al., with absorbance measured at 660 nm.



**Figure 6.1.3.** A GC-FID chromatogram of whole cell extracts for an initial fatty acid analysis of the SMA yeast grown in YEPD broth. Yeast culture, fatty acid extraction, fatty acid methyl ester production and instrumental conditions were conducted as indicated in the methods section. Above, peaks A, B, C, D and E correspond to the 5 most abundant fatty acids C16:0, C16:1n-7, C18:0, C18:1n-9 and C18:1n-7, respectively.

The same sample of the SMA yeast grown in YEPD analyzed with the GC-FID apparatus was then analyzed with the GC-MS apparatus (Figure 6.1.4). A thorough examination of the peaks and spectra produced and comparison to known structures with the National Institute of Standards and Technology (NIST) MS Search 2.0 program did not reveal any structures that appeared to be 3-OH oxylipins, principally the  $\text{CH}_3\text{COOCHOH}$  fragment first reported by Vesonder et al. (1968).

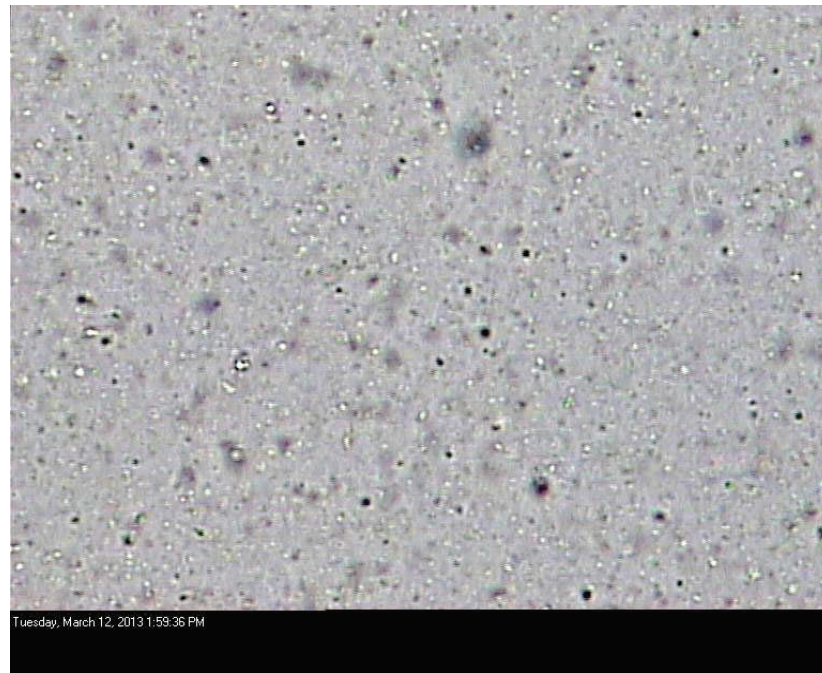
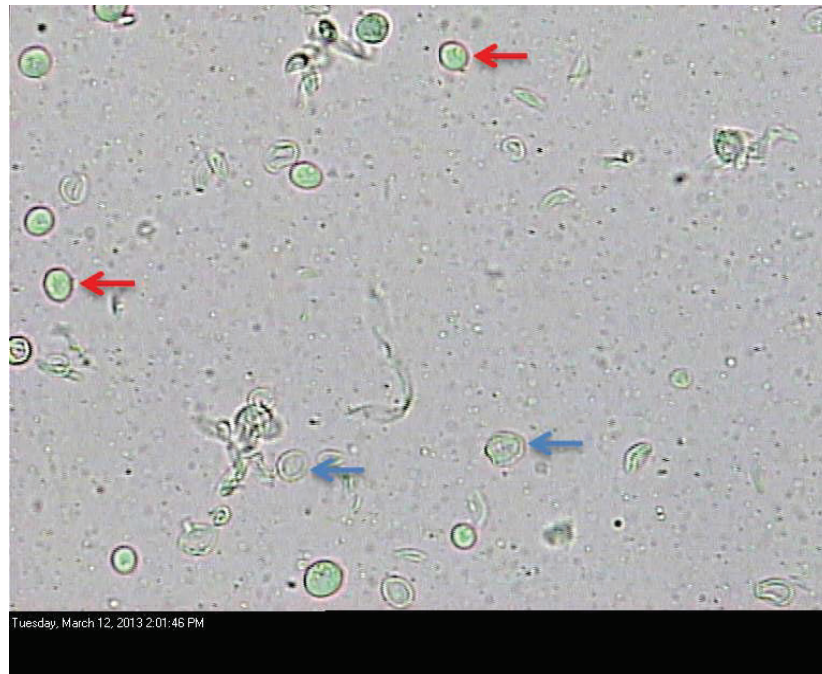


**Figure 6.1.4.** A GC-MS chromatographic (above) and mass spectral (below) analysis of whole cell extracts of the SMA yeast strain grown in YEPD broth. Yeast culture, fatty acid extraction, fatty acid methyl ester production and instrumental conditions were as indicated in the methods section.

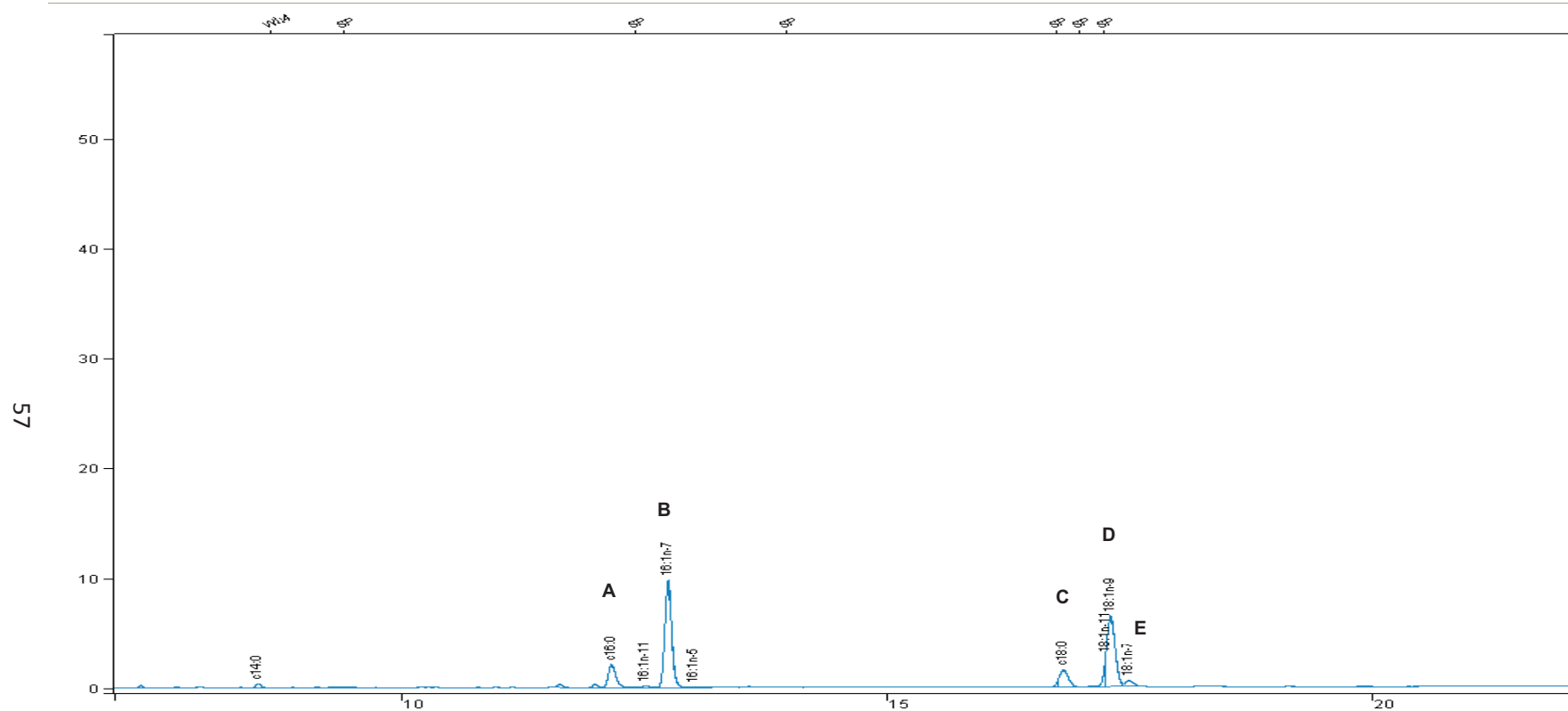
### *Oxylipin Analysis of Yeast Cell Wall Isolates*

It was hypothesized that cell rupture with a French press and subsequent isolation of the cell wall ghosts would concentrate the cell wall-associated lipids. In Figure 6.1.5 it is clear that while French pressing and fraction isolation were a successful means to create cell ghosts, the process was not 100 % efficient. There are clearly un-ruptured cells which were centrifuged down into the pellet. This is not a unique observation though. French and Milner (1955) reported that rupture of yeasts was indeed incomplete with their device, and that rupture of certain bacteria, such as *Chlorella*, required multiple passes through the apparatus. Further, the initial culture volume used to collect cells for whole cell fatty acid extraction was less than half the culture volume for cell wall isolation (160 mL vs. 360 mL), yet the peaks for the cell wall isolates are much smaller (Figure 6.1.6). Thus, French pressing was deemed to be an unsuccessful method of concentrating cell wall-associated lipids.

It is believed that the extremely high pressures of the French press apparatus (8000 psi) could have liberated the lipids from the yeast cell into the surrounding YEPD broth. While lipids would typically be non-soluble in an aqueous environment, the primary oxylipins previously implicated in increasing CSH, 3-OH 8:0 and 3-OH 10:0 (Kock et al., 2000), are short-chained and therefore amphiphilic in nature. Conceivably, these compounds could have been solubilized into the aqueous surroundings and accidentally discarded during disposal of the supernatant following centrifugation. However, because examination of the GC-MS analysis of the whole cell isolates did not reveal any structures that appeared to be 3-OH oxylipins, the chance that this accidental disposal occurred is small.

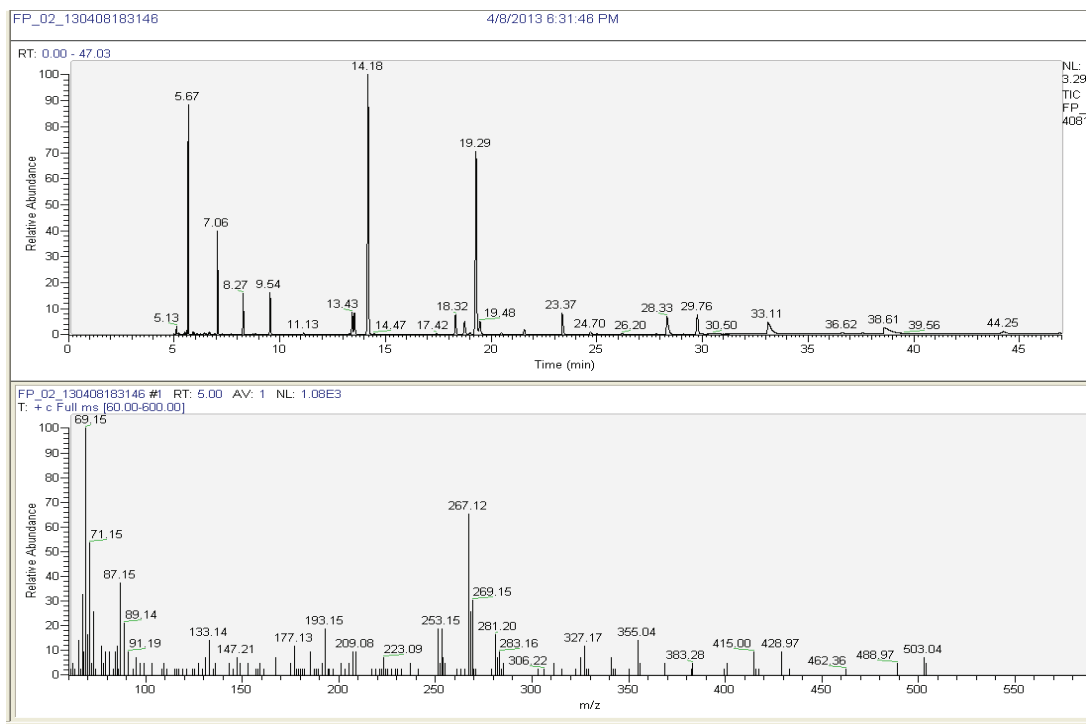


**Figure 6.1.5.** A sample of a SMA yeast culture French Pressed twice at 8000 psi. The pressed product was centrifuged at 3870 xg for 10 mins to isolate the cell wall fractions, according to the method of Campbell & Duffus (1988). The picture above is the re-suspended pellet viewed at 400 X showing ghosts (blue arrows) and whole cells (red arrows) and the picture below is the supernatant at 400 X showing no ghosts or whole cells, just cellular debris.



**Figure 6.1.6.** A GC-FID chromatogram of cell wall isolates (French Pressed cultures) of the SMA yeast strain grown in YEPD broth. Yeast culture, French pressing, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions were conducted as indicated in the methods section. Above, peaks A, B, C, D and E correspond to the 5 most abundant fatty acids C16:0, C16:1n-7, C18:0, C18:1n-9 and C18:1n-7, respectively.

As with the analysis of whole yeast cells, the cell wall isolates sample was analyzed with a GC-MS apparatus (Figure 6.1.7). Again, a thorough examination of the peaks and spectra produced and comparison to known structures with NIST MS Search 2.0 program did not reveal any structures that appeared to be 3-OH oxylipins.



**Figure 6.1.7.** A GC-MS chromatographic (above) and mass spectral (below) analysis of cell wall isolates (French pressed cultures) of the SMA yeast grown in YEPD broth. Yeast culture, French pressing, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions were as indicated in the methods section.

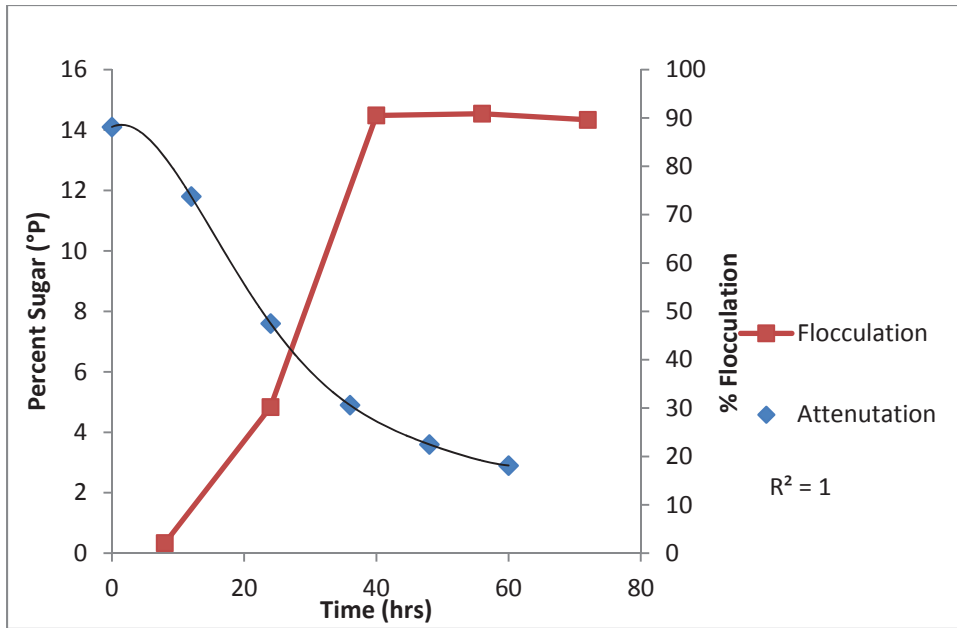
## 6.1.2 Part 2.) Growth in Miniature Fermentation Assay

### *Flocculation and Cell Surface Hydrophobicity Measurements*

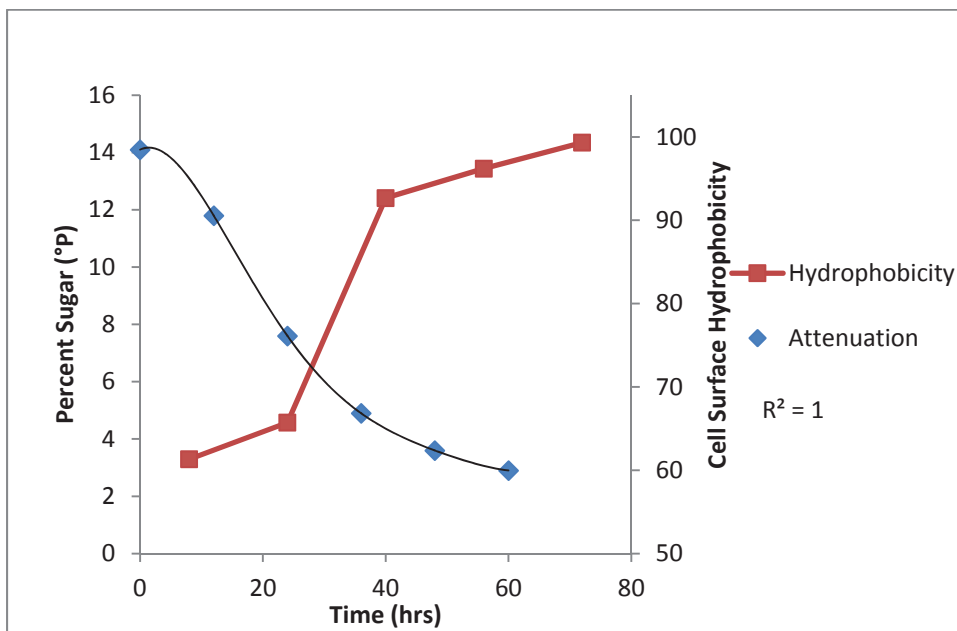
Like the growth in YEPD broth, the flocculation behaviour (Figure 6.1.10) and level and the cell surface hydrophobicity of the SMA yeast strain grown in the miniature fermentation assay increased distinctly over the course of the fermentation (Figure 6.1.8 and Figure 6.1.9). However, unlike the growth in YEPD broth, the change in flocculation and CSH in the miniature fermentation assay was approximately sigmoidal. Despite the dissimilar rates of change, in both growth setups (Erlenmeyer flask and miniature fermentation assay), flocculation level and CSH increased concurrently with respect to time.

As to why the rates of change in flocculation and cell surface hydrophobicity were dissimilar in the two phases, there are a number of potential causes. The first was that the composition of the two growth mediums were different, notably the oxygen and the initial sugar level; the wort was approximately 14.1 % glucose (°P) and the broth approximately 20 % (w/v) glucose. Secondly, the over pressure of the two vessels would not have been the same, with the 250 mL flask having much more head space for CO<sub>2</sub> to dissipate than the test tube, and therefore less over pressure. Thirdly, the growth in the 250 mL Erlenmeyer flask was on an orbital shaker, whereas the test tubes were static. In fact, growth on an orbital shaker was actually a preferred method of flocculation quantification in past laboratory studies (e.g., Stratford & Keenan; 1988).

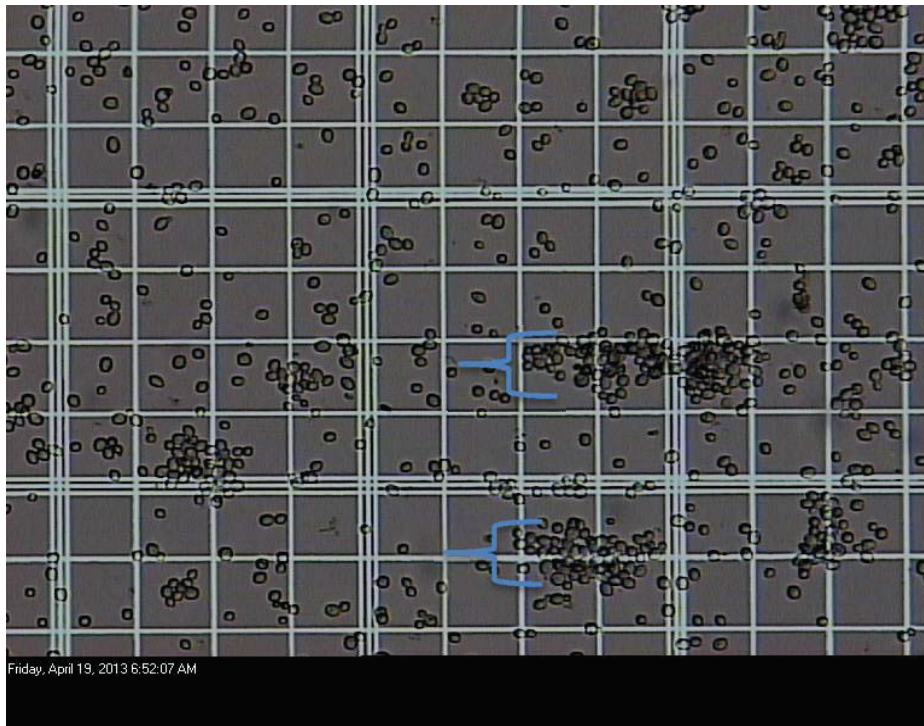




**Figure 6.1.8.** Attenuation and flocculation level as determined by ASBC Yeast-11-B method, with absorbance measured at 600 nm, during a miniature fermentation assay using the SMA yeast strain.



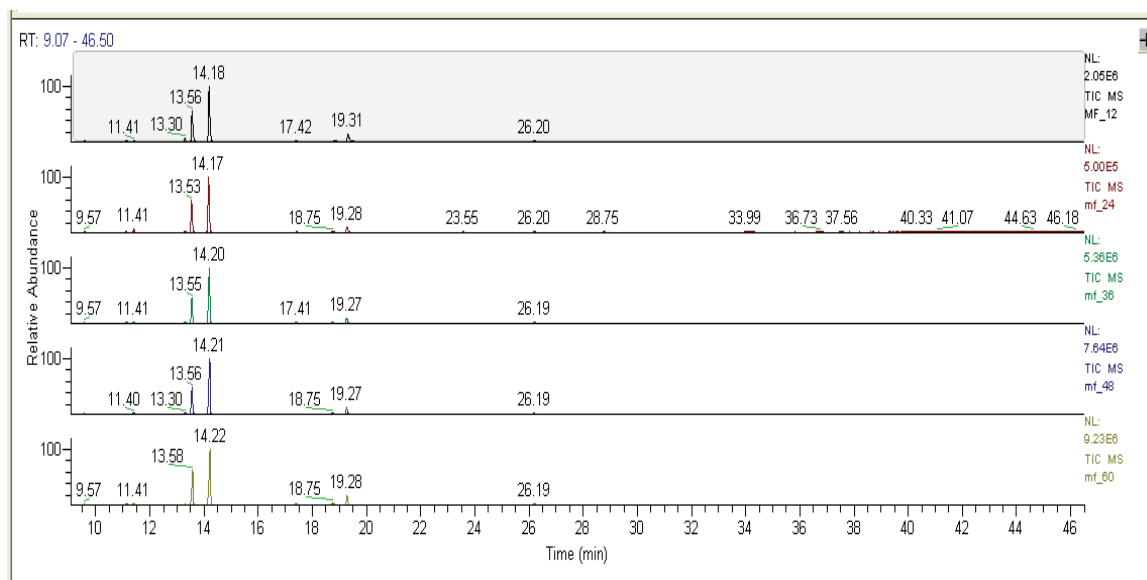
**Figure 6.1.9.** Attenuation and cell surface hydrophobicity as determined by the method of Akiyama-Jibiki et al., with absorbance measured at 660 nm, during a miniature fermentation assay using the SMA yeast strain.



**Figure 6.1.10.** Distinct flocculation/aggregate (blue arrow) of the SMA yeast strain as observed during ASBC Yeast-11-B method assay, taken from a culture grown in the miniature fermentation assay for 36 hours.

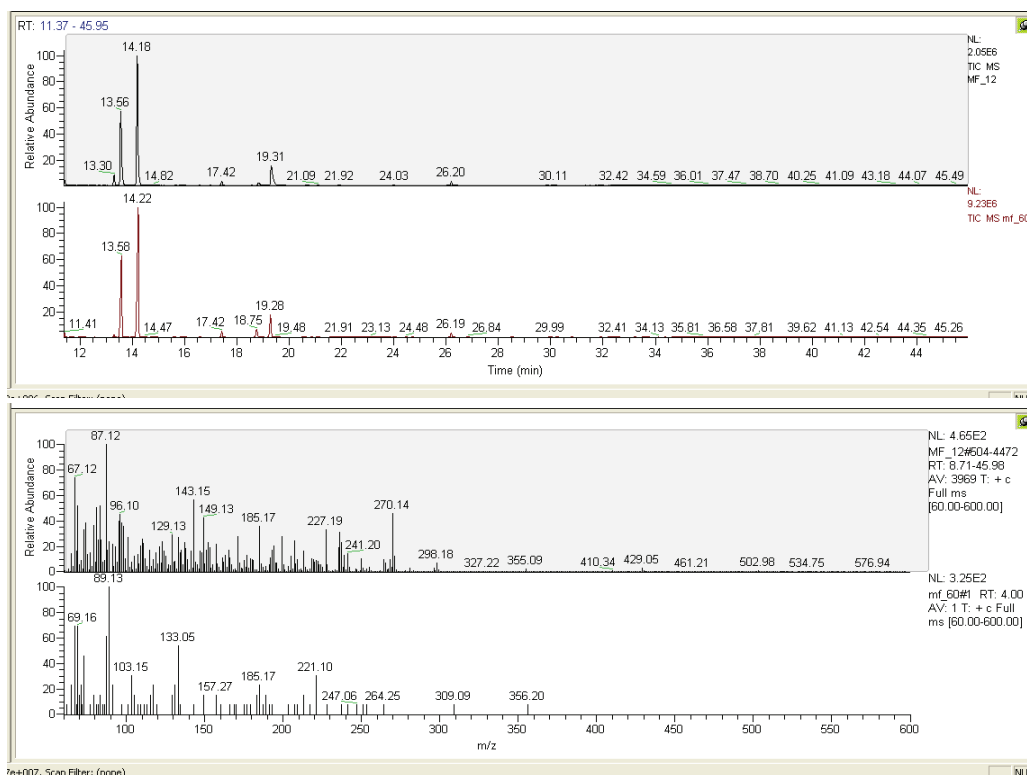
## Oxylipin Analysis of Whole Yeast Cells

The fatty acid analysis during growth in the miniature fermentation assay was conducted using a GC-MS apparatus, with yeast samples collected at 12, 24, 36, 48 and 60 hours in the fermentation. The chromatographs for each sampling time appear similar (Figure 6.1.11) indicating a fairly constant cell fatty acid composition over the course of the fermentation.



**Figure 6.1.11.** Chromatographic analysis from the GC-MS of whole cell extracts of the SMA yeast grown in the miniature fermentation assay from growth at 12, 24, 36, 48 and 60 hours. Yeast culture, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions as indicated in the methods section.

When the samples from 12 hours into the fermentation are compared to those from 60 hours into the fermentation (Figure 6.1.12), the chromatograms are close in appearance, except there is one small peak in the 60 hour sample at retention time 18.75 minutes that doesn't appear in the 12 hour sample. While this does imply a small increase in either or all of monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs), phospholipids and glycolipids content in the cells (Jones et al., 2012) over the course of the fermentation, the relative size of this peak is too small for it alone to have driven such an increase in CSH.

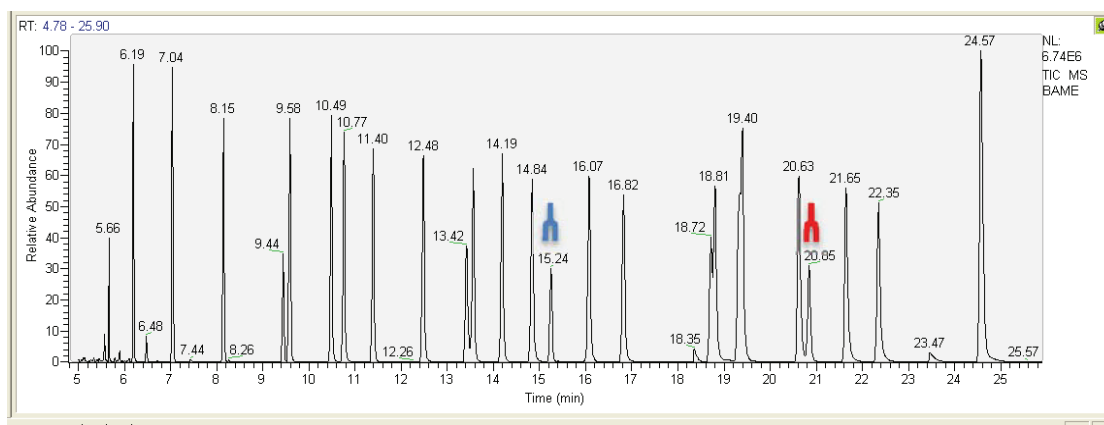


**Figure 6.1.12.** A GC-MS chromatographic (above) and mass spectral (below) analysis of whole cell extracts of the SMA yeast grown in the miniature fermentation assay at 12 and 60 hours. Yeast culture, fatty acid extraction, fatty acid methyl ester production and instrumental conditions as indicated in the methods section.

### 6.1.3 Part 3.) Use of Mixed BAME Standard

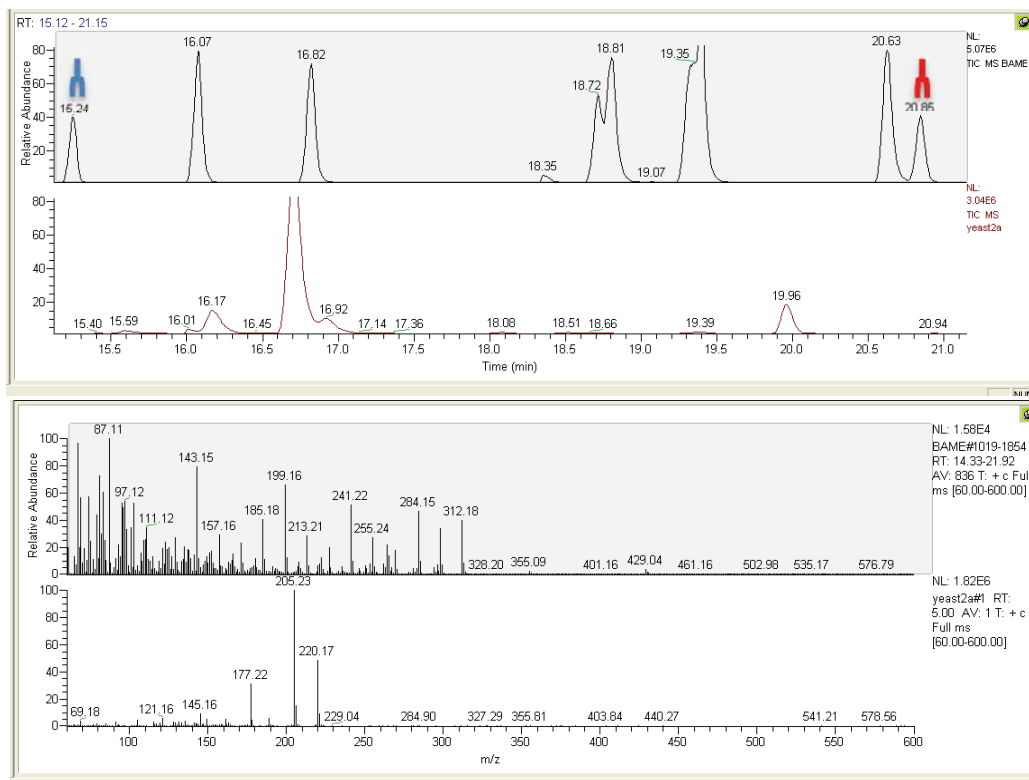
#### *Oxylipin Analysis of Whole Yeast Cells from Growth in YEPD Broth*

In part 3, a mixed BAME standard was used in an attempt to improve detectability of 3-OH oxylipins. The BAME mix contained, amongst other methyl esters, 3-OH 12:0 and 3-OH 14:0. Initially, the BAME standard was run on its own to identify retention times of the oxylipins of interest. On the GC-MS, the retention times of the two oxylipins are as indicated in Figure 6.1.13.



**Figure 6.1.13.** A GC-MS chromatographic analysis of the BAME standard, with peaks corresponding to 3-OH 12:0 (blue arrow) and 3-OH 14:0 (red arrow). Yeast culture, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions as indicated in the methods section.

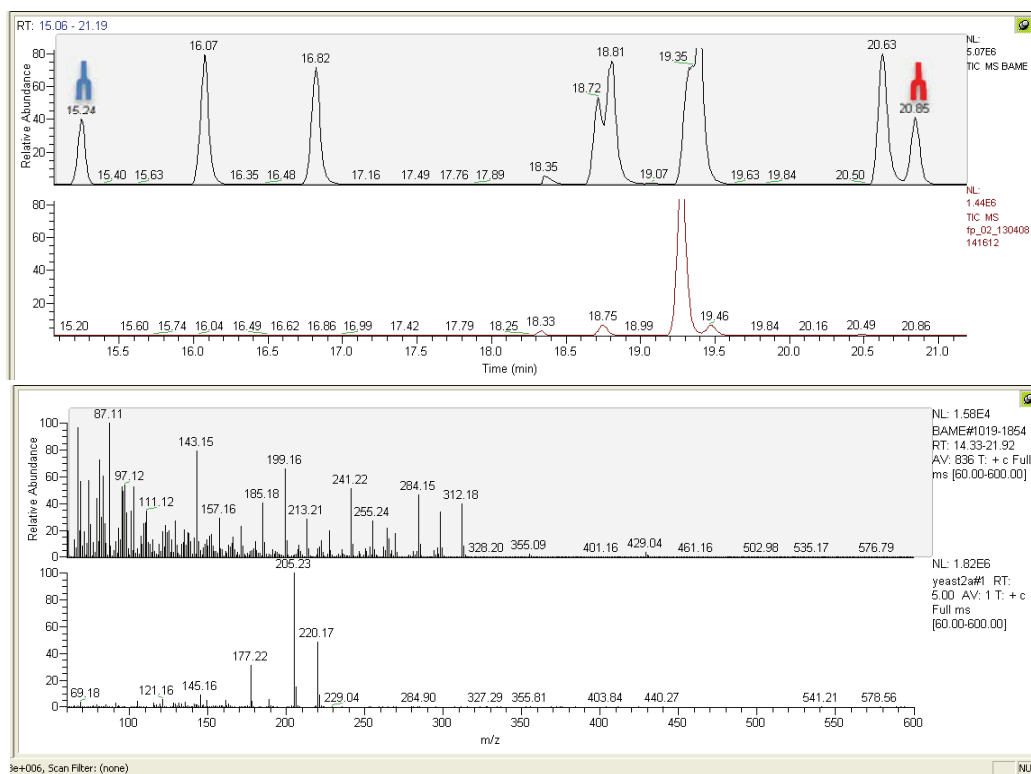
Next a window of retention times which included both the 3-OH 12:0 and 3-OH 14:0 was compared to the same window for whole yeast cell extractions from growth in YEPD broth (Figure 6.1.14). There were no matching retention times or matching fragmentations in the whole yeast cell extract samples, or substantial peaks that eluted earlier than these 3-OH oxylipins times suggesting shorter chain 3-OH oxylipins (i.e., 3-OH 8:0 and 3-OH 10:0).



**Figure 6.1.14.** A GC-MS chromatographic (above) and mass spectral (below) analysis of the BAME standards and whole cell extracts of the SMA yeast grown in YEPD broth. The chromatographic peaks corresponding to 3-OH 12:0 (blue arrow) and 3-OH 14:0 (red arrow) are as shown. Yeast culture, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions as indicated in the methods section.

## Oxylipin Analysis of Yeast Cell Wall Isolates from Growth in YEPD Broth

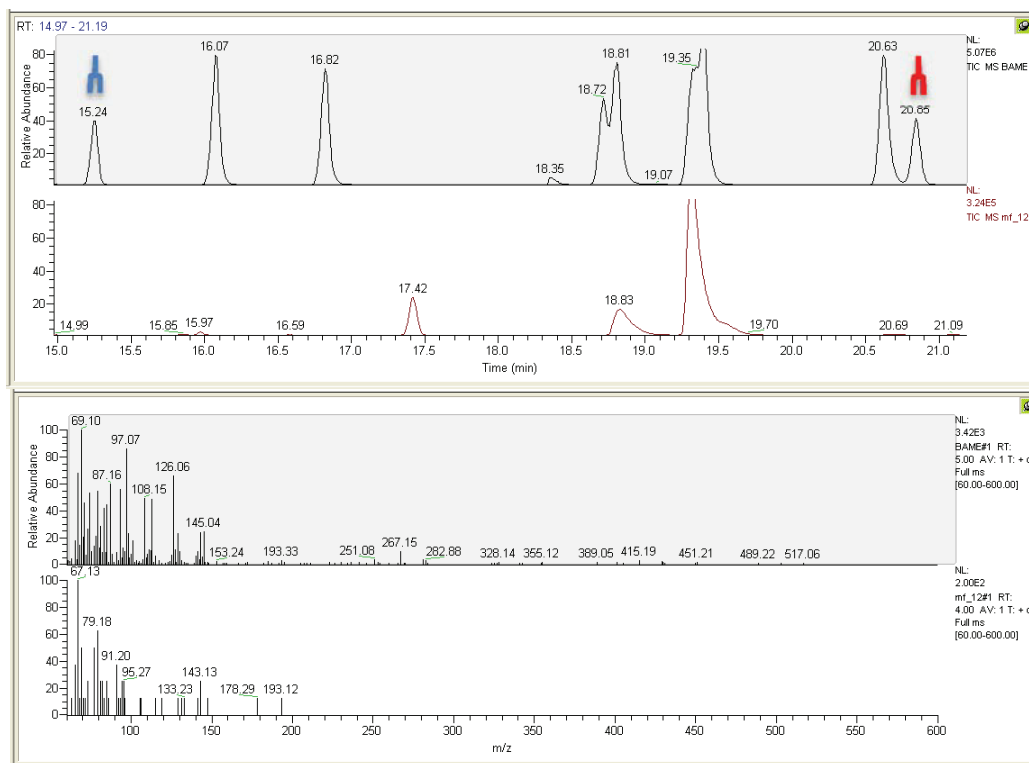
Again, a window of retention times which included both the 3-OH 12:0 and 3-OH 14:0 was compared to the same window for yeast cell wall isolates from growth in YEPD broth (Figure 6.1.15). There were no matching retention times or matching fragmentations in the whole yeast cell extract samples, or substantial peaks that eluted earlier than these 3-OH oxylipins times suggesting shorter chain 3-OH oxylipins (i.e., 3-OH 8:0 and 3-OH 10:0).



**Figure 6.1.15.** A GC-MS chromatographic (above) and mass spectral (below) analysis of the BAME standards and cell wall isolates of the SMA yeast grown in YEPD broth. The chromatographic peaks corresponding to 3-OH 12:0 (blue arrow) and 3-OH 14:0 (red arrow) are as shown. Yeast culture, French pressing, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions as indicated in the methods section.

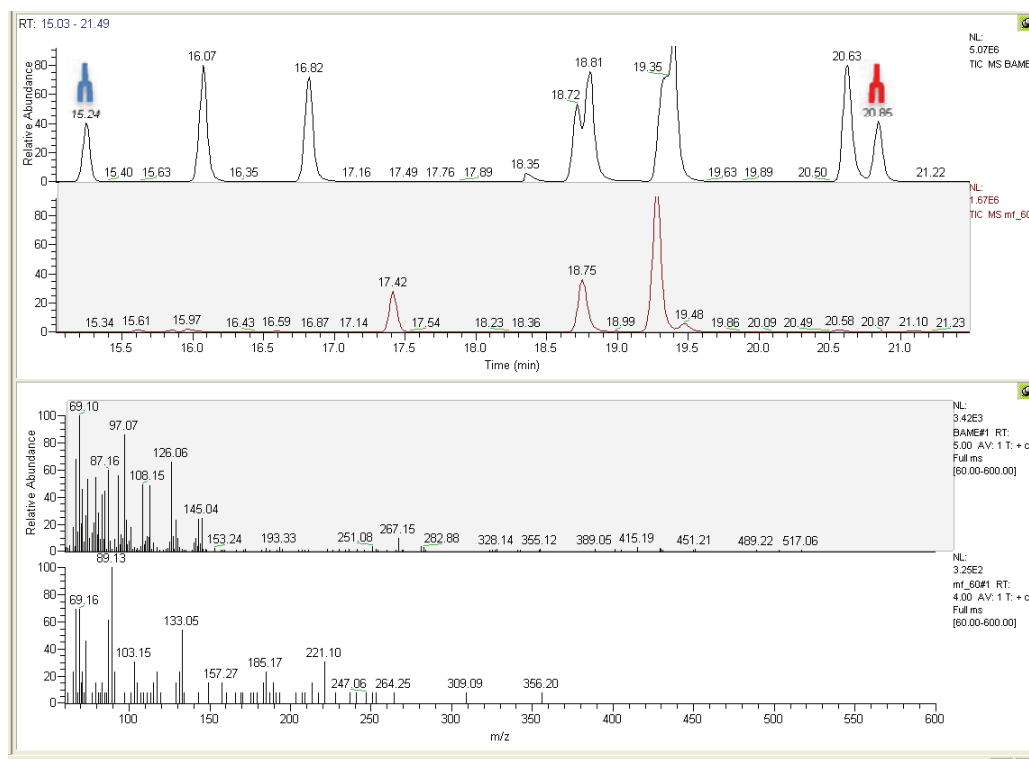
## Oxylipin Analysis of Whole Yeast Cells from Growth in the Miniature Fermentation Assay

The window of retention times that included 3-OH 12:0 and 3-OH 14:0 were also compared to chromatograms for yeast cell isolates 12 hours and 60 hours into the fermentation (Figure 6.1.16 and Figure 6.1.17). Again neither sample had substantial peaks corresponding to 3-OH oxylipins.



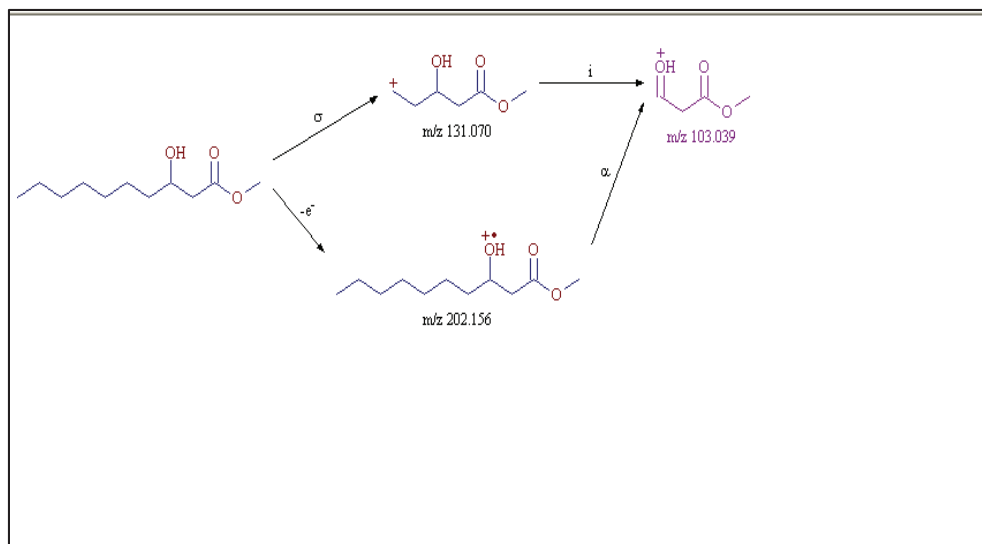
**Figure 6.1.16.** A GC-MS chromatographic (above) and mass spectral (below) analysis of the BAME standard and whole cell extracts of the SMA yeast grown in the miniature fermentation assay at 12 hours. The chromatographic peaks corresponding to 3-OH 12:0 (blue arrow) and 3-OH 14:0 (red arrow) are as shown. Yeast culture, French pressing, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions as indicated in the methods section.





**Figure 6.1.17.** A GC-MS chromatographic (above) and mass spectral (below) analysis of the BAME standard and whole cell extracts of the SMA yeast grown in the miniature fermentation assay at 60 hours. The chromatographic peaks corresponding to 3-OH 12:0 (blue arrow) and 3-OH 14:0 (red arrow) are as shown. Yeast culture, French pressing, fatty acid extraction, production of fatty acid methyl esters and instrumental conditions as indicated in the methods section.

In a further and final effort to be confident of the absence of oxylipins, the GC-MS was set to selective ion monitoring mode (SIM). With GC-MS-SIM mode, the detector is set to only detect particular ions which are indicative of the compound of interest. In reports first by Vesonder et al. (1968) and later by Van Dyk et al. (1991), a fragment with a  $m/z$  of 103 was shown to be characteristic of 3-OH fatty acids, as shown in Figure 6.1.18. The GC-MS was set in SIM mode to detect only fragments with 103  $m/z$ . However, there were no 103  $m/z$  fragments that corresponded to compounds that eluted at all close to the oxylipins in the BAME standard in any of the samples previously described. The machine was in fact working properly, as the BAME standard produced a positive signal when run on the apparatus in SIM mode.



**Figure 6.1.18.** Fragmentation of the oxylipin 3-OH 10:0 to give the characteristic ion with a  $m/z$  of 103, as reported by Vesonder et al. (1968) and later van Dyk et al. (1991).

## 6.2 Phase 2

**\*Note:** A thorough explanation of all the Materials and Methods used in this study were provided so the reader is aware of its breadth and was not left questioning its validity. This thesis will not be reporting or discussing those aspects of the collaborative project mentioned in Chris Stanton, Jr.'s thesis, such as the Standard Curve Measurement and the Langmuir Analysis of the Avidin, Texas Red® conjugate. Instead, what follows will focus on the statistical work the author conducted to correlate the data with cell size, cell granularity and zymolectin density over the course of a fermentation. If the reader is interested in the details of the Standard Curve Measurement or the Langmuir Analysis they are directed to an electronic copy of Chris Stanton, Jr.'s thesis (Available upon request from School of Life Sciences at Heriot-Watt University).

### 6.2.1 Overall Data Analysis

A considerable initial focus of this collaborative study was to design a protocol to more accurately determine zymolectin density on individual cells. However, because of the versatility of flow cytometry and its capacity for multiparametric analysis, much more information than just zymolectin density was produced. During each sampling time, large volumes of data were gathered which also gave information on cell size, as FSC light and cell granularity, as SSC light. In examining the data for each parameter, it was of interest to assess whether or not the approach used was able to detect multiple populations within the data. For example, distinct populations of larger mother and smaller daughter cells in the FSC data, older cells with more bud scars and younger cells with less bud scars in the SSC data and any populations with higher or lower zymolectin densities in the emission at 630 nm data. Few apparent trends could be gleaned when the average of FSC, SSC and 630 readings at each of 24, 48 and 72 hours (see Appendix V) culture time were determined, so information about the size, granularity, zymolectin density and relative sizes of potential sub-populations in the data was examined.

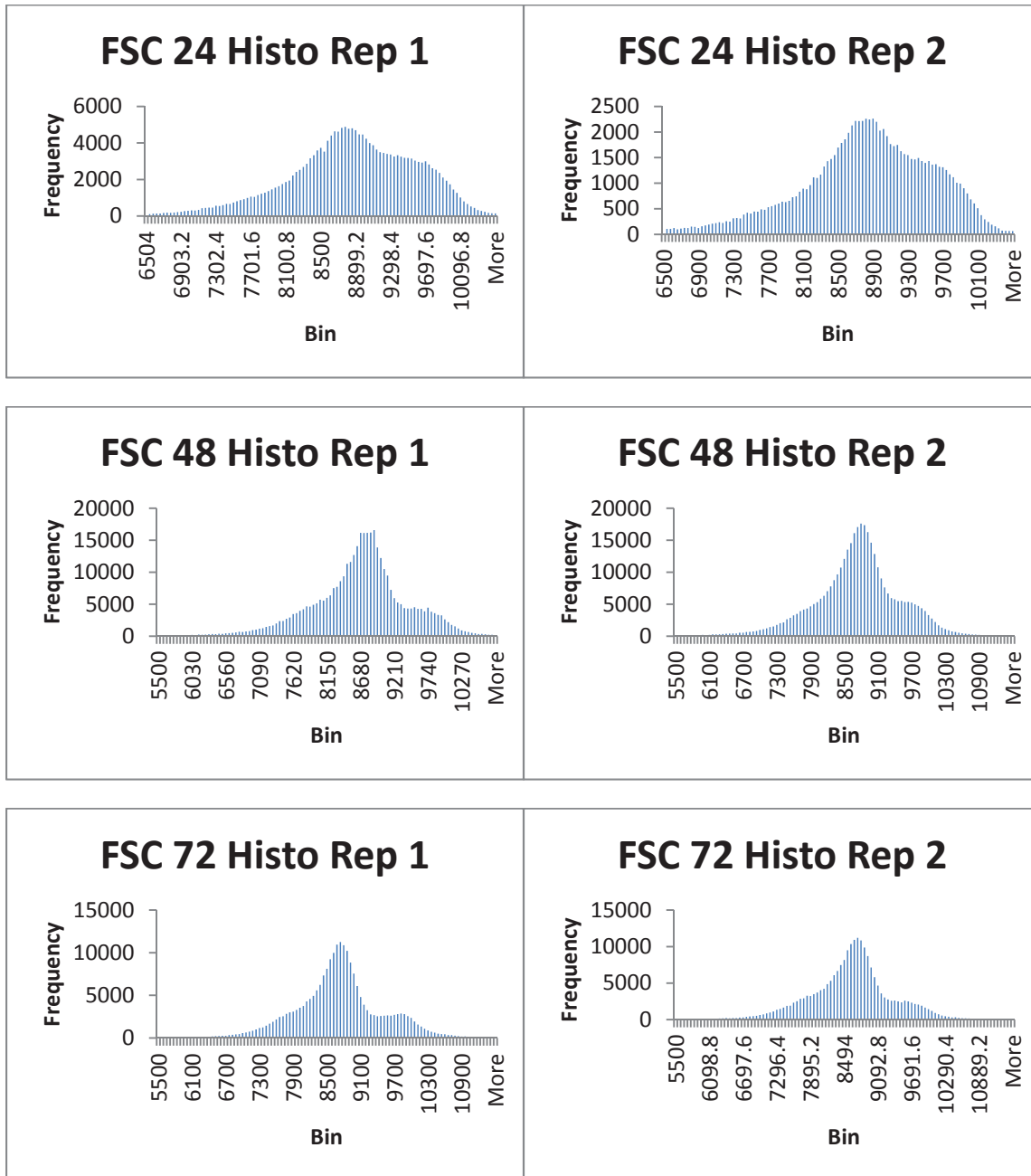
To check for multiple populations within the data, histograms of each replicate for each measure at each sampling time had to be prepared. As previously mentioned, in processing the 9 samples with different concentrations of the Avidin, Texas Red® conjugate probe, the sample

with a probe concentration of 1.5 mg/mL was always processed first. The remaining 8 samples were processed in the flow cytometer after, but this may have been up to several hours after removal from the main culture and the cells were not fixed at extraction. Therefore, the aliquot with the 1.5 mg/mL probe concentration was the most representative of the culture at the sampling time, and it was always used to produce the histograms of each measured parameter.

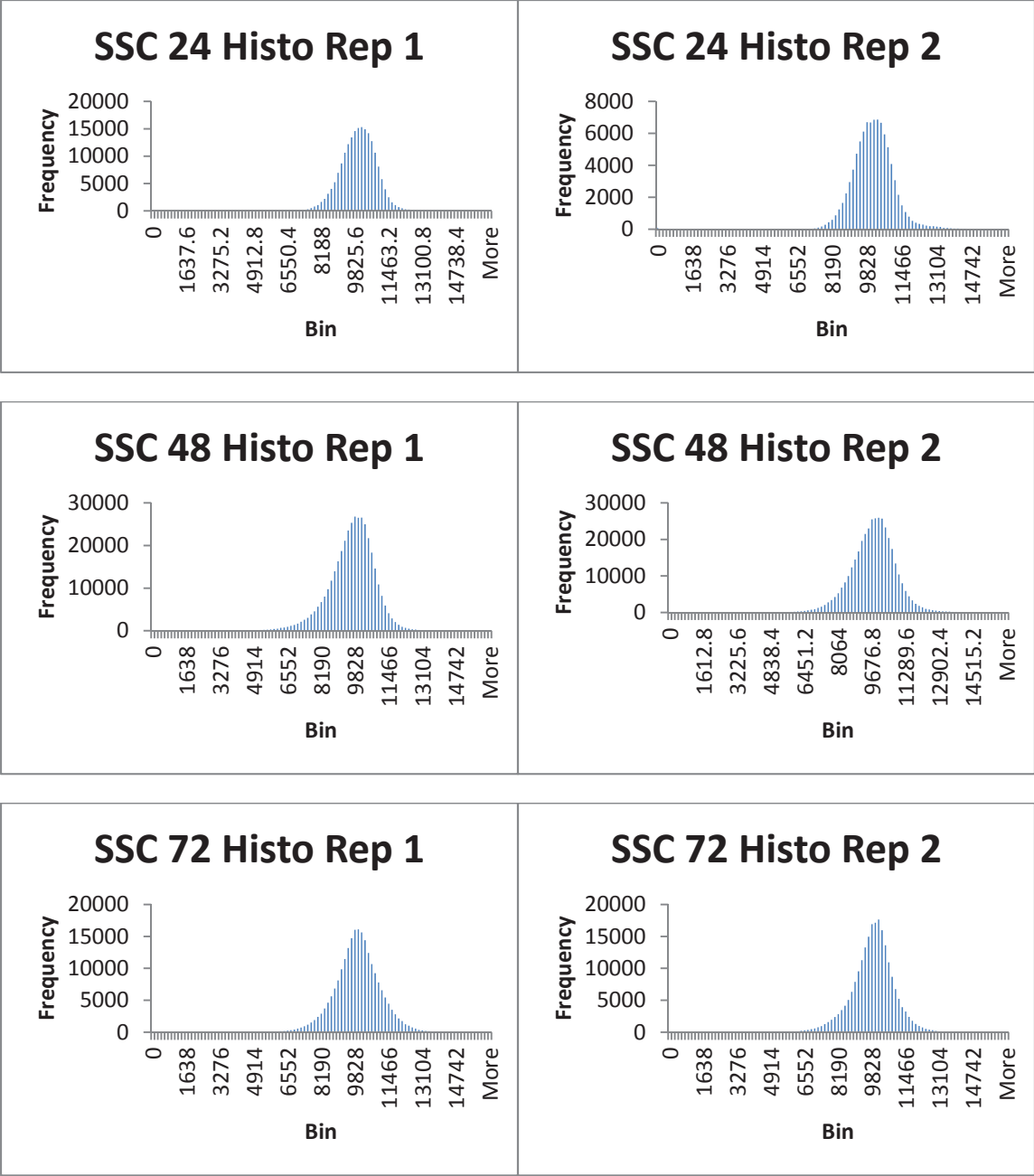
When constructing the histograms, an appropriate number of bins had to be chosen. In the literature it was clear that there was some subjectivity and lack of consensus in choosing the appropriate width, height and number of bins. For example, each of Scott (1979), Wand (1997) and Birgé and Rozenholc (2006) suggested different approaches to optimizing bin number. However, it was generally agreed that a bin number be chosen that did not produce “comb-like” projections in the histogram (Sawin, 2013), but also did not “oversmooth” or “undersmooth” the data (Wand, 1997). After several attempts, it was found 100 bins produced histograms without “comb-like” projections yet still conveyed the data trends, and so all histograms that follow were constructed with 100 bins.

Upon initial visual inspection of the histograms produced, it seemed that there were multiple populations of cells captured in the FSC measures at each time interval (Figure 6.2.1). All FSC measurements looked non-normally distributed, and there appeared to be a second smaller mode to the right of each histogram apex (Figure 6.2.1). The SSC measurements looked more unimodal initially (Figure 6.2.2), especially relative to the FSC measurements and it seemed less evident that there were multiple populations in this data. That being said the earlier SSC histograms at 24 hours had a distinctly different shape than those SSC histograms at 72 hours (Figure 6.2.2). Similarly, the histograms from the emission at 630 nm looked more unimodal than the FSC histograms, but they also did not all resemble perfectly symmetrical

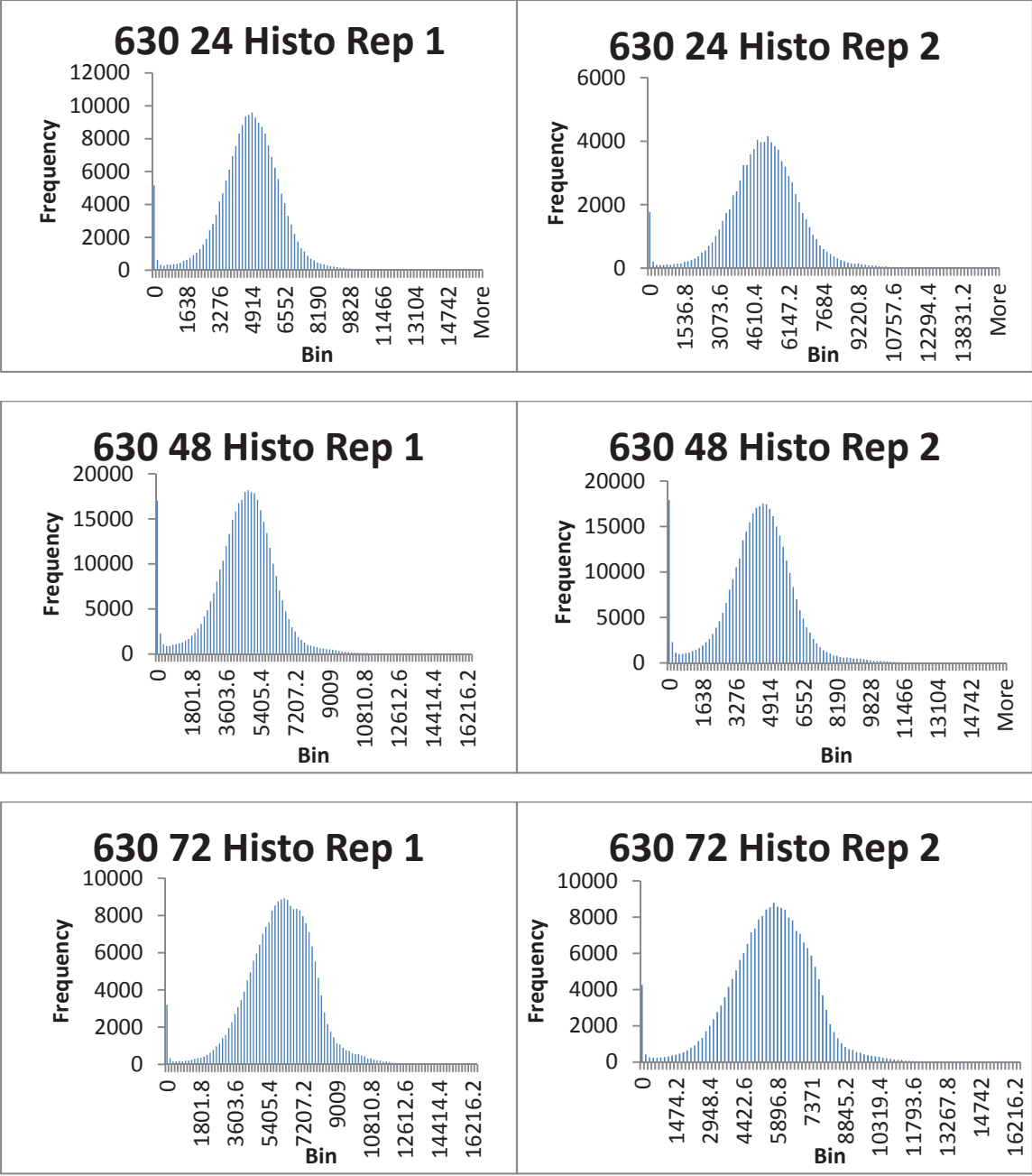
Gaussian distributions (Figure 6.2.3). In particular, the histogram for Rep 1 for emission at 630 nm 72 hours into the fermentation looked to have a second mode just to the right of the apex (Figure 6.2.3).



**Figure 6.2.1** Histograms of the forward scatter (FSC) of a population of the SMA yeast strain extracted from a fermentation at 24, 48 and 72 hours and analyzed by a flow cytometer.



**Figure 6.2.2.** Histograms of the side scatter (SSC) of a population of the SMA yeast strain extracted from a fermentation at 24, 48 and 72 hours and analyzed by a flow cytometer.



**Figure 6.2.3.** Histograms of the emission at 630 nm (630) of a population of the SMA yeast strain extracted from a fermentation at 24, 48 and 72 hours and analyzed by a flow cytometer.

While the FSC data histograms looked distinctly multimodal, it was less obvious whether the SSC and 630 nm data had second modes or included multiple populations. Unequivocally, the most well-known of the unimodal distributions is the normal distribution. As a means to further investigate whether there were multiple populations within the data for each parameter, every data set was tested for normality. The rationale for this being that any data set that could have had multiple populations or multiple modes would have been non-normal. Every data set was tested for normality by the Jarque-Bera, Anderson-Darling and Kolmogorov-Smirnov tests. The Jarque-Bera test was performed with the NumXL Microsoft Excel add-in (Spider Financial Corp, Chicago, IL) and an  $\alpha = 0.05$  was used. The Anderson-Darling and Kolmogorov-Smirnov tests were performed with the Systat 13 software package (Systat Software, Inc., Chicago, IL).

In an in depth study by Yazici and Yolacan (2007), these three methods and eight others were compared. The Jarque-Bera test was deemed the most powerful with a large sample size and when the distribution of the population was not known (Yazici & Yolacan, 2007). Nonetheless, all three tests were employed to be thorough. Every data set was found to be non-normal by all three tests (Table 6.2.1) at the 1 % significance level. It is notable that in studies of brewing yeast cell volume during fermentations in tall tube fermenters, the distribution of cell volume was also found to be predominantly non-normal by the Kolomogorov-Smirnov test (Speers et al., 2006).

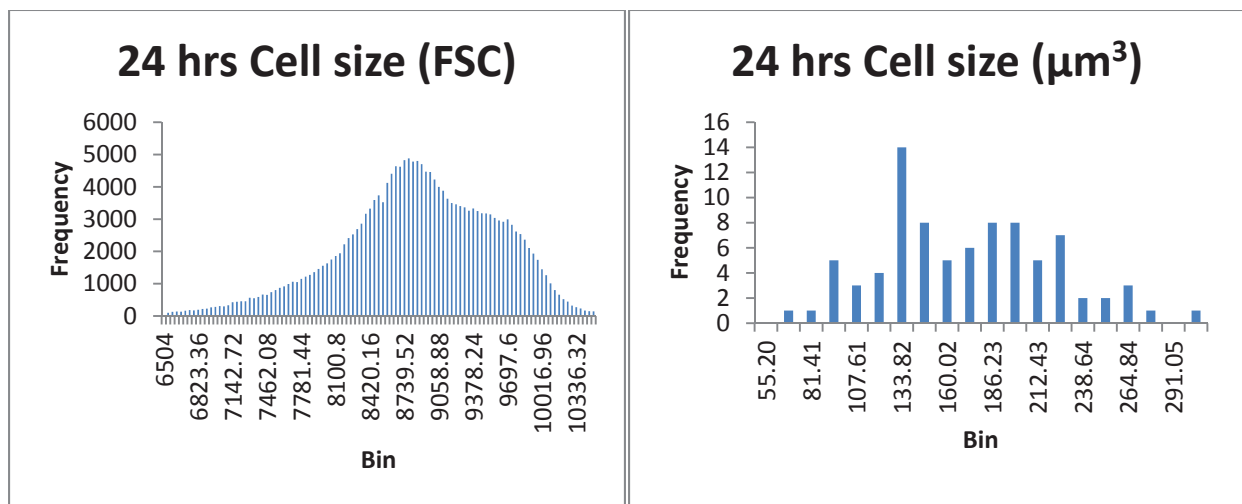


**Table 6.2.1.** Normality tests of the data collected for Forward Scatter (FSC), Side Scatter (SSC) and emission at 630 nm (630) of the SMA yeast strain as measured by a Partec Cyflow flow cytometer.

Measurement	Jarque-Bera	Anderson-Darling		Kolmogorov-Smirnov	
	P-value	A-D Stat.	P-value	Max. Diff.	P-value (2-tail)
FSC 24 Rep1	0.000	239 808.646	<0.01	1.000	0.000
FSC 24 Rep 2	0.000	28 821.256	<0.01	1.000	0.000
FSC 48 Rep 1	0.000	632 878.570	<0.01	1.000	0.000
FSC 48 Rep 2	0.000	604 948.524	<0.01	1.000	0.000
FSC 72 Rep 1	0.000	240 113.212	<0.01	1.000	0.000
FSC 72 Rep 2	0.000	196 341.061	<0.01	1.000	0.000
SSC 24 Rep 1	0.000	236 712.876	<0.01	1.000	0.000
SSC 24 Rep 2	0.000	20 377.305	<0.01	0.999	0.000
SSC 48 Rep 1	0.000	353 459.705	<0.01	1.000	0.000
SSC 48 Rep 2	0.000	461 353.527	<0.01	1.000	0.000
SSC 72 Rep 1	0.000	127 423.922	<0.01	0.999	0.000
SSC 72 Rep 2	0.000	89 218.039	<0.01	0.999	0.000
630 24 Rep 1	0.000	101 962.311	<0.01	0.973	0.000
630 24 Rep 2	0.000	20 524.925	<0.01	0.981	0.000
630 48 Rep 1	0.000	992 040.200	<0.01	0.956	0.000
630 48 Rep 2	0.000	1 144 013.322	<0.01	0.953	0.000
630 72 Rep 1	0.000	176 321.376	<0.01	0.986	0.000
630 72 Rep 2	0.000	172 980.411	<0.01	0.981	0.000

### 6.2.2 Forward Scatter Measurements

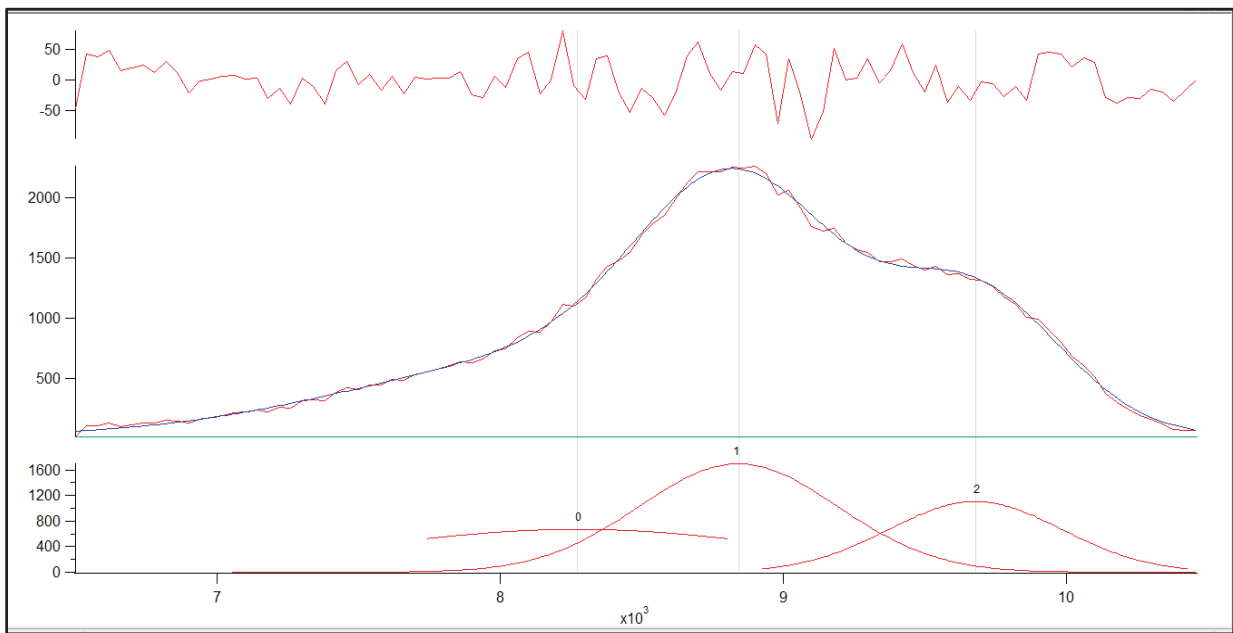
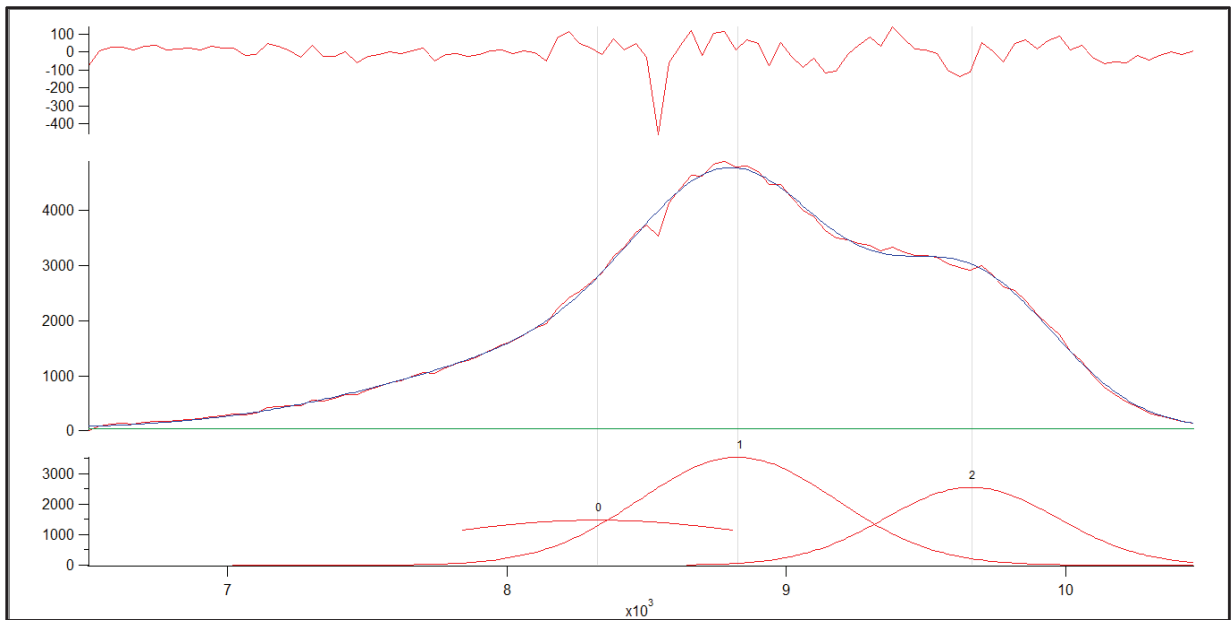
Because this study was somewhat limited by time, resources and student availability, manual measurements of cell size (using a micrometer) were only determined at 24 and 48 hours into the previously described fermentation. Furthermore, a large number of measures ( $\geq 100$  cells) were only collected at 24 hours into the fermentation. Nonetheless, when the histograms of manual cell size (in  $\mu\text{m}^3$ ) measures are compared to the histograms of cell size determined by the flow cytometer (as FSC) (Figure 6.2.4), both appear to have a roughly similar shape and therefore similar distribution of cell sizes. From the two histograms it would appear that a forward scatter of 8740 corresponded to a cell volume of  $134 \mu\text{m}^3$ . The fact the two histograms looked similar was encouraging as numerous studies have shown there is a linear relationship between cell size and forward scatter (Robertson and Button, 1989; Robertson et al., 1998). It is likely with many more manual measures of cell size both histograms would have resembled each other even further.



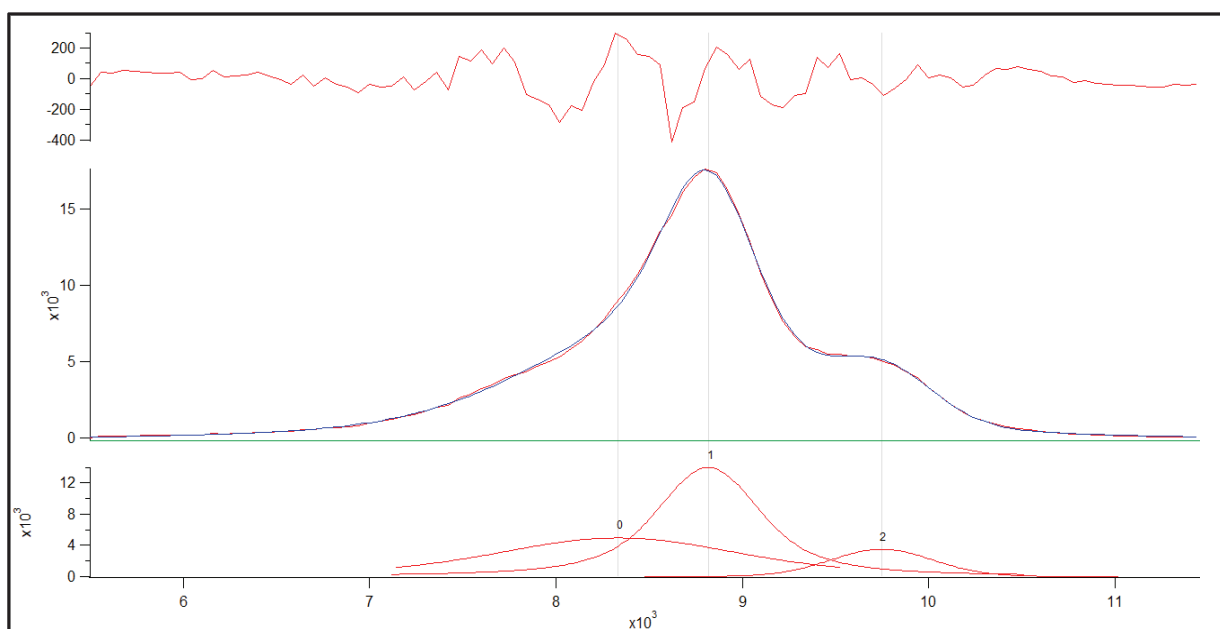
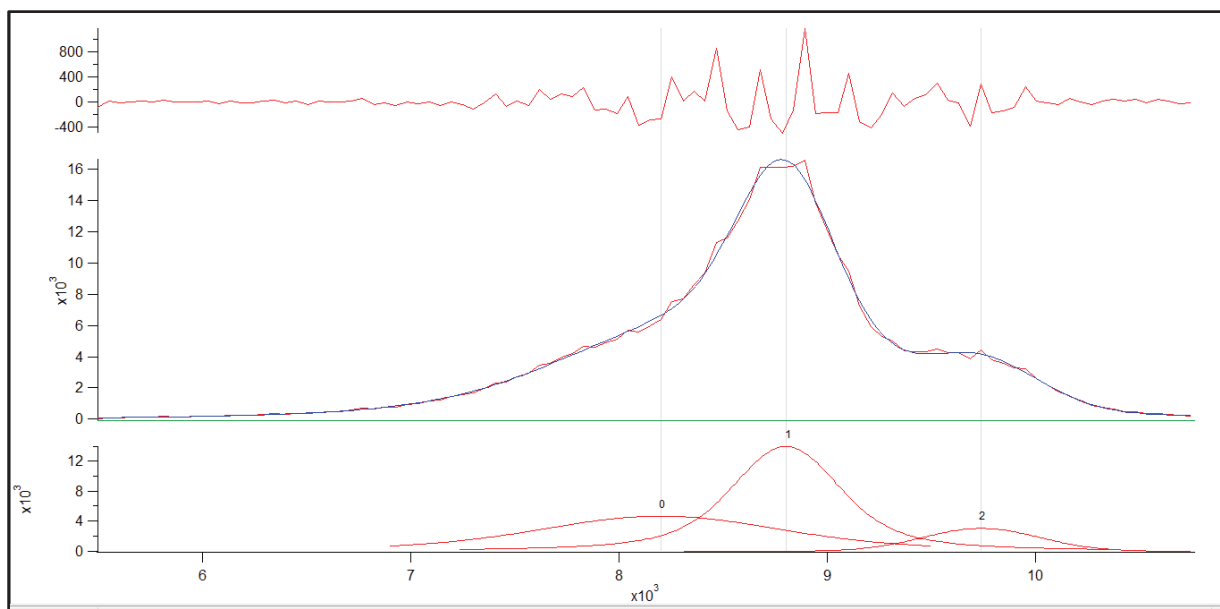
**Figure 6.2.4.** Histograms of cell size determined by a flow cytometer (as forward scatter) and cell size measured with a Zeiss Axioplott microscope equipped with an AxioCam ERc 5s camera and micrometer. Both sets of measurements were taken after cultures of the SMA yeast strain had fermented YEPD broth for 24 hours.

It was apparent that the distribution and spread of forward scatter/cell sizes changed over the course of the fermentation (Figure 6.2.1). Determining whether or not sub-populations of cells, such as larger mother and smaller daughter cells, could be detected within the total cell population was of interest. In an effort to detect potential sub-populations, and because the forward scatter data at each sampling time did not appear to be normally distributed, all forward scatter data sets were analyzed with Igor Pro 6.2. Among its many functionalities, Igor Pro 6.2 is capable of fitting multiple curves to graphs and data sets best modelled this way. When the curves that bound each of the forward scatter histograms from 24, 48 and 72 hours into the fermentation were analyzed with Igor Pro 6.2, all were best fit with 3 smaller Gaussian curves (Figures 6.2.5, 6.2.6 and 6.2.7).

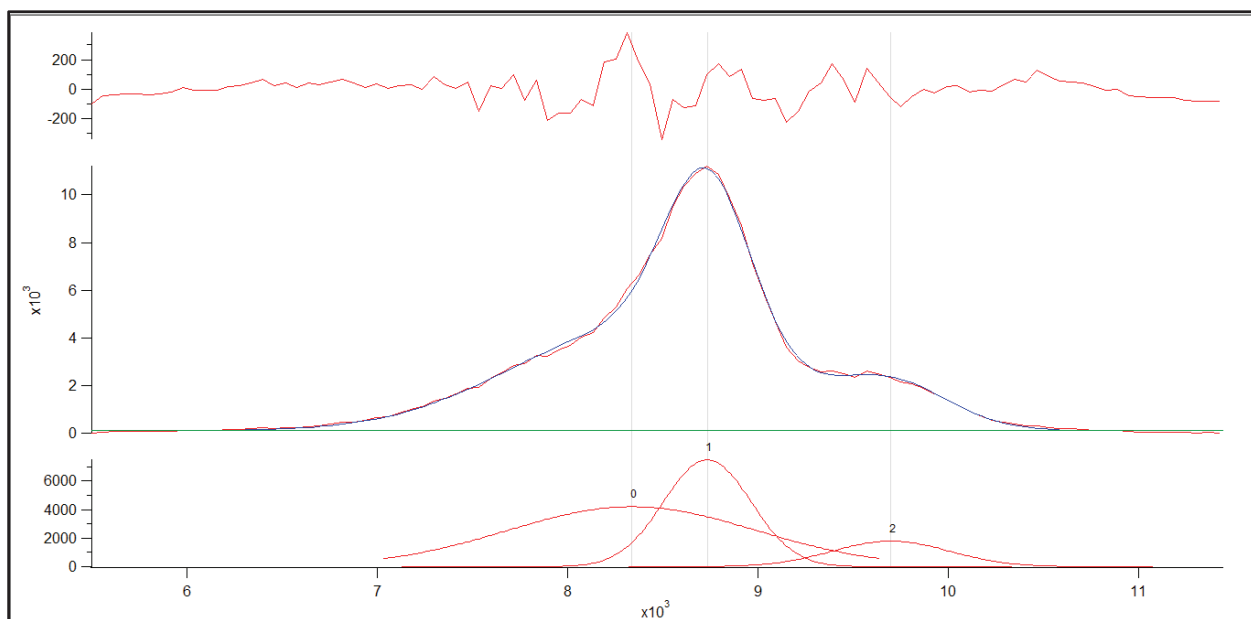
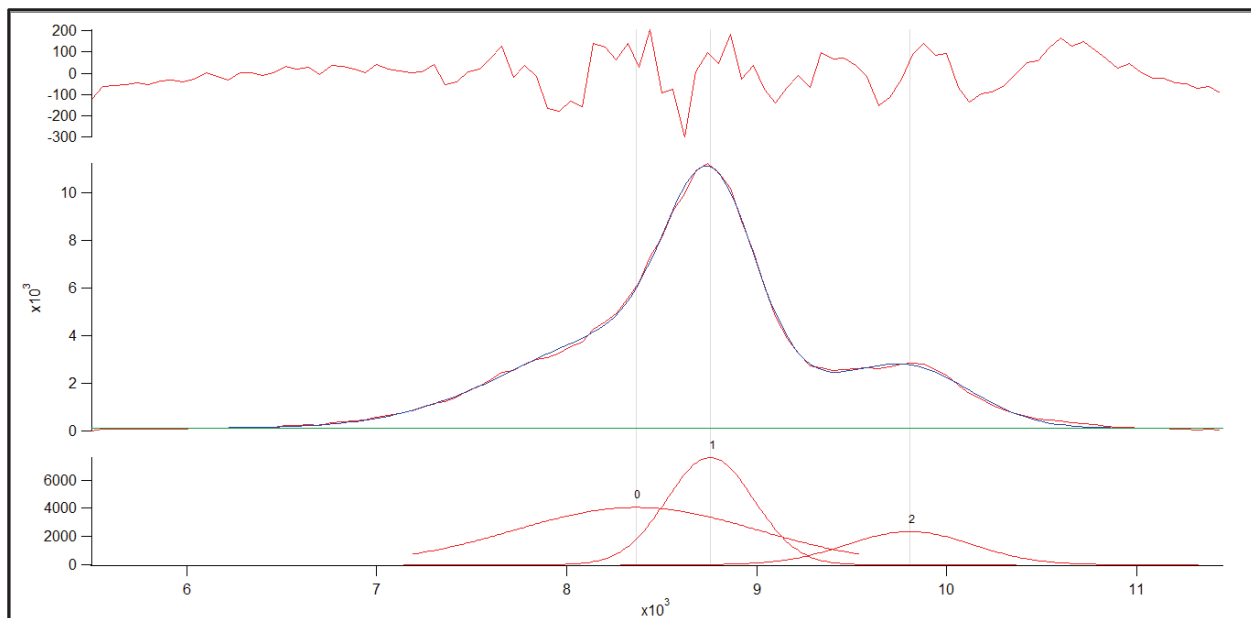
During determination of cell size with the microscope and micrometer, three types of cell morphotypes were regularly observed; smaller daughter cells, larger mother cells and mother cells with a bud still attached. In each multiple fitting, the fitted curves denoted as 0, 1 and 2 could have conceivably represented these 3 types of cell morphotypes (before cells were processed in the flow cytometer, they were vortexed vigorously to break up any floccs). Qualitatively, the degree to which each of the fitted curves overlap each other appears to increase as the fermentation proceeded (Figures 6.2.5, 6.2.6 and 6.2.7). This would suggest that as the fermentation progressed, the variability of cell sizes decreased and they became more uniform. In a similar study by Portell et al. (2010) batch cultures of *Saccharomyces cerevisiae* tended towards cell size uniformity after 29 hours culture time once the cells had entered stationary phase. The area bounded by curve 0 also looked to increase over the course of the fermentation suggesting that a higher proportion of the total cell population were smaller cells later in the fermentation.



**Figure 6.2.5.** Multiple curves fitted to the histograms for the first replicate (top) and the second replicate (bottom) for the forward scatter data 24 hours into the fermentation. The fits were done with the multiple-peak fit function of the Igor Pro 6.2 software package. In each picture the top line in red is the difference between the predicted curve and the actual curve, the middle picture is the predicted curve overlaid on the data and the bottom section shows the three fitted curves.



**Figure 6.2.6.** Multiple curves fitted to the histograms for the first replicate (top) and the second replicate (bottom) for the forward scatter data 48 hours into the fermentation. The fits were done with the multiple-peak fit function of the Igor Pro 6.2 software package. In each picture the top line in red is the difference between the predicted curve and the actual curve, the middle picture is the predicted curve overlaid on the data and the bottom section shows the three fitted curves.



**Figure 6.2.7.** Multiple curves fitted to the histograms for the first replicate (top) and the second replicate (bottom) for the forward scatter data 72 hours into the fermentation. The fits were done with the multiple-peak fit function of the Igor Pro 6.2 software package. In each picture the top line in red is the difference between the predicted curve and the actual curve, the middle picture is the predicted curve overlaid on the data and the bottom section shows the three fitted curves.

To assess the size of each assumed sub-population in the forward scatter data, the proportion of the total area each of the smaller curves occupied at each sampling time (Table 6.2.2 (a)-(f)) were examined. Because the total area predicted by Igor Pro 6.2 included a large error range in some cases, a second software package, an “Area Under the Curve” Excel add-in produced by FTC Innovations (Franklin-Thomas Co., Inc., Orlando, FL) was also used. With this software an 8<sup>th</sup> order polynomial was fit to the curve that bound each histogram and the area beneath the curve was determined using an integral calculus-based algorithm. The area bounded by curve 0 increased over the course of the fermentation, again suggesting that a higher proportion of the total cell population were smaller cells later in the fermentation (Tables 6.2.2 (a)-(f)). Meanwhile, the area bound by curve 2, presumed to be either a population of mother cells with buds attached or just larger cells, also generally decreased as the fermentation proceeded (Tables 6.2.2 (a)-(f)).

**Tables 6.2.2 (a)-(f).** The area beneath the curve that bounds the histograms of the FSC data from 24, 48 and 72 hours (Rep 1 and Rep 2) into the fermentation. The area beneath each curve was determined using the Igor Pro 6.2 software and a second package (an “Area Under the Curve” Excel add-in produced by FTC Innovations) because the error from Igor was high. The area of the fitted curves for each data set are also shown, as well as the proportion of total area occupied by each fitted curve determined with the total area predicted by Igor Pro 6.2 and the area predicted by the FTC Innovations software.

(a)-FSC 24 hrs Rep 1

<b>Igor Area-7 608 600 ± 2 272 200    FTC Area-7 770 540.70</b>		
Fitted Curve Areas (Igor)	% Total Igor	% Total FTC
<i>Curve 0-2</i> 530 400 ± 1 850 900	33.26	32.56
<i>Curve 1-3</i> 116 300 ± 1 271 100	40.96	40.10
<i>Curve 2-1</i> 961 900 ± 347 940	25.78	25.25

(b)-FSC 24 hrs Rep 2

<b>Igor Area-3 611 600 ± 839 380    FTC Area-3 652 830.88</b>		
Fitted Curve Areas (Igor)	% Total Igor	% Total FTC
<i>Curve 0-1</i> 276 000 ± 719 730	35.33	34.93
<i>Curve 1-1</i> 490 000 ± 408 620	41.26	40.79
<i>Curve 2-845 640 ± 139 960</i>	23.41	23.15

(c)-FSC 48 hrs Rep 1

<b>Igor Area-19 230 000 ± 1 849 200    FTC Area-20 336 386.99</b>		
Fitted Curve Areas (Igor)	% Total Igor	% Total FTC
<i>Curve 0-10</i> 171 000 ± 1 528 800	52.89	50.01
<i>Curve 1-6</i> 723 600 ± 838 970	34.96	33.06
<i>Curve-2</i> 336 100 ± 615 120	12.15	11.49

(d)-FSC 48 hrs Rep 2

<b>Igor Area-21 467 000 ± 1 227 700    FTC Area-22 726 214.20</b>		
Fitted Curve Areas (Igor)	% Total Igor	% Total FTC
<i>Curve 0-12</i> 731 000 ± 1 025 800	59.30	56.02
<i>Curve 1-6</i> 587 600 ± 465 470	30.69	28.99
<i>Curve 2-2</i> 147 500 ± 488 280	10.00	9.45

(e)-FSC 72 hrs Rep 1

<b>Igor Area-12 846 000 ± 462 610    FTC Area-13 674 519.58</b>		
Fitted Curve Areas (Igor)	% Total Igor	% Total FTC
<i>Curve 0-6</i> 505 500 ± 375 210	50.64	47.57
<i>Curve 1-4</i> 385 100 ± 222 120	34.13	32.07
<i>Curve 2-1</i> 955 100 ± 154 550	15.22	14.30

(f)-FSC 72 hrs Rep 2

<b>Igor Area-12 533 000 ± 668 130    FTC Area-13 215 788.38</b>		
Fitted Curve Areas (Igor)	% Total Igor	% Total FTC
<i>Curve 0-6</i> 844 500 ± 548 050	54.61	51.79
<i>Curve 1-4</i> 309 400 ± 304 920	34.38	32.61
<i>Curve 2-1</i> 379 200 ± 230 350	11.00	10.44



### 6.2.3 Side Scatter Measurements

While the forward scatter data looked distinctly bimodal, the side scatter data at each sampling time looked more unimodal. When each side scatter data set was analyzed by the Multi-peak package, each set was predicted to be modelled by just 1 Gaussian distribution. This would suggest there were not any sub-populations in the side scatter data. With Igor Pro 6.2, additional curves can be manually added to the model to improve the fit, and thus reduce the size of the residuals. It was possible to add a second curve to all the data sets except one, such that the second curve was not completely bound by the first (if one curve completely bound the other that would not suggest distinct sub-populations). The residual range was improved for all data sets except one when a second Gaussian curve was fit to each model (Table 6.2.3).

**Table 6.2.3.** The residual ranges between the curve fitted by Igor Pro 6.2 and actual distribution. The new residual range is also shown for those curves for which it was possible to manually add a second curve to the model. The residual range is shown for each appropriate data set after a second curve was added to the data.

Measurements	Residual Range	Second Peak Possible	New Residual Range
SSC 24 Rep 1	-800 to +800	Yes	-200 to +200
SSC 24 Rep 2	-200 to +200	No	-100 to +100
SSC 48 Rep 1	-1000 to +2000	Yes	-400 to +400
SSC 48 Rep 2	-1000 to +1000	Yes	-800 to +800
SSC 72 Rep 1	-500 to +1000	Yes	-1000 to +500
SSC 72 Rep 2	-1000 to +1500	Yes	-400 to +800

It is clear when considering the Igor Pro 6.2 fits and the residual ranges that the granularity of the SMA yeast population changed over the course of the 72 hour fermentation. Because most of the fits could be improved by adding an additional curve, it is possible that there were smaller sub-populations of cells with differing granularities but it is not possible to be absolutely sure. Certainly, the change in the distribution of the population granularity during the

fermentation was not nearly as distinct as observed by Portell et al. (2010) though. As to why the granularity of the SMA yeast strain did not change like the Portell et al. (2010) study and why distinct sub-populations of cells were not so readily observed, there are several potential explanations.

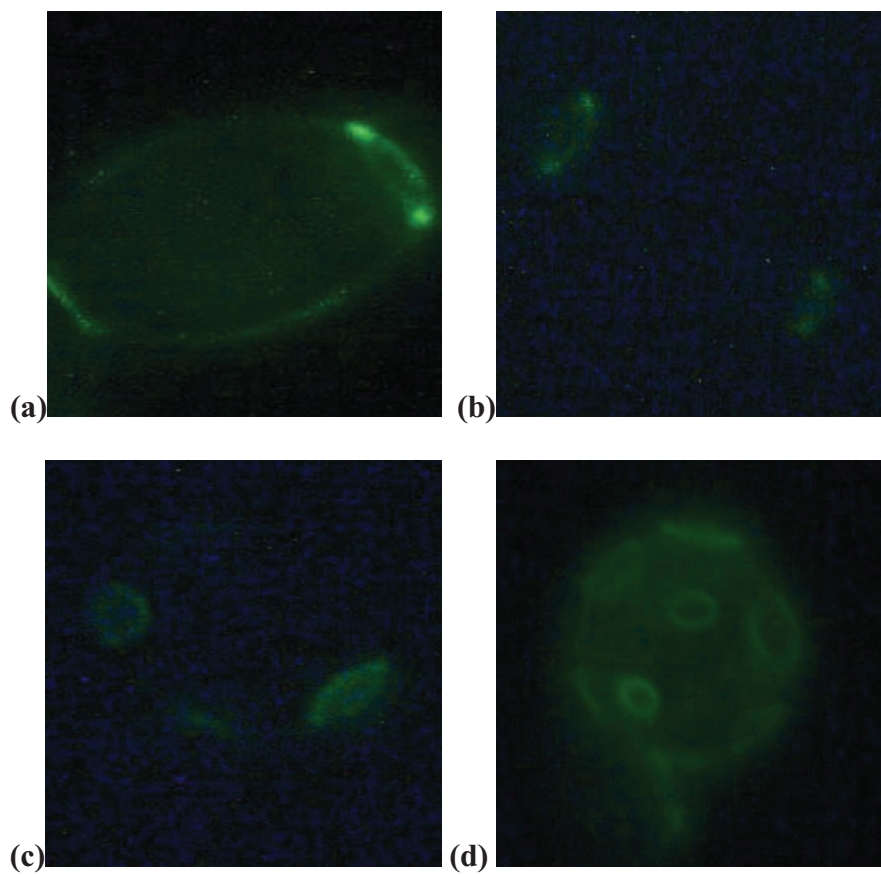
Firstly, the strain used in the study by Portel et al. (2010) was a wine yeast, while the strain used in this study was a brewing yeast. Wine yeasts generally experience higher alcohol environments than brewing yeasts, and they have adapted numerous defenses against ethanol challenge including altered membrane fluidity and structure (Pretorius, 2000). Therefore, it is conceivable that the wine yeast had a distinctly different cellular granularity than the SMA strain to begin with. A second possible explanation relates to the methods employed in this study. After each 24 hour period, the yeast was transferred to a new flask with fresh broth. It is quite possible this transfer would have allowed certain cells to re-start replication and leave stationary phase. In stationary phase cell walls are known to thicken (Chaudhari et al., 2012), thus we may have altered the natural changes in cellular granularity by transferring the cells to a fresh growth medium.

#### ***6.2.4 Fluorescent Microscopy***

Because the distribution of the side scatter data did not appear to have the distinct sub-populations observed by Portell et al. (2010), the level of bud scars on cells of the SMA yeast strain over the course of our 72 hour shaker flask fermentation was examined. Side scatter is believed to be affected by both external and internal cellular structure (Allman et al., 1990). Each time *Saccharomyces cerevisiae* replicates asexually a chitin scar ring or bud scar is left on the cell surface. Those “older” cells which have undergone more replications are known to have both more abundant and larger bud scars than those cells which have experienced fewer replicative

cycles (Powell et al. A, 2003). Therefore, it was expected that as the fermentation progressed there would be a higher proportion of cells with more bud scars, and therefore more cellular complexity, or at the very least the distribution of the population side scatter would have changed.

At each sampling time, the bud scar number on at least 40 cells was determined. The cells were grouped according to the method described by Chaudhari et al. (2012); that is daughter cells having only a birth scar (larger than bud scars) (Figure 6.2.8a), cells with a single bud scar (Figure 6.2.8b), cells with two bud scars (Figure 6.2.8c) and cells with three or more bud scars (Figure 6.2.8d). Ideally, confocal microscopy would have been used as this allows visualization of the whole cell (Powell et al. A, 2003). However, whenever each cell was visualized the fine focus on the microscope was adjusted back and forth which gave a better picture of the bud scars on the whole cell surface. During each successive sampling time, the percentage of cells with just a birth scar, one bud scar and two bud scars all decreased. Meanwhile, the percentage of those cells with three or more bud scars increased (Table 6.2.4).



**Figure 6.2.8. (a)-(d).** Examples of the four groups of bud-scarred yeast cells described by Chaudhari et al. (2012); daughter cells having only a birth scar (a), cells with a single bud scar (b), cells with two bud scars (c) and cells with three or more bud scars (d). The samples were taken from 24, 48 and 72 hours into a shaker-flask fermentation and visualized with fluorescent microscopy as described in the Materials and Methods section.

**Table 6.2.4.** The proportions of yeast cells with just a birth scar, a single birth scar, two bud scars and three or more bud scars taken from 24 hours, 48 hours and 72 hours in a shaker-flask fermentation. The cells were stained and visualized with fluorescent microscopy as described in the Materials and Methods section.

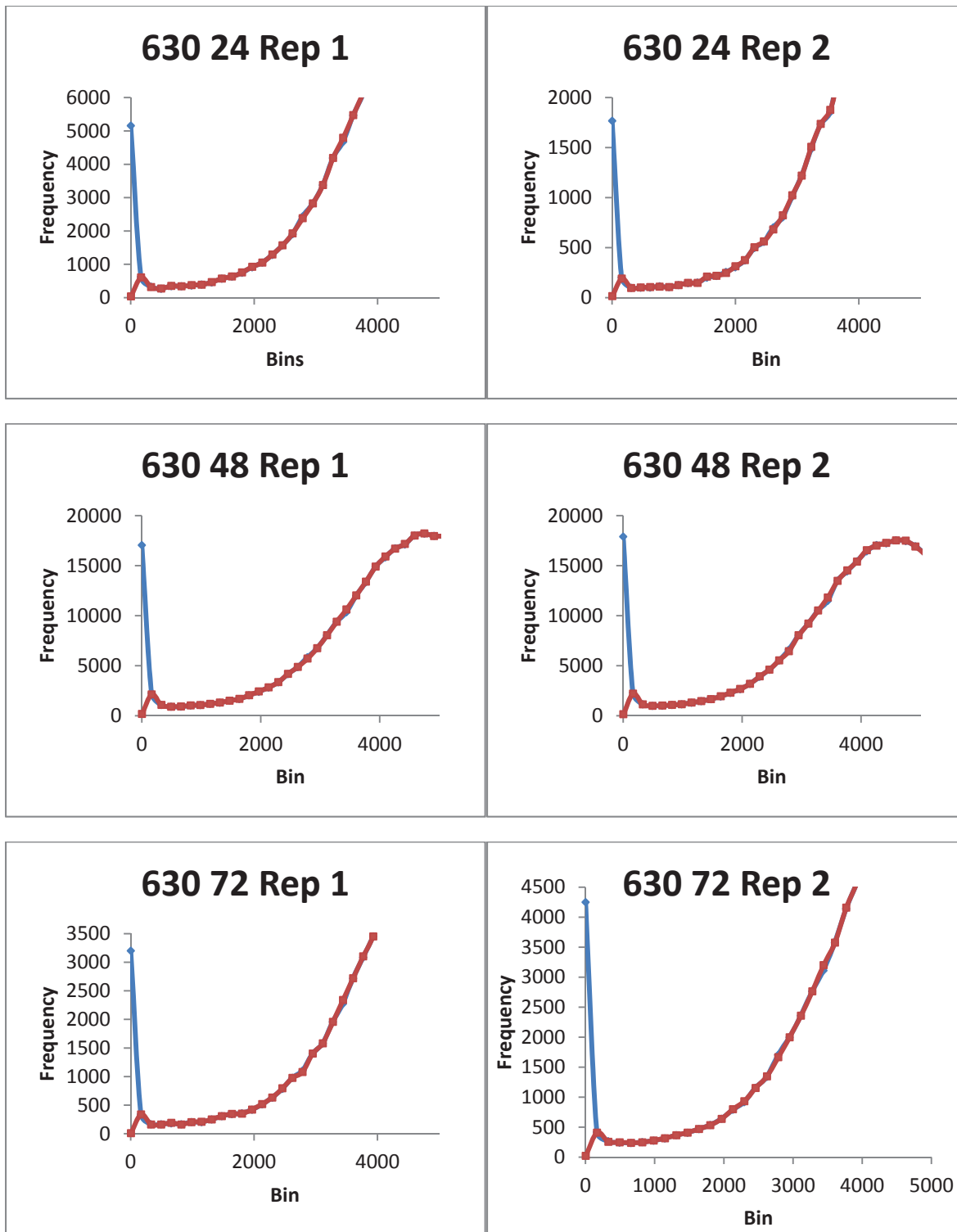
Sample Time	% Birth Scar	% Single Bud Scar	% Two Bud Scars	% Three or More Bud Scars
24 hours	10	35	42.5	12.5
48 hours	2.6	20.5	23.1	53.8
72 hours	2.5	2.5	17.5	77.5

Laboratory batch cultures and cells in stationary phase typically contain defined proportions of differently aged cells. These cultures usually consist of 50 % virgin cells (cells with a birth scar), 25 % first-generation mothers (cells with 1 bud scar), 12.5 % second-generation mothers (cells with 2 bud scars), etc. (Powell et al A., 2003). In the shaker-flask fermentation the virgin cells/cells with a birth scar were never the predominant cell type at any of the sampling times. However, this is not to say that this study was invalid. After each 24 hour period the cells were transferred into a fresh growth medium which would have reduced what is a significant barrier to cell growth and a predominant factor that forces cells into stationary phase-nutrient depletion (Werner-Washburne et al., 1993). Furthermore, in fermentations cell growth and division is not typically synchronous (Powell et al. B, 2003). Asynchronous cell division and growth in fermentations, assumed to be the norm, gives rise to a heterogeneously aged population of cells (Powell et al. B, 2003), which could have explained the cell age proportions observed (Table 6.6) in our experiments.

### ***6.2.5 Zymolectin Density***

Like the side scatter data, the emission at 630 nm data at each sampling time looked more unimodal (Figure 6.2.3). However, there was an accumulation of measures in each sample set which appeared to have very little or no fluorescence and were separate from the rest of the histogram. It was believed (but could not be confirmed) that this part of the data separated from the rest of the histogram represented material other than intact cells bound with a FITC-avidin conjugate. As such, those measurements with an emission of 0 were excluded from the data set when the histograms were analyzed by Igor Pro 6.2 with the Multi-peak package. When each emission at 630 nm data set was analyzed by the Multi-peak package, each set was best modelled by just 1 Gaussian distribution.

At the surface, this would suggest there were not any distinct sub-populations, i.e.,) cells expressing higher levels and lower levels of zymoelectins in the emission at 630 nm data. However, even when the data was filtered to exclude those measures with a fluorescence of 0, a small “bump” remained at the left most part of the 630 nm histograms with every data set (Figure 6.2.9). It is possible that this small “bump” did represent a small sub-population of cells with less zymoelectins. Ideally, the flow cytometric analysis would have been coupled with microscopic examination of the same sample to check for the presence of cellular debris or other material which wouldn't have bound the FITC-avidin probe. Unfortunately, both time and resources were limited.



**Figure 6.2.9.** The left most part of the histograms of the emission at 630 nm data at each sampling time showing those produced when the 0 measures were included (blue), and those for when the 0 measures were filtered out (red).

# Chapter 7: Conclusion and Future Work

## 7.1 Phase 1

Both the flocculation level and CSH increased for the NewFlo phenotypic SMA yeast strain during fermentation in YEPD broth and the miniature fermentation assay (ASBC, 2013, Yeast-14). Two other industrial strains, LCC1209 (Jin et al., 2001) and LCC125 (Speers et al., 2006), have exhibited this same behaviour during previous studies in the brewing science lab. Regrettably, 3-OH oxylipins could not be isolated from SMA whole cells or cell wall isolates from cultures grown in YEPD broth or from whole cells grown in the miniature fermentation assay (ASBC, 2013, Yeast-14) using the methods described. Therefore, an increase in 3-OH oxylipin concentration over the course of a fermentation was not detected, nor was a concurrent increase in flocculation level, CSH and 3-OH oxylipin concentration observed.

Unfortunately, after the 3-OH oxylipin portion of this work was abandoned it became clear that acid-catalyzed transesterification was most likely a poor choice to derivatize the oxygenated free fatty acids, 3-OH oxylipins. Kock et al. (2013) advised the brewing science lab that his group had only been able to qualitatively detect 3-OH oxylipins in most species of yeast. In addition, he said when his group tried transesterifying total lipid extract as a derivitization approach, 3-OH oxylipins present were generally masked by all the other previously esterified fatty acids to such an extent that detection with GC-MS was not possible. Kock et al. (2013) then suggested the brewing science group should try a derivitization approach that selectively methylated free fatty acids while leaving esterified fatty acids unaffected. Had the selective derivitization of free fatty acids been utilized at the outset of this project, it may have been possible to isolate 3-OH oxylipins from the SMA yeast strain.



Brewing yeast lipid quantification and classification studies by Blagovic et al. (2001) have demonstrated fatty acids were far from the most abundant lipid species in the yeast cell. TAGS, squalene, ergosterol and sterol esters alone made up of 51 % of total lipids. Furthermore, Kock et al. (2013) mentioned they could only qualitatively detect 3-OH oxylipins in most species of yeast. Based on these observations and our inability to isolate 3-OH oxylipins in the SMA strain, it could be reasonable to conclude that 3-OH oxylipins do not drive cell-to-cell aggregation and flocculation by increasing CSH when they localize to the cell wall. An alternate theory is to suggest that 3-OH oxylipins cause flocculation and aggregation via a “chemo-attractant” or quorum sensing model.

In this study a conservative estimate of the detection limits of 3-OH oxylipins was  $> 50$  ng/0.5 g wet yeast (as calculated in Appendix VI). This concentration is comparable to the concentration of other signalling molecules implicated in quorum sensing. For example, a recent study of the Proteobacteria *Rhodospseudomonas palustris* acyl-homoserine lactone (HSL) quorum sensing system found that the organism elicits a response to the fatty signal molecule *p*-coumaroyl-HSL at concentrations as low as several nanomolar and achieves saturation at 20-50 nM (Schaefer et al, 2008). Furthermore, a 3-OH oxylipin (3-OH 14:2) has been implicated as potent mediator of quorum sensing in the yeast *Candida albicans* (Nigam et al., 2010).

It would clearly be of interest to attempt the experiments again while selectively derivatizing free fatty acids in accordance with the suggestion of Kock et al. (2013). As described in works by Blagovic et al. (2001, 2005a and 2005b), brewing yeasts are comprised of many lipid species including TAGs, DAGs, MAGs, squalene, ergosterol, sterol esters, fatty acids, phospholipids and glycolipids. Acid-catalyzed transesterification only produces FAME from TAGs, DAGs, MAGs, phospholipids and glycolipids (Jones et al, 2012). Therefore, a

second interesting future study would be to monitor the concentrations of all lipid classes in brewing yeast during a fermentation and in turn correlate these with flocculation level and CSH. In this way it may be possible to discern which lipid classes, if any, are principally responsible for driving an increase in CSH.

## **7.2 Phase 2**

As this project was the first time a flow cytometer had been employed by the Brewing Group, it is not possible to compare the results to past studies. However, it was apparent that the distribution of cell size, granularity and emission at 630 nm were non-normally distributed at each sampling time. Only the histograms of the forward scatter measurements looked to capture distinct sub-populations of cells though. Furthermore, cell size determined by forward scatter appeared to be correlated with cell size manual measures as histograms of both appeared similar at 24 hours into the shaker flask fermentation. Based on the forward scatter measures, it is fair to conclude that cell size tended towards uniformity after 72 hours during the lab scale fermentations.

Histograms of the cellular complexity (SSC) during each sampling time were not predicted to be best modelled by multiple curves by Igor Pro 6.2., and thus did not likely encompass multiple populations. This apparent lack of multimodality in the distributions did not align with a previous study (Portell et al., 2010) and this may be attributable to the fact that different yeast strains were used in this project and the previous study. Fluorescent microscopy experiments revealed that the level of bud scars on the SMA yeast strain was not typical of most batch cultures (Powell et al A., 2003), but this was most likely due to the methods employed.

The distribution of emission at 630 nm data was also only best modelled by just one Gaussian distribution, suggesting only a single population of cells with respect to zymolectin density.

However, there was an accumulation of measures with no or very little fluorescent intensity in each histogram. Because cell size standards were unavailable during flow cytometry experiments, it is not possible to determine whether these accumulations were sub-cellular debris or a sub-population of cells with fewer zymolectins. Therefore, it is not possible to conclusively affirm or deny the hypothesis that zymolectin densities for a fermenting yeast cell population, as determined by a flow cytometer, should be bi-modal. However, this preliminary data is encouraging and definitely warrants further investigation.

Size standards were unavailable during the flow cytometry experiments, so it was not possible to distinguish between whole cells and sub-cellular debris with absolute certainty. It would be of interest to repeat the aforementioned experiments while employing flow cytometry size standards. These are typically unstained polystyrene microspheres of known diameter and they would have allowed for more definitive gating of the, in certain instances, confoundingly large data sets.

The aggregation of measures with very little fluorescence at the left most portion of the emission at 630 histograms (Figure 35) were promising results, but, again, it was not possible to say whether these were cells with fewer zymolectins or other debris that did not bind the FITC-avidin probe. Eglimez et al. (1990) describe a method to create age-synchronized populations of *Saccharomyces cerevisiae* by both growth-synchronization methods and by rate-zonal sedimentation in density gradients. This method was further reported to be straightforward and to produce bulk quantities of cells. It would be of interest to employ the techniques of Eglimez et

al. (1990) to create homogenous populations of virgin cells (daughter cells) and older cells (mother cells) and measure the zymolectin level on each of these respective populations with a flow cytometer. In this way it may be possible to better understand whether zymolectin activity is constitutive or induced.

## References

- Akiyama-Jibiki, M., Ishibiki, T., Yamashita, H. and Eto, M. (1997). A rapid and simple assay to measure flocculation in brewer's yeast. *Master Brewers Association of the Americas Technical Quarterly*, 34, 278-281.
- Albrecht-von-Haller Institute for Plant Sciences-Oxylipin Profiling Database. (2005). HIP Extraction. Retrieved January, 2013 from [http://www.oxylipins.uni-goettingen.de/methods.php?path=hip\\_f&caption=HIP%20Extraction](http://www.oxylipins.uni-goettingen.de/methods.php?path=hip_f&caption=HIP%20Extraction)
- Allman, R., Hann, A. C., Phillips, A. P., Martin, K. L. and Lloyd, D. (1990). Growth of *Azotobacter vinelandii* with correlation cell size, flow cytometric parameters, and ultrastructure. *Cytometry Part A*, 11, 822-831.
- Amory, D. E. and Rouxhet, P. G. (1988). Flocculence of brewery yeasts and their surface properties: Chemical composition, electrostatic charge and hydrophobicity. *Journal of the Institute of Brewing*, 94, 79-84.
- Amri, M. A., Bonaly, R., Duteurtre, B. and Moll, M. (1982). Yeast flocculation: Influence of nutritional factors on cell wall composition. *Journal of General Microbiology*, 128, 2001-2009.
- Anderson, T. W. and Darling, D. A. (1954). A test of goodness of fit. *Journal of the American Statistical Association*, 49, 765-769.
- Andleigh, H. S. (1964). Rapid identification of *Candida albicans*. *Mycopathologia et Mycologia Applicata*, 23, 81-84.
- ASBC (2013). *Methods of Analysis*, 14<sup>th</sup> ed. Yeast-4 Microscopic yeast cell counting, -11B Absorbance method, -14 Miniature fermentation assay. American Society of Brewing Chemists. The Society, St Paul, MN.

- Baker, D. A. and Kirsop, B. H. (1972). Flocculation in *Saccharomyces cerevisiae* as influenced by the wort composition and by actidione. *Journal of the Institute of Brewing*, 78, 454-458.
- BD Biosciences. (2000). *Introduction to flow cytometry: A learning guide*. San Jose, CA: Becton Dickson and Company.
- Beaven, M. J., Belk, D. M., Stewart, G. G. and Rose, A. H. (1979). Changes in electrophoretic mobility and lytic enzyme activity associated with development of flocculating ability in *Saccharomyces cerevisiae*. *Canadian Journal of Microbiology*, 25, 888-895.
- Birgé, L. and Rozenholc, Y. (2006). How many bins should be put in a regular histogram. *Probability and Statistics*, 10, 24-45.
- Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7, 1513-1523.
- Blagovic, B., Rupcic, J., Mesaric, M., Georgiu, K. and Maric, M. (2001). Lipid composition of brewer's yeast. *Food Technology and Biotechnology*, 39, 175-181.
- Blagovic, B., Mesaric, M., Maric, V. and Rupcic, J. (2005a). Characterization of lipid components in the whole cells and plasma membranes of baker's yeast. *Croatica Chemica Acta*, 78, 479-484.
- Blagovic, B., Rupcic, J., Mesaric and Maric, V. (2005b). Lipid analysis of the mitochondria and plasma membrane of brewer's yeast. *Folia Microbiologica*, 50, 24-30.
- Bhatt, R. K., Falck, J. R. and Nigam, S. (1998). Enantiospecific total synthesis of a novel arachidonic acid metabolite 3-hydroxyeicosatetraenoic acid. *Tetrahedron Letters*, 39, 249-252.

- Carstens, E., Lambrechts, M. G. and Pretorius, I. S. (1998). Flocculation, pseudohyphal development and invasive growth in commercial wine yeast strains. *South African Journal of Enology and Viticulture*, 19, 52-61.
- Campbell, I. (Ed.) & Duffus, J. H. (Ed.). (1988). *Yeast-a practical approach*. Oxford, GBR: IRL Press Limited.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as marker for gene expression. *Science*, 263, 802-805.
- Champe, S. P. and El-Zayat, A. A. (1989). Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *Journal of Bacteriology*, 171, 3982–3988.
- Chaudhari, R. D., Stenson, J. D., Overton, T. W. and Thomas, C. R. (2012). Effect of bud scars on the mechanical properties of *Saccharomyces cerevisiae* cell walls. *Chemical Engineering Science*, 84, 188-196.
- Christie, W. W. (ed.). (1989). *Gas chromatography and lipids: A practical guide*. Dundee, GBR: The Oily Press.
- Christie, W. W. (ed.). (1993). *Preparation of lipid extracts from tissues. Advances in Lipid Methodology-Two*. Dundee, GBR: The Oily Press.
- D'Hautcourt, O. and Smart, K. A. (1999). Measurement of brewing yeast flocculation. *Journal of the American Society of Brewing Chemists*, 57, 123-128.
- Eglimez, N. K., Chen, J. B. and Jazwinski, S. M. (1990). Preparation and partial characterization of old yeast cells. *Journal of Gerontology*, 45, B9-B17.
- Faulkner, F. and Robb, D. C. (1876). *Studies on Fermentation. Translation of Pasteur, L* (pp. 202-20). London, GBR: MacMillan and Company.

- Finnerty, W. R. (1989). Microbial lipid metabolism. In *Microbial Lipids*, Vol. 2. Edited by C. Ratledge and S. G. Wilkinson. London, GBR: Academic Press.
- Folch, J., Lees, M. and Stanley, G. H. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry*, 226, 497-509.
- French, C. S. and Milner, H. W. (1955). Disintegration of bacteria and small particles by high-pressure extrusion. In S. P. Colowick and N. O. Kaplan (Ed.), *Methods in Enzymology* Vol. I. New York, NY: Academic Press.
- Göbel, C. and Feussner, I. (2009). Methods for analysis of oxylipins in plants. *Phytochemistry*, 70, 1485-1503.
- Goldblatt, M. E. (2007). Ascospore release and oxylipin production in the yeast *Dipodascopsis*. (Published masters dissertation). University of the Free State, Bloemfontein, SA.
- Gilliland, R. B. (1951). The flocculation characteristics of brewing yeasts during fermentations. In *Proceedings of the 8<sup>th</sup> Congress, Brighton, GBR. European Brewery Convention*.
- Graham, S. and Fryhle, C. (2002). *Organic Chemistry (7th Edition ed.)*. New York, NY: John Wiley and Sons Inc.
- Hara, A. and Radin, N. S. (1978). Lipid extraction of tissues with a low toxicity solvent. *Analytical Biochemistry*, 90, 420-426.
- Helm, E., Nohr, B. and Thorne, R. S. W. (1953). The measurement of yeast flocculence and its significance in brewing. *Wallerstein Laboratory Communications*, 16, 315-325.
- Hobden, C., Teevan, C., Jones, L. and O'Shea, P. (1995). Hydrophobic properties of the cell surface of *Candida albicans*: A role in aggregation. *Microbiology*, 141, 1875-1881.



Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., Dussault, P. and Nickerson, K.W. (2001). Quorum sensing in the dimorphic fungi *Candida albicans* is mediated by farnesol. *Applied and Environmental Microbiology*, 67, 2982-2992.

Hunter, K. and Rose, A. H. (1972). Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. *Biochimica et Biophysica acta-Lipids and lipid metabolism*, 260, 639-653.

Huuskonen, A., Rautio, J. and Londesborough, J. (2012). Flocculation assay: Utilization of TRAC to monitor yeast-flocculation related gene expression. In R.A. Speers (Ed.), *Proceedings of the 2<sup>nd</sup> International Brewers Symposium: Yeast Flocculation, Vitality and Viability*. St. Paul, MN. Master Brewers Associations of the Americas.

Imura, Y., Hara, S. and Otsuka, K. (1980). Fatty acids as hydrophobic substance on cell surface of film strain of *Saccharomyces*. *Agricultural and Biological Chemistry*, 44, 1223-1229.

Jarque, C. M. and Bera, A. K. (1987). A test for normality of observations and regression residuals. *International Statistical Review*, 55, 163-172.

Jin, Y-L and Speers, R. A. (1998). Flocculation of *Saccharomyces cerevisiae*. *Food Research International*, 31, 421-440.

Jin, Y-L. and Speers, R. A. (2000). Effect of environmental conditions on the flocculation of *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists*, 58, 108-116.

Jin, Y-L., Ritcey, L. L., Speers, R. A. and Dolphin, P. J. (2001). Effect of cell surface hydrophobicity, charge and zymolectin density on the flocculation of *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists*, 59, 1-9.

- Jones, J., Manning, S., Montoya, M., Keller, K. and Poenie, M. (2012). Extraction of algal lipids and their analysis by HPLC and mass spectrometry. *Journal of the American Oil Chemists' Society*, 89, 1371-1381.
- Kamada, K and Murata, M. (1984). On the mechanism of brewer's yeast flocculation. *Agricultural Chemical Society of Japan*, 48, 2423-2433.
- Klis, F.M. (1994). Review: Cell wall assembly in yeast. *Yeast*, 10, 851-869.
- Klose, J., de Sa, M. M. and Kronstad, J. W. (2004) Lipid-induced filamentous growth in *Ustilago maydis*. *Molecular Microbiology*. 52, 823–835.
- Knapp, D. R. (1979). *Handbook of analytical derivatization reactions*. New York, NY: John Wiley and Sons, Inc.
- Kock, J. L. F., Van Wyk, P. W. J., Venter P., Coetzee D. J., Smith D. P., Viljoen, B. C. and Nigam, S. (1999). An acetylsalicylic acid-sensitive aggregation phenomenon in *Dipodascopsis uninucleata*. *Antonie van Leeuwenhoek*, 75, 261–266.
- Kock, J. L. F., Venter, P., Smith, D. P., Van Wyk, P. W. J., Coetzee, D. J., Pohl, C. H., Botha, A., Ridel, K-H. and Nigam, S. (2000). A novel oxylipin-associated “ghosting” phenomenon in yeast flocculation. *Antonie van Leeuwenhoek*, 77, 401-406.
- Kock, L., Swart, C. and Pohl, C. (2013). Response: 3-OH oxylipins in *Saccharomyces cerevisiae*. *Journal of the Institute of Brewing*, 119, (4), 201.
- Kolmogorov, A. N. (1933). Sulla determinazione empirica di una legge di distribuzione. *Giornale dell'Intituto Italiano degli Attuari*, 4, 83-91.
- Laerum, O. D. and Farsund, T. (1981). Clinical application of flow cytometry: A review. *Cytometry*, 2, 1-13.

- Lake, J. (2008). Detection of malt inducing premature yeast flocculation: Mechanisms and composition. (Published doctoral dissertation). Dalhousie University, Halifax, NS.
- Lehnhart, R., Kuřec, M., Brányik, T., Mota, A. and Teixeira, J. A. (2007). Assesment of brewing yeast age based on selective bud scar staining and subsequent fluorescence measured by flow cytometry. In Proceedings of the 31<sup>st</sup> European Brewery Convention Congress, Venice, Italy, 6-10, May, 2007. Paper 130.
- Lesage, G. and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews, 70, 317-343.
- Lipke, P. N. and Ovalle, R. (1998). Cell wall architecture in yeast: New structure and new challenges. Journal of Bacteriology, 180, 3735-3740.
- Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., ... Darnell, J. (2004). Molecular Cell Biology 5<sup>th</sup> Edition. New York, NY: W. H. Freeman and Company.
- Macintosh, A.J., Adler, J., Eck, E. and Speers, R.A. (2012). Suitability of the miniature fermentability method to monitor industrial fermentations. Journal of the American Society of Brewing Chemists, 70, 205-211.
- Marine Lipids Lab (2013). Lab Protocol: Transesterification: production of fatty acid methyl esters or “FAME” for GC. Halifax, NS. Dalhousie University.
- Masters, B. R. (2010). The development of fluorescence microscopy. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester, GBR. DOI: 10.1002/9780470015902.a0022093
- Masy, C. L., Henquinet, A. and Mestdagh, M. M. (1998). Flocculation of *Saccharomyces cerevisiae*: Inhibition by sugars. Canadian Journal of Microbiology, 32, 1298-1306.
- Menzies, K.L. and Jones, L. (2010). The impact of contact angle on the biocompatibility of biomaterials. Optometry and Vision Science, 87, 387-399.

- Mestdagh, M. M., Rouxhet, P. G. and Dufour, J. P. (1990). Surface chemistry and flocculation of brewery yeast. *Ferment*, 3, 31-37.
- Miki, B. L. A., Poon, N. H., James, A. P. and Seligy, V. L. (1982a). Possible mechanisms for flocculation interactions governed by the gene FLO1 in *Sachharomyces cerevisiae*. *Journal of Bacteriology*, 150, 878-889.
- Miki, B. L. A., Poon, N. H. and Seligy, V. L. (1982b). Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, 150, 890-899.
- Mozes, N. and Rouxhet, P. G. (1987). Methods for measuring hydrophobicity of microorganisms. *Journal of Microbiological Methods*, 6, 99-112.
- Mozes, N., Amory, D.E., Leonard, A.J. and Rouxhet, P.G. (1989). Surface properties of microbial cells and their role in adhesion and flocculation. *Colloids and Surfaces*, 42, 313-329.
- Muirhead, K. A., Horan, P. K. and Poste, G. (1985). Flow cytometry-Present and future. *Nature Biotechnology*, 3, 337-356.
- Murphy, E. A. (1964). One cause? Many causes?: The argument from the bimodal distribution. *Journal of Chronic Diseases*, 17, 301-324.
- National Institute of Standards and Technology. (2013). *Engineering Statistics Handbook*. Retrieved September, 2013 from <http://www.itl.nist.gov/div898/handbook/eda/section3/histogr4.htm>
- Nevoigt, E. (2008). Progress in metabolic engineering of *Saccharmyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, 72, 379-412.

- Nigam, S., Ciccoli, R., Ivanov, I., Szczipanski, M., and Deva, R. (2010). On mechanism of quorum sensing in *Candida albicans* by 3(R)-hydroxy-tetradecaenoic acid. *Current Microbiology*, 62, 55-63.
- Pasteur, L. (1876). Page 196 in: *Etudes sur la Biere*. Gauthier-Villars, Paris, FRN.
- Patelakis, S. J. J., Ritcey, L. L. and Speers, R. A. (1998). Density of lectin-like receptors in the FLO1 phenotype of *Saccharomyces cerevisiae*. *Letters in Applied Microbiology*, 26, 279-282.
- Pembrey, R. S., Marshall, K. C. and Schneider, R. P. (1999). Cell surface analysis techniques: What do cell preparation protocols do to cell surface properties? *Applied and Environmental Microbiology*, 65, 2877-2894.
- Pierce, A. E. (1968). *Silylation of organic compounds: A technique for gas phase analysis*. Rockford, NY: Pierce Chemical Company.
- Portell, X., Ginovart, M., Carbo, R., Gras, A. and Vives-Rego, J. (2010). Population analysis of commercial *Saccharomyces cerevisiae* wine yeast in a batch culture by electric particle analysis, light diffraction and flow cytometry. *FEMS Yeast Research*, 11, 18-28.
- Porter, A. (2007). *Influence of wort composition on the incidence of premature yeast flocculation*. (Published masters dissertation). Dalhousie University, Halifax, NS.
- Powell, C. D., Quain, D. E. and Smart, K. A. A. (2003). Chitin scar breaks in aged *Saccharomyces cerevisiae*. *Microbiology*, 149, 3129-3127
- Powell, C. D., Quain, D. E. and Smart, K. A. B. (2003). The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation. *FEMS Yeast Research*, 3, 149-157.

- Pretorius, I.S. (2000). Tailoring wine yeast for the new millenium: novel approaches to the ancient art of wine making. *Yeast*, 16, 675-729.
- Priest, F. G. and Stewart, G. G. (2006). *Handbook of Brewing* (2nd ed.). Boca Raton, FL: CRC Press.
- Rees, E. M. R. and Stewart, G. G. (1998). Strain specific response of brewer's yeast strain to zinc concentrations in conventional and high gravity worts. *Journal of the Institute of Brewing*, 104, 221-228.
- Rhymes, M. R. and Smart, K. A. (2000). The relationship between flocculation and cell surface physical properties in a FLO1 ale yeast. *Brewing Yeast Fermentation Performance* (1st ed., pp. 152-15). Oxford, GBR: Blackwell Science.
- Robertson, B. R. and Button, D. K. (1989). Characterizing aquatic bacteria according to population, cell size, and apparent DNA content by flow cytometry. *Cytometry*, 10, 70-76.
- Robertson, B. R., Button, D. K. and Koch, A. L. (1998). Determination of biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. *Applied and Environmental Microbiology*, 64, 3900-3909.
- Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters*, 9, 29-33.
- Sawin, S. Fairfield University. (2013). Histograms and the shape of distributions. Retrieved September, 2013 from <http://cs.fairfield.edu/~sawin/stats/notes/histogram.pdf>.

- Samaranayake, Y. H., Wu, P. C., Samaranayake, L. P. and So, M. (1995). Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelial and denture acrylic surfaces. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, 103, 707-713.
- Schaefer, A. L., Greenberg, E. P., Oliver, C. M., Oda, Y., Huang, J. J., Bittan-Banin, G., Peres, C. M., Schmidt, S., Jushaszova, K., Sufrin, J. R. and Harwood, C. S. (2008). A new class of homoserine lactone quorum-sensing signals. *Nature*, 454, 595-599.
- Sebolai, O. M., Pohl, C. H., Kock, J. F. L., Chaturvedi, V. and del Poeta, M. (2012). The presence of 3-hydroxy oxylipins in pathogenic microbes. *Prostaglandins and other Lipid Mediators*, 97, 17-21.
- Scott, D. W. (1979). On optimal and data-based histograms. *Biometrika*, 66, 605-610.
- Selitrennikoff, C. P. (2001). Antifungal proteins. *Applied and Environmental Microbiology*, 67, 2883-2894.
- Seyffert, H. (1896). Remarks on pure culture yeasts and their nourishment. *Zeitschrift fur das Gesamte Brauwesen*, 19, 318.
- Smit, G., Straver, M. H., Lugtenberg, B. J. J. and Kijne, J. W. (1992). Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Applied and Environmental Microbiology*, 58, 3709-3714.
- Smukalla, S., Caldar, M., Pochet, N., Beauvais, A., Guadagini, S., Yan, C., Vinces, M. C., Jansen, A., Prevost, M. C., Latge, J., Fink, G. R., Foster, R. and Versteppen, K. J. (2008). Flo1 is a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell*, 135, 726-737.

- Soares, E.V. and Duarte, A.A. (2002). Addition of nutrients induce a fast loss of flocculation in starved cells of *Saccharomyces cerevisiae*. *Biotechnology Letters*, 24, 1957-1960.
- Sparkman, O. D., Penton, Z. E. and Kitson, F. G. (2011). *Gas chromatography and mass spectrometry: A practical guide*. Oxford, GBR: Academic Press.
- Speers, R. A., Tung, M. A., Durance, T. D. and Stewart, G. G. (1992). Colloidal aspects of yeast flocculation: A review. *Journal of the Institute of Brewing*, 98, 525-531.
- Speers, R. A., Smart, K., Stewart, R. and Jin, Y-L. (1998). Zymolectins in *Saccharomyces cerevisiae*. *Letter to the Journal of the Institute of Brewing*. 112, 246-254.
- Speers, R. A., Wan, Y-Q., Jin, Y-L. and Stewart, R. J. (2006). Effects of fermentation parameters and cell wall properties on yeast flocculation. *Journal of the Institute of Brewing*, 112, 341-342.
- Speers, R. A. (2012). A review of yeast flocculation. In R. A. Speers (Ed.), *Proceedings of the 2<sup>nd</sup> International Brewers Symposium: Yeast Flocculation, Vitality and Viability*. St. Paul, MN. Master Brewers Associations of the Americas.
- Stanton, C. (2013). Preliminary exploration into measuring zymolectin concentration using flow cytometry. (Unpublished masters dissertation). Heriot-Watt University, Edinburgh, UK.
- Stephens, M. A. (1992). An appreciation of Kolmogorov's 1933 paper. (Technical Report #453). Retrieved September, 2013 from Defence Technical Information Center:  
<http://www.dtic.mil/dtic/tr/fulltext/u2/a253575.pdf>
- Stewart, G. G. and Russell, I. (1981). In *Brewing Science*. J. R. A. Pollock ed. (Vol. 2). New York, NY: Academic Press Inc.



- Stewart, G. G., Goring, T. E. and Russell, I. (1983). Yeast strain for fermenting high Plato value wort. U.S. Patent 4 409 246.
- Stewart, G. G., Garrison, I. F., Goring, T.E., Meleg, M., Pipasts, P. and Russell, I. (1976). Biochemical and genetic studies on yeast flocculation. *Kemia Kemi*, 10, 465-479.
- Strauss, C.J. (2005). The role of lipids in the flocculation of *Saccharomyces cerevisiae*. (Published doctoral dissertation). University of the Free State, Bloemfontein, SA.
- Stratford, M. and Keenan, M. H. J. (1988). Yeast flocculation: quantification. *Yeast*, 4, 107-115.
- Stratford, M. (1989). Yeast flocculation: Calcium specificity. *Yeast*, 5, 487-496.
- Stratford, M. and Assinder, S. (1991). Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. *Yeast*, 7, 559-574.
- Stratford, M. (1992). Yeast flocculation: Restructuring the theories in line with research. *Cerevisia*, 21, 38-45.
- Stratford, M. (1992). Yeast flocculation: A new perspective. *Advances in Microbial Physiology*. 33, 1-71.
- Stratford, M. and Carter, A. T. (1993). Yeast flocculation: lectin synthesis and activation. *Yeast*, 9, 371-378.
- Straver, M. H., Aar, P. C. V. D., Smit, G. and Kijne, J. W. (1993). Determinants of flocculence of brewer's yeast during fermentations in wort. *Yeast*, 94, 527-532.
- Straver, M. H., Kijne, J. W. and Smit, G. (1993). Cause and control of flocculation in yeast. *Trends in Biotechnology*, 11, 228-232.

- Straver, M. H. and Kijne, J. W. (1996). A rapid and selective assay for measuring cell surface hydrophobicity of brewer's yeast cells. *Yeast*, 12, 207-213.
- Supelco Analytical. (2013). Fatty acid/FAME application guide: Analysis of food for nutritional needs. Retrieved May, 2013 from [http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/General\\_Information/t408126.Par.0001.File.tmp/t408126.pdf](http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/General_Information/t408126.Par.0001.File.tmp/t408126.pdf)
- Taylor, N. W. and Orton, W. L. (1978). Aromatic compounds and sugars in flocculation of *Saccharomyces cerevisiae*. *Journal of the Institute of Brewing*, 84, 113-114.
- University of California Davis Chemwiki. (2013). Heavy atom NMR: Non  $\frac{1}{2}$  Spin Systems. Retrieved October, 2013 from [http://chemwiki.ucdavis.edu/?title=Physical\\_Chemistry/Spectroscopy/Magnetic\\_Resonance\\_Spectroscopies/Nuclear\\_Magnetic\\_Resonance/NMR:\\_Experimental/Heavy\\_Atom\\_NMR:\\_Non\\_1%2F%2F2\\_Spin\\_Systems](http://chemwiki.ucdavis.edu/?title=Physical_Chemistry/Spectroscopy/Magnetic_Resonance_Spectroscopies/Nuclear_Magnetic_Resonance/NMR:_Experimental/Heavy_Atom_NMR:_Non_1%2F%2F2_Spin_Systems).
- University of California San Francisco School of Medicine. (2013). Flow cytometry and fluorescence-activated cell sorting. Retrieved October, 2013 from <http://missinglink.ucsf.edu/lm/molecularmethods/flow.htm>.
- Van Dyk, M. S., Kock, J. L. F., Coetzee, D. J., Augustyn, O. P. H. and Nigam, S. (1991). Isolation of a novel arachidonic acid metabolite 3-hydroxy-5, 8, 11, 14-eicosatetraenoic acid (3-HETE) from the yeast *Dipodascopsis unicleata* UOFs-Y128. *FEBS Journal*, 283, 195-198.
- Van Mulders, S., Daenen, L., Verbelen, P. J., Saerens, S. M. G., Verstrepen, K. J. and Delvaux, F. R. (2012). Flocculation assay: The genetics behind yeast flocculation: A brewer's perspective. In R. A. Speers (Ed.), *Proceedings of the 2<sup>nd</sup> International Brewers Symposium: Yeast Flocculation, Vitality and Viability*. St. Paul, MN. Master Brewers Associations of the Americas.

- Vesonder, R. F., Wickerham, L. J. and Rohwedder, W. K. (1968). 3-D-Hydroxypalmitic acid: a metabolic product of the yeast NRRL Y-6954. *Canadian Journal of Chemistry*, 46, 2628.
- Vidgren, V. and Londesborough, J. (2011). 125th anniversary review: Yeast flocculation and sedimentation in brewing. *Journal of the Institute of Brewing*, 117, 475-487.
- Vyas, A. P., Verma, J. L. and Subrahmanyam, N. (2010). A review on FAME production processes. *Fuel*, 89, 1-9.
- Wand, M. P. (1997). Data-based choice of histogram bin width. *The American Statistician*, 51, 59-64.
- Werner-Washburne, M., Braun, E., Johnston, G. C. and Singer, R. A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiological Reviews*, 57, 383-401.
- Wosten, H. A., Schuren, F. H. and Wessels, J. G. (1994). Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. *The EMBO Journal*, 13, 5848-5854.
- Wright, C. S. (1984). Structural comparison of the two distinct sugar binding sites in wheat germ agglutinin isolectin II. *Journal of Molecular Biology*, 178, 91-104.
- Yamauchi, Y., Okamoto, T., Murayama, H., Kajino, K., Amikura, T., Hiratsu, H., Nagara, A., Kamiya, T., & Inoue, T. (1995). Rapid maturation of beer using an immobilized yeast bioreactor. 1. heat conversion of  $\alpha$ -acetolactate. *Journal of Biotechnology*. 38, 101-108.
- Yazici, B. and Yolocan, S. (2007). A comparison of various tests of normality. *Journal of Statistical Computation and Simulation*, 77, 175-183.
- Yuste, R. (2005). Fluorescence microscopy today. *Nature Methods*, 2, 902-904.

Zhang, N. (2001). Identification and quantification of very-long chain methylene-interrupted polyunsaturated fatty acids (VLCPUFA) in fish oils and seal oils. (Published masters dissertation). Dalhousie University, Halifax, NS.

## Appendix I

Selitrennikoff, Claude <Claude.Selitrennikoff@ucdenver.edu>

Thu 5/23/2013 12:29 PM

To:

Greg Potter;

hello greg yes you may use the figure with attribution if that is ok?

best

claudeselitrennikoff, Ph.D.

Professor and Vice-Chair

Department of Cell and Developmental Biology

University of Colorado School of Medicine

---

From: Gregory Potter [GPOTTER@DAL.CA]

Sent: Thursday, May 23, 2013 7:09 AM

To: Selitrennikoff, Claude

Subject: Permission

Hello Dr. Selitrennikoff,

I am writing up my MSc thesis-it is on the influence of cell hydrophobicity in brewing yeast flocculation-and part of my literature review discusses the architecture and structure of the yeast plasma membrane/cell wall. I came across your article, and in particular Figure 1 which is a cross section of the plasma membrane and cell wall.

Selitrennikoff, C.P. (2001). Antifungal proteins. *Applied and Environmental Microbiology*, 67, (7), 2883-2894. (Figure 1)

I was just wondering if I could have your permission to include this figure in my thesis?

Thanks very much,

Greg

## Appendix II

makiko.jibiki@asahi-fh.jp  
Mon 6/3/2013 7:20 AM  
To:  
Greg Potter;

Dear Mr. Potter,

Thank you for your e-mail of 23 May 2013. Since I was transferred to a group company of Asahi Breweries, co Ltd., your e-mail takes time to reach me.

I am very happy that you are interested in our article in 1997. You can use the figure of my article as long as you follow the rules for citations. I hope it will be helpful for your MSc degree.

By the way, your e-mail suggest me that you are enrolled in a master's course of DALHOUSIE University. I wonder Dr.Alex Speers still belong to your University. If that is right, please give my best regards to him.

Sincerely,

Ms. Makiko Jibiki

♩ \*\*\*\*\* ♪♪

Makiko Jibiki  
Chief Producer  
Quality Assurance Department  
Quality Assurance Headquarters  
ASAHI FOOD & HEALTHCARE CO.,LTD.  
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### Appendix III

Arif Hussain <Arif.Hussain@ucsf.edu>

Thu 5/23/2013 8:19 PM

To:

Greg Potter;

Hi Greg,

You certainly have my permission but I don't know if that's worth anything, to be honest. I imagine the university (UCSF) owns the learning module, which we put together many years ago. That being said, if it's for a thesis, I don't think you'd be doing any wrong with an appropriate citation.

Cheers,

Arif

=====

Arif S. Hussain, MSTP Trainee

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On May 23, 2013, at 6:18 AM, Gregory Potter <[GPOTTER@Dal.Ca](mailto:GPOTTER@Dal.Ca)> wrote:

Hi guys,

I'm not sure who of you is the right person to ask (or if you are at all), but I am writing up my MSc thesis Lit Review and came across your tutorial on Flow Cytometry and Fluorescence-Activated Cell sorting.

I would like to include your schematic of FACS in my report (<http://missinglink.ucsf.edu/lm/molecularmethods/flow.htm>), and was wondering if I could get your permission to do that.

Thanks for your help,

Greg

## Appendix IV

dlarsen <delmarlarsen@gmail.com>

Wed 10/9/2013 12:31 PM

To:

Greg Potter;

You replied on 10/9/2013 12:41 PM.

Greg:

As the director of the ChemWiki, if you use this for your thesis, it is ok by me to use this figure (with attribution). Other uses will require more effort to confirm. If you have any content that you feel would complement the ChemWiki in this topic, feel free to contact me as we would love to have more people involved.

Delmar

Greg Potter

Wed 10/9/2013 11:37 AM

Sent Items

To:

copyright@ucdavis.edu;

Hello there,

I am wondering if I could have permission to use the attached Figure in my MSc thesis?

I obtained this picture from the website given below.

[http://chemwiki.ucdavis.edu/?title=Physical\\_Chemistry/Spectroscopy/Magnetic\\_Resonance\\_Spectroscopies/Nuclear\\_Magnetic\\_Resonance/NMR:\\_Experimental/Heavy\\_Atom\\_NMR:\\_Non\\_1%2F%2F2\\_Spin\\_Systems](http://chemwiki.ucdavis.edu/?title=Physical_Chemistry/Spectroscopy/Magnetic_Resonance_Spectroscopies/Nuclear_Magnetic_Resonance/NMR:_Experimental/Heavy_Atom_NMR:_Non_1%2F%2F2_Spin_Systems)

Thank you for your time and I hope to hear from you soon.

Greg



## Appendix V

Sample	Average FSC	Average SSC	Average 630
24 hr Rep 1	8845.80	9919.56	4675.66
24 hr Rep 2	8815.68	10027.96	4959.13
48 hr Rep 1	8645.94	9587.07	4380.66
48 hr Rep 2	8704.98	9657.50	4251.16
72 hr Rep 1	8684.63	9858.01	5926.66
72 hr Rep 2	8583.99	9781.23	5503.28

## Appendix VI

- i.) All FAME samples were originally made to 0.5 mg/mL or 0.5 ug/uL.
- ii.) Injected 1 uL onto the column and saw decent FAME peaks.
- iii.)  $0.5 \text{ ug/uL} * 1 \text{ uL} = 0.5 \text{ }\mu\text{g} = 500 \text{ ng}$ . Therefore, 500 ng total onto the column.
- iv.) Can easily detect fatty acids that are present at ~1% of total mass, ( $500 \text{ ng} * 0.01 = 5 \text{ ng}$ ), therefore 5 ng in this sample.
- v.) With selective ion monitoring mode, it would be possible to go considerably below the 5 ng in this sample.
- vi.) So we conservatively set 0.5 ng as the minimum detectable amount. In reality, it's probably quite a bit less than that.
- vii.) To determine the minimum detectable amount, had to consider dilutions. Above stated we could see 0.5 ng in 1 uL of FAME sample. But the total sample volume was ~100uL, ( $0.5 \text{ ng}/\mu\text{l} * 100 \mu\text{l} = 50 \text{ ng}$ ).
- viii.) Therefore we had 50 ng/starting sample mass which was always ~0.5 g. Thus ~ 50 ng FAME/0.5 g wet yeast.