SACCHAROMYCES HYBRIDS: GENERATION AND ANALYSIS

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By

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"All right, brain. You don't like me and I don't like you, but let's just do this and I can get back to killing you with beer." Homer Simpson

Abstract

Hybrids of *Saccharomyces* yeasts are found in many fermentative industries; without intentional selection they have been identified as key strains in fermentations of beer, wine, and cider. Hybrids appear to confer a selection advantage as they can inherit beneficial traits from both parent species. *S. pastorianus* is responsible for the fermentation of lager. In the UK; a country famed for its ale, lager accounts for two thirds of beer sold. Globally this is much higher. With the rise of craft brewing and pressure upon large breweries to increase efficiency, there is a growing demand for new and improved yeast strains. This pressure is a challenge, because most hybrids are sterile and cannot be improved through selective breeding. The sterility of hybrids also means they have not previously been the subject of quantitative genetic analysis. A key requirement of this analysis is a large and diverse segregating population created through mating.

This project opens the door for quantitative genetics on hybrids for the first time. Utilising higher levels of ploidy, hybrid sterility has been overcome to create a large set of new hybrids. For performing genetic analysis, huge genetically diverse populations were generated through multiple rounds of interbreeding. Phenotypes analysed here are industrially relevant, and we have begun to reveal the loci responsible for these traits.

S. pastorianus strains have been used in lager production for hundreds of years due to their strong fermentation performance at low temperatures, however diversity is lacking across the species. Through introgressing genetic material from *S. cerevisiae* isolates, it has been possible to increase the phenotypic diversity of *S. pastorianus*, as well as introduce fertility to these hybrids.

Using a high throughput phenotypic screen, we have been able to identify individuals with desirable brewing traits and assess their fermentation performance. Most candidates displayed an enhanced performance relative to the hybrid parent strains. This demonstrated that through the introduction of fertility it is possible to create new and improved industrial hybrids.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIL	Advanced Inter-cross Line
BSA	Bulk Segregant Analysis
CHEF	Clamped Homogeneous Electric Field
COM	Complete
CSH	Cold-Spring Harbour
EtBr	Ethidium Bromide
FACS	Fluorescence-Activated Cell Sorting
G418	Geneticin (Kanamycin)
GCR	Gross Chromosomal Rearrangement
GM	Genetically Modified
GMO	Genetically Modified Organism
GWAS	Genome Wide Association Studies
HML	Hidden MAT Left
HMR	Hidden MAT Right
HPLC	High Performance Liquid Chromatography
HYG	Hygromycin
I-QTL	Intercross Quantitative Trait Locus
ISA	Individual Segregant Analysis
ITS	Internal Transcribed Spacer
KAC	Potassium Acetate
KACmin	Potassium Acetate Minimal
LB	Lysogeny Broth
LOD	Logarithm of the Odds
MAT	Mating Type Locus
OD	Optical Density

ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PHENOS	Phenotyping On Solid Media
PIS	Protoplast Fusion Isolate Spores
QTL	Quantitative Trait Locus
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
SD	Synthetic Defined
TCA	Tricarboxylic Acid Cycle
VDK	Vicinal Diketones
VHG	Very High Gravity
X-QTL	Extreme Quantitative Trait Locus
YPD	Yeast Extract, Peptone, D-Glucose
YEPEG	Yeast Extract, Peptone, Ethanol, Glycerol

1 Introduction

1.1 The *Saccharomyces* Clade

The *Saccharomyces* clade consists of diverse budding yeast species, which have been isolated across the globe. The formation of the clade took place after a whole genome duplication event occurred in a common ancestral species. This duplication event took place shortly after *Saccharomyces* diverged from *Kluyveromyces* approximately 10^8 years ago (Wolfe and Shields, 1997, Scannell et al., 2011). Geographic isolation accompanied with rapid loss of redundant genes and sequence divergence then led to the formation of the distinct species found within the sensu stricto clade (Cliften et al., 2003, Cliften et al., 2006). The degree of nucleotide divergence between *Saccharomyces* species was found to be similar to the divergence between birds and humans (Dujon, 2006).

Until recently there were seven known closely related species that made up the *Saccharomyces* clade (Kellis et al., 2003) (**Figure 1.1.1**). Relative to *S. cerevisiae*, *S. eubayanus* and *S. uvarum* are the most genetically diverged, whereas the split of *S. paradoxus* from *S. cerevisiae* occurred most recently (Borneman and Pretorius, 2015). In 2017 a new species, *Saccharomyces jurei sp. Nov.*, was isolated in a mountainous region of France (Naseeb et al., 2017). *S. jurei* is closely related to both *S. mikatae* and *S. paradoxus*, with reproductive isolation from all *Saccharomyces* species distinguishing these isolates as a true new species.

Within the clade, *S. cerevisiae* has been the most widely domesticated and as a result the most studied. It takes its name from the Latin for ‘of beer’, due to its major role in the fermentation of beer. Its other uses range from baking, to wine making, to biofuel production. The small genome size and short life cycle make *S. cerevisiae* one of the most important model organisms for the study of genetics. The species was the first eukaryote genome to be fully sequenced (Goffeau et al., 1996). This opened the door to expansive genetic analysis,

elucidating the roles of most *S. cerevisiae* genes and an understanding of complex biochemical pathways, through reverse genetic approaches.

Within the *S. paradoxus* species, geographic location of strains closely correlates with observed genetic diversity and populations are "well delineated along geographic boundaries" (Liti et al., 2009). Unlike *S. paradoxus*, which has rarely been domesticated (Boynton and Greig, 2014), S*. cerevisiae* has been subject to human domestication for centuries (Liti et al., 2006). As a result of widespread domestication global populations of *S. cerevisiae* display much less differentiation. A comparative genomic analysis of > 35 isolates found that *S. cerevisiae* strains cluster into 5 distinct geographically isolated lineages. Many individuals possessed mosaic genomes, highlighting that domestication over centuries has brought together and allowed cross-breeding between these populations (Liti et al., 2009). Further analysis of domesticated industrial yeasts, found that these cluster in distinct groups depending on the degree of domestication and the purpose for which they were selected (Gallone et al., 2016). Ale yeasts in particular demonstrate high levels of aneuploidy as well as decreased ability to sexually reproduce. Other than as part of interspecific hybrids, there has been limited evidence for the domestication of the non *S. cerevisiae Saccharomyces* species.

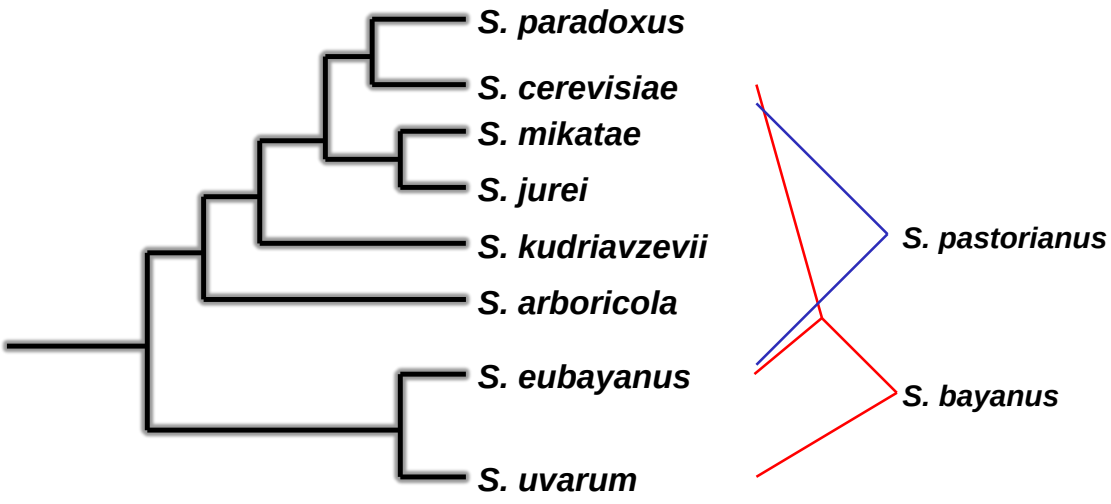

Figure 1.1.1 Phylogeny Tree of the *Saccharomyces* Clade

Phylogenetic tree presenting the current shape of the *Saccharomyces* clade. Adapted from (Dujon and Louis, 2017). The species contribution of two common hybrid species is also presented, *S. pastorianus*: *S. cerevisiae* x *S. eubayanus* (purple), and *S. bayanus*: *S. cerevisiae* x *S. eubayanus* x *S. uvarum* (red).

S. uvarum is a cryotolerant species, with three main subpopulations; Holarctic, South American and Australasian, separated by geographic location. The species was initially designated as *S. bayanus*, before the discovery that many strains were in fact complex hybrids between *S. uvarum*, *S. eubayanus* and *S. cerevisiae*. Hybrid strains were designated as *S. bayanus var. bayanus*, with clean *S. uvarum* strains designated as *S. bayanus var. uvarum (Vaughan-Martini and Martini, 2011),* before being confirmed as a distinct species (Nguyen and Gaillardin, 2005). Due to the nomenclature changes, there is often confusion in literature regarding these species. For clarity in this work, *S. uvarum* will solely be used to refer to the clean species; whereas *S. bayanus* will be used to refer to the hybrid species.

Most domestication of *S. uvarum* has been in the hybrid *S. bayanus* for fermenting wine, however there are numerous examples of *S. uvarum* wine strains (Masneuf-Pomarede et al., 2010, Masneuf-Pomarède et al., 2007, Naumov et al., 2002), hence its name being Latin for grapes. Rodriguez (Rodriguez et al., 2017) discovered that *S. uvarum* is involved in the fermentation of apple chicha, a traditional Patagonian drink. Interestingly they found strains of the Holarctic subpopulation, both as clean lineage and intraspecific hybrids with South American strains. Presumably the Holarctic strains were brought over when domesticated apple trees were introduced.

S. eubayanus, another cryotolerant species and closely related to *S. uvarum*, was first isolated in Patagonia (Libkind et al., 2011). The species has since been discovered in other locations including North America (David et al., 2014), New Zealand (Velimir and R., 2016), and China (Bing et al., 2014). Since the isolation and identification of *S. eubayanus* as a new species, it has been found to contribute to important domesticated hybrids *S. bayanus*, detailed above, and *S. pastorianus* the hybrid responsible for the production of lager. No wine or beer fermentations have been found to use a clean isolate of *S. eubayanus*, however since its characterisation Heineken has used a Patagonian isolate to brew a commercially available limited edition lager (Heineken, 2016). (Rodriguez et al., 2014), have also identified a role played by wild *S. eubayanus* strains in the production of Mudai, a fermented drink made from *Araucaria araucana* seeds.

1.2 Yeast Cell Cycle

Saccharomyces yeasts are capable of both asexual and sexual reproduction (Haber, 2012). During asexual reproduction, the maternal cell undergoes mitosis producing a clonal diploid daughter cell (**Figure 1.2.1**). For sexual reproduction the maternal cell undergoes meiosis, producing four haploid gametes that sit as a tetrad within a protective ascus (**Figure 1.2.2**). Diploids possess a copy of each mating type locus (*MAT*); *MATa* and *MATα*. Gametes will only inherit a single *MAT* locus, and will behave as a mater. Cells with a mating type will mate with cells of the opposing mating type. Gametes secrete mutually attractive mating pheromones ('a-factor' by *MATa* cells and 'α-factor' *MATα* cells), which stimulates cells to shmoo towards and mate with gametes of the opposite mating type. Due to proximity this most often occurs between gametes of the same tetrad (**Figure 1.2.3**b). Alternatively a gamete can mate with a spore of another tetrad. This may be of the same strain or a different strain and is the primary route of creating diversity (**Figure 1.2.3**a). Gametes are also capable of mating with gametes of another species to form a hybrid species. In the absence of the opposite mating type pheromone, gametes are capable of undergoing a mating type switch (**Figure 1.2.3**c). A haploid cell will undergo a mitotic division, the maternal cell will then switch mating type and then mate with the daughter cell to produce a diploid. Mating type switching is controlled by the action of the *HO* gene (Herskowitz et al., 1992, Haber, 2012). The *HO* gene encodes a DNA endonuclease, with sequence specificity to the *MAT* locus. The *MAT* locus is present on chromosome III, as well as two other loci involved in the determination of mating type, *HML* (Hidden MAT Left) and *HMR* (Hidden MAT Right). Epigenetically silenced copies of each *MAT* allele are present at these loci. Upon cleavage by the HO endonuclease, exonucleases degrade the active *MAT* locus. The site is then repaired using either the *HML* or *HMR* locus as a template. Mating type specific recombination enhancers promote a switch in mating type. Deletion of the *HO* gene removes the ability to switch mating type. This results in stable haploid cells unable to become diploid in the absence of cells of the opposite mating type. This can be beneficial in laboratory experiments as mating between strains can be controlled.

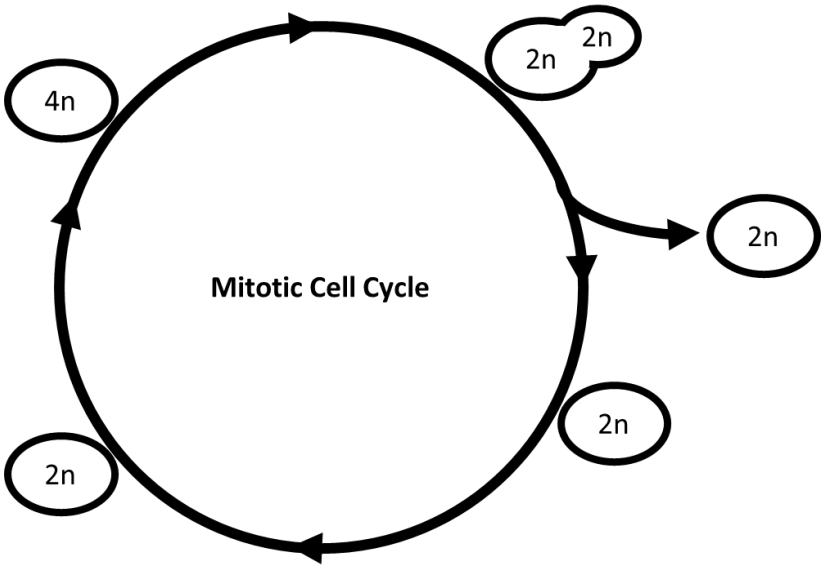

Figure 1.2.1 Yeast Mitotic Cell Cycle

Under normal growth conditions, yeast cells favour mitotic replication. Diploid cells (2n) replicate their genome content, to become tetraploid (4n). Budding occurs to produce two genetically identical diploid cells; the maternal and daughter cell.

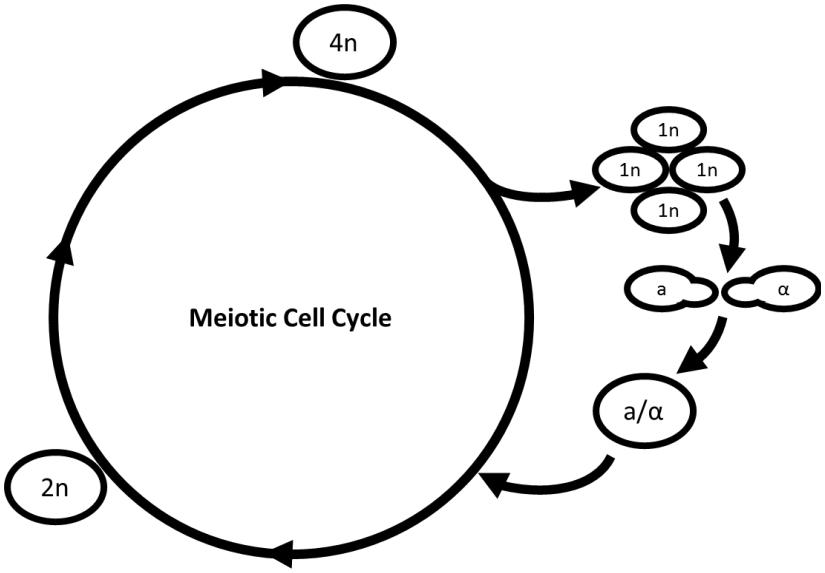

Figure 1.2.2 Yeast Meiotic Cell Cycle

Saccharomyces yeasts are capable of undergoing meiosis. Diploid cells (2n) undergo DNA replication to produce a tetraploid cell (4n). This tetraploid will sporulate producing a tetrad of four haploid spores (1n). Haploid spores have a defined mating type (*MATa* or *MATα*). Opposing mating types will shmoo towards each other and fuse to form a diploid.

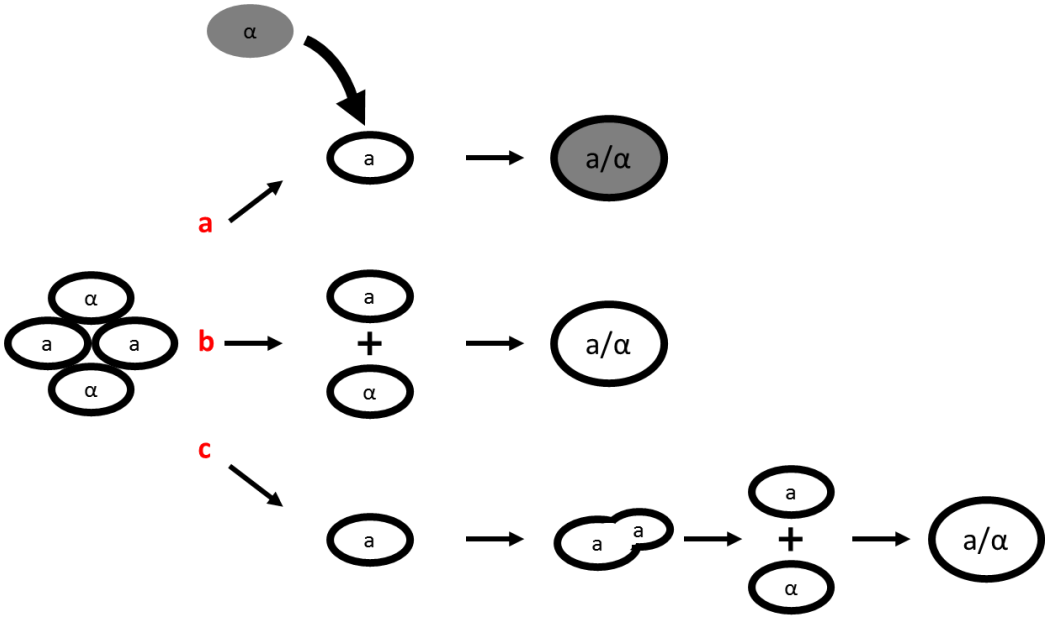

Figure 1.2.3 Gamete Mating

Gametes have several options for mating to become diploid. (a) Inter-tetrad mating; where a spore mates with a spore of opposing mating type from a different tetrad. (b) Intra-tetrad mating; where spores from the same tetrad, of opposing mating type mate. (c) In the absence of the opposing mating type pheromone the gamete will undergo mitosis producing a clonal daughter cell. The maternal cell will undergo a mating type switch and then mate with the daughter cell.

1.3 History of *Saccharomyces* Studies

S. cerevisiae has been one of the most desirable model organisms, particularly for research in genetics (Schneiter, 2004, Botstein et al., 1997). Highly favourable due to its simple and short life cycle, small genome size, and ease of manipulation. Another key factor in yeast as a model organism is that it possesses well conserved genes, processes, pathways, and other genomic features of higher multicellular eukaryotic organisms, including humans. Early mutagenic screens identified the roles that individual genes played on a range of cellular processes. Since *Saccharomyces* yeasts are stable in both haploid and diploid states, *S. cerevisiae* proved a helpful tool to study the cell cycle and production of gametes (Hartwell et al., 1970).

The work by Louis Pasteur; including investigations into preventing beer and wine spoilage, as well as the biochemistry of fermentation, dramatically advanced our knowledge of microbiology, benefitting research far beyond the brewing industry. As well as making discoveries in yeast taxonomy, Pasteur's

research also provided us with many tools used in yeast research, including cell culture techniques (Barnett, 2000, Gibson et al., 2017).

Sequencing of the genome revealed over 6000 open reading frames (ORFs), with only half of known function (Goffeau et al., 1996), opening up vast possibilities for functional studies through reverse genetic approaches. Sequencing also revealed a compact genome, with ORFs making up 72 % of the genome leaving little room for non-coding DNA (Dujon, 1996). At least a third of yeast genes possess mammalian orthologs, many of which are responsible for cell maintenance, DNA synthesis and repair, and homeostasis (Botstein et al., 1997). Utilising a single cell organism, it is easier to assess the role and function of these orthologous genes through deletion and overexpression, in reverse genetic screens. Within years of the genome being published, the yeast deletion collection was released (Giaever and Nislow, 2014, Winzeler et al., 1999, Brachmann et al., 1998). Utilising a PCR gene deletion strategy, each ORF was deleted and an array of individuals, each with a specific single gene deletion, was created. Gene deletion studies allowed for the study and assignment of function to most *S. cerevisiae* genes (Winzeler et al., 1999). These studies revealed the roles played by individual genes in yeast specific processes, but also helped to understand the role homologous genes play in human cells and in models of disease. As well as the gene deletion collection, overexpression libraries have been creating using plasmid vectors (Stevenson et al., 2001, Fleming and Gitler, 2011). Overexpression libraries can help reveal the role of essential genes, which cause cell death when deleted, or where there is gene redundancy in which a second gene compensates for the deletion of the gene of interest. Whilst deletion and overexpression have proven to be powerful tools in elucidating the role of many genes, they have a limited potential to help understand the genetic basis of complex traits. Complex traits do not conform to patterns of Mendelian inheritance, since they are not controlled by just one or a few genes. Instead complex traits are influenced by many loci, which individually confer an effect on the trait. These loci confer varying degrees of effect, from large effect to small effect. The deletion or overexpression of a locus that contributes a small effect, is likely to be missed in a phenotypic screen. It is not just the contribution of an individual locus, but the combined interaction

and effect of many loci (Nature-Education, 2018). A normal, bell-shaped, distribution curve is observed around the mean for complex traits in a population.

Since the first yeast genome was sequenced, S288C, in excess of 1000 isolates have been deep sequenced (Peter et al., 2018, Skelly et al., 2013, Bergström et al., 2014, Strope and Skelly, 2015). The magnitude of data creates the opportunity for comparative genomic analysis between strains, providing insight into evolutionary relationships as well as phenotypic differences (Peter et al., 2018, Pedro et al., 2015, Gallone et al., 2016). Comparative genomics between isolates reveals SNPs and copy number variations responsible for a phenotypic trait. Along with the sequence of *S. cerevisiae*, the sequences of other species within the *Saccharomyces* clade have now been assembled (Kellis et al., 2003, Cliften et al., 2003, Scannell et al., 2011, Liti et al., 2013, Baker et al., 2015, Liti et al., 2009) albeit with varying degrees of completion and annotation. Comparison of these provided insight into speciation within the clade, as well as events of horizontal gene transfer between species (Louis, 2011).

Most traits are not caused by just one or several genes, but are complex and determined by multiple quantitative trait loci (QTL) (Donnelly, 2008). A QTL is a region of the genome that is linked to conferring a specific trait, the effect each QTL has upon the trait varies with some being large effect and others small effect loci. The degree that an individual exhibits a complex trait depends upon the number and combination of different QTL. A typical quantitative trait will be determined by several large effect QTL, and a larger number of small effect QTLs (Manolio et al., 2009). It is often possible to detect large effect QTL by gene deletion or Genome Wide Association Studies (GWAS), however small effect QTL are difficult to identify. Traditional GWAS studies involve grouping strains by phenotype and linking alleles observed at higher frequencies to a given trait (Sardi et al., 2018). However in *S. cerevisiae* there is a high rate of calling false positives, due to strong correlation between phenotype and lineage (Connelly and Akey, 2012). This can be overcome through the creation of diverged populations from crossing of clean lineages (Cubillos et al., 2011, Kim and Fay, 2007).

Yeasts are highly suitable to study quantitative traits, owing to their short generation time and the ease of creating large genetically diverse populations for analysis. Quantitative traits can be investigated by phenotyping and sequencing individual segregants, or by sequencing entire pooled populations by bulk segregant analysis (Wilkening et al., 2014, Liti and Louis, 2012, Cubillos et al., 2013). Sequencing of pooled populations is only suitable for traits that are selectable, such as high temperatures or harsh environmental conditions. Comparison of the selection pool reveals enrichment of genomic regions. Phenotyping of individuals is less sensitive and is more labour intensive than pooled analysis. However it is possible to investigate traits that are not selectable, for example the production of off-flavours in brewing.

Typical QTL analysis in yeast will assess the F1 population generated from a cross between two diverged strains (Ehrenreich et al., 2010). The resolution of QTL mapping can be improved by generating advanced intercrossed lines (AILs) (Parts et al., 2011). By taking the F1 population and subjecting them to multiple rounds of sporulation and mating, genome shuffling occurs randomising closely associated variants. Breaking down linkage disequilibrium between loci improves the resolution of QTL analysis. This is important, firstly as low resolution mapping will reveal large regions contributing to a trait, when multiple QTLs may fall within this. Secondly it has been found that positive and negative effect QTLs can be found closely linked, these may cancel each other out and be hidden in low resolution QTL mapping. Thirdly, low resolution QTL mapping can highlight large genomic regions conferring a role in a given trait. However it is likely that a single gene or locus within that particular region is responsible. A large region may contain a multitude of genes, rendering the identification of the specific gene involved difficult.

1.4 Speciation and Hybrid Sterility

Species within the *Saccharomyces* clade are not pre-zygotically isolated, meaning hybrids form readily and frequently in nature. However they are post-zygotically sterile, producing non-viable gametes. The causes of post-zygotic reproductive isolation varies between differing phylogenetic groups (Greig et

al., 2002a). Typically in plants sterility is caused by changes in karyotype, including gross chromosomal rearrangements (GCRs) and changes in the level of ploidy (De Storme and Mason, 2014). Dicentric and acentric chromosomes can arise from inversion heterozygotes, both of which are inviable. Cross-overs in translocation heterozygotes generally result in unbalanced genomic content in 50% of resulting gametes which can be lethal (Osborn et al., 2003).

In the animal kingdom hybrid sterility is usually caused by genic incompatibilities, a model first described by William Bateson and later, independently, by Theodosius Dobzhansky (Orr, 1996, Bateson, 2009, Dobzhansky, 1934). This model is given the name Bateson Dobzhansky Muller (BDM) incompatibility. In its simplest two locus form; the ancestral species has the genotype AABB, a split in the population and change in environmental niche of each population sees the first population evolve over time to aaBB, whereas the second population evolves to AAbb. On hybridisation between the two populations the ‘a’ allele and the ‘b’ allele interact for the first time as AaBb. The combination of ‘a’ and ‘b’ together in a gamete may be incompatible (Wu and Ting, 2004). Within the Drosophila genus, examples of both GCRs and BDM incompatibilities causing hybrid sterility exist (J. A. Coyne, 2004).

Within the *Saccharomyces*, it has been shown that each species possesses a haploid set of 16 chromosomes and that the limited number of chromosomal rearrangements between species is not correlated to genetic distance (Naumov et al., 2000, Fischer et al., 2000). Examples of large reciprocal translocations have been found in closely related species within the clade, while distant species possess collinear genomes. A subset of the *S. paradoxus* species was previously designated as a separate species *S. cariocanus*. This subset exhibits post-zygotic isolation from other *S. paradoxus* strains despite minimal sequence differences. Isolation was induced by the accumulation of four reciprocal translocations (Liti et al., 2006). These findings ruled that the predominant cause of *Saccharomyces* hybrid sterility cannot be due to changes in karyotype. It has also been demonstrated that sterility of hybrids within the clade was not due to dominant genic incompatibilities. This was achieved by implementing a test proposed by

(Dobzhansky, 1936, Dobzhansky, 1934). This test theorised that any genic incompatibilities present in a diploid hybrid would still be present in a tetraploid hybrid and that the hybrid would remain sterile. (Greig et al., 2002a) created new hybrids, both diploid and tetraploid, and then assessed for F1 viability. As expected the interspecific diploid hybrids had low F1 viability (< 1 %), however the F1 viability was restored in the tetraploid hybrids. Molecular mechanisms have also been proposed as the cause of speciation and hybrid sterility (Dover, 1982, McMillan et al., 1997).

Sterility of hybrids is caused by sequence divergence between isolates and its effect on the mismatch repair mechanism (Hunter et al., 1996). The primary function of the mismatch repair mechanism is to recognise and correct mis-paired bases that arise during DNA replication. A number of mismatch repair proteins are also involved in the promotion of cross-overs. These cross overs provide a physical link between homologous chromosomes facilitating correct chromosomal disjunction during meiosis (**Figure 1.4.1**a) (Hunter and Borts, 1997, Wang et al., 1999). Other mismatch repair proteins, some performing a dual function, act to remove mismatches within the DNA heteroduplex (Rayssiguier et al., 1989). These proteins achieve this through gene conversion and anti-recombination (Borts et al., 2000, Alani et al., 1994). Anti-recombination involves the abortion of strand exchange through unwinding of the DNA heteroduplex, an intermediate structure required in the formation of cross-overs during meiosis. With increased sequence diversity anti-recombination results in an increased frequency of heteroduplex rejection (Greig et al., 2003, Chambers et al., 1996). As a consequence cross-overs are unable to form between homologous chromosomes in hybrids, where sequence divergence is high. This leads to non-disjunction and improper chromosomal segregation during meiosis, resulting in non-viable gametes (**Figure 1.4.1**b) and, in turn, reproductive isolation (Martini et al., 2006, Greig et al., 2002b, Roeder, 1997). Approximately 1 % of F1 spores are viable in hybrids, these are then highly aneuploid. It is likely that geographical isolation, followed by sequence divergence would have occurred first to promote speciation within the clade. The mismatch repair system is likely to have then complemented this to add to the hybrid sterility (Sniegowski, 1998). The finding by (Greig et al., 2002a) supports the theory of sterility being caused by

sequence divergence and abortion of cross-overs by the mismatch repair machinery. In the tetraploid hybrids there are four copies of each chromosome, two from each parental species. Crossovers between the homologous chromosomes of the same parental species will not be aborted by the mismatch repair system. Each gamete inherits a complete haploid set of 32 chromosomes, 16 from each of the hybrid parental species (**Figure 1.4.1**c).

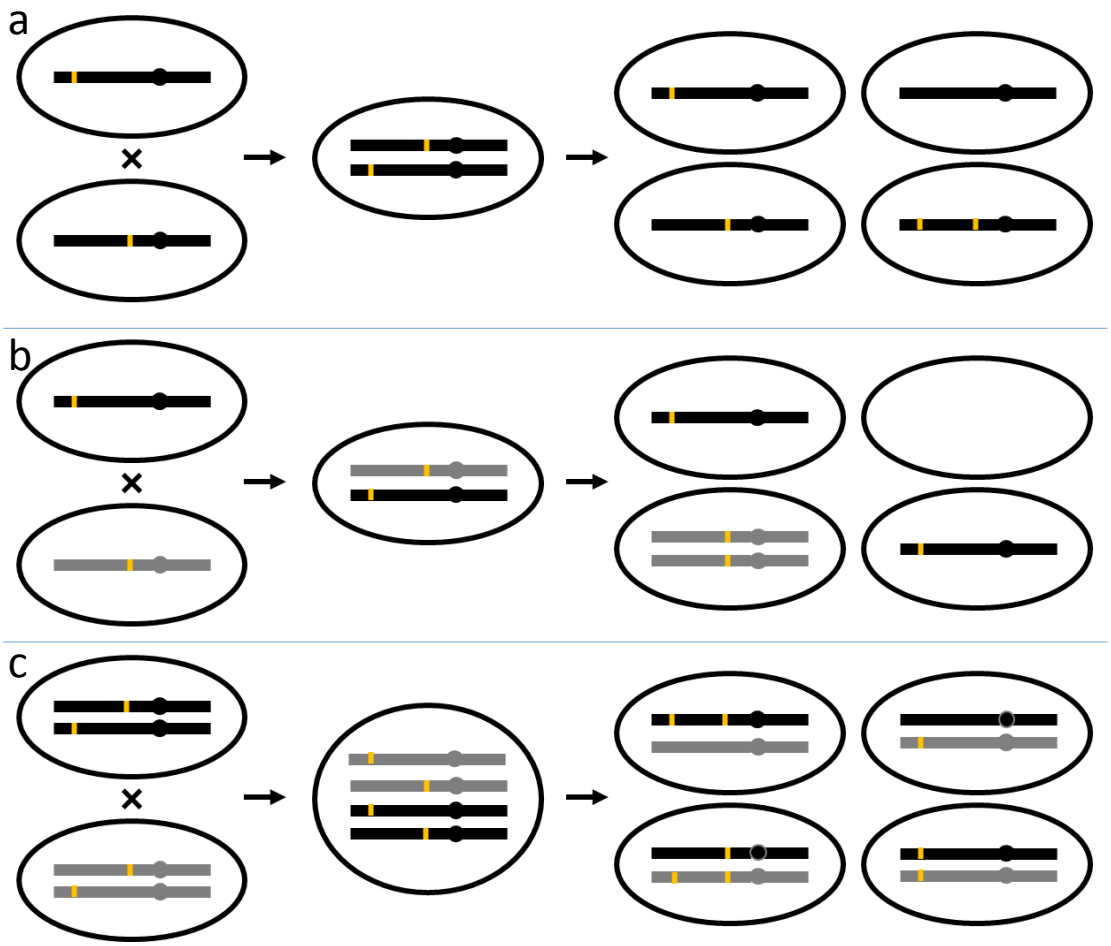

Figure 1.4.1 Hybrid Sterility

Hybrids produce non-viable gametes due to sequence variation disrupting chromosome segregation. (a) Mating of haploids within a species, results in a diploid that is capable of correctly pairing and segregating homologous chromosomes into gametes. (b) In a hybrid the sequence divergence between homologous chromosomes disrupts pairing of chromosomes, and so random segregation occurs giving non-viable highly aneuploid gametes. (c) In a tetraploid hybrid, there are two copies of each parental genome. Pairing of homologous chromosomes facilitates controlled segregation producing viable diploid hybrid gametes.

1.5 Strain Improvement

There are a variety of methods available for strain improvement: both classical genetic approaches and by genetic modification techniques. Classical genetic methods operate by selecting for individuals with a more desirable phenotype than the rest of the population. This is performed in a manner “naïve of genome sequences or the resulting genetic changes” (Crook and Alper, 2012). The individuals to be isolated have acquired beneficial mutations. The mutations are spontaneous arising naturally within the population or induced by mutagen such as exposure to UV radiation. Strain improvement by isolating random mutants is a slow process and only modest improvements to phenotypes are generally observed.

The method of selection is dependent upon the trait that is being improved. A number of traits are selectable, such as tolerance to heat and ethanol or resistance to antibiotics. Some conditions may prove toxic to individuals without beneficial mutations, therefore exposure to this condition will result in only those with these mutations growing. Alternatively growing the population through multiple generations under selection conditions will enrich the population with individuals that possess beneficial mutations. Isolating individuals with more desirable non-selectable traits is more time consuming than for selectable traits. Large numbers of individuals must be isolated from the population and then phenotyped independently. Some traits such as growth and colony size can easily be phenotyped using high throughput plate reader assays. However traits such as volatile production or sugar utilisation requires laborious analysis of gas and liquid chromatography respectively.

Breeding between individuals has long been utilised by humans in the domestication of plants for crops, animals for livestock, as well as microorganisms like yeast for baking and fermentation. A high rate of recombination is observed in *Saccharomyces* species resulting in extensive genome shuffling with each round of meiosis and mating. This shuffling mixes allele combinations and creates genetically diverse individuals distinct both genetically and phenotypically from the parental individuals. The review by (Steensels et al., 2014) provides examples of where mating has been used for strain improvement. These include intraspecific mating, for example to

improve fermentation performance of baking yeasts (Oda and Ouchi, 1990), as well as interspecific mating, such as combining the fermentation performance of *S. cerevisiae* with the flavour profile produced by *S. mikatae* for wine fermentations (Bellon et al., 2011). Breeding of individuals utilises natural diversity and avoids the potential damaging effects of exposure to mutagens like UV radiation. Furthermore, breeding of strains to combine two or more desirable traits can also give rise to an interesting phenomenon called heterosis. Heterosis, also known as hybrid vigour, is when the progeny of a mating event performs better than either parent for a given phenotype (Lippman and Zamir, 2007).

Strain improvement through genetic modification, involves the addition or manipulation genetic elements using recombinant DNA. This technique allows for a given trait to be altered or for strains to be developed with novel phenotypes not previously possible in yeast. Strain improvement can utilise the addition of a desirable gene, again (Steensels et al., 2014) provide extensive examples of where introduction of genes has been implemented. These include attempts to improve substrate utilisation efficiency (Kong et al., 2006, Nissen et al., 2000), as well as introducing microbial properties to make fermentations more resistant to contaminants (Boone et al., 1990).

Alternatively a gene that negatively contributes to a phenotype could have its function knocked out to prevent its expression in the strain. In fermentation this could be a gene which upon expression gives rise to undesirable aroma or flavour compounds. (Ni et al., 2007) used transposon induced mutagenesis to engineer individuals with improved growth and fermentation performance on a xylose substrate. Deletion of *PHO13* resulted in the overexpression of *TAL1*, giving rise to an observed increase in xylose utilisation.

The biochemistry for a trait of interest must be fully understood to enable selection of the correct elements to modify. If a locus is known to be involved in a trait site directed mutagenesis can be used to manipulate the locus (Storici et al., 2001). Targeted alterations of specific sequences ensures nonspecific undesired changes do not occur. For a gene encoding an enzyme the sequence coding for the active site may be manipulated to bind substrate more effectively. New techniques such as the CRISPR-Cas9 (Jinek et al.,

2012) system as well as HyPr (Alexander et al., 2016) have been developed which leave no trace of non-native DNA in the isolate, a key factor with regards to fears and regulation surrounding genetically modified organisms (GMOs). The absence of non-native DNA gave rise to hopes that regulation would be less stringent than conventional GMOs. However the Court of Justice of the European Union dashed these hopes after ruling that they should be treated in the same way as GMOs (Callaway, 2018). The targeting and altering of specific loci is possible for simple Mendelian traits; however most brewing traits of interest are complex and controlled by a multitude of genetic loci. It is the cumulative contribution of these loci that determine the extent of phenotype. The deletion of an undesirable gene may not always yield the desired strain. Many genes are pleiotropic and important for multiple cell functions (Attfield and Bell, 2003). Knocking out a gene may improve one phenotype but negatively affect another.

1.6 Lager Yeast

1.6.1 Origins of Lager Yeast

S. pastorianus is a relatively new hybrid species, first arising just 500 to 600 years ago. It Most likely formed in an ale fermentation, as a result of a non *S. cerevisiae* contaminant hybridising with the ale producing yeast. The conditions used by brewers of the time would have unintentionally favoured the new hybrid. Laws in Bavaria restricted brewing to the colder winter months. This was designed to avoid the production of poor quality beer with off-flavours, such as diacetyls, caused by fermentation at high temperatures. Inheriting cryotolerance from its *S. eubayanus* parent, *S. pastorianus* possessed a selective advantage over the ale yeast with its enhanced ability to ferment sugars at cooler temperatures.

Prior to 2011, the non *S. cerevisiae* part of the genome was thought to be of *S. uvarum*, or an unknown species closely related to *S. uvarum*. Upon isolation and sequencing of *S. eubayanus*, it was found that the genome had 99.5 % sequence identity to that of the non *S. cerevisiae* content in *S. pastorianus* (Libkind et al., 2011). No European isolate of *S. eubayanus* has yet been isolated, raising questions as to how the hybridisation event took

place (Gibson and Liti, 2015). Upon isolation of *S. eubayanus* in Patagonia, it was suggested that the species migrated to Europe via transatlantic trade, where the species met with ale yeast to form the hybrid. One problem with this theory is that lager production, presumably with *S. pastorianus*, occurred prior to Columbus' voyage to the Americas. This presents timeline inconsistency. Perhaps instead another cold tolerant European hybrid, possibly similar to the *S. kudriavzevii* x *S. cerevisiae* wine hybrids, was initially responsible for lager production before *S. pastorianus* was introduced and outcompeted the previous hybrid species. New isolates of *S. eubayanus* have since been isolated in China and Tibet (Bing et al., 2014), with the Tibetan strain exhibiting greater sequence identity to the *S. eubayanus* content in *S. pastorianus* (up to 99.82 %) than the sequence similarity of the Patagonian isolate (99.35 %) (Bing et al., 2014). This led to the suggestion that the *S. eubayanus* content was of East Asian origin, having been brought to Europe along the Silk trade routes. Another possibility is that *S. eubayanus* isolates of the North Americas were introduced to European fermentations, as transatlantic trade occurred here earlier than with the South American countries (David et al., 2014). Whilst the Tibetan strains have the closest phylogenetic relationship to lager strain, there are inconsistencies and no isolate can definitively be assigned as the *S. pastorianus* parental strain. It is also probable that European *S. eubayanus* populations exist, waiting to be isolated. Using environmental DNA samples, *S. eubayanus* rDNA sequences have been identified. Current efforts are afoot to isolate the first European isolate of the species (personal communication).

1.6.2 Classes of *S. pastorianus*

S. pastorianus strains can be assigned to one of two classes. The Saaz (Group 1) class, and the Frohberg (Group 2) class. These classes are genetically distinct, Saaz yeasts being allotriploid (3n-1), whereas Frohberg yeasts are allotetraploid (4n+2) (Wendland, 2014, Dunn and Sherlock, 2008, Nakao et al., 2009). The difference in ploidy translates into phenotypic differences. Frohberg yeasts demonstrating improved fermentation performance over Saaz strains at 22 °c, whereas Saaz strains display superior cold tolerance at 10 °c (Gibson et al., 2013). The Saaz genome is made up of two copies of the *S. eubayanus* parental genome and one copy of the *S. cerevisiae* genome, whereas the Frohberg strains possess an equal amount

of *S. cerevisiae* and *S. eubayanus* genomic material. Saaz yeasts have been found to produce lower levels of flavour molecules, which could be due to the lower *S. cerevisiae* genome content. Although interestingly a comparison of fermentations of *S. eubayanus* strains with both classes of *S. pastorianus* found *S. eubayanus* fermentations produced similar flavour profiles to that of Frohberg strains (Gibson et al., 2013). Saaz and Frohberg isolation correlates with geography and brewery group, with Saaz beers typically brewed around what is now the Czech Republic whereas Frohberg strains are predominant in Dutch breweries (Dunn et al., 2013).

Several models have been proposed as to how *S. pastorianus* came to exist, based upon sequence comparison of the species isolates. One hypothesis was that there was a single hybridisation event (Walther et al., 2014), between a diploid isolate of each parental species. Under this hypothesis the Saaz sub-group then lost one copy of the *S. cerevisiae* genome. Others proposed two isolated hybridisation events created each sub-group (Baker et al., 2015, Gallone et al., 2016, Goncalves et al., 2016). A hybridisation between two diploids isolates to produce the Frohberg class, whereas the Saaz group being formed as a result of a hybridisation event between a diploid *S. eubayanus* and a haploid *S. cerevisiae*. A third and now largely accepted hypothesis was for two successive hybridisation events: the first fusing a diploid *S. eubayanus* with a haploid *S. cerevisiae* to form the Saaz sub-group; the second hybridisation event between a Saaz isolate and another *S. cerevisiae* haploid, probably from a stout fermentation (Monerawela and Bond, 2017, Monerawela et al., 2015). This theory is supported by multiple shared sites of recombination, suggesting some recombination events had occurred prior to the second hybridisation event. Subsequent recombination events have occurred in both classes, with sub-groups within the Saaz yeasts sharing common recombination sites. Unique sites in individuals suggests continued recombination and reordering of the genome. The harsh brewing environment, with high osmotic and alcohol stresses, is thought to promote recombination and would have contributed to the rapid adaptation of *S. pastorianus* isolates (James et al., 2008).

1.6.3 Targets of Strain Improvement

Targets for lager strain improvement can be assigned into two groups. The final product and the production process. It is not just the grains and the hops used that contribute to the flavour and aroma profiles of lager. During the fermentation process yeast produces a broad spectrum of volatiles and aroma compounds. Many of these, such as vicinal diketones (VDK), are considered off-flavours and undesirable to the final product. The presence of VDKs for example contributes a rather buttery/butterscotch flavour. The creation of yeast strains which produce less aroma compounds would be desirable for the production of cleaner, typically more mass produced, lagers. In contrast, the craft brewing industry desire more complex flavour profiles. Flavours that may have been considered undesirable in a clean lager could help to create lagers with new and unique flavours. Craft breweries already experiment with the addition of fruits, herbs, and spices; new yeasts capable of producing a range of aromas may enhance these or create new lager flavours for an increasingly adventurous market. Another quickly expanding market, is that of low-alcohol beers (Maida, 2017). With consumers becoming more health conscious, the demand for healthier low-alcohol & low-calorie beers has greatly increased. Previously non-*saccharomyces* species, such as *Saccharomycodes ludwigii*, have been used to create these low-alcohol beers (Gibson et al., 2017, Huige, 1990). An issue with the production of low alcohol beers is that typically the fermentation is stopped early or alcohol is removed after fermentation. The resulting is a lack of desirable flavour compounds and a beer that tastes like raw wort. The generation of yeasts with a lower ability to utilise wort sugars such as maltose would allow fermentation to be completed with the desired flavour compounds produced and with low levels of alcohol (Gibson et al., 2017, Saison et al., 2010). This would however result in high sugar concentrations in the finished beer. On the production side of strain improvement, new strains may enable a brewery to increase its capacity without investing in larger fermenters and maturation tanks. This could be achieved with yeast that have faster metabolism, shortening fermentation cycles. Alternatively the ability of yeasts with higher tolerance to the environmental stresses of brewing, such as osmotic stress and high alcohol stress, could also improve capacity. Higher tolerance to osmotic stress means

higher gravity wort can be used in fermentation. Coupled with higher alcohol tolerance, new yeasts would be capable of utilising high gravity wort and producing higher alcohol beer. This beer would be suitable for dilution post fermentation, increasing the brewery capacity. Returning to the improvement of aroma and volatile compound production, new strains that produce fewer off flavours may also lead to an increase in brewery capacity. A large portion of the duration of the brewing process is post fermentation, during which the beer matures. Included in this maturation time is a period called diacetyl rest. During which the yeast reabsorbs diacetyl and enzymatically reduces it to acetoin and 2,3-butanediol, which have little contribution to the beer flavour (White, 2017). New yeast strains which produce less diacetyl, and/or are capable of consuming the produced diacetyl faster, would result in shorter maturation periods and therefore increase capacity. Since *S. pastorianus* is thermosensitive, lager has continued to be brewed at low temperatures, aiding production of lower off-flavour levels but also slowing the rate of fermentation. New strains with the ability to ferment in broader temperature ranges would increase capacity through increased rate of metabolism and faster fermentation completion.

1.6.4 History & Methods of Strain Improvement in Lager Yeasts

Despite the current legislation making genetically modified (GM) strains unsuitable for commercial use, there has been a large investment into creating such strains. Primarily these are proof of principle studies and done to investigate the effect of altering specific genetic elements on fermentation performance. GM strains are also being developed to be placed into storage collection in the expectation that legislation will relax and public perception of GMOs will become more positive. (Wendland, 2014) briefly outlines a number proof of principle studies where GM has been used to improve a range of important characteristics including maltose utilisation efficiency, and regulation of flocculation timing. The review by (Dequin, 2001) describes targets for strain improvement in the wine and brewing environment and highlights examples where groups have successfully made strains more desirable through GM approaches. This included the elimination of undesirable by-products, such as diacetyls, as well as increasing ability of strains to ferment dextrins.

Reverse engineering has been used in advancing the qualities of lager strains. Since knowledge of the genetics of *S. pastorianus* is in its infancy, compared to *S. cerevisiae* for example, it would appear the potential to use this technique to improve lager strains has not yet been utilised to its fullest. However using the extensive knowledge known about *S. cerevisiae* biochemistry and genetics, it is possible to take a pathway of interest and genetically engineer it into *S. pastorianus* strains of interest. (Yoshida et al., 2008) benefitted from metabolomics of *S. cerevisiae*, to identify factors that limit sulphite production. They then developed lager strains with increased sulphite levels without affecting other desirable fermentation properties. Another excellent example is the work of (Vidgren et al., 2009) who improved the fermentation performance of lager yeast by repairing the strains *ATG1* gene using the sequence of an ale strain *ATG1* gene. This resulted in improved uptake and utilisation of maltose and maltotriose during fermentation.

One technique currently used to improve lager strains is evolutionary engineering. Populations are subjected to selection pressures for multiple generations. Random mutations arise over time, with some conferring an adaptive advantage to growth under the selection pressures (Butler et al., 1996). Due to yeast's short life cycle, it is possible to obtain improved strains with relative pace using this method (Steensels et al., 2014). It is possible to speed up the process further through combining mutagenesis with selection. (Huuskonen et al., 2010) provides one example of where this method has been utilised to improve lager strains. Mutants of an industrial yeast strain were generated by treatment with ethyl methanesulfonate (EMS). The mutagenised population were placed in very high gravity (VHG) 25 °P (Plato) wort, and allowed to ferment. Individuals were isolated and the end of fermentation and found to possess improved fermentation performance in VHG wort relative to the original industrial strain. Lager strains have also been selected for to have greater tolerance to high ethanol concentrations and strong rates of growth under high osmolarity (Steensels et al., 2014, Ekberg et al., 2013). This method is only beneficial to improve phenotypes that can be selected for, and whilst the process can be done quickly with ease the improvements to strains are only moderate. This is because each mutation is

usually to a single nucleotide; large strain improvements tend to require more extensive genetic alterations. Mating between strains is able to provide this.

Several attempts have been made to breed with hybrids, despite their lack of spore viability (Krogerus et al., 2017, Gibson et al., 2017). Both in *S. pastorianus* and other hybrids this has previously been achieved through rare mating and protoplast fusion, both methods favoured since the ability to produce viable spores is not a necessity. Rare instances of loss of heterozygosity at the mating type locus can be screened for, with the resulting individuals capable of mating with those of opposing mating types (Hiraoka et al., 2000). Rare mating between *S. bayanus* and *S. cerevisiae* has been used to produce a more cryotolerant brewing yeast (Sato et al., 2002). Despite the lack of fertility in lager strains, it is possible to isolate viable gametes. When screened some of these are identified as possessing a mating type suitable for breeding (Gjermansen and Sigsgaard, 1981). When breeding with rare spores of *S. pastorianus*, *S. cerevisiae* ale strains are often selected for crossing. Ale strains are favoured due to their already strong fermentation performance and the possessing of other desirable traits. (Garcia Sanchez et al., 2012) have improved both fermentation performance and resistance to stresses, including high osmolarity using this technique.

1.6.5 Future Direction of Lager Yeast

Within the Louis research group in as yet unpublished data, a tetraploid hybrid capable of producing diploid gametes was generated through rare mating of a triploid *S. kudriavzevii* x *S. cerevisiae* wine hybrid with a haploid *S. kudriavzevii* strain. These could then be screened for improved desirable phenotypes. Having demonstrated the possibility of introducing fertility to previously sterile hybrids, it may be possible to introduce fertility to existing lager yeasts. The allotriploid Saaz yeasts appear to be prime candidates for attempting this. The introduction of fertility to lager hybrids would open the door to dramatic strain improvement, through the production of large genetically diverse populations and the subsequent selection of individuals with desired phenotypes.

Rare mating, as the name suggests, occurs at low frequencies. It also requires markers, either auxotrophic or antibiotic resistance, to isolate the individuals

of rare mating events. Antibiotic resistance markers render these individuals GMOs, and auxotrophies may impact the performance of the strains (Swinnen et al., 2015). A new gene editing technique termed HyPr (Hybrid Production), uses plasmids which induce loss of heterozygosity in individuals, creating diploid maters which can be co-cultured with another species to create tetraploid hybrids (Alexander et al., 2016). Since the resulting tetraploids will readily lose the selection plasmids, these *de novo* hybrids might avoid the classification as a GMO.

With increasing availability of genomic data, marker assisted breeding will become more possible. Marker assisted breeding is widely utilised in the improvement of livestock and crops (Takeda and Matsuoka, 2008). Phenotypes can be assigned to genotypes, which can in turn be screened for in mated segregants. A proof of principle study was performed using *S. cerevisiae* strains, which resulted in major aroma profile changes in selected segregants (Gallone et al., 2016). With improved ability to breed within the *S. pastorianus* species, marker assisted breeding may become feasible for improving lager strains.

The use of gene editing techniques, such as CRISPR, may not see their products branded as GMOs, and so the tight regulation of such an organism may not be applied to these (Brodwin, 2018, Rotman, 2017). If gene edited plants are exempt from GMO labelling, it is likely that yeasts will also be open to improvement using these techniques. Targets of allele swap or gene deletion strategies that previously would require genetic modification could be targeted using a non-GM methodology. Some gene editing in *S. pastorianus* has already been achieved using the CRISPR-Cas9 system (de Vries et al., 2017). Here multiple genes were precisely deleted simultaneously, proving the ability of the system in *S. pastorianus*.

1.7 Aims and Objectives

The first objective of this project is to create a new set of *de novo* hybrids, which have overcome post zygotic sterility (Chapter 3). Primarily focusing on producing *S. pastorianus*-like hybrids, *S. cerevisiae* strains will be crossed with *S. eubayanus* strains. However other crosses will be performed including

between *S. cerevisiae* and *S. uvarum*, as well as crosses between the closely related *S. eubayanus* and *S. uvarum*, producing hybrids with similar parental contributions as the natural hybrid *S. bayanus*.

These newly synthesised hybrids will undergo multiple generations of intercrossing to produce large genetically and phenotypically diverse populations. These hybrid populations will be suitable to perform, for the first time, detailed quantitative genetics on *Saccharomyces* hybrids (Chapter 4).

The second objective of this project is to introduce fertility in existing lager producing hybrids (Chapter 5). Due to the differing ploidy of the Saaz and Frohberg classes of *S. pastorianus*, different approaches will be required for each. However it is hoped that a moderate level of fertility can be achieved that will allow us to breed both between *S. pastorianus* strains and with other *Saccharomyces* species. Breeding will allow for the introduction of increased phenotypic diversity and eventually facilitate quantitative genetics to be performed on lager producing hybrids.

The final objective is to assess the newly created hybrids, both *de novo* and manipulated existing hybrids for brewing potential (Chapter 6). As candidate brewing strains, individuals will need to be able to utilise the key sugars in wort as well as being able to tolerate and perform well under the various environmental stresses that occur in a fermentation vessel.

2 Media and Methods

2.1 Media

Unless stated, media was adjusted to pH 6-6.5 using either 1 M HCl or 2.5 M NaOH prior to the addition of bio-agar (Biogene), henceforth referred to as agar. All media was autoclave sterilised at 20 psi for 20 minutes at 121 °C. Recipes provided below are for solid media. Liquid media preparation followed the same recipes, without the addition of bio-agar. Media reagents were obtained from Sigma Aldrich unless otherwise stated. All media was made using deionised water.

2.1.1 YPD

1 % (w/v) yeast extract (BD biosciences)

2 % (w/v) Bactopeptone (BD Biosciences)

2 % (w/v) D-glucose (Fisher Scientific)

1 % adenine solution (0.5 % (w/v) adenine in 0.05 M HCl)

2 % (w/v) agar (Biogene)

2.1.2 Synthetic Defined (SD)

2.1.2.1 Drop-out powder

The (mg) ratio of each amino acid in synthetic defined (SD) dropout powders were as follows. 1 adenine: 1 arginine: 5 aspartic acid: 1 histamine: 1 leucine: 1.5 lysine: 1 methionine: 2.5 phenylalanine: 10 threonine: 1 tryptophan: 1.5 tyrosine: 1 uracil. Each respective drop out powder lacked the respective amino acids. Synthetic defined media lacking uracil will be termed SD-uracil, for example. Complete (COM) SD contained all amino acids.

2.1.2.2 SD media recipe

0.675 % (w/v) yeast nitrogen base (w/o amino acids)

2 % D-glucose

2 % (w/v) bio-agar

6.25 ml/l 1 % leucine

3 ml/l 1 % lysine

0.0875 g/l COM or Drop-out powder

2.1.3 Potassium acetate sporulation medium (KAC)

2 % (w/v) potassium acetate

0.22 % (w/v) yeast extract

0.05 % (w/v) D-glucose

0.0870 % (w/v) COM SD powder

2 % (w/v) agar

pH 7

2.1.4 Potassium acetate minimal sporulation medium (KAC Min)

2 % (w/v) potassium acetate

0.0870 % (w/v) COM SD powder

2 % (w/v) bio-agar

pH 7

2.1.5 Cold-Spring Harbour Sporulation media (CSH)

0.05 % (w/v) D-glucose

1 % (w/v) potassium acetate

0.1 % (w/v) yeast extract

2 % (w/v) bio-agar

pH 7

2.1.6 YEPEG

1 % (w/v) yeast extract (BD biosciences)

2 % (w/v) Bactopeptone (BD Biosciences

2 % (w/v) Glycerol

2 % (w/v) bio-agar

2.1.7 Antibiotic selection medium

Two antibiotics are used here for selection; Hygromycin B (HYG), Geneticin (G418) (Goldstein and McCusker, 1999).

1 % (w/v) yeast extract

2 % (w/v) Bactopeptone

2 % (w/v) D-glucose

1 % adenine solution (0.5 % (w/v) adenine in 0.05 M HCl)

2 % (w/v) agar

After autoclaving, media was cooled to approximately 50 °C, before the addition of either:

300 µg/ml HYG, 200 µg/ml G418, or 100 µg/ml NAT

2.1.8 Protoplast regeneration medium

Synthetic defined uracil drop out media (SD-uracil)

300 µg/ml Hygromycin B

18.2 % (w/v) sorbitol

2 % (w/v) bio-agar for plate preparation or 0.5 % (w/v) bio-agar for molten regeneration medium

2.1.9 Lysogeny broth (LB)

0.5 % (w/v) yeast extract

1 % (w/v) tryptone

0.5 % (w/v) NaCl

2 g/l agarose

100 µg/ml ampicillin added post autoclave

2.2 General Methods

2.2.1 Yeast culture conditions

Strains were recovered from 25 % glycerol stocks, stored at -80 °C, and streaked onto YPD plates (**2.1.1**). The thermo-sensitive species; *S. eubayanus*, *S. uvarum*, and *S. pastorianus*, were cultured at 23 °C. *S. cerevisiae* and *S. paradoxus* were cultured at 30 °C.

2.2.2 Sporulation

For sporulation, strains were patched onto KAC, KAC min or CSH media (**2.1.3 – 2.1.5**) and cultured at 23 °C for 4 - 7 days until spores formed. The choice of sporulation media was strain specific. Most strains showed optimal sporulation on KAC and so this media was favoured. However all three recipes were used in the isolation of rare viable Frohberg spore isolates.

2.2.3 Tetrad dissection

Spores were treated with 100 µg/ml zymolase in dissection buffer for 15 minutes at 37 °C to digest the cell ascus. Excess dissection buffer was used to terminate digestion. Spores were then streaked onto YPD, and tetrads dissected by micromanipulation (using Singer Instruments Zeiss Axioskop 40 Tetrad platform). Spore viability is given as a percentage of total spores isolated.

Spore viability = (Number of colonies formed / (4 * number of tetrads dissected))* 100

2.2.3.1 Dissection buffer

1 M sorbitol

10 mM EDTA

10 µM sodium phosphate

pH 7.2

2.2.4 Plasmid DNA extraction

A single *E. coli* colony, was incubated overnight overnight in LB media (**2.1.9**) (with ampicillin) at 37 °C. Plasmid extraction was carried out using the

OMEGA biotech plasmid mini kit 1, using the supplied manufacturers' protocol. Plasmids used are listed in (**Table 2.3.2**).

2.2.5 Primer Design

Primers were designed using primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Primers used are listed in tables (**Table 2.3.1** and **Table 2.5.1**).

2.2.6 Polymerase Chain Reaction (PCR)

PCR amplification to produce transformation product used 1 µM gDNA per 50 µl reaction. The master mix consisted of: 5 µl 10x Kappa Taq A buffer (with Mg2+), 1 µl 10 mM dNTPs, 2.5 µl 10 µM forward primer and reverse primer, 0.5 µl Kappa Taq polymerase, 36.5 µl PCR grade water.

Amplification of product was as follows: initiation at 94 °C for 120 seconds, before 30 cycles of; 30 s denaturation at 94 °C, 30 s primer annealing at 55 °C, and 120 s extension at 72 °C. After 30 cycles a final extension step is performed at 72 °C for 10 minutes. For products >2 kb, the extension step time is increased, a conservative time of 1 minute per 1000 base pairs was used.

2.2.7 Colony PCR

This technique was used as a quick method of checking for the correct insertion of transformation products. A single colony is picked and suspended in 0.02 M NaOH, incubated at room temperature for 5 minutes, before vortexing. After brief centrifugation, the supernatant was used as the PCR template DNA. The reaction mix was as follows: 0.4 µl 10 mM dNTPs, 2 µl 10x Kappa Taq A buffer (with Mg2+), 1 µl of both forward and reverse primer (10 µm), 2 µl 10x rediLoad buffer, 0.2 µl Taq polymerase and 1 µl template DNA, made up to a volume of 20 µl with PCR grade H2O. Amplification followed the same steps as the regular PCR technique.

2.2.8 Agarose Gel Electrophoresis

To check PCR product, samples are run at 100 V for 40 minutes through a 1 % agarose gel. The agarose gel is made up in in 0.5 x TBE (Tris base 0.54

g/l, boric acid 2.75 g/l, 0.5 M EDTA (pH 8.0) 0.2 %). 3 µl Ethidium Bromide (EtBr) is added per 100 ml agarose solution.

2.2.9 Flow Cytometry

2.2.9.1 Sample Staining

Sytox Green (BioLegend, 1 µm working concentration) was used to stain DNA (Haase, 2003), and Cell Tracker DiD (ThermoFisher Scientific, 10 µm working concentration) was used as a membrane stain (Bell et al., 1998). To reduce cell clumping, all solutions used in the staining of samples were supplemented with EDTA (5 mM). Fresh overnight YPD cultures were grown and samples were stained with Cell Tracker DiD at a cell density of $1*10^8$ cells/ml at 23 °C for 45 minutes. Stained samples were washed three times in YPD, followed by a 30 minute incubation to remove unbound Cell Tracker DiD. Samples were resuspended in 1.5 ml H_2O and 3.5 ml 100 % ethanol was added slowly and incubated overnight at 4 °C to fix cells. Samples were treated with 2 mg/ml RNase A for at least 2 hours at 37 °C, before treatment with 5 mg/ml proteinase K for 45 minutes at 37 °C. Cells were fixed for a second time in formalin overnight, a prerequisite to guest access to the flow cytometry equipment. An aliquot of each sample, approximately $1*10^6$ cells/ml was then stained using Sytox Green immediately prior to assessing ploidy using flow cytometry. Samples were vortexed vigorously to break up clumps.

2.2.9.2 Sample Analysis

Samples were analysed within the University of Leicester Flow Cytometry Facility, using the Accuri C6 Plus flow cytometer (BD Biosciences). For measurement of the Sytox Green stain, excitation was achieved with the blue laser (488 nm) with absorbance detected using a 533/30 standard filter. The membrane stain cell tracker DiD was measured in the APC channel, excitation using the red laser (640 nm) and absorbance detected using the 675/25 standard filter. Cell tracker DiD stain was used to gate cells, to exclude debris from the ploidy analysis. Histograms were produced for each sample of Sytox Green stain intensity against cell number. Control strains of known ploidy were used to draw gates around the C1 and C2 peak for each ploidy level (2n, 3n, and 4n).

2.2.10 Phenotyping on Solid Media (PHENOS)

Phenotyping of individuals for Multipool QTL analysis, and assessment of variation in protoplast fusion isolates was performed using the PHENOS platform (Barton et al., 2018). Each plate analysed consisted of 96 individuals in quadruplicate in an array of 384 colonies. Arrays were replicated using the Rotor HDA (Singer Instruments), using long pin 384 repads. Arrays were maintained on YPD, before replicating to soft agar YPD plates (5 g/l agar) and incubated at 23 °C for 16 hours. After 16 hours arrays were replicated to phenotyping plates using the “punch-in” technique to normalise cell mass printed on each spot. The punch-in method involves using the maximum pinning pressure of the Rotor HDA with an overshoot set. The pin pad punctures the soft agarose, pushing excess cell mass up the individual pins. On replication to the experimental plate, only the cell mass on the pin tips is deposited.

Colony growth was measured using the FLUOstar Omega microplate reader. Absorbance readings were measured as optical density (OD) using the 595 nm filter. Prior to array pinning, a blank measurement was taken for each phenotyping plate and subtracted from all future measurements. After pinning, an initial OD measurement was taken for time zero and then subsequent measurements taken daily. Time points were combined into a single data file using the PHENOS software.

2.2.11 Mating Type PCR

The mating type of each strain was assessed by PCR (**2.2.6**) using three primers (**Table 2.2.1**) (Illuxley et al., 1990). If a strain contained an active MATα locus, the primers G156 and G157 would anneal to enable to amplification of a PCR product approximately 400 bp long. If a strain contained an active MATa locus the primers G156 and G158 would be able to anneal to and a PCR product of approximately 600 bp long would be produced (**Figure 2.2.1**). Strains heterozygous for the MAT locus would have both PCR products synthesised with a two band pattern produced on an electrophoresis gel (**2.2.8**).

Primer	Sequence	Target
G156	agtcacatcaagatcgtttatgg	MAT locus
G157	gcacggaatatgggactacttcg	MAT α specific
G158	actccacttcaagtaagagtttg	MAT a specific

Table 2.2.1 Primer Table for Mating Type PCR

Three primers were used to assess the mating type of each strain. G156 corresponded a region of the *MAT* locus unrelated to mating type. G157 was specific to a sequence of the *MATα* region, while G158 was specific to the *MATa* locus.

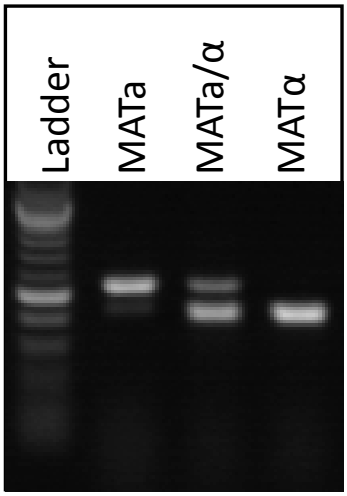

Figure 2.2.1 Mating Type PCR

PCR assay to determine the mating type of isolates. The presence of *MATa* gives rise to a band approximately 600 bp long. The presence of *MATα* gives rise to a band approximately 400 bp long. Both products will be amplified in strains that are heterozygous for the *MAT* locus.

2.3 Methods Specific to Chapter 3

2.3.1 Lithium Acetate Transformation

Transformations were carried out using the lithium acetate protocol (Daniel Gietz and Woods, 2002) (**Figure 2.3.1**). Briefly: 5 ml YPD (**2.1.1**) was inoculated with a single colony and incubated overnight, at the species specific growth temperature (see yeast culture conditions), in a shaking incubator (250 rpm). The following morning 4.5 ml YPD was inoculated with 500 µl overnight culture and incubated for 3.5 - 4 hours until cells reached mid exponential growth phase. Cells were harvested by centrifugation, and washed in 1 ml H_2O, before pelleted again. Cells were then resuspended in 1

ml 100 mM lithium acetate (LiAc), pelleted again by centrifugation and resuspended in 100 µl 100 mM LiAc. Cells were pelleted and supernatant removed once more before the transformation mix was added in the sequence of; 240 µl 50 % polyethylene glycol (PEG), 36 µl 1 M LiAc,40 µl 2 mg/ml single stranded carrier DNA (ssDNA), 1 µg DNA (transformation product), and H_2O to a total volume of 360 µl. After vigorous re-suspension of cell pellet, cells were incubated for 30 minutes, at the normal growth temperature of each species (23 °C or 30 °C). This was followed by a heat shock incubation to aid DNA uptake into cells. Due to variation between species, including the heat sensitive nature of multiple species, the heat shock temperature had to be optimised. For *S. cerevisiae* and other non-heat sensitive species, 30 minutes at 42 °C was used as standard. For heat sensitive species transformation was most efficient with 30 minutes incubation at 37 °C. (Scannell et al., 2011) provided a starting point for optimisation of this step. After heat shock, cells were pelleted and supernatant removed before being re-suspended in 1 ml YPD and incubated overnight at 4 °C. After incubation, cells were seeded onto selection media (**2.1.7**) for 4 – 6 days before transformants were picked.

2.3.2 Creation of PCR based transformation product

Primers (**Table 2.3.1**), between 63 and 79 base pairs (bp) long, were designed to amplify the selectable marker (either the hygromycin resistance gene or the kanamycin resistance gene), from the drug resistance cassettes used (Goldstein and McCusker, 1999, Wach et al., 1994). The 3' end of each primer was specific to the flanking region of the selectable marker (**Figure 2.3.1**a.1). The other portion of the primer conferred homology to the flanking region of the target gene. The species specific gene sequences were obtained from the *Saccharomyces* Genome Database (https://www.yeastgenome.org/). On PCR amplification of the drug cassette, a product was created which contained the gene conferring the antibiotic resistance. This gene was flanked on each end with homologous regions to those flanking the gene to be knocked out. A long region of homology increased the probability of recombination, resulting in a higher frequency of transformation.

2.3.3 Creation of Transformation Product by Plasmid Digest

The KanMX4 marker was previously used to delete the *URA3* gene in the *S. cerevisiae* haploids used here (Cubillos et al., 2009). For the *MAT* locus

deletion in *S. cerevisiae* strains, the *URA3* gene was used as a marker to identify transformants. The transformation product was created through the digestion of plasmid pRED266 (**Table 2.3.2**) by PvuII (**Figure 2.3.1**a.2). The fragment possessed a functional *URA3* gene flanked by the X, W, and Z2 sequences. These sequences also flank the mating type locus. This method was previously described by Greig (Greig et al., 2002a).

Primer	Sequence	Species	Usage
AH1	ACTATATTGATGGCCAATGGCGAAATTAAAGACATCGCTAATGTCc gtacgctgcaggtcgac	*S. uvarum* & *S. eubayanus*	*ho::HYG* forward
AH2	ACTCTTATGGGGTCCACGAACAGCGTCAAATTGCAAAATCCCCCCa tcgatgaattcgagctcg	*S. uvarum* & *S. eubayanus*	*ho::HYG* reverse
AH4	TCTCTTGCTTCCGTCGAA	*S. uvarum* & *S. eubayanus*	*ho::HYG* check primer upstream forward
A521	CGGCGGGAGATGCAATAGG	*S. uvarum* & *S. eubayanus*	*ho::HYG* check primer upstream reverse
AH6	CGATCAATGGAGTGACCGTA	*S. uvarum* & *S. eubayanus*	*ho::HYG* check primer downstream reverse
A522	TCGCCCGCAGAAGCGCGGCC	*S. uvarum* & *S. eubayanus*	*ho::HYG* check primer downstream forward
A560	AAAGAGGAGAATGTCGTTCAAGGTATGGTTGGTATCTAGCCAATA GGATTGTAGCCAGAGcgtacgctgcaggtcgac	*S. eubayanus*	*mat::KanMX* forward
A561	GTTCAAATTATAAAAGTCACATCAAGATCATTTATGGTCAAGATAA AGGCAAAGAAAGACatcgatgaattcgagctcg	*S. eubayanus*	*mat::KanMX* reverse
AH24	ACGCTACATACAAAGAACGTGCTGCTACTCATCCTAGCCCAGTTGc gtacgctgcaggtcgac	*S. uvarum*	*mat::KanMX* forward
AH25	CCCGCATCTCTTCAAGTAAGCTTCCCAGCCTGCTTTTCTGTAACGat cgatgaattcgagctcg	*S. uvarum*	*mat::KanMX* reverse

Table 2.3.1 Table of Primers

http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi was used to design primers for the deletion of the *HO* and *MAT* loci in *S. uvarum* and *S. eubayanus*. Sections of primer complementary to the flanking region of genes targeted for deletion are capitalised. Sections of primer complementary to the flanking region of the marker gene on the plasmid are designated in lower case. Primers to confirm successful *ho::HYG* transformants were also designed.

Plasmid	Alias	Description
pRED231	pS30 pFA6-KanMX4	Derived from pFA6 with KanMX cloned into the *PmeI* site. Confers resistance to Geneticin.
pRED460	pAG32	Derived from pRED231, with KanMX substituted by hph at the *PmeI* site. Confers resistance to hygromycin B.
pRED266	pFP18	Contains a URA3 gene flanked by the sequences of the X, W, and Z2 portions of the mating type locus.

Table 2.3.2 Table of Plasmids

Three plasmids were used in the generation of *de novo* hybrids. pRED460 was used to create stable haploids. pRED231 was used to create diploid maters in *S. eubayanus* and *S. uvarum* strains, whilst pRED266 was used to this effect in *S. cerevisiae* strains.

2.3.4 Generation of Petites

For each diploid mater, a petite version was generated by exposure to EtBr (Goldring et al., 1971). Ethidium bromide treatment induces mtDNA damage as well as complete loss of mtDNA (Goldring et al., 1970).

Isolates were seeded at an approximate density of 300 individuals per plate. A 3 µl drop of EtBr (10 mg/ml) was spotted onto each plate. A ring around the spot formed where all cells were killed due to the toxic effects of EtBr. Surrounding this kill zone, a ring of petite colonies form. Loss of mitochondria enables petites to grow faster than colonies with functional mitochondria, in the presence of EtBr. These individuals were confirmed as petites by their inability to grow on YEPEG plates (**2.1.6**) containing ethanol and glycerol, non-fermentable carbon sources (Day, 2013).

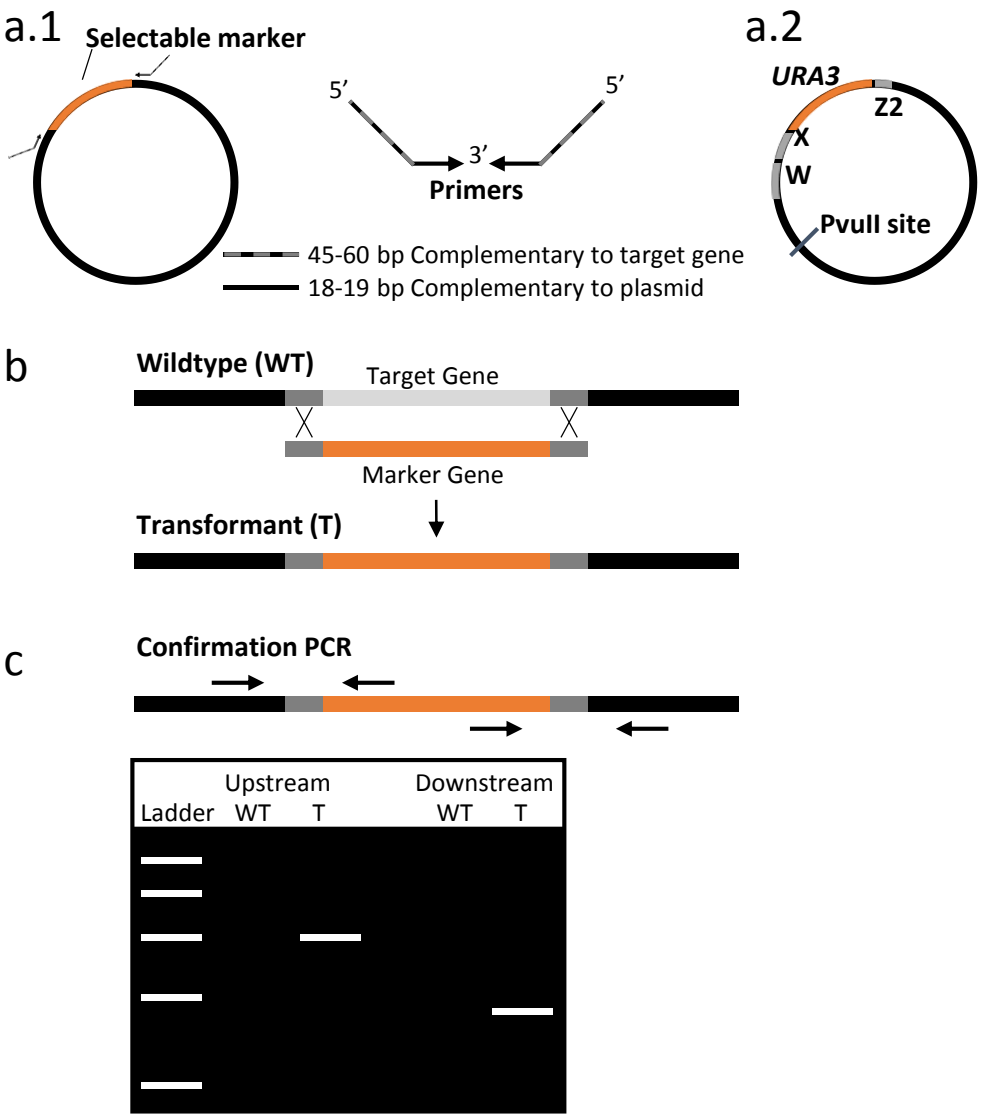

Figure 2.3.1 Transformation Methodology

Overview of methods involved in deletion of target genes. (a.1) selectable marker is amplified from plasmid construct. Primers were designed with 45 – 60 bp homology to target gene at their 5' end. (a.2) For deletion of MAT locus in *S. cerevisiae* pRED266 was digested by *PvuII* yielding a transformation product containing *URA3* flanked by complementary sequences of the W, X and Z2 portions of the mating type locus. (b) Homologous recombination occurs during transformation with the target gene switched out for the marker gene. (c) ho::HYG transformation is confirmed by PCR. Two sets of primers were designed. In the first set the forward primer was specific to the sequence upstream of the target gene the reverse primer was specific to a site within the selectable marker. The second set, the forward primer was specific to a site within the selectable marker, and the reverse primer was specific to the downstream region flanking the target gene.

2.3.5 Patched Mating

Intraspecific diploids were created by mating two haploid strains. Interspecific tetraploid hybrids were created by mating of two diploid maters. Mating was performed by patch mating. A small amount of each strain was mixed together on YPD, and incubated at 23 °C overnight. The following day the patch was streaked for singles, with characteristically large colonies picked (tetraploid colonies being larger than diploid colonies, which in turn being larger than haploid colonies). Colonies were confirmed as successfully mated isolates by renewed ability to sporulate. Haploids and diploid maters will not undergo sporulation, whereas diploids and tetraploids will. These isolates were also confirmed by mating type PCR (**Figure 2.2.1**). Successfully mated isolates will have a copy of each mating type locus.

2.3.6 Generation of Advanced Intercrossed Cell Lines (AILs)

Hybrids from each species cross were selected to be pushed through multiple rounds of intercrossing to reach the F12 generation (**Figure 2.3.2**). Each round of mating involved seeding a lawn of cells onto KAc (**2.1.3**) until > 80 % sporulation was achieved (5-7 days). Unsporulated cells were destroyed by Ethyl Ether treatment. Asci of tetrads were digested with zymolase, before vigorous vortexing to disperse spores to ensure inter-tetrad mating. Spores were plated onto YPD for two days to allow for germination. After 24 hours of germination, cells were collected and re-plated onto fresh YPD, in order to maximise chance of spore to spore mating. Ten percent of cell mass at each generation was collected in 25 % glycerol and stored at -80 °C, ready for future investigation. An Array of 384 F12 individuals for each hybrid was generated to allow for future analysis of individuals.

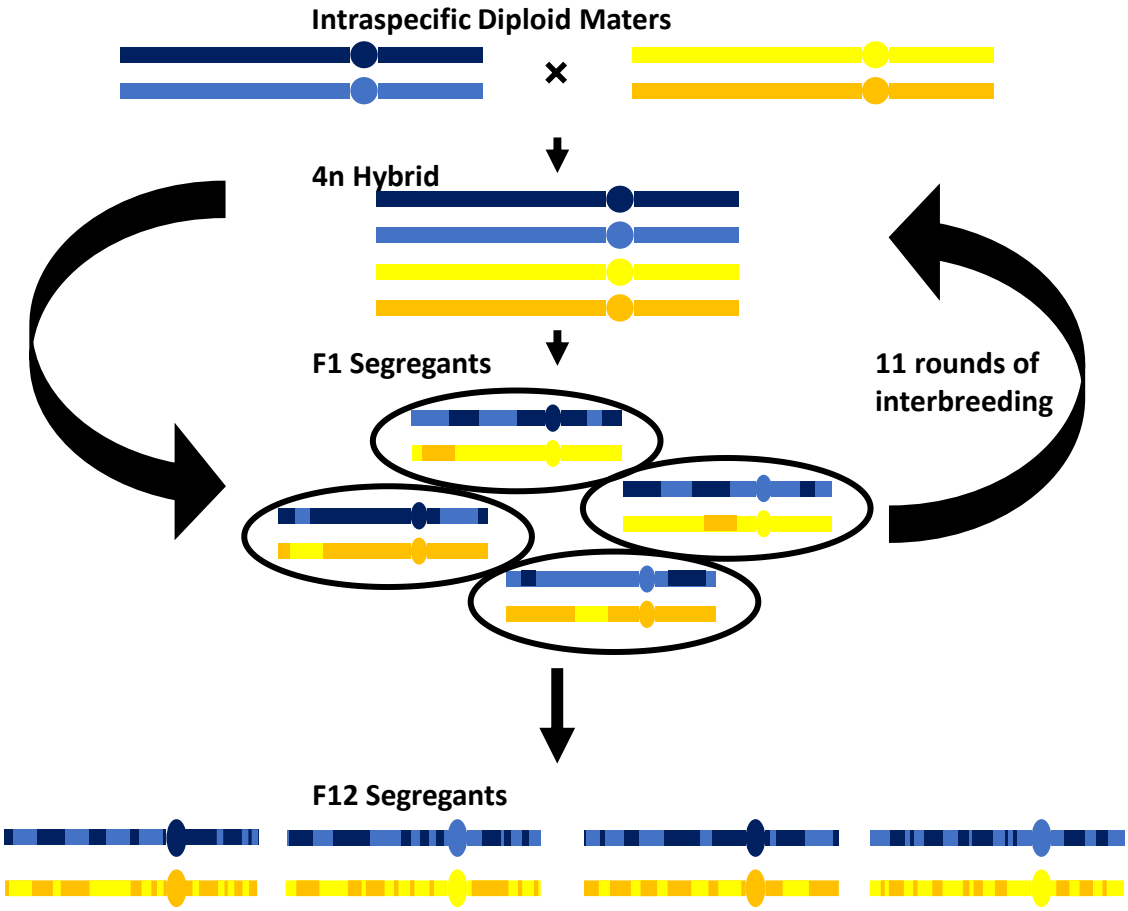

Figure 2.3.2 Multigenerational Interbreeding

An overview of the generation of Advanced Intercrossed Cell Lines. Intraspecific diploids are crossed generating a tetraploid hybrid. Sporulation produces diploid gametes, with crossovers occurring between sister chromosomes of the same parental species. Tetrads are broken up by zymolase treatment and vortexing. F1 gametes are allowed to mate within the population creating a new tetraploid population. This is repeated eleven times to create a genetically diverse F12 population, with each individual genome unique, after extensive rearrangements between sister parental genomes.

2.4 Methods Specific to Chapter 4

2.4.1 Pooled Selection

A pool of 1 * 10^8 F12 cells were seeded onto each selection condition as well as the YPD control (**Table 2.4.1**). The same starting population pool was serial diluted onto selection plates to obtain individuals. For each condition, 384 individuals were arrayed and stored in 20 % glycerol. Selection conditions were prepared so that only the top 0.1 – 1 % of the pool would be capable of growth (**Figure 2.4.1**). After four days the cells were washed off the selection

plates and collected in H_2O. 50 % was stored in glycerol, with DNA extracted for sequencing of the other 50 %.

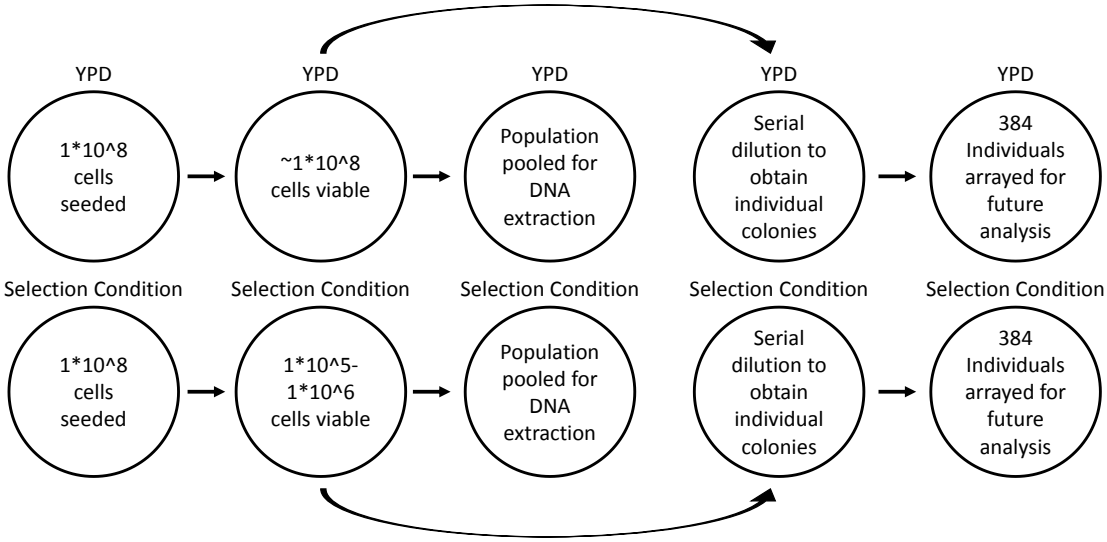

Figure 2.4.1 Selection methodology

1 * 10^8 F12 individuals were seeded onto either an YPD control plate or a selection plate. The selection condition was determined based upon 0.1 – 1 % of the total population capable of growth. Individuals were grown for 3 days before being pooled for DNA extraction. A fraction of the population was taken and serial diluted to obtain individual colonies that could be picked and arrayed for future analysis.

Strain	Selection Condition					
H51	Control YPD, 23 °C	Levulinic Acid 50 mM	High temperature 40 °C	Acetic Acid 0.35 %		
H179	Control YPD, 23 °C	Glucose 35 %	High Temperature 40 °C	Low Temperature 4 °C	H2O2 4 mM	Maltose 35 %
H188	Control YPD, 23 °C	Glucose 35 %	High Temperature 40 °C	Low Temperature 4 °C	H2O2 4 mM	Maltose 35 %

Table 2.4.1 Selection conditions

The selection conditions for each strain that were used for QTL analysis by pooled selection. The conditions for H179 and H188 were the same, to allow for direct comparison.

2.4.2 Multipool

Phenotyping was performed using the PHENOS platform (**2.2.10**). 384 F12 individuals were phenotyped for both H179 and H188. For each hybrid, four plates of 96 individuals arrayed in quadruplicate were grown on YPD. Using the Singer Rotor HDA, each array was replicated onto ‘soft’ YPD plates (standard YPD plates, with 0.5 g/l agarose instead of the normal 2 g/l) and

incubated at 23 °C overnight. The arrays were then replicated onto selection plates. To ensure an even seeding density, long pin re-pads were used at high pressure (100 %). This pressure causes the re-pad pins to pierce through the soft YPD plates, excess cell mass is pushed up the pins. When the re-pads are subsequently depositing cells onto the selection plates, an approximately equal amount is present on each pin. Change in absorbance (600 nm) was measured using the FLUOstar Omega plate reader (BMG Labtech). Prior to seeding cells on selection plates a blank reading was taken, with values subtracted from each time course reading. Plates were incubated at 23 °C (except for temperature selection plates) and absorbance measured frequently over the course of three days.

Individuals were ranked by the maximum change in absorbance, after normalising to the maximum change in absorbance under control conditions. In order to avoid plate to plate variation, plates were analysed individually. Each plate had 96 individuals in quadruplicate, from each plate the top five performing individuals, with the highest maximum change in absorbance, and the five lowest performing individuals were picked for pooling. Combining the top individuals from the four plates gave a pool of 20 individuals. Likewise there was a pool of 20 poor performing individuals.

2.4.3 DNA Extraction and Quality Control

High quality genomic DNA was obtained for sequencing using the E.Z.N.A.® Yeast DNA Kit (Omega Bio-tek), following the supplied column centrifugation protocol. In brief samples were cultured in liquid YPD to an OD_{600} of 10. Cells were treated with lyticase and β-mercaptoethanol for 30 minutes at 30 °C to generate spheroblasts. Cells were washed in TL buffer, before physical release of cell content by vortexing with glass beads for 5 minutes. A one hour proteinase K treatment at 55 °C enzymatically digested protein content. Supernatant was treated with RNase A, before vortexing at max speed to pellet remaining cell debris. BL buffer was added and incubated at 65 °C, followed by addition of pure ethanol. A vigorous vortex breaks up DNA precipitates. BL buffer and ethanol treatment prepare samples for optimal DNA binding to the HiBind® DNA Mini column. Column equilibration was performed by addition of 3 M NaOH, and centrifugation at max speed. Supernatant is loaded into the column and washed first with HBC buffer and

secondly twice with DNA wash buffer, to remove protein and salt contaminants. DNA was eluted in 60 µl elution buffer pre-heated to 65 °C.

The concentration and quality of DNA samples were analysed using the ThermoFisher Scientific NanaDrop™ 8000 spectrophotometer. Samples were required by the Earlham institute to be a minimum of 2-5 µg, to have a ratio of absorbance at 260 nm and 280 nm to be between 1.8 and 2.0 nm, as well as a ratio of 260 nm and 230 nm to be 1.8-2.4 nm. A 260/280 ratio of 1.8 nm is considered pure DNA, with lower ratios indicating presence of contaminants such as protein. The secondary indicator of nucleic acid purity is the 260/230 ratio. A lower ratio than 1.8 nm indicates possible contamination by salts.

For pooled selection experiments, the population was collected en-masse from the selection plate. The pooled sample was resuspended and diluted to an OD_{600} reading of 10 in H_2O. 3 ml of each sample was then taken for DNA extraction. For sequencing by multipool, the DNA of each individual was extracted and quantified, before pooling an equal DNA quantity from each individual.

2.4.4 Genome Sequencing and Analysis Script

Whole genome sequencing was performed using Illumina sequencing at the Earlham Institute. Data processing was performed by Yue Hu, a bioinformatician within the lab group. FastQC reports were produced for each sample, to assess the sequencing quality including base sequence quality, read depth, GC content and level of sequence duplication (**Figure 2.4.2**).FASTQ sequence files were aligned to the reference genome using the program BWA 0.7.17a (Li and Durbin, 2009). As hybrids were being sequenced the reference genome constituted a reference genome for each species that were stitched together. The *S. cerevisiae* reference genome was YPS128 (Liti et al., 2009). The *S. eubayanus* reference genome was Seub3.0, which was the sequence of the strain CBS12357 (Libkind et al., 2011). The *S. uvarum* reference genome was of ZP555 (Sampaio and Goncalves, 2008), which exists as a scaffold. Reads were subsequently locally realigned using the program Samtools (Li et al., 2009); reads were filters and base quality scores recalibrated using a reference set of known variant sites (Bergström et

al., 2014). The Program Picard 2.6.0 was used to mark duplications for deletion, and GATK was used to measure the depth of coverage.

Variants were identified in pooled samples using Freebayes/1.1.0, with ploidy set to 1 (the combined reference genome is for a diploid), the minimum alternate fraction was set at 0, the minimum mapping quality set to 30, and the minimum base quality set at 20. Allele frequencies in each pool were calculated using R, with analysed allele frequency differences between control and treatment used to define QTL intervals. Intervals were assigned when the allele frequency differed equal or greater than 0.2. Curves were smoothed using GGplot2/R. Fisher's exact test was used to confirm that the allele frequency at each interval peak was significantly different from the control allele frequency (Fisher, 1922). Each interval identified by sequencing of the selection pools (**4.2.1**) was significant ($p < 0.05$).

2.4.5 Candidate Gene Analysis

Genes that fell within each QTL interval were screened for reported function, and whether previous studies such as gene deletion or overexpression identified a change in a relevant phenotype to the selection condition. Genes were screened using the Saccharomyces Genome Database (SGD) YeastMine https://yeastmine.yeastgenome.org/yeastmine/bag.do.

Sequences of *S. cerevisiae* candidate genes were obtained from the SGD (https://www.yeastgenome.org/). The parental strain specific gene sequences were obtained by BLAST search, using the Saccharomyces Genome Resequencing Project BLAST server (Bergström et al., 2014, Liti et al., 2009) (http://www.moseslab.csb.utoronto.ca/sgrp/blast_original/). Sequences of genes identified within intervals of the *S. eubayanus* portion of the hybrid genomes were obtained from Ensembl Fungi (http://fungi.ensembl.org/index.html). This website only gave the species reference sequence (CBS12357, OS578) for each gene. The other *S. eubayanus* parental strain, OS626, was re-sequenced alongside the pooled selection samples. The gene sequences from these strains were then obtained from the consensus sequence after aligning reads to the reference sequence.

Nucleotide sequences were then translated into amino acid sequences (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Pairwise sequence alignment was performed using the EMBOSS Needle global alignment tool (https://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=emboss_needle-I20180822-154249-0005-53952414-p1m).

a

Measure	Value
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File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	3961936
Sequences flagged as poor quality	0
Sequence length	251
%GC	38

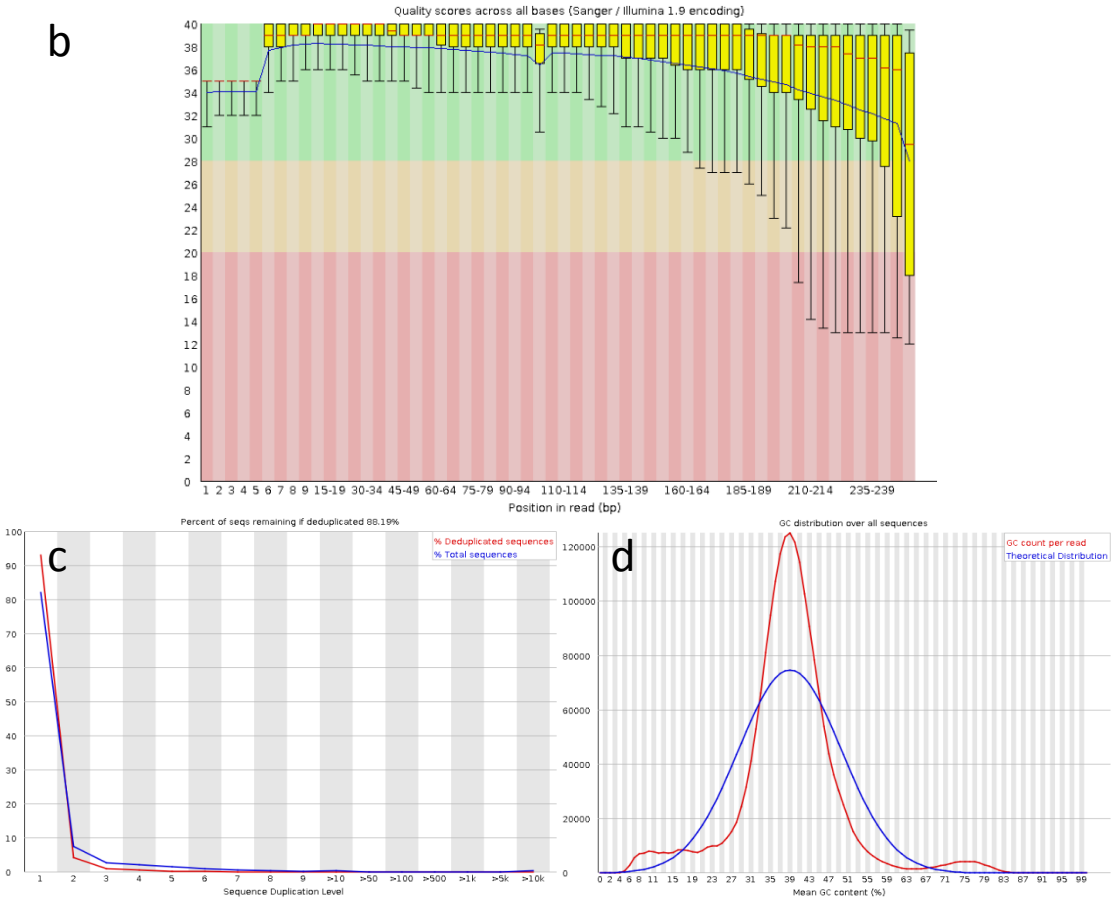


Figure 2.4.2 Quality Control Statistics

An example FastQC output using the control condition for H179. (a) Summary report of sequence reads, showing the total number of sequence reads; 3961936, and individual sequence length; 251. This gave an approximate sequencing coverage of 40x. (b) Fast QC per base sequencing quality. Sequence reads below a quality score of 20 (y axis) were excluded. (c) Graph of sequence duplication levels, excluding duplicates left 88.18 % of sequence reads. (d) Graph of sequence GC content, *S. cerevisiae* has approximately 40 % GC content, similar to each sample.

2.5 Methods Specific to Chapter 5

2.5.1 Rare Mating

Saaz strains and Haploid *S. cerevisiae* strains were cultured overnight in 5 ml YPD to give fresh cultures. Cultures were diluted 1/10 with fresh YPD and incubated for 3 – 4 hours until in exponential growth. 2 ml of the Saaz culture was mixed with 2 ml of either the *MATa* or the *MATα S. cerevisiae* haploid culture. Static incubation of the culture mix for 6 - 8 hours allowed for mating type pheromone gradients to arise and mating to occur. Cultures were pelleted, washed twice in sterile H_2O, plated onto SD-uracil media supplemented with G418, and grown at 23 °C for 4 - 6 days to select for mated isolates. The haploid *S. cerevisiae* are *ura3*$^-$, and incapable of growth on media that isn't supplemented with uracil. They however carry the *KanMX* gene that confers resistance to G418. The wildtype Saaz strains have a functional *URA3*, and are sensitive to G418. Only the isolates of rare mating (and protoplast fusion below) will be capable of growth on SD-uracil (**2.1.2.2**) + G418 media.

2.5.2 Protoplast Fusion

Protoplast fusion was attempted between Saaz strains and haploid *S. cerevisiae* strains (**Table 2.5.2**). The protocol used was adapted from (Curran and Bugeja, 1996), as follows. Overnight cultures were pelleted and washed in H_2O prior to resuspension in protoplasting solution, and incubated for 15 minutes at 23 °C. Protoplasts were collected by centrifugation at 3000g, and washed twice in 10 ml MP buffer. 2 x 10^7 protoplasts of each strain were mixed and divided into two tubes. One tube was resuspended in 2 ml 60 % (w/v) polyethylene glycol (PEG) and 0.1 M $CaCl_2$. The second tube, the control, was resuspended in 2 ml MP buffer and 0.1 M $CaCl_2$. Tubes were incubated at room temperature for three minutes, before the addition of 6 ml MP buffer and a further 6 minute incubation. Cells were washed and resuspended in MP buffer. Aliquots were added to 10 ml molten regeneration medium (kept at 40 °C, to avoid the agar setting), and plated onto pre-prepared plates of regeneration medium (**2.1.8**). Plates were incubated for six days to allow for cell wall reconstruction and colony growth. Colonies were picked and re-plated onto SD-uracil media, supplemented with G418.

2.5.2.1 MP Buffer and Protoplasting Solution

MP buffer was made up of 1 M sorbitol, 0.1 M NaCl, and 0.01 M acetic acid. The pH was adjusted to pH 5.5 by 1 M HCl or 2.5 M NaOH. Protoplasting solution was filter sterilised, and consisted of 3 mg/ml Zymolase 20T, in MP buffer and 1 M β-mercaptoethanol.

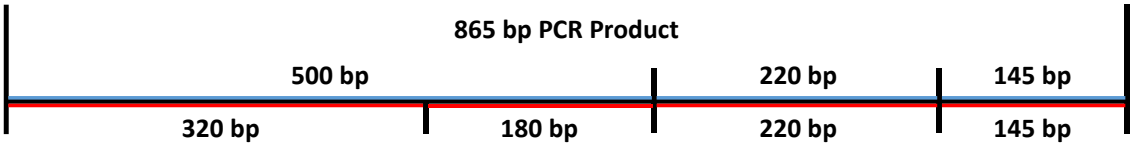
2.5.3 Species Identification by RFLP

Strain species and the genome content of hybrids were analysed by restriction fragment length polymorphisms (RFLP) of the rRNA intergenic transcribed spacer (ITS) region (McCullough et al., 1998, Esteve-Zarzoso et al., 1999, Guillamón et al., 1998). The internal transcribed spacer (ITS) region was amplified by PCR using primers ITS1 and ITS4 (**Table 2.5.1**) given a product 865 bp in length. The PCR product was digested by the *HaeIII* endonuclease, for 80 minutes at 37 °C. Restriction fragments were separated on a 2 % agarose gel (0.5x TBE) at 120 volts for 45 minutes. Saaz strains were distinguishable from *S. cerevisiae* strains by differences in their restriction fragments (**Figure 2.5.1**).

Primer	Sequence	Function
ITS1	TCCGTAGGTGAACCTGCGG	Species identification by RFLP
ITS4	TCCTCCGCTTATTGATATGC	Species identification by RFLP

Table 2.5.1 Primer Table

Primers used in the amplification of the ITS region to determine species of each strain. These sequences were obtained from Guillamón (Guillamón et al., 1998).



Yeast type	Bands	Restriction fragment lengths (bp)
Saaz	3	500, 220, 145
S. cerevisiae	4	320, 220, 180, 145
S. cerevisiae * Saaz	5	500, 320, 220, 180, 145

Figure 2.5.1 *HaeIII* Digest of the rRNA ITS Region.

Sequence variation in the ITS region can be used to identify the species of a strain by restriction fragment length polymorphisms (RFLP). An 865 bp sequence is amplified by PCR. When digested with *HaeIII*, four fragment lengths are observed for *S. cerevisiae* strains; 320, 220, 180, and 145 bp in length. Strains of the Saaz class give rise to three fragments; 500, 220, and 145 bp in length. Five fragment lengths are observed for protoplast fusion isolates; 500, 320, 220, 180, and 145 bp in length.

2.5.4 Strains Used

Using lager hybrid isolates stored in the OS strain collection, *S. pastorianus* strains were identified as belonging to either the Saaz class or Frohberg class based on RFLP of the ITS region (**Table 2.5.2**). Upon digestion by HaeIII, the Saaz strains gave a characteristic three band pattern (**Figure 2.5.1**), whereas Frohberg strains possessed the same banding pattern as *S. cerevisiae* strains. Frohberg strains appear to have inherited the ITS region from the *S. cerevisiae* parent whereas Saaz strains inherited this region from the *S. eubayanus* parent (Pham et al., 2011). Previously used *S. cerevisiae* haploid strains were used to cross with Saaz strains (**Table 2.5.2**).

Species	Strain Number	Alias	Genotype	Origin
S. cerevisiae	OS60a	CBS405	*MATa ho::HYG ura3::KanMX*	West Africa
	OS60α	CBS405	*MATα ho::HYG ura3::KanMX*	West Africa
Saaz	OS57	CBS1538	Wild Type	Netherlands
	OS320	CBS1462	Wild Type	London
Frohberg	OS56	DBVPG6560	Wild Type	Denmark
	OS311	CBS1483	Wild Type	Netherlands
	OS326	CBS2156	Wild Type	Netherlands
	OS327	CBS6903	Wild Type	Unknown

Table 2.5.2 Parental Strains

Strains used for attempting to introduce fertility to existing lager hybrids. Haploid *S. cerevisiae* strains OS60a and OS60α with genetic markers associated were crossed with the Saaz class *S. pastorianus* strains. Rare viable spores were isolated from four Frohberg strains.

2.5.5 Isolation of Rare Viable Spores

Frohberg strains were incubated in 5 ml pre-sporulation media (Guthrie and Fink, 1991) at 23 °C, 200 rpm for five days. Cells were then seeded onto three types of sporulation media; KAc, KAc Min, and CSH media (**2.1.3 – 2.1.5**). After seven days, cell mass was collected in H_2O using glass beads to gently resuspend cells from the plates. Cells were ether treated (0.5 ml ether : 0.5 ml H_2O) and vortexed for 10 minutes to destroy any unsporulated cells. Spores are protected from ether treatment by the thick ascus that surrounds tetrads. Spores were then treated with 1 mg/ml zymolase for 30 minutes at 37 °C, and vortexed to release spores from the digested ascus tetrads. Spores were then cultured on YPD at 23 °C for five days with colonies picked and stored.

2.5.5.1 Pre-sporulation Media

0.8 % (w/v) Yeast Extract

0.3 % (w/v) Bacto-peptone

0.25 % (w/v) D- Glucose

pH 7

2.6 Methods Specific to Chapter 6

2.6.1 Lager wort

The lager wort used was provided and produced on site by the AB-INBEV pilot brewery at Sutton Bonington, England. This wort sugar content was measured as 15 ° Plato (°P), and was supplemented with zinc (1 ppm). The Plato scale

is a measure of dissolved solids, which in wort is predominantly sugars, for every 1 °P there is 1g of sugar per 100g wort (Phillisкirk). Typical lager wort ranges from 7.5 °P to 25 °P in VHG wort (Boulton and Quain, 2007). The sugar composition was measured by High Performance Liquid Chromatography (HPLC) (**Figure 6.2.11**). The relative amounts of the key sugars were quantified as: 68 % maltose, 16 % maltotriose, 11 % glucose, 3 % sucrose, and 2 % fructose.

2.6.2 Biolog

For each experiment run, the SMCC strain was run in parallel for comparison and to normalise individuals within the experiment as well as between experiments, reducing read variation caused by any minor differences in media composition between plates. SMCC is an *S. pastorianus* lager strain, used industrially, and is a proprietary strain owned by AB-Inbev.

2.6.3 Plate Preparation

For all individuals other than H179 two plates were prepared per individual. One for the analysis of sugar metabolism, and the second for analysis of metabolic activity under stress conditions and in lager wort. Stock solutions were made for each condition. Each sugar solution consisted of YP (**2.1.1**, without glucose) plus the addition of each sugar at the specific concentration. To account for the volume of cell suspension to be added, each stock was prepared as 6/5th concentrated. Each stress condition consisted of YPD plus the appropriate concentration of each stress agent (ethanol, sorbitol, H_2O_2, menadione, and ISO-Hop). Previous studies found that 100 % wort was too turbid for quality measurement of metabolic activity. The measurement of the change in dye was disrupted by the deep wort colour. Instead the wort was diluted in H_2O to 50 %.

Adjustments to the conditions and plate layout were made for the experiments that analysed H179 individuals. From analysing the data of the other experiments, it was possible to reduce the number of concentrations for each condition analysed. This allowed all conditions to be run on one plate instead of two, reducing both time and cost. Two concentrations were run for each sugar and stress condition. The concentration of sugars was increased, allowing for analysis in higher gravity media. The higher concentration of

ethanol, 8 %, from previous experiments was kept. The second ethanol concentration was set to 12 % to increase the stress upon the individuals. Likewise the high concentration of sorbitol was kept, with the second concentration set to 40 % sorbitol. This would mimic the osmotic stress of a VHG wort. The two concentrations chosen for the oxidative stress agents were equivalent to the upper concentrations of the other experiments.

2.6.3.1 Cell Preparation

Fresh cell stocks were prepared through overnight culture in YPD at 23 °C. Cells were harvested, washed in sterile H_2O, and resuspended in 0.5 ml IFY-0 buffer (Biolog). Each cell suspension was added to 10 ml H_2O, in a sterile turbidometer tube, incrementally until 62 % transmittance was achieved. Prior to dilution, the cells in 0.5 ml buffer were vortexed to resuspend and separate any clumps.

2.6.3.2 Analysis

Biolog Redox Dye Mix D was added at a 1x concentration to each stock solution immediately prior to loading onto the Biolog MicroPlate. 100 µl media was added to each well prior to addition of 20 µl cell suspension – which was vortexed again prior to addition, giving a final volume of 120 µl. The outer edge wells were loaded with 100 µl YPD and used to measure the blank reading. Inoculated plates were loaded into the Omnilog, set to 20 °C, and incubated for 48 hours with absorbance measurements taken every 15 minutes. Each condition was run in triplicate, with the mean maximum metabolic activity of the individuals normalised to SMCC. Heat maps were created based upon raw absorbance readings (see Appendix 8.2) as well as after normalising to the SMCC lager strain (within text). The colour coding for the normalised heat maps is shown below (**Figure 2.6.1**).

Colour	Growth Relative to SMCC
	> 30 % stronger
	10 – 30 % stronger
	+/- 10 %
	10 - 25 % weaker
	25 – 50 % weaker
	> 50 % weaker

Figure 2.6.1 Colour code for heat maps

Colour code for each of the heat maps (below) displaying the phenotypic qualities of individuals normalised to the lager strain SMCC. Heat maps range from dark green in colour; when the metabolic activity of an individual is over 30 % higher than that of SMCC; to dark red, when individuals are over 50 % weaker than SMCC.

2.6.4 High Performance Liquid Chromatography (HPLC)

Wort sugar content was analysed by HPLC using the Dionex platform (Thermo Scientific). Chromatograms were analysed using the Chromeleon 6.70 QNT editor (Thermos Scientific). HPLC separates sugars from each other by their specific retention times. Peaks for each sugar were automatically assigned and quantified based on a program created and used routinely at the research brewery. This program was created using individual sugar standards to measure the specific retention time of each. All chromatograms were checked and erroneous peaks were reassigned manually. The stock calibration standard was created (Maltose 3 g/l, Sucrose 2 g/l, Fructose 2 g/l, Maltotriose 2 g/l, and Glucose 1 g/l, made up in ultra-pure H2O). Serial dilutions were made of the standard (100 %, 50 %, 25 %, 12.5 %, 6,25 %, 3.125 %), before each being diluted 1:100. The calibration standards were run in each experiment, to produce a standard concentration curve for each sugar. 1:1000 and 1:10000 dilutions of the raw wort were analysed, with 1:100 and 1:1000 dilutions of the end of fermentation wort being analysed. The sugar calibration curves are not linear above 4 g/l and so the final maltose and maltotriose content was analysed using the 1:1000 dilution. Sucrose, glucose and fructose content was analysed using the 1:100 sample dilution.

2.6.5 Fermentation

Samples were grown in liquid YPD at 15 °C shaking at 180 rpm for 3 days. Fresh YPD was added daily. An aliquot of each sample was stained with

methylene blue (1 %, Merck) and cell number was calculated using the Countstar (Aber instruments) automated yeast cell counter. Cells were pitched into 100 ml 15 °P lager wort; which was pre-cooled to 15 °C, at a rate of 1 * 10^6 /°P /ml. The viability of each strain was measured (**Figure 2.6.2**) to ensure that the cell number pitched referred to viable cells. The fermentation vessels were flat bottomed, and cylindrical narrowing sharply at the top. Fermentation vessels were sealed with rubber bungs, and fitted with a Bunsen valve to allow pressure to be released. A magnetic flea to maintain homogeneity of the sample was used with fermentation vessels were placed on magnetic plates. The weight was measured at time 0, and at regular intervals (3 – 4 times per day for the first 48 hours, before once daily) until no further weight loss was observed.

On completion of fermentation, cell number and viability was calculated using methylene blue and the Countstar cell counter. Aliquots of the finished wort were filter sterilised and stored at - 80 °C, before analysing the sugar content by HPLC. The wort alcohol content was measured using the Alcolyzer beer analysing system (Anton-Paar). The Alcolyzer uses densitometry to accurately measure alcohol content, repeatable to 0.01 % v/v. Wort pH was also measured using the Alcolyzer.

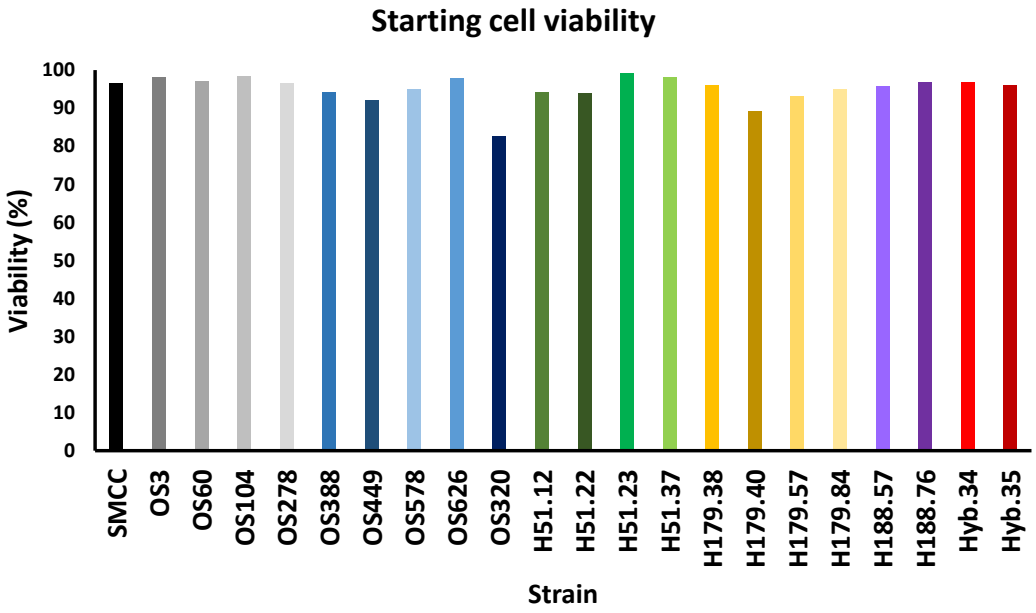


Figure 2.6.2 Pre-fermentation Cell Viability

Cell viability was assessed by methylene blue staining and quantification by the cellstar cell counter. For fermentation assessment, each individual was assigned a colour that remained consistent between figures.

3 Generation of De Novo Hybrids

3.1 Introduction

3.1.1 Chapter Aims

The first objective is to create a large set of fertile interspecific hybrids which will be generated through pairwise species crossing. Secondly isolates from each species cross will be put through a multigenerational mating regime to generate AILs. The genetic diversity generated through interspecies breeding and mass mating will open the door to QTL analysis (**Chapter 4**) as well as screening for individuals with desirable brewing traits (**Chapter 6**).

3.1.2 Application of Aims

In order to overcome hybrid sterility, each hybrid created possessed the genomes of four parental strains (**Table 3.1.1**). Two strains of species A, and two from species B (**Figure 3.1.2**). During meiosis, each chromosome pairs with the homologous chromosome of the same parental species. This will facilitate correct segregation as well as rearranging the genome creating genetic diversity in the progeny.

The mitochondria present in all *S. pastorianus* strains was inherited from the *S. eubayanus* parent. This mitotype is thought to have conferred a selection advantage over the existing brewing yeasts at the time. Low temperatures were used for brewing to reduce off-flavours. With the *S. eubayanus* mitotype, *S. pastorianus* was more efficient and so was likely inadvertently selected. To investigate the effect of mitochondrial origin, three sets of each cross will be created. One with the ‘species A’ mitotype, another with ‘species B’ mitotype and a third set possessing a mixed mitotype. In the third set competitive selection between the two species mitochondria will occur (Verspohl et al., 2018). Inheritance of mitochondria in yeast is biparental (Westermann, 2014), and new hybrids will initially be heteroplasmic. Through rounds of mitosis this becomes homoplasmic due to the bottle neck caused by only limited mtDNA inheritance into budding cells. Early isolation of individuals finds each with a unique mitochrondrial sequence, suggesting mitochondrial recombination is common. This was observed in the data collected by Parts, however was not discussed (Parts et al., 2011).

3.1.3 Strain Selection

S. cerevisiae, *S. uvarum*, and *S. eubayanus* were selected as Parental species due to two primary factors. Firstly, the two cold tolerant species are the most genetically distinct from *S. cerevisiae* within the *Saccharomyces* clade. The hybrids generated between these as a result have the broadest phenotypic range. Secondly, one of the key project aims was to create fertile hybrids similar to the lager brewing hybrid *S. pastorianus*. Using the parental species of *S. pastorianus* in the new hybrids was therefore essential.

Strains within each species were selected (**Table 3.1.1**) to maximise the phenotypic and genotypic diversity in the hybrids, other important factors were taken into consideration including the ability of strains to sporulate, spore viability, and good growth rate. Five *S. cerevisiae* strains were selected, one from each of the clean lineages (**Figure 3.1.1**). *S. uvarum* strains were picked based on geographic origin. On commencement of the project three *S. eubayanus* strains were available to the group; the type strain isolated in Patagonia (Libkind et al., 2011) and, conveniently for broadening diversity, two Asian isolates (Bing et al., 2014).

Species	Strain Number	Alias	Origin
S. cerevisiae	OS3	DBVPG6765	(Wine European) France
	OS60	CBS405	West Africa
	OS104	YPS128	(North America) Pennsylvania
	OS253	NRRL-Y12663	(Sake) African Palm wine strain
	OS278	UWOPS03-461.4	Malaysia
S. uvarum	OS274	UWOPS99-807.1.1	Argentina
	OS388	ZP 555	Canada
	OS449	A1	New Zealand
	OS471	A4	New Zealand
S. eubayanus	OS578	CBS12357	Patagonia
	OS626	LZSP32.1	West China
	OS627	CDFM212.1	Tibet

Table 3.1.1 Strains

The strains above were chosen for the creation of *de novo* interspecific hybrids; five *S. cerevisiae* strains, four *S. uvarum* and three *S. eubayanus* strains were picked. Each of the starting parental strains in the table were diploid. Strain number refers to the culture collection of the research group, and is used to refer to individuals throughout. The alias for each strain can be used to find information online regarding each. The geographic origin of each strain was given.

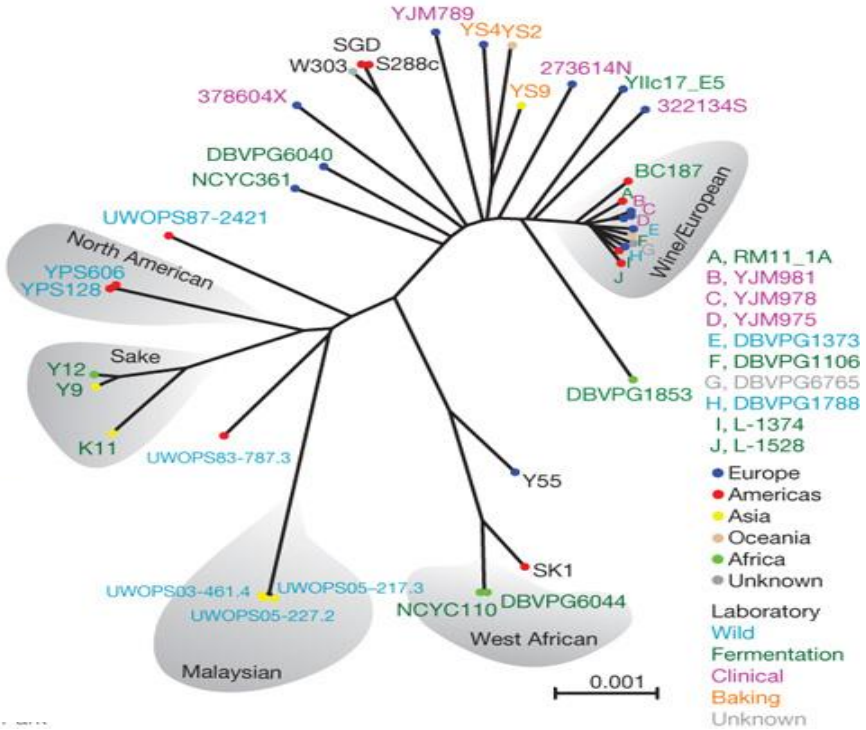


Figure 3.1.1 Phylogenetic Tree of the *S. cerevisiae* species

Neighbour-joining tree based upon SNP differences of *S. cerevisiae* strains. From each of the clean lineages (shaded in grey), a single strain has been taken for hybrid creation in this project. This tree has been borrowed from Liti, (Liti et al., 2009).

3.1.4 Hybrid Generation

It was necessary to manipulate the yeast strains, in order to direct the hybridisation process ensuring correct parental contribution to the desired hybrids. Stable hybrids of both mating types were generated for each parental strain, by deletion of the *HO* gene (**2.3.1**). Diploids were then made by mating between haploids opposing mating types (**2.3.5**). Diploid maters were subsequently created through the deletion of one *MAT* locus in each diploid (**2.3.1**). Since both copies of the HO gene had previously been deleted, these diploid maters were unable to switch mating type to self-mate. Finally, crossing between diploids of opposing mating types created fertile tetraploid hybrids. The mating types of each new isolate generated was confirmed by mating type PCR (**2.2.11**).

There were two options suitable for the generation of hybrids. The first option involved the creation of intraspecific diploid hybrids, the deletion of one mating type locus in each, before crossing diploid maters of differing species to create

interspecific tetraploid hybrids (**Figure 3.1.2**). There were four mating type configurations of the diploid gametes produced from sporulation of the hybrid. These were *MATa*, *MATα*, *MATa/α* – where both mating type loci were present, and *MAT-/-* – where neither mating type loci were inherited. The latter type behaves as if it were *MATa* when exposed to a *MATα* strain.

The alternative option involved creating interspecific diploids (**Figure 3.1.3**). Unlike the intraspecific diploids created in option 1, these diploids were sterile producing non-viable gametes. The *MAT* locus, from one species, was deleted in each hybrid. One diploid mater was then crossed to another diploid, which was formed from two different strains of the same parental species as the first diploid. The diploid gametes formed from this tetraploid hybrid were either *MATa* or *MATα*.

The first technique option was chosen for the creation of the hybrids in this project. Justification for this choice came from the resulting variation of gamete mating type. Diploid maters need to be produced to facilitate multigenerational mating. Both techniques achieve this, however the first also produced diploids that are heterozygous for the *MAT* locus. These are desirable products at the end of multigenerational interbreeding. These are stable diploids that whilst capable of sporulation, produce non-viable gametes. Thus when isolated they will not undergo further sporulation and mating, and so should be phenotypically stable. This would be a desirable trait for individuals chosen for production of lager for example, as it would be important for the new strain isolate to maintain consistency. In each round of the interbreeding process, the population is sporulated, and ether treated before allowing for mating between spores. Tetrad spores are encapsulated in a thick ascus. This ascus protects the spores from the ether treatment. Unsporulated cells are unprotected by this ascus and so are destroyed when exposed to ether treatment. When sporulated, the *MATa/α* diploids produce non-viable gametes and so will not be present in the next generation (i.e. F1 heterozygous diploids will not be carried forward to the F2 population). Those diploids with neither copy of the *MAT* locus will be unable to sporulate and will therefore be destroyed by ether treatment. A third, and more technical, reason for choosing the first method is that fewer diploids needed to be created. For a complete set of tetraploid hybrids between the three species, 19 diploids

were required. In contrast, creation method 2 required the creation of 47 diploids. The 19 created by method 1 would be suitable for making hybrids with further species, whereas new diploid hybrids would be needed using method 2.

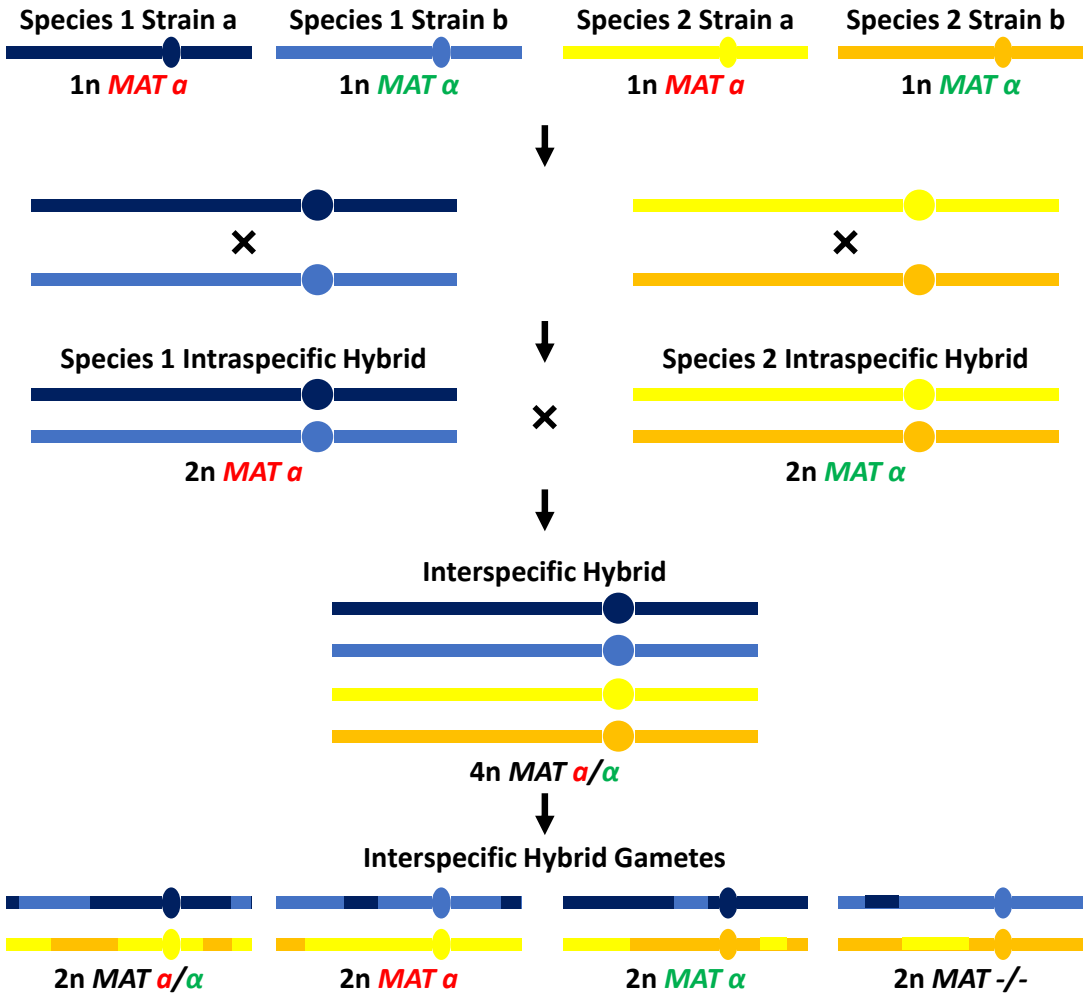


Figure 3.1.2 Hybrid Generation from Intraspecific Diploids

Option 1 for fertile hybrid generation. Each strain in the example cross is represented as a single chromosome. The cross involved two strains of 'species 1' (shades of blue), and two strains of 'species 2' (yellow and orange). The two haploid strains of species 1 were crossed to create an intraspecific diploid. The same was performed between haploids of species 2. In the species 1 diploid the *MATα* locus was deleted to create a diploid with a *MATa* mating type (red). The *MATa* locus was deleted in the species 2 diploid, which resulted in a *MATα* mating type (green). The two diploid maters were crossed to create an interspecific tetraploid with a haploid copy of each of the four parent strains. Diploid gametes had one copy of each parental species genome. At the MAT locus gametes were either heterozygous MATa/*α*, homozygous *MATa* or *MATα*, or lacking in either copy.

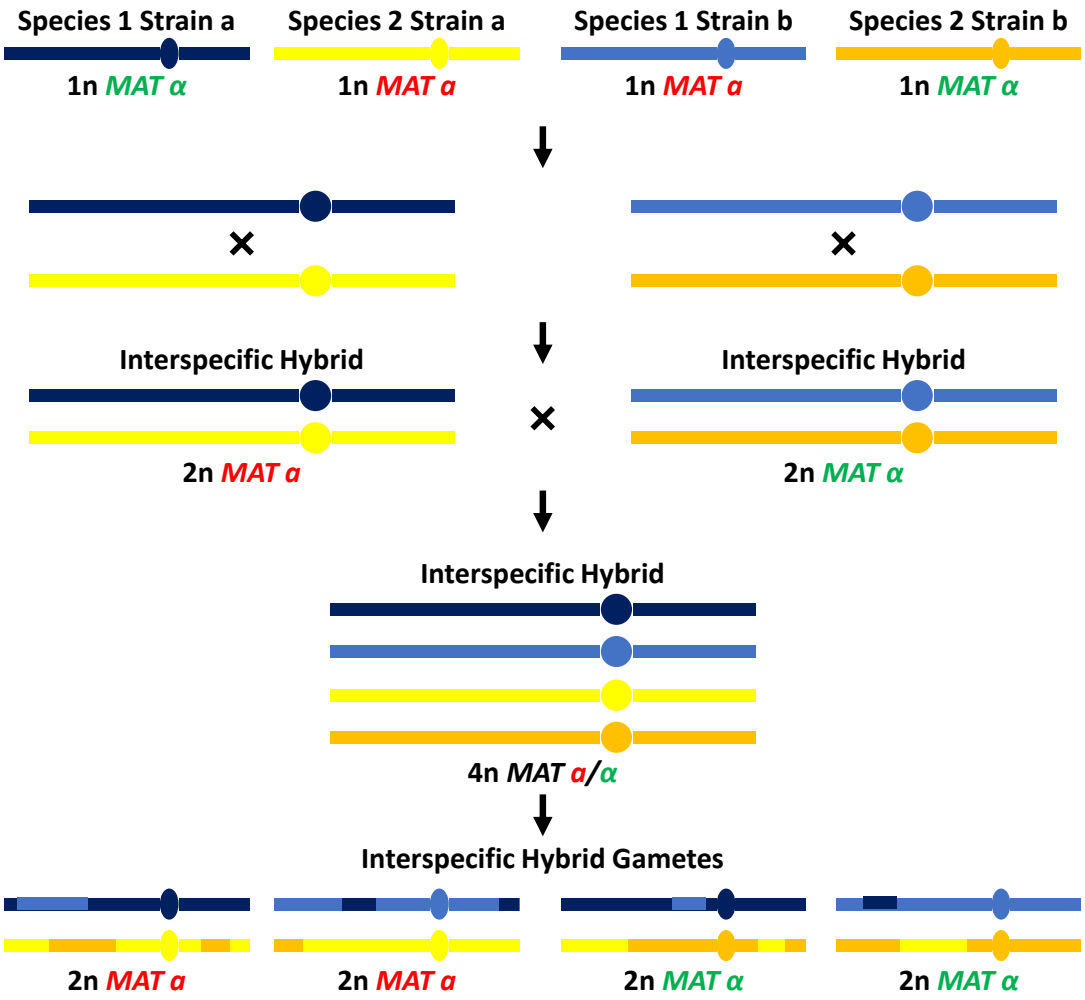


Figure 3.1.3 Hybrid Generation from Intraspecific Diploids

Option 2 for fertile hybrid generation. Each strain in the example cross is represented as a single chromosome. The cross involved two strains of 'species 1' (shades of blue), and two strains of 'species 2' (yellow and orange). Two diploid hybrids were created, each with one strain from each parental species. The *MAT* locus of the strain from species 1 was deleted. The two diploid maters were crossed to create an interspecific tetraploid with a haploid copy of each of the four parent strains. Diploid gametes had one copy of each parental species genome and were either *MATa* (red) or *MATα* (green).

3.2 Results

3.2.1 Haploid Generation

Haploids were generated through the deletion of the *HO* locus using the hygromycin B phosphotransferase gene (*hph*) as a selectable marker (Goldstein and McCusker, 1999) (**2.3.2**). Hph confers resistance to the antibiotic Hygromycin B. HYG interferes with tRNA recognition, resulting in misreading and disrupted protein synthesis due to incorrect amino acid incorporation. Hygromycin B phosphotransferase phosphorylates the cyclitol ring of HYG inhibiting its action upon tRNAs (Rao et al., 1983).

Diploid HYG^r colonies were dissected to isolate haploids with the *ho::HYG* genotype. Confirmation of the knockout was achieved by PCR (**Figure 2.2.1**) as well as the inability of isolates to sporulate. Haploids of both mating types were generated for each parental strain (**Table 3.2.1**). Haploids of the *S. cerevisiae* parental strains were created previously in the research group, as part of a set of genetically tractable strains (Cubillos et al., 2009).

Species	Strain Number	Collection Number	Genotype
S. cerevisiae	OS3	FEMS OS3a	*MATa ho::HYG ura3::KanMX-barcode[GGCCAT]*
		FEMS OS3α	*MATα ho::HYG ura3::KanMX-barcode[GGCCAT]*
	OS60	FEMS OS60a	*MATa ho::HYG ura3::KanMX-barcode[GCTAGC]*
		FEMS OS60α	*MATα ho::HYG ura3::KanMX-barcode[GCTAGC]*
	OS104	FEMS OS104a	*MATa ho::HYG ura3::KanMX-barcode[GGTACC]*
		FEMS OS104α	*MATα ho::HYG ura3::KanMX-barcode[GGTACC]*
	OS253	FEMS OS253a	*MATa ho::HYG ura3::KanMX-barcode[GTCGAC]*
		FEMS OS253α	*MATα ho::HYG ura3::KanMX-barcode[GTCGAC]*
	OS278	FEMS OS278a	*MATa ho::HYG ura3::KanMX-barcode[CCCGGG]*
		FEMS OS278α	*MATα ho::HYG ura3::KanMX-barcode[CCCGGG]*
S. uvarum	OS274	Q185	*MATa ho::HYG*
		Q188	*MATα ho::HYG*
	OS388	AM524	*MATa ho::HYG*
		AM525	*MATα ho::HYG*
	OS449	Q216	*MATa ho::HYG*
		Q215	*MATα ho::HYG*
	OS471	AM554	*MATa ho::HYG*
		AM555	*MATα ho::HYG*
S. eubayanus	OS578	Q183	*MATa ho::HYG*
		Q184	*MATα ho::HYG*
	OS626	Q261	*MATa ho::HYG*
		Q260	*MATα ho::HYG*
	OS627	Q218	*MATa ho::HYG*
		Q217	*MATα ho::HYG*

Table 3.2.1 Table of Haploids Generated

Haploids generated and used in the creation of intraspecific diploids. *S. cerevisiae* haploids were created previously, and have an *ura3::KanMX* deletion as well as *ho::HYG*. *S.eubayanus* and *S. uvarum* haploids were created here by deletion of the HO gene.

3.2.2 Intraspecific Diploid and Diploid Mater Generation

Using the haploids generated, all the possible intraspecific diploid hybrid combinations were produced (**Table 3.2.2**) by patched mating and picking of singles (**2.3.5**). Diploid colonies were characteristically larger than haploid

colonies. Diploids were confirmed by their ability to sporulate, and by mating type PCR. Successfully mated isolates possessed both mating types.

Diploid maters were generated through the deletion of one copy of the *MAT* locus. For each strain a *MATa* and a *MATα* isolate was achieved. *S. eubayanus* and *S. uvarum* diploids maters were confirmed by their resistance to Kanamycin, and inability to sporulate. *S. cerevisiae* diploid maters were isolated by their ability to grow on SD-uracil selection media, their inability to grow on FOA, as well as their inability to sporulate. The mating type of each was confirmed by mating type PCR. The *S. cerevisiae* cross between OS60 and OS253, failed to give rise to any diploid colonies. This cross was unsuccessful on three repeat attempts to mate. All others were successful.

Petites of *MATa S. cerevisiae* diploids were generated by treatment with EtBr (**2.3.4**). Using the same method *MATα S. eubayanus* and *S. uvarum* petites were created.

3.2.3 Generation of Interspecific Tetraploid Hybrids

All possible interspecific hybrids were generated, creating a set of 252 hybrids, through crossing between the intraspecific diploids of each species (**Table 3.2.3**). Hybrids were created by patched mating, and picking of colonies. Tetraploid colonies were characteristically larger than diploid colonies. Successful mating was confirmed through ability to sporulate along with viable gametes. These were confirmed a second time through mating type PCR, with both mating types observed. Three sets were created per species cross. One set had the mitochondria of ‘species A’, generated by mating with petites of ‘species B’. Another set was the reciprocal of the previous, generated by mating with petites of ‘species A’ to the diploids of ‘species B’ containing functional mitochondria. The third set was created by crossing diploids strains with functional mitochondria in both species. In crosses involving *S. cerevisiae*, the *MATa* strains were crossed with *MATα* strains, either petite or those possessing functional mitochondria, of *S. uvarum* and *S. eubayanus*. In crosses between *S. eubayanus* and *S. uvarum* diploids, MATa strains possessing functional mitochondria were mated with *MATα* petites. In the third

set between these two species *S. eubayanus MATa* strains were crossed with *MATα S. uvarum* strains.

From the set of 252 hybrids 28 were selected for multigenerational interbreeding (**2.3.6**) (**Table 3.2.4**). Selection was based upon an observation of strong spore viability (10 tetrads were dissected per hybrid), strong spore growth under control conditions (16 spores analysed from each), and fast sporulation time (majority of cells underwent sporulation within five days). Eight hybrids were selected from a *S. cerevisiae* x *S. uvarum* cross, and ten each from the crosses of *S. cerevisiae* x *S. eubayanus*, and *S. eubayanus* x *S. uvarum*. Hybrid ploidy was confirmed by flow cytometry analysis of DNA content using the DNA binding Sytox green dye. The *de novo* hybrids had two distinct peaks of approximately the same intensity as the peaks observed in the tetraploid control (**Figure 3.2.1**-d and -e respectively). The gametes of the de novo hybrids had two distinct peaks of equal intensity to the peaks in the diploid control (**Figure 3.2.1**-f and -b respectively). Some unexpected peaks were observed in both the ploidy control samples and the constructed hybrids and their gametes. The haploid control (**Figure 3.2.1**a) had a small peak approximately twice the intensity of the C2 peak. The tetraploid control (**Figure 3.2.1**d) had a third peak half the intensity of the expected C1 peak. The de novo hybrids generally had a small third peak of half the stain intensity to the expected tetraploid C1 peak, and a third peak was observed in the gamete samples at twice the intensity of the expected diploid C2 peak.

The viability of the F12 population was assessed for each. Interestingly, despite the two species being closely related within the *Saccharomyces* clade, the hybrids of *S. eubayanus* and *S. uvarum* in general displayed lower viability than hybrids from the other two species crosses. The average viability of these hybrids was 74 %, compared with 79 % in those between *S. cerevisiae* and *S. uvarum,* and 85 % in those between *S. cerevisiae* and *S. eubayanus*. The observed lower viability may be due to their close genetic relationship. *S. cerevisiae* tetraploids have lower viability than any of the interspecies tetraploids created, as well as the *S. cerevisiae* diploid equivalent (Greig et al., 2002a). This was thought to be due to all four homologous chromosomes interacting, instead of a single pair. Recombination is likely to occur more frequently between closely related chromosomes, than more distantly related.

This occurring between the *S. uvarum* and *S. eubayanus* chromosomes will decrease the spore viability of the hybrids.

Attempts to synthesise tetraploid hybrids using the diploid maters of OS626 and OS627; Q387, Q383, and Q384, were unsuccessful (**Table 3.2.2**). Without any clear indication as to the cause of this, the project proceeded with the set of newly created hybrids, which exceeded 250 unique isolates.

Species	Strains Crossed	Collection Number	*MAT*a Collection Number	*MAT*α Collection Number	*MAT*a Petite	*MAT*α Petite
S. cerevisiae	OS3 x OS60	Q368	Q324	Q325	Q345	
	OS3 x OS104	Q372	Q332	Q333	Q349	
	OS3 x OS253	Q367	Q322	Q323	Q341	
	OS3 x OS278	Q373	Q334	Q335	Q350	
	OS60 x OS104	Q369	Q326	Q327	Q346	
	OS60 x OS253					
	OS60 x OS278	Q370	Q328	Q329	Q347	
	OS104 x OS253	Q374	Q336	Q337	Q351	
	OS104 x OS278	Q371	Q330	Q331	Q348	
	OS253 x OS278	Q375	Q338	Q339	Q352	
S. uvarum	OS274 x OS388	AM651	AM677	AM661		AM678
	OS274 x OS449	AM652	AM664	AM665		AM680
	OS274 x OS471	AM653	AM662	AM663		AM679
	OS388 x OS449	AM655	AM670	AM671		AM683
	OS388 x OS471	AM654	AM666	AM667		AM681
	OS449 x OS471	AM656	AM668	AM669		AM682
S. eubayanus	OS578 x OS626	Q278	Q342	Q340		Q344
	OS578 x OS627	Q279	Q376	Q343		Q355
	OS626 x OS627	Q280	Q387	Q383		Q384

Table 3.2.2 Generation of Diploids and Derivatives

Diploids were generated by crossing haploids within each species. Nine *S. cerevisiae* diploids were created, with a tenth (OS60 x OS253) failing to be synthesised. Six *S. uvarum* and three *S. eubayanus* diploids were made. Deletion of a single MAT locus created diploid maters of each diploid. Petites were made by EtBr treatment.

Collection of *De Novo* Hybrids			
Species Crossed	**Collection number**	**Diploids Crossed**	**Mitotype**
S. cerevisiae* x *S. uvarum	H1	(ELOS274 x ELOS388) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H2	(ELOS274 x ELOS388) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H3	(ELOS274 x ELOS388) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H4	(ELOS274 x ELOS388) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H5	(ELOS274 x ELOS388) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H6	(ELOS274 x ELOS388) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H7	(ELOS274 x ELOS388) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H8	(ELOS274 x ELOS388) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H9	(ELOS274 x ELOS388) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H10	(ELOS274 x ELOS471) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H11	(ELOS274 x ELOS471) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H12	(ELOS274 x ELOS471) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H13	(ELOS274 x ELOS471) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H14	(ELOS274 x ELOS471) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H15	(ELOS274 x ELOS471) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H16	(ELOS274 x ELOS471) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H17	(ELOS274 x ELOS471) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H18	(ELOS274 x ELOS471) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H19	(ELOS274 x ELOS449) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H20	(ELOS274 x ELOS449) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H21	(ELOS274 x ELOS449) x (ELOS60 x ELOS104)	*S. cerevisiae*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H22	(ELOS274 x ELOS449) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H23	(ELOS274 x ELOS449) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H24	(ELOS274 x ELOS449) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H25	(ELOS274 x ELOS449) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H26	(ELOS274 x ELOS449) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H27	(ELOS274 x ELOS449) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H28	(ELOS388 x ELOS471) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H29	(ELOS388 x ELOS471) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H30	(ELOS388 x ELOS471) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H31	(ELOS388 x ELOS471) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H32	(ELOS388 x ELOS471) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H33	(ELOS388 x ELOS471) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H34	(ELOS388 x ELOS471) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H35	(ELOS388 x ELOS471) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H36	(ELOS388 x ELOS471) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H37	(ELOS449 x ELOS471) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H38	(ELOS449x ELOS471) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H39	(ELOS449 x ELOS471) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H40	(ELOS449 x ELOS471) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H41	(ELOS449 x ELOS471) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H42	(ELOS449 x ELOS471) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H43	(ELOS449 x ELOS471) x (ELOS3 x ELOS278)	*S. cerevisiae*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H44	(ELOS449 x ELOS471) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H45	(ELOS449 x ELOS471) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H46	(ELOS388 x ELOS449) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H47	(ELOS388 x ELOS449) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H48	(ELOS388 x ELOS449) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H49	(ELOS388 x ELOS449) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H50	(ELOS388 x ELOS449) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H51	(ELOS388 x ELOS449) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H52	(ELOS388 x ELOS449) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H53	(ELOS388 x ELOS449) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H54	(ELOS388 x ELOS449) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H55	(ELOS274 x ELOS388) x (ELOS3 x ELOS253)	*S. uvarum*
	H56	(ELOS274 x ELOS388) x (ELOS3 x ELOS60)	*S. uvarum*
	H57	(ELOS274 x ELOS388) x (ELOS60 x ELOS104)	*S. uvarum*
	H58	(ELOS274 x ELOS388) x (ELOS60 x ELOS278)	*S. uvarum*
	H59	(ELOS274 x ELOS388) x (ELOS104 x ELOS278)	*S. uvarum*
	H60	(ELOS274 x ELOS388) x (ELOS3 x ELOS104)	*S. uvarum*
	H61	(ELOS274 x ELOS388) x (ELOS3 x ELOS278)	*S. uvarum*
	H62	(ELOS274 x ELOS388) x (ELOS104 x ELOS253)	*S. uvarum*
	H63	(ELOS274 x ELOS388) x (ELOS253 x ELOS278)	*S. uvarum*
	H64	(ELOS274 x ELOS471) x (ELOS3 x ELOS253)	*S. uvarum*
	H65	(ELOS274 x ELOS471) x (ELOS3 x ELOS60)	*S. uvarum*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H66	(ELOS274 x ELOS471) x (ELOS60 x ELOS104)	*S. uvarum*
	H67	(ELOS274 x ELOS471) x (ELOS60 x ELOS278)	*S. uvarum*
	H68	(ELOS274 x ELOS471) x (ELOS104 x ELOS278)	*S. uvarum*
	H69	(ELOS274 x ELOS471) x (ELOS3 x ELOS104)	*S. uvarum*
	H70	(ELOS274 x ELOS471) x (ELOS3 x ELOS278)	*S. uvarum*
	H71	(ELOS274 x ELOS471) x (ELOS104 x ELOS253)	*S. uvarum*
	H72	(ELOS274 x ELOS471) x (ELOS253 x ELOS278)	*S. uvarum*
	H73	(ELOS274 x ELOS449) x (ELOS3 x ELOS253)	*S. uvarum*
	H74	(ELOS274 x ELOS449) x (ELOS3 x ELOS60)	*S. uvarum*
	H75	(ELOS274 x ELOS449) x (ELOS60 x ELOS104)	*S. uvarum*
	H76	(ELOS274 x ELOS449) x (ELOS60 x ELOS278)	*S. uvarum*
	H77	(ELOS274 x ELOS449) x (ELOS104 x ELOS278)	*S. uvarum*
	H78	(ELOS274 x ELOS449) x (ELOS3 x ELOS104)	*S. uvarum*
	H79	(ELOS274 x ELOS449) x (ELOS3 x ELOS278)	*S. uvarum*
	H80	(ELOS274 x ELOS449) x (ELOS104 x ELOS253)	*S. uvarum*
	H81	(ELOS274 x ELOS449) x (ELOS253 x ELOS278)	*S. uvarum*
	H82	(ELOS388 x ELOS471) x (ELOS3 x ELOS253)	*S. uvarum*
	H83	(ELOS388 x ELOS471) x (ELOS3 x ELOS60)	*S. uvarum*
	H84	(ELOS388 x ELOS471) x (ELOS60 x ELOS104)	*S. uvarum*
	H85	(ELOS388 x ELOS471) x (ELOS60 x ELOS278)	*S. uvarum*
	H86	(ELOS388 x ELOS471) x (ELOS104 x ELOS278)	*S. uvarum*
	H87	(ELOS388 x ELOS471) x (ELOS3 x ELOS104)	*S. uvarum*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H88	(ELOS388 x ELOS471) x (ELOS3 x ELOS278)	*S. uvarum*
	H89	(ELOS388 x ELOS471) x (ELOS104 x ELOS253)	*S. uvarum*
	H90	(ELOS388 x ELOS471) x (ELOS253 x ELOS278)	*S. uvarum*
	H91	(ELOS449 x ELOS471) x (ELOS3 x ELOS253)	*S. uvarum*
	H92	(ELOS449x ELOS471) x (ELOS3 x ELOS60)	*S. uvarum*
	H93	(ELOS449 x ELOS471) x (ELOS60 x ELOS104)	*S. uvarum*
	H94	(ELOS449 x ELOS471) x (ELOS60 x ELOS278)	*S. uvarum*
	H95	(ELOS449 x ELOS471) x (ELOS104 x ELOS278)	*S. uvarum*
	H96	(ELOS449 x ELOS471) x (ELOS3 x ELOS104)	*S. uvarum*
	H97	(ELOS449 x ELOS471) x (ELOS3 x ELOS278)	*S. uvarum*
	H98	(ELOS449 x ELOS471) x (ELOS104 x ELOS253)	*S. uvarum*
	H99	(ELOS449 x ELOS471) x (ELOS253 x ELOS278)	*S. uvarum*
	H100	(ELOS388 x ELOS449) x (ELOS3 x ELOS253)	*S. uvarum*
	H101	(ELOS388 x ELOS449) x (ELOS3 x ELOS60)	*S. uvarum*
	H102	(ELOS388 x ELOS449) x (ELOS60 x ELOS104)	*S. uvarum*
	H103	(ELOS388 x ELOS449) x (ELOS60 x ELOS278)	*S. uvarum*
	H104	(ELOS388 x ELOS449) x (ELOS104 x ELOS278)	*S. uvarum*
	H105	(ELOS388 x ELOS449) x (ELOS3 x ELOS104)	*S. uvarum*
	H106	(ELOS388 x ELOS449) x (ELOS3 x ELOS278)	*S. uvarum*
	H107	(ELOS388 x ELOS449) x (ELOS104 x ELOS253)	*S. uvarum*
	H108	(ELOS388 x ELOS449) x (ELOS253 x ELOS278)	*S. uvarum*
	H109	(ELOS274 x ELOS388) x (ELOS3 x ELOS253)	*mixed*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H110	(ELOS274 x ELOS388) x (ELOS3 x ELOS60)	*mixed*
	H111	(ELOS274 x ELOS388) x (ELOS60 x ELOS104)	*mixed*
	H112	(ELOS274 x ELOS388) x (ELOS60 x ELOS278)	*mixed*
	H113	(ELOS274 x ELOS388) x (ELOS104 x ELOS278)	*mixed*
	H114	(ELOS274 x ELOS388) x (ELOS3 x ELOS104)	*mixed*
	H115	(ELOS274 x ELOS388) x (ELOS3 x ELOS278)	*mixed*
	H116	(ELOS274 x ELOS388) x (ELOS104 x ELOS253)	*mixed*
	H117	(ELOS274 x ELOS388) x (ELOS253 x ELOS278)	*mixed*
	H118	(ELOS274 x ELOS471) x (ELOS3 x ELOS253)	*mixed*
	H119	(ELOS274 x ELOS471) x (ELOS3 x ELOS60)	*mixed*
	H120	(ELOS274 x ELOS471) x (ELOS60 x ELOS104)	*mixed*
	H121	(ELOS274 x ELOS471) x (ELOS60 x ELOS278)	*mixed*
	H122	(ELOS274 x ELOS471) x (ELOS104 x ELOS278)	*mixed*
	H123	(ELOS274 x ELOS471) x (ELOS3 x ELOS104)	*mixed*
	H124	(ELOS274 x ELOS471) x (ELOS3 x ELOS278)	*mixed*
	H125	(ELOS274 x ELOS471) x (ELOS104 x ELOS253)	*mixed*
	H126	(ELOS274 x ELOS471) x (ELOS253 x ELOS278)	*mixed*
	H127	(ELOS274 x ELOS449) x (ELOS3 x ELOS253)	*mixed*
	H128	(ELOS274 x ELOS449) x (ELOS3 x ELOS60)	*mixed*
	H129	(ELOS274 x ELOS449) x (ELOS60 x ELOS104)	*mixed*
	H130	(ELOS274 x ELOS449) x (ELOS60 x ELOS278)	*mixed*
	H131	(ELOS274 x ELOS449) x (ELOS104 x ELOS278)	*mixed*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H132	(ELOS274 x ELOS449) x (ELOS3 x ELOS104)	*mixed*
	H133	(ELOS274 x ELOS449) x (ELOS3 x ELOS278)	*mixed*
	H134	(ELOS274 x ELOS449) x (ELOS104 x ELOS253)	*mixed*
	H135	(ELOS274 x ELOS449) x (ELOS253 x ELOS278)	*mixed*
	H136	(ELOS388 x ELOS471) x (ELOS3 x ELOS253)	*mixed*
	H137	(ELOS388 x ELOS471) x (ELOS3 x ELOS60)	*mixed*
	H138	(ELOS388 x ELOS471) x (ELOS60 x ELOS104)	*mixed*
	H139	(ELOS388 x ELOS471) x (ELOS60 x ELOS278)	*mixed*
	H140	(ELOS388 x ELOS471) x (ELOS104 x ELOS278)	*mixed*
	H141	(ELOS388 x ELOS471) x (ELOS3 x ELOS104)	*mixed*
	H142	(ELOS388 x ELOS471) x (ELOS3 x ELOS278)	*mixed*
	H143	(ELOS388 x ELOS471) x (ELOS104 x ELOS253)	*mixed*
	H144	(ELOS388 x ELOS471) x (ELOS253 x ELOS278)	*mixed*
	H145	(ELOS449 x ELOS471) x (ELOS3 x ELOS253)	*mixed*
	H146	(ELOS449x ELOS471) x (ELOS3 x ELOS60)	*mixed*
	H147	(ELOS449 x ELOS471) x (ELOS60 x ELOS104)	*mixed*
	H148	(ELOS449 x ELOS471) x (ELOS60 x ELOS278)	*mixed*
	H149	(ELOS449 x ELOS471) x (ELOS104 x ELOS278)	*mixed*
	H150	(ELOS449 x ELOS471) x (ELOS3 x ELOS104)	*mixed*
	H151	(ELOS449 x ELOS471) x (ELOS3 x ELOS278)	*mixed*
	H152	(ELOS449 x ELOS471) x (ELOS104 x ELOS253)	*mixed*
	H153	(ELOS449 x ELOS471) x (ELOS253 x ELOS278)	*mixed*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae* x *S. uvarum	H154	(ELOS388 x ELOS449) x (ELOS3 x ELOS253)	*mixed*
	H155	(ELOS388 x ELOS449) x (ELOS3 x ELOS60)	*mixed*
	H156	(ELOS388 x ELOS449) x (ELOS60 x ELOS104)	*mixed*
	H157	(ELOS388 x ELOS449) x (ELOS60 x ELOS278)	*mixed*
	H158	(ELOS388 x ELOS449) x (ELOS104 x ELOS278)	*mixed*
	H159	(ELOS388 x ELOS449) x (ELOS3 x ELOS104)	*mixed*
	H160	(ELOS388 x ELOS449) x (ELOS3 x ELOS278)	*mixed*
	H161	(ELOS388 x ELOS449) x (ELOS104 x ELOS253)	*mixed*
	H162	(ELOS388 x ELOS449) x (ELOS253 x ELOS278)	*mixed*
S. cerevisiae x S. eubayanus	H163	(ELOS578 x ELOS626) x (ELOS3 x ELOS253)	mixed
	H164	(ELOS578 x ELOS626) x (ELOS3 x ELOS60)	mixed
	H165	(ELOS578 x ELOS626) x (ELOS60 x ELOS104)	mixed
	H166	(ELOS578 x ELOS626) x (ELOS60 x ELOS278)	mixed
	H167	(ELOS578 x ELOS626) x (ELOS104 x ELOS278)	mixed
	H168	(ELOS578 x ELOS626) x (ELOS3 x ELOS104)	mixed
	H169	(ELOS578 x ELOS626) x (ELOS3 x ELOS278)	mixed
	H170	(ELOS578 x ELOS626) x (ELOS104 x ELOS253)	mixed
	H171	(ELOS578 x ELOS626) x (ELOS253 x ELOS278)	mixed
	H172	(ELOS578 x ELOS626) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H173	(ELOS578 x ELOS626) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H174	(ELOS578 x ELOS626) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H175	(ELOS578 x ELOS626) x (ELOS60 x ELOS278)	*S. cerevisiae*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae x S. eubayanus	H176	(ELOS578 x ELOS626) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H177	(ELOS578 x ELOS626) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H178	(ELOS578 x ELOS626) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H179	(ELOS578 x ELOS626) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H180	(ELOS578 x ELOS626) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H181	(ELOS578 x ELOS626) x (ELOS3 x ELOS253)	*S. eubayanus*
	H182	(ELOS578 x ELOS626) x (ELOS3 x ELOS60)	*S. eubayanus*
	H183	(ELOS578 x ELOS626) x (ELOS60 x ELOS104)	*S. eubayanus*
	H184	(ELOS578 x ELOS626) x (ELOS60 x ELOS278)	*S. eubayanus*
	H185	(ELOS578 x ELOS626) x (ELOS104 x ELOS278)	*S. eubayanus*
	H186	(ELOS578 x ELOS626) x (ELOS3 x ELOS104)	*S. eubayanus*
	H187	(ELOS578 x ELOS626) x (ELOS3 x ELOS278)	*S. eubayanus*
	H188	(ELOS578 x ELOS626) x (ELOS104 x ELOS253)	*S. eubayanus*
	H189	(ELOS578 x ELOS626) x (ELOS253 x ELOS278)	*S. eubayanus*
	H190	(ELOS578 x ELOS627) x (ELOS3 x ELOS253)	mixed
	H191	(ELOS578 x ELOS627) x (ELOS3 x ELOS60)	mixed
	H192	(ELOS578 x ELOS627) x (ELOS60 x ELOS104)	mixed
	H193	(ELOS578 x ELOS627) x (ELOS60 x ELOS278)	mixed
	H194	(ELOS578 x ELOS627) x (ELOS104 x ELOS278)	mixed
	H195	(ELOS578 x ELOS627) x (ELOS3 x ELOS104)	mixed
	H196	(ELOS578 x ELOS627) x (ELOS3 x ELOS278)	mixed
	H197	(ELOS578 x ELOS627) x (ELOS104 x ELOS253)	mixed

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. cerevisiae x S. eubayanus	H198	(ELOS578 x ELOS627) x (ELOS253 x ELOS278)	mixed
	H199	(ELOS578 x ELOS627) x (ELOS3 x ELOS253)	*S. cerevisiae*
	H200	(ELOS578 x ELOS627) x (ELOS3 x ELOS60)	*S. cerevisiae*
	H201	(ELOS578 x ELOS627) x (ELOS60 x ELOS104)	*S. cerevisiae*
	H202	(ELOS578 x ELOS627) x (ELOS60 x ELOS278)	*S. cerevisiae*
	H203	(ELOS578 x ELOS627) x (ELOS104 x ELOS278)	*S. cerevisiae*
	H204	(ELOS578 x ELOS627) x (ELOS3 x ELOS104)	*S. cerevisiae*
	H205	(ELOS578 x ELOS627) x (ELOS3 x ELOS278)	*S. cerevisiae*
	H206	(ELOS578 x ELOS627) x (ELOS104 x ELOS253)	*S. cerevisiae*
	H207	(ELOS578 x ELOS627) x (ELOS253 x ELOS278)	*S. cerevisiae*
	H208	(ELOS578 x ELOS627) x (ELOS3 x ELOS253)	*S. eubayanus*
	H209	(ELOS578 x ELOS627) x (ELOS3 x ELOS60)	*S. eubayanus*
	H210	(ELOS578 x ELOS627) x (ELOS60 x ELOS104)	*S. eubayanus*
	H211	(ELOS578 x ELOS627) x (ELOS60 x ELOS278)	*S. eubayanus*
	H212	(ELOS578 x ELOS627) x (ELOS104 x ELOS278)	*S. eubayanus*
	H213	(ELOS578 x ELOS627) x (ELOS3 x ELOS104)	*S. eubayanus*
	H214	(ELOS578 x ELOS627) x (ELOS3 x ELOS278)	*S. eubayanus*
	H215	(ELOS578 x ELOS627) x (ELOS104 x ELOS253)	*S. eubayanus*
	H216	(ELOS578 x ELOS627) x (ELOS253 x ELOS278)	*S. eubayanus*
S. uvarum* x *S. eubayanus	H217	(ELOS578 x ELOS626) x (ELOS274 x ELOS388)	*mixed*
	H218	(ELOS578 x ELOS626) x (ELOS274 x ELOS449)	*mixed*
	H219	(ELOS578 x ELOS626) x (ELOS274 x ELOS471)	*mixed*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. uvarum* x *S. eubayanus	H220	(ELOS578 x ELOS626) x (ELOS471 x ELOS388)	*mixed*
	H221	(ELOS578 x ELOS626) x (ELOS449 x ELOS471)	*mixed*
	H222	(ELOS578 x ELOS626) x (ELOS449 x ELOS388)	*mixed*
	H223	(ELOS578 x ELOS626) x (ELOS274 x ELOS388)	*S. uvarum*
	H224	(ELOS578 x ELOS626) x (ELOS274 x ELOS449)	*S. uvarum*
	H225	(ELOS578 x ELOS626) x (ELOS274 x ELOS471)	*S. uvarum*
	H226	(ELOS578 x ELOS626) x (ELOS471 x ELOS388)	*S. uvarum*
	H227	(ELOS578 x ELOS626) x (ELOS449 x ELOS471)	*S. uvarum*
	H228	(ELOS578 x ELOS626) x (ELOS449 x ELOS388)	*S. uvarum*
	H229	(ELOS578 x ELOS626) x (ELOS274 x ELOS388)	*S. eubayanus*
	H230	(ELOS578 x ELOS626) x (ELOS274 x ELOS449)	*S. eubayanus*
	H231	(ELOS578 x ELOS626) x (ELOS274 x ELOS471)	*S. eubayanus*
	H232	(ELOS578 x ELOS626) x (ELOS471 x ELOS388)	*S. eubayanus*
	H233	(ELOS578 x ELOS626) x (ELOS449 x ELOS471)	*S. eubayanus*
	H234	(ELOS578 x ELOS626) x (ELOS449 x ELOS388)	*S. eubayanus*
	H235	(ELOS578 x ELOS627) x (ELOS274 x ELOS388)	*mixed*
	H236	(ELOS578 x ELOS627) x (ELOS274 x ELOS449)	*mixed*
	H237	(ELOS578 x ELOS627) x (ELOS274 x ELOS471)	*mixed*
	H238	(ELOS578 x ELOS627) x (ELOS471 x ELOS388)	*mixed*
	H239	(ELOS578 x ELOS627) x (ELOS449 x ELOS471)	*mixed*
	H240	(ELOS578 x ELOS627) x (ELOS449 x ELOS388)	*mixed*
	H241	(ELOS578 x ELOS627) x (ELOS274 x ELOS388)	*S. uvarum*

Species Crossed	Collection number	Diploids Crossed	Mitotype
S. uvarum* x *S. eubayanus	H242	(ELOS578 x ELOS627) x (ELOS274 x ELOS449)	*S. uvarum*
	H243	(ELOS578 x ELOS627) x (ELOS274 x ELOS471)	*S. uvarum*
	H244	(ELOS578 x ELOS627) x (ELOS471 x ELOS388)	*S. uvarum*
	H245	(ELOS578 x ELOS627) x (ELOS449 x ELOS471)	*S. uvarum*
	H246	(ELOS578 x ELOS627) x (ELOS449 x ELOS388)	*S. uvarum*
	H247	(ELOS578 x ELOS627) x (ELOS274 x ELOS388)	*S. eubayanus*
	H248	(ELOS578 x ELOS627) x (ELOS274 x ELOS449)	*S. eubayanus*
	H249	(ELOS578 x ELOS627) x (ELOS274 x ELOS471)	*S. eubayanus*
	H250	(ELOS578 x ELOS627) x (ELOS471 x ELOS388)	*S. eubayanus*
	H251	(ELOS578 x ELOS627) x (ELOS449 x ELOS471)	*S. eubayanus*
	H252	(ELOS578 x ELOS627) x (ELOS449 x ELOS388)	*S. eubayanus*

Table 3.2.3 Table of Tetraploid Hybrids Generated

A total of 252 tetraploid hybrids were created. Each hybrid had four parental strains contributing a single genome copy each. Petites diploid maters were mated with diploid maters possessing functional mitochondria in order to control the tetraploid mitotype. Hybrids with *S. cerevisiae* mitotype are highlighted in blue, *S. uvarum* mitotype in yellow, and *S. eubayanus* mitotype in red. Hybrids of a mixed mitotype between *S. cerevisiae* and *S. uvarum* are highlighted in green, a mix between *S. cerevisiae* and *S. eubayanus* in purple, and finally a mixed mitotype between *S. uvarum* and *S. eubayanus* in orange.

Species Crossed	Collection Number	Spore Viability (%)
S. cerevisiae* x *S. uvarum	H28	75
	H35	78.8
	H51	**92.5**
	H73	77.5
	H96	73.8
	H100	81.3
	H105	88.8
	H107	66.3
S. cerevisiae* x *S. eubayanus	H163	86.5
	H172	81.7
	H179	**89.0**
	H181	81.7
	H186	81.7
	H188	**87.5**
	H199	80.8
	H201	78.8
	H204	98.0
	H210	88.0
S. eubayanus* x *S. uvarum	H224	70.7
	H227	56.0
	H230	**76.0**
	H235	82.5
	H236	81.5
	H237	83.3
	H239	83.9
	H242	72.5
	H243	61.0
	H245	71.7

Table 3.2.4 Advanced Generation Intercrossed Cell lines

28 strains, from the three species crosses, underwent multigenerational interbreeding to obtain F12 populations. The 12th generation spore population was dissected to calculate percentage viability (n = 80). Four strains (in bold) were selected for phenotypic assessment. H51, H179 and H188 were selected for QTL analysis.

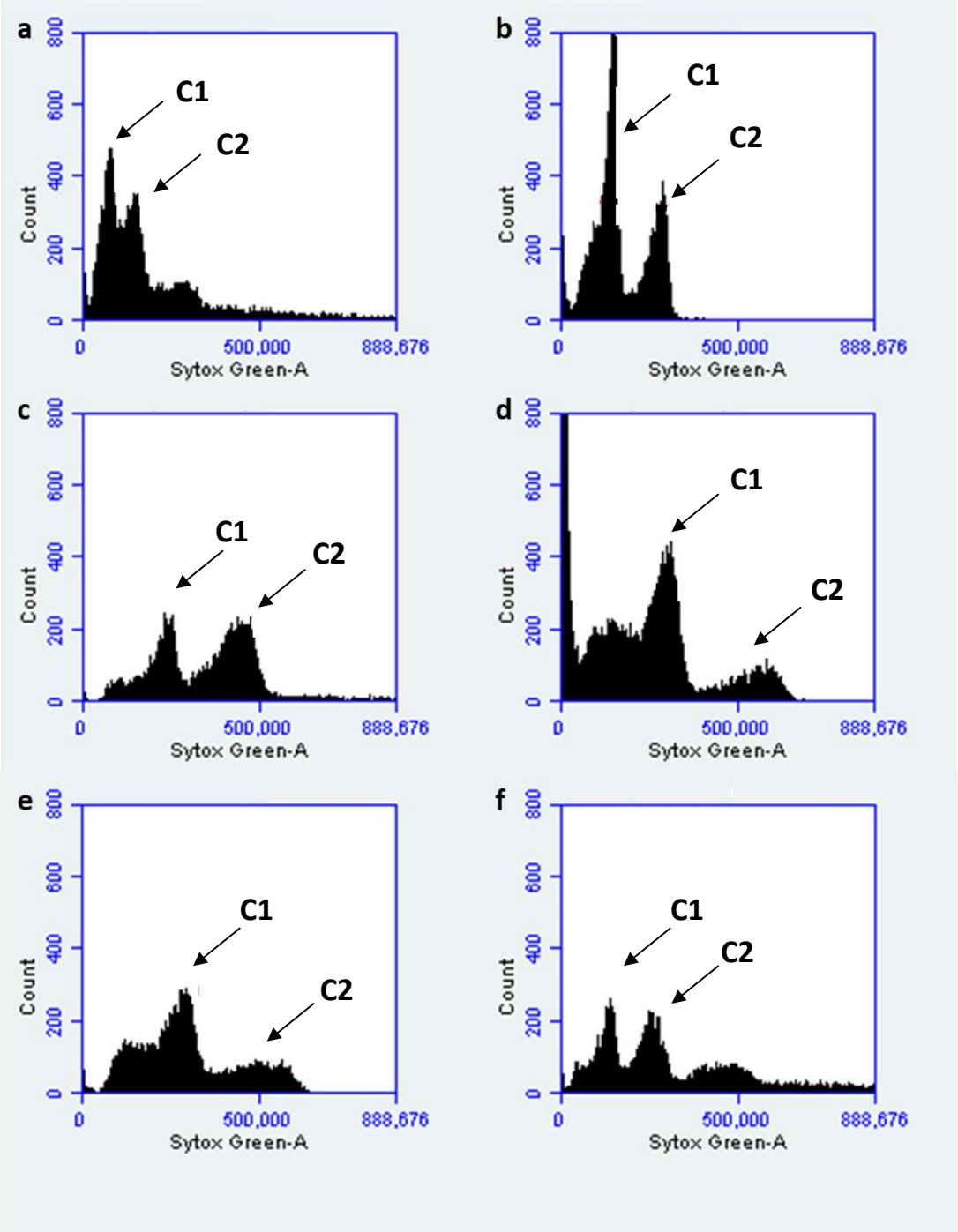


Figure 3.2.1 Ploidy Analysis by Flow Cytometry

The tetraploid hybrids chosen for multi-generation interbreeding were assessed for ploidy, along with their F12 spores. (a-d) Control samples of were used to set gates for the C1 and C2 peaks, corresponding to the G1/0 and G2 phase of the cell cycle respectively: - (a) haploid, (b) diploid, (c) triploid, and (d) tetraploid. (e) H179 was used as the example tetraploid hybrid, with two peaks in the gates corresponding to tetraploid gates. (f) F12 spore of H179 with two peaks within the diploid gates.

3.3 Discussion

3.3.1 Summary

In this study a set of 252 hybrids were successfully created. Each of these gave rise to viable gametes, having overcome sterility through higher ploidy. Eight *S. cerevisiae* x *S. uvarum* hybrids, ten *S. cerevisiae* x *S. eubayanus* hybrids, and ten *S. eubayanus* x *S. uvarum* hybrids underwent multigenerational mating to create AILs. The ploidy of these hybrids and F12 spores was confirmed by flow cytometry as tetraploid and diploid respectively These AILs will be used to study the genetics of hybrids by QTL analysis. These F12 diploids will also be phenotypically assessed for industrially relevant traits, such as sugar utilisation and resistance to a variety of stresses present in brewing and other fermentation processes.

3.3.2 Flow Cytometry

The assessment of sample ploidy using Sytox Green staining and flow cytometry was useful to confirm that the new strains were of the ploidy that was expected. However additional peaks to what was expected made the confidence of these conclusions less strong. Causes of additional peaks can arise due to cell clumping, whereby two cells pass through the detector at the same time registering as a single cell. If a sufficient number of doublets passed across the detector an additional peak of twice the expected intensity would be observed. A cause of peaks lower in intensity than expected could be caused by aneuploidy, which is particularly likely in yeast cells of high ploidy such as the tetraploids created here. It has been found that increasing the ploidy of yeast strains increases chromosome instability resulting in higher levels of aneuploidy (Mayer and Aguilera, 1990). An alternative method of assessing ploidy that could support this flow cytometry data is by measuring the DNA content per cell (Aigle et al., 1983).

3.3.3 Alternative Hybrids

The hybrids created here were designed to have a similar parental contribution as the industrial hybrids *S. pastorianus* and *S. bayanus*. It would also be possible to make a wide variety of other hybrids, utilising the full *Saccharomyces* clade. Other hybrids exist in industrial processes such as the *S. cerevisiae* x *S. kudriavzevii* hybrids used in some wine fermentations. While

S. cerevisiae has been utilised and domesticated over millennia, other *Saccharomyces* species have had little to no examples of domestication. Consequently the vast diversity of these species remain largely untapped. Creating new hybrids between these may unveil novel traits, highly beneficial for various processes. For example *S. cerevisiae* is unable to utilise C5 sugars, xylose and arabinose, found in lignocellulose, a key sugar in plant cell walls. Normally the lignocellulose requires enzymatic digestion prior to yeast fermentation. This inability to digest lignocellulose is a major limitation of *S. cerevisiae* to produce biofuels. During pre-treatment by-products, such as acetic and levulinic acid, are formed. These can have an inhibitory effect upon the fermentative action of yeast (Jönsson and Martín, 2016). A hybrid that was capable of this digestion would be hugely beneficial (Larsson et al., 2001).

4 QTL Analysis of *de Novo* Hybrids

4.1 Introduction

4.1.1 Chapter aims

The primary objective of this chapter is to demonstrate the potential for performing QTL analysis on the *de novo* hybrids generated (**Chapter 3**). This will be performed using industrially relevant traits, primarily these will be traits desirable in brewing and fermentation. Two methods of bulk segregant analysis; pooled selection, and multipool analysis will be used in order to compare their efficacy at identifying loci responsible for each trait. In assessing the capacity for QTL analysis in hybrids, numerous genes will be identified as being involved in conferring a selection advantage and the observed phenotype. Pooled selection (**2.4.1**) involves a large population pool placed under selection pressure, after selection the pool is sequenced and compared against a control population pool which has not been placed under selection pressure. Multipool (**2.4.2**) is “a computational method for genetic mapping in model organism crosses that are analyzed by pooled genotyping” (Edwards and Gifford, 2012). Comparing the pooled sequences of the top performing individuals against the pooled sequences of the low performing individuals, allele frequency differences identify QTL for the given trait.

The influence of mitotype is assessed by analysing two hybrids H179 and H188 under the same conditions. These two hybrids are genetically identical, differing solely in the inheritance of the mitochondria. H179 has a *S. cerevisiae* mitotype, whereas H188 has a *S. eubayanus* mitotype. Intervals identified in one hybrid but not the other may be due to the role or interaction the candidate gene has with the mitochondria. Here the term interval has been used to describe a region of the genome where the allele frequency in one population differs significantly with the allele frequency of the control population. In this analysis intervals were assigned when the allele frequency difference exceeded 0.2.

4.1.2 Background

Mating is an essential requirement to perform QTL analysis. Mating shuffles the genome, disrupting linkage groups, and creates a broad phenotypic range

for each trait. This phenotypic continuum allows for selection of high performers, which when sequenced in comparison to the population reveals enhanced frequencies of specific loci that contribute to the trait. AILs, which have gone through multiple rounds of mating have the capacity for higher resolution QTL analysis. Each round of mating further unlinks neighbouring loci. This allows for the identification of smaller regions, often down to a single gene, to be identified as a QTL. With higher resolution small effect loci are also able to be identified, which may have previously been hidden by a nearby locus conferring a large contribution to the trait. Due to their sterility, QTL analysis of *Saccharomyces* hybrids has previously not been possible. The understanding of hybrid genetics as a consequence remains poor.

4.1.3 QTL analysis Techniques

There are several methods available for QTL analysis. Pooled selection uses these large genetically diverged populations, to identify loci responsible for a given selectable trait. QTL analysis from pooled selection should give the most detailed results. Selection is occurring on a population size of over 100 million individuals. With a larger sample size, the statistical power of the analysis is increased. Extreme QTL (X-QTL) mapping (Ehrenreich et al., 2010) involves the generation of a large F1 segregating population. This population is placed under selection pressure where only a fraction of the individuals are capable of growth. Allele frequency differences between the selection pool and the control pool are then identified. QTL mapping resolution was improved in a method termed intercross QTL (i-QTL) mapping (Parts et al., 2011, Cubillos et al., 2013). The creation of AILs decreased linkage disequilibrium between adjacent loci reducing lengths of QTL intervals. Having created AILs of the *de novo* hybrids, we have been able to perform detailed i-QTL mapping on hybrids for the first time.

Pooled selection is only suitable for selectable traits, however there are a multitude of traits which are non-selectable. These include brewing specific traits such as flavour compound production, rate of growth at low temperature, and flocculation; the process of cell aggregation in order to sediment out of solution (Boulton and Quain, 2007). Individual segregant analysis (ISA) can be used to identify QTL of both selectable and non-selectable traits. Ranking of individuals by performance and sequencing of each individual can identify

causal gene variants by single QTL mapping (Brem et al., 2002). This has been achieved using the PHENOS platform (Barton et al., 2018) (**2.2.10**). Individuals segregants are phenotyped as well as sequenced individually. The two data sets are combined using the R-QTL analysis script (Broman et al., 2003). The financial cost of sequencing an array of 384 individuals per hybrid, precluded our ability to perform this here. With the rapidly falling cost of sequencing, it will not be long before this is a viable alternative to bulk segregant analysis.

The multipool computational technique was developed by (Edwards and Gifford, 2012). Like ISA, multipool can be used for both selectable and non-selectable traits. Phenotyping is performed on an array of individuals, which are then ranked according to phenotypic strength. DNA is extracted from individuals with extreme phenotypes, with sequencing performed upon the pools. The two extreme pools are compared to identify reciprocal changes in allele frequency. Within the Louis group, multipool has previously been utilised to identify QTL intervals containing seven candidate genes, involved in the regulation of telomere length (Aryal, 2016).

4.1.4 Selection Conditions

Five conditions were chosen for pooled selection of H179 and H188; temperature extremes, high glucose and maltose concentration, and hydrogen peroxide. Barring high temperature, each of these selection conditions are relevant to the production of lager. Maltose and glucose are two key wort sugars, and the high concentration of each mimics the osmotic pressure exerted upon yeast in high gravity wort. Hydrogen peroxide is an oxidative stress agent. Yeast cells are placed under a number of stresses during fermentation, which in turn increases the production of reactive oxygen species (ROS) raising the level of oxidative stress (Perrone et al., 2008, Jamieson, 1998). Individuals isolated from H_2O_2 selection have been shown to be multi-stress resistant (Nakagawa et al., 2013). Brewing yeast is stored at low temperatures, and it is desirable for strains to be recover quickly from storage. Lager is also fermented at low temperatures to minimise off-flavour production. A fast rate of growth and fermentation at low temperatures is therefore a desirable trait. Selection at both high and low temperatures has been performed for these two strains here. Observed differences could be due

to the influence of the mitochondria. H179 possessing the *S. cerevisiae* mitochondria, should be less sensitive to high temperature than H188 which possesses the *S. eubayanus* mitochondria. Conversely H188 should be less sensitive to the low temperature selection. Whilst the other selection conditions result in a fraction of the population being capable of growth, most individuals are capable of growth at 4 °C. In this selection pool, individuals that are capable of fast growth at the low temperature are being selected for over individuals with a slow rate of growth.

Three selection conditions were assessed for the hybrid H51; high temperature, acetic acid and levulinic acid. Previously high temperature has been used as a selection condition for QTL analysis in *S. cerevisiae* populations (Cubillos et al., 2013, Cubillos et al., 2011, Parts et al., 2011), here it will be possible to compare those findings with the QTL analysis in hybrids. Acetic acid is present in grape must. Wine producing yeasts, some with a not dissimilar genetic composition to H51, require resistance to this stress. Levulinic acid is produced in the pretreatment of lignocellulosic biomass for the production of bioethanol (Oshoma et al., 2015). Hydrolysis of hemicellulose gives monomeric hexose and pentose sugars, dehydration of hexose sugars produces hydroxymethylfuraldehyde (HMF). Levulinic acid arises from the breakdown of HMF (Almeida et al., 2007). Levulinic acid is also produced as a platform for the production of high value chemicals (Silveira et al., 2018). Strains used in the production of bioethanol must confer resistance to this weak acid.

For multipool analysis, high and low temperature was assessed for a second time for strains H179 and H188. This should allow for a direct comparison between the two approaches to QTL analysis in hybrids. An Individuals ability to grow in high ethanol was also assessed, since yeasts fermenting super high gravity wort will produce more ethanol and subsequently be exposed to higher ethanol concentration.

4.2 Results

4.2.1 Pooled Selection

Intervals were identified for each species under selection pressures. The intervals are shown in the appendix (**Figure 8.1.1** - **Figure 8.1.10**).

4.2.1.1 Identification of Quantitative Trait Loci in H51

Nine intervals were detected in the pooled selection experiments involving the hybrid H51 (**Table 4.2.1**). A single region was identified as conferring tolerance to acetic acid, four conferred a selection advantage at high temperature, and four giving tolerance in an environment containing levulinic acid. All nine intervals were found within the *S. cerevisiae* portion of the genome, with no intervals detected within the *S. uvarum* portion. The smallest interval was 6321 bp, conferring resistance to levulinic acid, and contained just two genes. The largest interval was 30030 in length, containing 12 genes, and conferred acetic acid tolerance. Several peaks overlapped with peaks found in other selection conditions. The six genes within the interval conferring acetic acid tolerance on chromosome (chr) 7 were also observed in the interval on chr 7 for levulinic acid selection. Intervals overlapped on chr 2 and chr 6 for the high temperature selection and levulinic acid selection, sharing two and five genes respectively.

Candidate genes were identified in each of the intervals based on previous phenotypic studies. These studies typically involved gene deletion, reduction of function, and overexpression. A total of 15 candidate genes were shortlisted as conferring a selection advantage for the three conditions (**Table 4.2.2**). The amino acid sequence for each parental allele was aligned and amino acid differences were identified. No sequence differences were identified between the alleles of *EMP24*, *SUP45*, *SPB4* and *CAB2*. The complete sequence of the OS104 allele of the *FLO1* gene was not available, so a comparison was not possible for this gene. Neither gene within the chr 2 interval for levulinic acid tolerance had been reported as altering the phenotype in varying pH conditions. *HOS2* was shortlisted for acetic acid tolerance and levulinic acid tolerance. *BLM10* was shortlisted as a candidate for both resistance to high temperature and levulinic acid.

The 15 genes shortlisted based upon reported function, were able to be cut down to nine genes based upon the amino acid sequence similarity and the presence of major amino acid changes (**Table 4.2.2**). Major amino acid changes designated based upon their Gonnet PAM (point accepted mutation) score. These changes include from a polar to a non-polar amino acid; e.g. serine to glycine, as well as structural changes; e.g. valine to leucine.

The *MAK5* sequence length of the OS104 allele is 12 bases longer than the OS3 allele. This translated into four amino acids; E139, D140, V141, and D142. This is a duplication of the subsequent 12 bases, present in both allele sequences.

Condition	Chromosome	Interval Length	Peak	Gene Names
Acetic Acid	chr7	30030	121427	**EMP24** YIP4 MDS3 DSD1 GCN1 **HOS2** IME4 COX13 CDC55 RPS26A COX4 **TPN1**
High Temperature	chr1	17660	191647	**FLO1**
High Temperature	chr2	31627	509833	ATG42 IRA1 BMT2 **MAK5 SUP45** ADH5 MRPS9 RTC2 YSW1 ARA1 TBS1
High Temperature	chr6	21320	157318	**SMC1** MSH4 **BLM10 DEG1 SPB4** SEC4 VTC2
High Temperature	chr9	28354	213965	UTP25 ICE2 AVT7 AIM19 KTR7 SDS3 **CAB2** AIR1 THS1 RCI37 SEC28 RPN2 SER33 SPO22
Levulinic acid	chr2	6321	511061	IRA1 ATG42
Levulinic acid	chr6	17428	153141	SMC1 MSH4 **BLM10** SEC4 VTC2
Levulinic acid	chr7	18325	138112	MDS3 DSD1 **GCN1** CDC55 AME4 COX13 HOS2
Levulinic acid	chr11	11039	295410	**PSG1** MUD2 STB6 LHS1

Table 4.2.1 Quantitative Trait Loci Identified in H51

QTL summary table of each region identified for H51 within the *S. cerevisiae* portion of the hybrid genome. The table gives the selection condition for each region, the chromosome number, the interval length, the peak for each QTL and the genes identified within the region. Candidate genes, based upon phenotypic findings are in bold. Genes that were found at the interval peak in red font. Genes that were identified in regions for other phenotypes are highlighted in colour.

Chromosome	Gene	Implication	A.A Similarity	A.A changes
			Acetic Acid	
chr7	**TPN1**	Acetic acid tolerance	302/303	**V123L**
	HOS2	Acetic acid tolerance	452/453	M366V
	EMP24	Lactic acid tolerance	204/204	
			High Temperature	
chr1	FLO1	Heat Tolerance		
chr2	**MAK5**	Heat Tolerance	748/756	**E139_, D140_, V141_, D142_**, Q239R, **S367G, S575C, P613S**
	SUP45	Heat Tolerance	438/438	
	IRA1	Heat Tolerance	1002/1005	**P6L, H7Q, N841D**
chr6	SPB4	Heat Tolerance	502/502	
	DEG1	Heat Tolerance	442/443	I244V
	BLM10	Heat Tolerance	2134/2144	Q98R, S729N, R1102K, **G1315S**, D1444N, **S1546F, R1592C, A1698T, G1782D, D1861Y**
	SMC1	Heat Tolerance	1036/1042	**R313L, T428A, E778V,** N794S, **S923R,** S948N
chr9	CAB2	Heat Tolerance	366/366	
			Levulinic Acid	
chr6	**BLM10**	H_2O_2 tolerance	2134/2144	Q98R, S729N, R1102K, **G1315S**, D1444N, **S1546F, R1592C, A1698T, G1782D, D1861Y**
chr7	**GCN1**	Propanoic acid tolerance	765/772	**T59P,** V73I, **A139V,** T178A, **P320S, A330T, T415I**
	MDS3	H_2O_2 tolerance	1472/1488	**T8A, T22A,** R144K, E159D, **A303T, D320Y, S321P, A349T,** K470R, **G885C, E1282G, N1317K,** T1398S, **S1400P,** I1459M,**S1483C**
chr11	**PSG1**	Acetic acid tolerance	392/393	**Y115S**

Table 4.2.2 Top Candidate Genes Within H51 QTL Regions

Candidate genes were picked based upon analysis of gene function, and whether implicated in contributing to a trait in previous studies. Alignment of parental allele sequences identified differences. For each candidate, the amino acid (A.A) sequence similarity is given, along with the amino acid changes. Changes are shown as a switch from the OS104 to the OS3 sequence. The Gonnet PAM 250 matrix was used to determine whether amino acid changes resulted in a conservation of properties. Major amino acid changes that lack property conservation, with a Gonnet PAM score of > 0.5, are highlighted in bold. Genes that were shortlisted further based upon major amino acid changes have been highlighted in green.

4.2.1.2 Identification of Quantitative Trait Loci in H179 and H188

There were sixteen intervals identified through selection experiments involving the hybrid H179. Six were identified in the *S. cerevisiae* portion of

the genome (**Table 4.2.3**); one conferring tolerance to H_2O_2 on chr 12, and five conferring a selection advantage on rich medium containing 35 % maltose. The interval on chr 12 was present in both maltose and H_2O_2 selection sharing three common genes. Ten intervals were identified in the *S. eubayanus* portion of the genome (**Table 4.2.7**), all of these conferred a selection advantage to growth on high maltose concentration. No intervals were identified in the selection at low temperature, high glucose concentration, or H_2O_2 treatment. The sequencing quality for the high temperature selection of H179 was too poor for analysis. Only 7,383 reads of 239 bp in length were acquired, giving less than one fold coverage. This is in comparison to the average of approximately 7 million reads at 245 bp in length, which is roughly equal to 70 x coverage for other samples.

A total of 25 intervals were found from the selection events involving the hybrid H188. 19 intervals were identified within the *S. cerevisiae* portion of the hybrid genome (**Table 4.2.5**). Four contributed to the selection advantage on high glucose, and fifteen; one on each chromosome except chr 3, conferred an advantage to growth on maltose. For both conditions there was an interval on chr 2, which shared 8 genes in common. A further six intervals were identified within the *S. eubayanus* portion of the genome (**Table 4.2.9**); five conferring the selection advantage on maltose, and a single interval conferring a selection advantage to low temperature. No intervals were identified in the selection pools at high temperature, or for oxidative stress caused by H_2O_2.

Maltose selection gave rise to four overlapping intervals between H179 and H188, in the *S. cerevisiae* genome portion. These were found on chr 1, chr 2, chr 12 and chr 13. Three genes were present in both intervals on chr 1. Seven genes were found within the interval section in common on chr 2. These genes were also found within the interval for glucose selection for H188. Three genes were shared between the intervals on chr 12, and a single gene was in common between the overlapping intervals on chr 13. The fifth and final interval, on chr 8, found within the *S. cerevisiae* portion of H179 conferring an advantage on maltose, overlapped with an interval on chr 8 of the *S. eubayanus* portion of H188. This overlap shared two genes in common. This interval on the H188 *S. eubayanus* chr 8 also overlapped with the interval on the H188 *S. cerevisiae* chr 8, sharing four genes in common. Interestingly

these four genes were different to the two genes shared between H179 and H188. There was a single interval on the *S. cerevisiae* chr 6 in H188, conferring a selection advantage on maltose, that shared a single gene in common with an interval on the *S. eubayanus* chr 6. There was no shared intervals within the H179 and H188 *S. eubayanus* portion of the genome.

The list of genes within each interval was narrowed as described previously. For the *S. cerevisiae* portions of the genomes, four genes were identified in the intervals of H179 (**Table 4.2.4**) and 18 genes within the H188 intervals (**Table 4.2.6**). For several intervals, for example H179 chr 12 conferring resistance to H_2O_2, no clear candidate gene was identified based upon prior knowledge of phenotype. Variation in the two parental strain amino acid sequences was assessed. The sequence difference between the two strains of H179 and H188 was less so than the two *S. cerevisiae* parental strains of H51. This correlates with the genetic distance between the strains (Liti et al., 2009). The North American *S. cerevisiae* lineage; which OS104 belongs, is more closely related to the Sake lineage; which OS253 belongs, than the Wine European lineage, which OS3 belongs. Six genes were shortlisted as conferring a selection advantage to maltose from the *S. eubayanus* portion of the H179 genome (**Table 4.2.7**). From the S. eubayanus portion of the H188 genome, two candidate genes were identified as being involved in selection at low temperature, and 12 genes were shortlisted from maltose treatment across five intervals (**Table 4.2.9**).

In total there were eight intervals identified in the H179 selection that no candidates were identified based upon prior knowledge of phenotypes. Five were identified in the *S. eubayanus* portion, and three in the *S. cerevisiae* portion. There were six intervals found in H188 without obvious candidate genes. All six were within the S. cerevisiae portion, and all conferred a selection advantage on maltose rich media.

The candidate genes found within the *S. cerevisiae* portion of the genome were able to be shortlisted further based upon major amino acid changes (**Table 4.2.4**, **Table 4.2.6**). *PIG1* appears to be the most promising candidate identified in the H179 maltose selection. In the H188 selection the shortlist was reduced to four genes conferring an advantage on glucose, and five

genes conferring an advantage on maltose. It was not possible to decrease the size of the shortlist of genes found within the *S. eubayanus* portion of the genome. For each gene the parental allele sequence differences were high, resulting in a large number of amino acid differences. The sequence disparity resulted in an average of 10 percent difference in the amino acid sequences.

Condition	Chromosome	Interval Length	Peak	Gene Names
H_2O_2	chr12	15971	653698	YCS4 PIG1 MCM5
Maltose	chr1	21478	207056	FLO1 PHO11/PHO12
Maltose	chr2	18594	532971	**ADH5** MRPS9 RTC2 YSW1 **ARA1** TBS1 APD1 SPP381 RIB7 RPB5 CNS1 CLI15
Maltose	chr8	25332	96956	**STE20** SHU1 MRP4 LAG1 HSE1 RPL14B
Maltose	chr12	17009	668162	YCS4 **PIG1** MCM5 SMD2 DBP9
Maltose	chr13	6358	691683	TRS130 ESC1

Table 4.2.3 QTL Identified in *S. cerevisiae* portion of H179

QTL summary table of each region identified within the *S. cerevisiae* portion of the hybrid genome of H179. The table gives the selection condition for each region, the chromosome number, the interval length, the peak for each QTL and the genes identified within the region. Candidate genes, based upon phenotypic findings are in bold. Genes that were found at the interval peak in red font. Genes highlighted in colour were identified in other selection conditions, or in the selection pools of other strains.

Chromosome	Gene	Implication	A.A similarity	A.A changes
Maltose				
chr2	ADH5	Glycolysis	352/352	
	ARA1	Carbohydrate metabolism	344/345	K140R
chr8	STE20	Osmotic stress	338/339	I337V
chr12	**PIG1**	Glycogen Synthesis	646/649	E376K, **T504A, A578T**

Table 4.2.4 Top Candidate Genes Within *S. cerevisiae* portion of H179

Four canditate genes were picked based upon analysis of gene function, and whether they were implicated in contributing to a trait in previous studies. Alignment of parental allele sequences identified differences. For each candidate, the amino acid (A.A) sequence similarity is given, along with the amino acid changes. Changes are shown as a switch from the OS104 to the OS253 sequence. The Gonnet PAM 250 matrix was used to determine whether amino acid changes resulted in a conservation of properties. Major amino acid changes that lack property conservation, with a Gonnet PAM score of > 0.5, are highlighted in bold. The alleles of ADH5 showed no amino acid variation. The PIG1 gene was further shortlisted based upon major amino acid changes (highlighted in green).

Condition	Chromosome	Interval Length	Peak	Gene Names
Glucose	chr2	18594	532971	**ADH5** MRPS9 RTC2 YSW1 **ARA1** TBS1 APD1 SPP381 RIB7 RPB5 CNS1 SLI5
Glucose	chr12	13744	664542	YCS4 **PIG1** MCM5
Glucose	Chr14	14229	251278	MEP2 AAH1 THO2 **SRV2** NAM9 **EAF7 FPR1**
Glucose	Chr15	26034	737870	RET1 PTP2 RFC1 NPT1 RPB10 MGM1 STE4 SAS5 SPR2 AIM41 RUD3 STE13 RCN2 **MCT1** ODC2
Maltose	Chr1	21622	226805	FLO1 PHO11/PHO12 IMD2
Maltose	Chr2	29900	546683	YSW1 **ARA1** TBS1 APD1 SPP381 RIB7 RPB5 CNS1 SLI15 ICS2 AMN1 **IFA38** CDC28 CSH1 TOS1 EXO5 YSY6
Maltose	chr4	22520	96164	HEM3 RTN2 ACK1 MRPL11 TRM8 MGT1 GGC1 ASF2 SEC31 **SNF3**
Maltose	chr5	11921	269294	PIC2 GIP2 HIS1 FCY2 HMF1
Maltose	chr6	12442	78128	RPO41 MOB2 RIM15 RPL22B MIL1
Maltose	chr7	7667	132749	**GCN1** HOS2
Maltose	chr8	10089	81144	YLF2 **OTU2 YAP3** ETP1 PRS3
Maltose	chr9	5554	49666	GUT2 **IMP2'** RRD1 ESL1
Maltose	chr10	5865	348984	GYP6 MHP1
Maltose	chr11	17436	55279	LOS1 EAP1 **TOR2** MNN4
Maltose	chr12	5442	487942	TFS1 SAM1 VTA1 SWI6
Maltose	Chr13	23458	695466	ESC1 ERG8 FMP42 FSH2 UBP8 MRE11 MRPL44 TAF7 MTF1 RRP5
Maltose	chr14	18633	137732	BNI1 ALP1 LYP1 PIK1 IST1 PDR17 YIF1 POL2
Maltose	chr15	17921	159675	HAL9 MPD2 DUF1 MHF1 **ADH1** PHM7 ATG34
Maltose	chr16	6800	46793	**FUM1** CUB1 APM1 THI21

Table 4.2.5 QTL Identified in *S. cerevisiae* portion of H188

QTL summary table of each region identified within the *S. cerevisiae* portion of the hybrid genome of H188. The table gives the selection condition for each region, the chromosome number, the interval length, the peak for each QTL and the genes identified within the region. Candidate genes, based upon phenotypic findings are in bold. Genes that were found at the interval peak in red font. Genes highlighted in colour were identified in other selection conditions, or in the selection pools of other strains.

Chromosome	Gene	Implication	A.A similarity	A.A changes
Glucose				
chr2	ADH5	Glycolysis	352/352	
	ARA1	Carbohydrate metabolism	344/345	K140R
chr12	**PIG1**	Glycogen synthesis	646/649	E376K, **T504A**, **A578T**, N631D
chr14	EAF7	Osmotic stress	240/241	K210M
	SRV2	Osmotic stress	526/527	**V8M**
	FPR1	Osmotic stress	113/115	**A76T, F92L**
chr15	**MCT1**	Glucose utilisation	358/361	**T81A, P91S, I310T**
Maltose				
chr2	IFA38	Carbohydrate metabolism	348/348	
	ARA1	Carbohydrate metabolism	344/345	K140R
chr4	**SNF3**	Glucose transport	693/700*	**N204K,** V442I, S472T**, S474L**, L626V, R638Q, I689S
chr6	RPO41	Osmotic stress	1351/1352	L1309V
chr7	GCN1	Osmotic stress	771/772	I450V
chr8	OTU2	Osmotic stress	308/308	
	YAP3	Osmotic stress	161/163	**T15I, T109D**
chr9	**IMP2'**	Osmotic stress	228/229	**G63D**
chr11	**TOR2**	Osmotic stress	2470/2475	**E122G, P607S,** I139M, **I1856T, I1871L**
chr15	**ADH1**	Glycolysis	490/494	**G173D,** S412N, **N465K, A491V**
chr16	FUM1	TCA cycle	488/489	S375A

Table 4.2.6 Top Candidate Genes Within *S. cerevisiae* portion of H188

Candidate genes were picked based upon analysis of gene function, and whether they were implicated in contributing to a trait in previous studies. Alignment of parental allele sequences identified differences. For each candidate, the amino acid (A.A) sequence similarity is given, along with the amino acid changes. Changes are shown as a switch from the OS104 to the OS253 sequence. The Gonnet PAM 250 matrix was used to determine whether amino acid changes resulted in a conservation of properties. Major amino acid changes that lack property conservation are highlighted in bold. These alleles had a Gonnet PAM score of > 0.5. Genes that were shortlisted further based upon major amino acid changes have been highlighted in green. * The OS253 sequence of SNF3 was missing the first 554 bases. Conversion of nucleic acid sequence to amino acids was performed from the 556th base to avoid shifting the read frame (sequence similarity in red)

Condition	Chromosome	Interval Length	Peak	Gene Names
Maltose	chr2	28533	547033	RUB1 MTQ2 DOP1 PEX7 SAN1 TAF12 SWI5 EKI1 **KGD2**
Maltose	chr3	9869	175146	RPS14A BPH1
Maltose	chr6	20878	42964	RGD2 FMP32 SEC53 OTU1 FET5 **ACT1** TUB2 RPO41
Maltose	chr7	16485	996766	YTA7 SLH1 TAF1 RTT102
Maltose	chr8	24745	680586	SPS4 SFG1 COT1 FAA1 HSH49 GNT1 **PMT3** LDB19 **PRO2** FRT1 MYO2
Maltose	chr10	13293	216183	UTP10 PRM10 IME2 SET4 PAM16 **GSM1**
Maltose	chr11	4184	63057	MST1 SDS22 ACP1 DPH2
Maltose	chr13	14227	876156	UBP15 SCW10 FKS3
Maltose	chr14	6658	461838	TCB2 SNN1 MKT1
Maltose	chr16	9986	202501	TRE1 SPT14 NIP100 **COX10**

Table 4.2.7 QTL Identified in *S. eubayanus* portion of H179

QTL summary table of each region identified within the *S. eubayanus* portion of the hybrid genome of H179. The table gives the selection condition for each region, the chromosome number, the interval length, the peak for each QTL and the genes identified within the region. Candidate genes, based upon phenotypic findings are in bold. Genes that were found at the interval peak in red font. Genes highlighted in colour were identified in other selection conditions, or in the selection pools of other strains.

Chromosome	Gene	Implication	A. A similarity
Maltose			
chr2	KGD2	TCA cycle	389/452
chr6	ACT1	Osmotic stress	336/361
chr8	PRO2	Osmotic stress	455/475
	PMT3	Glucose utilisation	733/754
chr10	GSM1	Regulation of metabolism	529/621
chr16	COX10	Respiration	403/464

Table 4.2.8 Top Candidate Genes Within H179 *S. eubayanus* portion

Six candidate genes were picked based upon analysis of gene function, and whether they were implicated in contributing to a trait in previous studies. Allele nucleic acid sequences were translated into amino acid sequences, which were then aligned to assess the degree of sequence similarity.

Condition	Chromosome	Interval Length	Peak	Gene Names
Low Temperature	chr16	21560	134162	BMS1 CBP3 THI6 **CBP3** NIP7 SRP72 **RKM1** TYW1 PGC1
Maltose	chr2	11250	385444	RPS13 RRG1 RTR2 DOS2 **DOA4** FMP16 PAA1 IPT1
Maltose	chr7	37414	541782	**RPS25A** MSP1 POP6 IMO32 **GSC2** **RPL26B** **TIM21** ACB1 ORM1 KSS1 **BUD9** NQM1 TAM41 TFC4
Maltose	chr8	14350	107418	**OTU2** PRS3 ETP1 **YAP3** **STE20** SHU1
Maltose	chr14	12844	315004	APC1 PSD1 FMP41 **SKO1** BNI5 IBD2
Maltose	chr16	23730	709776	NVJ2 ASR1 RDS3 SYT1 **MRPL51** RPL11A PRE2 FHL1 COG4 **ISR1** YTH1

Table 4.2.9 QTL Identified in *S. eubayanus* portion of H188

QTL summary table of each region identified within the *S. eubayanus* portion of the hybrid genome of H188. The table gives the selection condition for each region, the chromosome number, the interval length, the peak for each QTL and the genes identified within the region. Candidate genes, based upon phenotypic findings are in bold. Genes that were found at the interval peak in red font. Genes highlighted in colour were identified in other selection conditions, or in the selection pools of other strains.

Chromosome	Gene	Implication	A.A Differences
		Low Temperature	
chr16	RKM1	Innate thermotolerance	539/583
	CBP3	Innate thermotolerance	341/357
		Maltose	
chr2	DOA4	Osmotic stress	789/928
chr7	RPL26B	Osmotic stress	162/179
	RPS25A	Osmotic stress	104/109
	TIM21	Osmotic stress	213/246
	BUD9	Osmotic stress	322/366
	GSC2	Sugar utilisation	1842/1896
chr8	YAP3	Osmotic stress	252/331
	OTU2	Osmotic stress	265/306
	STE20	Osmotic stress	896/937
chr14	SKO1	Osmotic and oxidative stress	554/654
chr16	MRPL51	Osmotic stress	139/141
	ISR1	Osmotic stress	407/441

Table 4.2.10 Top Candidate Genes Within H188 *S. eubayanus* portion

Six candidate genes were picked based upon analysis of gene function, and whether they were implicated in contributing to a trait in previous studies. Allele nucleic acid sequences were translated into amino acid sequences, which were then aligned to assess the degree of sequence similarity.

4.2.2 Pooling of Individuals for Multipool

F12 arrays of H179 and H188 individuals were phenotyped under three stress conditions. These were; high ethanol concentration (15 %), low temperature (4 °C) and high temperature (40 °C). 384 individuals were phenotyped for each hybrid strain. The twenty best performing and twenty worst performing individuals were pooled after treatment. Under ethanol stress there was a larger difference in maximum growth between the extremes of H179 individuals (**Figure 4.2.1**) - t = 11.8, than that of the H188 individuals (**Figure 4.2.4**) t = 9.5. Under control conditions the H179 low performers at low temperature outperformed the high performers (**Figure 4.2.2**), – $p < 0.05$, t = -2.2. There was a greater difference between the two H179 pooled individuals at 4 °C – t = 15.1, than the two H188 pooled individuals (**Figure 4.2.5**) – t = 8. At 40 °C the difference between the two sets of pooled individuals in H179 (**Figure 4.2.3**) was similar to the difference between the H188 sets (**Figure 4.2.6**) – t = 8.8 and t = 8.4 respectively. As the H188 hybrid possessed the cryotolerant *S. eubayanus* mitochondria, it was expected that most individuals would be well adapted at low temperature and so less variation would be

observed. This evidentially occurred. Likewise it would be expected that the H188 hybrid would be less well adapted than H179 at high temperatures, and therefore show greater variation under this condition. This did not occur, instead the two sets of individuals displayed a similar phenotypic range to each other.

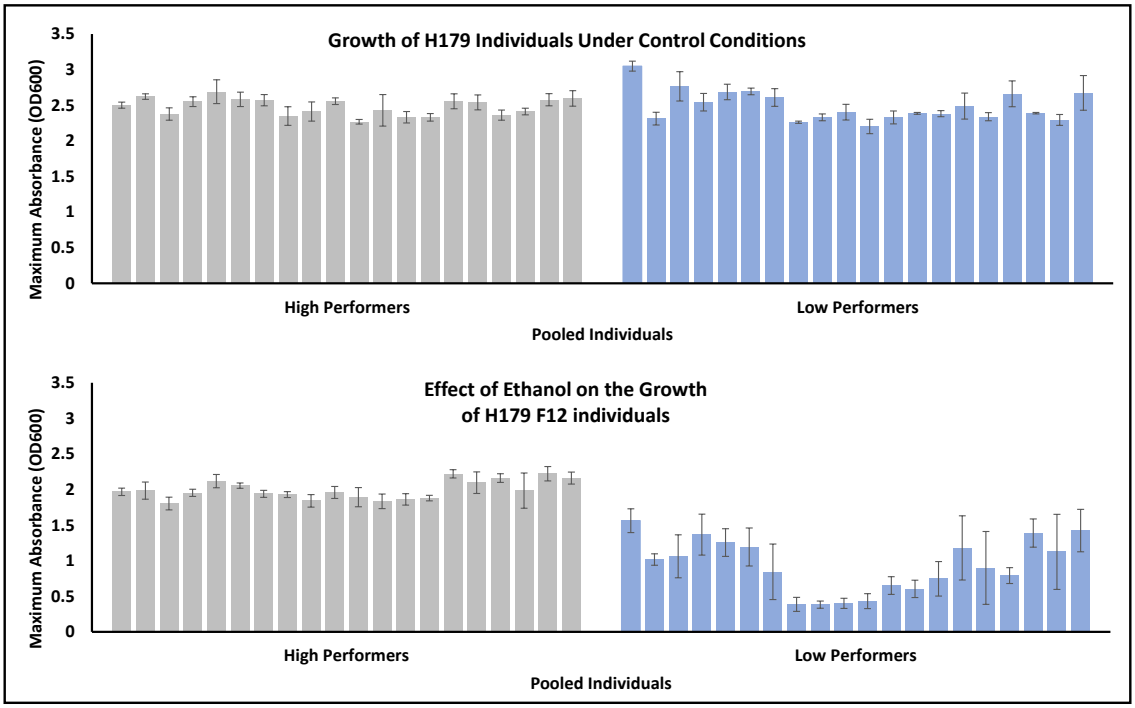


Figure 4.2.1 Phenotypic Variance of H179 Individuals Under Ethanol Stress

Phenotypic assessment of H179 individuals growth ability under stress conditions. Graphs show the maximum growth after 72 hours, of the twenty high performing individuals and the 20 lowest performing individuals, when in the presence of high ethanol concentration (15 %). (A) shows maximum growth of each individual on YPD at 23 °C. (B) shows maximum growth of each individual on YPD + 15 % ethanol at 23 °C. Plate to plate variation can be observed in particular between low performing individuals. There was no significant difference between the two pools under control conditions. Under ethanol stress there was a significant difference ($p < 1*10^{-10}$, $t = 11.8$)

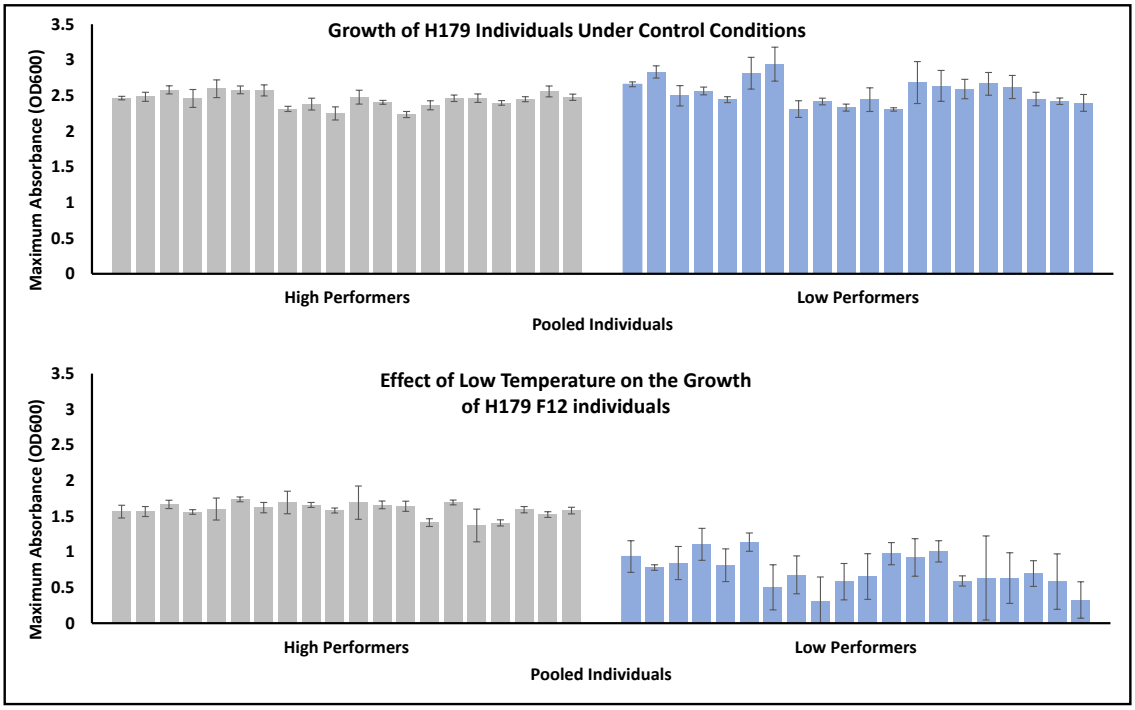


Figure 4.2.2 Phenotypic Variance of H179 Individuals at 4 °C

Phenotypic assessment of H179 individuals growth ability under stress conditions. Graphs show the maximum growth after 72 hours, of the twenty high performing individuals and the 20 lowest performing individuals, when incubated at low temperature. (A) shows maximum growth of each individual on YPD at 23 °C. (B) shows maximum growth of each individual on YPD at 4 °C. Under control conditions the low performing pool, demonstrated stronger growth than the high performers ($p < 0.05$, $t = -2.2$). At 4 °C the growth of the low performers was significantly weaker ($p < 1 * 10^{-13}$, $t = 15.1$).

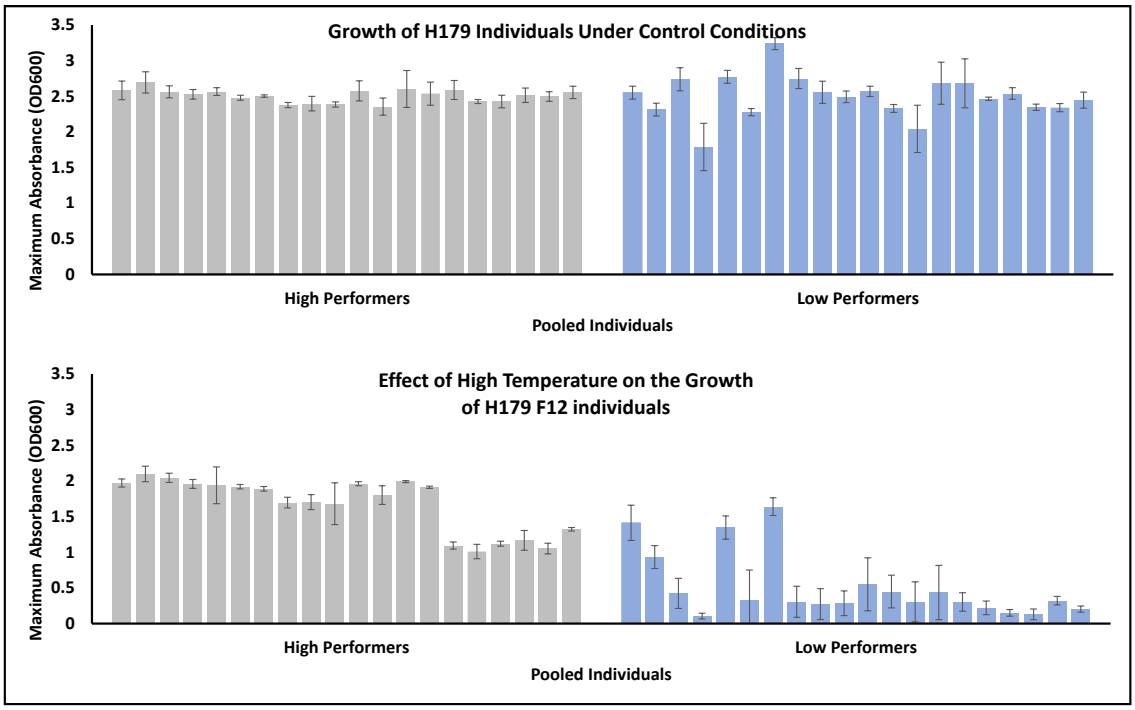


Figure 4.2.3 Phenotypic Variance of H179 Individuals at 40 °C

Phenotypic assessment of H179 individuals growth ability under stress conditions. Graphs show the maximum growth after 72 hours, of the twenty high performing individuals and the 20 lowest performing individuals, when incubated at high temperature. (A) shows maximum growth of each individual on YPD at 23 °C. (B) shows maximum growth of each individual on YPD at 40 °C. Under control conditions there was no significant difference between the two pools. At 40 °C the growth of the low performers was significantly weaker ($p < 1 * 10^{-9}$, $t = 8.8$).

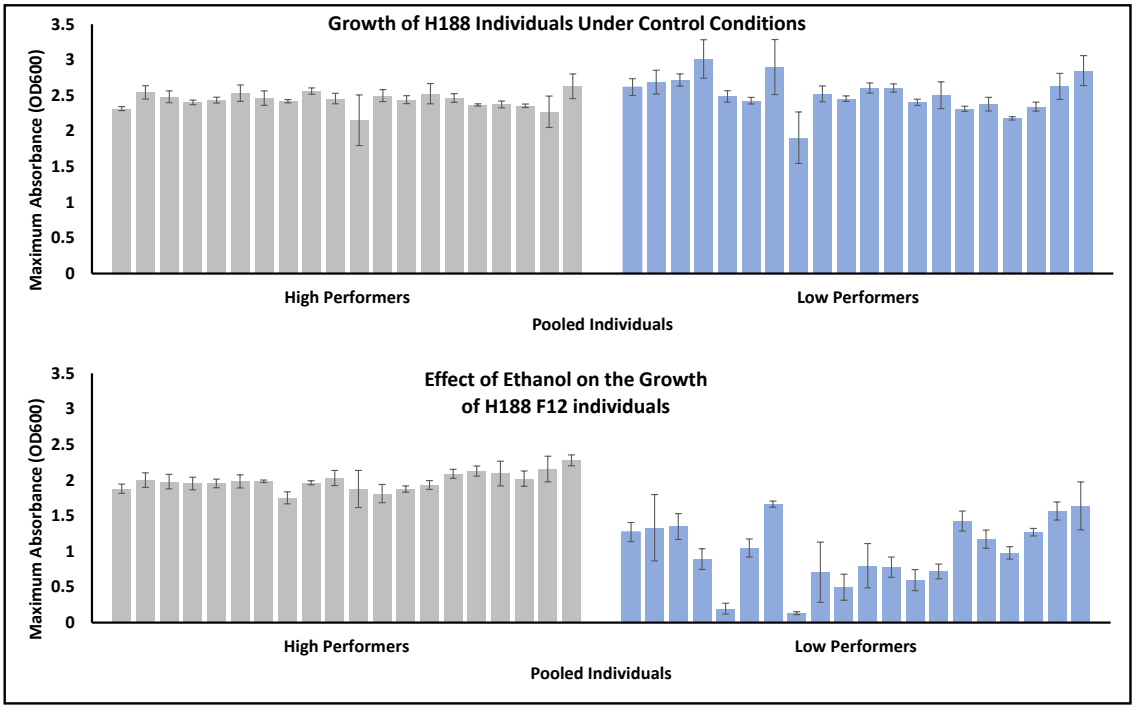


Figure 4.2.4 Phenotypic Variance of H188 Individuals Under Ethanol Stress

Phenotypic assessment of H188 individuals growth ability under stress conditions. Graphs show the maximum growth after 72 hours, of the twenty high performing individuals and the 20 lowest performing individuals, when in the presence of high ethanol concentration (15 %). (A) shows maximum growth of each individual on YPD at 23 °C. (B) shows maximum growth of each individual on YPD + 15 % ethanol at 23 °C. Under control conditions there was no significant difference between the two pools. Under ethanol stress the growth of the low performers was significantly weaker ($p < 1 * 10^{-8}$, $t = 9.5$).

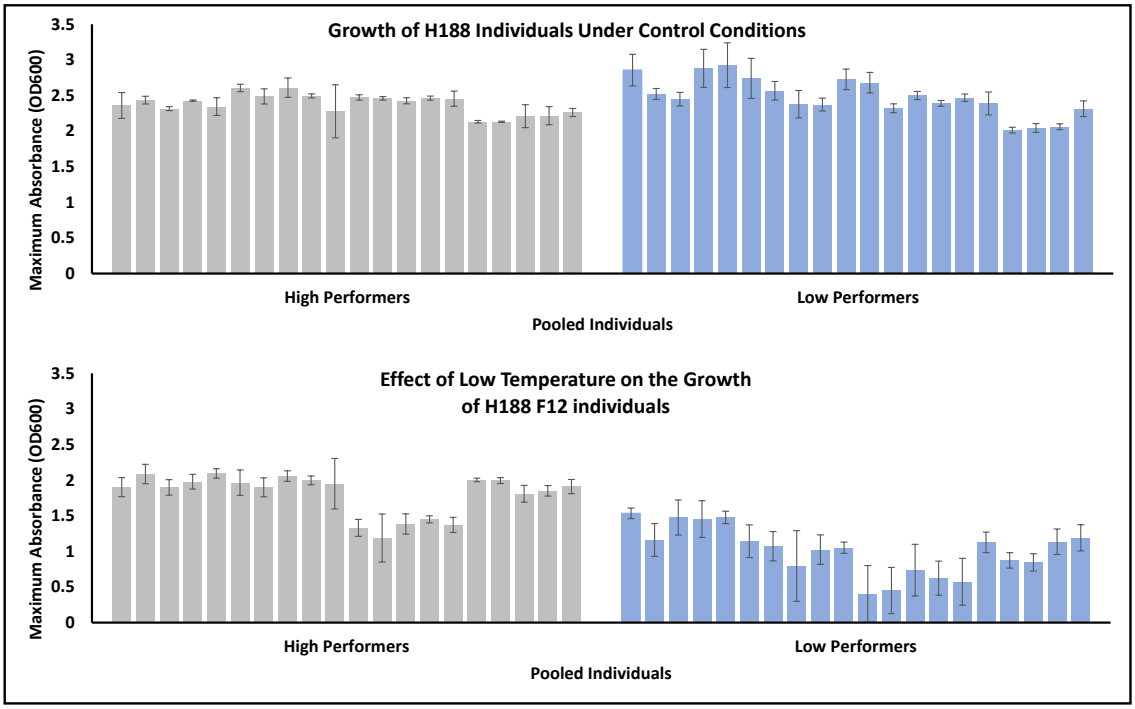


Figure 4.2.5 Phenotypic Variance of H188 Individuals at 4 °C

Phenotypic assessment of H188 individuals growth ability under stress conditions. Graphs show the maximum growth after 72 hours, of the twenty high performing individuals and the 20 lowest performing individuals, when incubated at low temperature. (A) shows maximum growth of each individual on YPD at 23 °C. (B) shows maximum growth of each individual on YPD at 4 °C. Under control conditions there was no significant difference between the two pools. At 4 °C, the growth of the low performers was significantly weaker ($p < 1 * 10^{-8}$, $t = 8$).

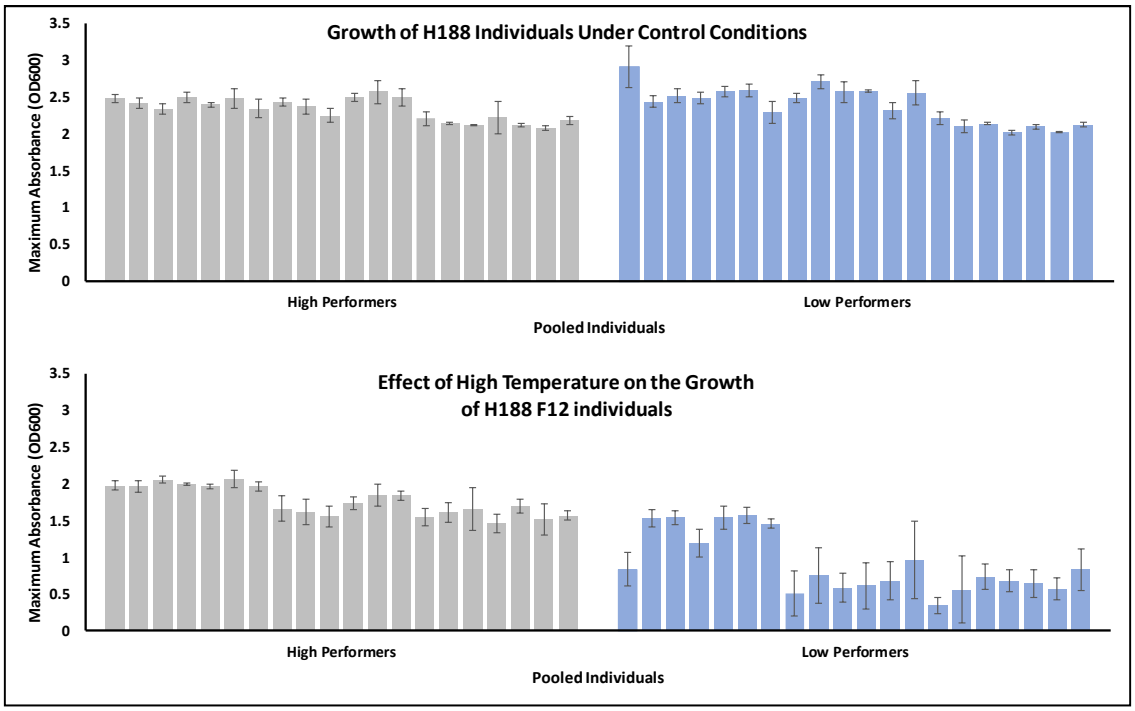


Figure 4.2.6 Phenotypic Variance of H188 Individuals at 40 °C

Phenotypic assessment of H188 individuals growth ability under stress conditions. Graphs show the maximum growth after 72 hours, of the twenty high performing individuals and the 20 lowest performing individuals, when incubated at high temperature. (A) shows maximum growth of each individual on YPD at 23 °C. (B) shows maximum growth of each individual on YPD at 40 °C. Under control conditions there was no significant difference between the two pools. At 40 °C the growth of the low performers was significantly weaker ($p < 1 * 10^{-8}$, $t = 8.4$).

4.2.3 Multipool QTL Identification

DNA samples of pooled individuals were sent for sequencing at the Earlham Institute. Unfortunately the sequencing data was not returned in time, for the complete analysis and identification of QTL by the multipool method. This data has subsequently been analysed and will be available in future publications.

4.3 Discussion

4.3.1 Pooled Selection

4.3.1.1 H51

Intervals were only observed in the *S. cerevisiae* portion, with the allele frequency difference not exceeding 0.2 across the whole genome. It would be interesting to perform the same selection experiment of the equivalent hybrid possessing the *S. uvarum* mitotype (H102). In the same way that H179 and

H188 were compared. If this was performed and intervals were found on the *S. uvarum* portion, possibly instead of the *S. cerevisiae* portion, it would suggest that the mitochondria are interacting preferentially with the genome section that the mitochondria were inherited alongside.

The interval conferring acetic acid tolerance on chr 7 contained three candidate genes, *TPN1*, *HOS2* and *EMP24*. Previously null mutants of *TPN1* and *HOS2* had been reported as having decreased resistance to acetic acid in *S. cerevisiae* strains (Rieger et al., 1999, Kawahata et al., 2006). Whereas the null mutant of *EMP24* was reported to be more resistant to lactic acid than the wildtype strain (Kawahata et al., 2006). This gene appears to operate antagonistically to the *TPN* and *HOS2* genes within the interval. The two parental alleles of *EMP24* had no differences in amino acid sequence, and so neither allele would confer an advantage. Of these three candidate genes, only *HOS2* is observed in the interval conferring levulinic acid tolerance. Two of the five genes identified as being involved in levulinic acid tolerance, did not have previous reports that null mutants resulted in a change in phenotype under acidic conditions. Instead *BLM10* and *MDS3* were reported to confer tolerance to hydrogen peroxide (Higgins et al., 2002). As mentioned earlier, H_2O_2 treatment has been used to isolate multi-stress resistant mutants. Environmental stress of low pH, increases the production of intracellular ROS, inflicting oxidative stress upon the cell. Accumulation of ROS triggers cellular responses from survival to programmed cell death (Farrugia and Balzan, 2012). Increased tolerance of oxidative stress may therefore increase tolerance to conditions of low pH.

Within the four intervals conferring tolerance to high temperature, nine genes were previously reported as being involved in heat tolerance. One locus identified in the pooled selection of H51 included the gene *IRA1*. This gene was within the intervals on chr 2 contributing to heat tolerance and levulinic acid resistance. *IRA1* has previously been identified as contributing to thermotolerance by QTL analysis of non-hybrid species (Cubillos et al., 2013, Parts et al., 2011). The finding of previously reported genes gives assurance to the confidence that the intervals identified in these hybrids are real, and play important roles cellular response to these stresses.

4.3.1.2 H179 and H188

Most the QTL intervals identified were for the selection in maltose rich media. The number of intervals anticipated for the other conditions were lacking. One would hypothesise that the selection conditions were not strong enough, and that a higher concentration of the stressing agent was required. Instead of selecting for the 0.1 - 1 % of individuals, a smaller fraction of the population could have been selected for. This should increase the power of the QTL analysis, identifying possible smaller effect loci. Initially attempts were made to increase the strength of selection, however for many of the conditions increasing the concentration of the stressing agent by just small amounts resulted in complete death of the population. This suggests the possibility that for these selection conditions there was little variation between the parental strains contributing to the segregating population. Alternatively most of the trait is made up of small effect QTLs which don't meet the threshold to be identified, with a lack of major effect QTLs.

The resolution varied between intervals with the smallest interval found in each hybrid analysed being 4 – 6 kb in length; containing as few as 1 or 2 genes. The largest intervals were 25 – 38 kb in length. 17 was the highest number of genes within a single interval. LOD score calculation was not possible, as the exact number of individuals in each pool was required but not known. Some intervals, that contribute to a trait, may have been missed as their allele frequency difference was below the threshold difference of 0.2. In the analysis of a four parent intercross population (Cubillos et al., 2013), the gene *VPS53* was identified as contributing towards heat and arsenite. This was despite the interval containing *VPS53* having a LOD score below the threshold set. The threshold difference was set in order to minimise the appearance of false positive intervals. Lowering the threshold for allele frequency difference, from 0.2 to 0.15 for example, may increase the number of intervals identified. Possibly identifying smaller effect QTL. In doing so the chance of marking intervals that are false positive increases. Decreasing the threshold would also increase the interval size of those found at the original threshold, lowering the analysis resolution. A tandem analysis could therefore be performed where primary intervals are identified at the 0.2 allele difference threshold, with secondary intervals identified at the lower threshold.

Considering that H179 and H188 have an identical parental genetic contribution, barring the difference in mitochondrial origin, it was expected that most QTL intervals would be shared. The five intervals observed on the *S. cerevisiae* portion of H179 that confer an advantage on maltose, had equivalents in the H188 selection pool. However none of the intervals of the *S. eubayanus* portion in H179 pools showed equivalents in the H188 pools. There was also a greater number of QTL intervals in the H188 selection on maltose that in the H179 selection. It could be hypothesised that the H179 pool did not experience the selection pressure to that same extent resulting in the control and treatment allele frequency differences to be less extreme. One of the *S. cerevisiae* parental strains is OS253, which is found within the Sake lineage and was isolated from a palm wine fermentation (**Table 3.1.1**). This strain will be adapted to an osmotic stress environment created by palm sugars, and this mitochrondrial contribution to the H188 hybrid may have resulted in the strain being less affected by the selection condition than the H179 hybrid.

From the H179 population selection on maltose, four *S. cerevisiae* genes were selected as candidates based on previously reported phenotypic studies. *ADH5* - an alcohol dehydrogenase involved in glycolysis (Smith et al., 2004), and *ARA1* – arabinose dehydrogenase involved in carbohydrate metabolism (Kim et al., 1998) appear to be strong candidates genes. However no amino acid differences were observed between the two parental alleles of the *ADH5* gene. It could be that there is base variation in the sequence upstream of the gene, for example in a promoter sequence. In future candidate gene validation studies, this should be taken into account. Rather than performing an allele swap, the promotor region could be swapped instead to investigate whether there are any observed changes in phenotype.

PIG1 has a role in glycogen synthesis, encoding a protein phosphatase that interacts with the glycogen synthase *Gsy2p (CHENG et al., 1997)*. Loss of function impairs glycogen synthesis. Improved sugar uptake and storage could be advantageous in an environment high in sugars. The first three candidates involved sugar utilisation or storage, whereas the fourth *STE20* has been reported to confer resistance to osmotic stress (Yoshida et al.,

2008). None of the three genes in the interval on chr 12 giving H_2O_2 tolerance, have previously been implicated in oxidative stress resistance.

From selection of the H188 population, seven *S. cerevisiae* genes were identified as candidates on high glucose media, and 11 candidates on high maltose media. Of the glucose candidate genes, *ADH5*, *ARA1* and *PIG1* were also identified in maltose selection in the H179 population. *ARA1* was also short listed as a candidate on maltose selection in the H188 population. Three others conferred resistance to osmotic stress, whilst *MCT1* is involved in glucose utilisation (VanderSluis et al., 2014). The null mutant shows a decreased rate of glucose utilisation. Six of the maltose selection candidate genes were involved in osmotic stress (Pastor et al., 2009). Null mutants of five of the genes showed decreased tolerance to osmotic stress, whereas *GCN1* showed a decrease in osmotic stress sensitivity (Goossens et al., 2001). Overexpression of *YAP3* also showed increased resistance to rapamycin, which is a TOR inhibitor (Butcher et al., 2006). Both YAP3 and *TOR2* are candidate genes for having a role in the selection advantage on high maltose media. Null mutants have previously been identified as having decreased osmotic stress tolerance. The final three candidates for maltose utilisation were *SNF3*, *ADH1* and *FUM1*. *SNF3* regulates the transport of glucose across the plasma membrane, with high intracellular glucose resulting in inhibition of transport (Karhumaa et al., 2010). ADH1 – Alcohol DeHydrogenase is involved in the final step of the glycolytic pathway reducing acetaldehyde to ethanol. ADH1 is a paralog of ADH5 which was shortlisted as a candidate in another interval (de Smidt et al., 2008). *FUM1* acts in the tricarboxylic acid cycle (TCA), releasing stored energy through the conversion of fumaric acid into malic acid. The null mutant of *FUM1* shows a decreased ability to utilise a range of sugars including glucose, lactate, and pyruvate (Kaclíková et al., 1992).

Of the *S. cerevisiae* genes identified in the H188 selection but not in the H179 maltose selection pools, *IMP2'* and *RPO41* interact with the mitochondria. *RPO41* is the mitochondrial RNA polymerase and required for transcriptional maintenance of the mitochondrial genome (Greenleaf et al., 1986). *IMP2'* is required for the de-repression of glucose repressed genes, including genes involved in maltose utilisation (Lodi et al., 1995), making *IMP2'* a clear

candidate for providing a selection advantage on maltose. Since these *S. cerevisiae* genes have an interaction with the mitochondria, it is interesting that they appear in H188 QTL intervals but not H179. It could be reasoned that neither allele is at an advantage when interacting with the mitochondria of the same species, but one is interacting more favourable than the other with mitochondria of *S. eubayanus* origin.

Shortlisting of *S. eubayanus* genes was based upon studies of gene function in *S. cerevisiae*. Of the six shortlisted genes within the *S. eubayanus* portion of H179, two genes; *ACT1* and *PRO2*, have been identified as conferring resistance to osmotic stress (Yoshikawa et al., 2009, Tedrick et al., 2004). *KGD2* acts upon the TCA cycle, catalysing alpha-ketoglutarate to succinyl-CoA (Reinders et al., 2007). The TCA cycle takes place within the matrix of the mitochondria. The null mutant of *COX10* results in respiration failure. The COX10 protein is a transmembrane protein of the mitochondrial inner membrane (Murakami et al., 1997). *GSM1* has been proposed to regulate energy metabolism, as well as being a modulator of glucose starvation (van Bakel et al., 2008). *KGD2* and *COX10* have a close relationship with the mitochondria, both in function and localisation. The absence of intervals containing these genes in H188 may indicate one allele is favoured over the other when interacting with the *S. cerevisiae* mitochondria.

RKM1 and *CBP3* were shortlisted from the *S. eubayanus* portion of H188, as being involved in growth at low temperature. They had been reported to be involved in innate thermotolerance. The null mutant of *RKM1* showed increased tolerance to heat shock of 50 °C, whereas the null mutant of *CBP3* showed decreased tolerance to the heat shock (Jarolim et al., 2013). These two genes, found within the same interval, appear to operate antagonistically at high temperatures. Further analysis, by individual gene deletion and allele swapping, will reveal whether two trait loci are within the single locus with separation hidden by insufficient resolution mapping. Of the 12 genes shortlisted from the maltose rich selection, 11 were reported to be involved in osmotic stress resistance (Proft et al., 2001, Auesukaree et al., 2009, Yoshikawa et al., 2009). The null mutant of all 11 genes showed decreased osmotolerance. The twelfth gene, *GSC2*, was reported to be involved in sugar utilisation (VanderSluis et al., 2014).

Under maltose selection, a much greater number of candidate genes were identified in H188 that had an effect upon the osmotolerance than in H179. These genes were identified in both parental regions of the genome. One might speculate that since *S. cerevisiae* has been widely domesticated, this species mitochondria have adapted to become more innately tolerant to osmotic stress than *S. eubayanus* mitochondria. This speculation owes to the fact that many of the processes that *S. cerevisiae* has been domesticated in; including wine, beer, and sake production, involve exposing yeast to high sugar concentrations.

The amino acid sequence similarity between parental alleles was much less in proteins coded by *S. eubayanus* genes, than *S. cerevisiae* genes. On average there was a difference in 1 in 10 amino acids coded by *S. eubayanus* sequences, whereas between the two *S. cerevisiae* alleles on average there was a single change in amino acid in every 200. Based on multilocus sequence comparison the West China *S. eubayanus* isolate (OS626) was found to have a genetic distance from the Holarctic isolates of 7.4 % (Peris et al., 2016), which includes the reference strain (OS578). This difference is similar to that between the *S. eubayanus* and *S. uvarum* reference genomes (Libkind et al., 2011). Introgressions between species have been reported; particularly in the Holarctic groups of each species, and this may be a primary cause of the observed divergence (Albertin et al., 2018). As the sequence for the West China strain was not available, it was sequenced alongside the pooled individuals. The depth of sequencing may not have been sufficient, and as a result may have contributed to the high sequence diversity observed between the two parental strains.

4.3.2 QTL Validation

Most previous QTL analysis has been performed on haploid segregants. Confirmation of candidate genes is performed by reciprocal-hemizygosity analysis (Steinmetz et al., 2002). From the parental cross two isogenic strains were created. One with the allele of parent 1 deleted, the second with the allele of parent 2 deleted. This produced hemizygous diploids carrying a single parental copy of the candidate gene. These strains were then tested to confirm whether one allele is advantageous over the other. Confirmation by reciprocal hemizygosity in the *de novo* hybrids is possible but more difficult, owing to the

higher ploidy. To achieve this, deletion of one copy of the candidate gene in the original tetraploid hybrid is required. The locus would be genotyped to determine which parental allele was deleted. Two deletion isolates would be required, differing only by the allele deleted. Finally phenotypic comparison would confirm whether one allele confers a selection advantage over the other.

Another option would be to return to the parental haploid strains, and simply delete the candidate locus in each. Observation of a change in the specific phenotype, in any direction, would indicate the locus acted in some way towards the trait. The next step would be to perform an allele swap between the parental strains (Erdeniz et al., 1997), by inserting the allele suspected of conferring a phenotypic advantage the other parental strain. In the past few years a variety of techniques have been developed that enable high throughput allele swapping, including utilising the CRISPR-Cas9 system (Stovicek et al., 2017). Another method termed HERP (Alexander et al., 2014), enables efficient swapping of multiple allele copies allowing for allele replacement in diploids. This method has been validated using alleles from across the *Saccharomyces* clade, making it highly applicable to these hybrids.

An alternative method of validation is achieved using the individuals arrayed at the end of the pooled selection experiments (**Figure 2.4.1**). Each individual will be genotyped for each candidate gene to determine which parental allele is present. The allele frequency amongst individuals should correlate with the frequency difference at the interval observed. It is also possible to genotype the array of individuals from the control population. Ranking control individuals by phenotype, should see an increase frequency of the candidate allele amongst individuals at the phenotypic extreme (Ehrenreich et al., 2010).

The difference in identified QTLs between H179 and H188 could be due to the influence of the mitotype. This should be investigated thoroughly in an attempt to prove this hypothesis. For both hybrids, arrays of *MATa* and *MATα* F12 individuals should be created. Individuals should then be genotyped at the loci of several major QTLs. Individuals need to be found with identical genotypes in both strains. Petites of each should be made, and then crossed with the individual of the other strain to effectively switch mitotype. A phenotypic

comparison of these individuals will shed light on the involvement the mitochondria has upon these QTLs.

4.3.3 QTL Analysis Improvement

In QTL analysis by pooled selection, the analysis of strains under selection on maltose rich media should be considered a success. A large number of intervals were identified as contributing a selection advantage. Other conditions yielded only a moderate number of intervals in each strain. Only one interval was identified in H179 that contributed to a trait, H_2O_2 tolerance, other than growth on maltose. The high temperature selection only identified QTL in H51, with no intervals found in H188. Only one interval was identified in selection at low temperature. Future experiments should involve greater selection pressures to improve the number of intervals identified. This would be achieved through higher reagent concentrations, and increased extreme temperature. At 4 °C, it appeared that little selection occurred in the number of individuals capable of growth. Instead, the population experienced differential rates of growth. It was hoped that pooling the population at a specific time point, would collect a population concentrated with individuals that are capable of a fast rate of growth. Decreasing the temperature is unlikely to improve the number of intervals identified, as the rate of growth of all individuals within the population becomes too slow. Instead to improve the hit rate, collecting the pooled population after an earlier time point should be attempted.

4.3.4 Summary

Detailed QTL analysis has been achieved for the first time in *Saccharomyces* hybrids. This has been achieved using two methods of bulk segregant analysis. A large number of candidate genes have been identified as contributing to a range of industrially relevant traits. These candidates now need to be individually validated using a variety of techniques outlined above. It was not possible for validation to be performed during the project timeline, due to the large number of candidate genes identified and the added difficulty that higher ploidy creates with regard to QTL validation. However there can be confidence over the legitimacy of the QTL intervals identified. This is in part due to the identification of genes that had previously been identified (e.g. IRA1). Furthermore many genes within the intervals have been reported in

previous literature to contribute to the traits studied, or related phenotypes. The large number of candidate genes based on previous literature also suggest that the intervals identified that lack candidate genes are likely to contain loci that contribute to the phenotype. These novel loci would further demonstrate the effectiveness of i-QTL analysis and our ability to perform QTL analysis in hybrid species.

The comparison between H179 and H188 has identified a number of unique intervals in each hybrid. Many of these intervals contain genes that are tied to mitochondrial processes including respiration and the TCA cycle. Further investigation is required to demonstrate that the hybrid mitotype influences these loci.

Shortlisting of genes was based upon previous reported phenotypes relating to those investigated here. Some intervals did not contain a candidate gene that had previously been implicated. Genes within these intervals should also be considered. The phenotypes of these may not have been identified by classical genetic techniques and they are being identified as playing a role in the trait for the first time here.

Another factor to consider is the impact of gross chromosomal changes. This investigation has looked at changes in allele frequency, however this would not identify GCRs such as changes in gene copy number which would also have an influence upon observed phenotype.

5 Introduction of Fertility to Existing Lager Hybrids

5.1 Introduction

5.1.1 Aims

The primary objective of this chapter is to manipulate *S. pastorianus* strains to introduce fertility. Restored fertility will enable breeding to be performed, which will improve the level of phenotypic variation, as well as create the potential for performing quantitative genetic analysis.

Fertility is to be introduced into Saaz strains through crossing with a haploid *S. cerevisiae* isolate, to create a near tetraploid individual (**2.5.1** and **2.5.2**). Rare viable spores of Frohberg strains will be created (**2.5.5**), that are suitable for crossing with other diploid isolates.

5.1.2 Background

Genetic modification has been performed to improve lager yeasts. However these improved strains have as yet not been commercialised due to negative public perception and strict regulation surrounding genetically modified organisms (GMOs) (Saerens et al., 2010, Dequin, 2001). Targets for improvement range from higher fermentation rate, and improved sugar utilisation, to reduction in off-flavour production (Wendland, 2014). A specific example improved the fermentation performance by repairing the lager yeast *AGT1* gene, a maltose and maltotriose transporter (Vidgren et al., 2009). Optimism that new gene editing techniques would be considered exempt from the extensive GMO regulation was damped by the recent EU Court ruling, as mentioned earlier (Callaway, 2018).

In attempts to create new *S. pastorianus*-like strains, attempts have looked at crossing *S. cerevisiae* strains; which have strong fermentative properties, to other cold tolerant species such as *S. kudriavzevii*, and *S. uvarum* (Bellon et al., 2011, Lopandic et al., 2016, Nikulin et al., 2018). These studies succeeded in creating new hybrids with strong fermentation performance at low temperature and improved flavour profiles in wine and lager.

Since the identification of *S. eubayanus* as the non-*S. cerevisiae* parental species to *S. pastorianus*, there have been numerous attempts to recreate the

lager hybrid using a variety of strains from both parental species (Krogerus et al., 2017, Krogerus et al., 2015, Mertens et al., 2015, Magalhaes et al., 2017). Without introducing fertility, this could be considered equivalent to breeding a horse and a donkey: combining two species, each with desirable traits, to yield a mule in the hope the mule inherits the desirable traits from each. Progeny from these crosses must then be extensively phenotyped to identify individuals that have inherited all the required traits. The Verstrepen group have been pioneers in improving high-through put screening, and are capable of screening individuals at a nanolitre scale (Vervoort et al., 2017).

Using the existing lager strains as a starting point is advantageous over creating *de novo* hybrids. Lager strains already possess a phenotypic profile desirable to brewers. They have strong fermentation properties and produce low levels of off-flavour compounds (Hebly et al., 2015). Previous studies have introduced additional characteristics to lager strains through breeding with *S. cerevisiae* strains (Garcia Sanchez et al., 2012).

Spore clones have been isolated previously from Frohberg strains (Gjermansen and Sigsgaard, 1981). These have also been used to introduce new traits though breading with both lab *S. cerevisiae* strains and ale yeasts (Garcia Sanchez et al., 2012). However, so far no genetic analysis has been performed to identify loci involved in brewing specific traits, or to further the understanding of why hybrids perform so well in fermentation industries. This is despite identification of this potential over 40 years ago (Spencer and Spencer, 1977).

Rare mating and protoplast fusion has been used previously to facilitate breeding in lager yeasts (Sato et al., 2002, Krogerus et al., 2018). Successful introduction of fertility will open the door to classical selective breeding. Breeding would be possible between different lager strains, exploiting the limited diversity within the species though rearranging the genome creating new allele combinations. It will also be possible to breed with other species, including *S. cerevisiae* ale strains, incorporating new traits into lager strains.

5.1.3 Application of chapter aims

In attempts to breed with, and introduce fertility to the Saaz subgroup of *S. pastorianus*, Saaz strains were crossed with *S. cerevisiae* strains (**2.5.4**). Since the ploidy of Saaz strains is allotriploid, haploid *S. cerevisiae* strains

were used for the crosses. This would create individuals with a ploidy close to tetraploid, which may be able to produce viable near diploid gametes. Isolates of crosses were produced from methods of rare mating (**2.5.1**), and protoplast fusion (**2.5.2**). Since *S. pastorianus* strains typically exhibit a very low sporulation frequency, isolates were assessed for their ability to sporulate. Isolates that displayed an improved ability to sporulate were dissected and the viability of these spores were assessed.

The Frohberg class of lager yeasts, demonstrate poor sporulation ability (Hansen and Kielland-Brandt, 2003). When they do produce spores they are rarely viable due to being highly aneuploid. It is expected that those which are viable would have a ploidy level close to a diploid state. By utilising various techniques to promote sporulation in various Frohberg strains (**2.5.4**), a number of rare viable spores were isolated (**2.5.5**). Since the *HO* gene has not been deleted, it would be expected that any viable spores would be non-maters. A spore mater would be able to undergo a mating type switch and become near tetraploid again. It has previously been reported that this is not the case, with near diploid maters being (Gjermansen and Sigsgaard, 1981), suggesting mating type switching is interrupted in Frohberg strains. This shall be utilised to our advantage to facilitate breeding with Frohberg yeasts. The mating type of rare viable spores will be determined by PCR (**2.2.11**) and their ploidy assessed by flow cytometry (**2.2.9**). Those with a defined mating type can then be crossed with a variety of other diploid mater strains.

5.2 Results

5.2.1 Rare Mating

Rare mated Saaz strains were selected for on SD-uracil media (**2.1.2**) supplemented with G418. The haploid *S. cerevisiae* isolates carried the *KanMX* resistance gene conferring resistance to G418. However, these haploid isolates lacked the *URA3* gene, requiring synthetic defined media to be supplemented with uracil for growth. Conversely the wild type Saaz strains lacked resistance to G418, but carried the functional *URA3* gene. As a result only individuals that underwent mating between the two species were capable of growth on the selection medium.

In total 37 rare mating isolates were found between OS60 haploids and OS57 (**Table 2.5.2**). Confirmation of successful mating between the two parental species and incorporation of the two genomes was performed by RFLP analysis of the ITS region (**Figure 5.2.1**) (**2.5.3**). Isolates that gave a five band restriction pattern, characteristic of mated isolates, were assessed for their ability to sporulate and the viability of the F1 gametes in individuals that demonstrated sporulation ability. There was no increase in the observed spore viability of these individuals relative to the wildtype lager strain (0 – 1 %).

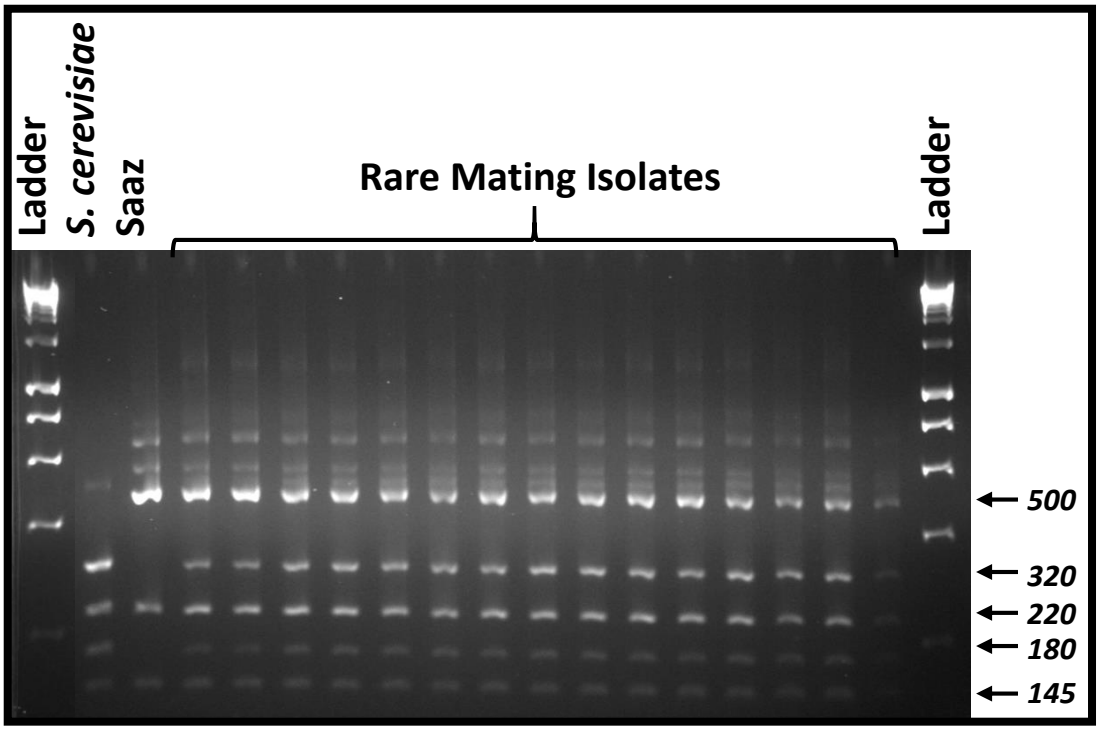


Figure 5.2.1 ITS PCR of Rare Mating Isolates

Isolates of rare mating events between Saaz strains and *S. cerevisiae* haploids were assessed for inheritance of both parental genomes. Saaz strains have a characteristic RFLP of three bands; 500, 220, and 145 bp in length. The banding pattern of *S. cerevisiae* strains is four bands; 320, 220, 180 and 145 bp in length. Successful rare maters give rise to a five restriction band pattern.

5.2.2 Protoplast Fusion

24 protoplast fusion isolates were obtained through crossing either OS57 or OS320 with OS60 haploids. Of these 24 isolates only five demonstrated an ability to sporulate. These were all from the fusion between OS320 and OS60. Of those which were able to sporulate, spore viability was assessed (**Table 5.2.1**). Only the fusion isolate 4 (henceforth referred to as the protoplast fusion

isolate) demonstrated viability of any significance (45.6 %). From the protoplast fusion isolate, each of the tetrads dissected that gave rise to viable spores showed two viable spores and two non-viable spores. The F1 protoplast fusion isolate spores (PIS), were confirmed as possessing both parental ITS regions by RLFP analysis (**Figure 5.2.2**) (**2.5.3**). The mating type of the PIS individuals was assessed by PCR with both mating type bands observed (**2.2.11**). The PIS were then checked for their ability to sporulate, of which none displayed this ability on KAC, KAC min, or CSH sporulation media (**2.1.3 – 2.1.5**). The ploidy of the fusion isolate and its PIS were assessed by flow cytometry (**Figure 5.2.3**) (**2.2.9**). Gating was determined using strains of known ploidy, and shown in chapter 3 (**Figure 3.2.1**a-d). The haploid *S. cerevisiae* parental strain had two peaks C1 and C2 of similar intensity to the peaks observed in the haploid control (**Figure 3.2.2**a). C1 refers to the peak intensity when cells are in the G1/0 phase of the cell cycle, while the C2 peak is twice the C1 intensity and contains cells in the G2 phase after DNA replication before mitosis. The wild type Saaz strain OS320 had two distinct peaks falling within the C1 and C2 gates of the triploid control. The protoplast fusion isolate had three distinct peaks. Two peaks were of similar intensity to the tetraploid control C1 and C2 peaks. There was a third, unexpected, peak at half the tetraploid C1 peak. This isolate was expected to be near tetraploid. The two higher intensity peaks support this expectation. The low intensity peak may suggest that there is a mixed population of diploid and tetraploid individuals. The PIS have three peaks, the two high intensity peaks fall within the two diploid gates. The third peak is approximately half the intensity of the C1 gate. This suggests the presence of both haploid and diploid cells. Since samples for flow cytometry were prepared from an overnight culture of a single colony, this may be evidence of genome instability. Alternatively, the diploid cells may have been undergoing sporulation to produce haploid gametes.

Fusion Isolate	Tetrads Dissected	Viable Spores	Viability (%)
1	20	0	0.0
2	20	0	0.0
3	20	1	1.3
4	40	73	45.6
5	20	0	0.0

Table 5.2.1 Spore Viability of Fusion Isolates

Protoplast fusion isolates, which demonstrated the ability to sporulate, were dissected to assess spore viability. Of the five isolates, only Fusion isolate 4 demonstrated spore viability (45.6 %).

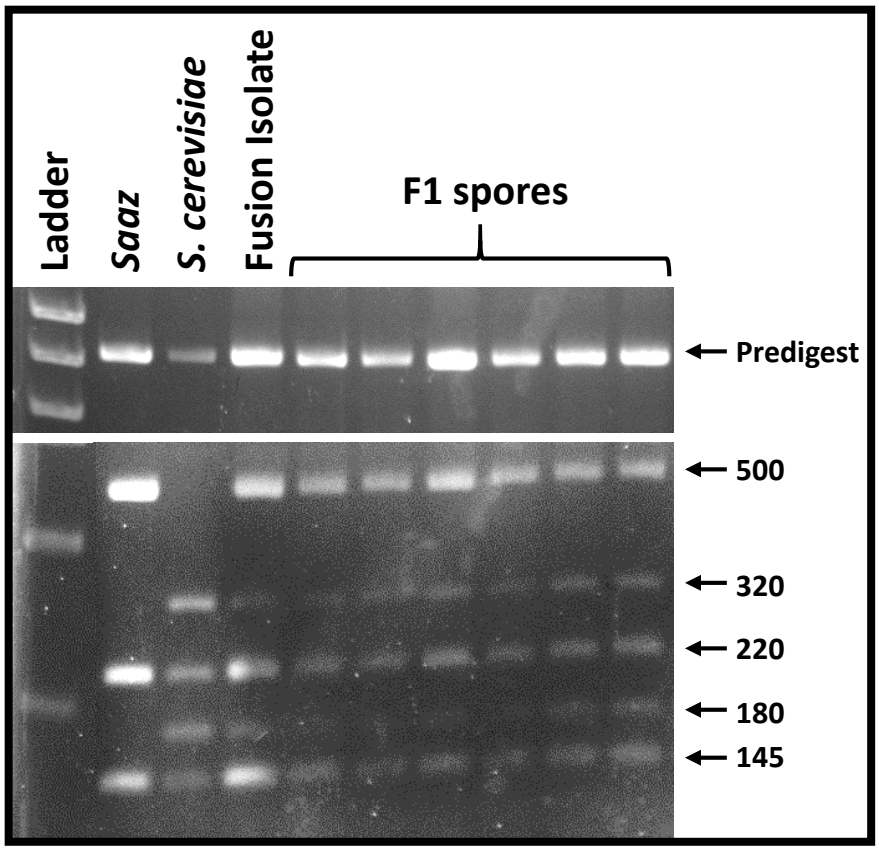


Figure 5.2.2 ITS PCR of Protoplast Fusion Isolate

Confirmation of fusion was performed by PCR amplification of the rRNA ITS region and subsequent digestion by *HaeIII*. Saaz strains have a characteristic RFLP of three bands; 500, 220, and 145 bp in length. The banding pattern of *S. cerevisiae* strains is four bands; 320, 220, 180 and 145 bp in length. The fusion isolate, and its viable spores inherited *HaeIII* restriction sites from both parents giving a characteristic 5 band restriction fragment pattern.

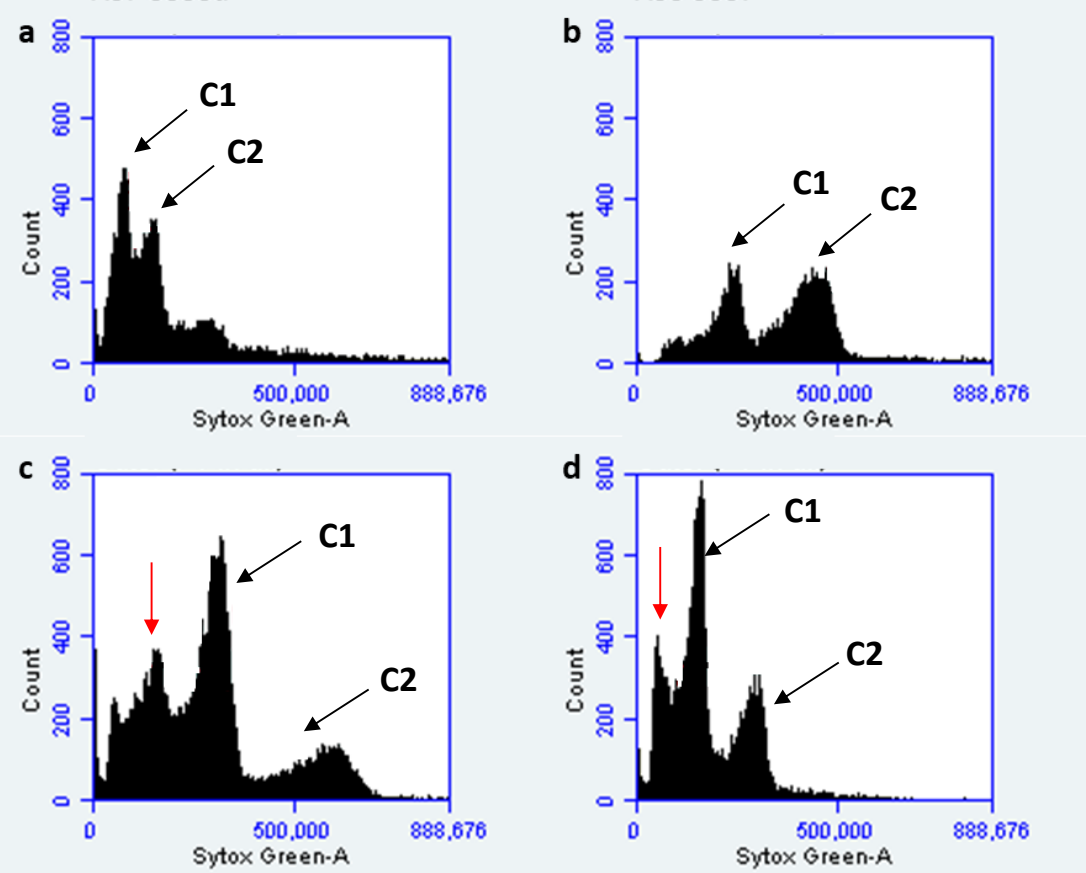


Figure 5.2.3 Ploidy Assessment of the Protoplast Fusion Isolate

Histograms showing the nuclear stain (Sytox Green) intensity of isolates (a) Haploid OS60 parental strain with two distinct peaks C1 and C2 these two peaks are of similar intensity to the peaks observed with the haploid control (**Figure 3.2.1**a). (b) Allotriploid Saaz strain OS320, with two distinct peaks, C1 and C2. These peaks are of similar intensity to the peaks observed with the triploid control (**Figure 3.2.1**c).(c) three peaks were observed in the analysis of protoplast fusion isolate 4. C1 and C2 were expected and of similar intensity to the tetraploid control peaks (**Figure 3.2.1**d) the third peak (red arrow) was half the intensity of the tetraploid C1 peak. (d) Histogram of protoplast fusion isolate spores. Three distinct peaks were observed, C1 and C2 were expected and match the peak intensities of the diploid control (**Figure 3.2.1**b). The third peak (red arrow) was half the intensity of the diploid C1 peak.

5.2.3 Phenotypic Analysis

The variation of the PIS individuals in tolerance to several stresses was assessed by phenotyping on solid media (**2.2.10**). The parental strains were also analysed and compared with the protoplast fusion isolate (fusion isolate 4). The environmental stresses assessed were, high temperature (40 °C), high salinity (7 % NaCl); relevant to bioethanol production, high ethanol concentration (15 %); relevant to brewing, and an environment with high acetic acid concentration (0.5 %); relevant to wine fermentation.

5.2.3.1 Parental analysis

Under control conditions (23 °C, on YPD) there was little variation between OS320, OS60a and the fusion isolate (**Figure 5.2.4**). The only significant difference observed was between the fusion isolate and the haploid *S. cerevisiae* strain ($p < 0.05$), with the fusion isolate having the higher maximum growth. At high temperature, the OS60a performed significantly better than OS320 ($p < 0.001$) and the fusion isolate ($p<0.01$). The original lager strain was the worst performing of the three under high temperatures. The second condition where OS60a was the best performing was in the acidic environment. The fusion isolate performed slightly better than OS320 ($p < 0.05$) at the low pH. Under ethanol stress, the fusion isolate was the highest performing ($p < 0.01$). This was followed by OS60a which was stronger than OS320 ($p < 0.05$). Under saline conditions the fusion isolate was stronger than both OS60a ($p < 0.01$) and OS320 ($p < 0.001$). OS320 had a higher maximum growth than OS60a ($p < 0.05$).

5.2.3.2 F1 spore analysis

92 individuals were phenotyped on solid media, in quadruplicate, and ranked by the maximum absorbance measured, normalised to the maximum absorbance under control conditions. For each stress the top five and the weakest five performers were pooled with means of replicates calculated. Differences between the two pools under control conditions were not significant, except for those pooled in the treatment with acetic acid. The individuals in the weak growing pool under acetic acid (**Figure 5.2.6**) outperformed the strong pool when under control conditions ($p < 0.05$). Under each condition, the difference between the strong and weak pools was significant. The difference between the strong and weak pools was greatest under acidic conditions ($p < 0.0001$). The p values for the difference in the pool in environments with NaCl (**Figure 5.2.8**) and ethanol (**Figure 5.2.7**) present were $p < 0.01$, whilst the smallest difference between pools was at 40 °C ($p < 0.05$) (**Figure 5.2.5**).

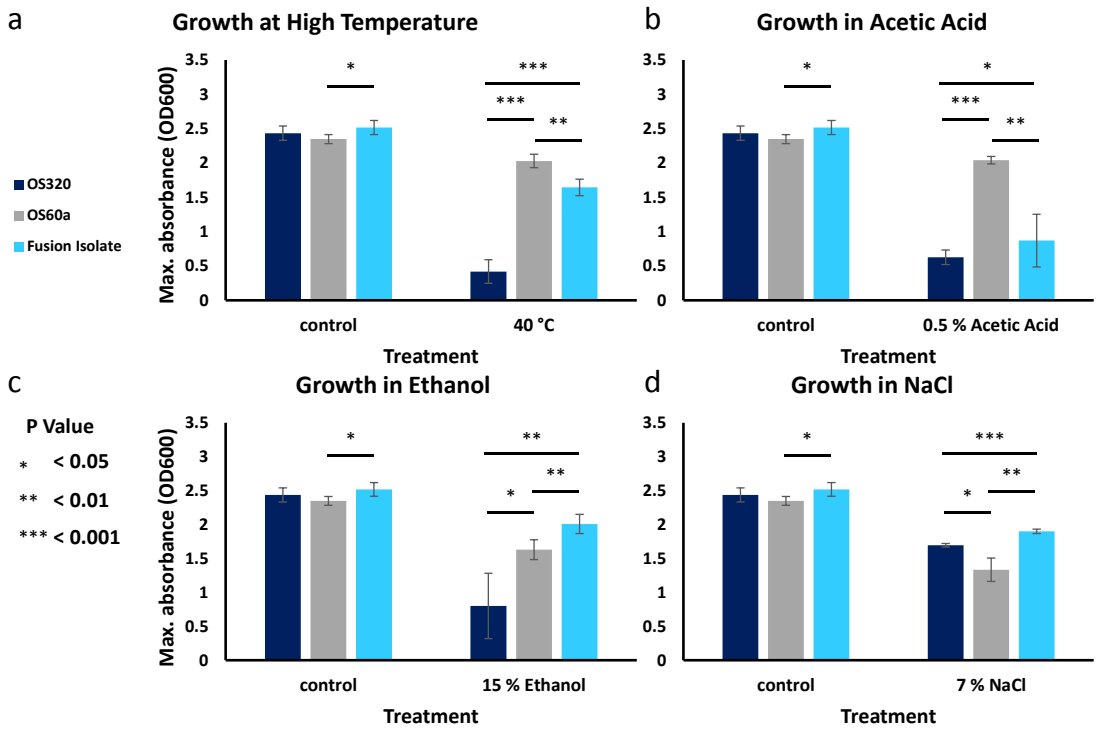


Figure 5.2.4 Phenotypic Assessment of Fusion Isolate and Parents

The fusion isolate (light blue) was assessed for tolerance to high temperature (a), acetic acid (b), ethanol (c), and NaCl (d); in comparison with its parental strains OS320 (dark blue) and OS60a (grey). Growth was measured over three days as absorbance (OD 600nm), with the maximum measurement compared. Pair wise t-test, assuming unequal variances was used to statistically assess observed differences.

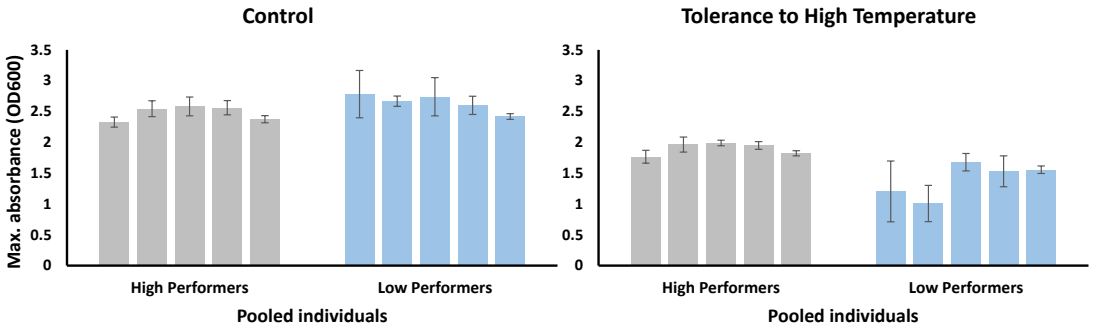


Figure 5.2.5 Variation Induced by High Temperature

Heat tolerance was assessed by growth at 40 °C. The five high performing individuals were pooled and compared against the five lowest performing individuals. Under control conditions (23 °C) there was no statistical difference between the two pools (two-tailed p value > 0.05). At 40 °C there was a statistical significance between the two pools ($p < 0.05$).

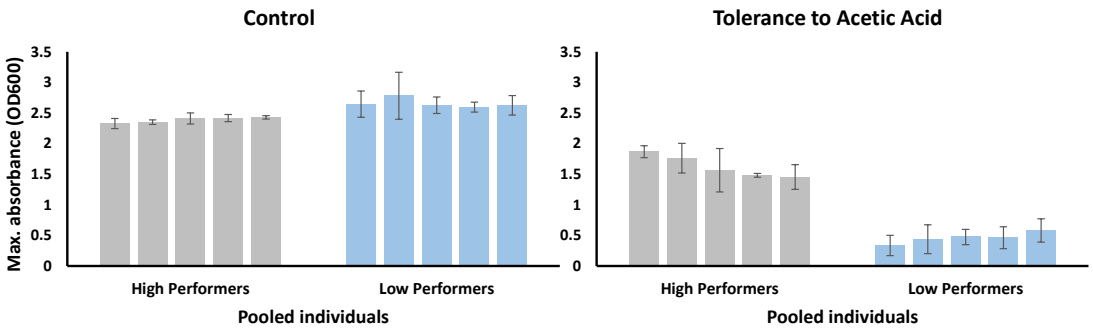


Figure 5.2.6 Variation in Tolerance to Acetic Acid

Individuals were grown on YPD + 0.5 % acetic acid. Growth was measured by absorbance. Five individuals with the greatest absorbance were pooled and compared against the five individuals with the lowest performance. Statistical analysis between the two pools was performed by t-test, assuming unequal variances. Under control conditions (YPD) the low performers, sensitive to acetic acid, outgrew the high performers (two-tailed p value < 0.01), with a mean max absorbance of 2.65 compared to the higher performers mean of 2.39. The mean for high performers under treatment was 1.63 compared with 0.46 for low performers, and was statistically significant ($p < 0.0001$)

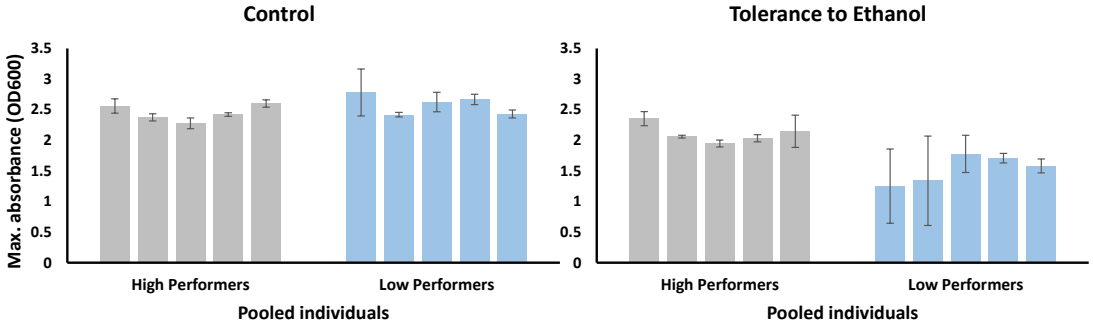


Figure 5.2.7 Variation induced by High Ethanol Concentration

Ethanol tolerance was assessed by growth at 23 °C on YPD + 15 % ethanol. The five high performing individuals were pooled and compared against the five lowest performing individuals. Under control conditions (23 °C) there was no statistical difference between the two pools (two-tailed p value > 0.1). Under ethanol stress there was a statistical significance between the two pools ($p < 0.005$).

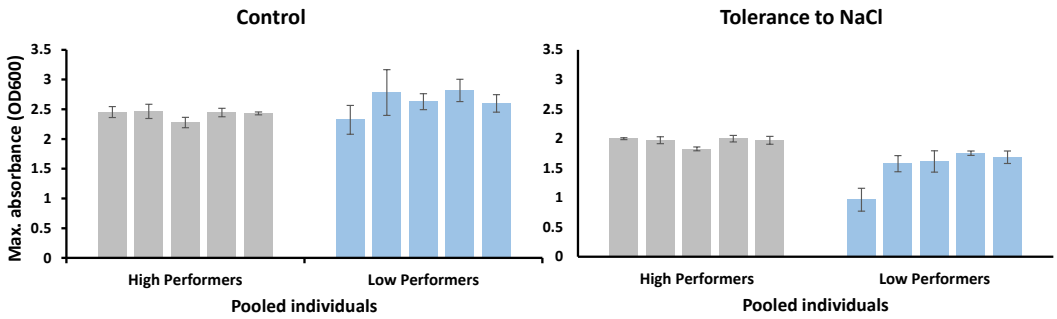


Figure 5.2.8 Variation induced by High NaCl Concentration

Tolerance to Sodium Chloride (NaCl) was assessed by growth at 23 °C on YPD + 7 % NaCl. The five high performing individuals were pooled and compared against the five lowest performing individuals. Under control conditions (23 °C) there was no statistical difference between the two pools (two-tailed p value > 0.1). Under ethanol stress there was a statistical significance between the two pools ($p < 0.01$).

5.2.4 Manipulation of Frohberg Strains

Frohberg strains were sporulated in an attempt to isolate rare viable spores (**2.5.5**). Incubation in pre-sporulation medium (**2.5.5.1**) increased the frequency of sporulation. Isolates were obtained from strains OS56, OS311, OS326, and OS327. The mating type of isolates was determined by PCR. Isolates from OS56, OS311, and OS326 possessed both mating type loci. The three spore isolates of OS327 lacked the *MATα* locus, functioning as *MATa* strains (**Figure 5.2.9**). The isolates of OS327 were mated, by patch mating, to the *S. cerevisiae* diploid mater Q323 created in chapter 3 (**Table 3.2.2**), (**Figure 5.2.10**). The mated isolates from each cross of the OS327 spores with Q323 were sporulated and dissected to assess the gamete viability. Each cross showed a moderate level of viability (13.7 – 20 %). The ploidy of the spore isolates was assessed by flow cytometry (**Figure 5.2.11**). The wild type OS327 produced two distinct peaks of similar intensity to the C1 and C2 peaks of the tetraploid control. This demonstrates that OS327 is near tetraploid and reconfirms that the strain is of the Frohberg class. The spore isolate of OS327 possessed three distinct peaks. It was expected that these would be near diploid. The C1 and C2 peaks (**Figure 5.2.11**b) support this expectation, being similar intensity to the diploid control C1 and C2 peaks (**Figure 3.2.1**b). The third peak was half the intensity of the C1 peak. These three peaks suggest the population contains diploid and haploid individuals, and therefore some level of genome instability.

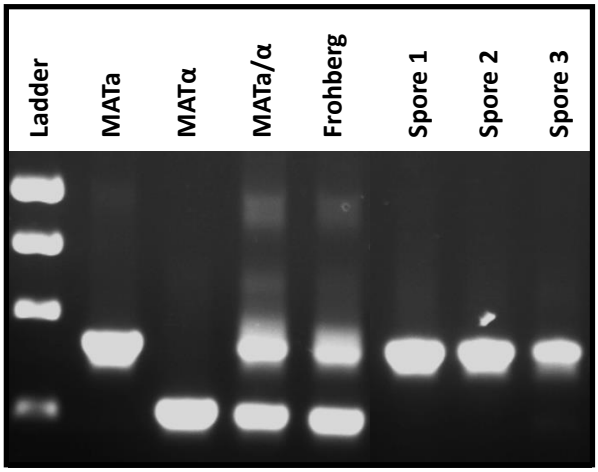
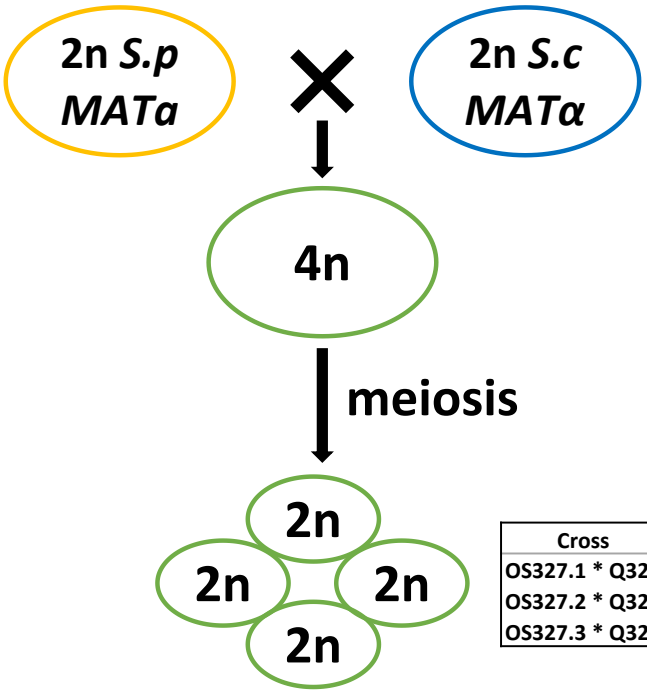


Figure 5.2.9 Mating Type PCR of Frohberg Rare Spore Isolates

The mating type PCR was performed on rare viable spores. Three S. cerevisiae controls were used, OS60 *MATa*, OS60 *MATα*, and the wildtype diploid OS60. The original Frohberg strain was confirmed as possessing both mating types. Three viable spores isolated each had a *MATa* mating type.



Cross	Tetrads Dissected	Viable Spores	Viability (%)
OS327.1 * Q323	20	12	15
OS327.2 * Q323	22	12	13.7
OS327.3 * Q323	19	15	19.7

Figure 5.2.10 Mating of Viable Frohberg Spores

The rare viable spores (*S.p*) identified as *MATa* were mated with *S. cerevisiae* (*S.c*) diploid mater Q323 generated in chapter 3 (Figure 3.1.2). Sporulation of the resulting tetraploid isolate, gave rise to low levels of viable diploid offspring.

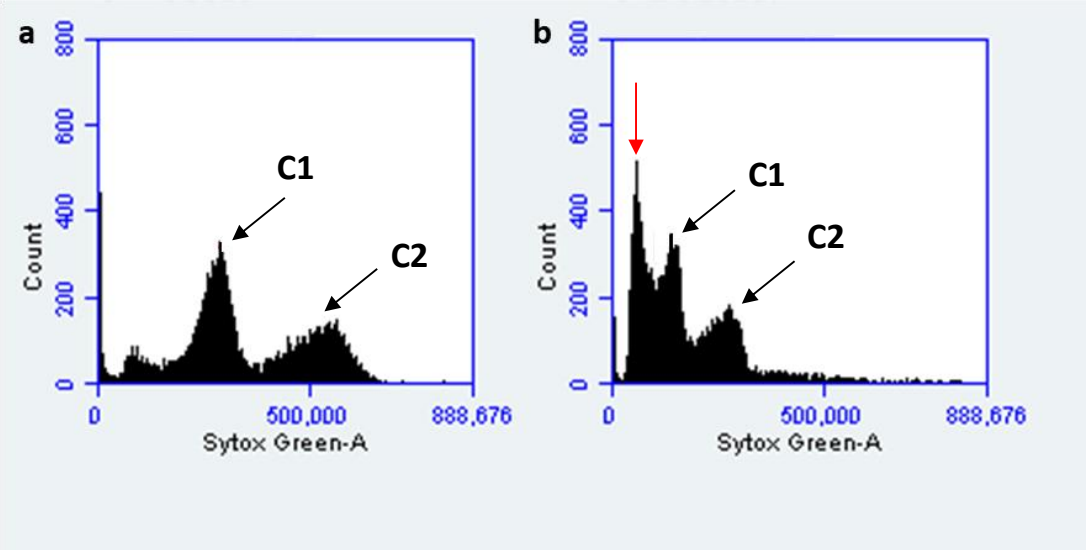


Figure 5.2.11 Ploidy Assessment of Frohberg Spores

Flow cytometry analysis of rare viable Frohberg spores. Histograms displaying the intensity of the nuclear stain (Sytox Green). (a) Wild type OS326 Frohberg strain, with two distinct peaks (C1 and C2) correlating with a tetraploid isolate. (b) Rare viable *MATa* spore isolate, with three clear intensity peaks. Peaks labelled C1 and C2 fit with what is expected of a diploid isolate. The peak labelled with the red arrow was unexpected and is half the Sytox Green intensity of the diploid C1 peak.

5.3 Discussion

5.3.1 Rare mating

The allotriploid Saaz strains presumably possess 3 active *MAT* loci, typically as either *MATa/a/α* or *MATα/α/a*. Since they are heterozygous for the *MAT* locus, they do not secrete mating type pheromones. One mode of becoming homozygous at the *MAT* locus involves a mitotic recombination event leading to homozygosity through gene conversion (e.g. *MATa/a/α* would become *MATa/a/a*) (Haber, 2012). On rare occasions a mating type switch occurs and an individual becomes homozygous at the locus. This individual will secrete the mating type pheromone. Alternatively a single *MAT* locus could have been lost, possible along with one copy of chromosome III, to create a *MAT*a/a or *MAT*α/α individual. When mixed with haploid maters of *S. cerevisiae*, the homozygous individual attracts individuals of the opposite mating type. 37 isolates were obtained from rare mating between OS60 haploids and OS57. In most cases, over two thirds, isolates were achieved from mating OS60a with OS57. This suggested OS57 was MATα/α/a since only one *MAT* locus was required to change or be lost to yield a homozygous individual. Upon

observation that the isolates gave rise to non-viable gametes, an alternative method of crossing between the two species, protoplast fusion, was utilised to investigate if different methods produce different outcomes.

5.3.2 Protoplast fusion

It appears that for the first time an isolate derived from a strain of *S. pastorianus* has been produced that gives rise to viable F1 progeny above the level considered as rare viable spores (~ 1 %) (Hunter et al., 1996, Louis, 2009). Other work that has bred between *S. pastorianus* and *S. cerevisiae* have yielded products lacking gamete viability. (Garcia Sanchez et al., 2012) successfully bred between Saaz and *S. cerevisiae* ale strains producing new hybrids that displayed improved fermentative traits.

Attempts to sporulate the F1 isolates were unsuccessful (**5.2.2**). If any were capable of sporulation, low viability would be expected since each diploid has just a single copy of each parental genome, with sequence divergence between homologous chromosomes disrupting recombination during meiosis.

Flow cytometry analysis revealed that the fusion isolate was approximately tetraploid, and the F1 isolates were near diploid. This confirmed the hypothesis that fusion of an allotriploid with a haploid would yield this level of ploidy. However flow cytometry was unable to provide detail of the exact karyotype. Future work should involve identifying the exact karyotype as it has been reported that during protoplast fusion, not all chromosomes are necessarily inherited into the fusion isolate (Steensels et al., 2014). Fusion isolates are often mitotically unstable, resulting in chromosome loss and aneuploidy (Attfield and Bell, 2003, Kavanagh and Whittaker, 1996). Karyotyping can be achieved through contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Schwartz and Cantor, 1984, Sheehan et al., 1991). This technique can be used to confirm the level of aneuploidy.

A possible future experiment would be to attempt to cross between the F1 spores. The mating type PCR could be applied to identify individuals with specific mating type. Crossing between the F1 spores would create new tetraploid individuals, which would give rise to an F2 population, with increased diversity. In developing new strains for lager production F1 spores with desirable traits would be selected for breeding. It is possible that the F1

isolates are heterozygous at the mating type locus, and therefore would not readily mate. In this instance, the individuals could be crossed by protoplast fusion. However genetic markers would need to be introduced to each individual, so that fusion products could be isolated through selection of both markers. Alternatively deletion of a *MAT* locus would be possible to artificially generate F1 diploid maters.

Not only were fusion events rare, in both mating and protoplast fusion experiments, but most isolates were unable to produce gametes. Just one isolate was identified that gave rise to viable offspring. Chromosomal mapping of the Saaz strain CBS1513 identified that Saaz strains are allotriploid (3n – 1), with 10 of the 47 chromosomes being chimeric (Walther et al., 2014). This genome complexity is likely to have impeded attempts to create more isolates with gamete viability. Furthermore, from the successful fusion isolate, only two of each tetrad set of spores were viable. To clarify why this is happening, the exact chromosomes inherited into the spores should be investigated. This could be done by CHEF gel electrophoresis. The expected ploidy of the fusion isolate is 4n – 1. One hypothesis is the viable gametes are 2n and the non-viable gametes would be 2n – 1.

5.3.3 Phenotypic analysis of fusion isolate spores

Phenotypic analysis of the F1 progeny has shown that variation has been introduced through the protoplast fusion. Through isolating and phenotyping a larger array of F1 individuals (e.g. n = 384), it will be possible to perform QTL analysis using the multipool method utilised in (**Chapter 4**). The greatest variation observed amongst the 92 individuals occurred when treated with acetic acid. This condition should yield the most detailed QTL analysis of the conditions trialled so far. The differences between the high performers and the low performers proved significant in the other stress environments. However, to improve the certainty of the genetic analysis, the temperature or stress concentration could be adjusted to increase the difference in the mean maximum absorbance between the two pools.

To investigate the potential of creating fertile strains from crossing Saaz and *S. cerevisiae* strains, 90 of the F1 spores were assessed for brewing traits.

Candidates showing promise were then selected for analysis of fermentation performance (**Chapter 6**).

5.3.4 Frohberg Isolates

The ability of the *MATa* viable spores of Frohberg isolates to mate with *MATα S. cerevisiae* diploids to produce near tetraploid isolates and subsequently produce viable offspring %) opens up an avenue of introducing new traits into lager strains.

The F1 progeny of these crosses should next be phenotyped and assessed for brewing performance, in the same manner that the Saaz protoplast fusion isolates were assessed (Heading 5.2.3). Flow cytometry analysis showed that these viable spores were near diploid. To confirm this, and identify the exact karyotype, CHEF gel electrophoresis should be performed.

These near tetraploid isolates had a 3 : 1 ratio of *S. cerevisiae* DNA to *S. eubayanus* DNA. Whilst gamete viability was introduced (13 – 20 %) (**Figure 5.2.10**), this was still rather low. The newly introduced *S. cerevisiae* chromosomes would be capable of forming crossovers with the existing *S. cerevisiae* chromosomes in the hybrid. The chromosomes of *S. eubayanus* origin however, lacked an equivalent to pair with during meiosis. The outcome of this would be 50 % of chromosomes segregating accurately, and 50 % segregating randomly creating aneuploid offspring. Increasing the viability of the F1 population may be achieved through mating the initial rare viable spores to *de novo* interspecific diploid hybrids of *S. cerevisiae* and *S. eubayanus*. The outcome of this mating event would be a new near tetraploid hybrid with 2 copies of each parental species genome. It would be expected that this ratio of parental chromosomes will result in a higher level of gamete viability than that of the cross with S. cerevisiae diploid maters (Figure 5.2.10).

Continuing to isolate further viable spores of Frohberg strains, would yield *MATα* spores. Possessing spore isolates of both mating types, from different Frohberg strains, would allow for mating between strains. Individuals from these crosses could then be screened for those which have inherited desirable traits from both parental strains.

5.3.5 Flow cytometry Analysis

In the histograms of the ploidy analysis, three peaks were observed within the populations of the protoplast fusion isolate, its spores, and the Frohberg spore isolate. For each sample two peaks were expected to correspond to the G1/0 and G2 phase of the cell cycle respectively. However, in each there was a third peak that was half the intensity of the expected G1/0 peak. It is possible that there is genome instability in these isolates, resulting in loss of chromosomes. One would not however expect a distinct peak to arise as a result, as it is unlikely that a uniform amount of DNA is being lost in each individual. An alternative hypothesis for the cause of this third peak, is that each population it has been observed in is a mixed population of cells with different ploidy. In the case of the Frohberg spore isolate, the population would appear to contain the expected near diploid individuals as well as a sub group of haploid individuals. Likewise for the protoplast fusion isolate spores. In the case of the protoplast fusion isolate, there appears to be a mixed population of diploid and the expected tetraploid individuals.

5.3.6 Future work

As well as performing breeding experiments within each individual class, it may be possible to perform crosses between Saaz and Frohberg strains. Since there are distinct phenotypic differences between the classes (Gibson et al., 2013), the opportunity to mate between them may prove to be a prosperous avenue in the pursuit of improved lager strains. Crossing between the classes would utilise the broadest phenotypic variation that exists within the hybrid species. An initial attempt could be made between the near diploid protoplast fusion isolate spores and the near diploid Frohberg gametes. Since the Frohberg isolates possess the *MATα* mating type, the mating type of the PIS should identified and a *MATa* isolate used in the cross. It is possible the PIS are all heterozygous for the *MAT* locus. In which case it would be possible to cross between the isolates by protoplast fusion.

One advantage of breeding within the species is that it avoids the addition of unwanted traits. For example, lager strains are characteristically POF negative (Mertens et al., 2017), which means they produce low levels of phenolic-off-flavours (POF). However most *S. cerevisiae* and *S. eubayanus* strains are POF positive. Breeding between *S. pastorianus* and *S. cerevisiae*

is likely to produce offspring that are POF positive. On the other hand, mating with non-lager producing species such as *S. cerevisiae* or *S. eubayanus* would enable the addition of new traits not already present in lager strains. This may still be possible without the introduction of POF. Recently strains of *S. eubayanus* were mutated through UV-radiation treatment, and isolates have been found that are POF negative (Diderich et al., 2018). This finding will enable strains with desirable traits, but are POF positive to be combined with *S. pastorianus* strains. As well as this new *S. pastorianus* strains could be created that are POF negative through crossing with naturally POF negative *S. cerevisiae* strains (Gallone et al., 2016), or mutagenized POF negative isolates of *S. cerevisiae*.

5.3.7 Options for Non-GMO Hybrids

The aim of creating isolates from both classes of *S. pastorianus* that are capable of breeding has been successful. Due to using selectable markers, all strains created here have been genetically modified and so unsuitable for use in industry. Protoplast fusion is also classed as a genetic modification technique for the brewing industry (Steensels et al., 2014). The decision to class this technique as one of genetic modification is somewhat confusing. Isolates are not genetically different to those arising from mating between strains, the technique simply overcomes the need for parent strains to be maters.

Alternative options are available for creating strains that could be used in industry. Non-GMO stable haploids can be generated by mutagenesis and screening for individuals with a loss of function mutation in the *HO* gene (Meiron et al., 1995). With difficulty, it is then possible to perform rare mating with Saaz strains without the resulting isolate being classed as a GMO. A method to select isolates of a mating event is required. One option would be to isolate auxotrophic mutants of each strain. This is simple in haploids, however in individuals of higher ploidy the function all copies of the prototrophic gene must be lost. Using mutagenesis this is possible (Hashimoto et al., 2005). However, in the case of Saaz strains, the intensity of the mutagenic agent to achieve loss of function mutations in three copies of the same gene is highly likely to result in additional mutations that will affect the strains phenotype. The generation of respiratory deficient Saaz strains

(petites), would be possible (Pretorius, 2000). Isolates of rare mating would be capable of growth on a non-fermentable carbon source (YEPEG), as well as being able to grow on the auxotrophic selection media.

Instead of using auxotrophic mutations to select for rare mating events, fluorescence-activated cell sorting (FACS) could be utilised. Using a specific colour stain for each parental strain, isolates of mating could be identified with both stains present. A cell sorter would then be able to isolate these from a mixed population (Bell et al., 1998).

The rare viable spores isolated from Frohberg strains remain non-GMOs. The act of introgressing variation from the *de novo* hybrids, resulted in their GMO status. It would be possible in the future to create diploid non-GMO *de novo* hybrids using haploids generated from loss of function mutations in the *HO* gene, described above. To facilitate mating these would also require the loss of heterozygosity at the mating type locus. Individuals that have lost heterozygosity could be screened for through use of the mating type PCR assay.

6 Assessment of Brewing Potential

6.1 Introduction

The third major project objective was to investigate the brewing potential of both the *de novo* hybrids created in (**Chapter 3**), and the existing lager strains manipulated in (**Chapter 5**). All created strains in this project were GM, due to the use of antibiotic resistance genes as genetic markers, and therefore are unsuitable for use in the food and beverage industry. However the strains synthesised were appropriate for performing a proof of principle study to assess the effectiveness of the methods used to create hybrids, and whether in the future new hybrids could be made that have real industrial potential. A pilot screen was performed searching for individuals with desirable brewing traits. This was performed at the University of Nottingham in collaboration with the industrial CASE studentship sponsor AB-InBev.

6.1.1 Application of chapter aims

The Omnilog (Biolog, US) is an “automated incubator-reader” that is used in the identification and characterisation of a range of microbial species. It can also be used for phenotypic analysis. Previously the system has been used to screen for non *S. cerevisiae* strains that possess formic acid tolerance for the production of bioethanol (Oshoma et al., 2015). The Omnilog is also suitable for assessing the phenotype of individuals in gene deletion microarrays (Bochner et al., 2001).

Using the Omnilog, it is possible to screen 46 strains/ individuals tandemly in triplicate, for up to 20 different conditions (**2.6.2** and **2.6.3**). The metabolic activity of each individual is measured using a redox sensitive tetrazolium dye (Wimalasena et al., 2014, Bochner, 1989). The tetrazolium dye is irreversibly reduced by NADH to formazan, which is purple in colour. The intensity of the purple colour correlates with the level of metabolic activity in the plate well. Utilisation and conversion of sugars into ethanol is a necessity of good brewing strains. The ability to utilise each of the five key brewing sugars (maltose, maltotriose, glucose, sucrose and fructose) was assessed. The ability of strains to utilise melibiose was also investigated, since lager strains

possess this ability whereas ale strains characteristically do not (Bokulich and Bamforth, 2013).

Throughout fermentation yeast is exposed to a harsh environment. Brewing strains must be able to tolerate the extreme environmental conditions present. Multiple stresses act at once as well as in succession. Ideal yeast strains must be capable of withstanding these stresses, which include osmotic stress, oxidative stress, and ethanol exposure. Strains that exhibit sensitivity to ethanol and osmotic stress, undergo a decrease in viability with increased cell death (Pratt et al., 2003). During fermentation there is the initially high osmotic stress created by large amounts of sugar in high gravity wort. In tandem with this, there is the potentially toxic effect of compounds associated with the addition of hops. Hops are used for creating beer aroma and bitterness. Certain hop-derived compounds have been shown to have anti-microbial properties. The iso-α-acids have an antibacterial effect on Gram-positive bacteria (Schurr et al., 2015), disrupting transmembrane proton gradients lowering the bacterial intracellular pH (Simpson, 1993). Previously yeast was considered immune to the anti-microbial properties of hops, however it has been suggested this may not be the case particularly at high concentrations (Hazelwood et al., 2010). As the fermentation advances, sugar is utilised and the osmotic stress decreases, however in doing so the alcohol level increases creating new environmental stress. Furthermore a side effect of being under stress conditions, is that yeast produce higher levels of ROS during metabolism (Pérez-Gallardo et al., 2013), creating an environment high in oxidative stress.

Candidates that performed well at utilising sugars and in response to stresses, using the Omnilog, were selected for assessment of fermentation performance. Speed of fermentation was measured and the composition of the final wort was assessed by high-performance liquid chromatography to investigate candidates' ability to utilise the wort sugars.

6.2 Results

6.2.1 Phenotypic assessment of individual traits

Using the Omnilog platform (**2.6.2**), each individual was assessed for their ability to metabolise six key brewing sugars, five stress conditions, and their performance in lager wort. 90 F12 individuals from four *de novo* hybrids; H51, H179, H188, and H230 were chosen for characterisation. From the protoplast fusion isolate, which gave rise to viable progeny, 90 F1 protoplast fusion isolate spores (PIS) were also characterised. So that comparisons could be made, the parental strains that contributed to these hybrids were also analysed. SMCC, an industrial lager strain, was used to normalise data and provided a reference point as to what was currently considered a desirably performance level in brewing. Heat maps produced using the raw data can be found in the appendix (**Figure 8.2.1** - **Figure 8.2.11**). Each coloured square corresponds to the mean of three technical replicates.

6.2.1.1 SMCC and the Parental strains

The metabolic activity level of the parental strains relative to that of the lager strain SMCC was investigated (**Figure 6.2.1**). The SMCC strain demonstrated strong performance across the six sugars, with its metabolic activity marginally lower with melibiose as the carbon source. SMCC performed well in the presence of ethanol, and to a slightly lesser extent under osmotic stress caused by sorbitol (**Figure 8.2.2**). Under oxidative stress, SMCC performed well at the lowest concentration of H_2O_2, but very poorly at the high 3 mM concentration. The oxidative stressed caused by menadione, resulted in a moderate performance, except at the higher concentration of 0.5 mM where a low metabolic activity was observed. In the presence of the ISO-Hop compound, which mimics the effect of hops on yeast, SMCC displayed slightly weak growth. Finally SMCC showed a moderate level of metabolic activity in lager wort.

The *S. eubayanus* strain OS626 displayed a moderate to poor performance under all conditions, barring in lager wort and in the presence of ISO-hop, where it was comparable to SMCC and the other parental strains.

Barring OS626, which was moderate in performance, all the parent strains showed strong metabolism when grown in sucrose, fructose and glucose.

Expectedly the lager strain OS320, along with OS3 showed the most similar performances to SMCC. The *S. uvarum* strains OS274 and OS449 had greater than 30 % increased maximum metabolic activity at the high concentrations of the three sugars relative to SMCC. The parental performance in Maltose was on the whole strong, but to a lesser degree than SMCC; in most cases individuals were between 0 and 10 percent lower in activity than SMCC. Most of the parents were weak to poor with maltotriose as the carbon source. OS274 however was moderate to strong. Interestingly the metabolic activity of OS320 with maltose and maltotriose was poor and very poor respectively. Like SMCC, OS320 is a lager strain and would be expected to perform well in the presence of maltose. The *S. cerevisiae* strains were similar to SMCC in melibiose, whereas the *S. uvarum* strains OS274 and OS388 were very poor. This contrasted with the other *S. uvarum* strain OS449 which showed very strong performance, which was 10-30 % stronger than SMCC. Tolerance to ethanol was good amongst the parents, and was mostly within a 10 % range of that of SMCC. The response to the two oxidative stress inducing agents was contrasting amongst the parents. In general, their performance was moderate to strong in the presence of H_2O_2, but weak to very poor in the presence of menadione. Parental performance under the osmotic stress of sorbitol was in general moderate to weak, between 0 and 25 % worse than SMCC. In general the parental performances in response to ISO-Hop and in lager wort was again similar to that of SMCC.

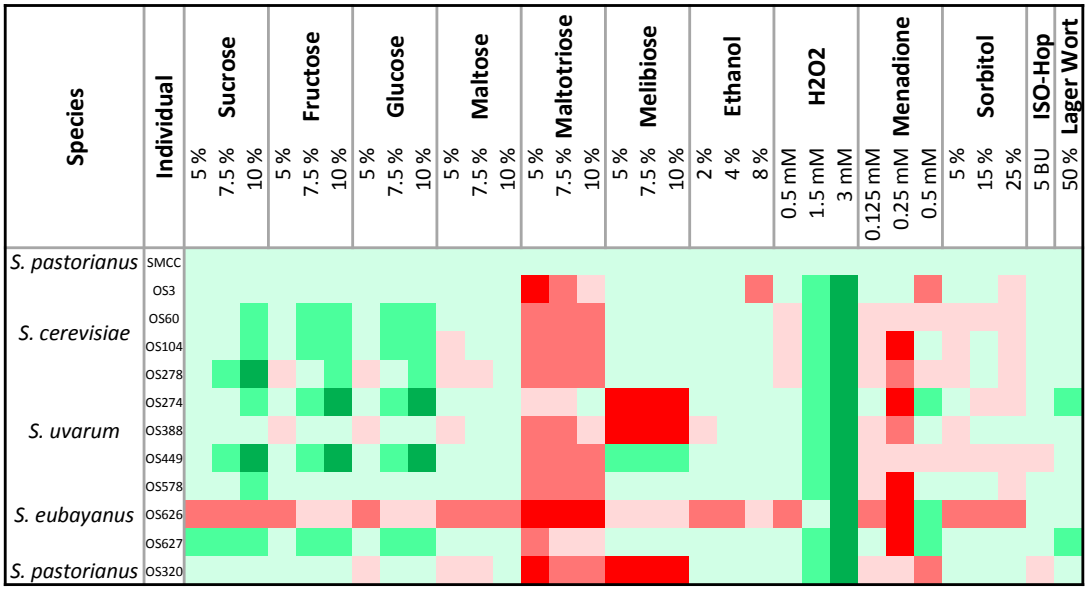


Figure 6.2.1 Phenotypic Heat Map of the Parental Strains

Heat map reveals trait variation between the parental strains of the hybrids assessed below, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC.

6.2.1.2 H51 – *S. cerevisiae* x *S. uvarum*

Most individuals were strong or very strong performers with sucrose, fructose and glucose as the carbon source (**Figure 6.2.2** and **Figure 6.2.3**). In the presence of maltose the raw reads were moderate and individuals were similar or stronger relative to SNCC. With maltotriose as the carbon source, H51 individuals showed low metabolic activity and most were over 25 % weaker than SMCC. Under osmotic stress most individuals were poor or equivalent to SMCC, decreasing in performance relative to SMCC at the higher concentration of sorbitol. In response to ethanol actual metabolic activity varied between weak, moderate and strong performing. Broadly this translated into most individuals being within a 10 % range either side of SMCC. Tolerance to the oxidative stress agents, was weak to moderate, and again not dissimilar to SMCC, albeit many showed performance more than 10 % weaker than the lager strain. There was a wide response to treatment with ISO-Hop, with one individual showing very weak metabolic activity, while others performing strongly compared to SMCC.

Individuals H51.12, H51.22, H51.23, and H51.37 were identified as strong candidates and chosen for further fermentation analysis. There were other

strong candidates, and with more time or fermentation equipment other individuals, such as H51.25 and H51.18, could have been assessed as well.

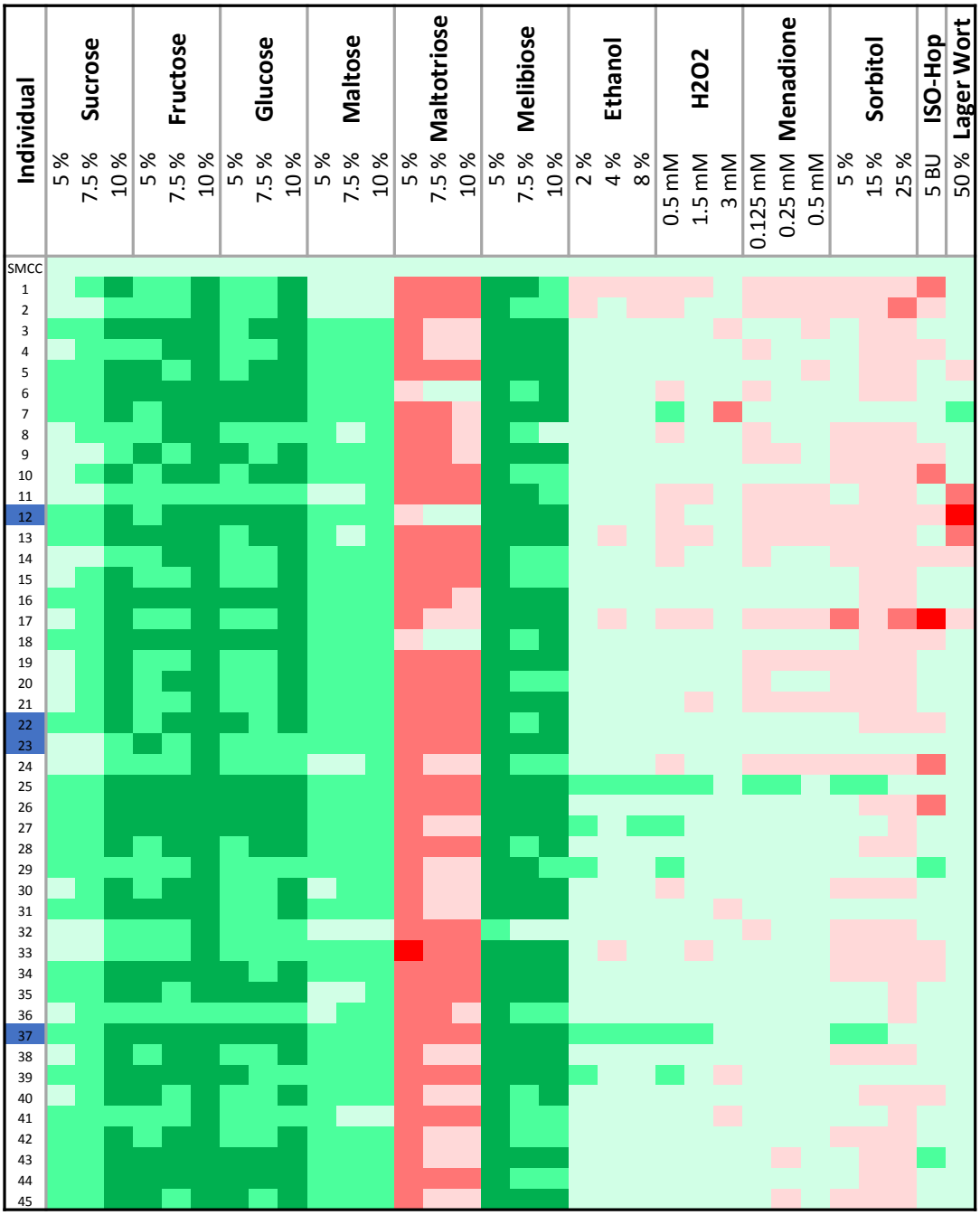


Figure 6.2.2 Phenotypic Heat Map of H51 individuals H51.1 - H51.45

Heat map reveals trait variation between F12 individuals of H51, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC. Individuals picked for fermentation assessment are highlighted in blue.

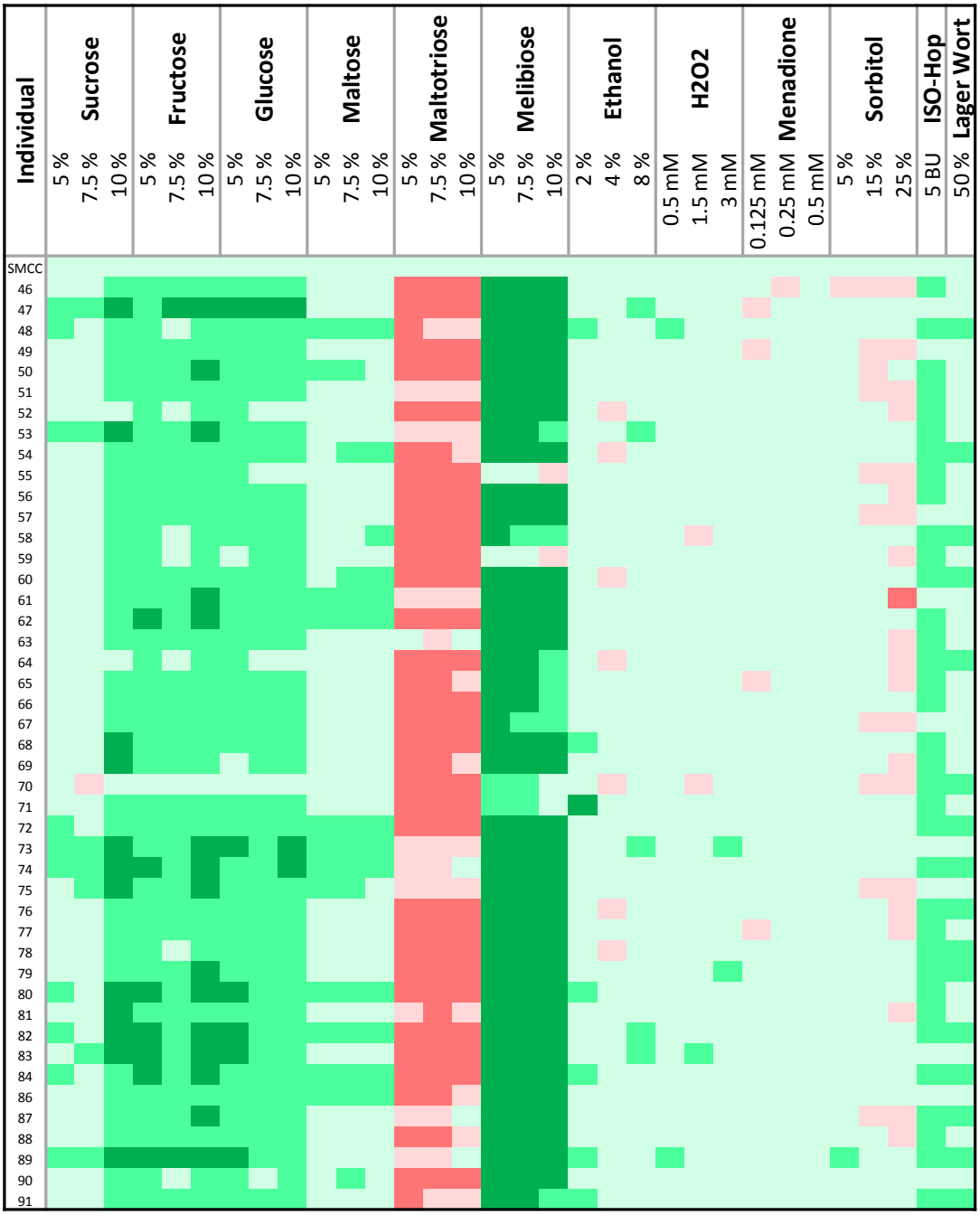


Figure 6.2.3 Phenotypic Heat Map of H51 individuals H51.46 - H41.90

Heat map reveals trait variation between F12 individuals of H51, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC.

6.2.1.3 H179 and H188 - *S. cerevisiae* x *S. eubayanus*, Differing in Mitotype

There were several H179 individuals that displayed poor performance under most conditions, including H179.8 and H179.33. Apart from these poor performers, individuals perform favourably when the carbon source was sucrose, fructose, glucose, maltose, or melibiose. Most individuals performed unfavourably in maltotriose, however several showed similar metabolic activity to SMCC, with several showing a performance over 10 % greater than SMCC (**Figure 6.2.4**).

H179 individuals were mostly equivalent to or better than SMCC, under ethanol and oxidative stress. Under osmotic stress individuals performed within 10 % of SMCC, or slightly worse at between 10 and 25 % lower peak metabolic activity.

H188 individuals performed favourably with sucrose, fructose, glucose and maltose as the carbon source (**Figure 6.2.5** and **Figure 6.2.6**). Like the H179 individuals, there was strong variation between individuals in the maltotriose rich media. Numerous H188 individuals performed very poorly. Whereas others such as H188.76 and H188.7, performed over 10 % greater than SMCC. Under the stress conditions H188 individuals showed similar or greater metabolic activity than SMCC, although the raw reads vary between moderate and poor.

Comparisons between experiment runs aren't optimal due to variation in actual read values. It could be stated that relative to SMCC, in general H188 individuals may outperform the H179 individuals under the oxidative stress of menadione, as well as in lager wort. It appears also that more H179 individuals have lower metabolic activity when maltotriose is the carbon source.

For further fermentation analysis H179.38, H179.40, H179.57, H179.84, H188.56, and H188.76 were chosen. These individuals performed well under the various stress conditions, and importantly appeared to be strong performers with maltose and maltotriose.

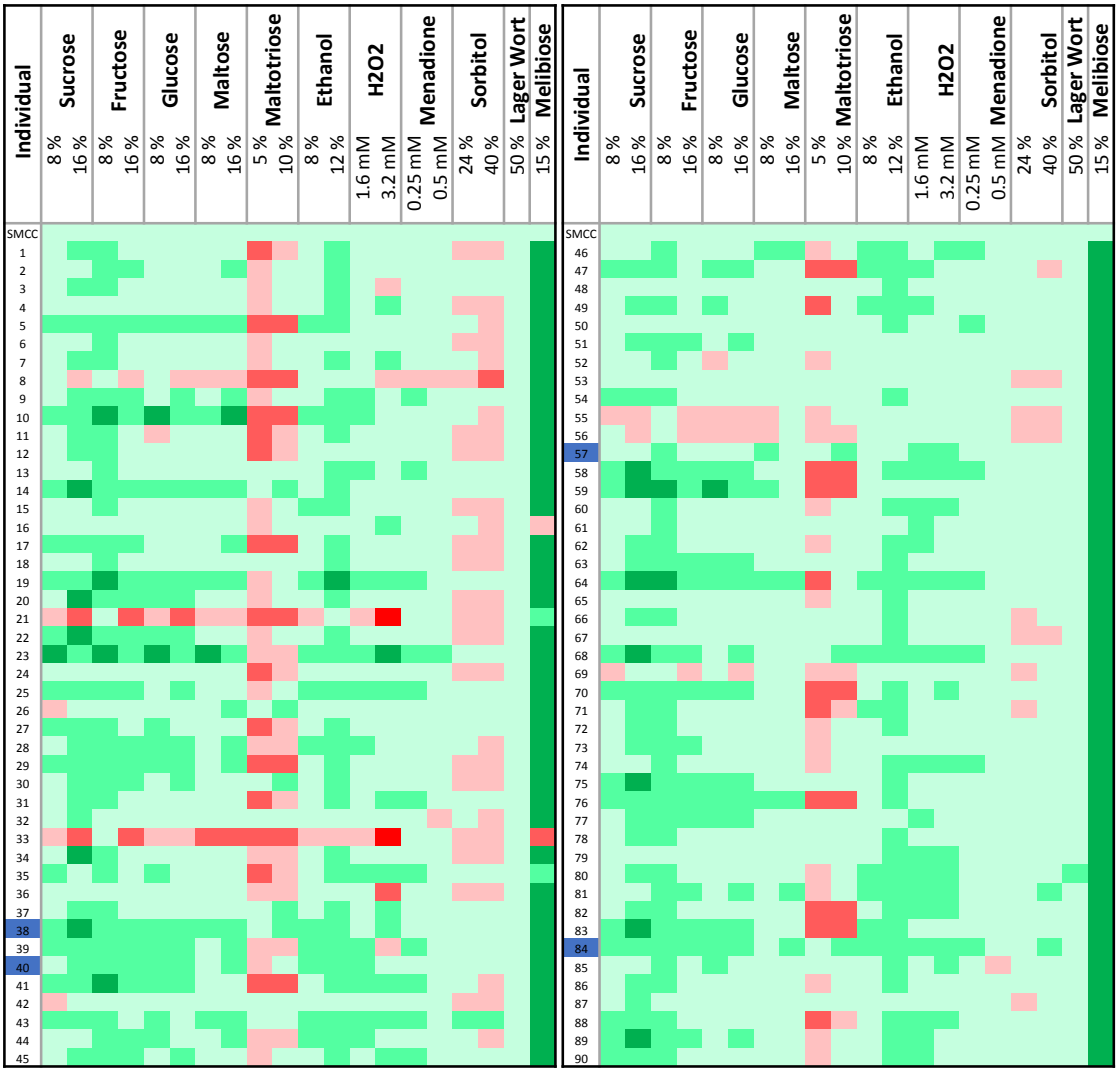


Figure 6.2.4 Phenotypic Heat Map of H179 individuals H179.1 - H179.90

Heat map reveals trait variation between F12 individuals of H179, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC. Individuals picked for fermentation assessment are highlighted in blue.

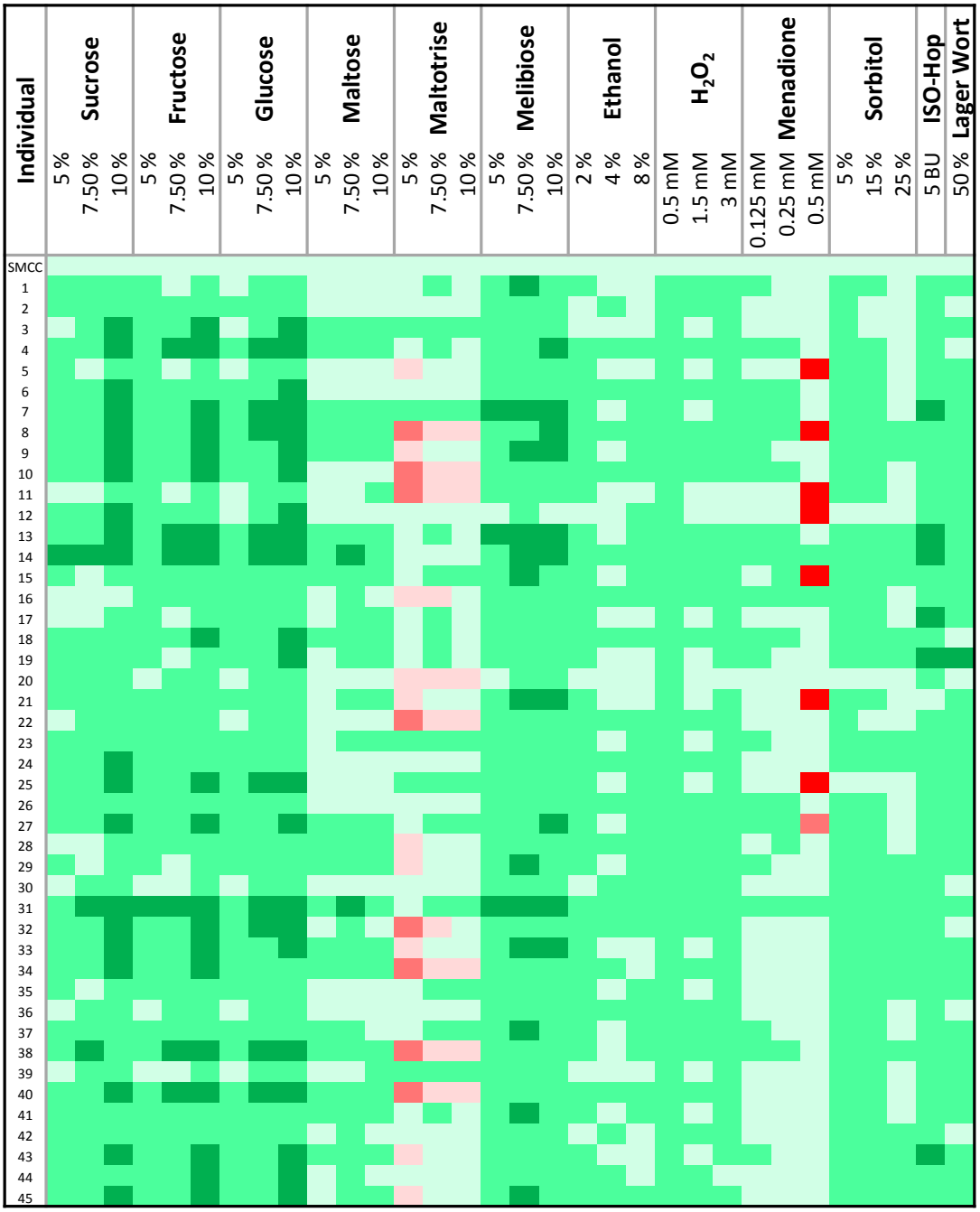


Figure 6.2.5 Phenotypic Heat Map of H188 individuals H188.1 - H188.45

Heat map reveals trait variation between F12 individuals of H188, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC.

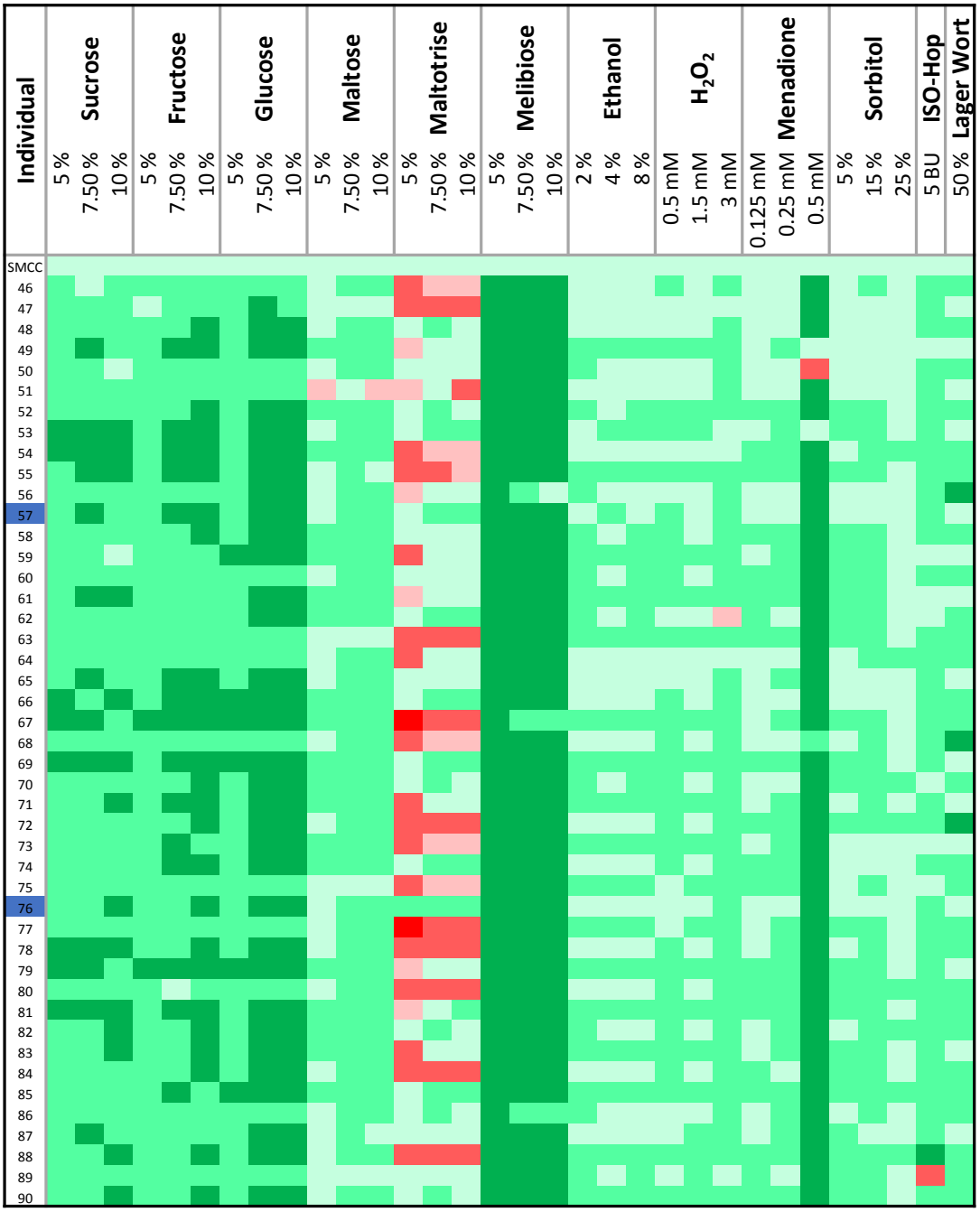


Figure 6.2.6 Phenotypic Heat Map of H188 individuals H188.46 - H188.90

Heat map reveals trait variation between F12 individuals of H188, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC. Individuals picked for fermentation assessment are highlighted in blue.

6.2.1.4 H230 – *S. eubayanus* x *S. uvarum*

The metabolic activity of H230 individuals was strong or very strong with sucrose fructose and glucose as the utilisable carbon source (**Figure 6.2.7** and **Figure 6.2.8**). Many individuals were over 30 % greater performing than SMCC, particularly at the higher sugar concentrations. The activity was in general moderate with maltose and maltotriose as the carbon source, although several individuals including H230.66 demonstrated very poor ability under these conditions.

The experiment run that assessed the H230 individuals 46-90 for performance in wort and under stress produced raw reads much lower than other experiment runs. Most of the read values would normally be considered very poor, this included the data produced for SMCC. When normalised, the individuals were mostly strong or similar to SMCC. This suggests that an issue arose with the particular experiment run, possibly degradation of the dye that measures metabolic activity. One exception to the performance within the 45 individuals was H230.60, which performed poorly under stresses. Numerous others struggled in the presence of ethanol. The first 45 individuals showed reasonable variation, with many similar in activity to SMCC under each of the stresses. Others performed more than 10 % better than SMCC. Over a dozen individuals showed sensitivity to the menadione and performed over 50 % worse than SMCC.

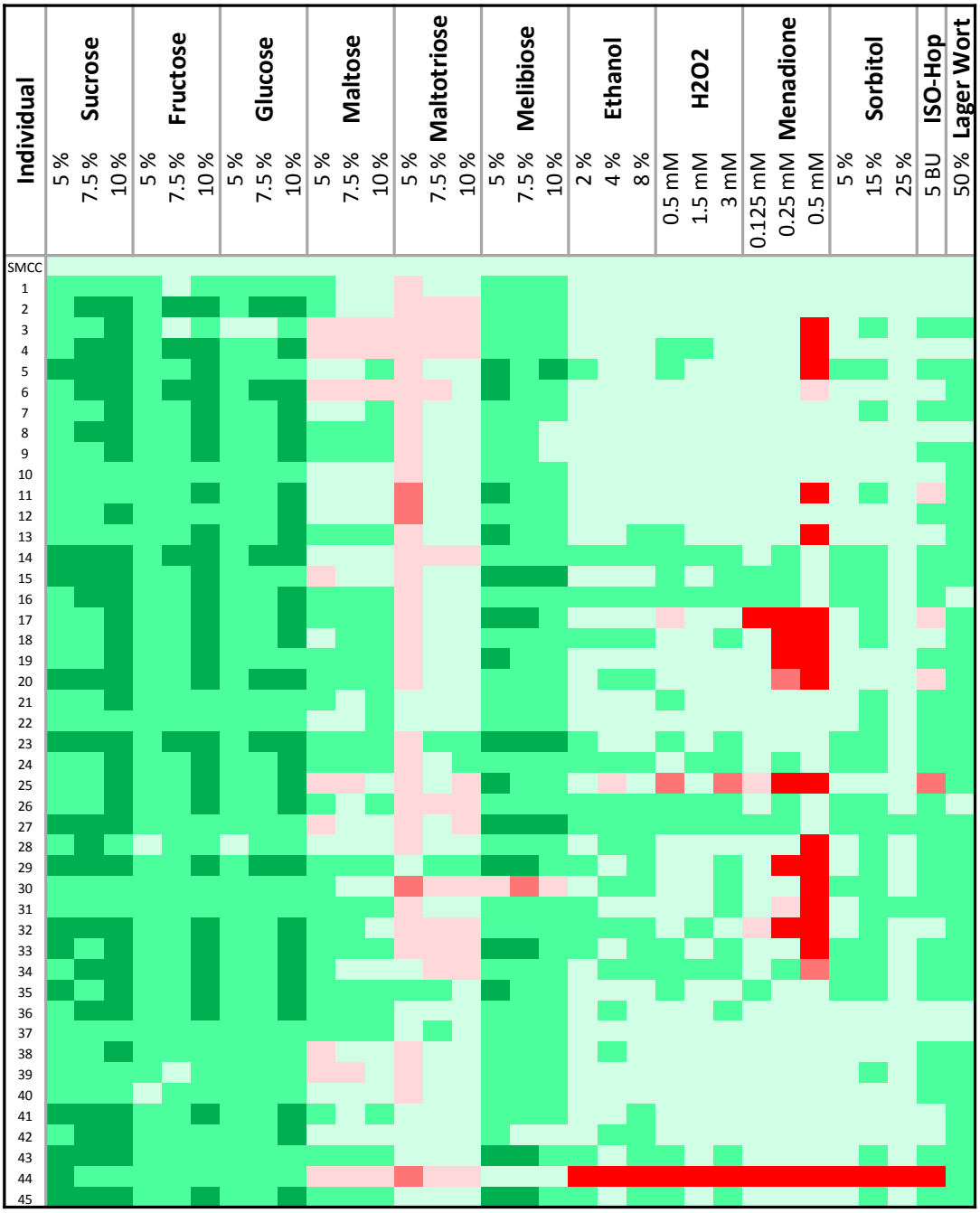


Figure 6.2.7 Phenotypic Heat Map of H230 individuals H230.1 - H230.45

Heat map reveals trait variation between F12 individuals of H230, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC.

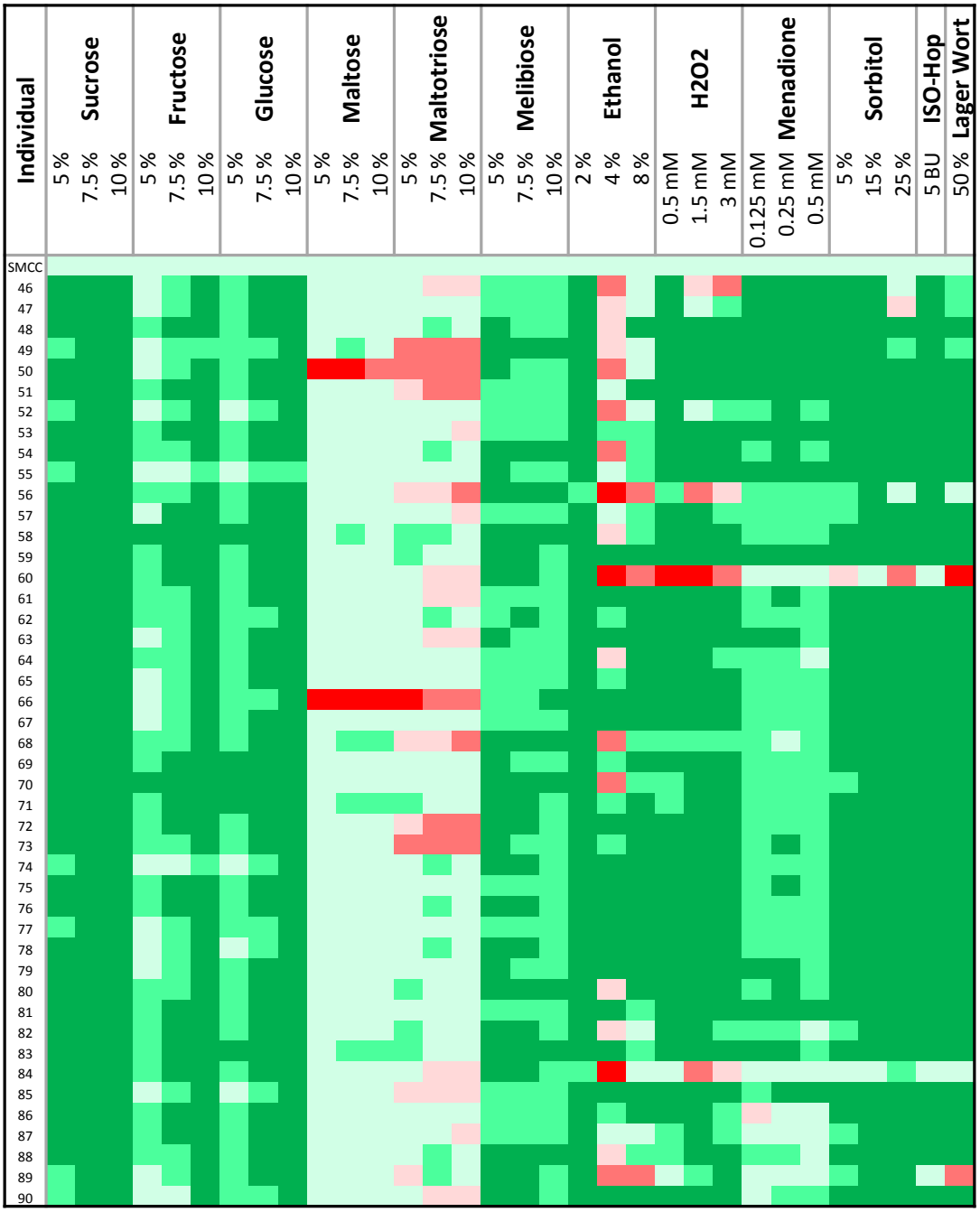


Figure 6.2.8 Phenotypic Heat Map of H230 individuals H230.46 - H230.90

Heat map reveals trait variation between F12 individuals of H230, relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC.

6.2.1.5 Protoplast fusion isolate

Most PIS individuals demonstrated strong or very high metabolic activity with fructose and glucose as the carbon source (**Figure 6.2.9** and **Figure 6.2.10**). Metabolic activity in sucrose, was very strong amongst most of the first 45 individuals. Their performance in ethanol and H_2O_2 was moderate to strong, with individuals 47-90 being comparable to SMCC, and the first 46 being comparable or slightly worse performing than SMCC. This is likely to be down to variation between plates. The raw values for SMCC as well as the PIS individuals were higher in the experimental run of 1- 46 than in the run of 47-90. The PIS individuals showed moderate to weak metabolism in maltose. PIS 1 to 45 showed a general weakness relative to SMCC in response to the various stresses, in particular in response to osmotic stress. PIS 46-90 were in general more similar to the SMCC, but were also weaker in response to osmotic stress and the many individuals were sensitive to the high concentration of menadione. The first 45 had very high metabolic activity in lager wort, with 46-90 showing poor raw reads but stronger relative to SMCC.

In contrast with the *de novo* hybrid individuals, the PIS's showed modest variation between individuals. At least, this was the case when the very poor performing individuals; such as 47, 48, 88, and 89, were excluded from the comparison. PIS.34 and PIS.35 were identified as performing better than SMMC under most conditions and therefore were selected for further analysis of fermentation capability.

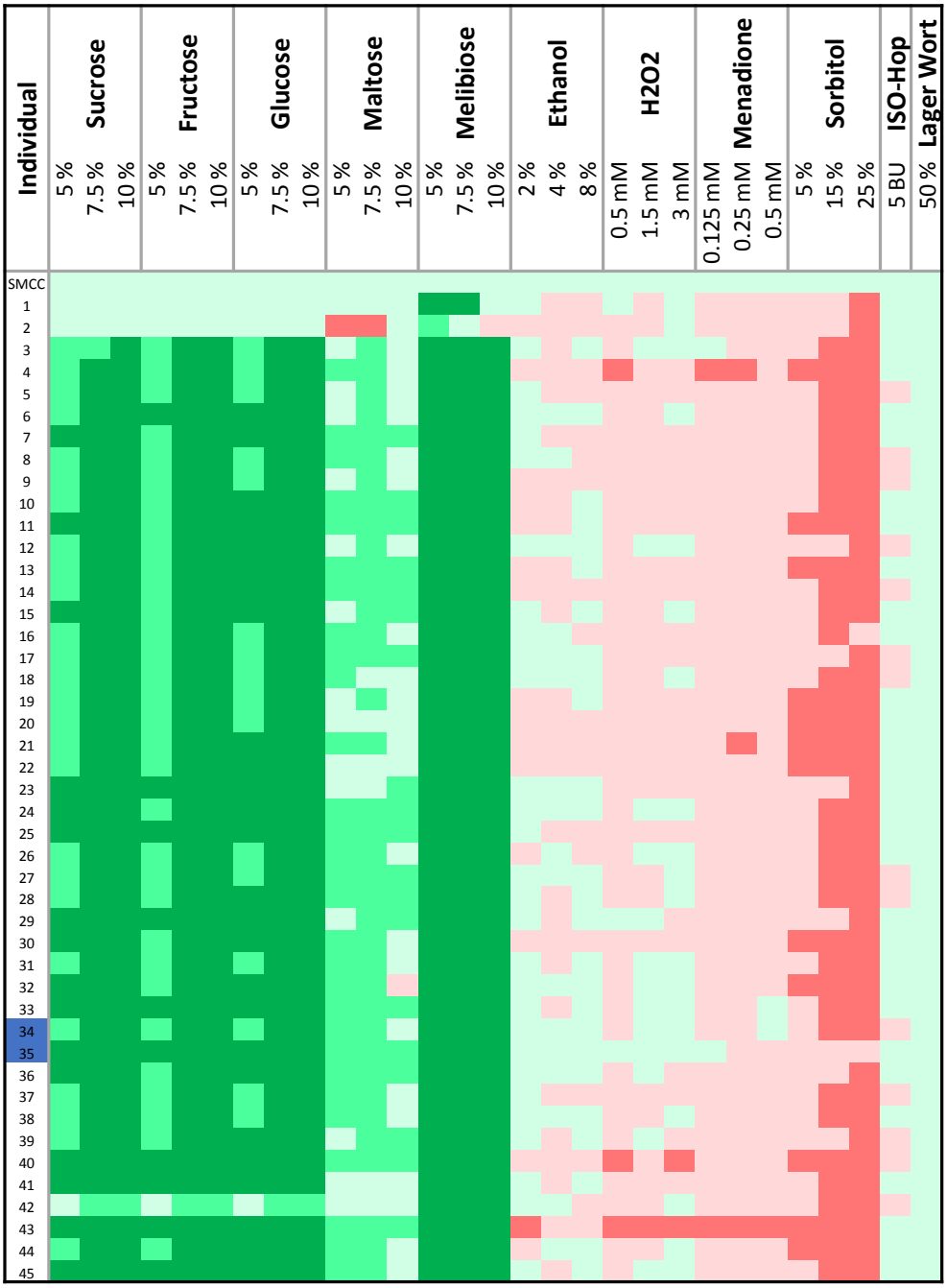


Figure 6.2.9 Phenotypic Heat Map of Protoplast Fusion Isolate Spores PIS.1 - PIS.45

Heat map reveals trait variation between protoplast fusion isolate spores (PIS'), relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC. Individuals picked for fermentation assessment are highlighted in blue.

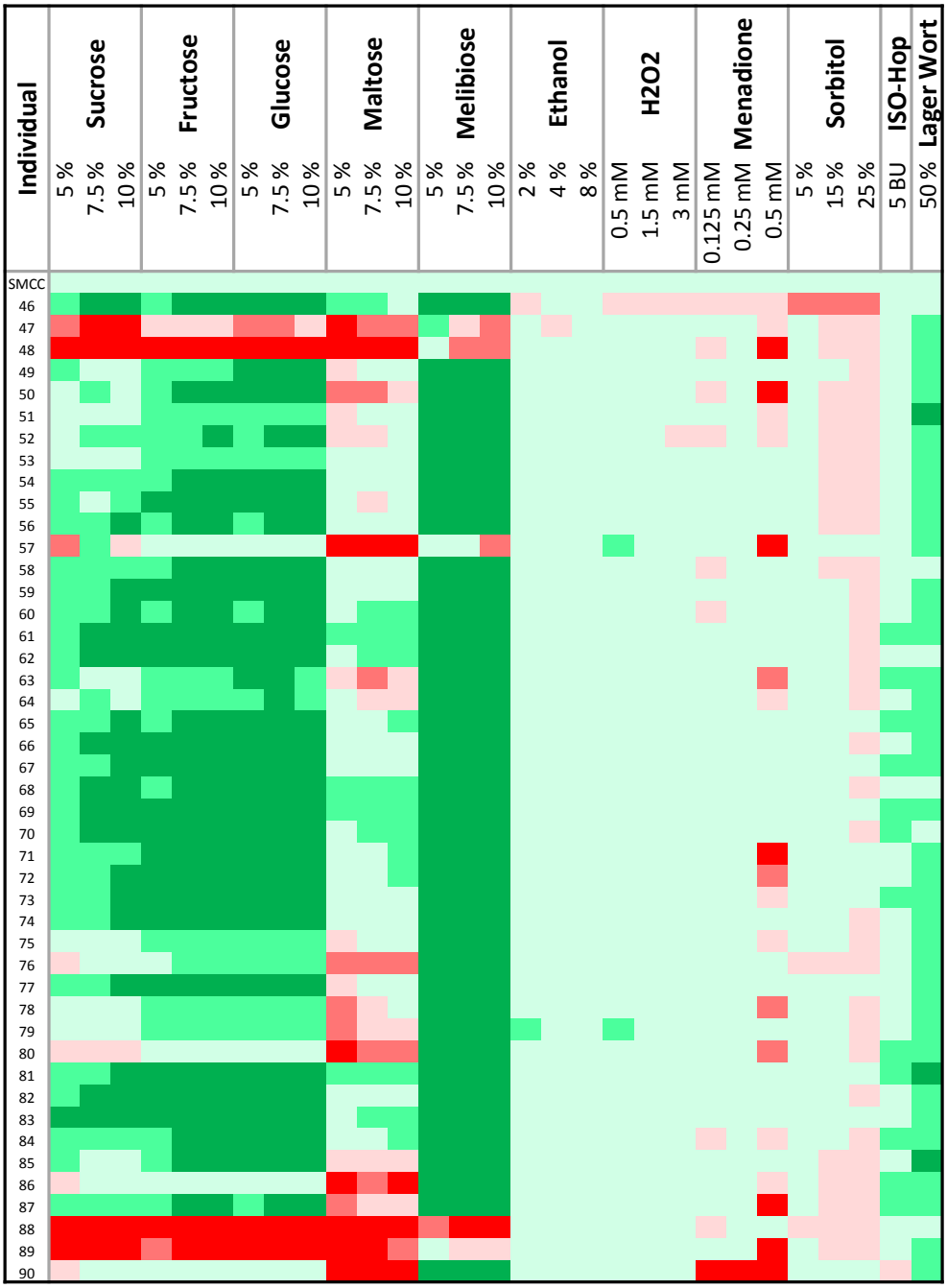


Figure 6.2.10 Phenotypic Heat Map of Protoplast Fusion Isolate Spores PIS.46 - PIS.90

Heat map reveals trait variation between protoplast fusion isolate spores (PIS'), relative to the reference lager strain SMCC. Metabolic activity was measured and normalised to SMCC. Colour coded according to **Figure 2.6.1**. Red = inferior to SMCC. Dark Green = superior to SMCC. Individuals picked for fermentation assessment are highlighted in blue.

6.2.2 Fermentation performance

Speed of fermentation was assessed through measuring weight loss over time (**2.6.5**). The quicker the weight loss was observed, the faster the rate of fermentation. At the end of fermentation, judged to be after no change in vessel weight between two successive time points, a number of factors were measured to assess fermentation performance. Yeast viability was measured as desirable strains would retain a high viability (**2.6.5**). In brewing, strains are routinely re-pitched to a fresh fermentation and so a high viability is required. Low cell viability post fermentation limits the number of times yeast can be re-pitched. The change in yeast cell number was quantified. A moderate increase in cell number is desired for a fast rate of fermentation, however an increased cell number comes at an opportunity cost. Wort sugars used for increasing biomass, cannot then be used for ethanol production. The sugars remaining in the wort post fermentation were investigated by HPLC (**2.6.4**). For comparison the sugar content of the raw wort prior to fermentation was also measured (**Figure 6.2.11**). Desirable brewing strains are able to utilise most of the wort sugar (**2.6.1**), during fermentation. Some residual sugar left over is usually desirable, as this contributes sweetness to the lager flavour. Strains that are unable to utilise maltose or maltotriose for example would produce beer with a flavour not dissimilar to Ovaltine (or other malt based beverage). The ethanol content of the finished wort was measured using an Alcolyser, and finally the wort pH was measured. During fermentation the wort pH typically drops from around pH 5.0 to around pH 4.0. From a 15 °P wort an ethanol yield of around 7 % would be considered desirable. This was calculated based upon a theoretical estimate of the conversion of sugar to ethanol of 88 % to account for use of sugars in biomass production (Boulton and Quain, 2007). As with the analysis of individual traits using the Omnilog, SMCC was used as a reference strain for what is currently considered desirable.

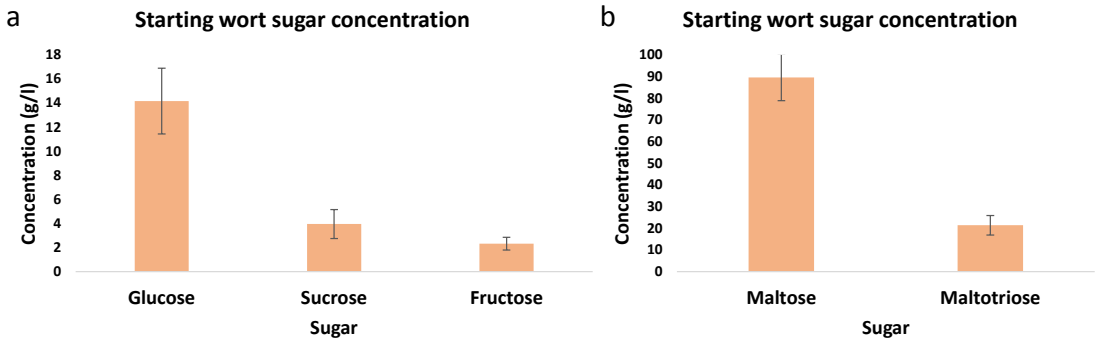


Figure 6.2.11 Starting Wort Sugar Concentrations

Sugar content of the raw wort, prior to fermentation. Concentration given as (g/l), quantified by HPLC.

6.2.2.1 Parental strains

Only the lager strain OS320 outperformed SMCC during fermentation, decreasing in weight at the same rate for longer (**Figure 6.2.12**). The performance of OS3 was similar to that of SMCC, however total weight loss was only 2.03 % compared to 2.32 %. The performance of the *S. uvarum* strains OS449, and OS388 and the *S. eubayanus* OS578 were similar to each other. Final weight loss was between 1.94 – 1.98 %. Of the three parental strains, OS449 had the fastest rate of fermentation. OS60, OS104, and OS626 performed very poorly. After an initial spurt of weight loss, fermentation terminated at less than 0.4 % weight loss. The profile of OS278 did not follow the typical fermentation progress of lag phase to exponential phase to stationary phase. After an initial fast rate of weight loss, the rate plateaued before increasing again. This could arise due to a change in the specific sugar being utilised. Collecting wort at time points throughout and analysing sugar composition by HPLC would reveal if this were the case. OS278 took 600 hours to complete fermentation, twice as long as any other parental strain.

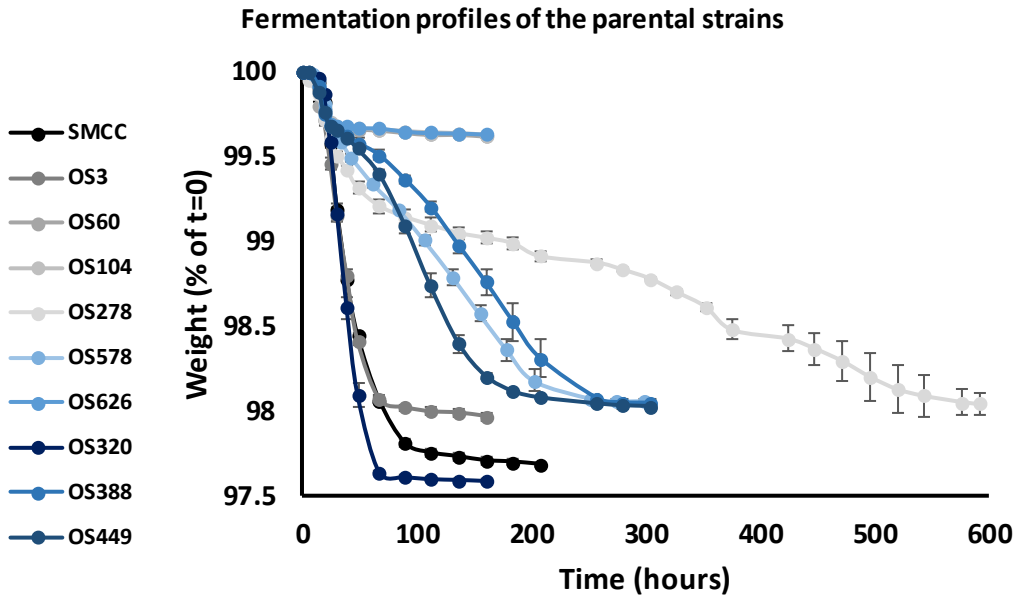


Figure 6.2.12 Fermentation profile of the parental strains

Assessment of fermentation profile. Weight loss (as a percentage of time 0) was measured over time, until weight ceased to decrease. Parental strains were compared against each other and SMCC. SMCC in black, *S. cerevisiae* strains in shades of grey, *S. uvarum* and *S. eubayanus* in shades of blue, and the *S. pastorianus* strain OS320 in what some might call a shade of deep purple. Individuals were analysed in triplicate with standard deviation plotted as error bars. OS60 and OS104 fermentation profiles overlap with OS626.

The viability of OS3 was the highest of the parental strains. However the change in cell number was significantly higher than all other strains, almost 15 fold compared to 7.5 fold observed with SMCC (**Figure 6.2.13**). The fold change in the other parental strains varied between 5.9 fold for OS449 and 9.5 fold seen in OS60 and OS278. The *S. uvarum* strains had the lowest post fermentation viability, however not significantly different from OS320 and *the S. eubayanus* strains. Despite premature fermentation termination with OS60 and OS104, the change in cell number was higher than SMCC, suggesting wort nutrients were used for increasing cell mass rather than ethanol production. This is confirmed in the amount of ethanol produced by these strains and OS626 (**Figure 6.2.14**). These strains produced an alcohol concentration of around just 1 percent, compared to the other strains which produced between 5.5 and 7 percent ethanol. The lager strain OS320 produced the highest volume of alcohol, 7 % abv, similar to the amount produced by SMCC, which was 6.8 % abv. During fermentation the pH

decreases, this can be observed in the high pH of the three strains that underwent a very brief fermentation with the wort of OS626 being the least acidic at pH 4.8. Excluding these three poor performing strains, OS320 produced the highest pH of pH 4.45. Most other parental strains fell within the pH range of the two lager strains, SMCC produced a final wort pH of 4.2. Only OS278 produced a lower final wort pH.

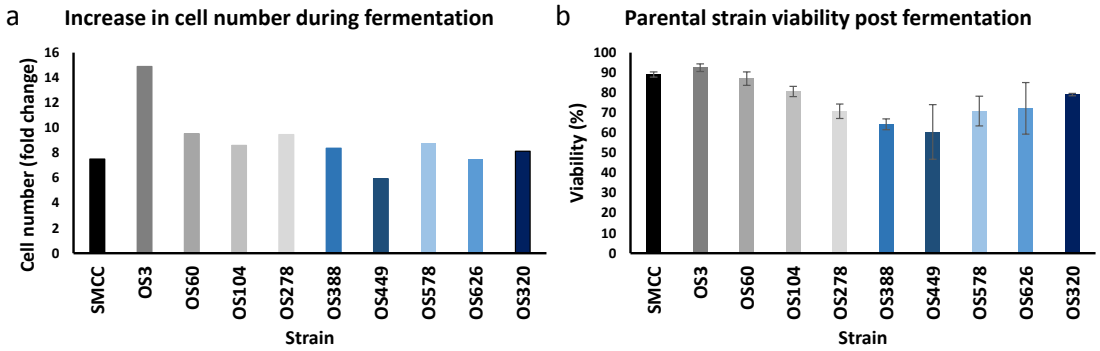


Figure 6.2.13 Cell Number and Viability of Parental strains

Quantification of the change in cell number during fermentation (a) and the post fermentation viability (b), of the parental strains.

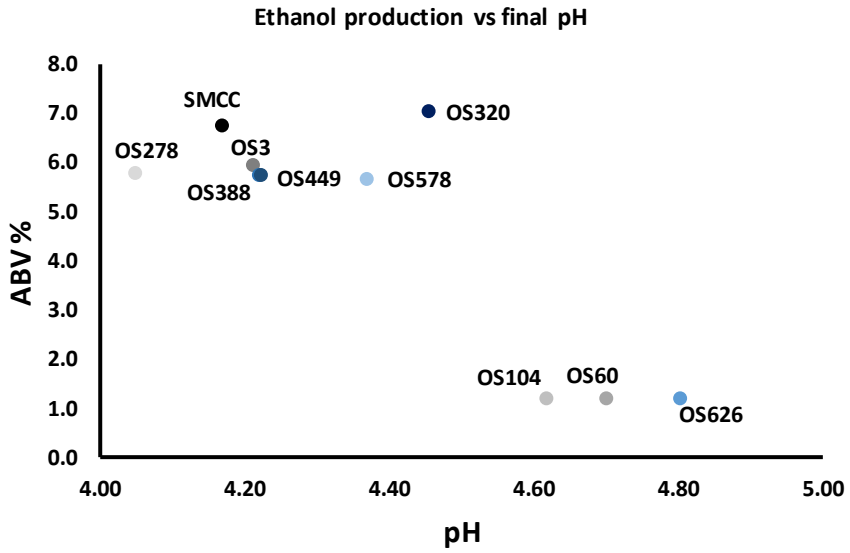


Figure 6.2.14 Production of Ethanol by Parental Strains and Final Wort pH

Post fermentation ethanol concentration and wort pH, produced by the parental strains. Both factors were quantified using the Alcolyser analysis system. Ethanol content was measured by densitometry. Strains producing low ethanol (~ 1 %) gave a wort pH of over 4.6. Strains producing high amounts of ethanol (5 - 7 %), also produced a lower pH wort (< pH 4.5).

The three strains that terminated fermentation so quickly had a very high concentration of maltose remaining, with over 70 g/l present in the final wort (**Figure 6.2.15**). The other strains showed strong ability to utilise maltose, with the concentration remaining in the wort negligible. Only SMCC and OS320 showed a strong ability to utilise maltotriose, with no maltotriose left in the wort. The other parental strains seemed unable to utilise maltotriose effectively with 15 – 20 g/l remaining. The two *S. uvarum* strain were able to utilise all of the fructose present, as was OS320. As these *S. uvarum* strains were isolated from wine fermentation, where fructose is the predominant sugar, their ability to utilise fructose was not unexpected. The concentration of glucose, sucrose, and fructose remaining in the wort from all strains was low, under 0.4 g/l. Other than the three strains that utilised all the fructose, the other strains left just over twice as much fructose than SMCC. OS626 left the most glucose in the wort, with OS3 utilising the most. OS278 used all the sucrose, while sucrose left by the other stains was in the range of the two lager strains.

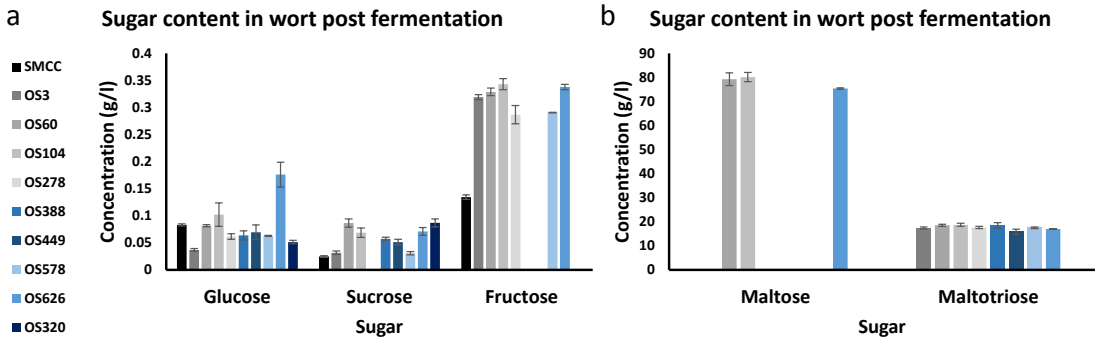


Figure 6.2.15 Sugar Utilisation of the Parental Strains

Comparison of the concentration (g/l) of key brewing sugars in the post fermentation wort. Glucose, sucrose and fructose (a). Maltose and maltotriose (b). The final wort produced by the parental strains was compared to their parental strains and SMCC.

6.2.2.2 H51

The four selected H51 individuals outperformed their *S. cerevisiae* parent OS104, however none of the four performed as well as their other *S. cerevisiae* parental strain OS3 (**Figure 6.2.16**). The fermentation of all four ended at a similar final weight loss as OS3, however the rate of weight loss was much slower. H51.23 and H51.37 were slower than either *S. uvarum*

parental, while H51.12 and H51.22 had a faster rate of fermentation. H51.37 had the most similar increase in cell number to SMMC, and its parents OS60 and OS388 (**Figure 6.2.17**). H51.12 had the highest increase in cell number, 11 fold, and the closest in similarity to OS3. The cell viability of the four individuals was very high, within a 1 % range of the viability of SMCC (97 %). Their viability was higher than all four parental strains. The amount of ethanol produced and pH of final wort was similar to the parental strains, barring the poor performing OS104 (**Figure 6.2.18**). SMCC produced approximately 1 % higher ethanol concentration, as well as the lowest final wort pH.

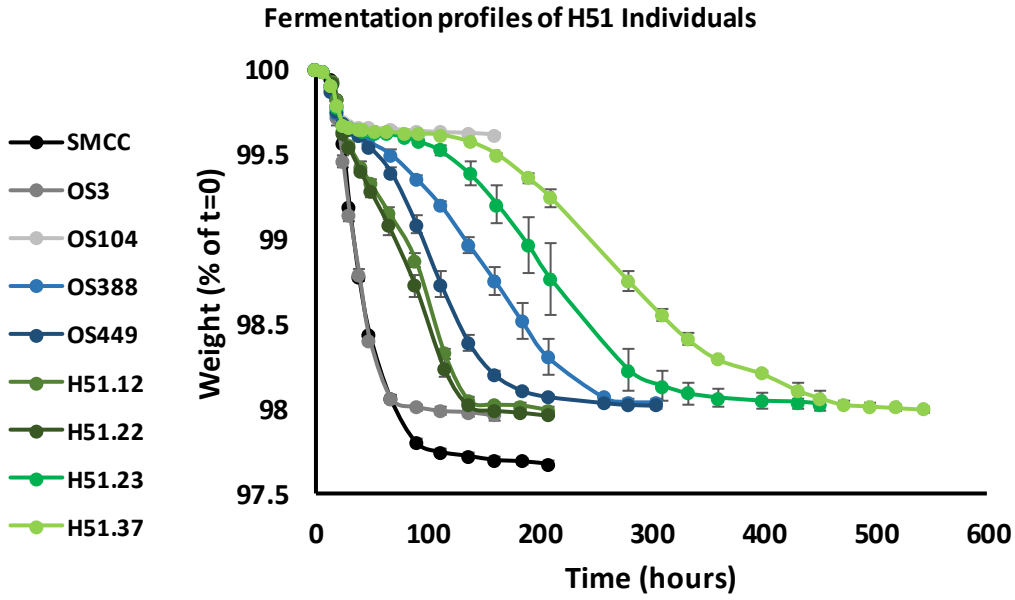


Figure 6.2.16 Fermentation profiles of H51 individuals

Assessment of fermentation profile. Weight loss (as a percentage of time 0) was measured over time, until weight ceased to decrease. Four H51 individuals were assessed (shades of green) and compared to their four parents, as well as SMCC. Individuals were analysed in triplicate with standard deviation plotted as error bars.

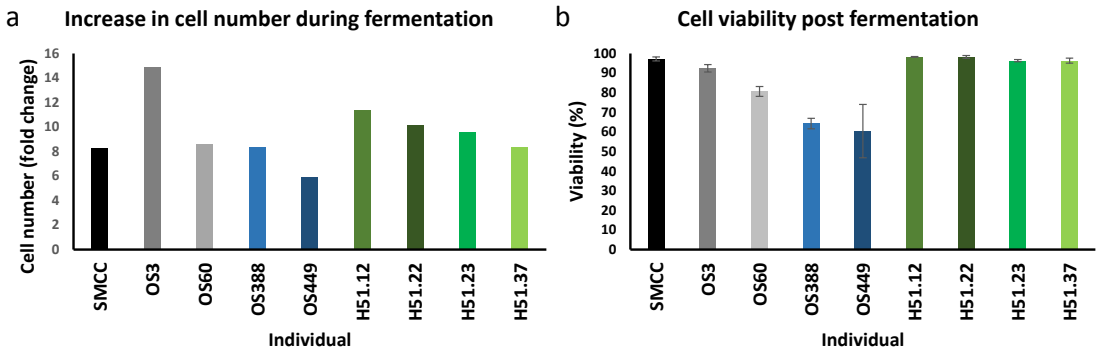


Figure 6.2.17 Cell Number and Viability of H51 Individuals

Quantification of the change in cell number during fermentation (a) and the post fermentation viability (b), of the H51 individuals compared with their parental strains.

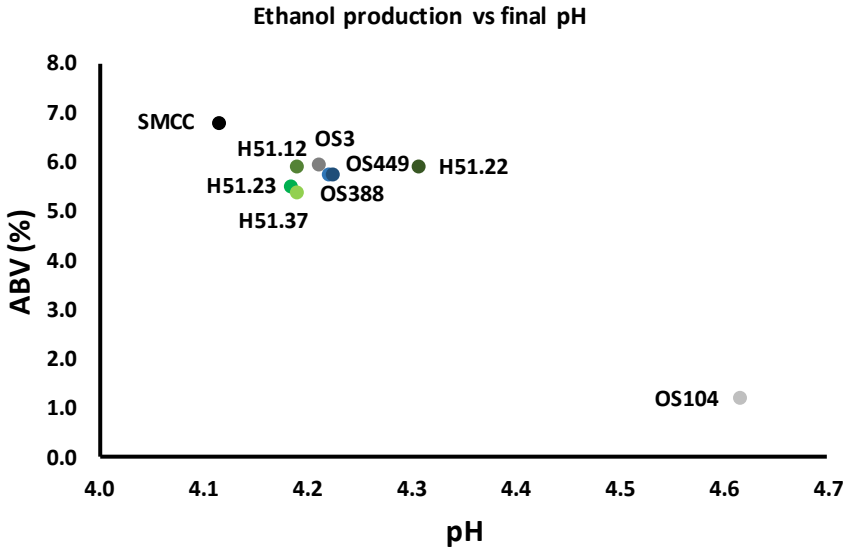


Figure 6.2.18 Production of Ethanol and Final Wort pH of H51 Individuals

Comparison of the post fermentation ethanol concentration and wort pH, produced by H51 individuals and their parental strains. Both factors were quantified using the Alcolyser analysis system. Ethanol content was measured by densitometry.

Sugar utilisation of the four individuals was similar to each other, only H51.23 left a small amount of residual maltose (**Figure 6.2.19**). All four used slightly more maltotriose, but less glucose, than the parent strains. Where the four strains differed was in their utilisation of fructose. H51.12 and H51.22 were able to fully use fructose, like their *S. uvarum* parents. Whereas H51.23 and H51.37 failed to use all the fructose. These two individuals left approximately four times more fructose than either the *S. cerevisiae* parents.

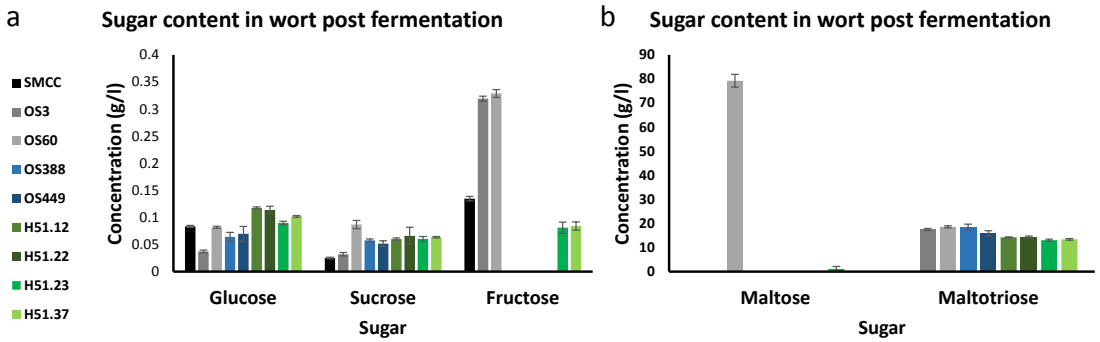


Figure 6.2.19 Sugar Utilisation of H51 Individuals

Comparison of the concentration (g/l) of key brewing sugars in the post fermentation wort. Glucose, sucrose and fructose (a). Maltose and maltotriose (b). The final wort produced by H51 individuals was compared to their parental strains and SMCC.

6.2.2.3 H179 and H188

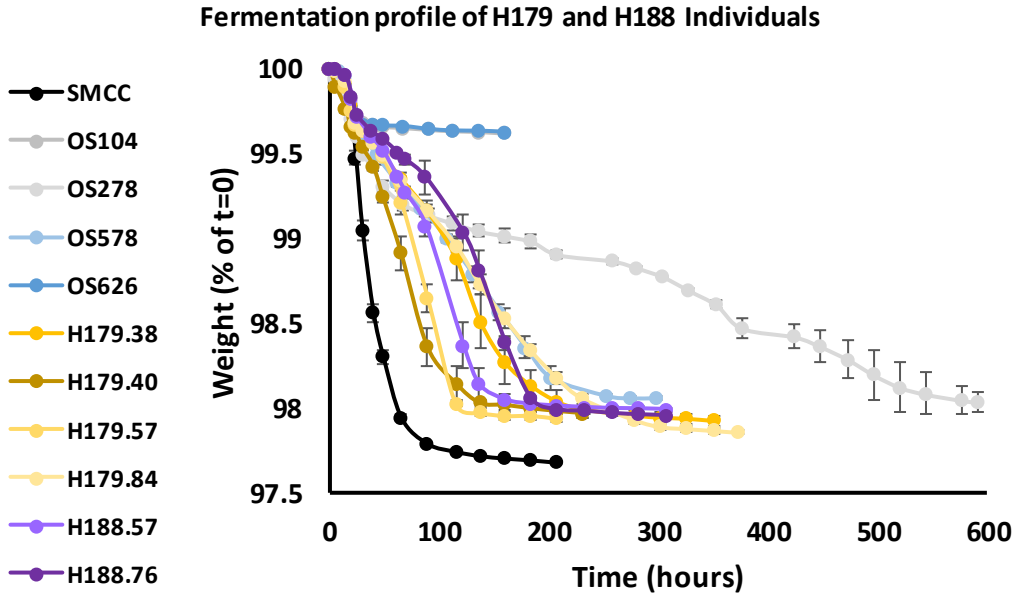


Figure 6.2.20 Fermentation profiles of H179 and H188 individuals

Assessment of fermentation profile. Weight loss (as a percentage of time 0) was measured over time, until weight ceased to decrease. Four H179 (shades of yellow) and two H188 (shades of purple) individuals were assessed and compared to their four parents, as well as SMCC. Individuals were analysed in triplicate with standard deviation plotted as error bars. The fermentation profile of OS104 overlap that of OS626.

Of the four parental strains that contribute to H179 and H188 individuals, only OS578 was similar to the six F1 individuals analysed. Even in comparison to the OS578 parental, each F1 isolate showed a faster rate of weight loss and

a higher total weight loss. The fermentation profiles of OS626 and OS104 were very poor, and OS278 also took significantly longer to complete fermentation (**Figure 6.2.20**). SMCC had both a faster rate of fermentation and a higher overall weight loss than any of the individuals. H179.57 and H179.40 had the best performances, followed by H188.76. The total weight loss of each individual was very similar, at approximately 2 %. The viability of each of the selected individuals was higher than all four parents, except for H188.57 which had a similar viability to OS278 and OS626 at 87 % (**Figure 6.2.21**). H188.76 had the highest viability at 98 %. There was broad variation in the post fermentation cell number. H179.57 had the lowest fold increase at 6 fold. H179.38 and H188.76 had the highest increase in cell number, close to 12 fold, similar to that of OS278. The finished wort pH of each individual was similar to that produced by OS578, with H179.38 being the most different and most acidic at pH 4.2 (**Figure 6.2.22**). There was little variation in the ethanol concentration ranging from 5.8 % abv to 6.3 % abv, and similar to both OS278 and OS578 but lower than the concentration produced by SMCC. Each of the selected individuals was able to fully utilise maltose, much like two of their parents OS278 and OS578 (**Figure 6.2.23**). Each individual utilised more maltotriose than any of the parents, with the H179 individuals utilising slightly more than the H188 individuals. H179.84 had the least maltotriose remaining post fermentation at 10.4 g/l. Their ability to utilise glucose was very similar, with around 0.1 g/l remaining in each, about two thirds more than OS278 and OS578. There were small differences in the residual sucrose concentration, with the H188 individuals utilising slightly more than the H179 individuals. Other than H188.57, which left similar amounts of fructose to OS278 and OS578, each of the individuals used more fructose than any parent. The two individuals that used the most fructose, and more than SMCC were H179.84 and H188.76.

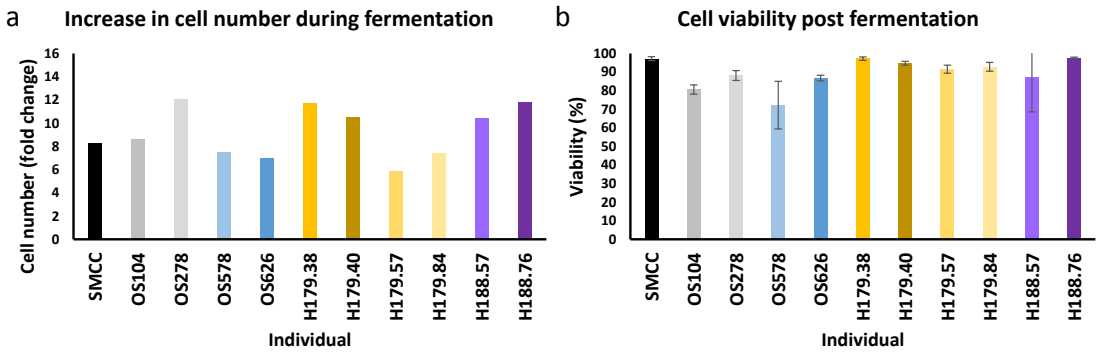


Figure 6.2.21 Cell Number and Viability of H179 and H188 Individuals

Quantification of the change in cell number during fermentation (a) and the post fermentation viability (b), of the H179 and H188 individuals compared with their parental strains.

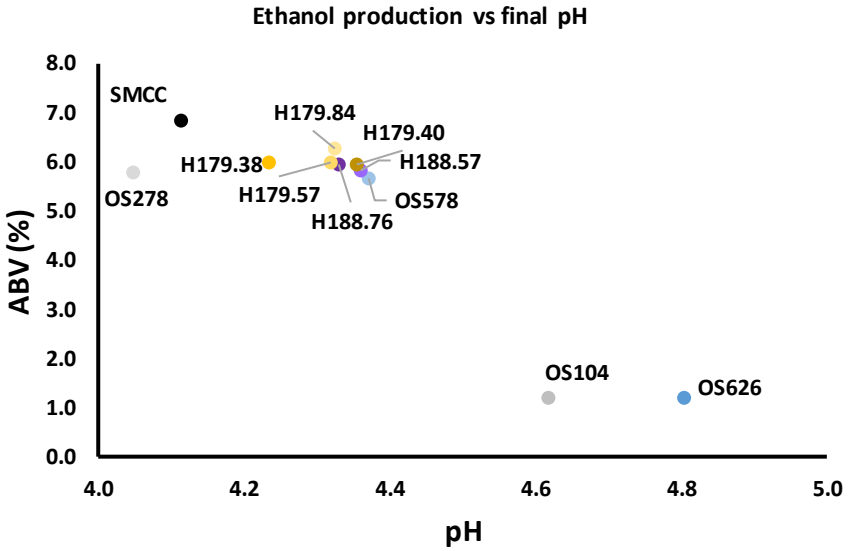


Figure 6.2.22 Production of Ethanol and Final Wort pH of H179 and H188 Individuals

Comparison of the post fermentation ethanol concentration and wort pH, produced by H179 and H188 individuals and their parental strains. Both factors were quantified using the Alcolyser analysis system. Ethanol content was measured by densitometry.

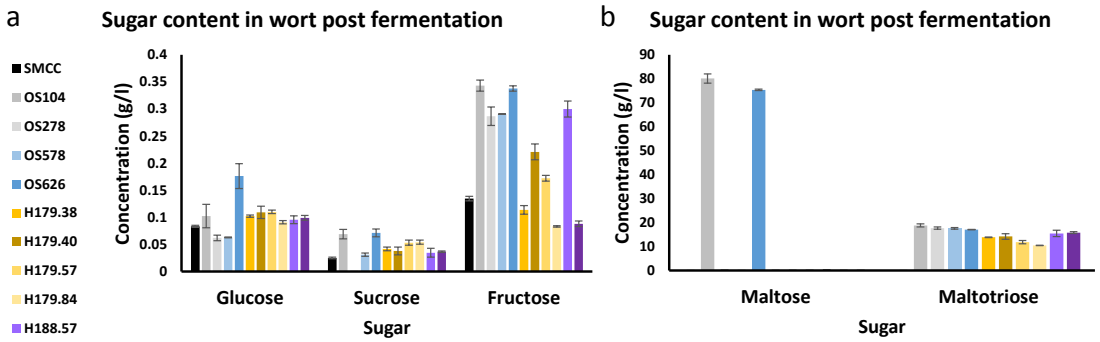


Figure 6.2.23 Sugar Utilisation of H179 and H188 Individuals

Comparison of the concentration (g/l) of key brewing sugars in the post fermentation wort. Glucose, sucrose and fructose (a). Maltose and maltotriose (b). The final wort produced by H179 and H188 individuals was compared to their parental strains and SMCC.

6.2.2.4 Protoplast fusion isolate

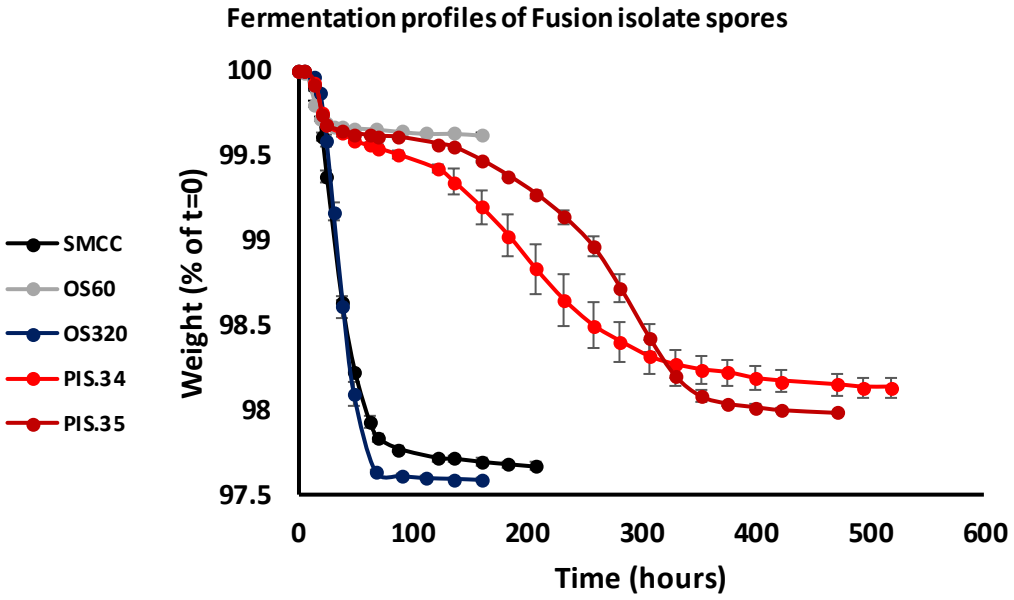


Figure 6.2.24 Fermentation profiles of PIS individuals

Assessment of fermentation profile. Weight loss (as a percentage of time 0) was measured over time, until weight ceased to decrease. Two PIS individuals were assessed (shades of red) and compared to their two parents, and SMCC. Individuals were analysed in triplicate with standard deviation plotted as error bars.

PIS.34 and PIS.35 performed better than their *S. cerevisiae* parent OS60, but worse than their *S. pastorianus* parent OS320 as well as SMCC (Figure 6.2.24). Their initial fermentation profile was similar to OS60, but instead of terminating their rate increased around 122 hours, with PIS.34 having the

faster rate early on than PIS.35. At the end of fermentation PIS.35 had lost the greater percentage weight, but the two individuals were similar. Their cell viability was higher than both parents, and similar to SMCC (**Figure 6.2.25**). The increase in the cell number of PIS.34 was lower than both parents and SMCC at 6.6 fold. Whereas PIS.35 underwent an 11.4 fold increase in cell number, significantly higher than both parent and SMCC. The final wort pH was lower than either parent, with PIS.35 producing a similar pH to SMCC (**Figure 6.2.26**). Both individuals produced a lower ethanol concentration than OS320 and SMCC, with PIS.34 producing fractionally less than PIS.35. They showed similar ability to utilise each of the sugars (**Figure 6.2.27**). More sucrose was utilised by both than by either parent, but less glucose was utilised. Both individuals fully utilised fructose, and while PIS.35 used all the maltotriose like OS320, PIS.34 did not fully utilise the sugar. Their ability to use maltotriose was a little improved relative to OS60.

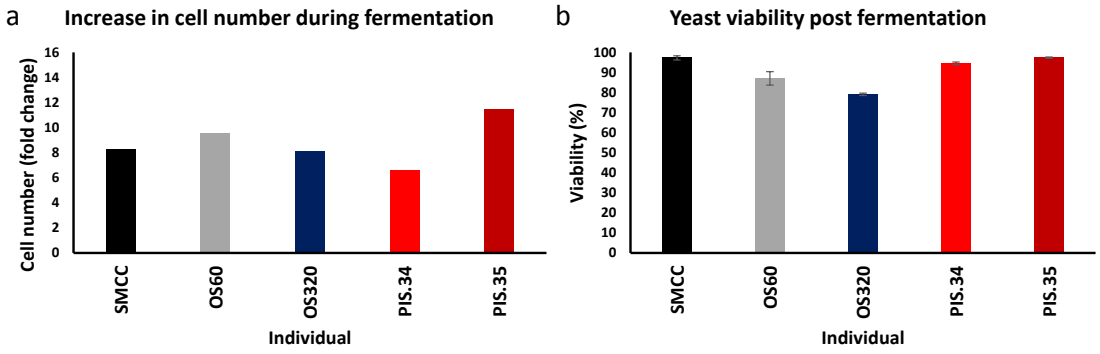


Figure 6.2.25 Cell Number and Viability of PIS Individuals

Quantification of the change in cell number during fermentation (a) and the post fermentation viability (b), of the PIS individuals compared with their parental strains.

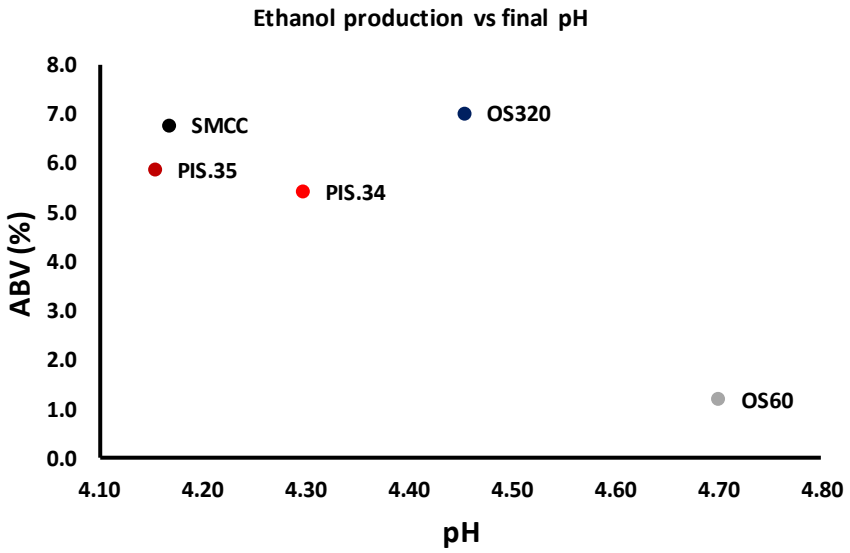


Figure 6.2.26 Production of Ethanol and Final Wort pH of PIS Individuals

Comparison of the post fermentation ethanol concentration and wort pH, produced by PIS individuals and their parental strains. Both factors were quantified using the Alcolyser analysis system. Ethanol content was measured by densitometry.

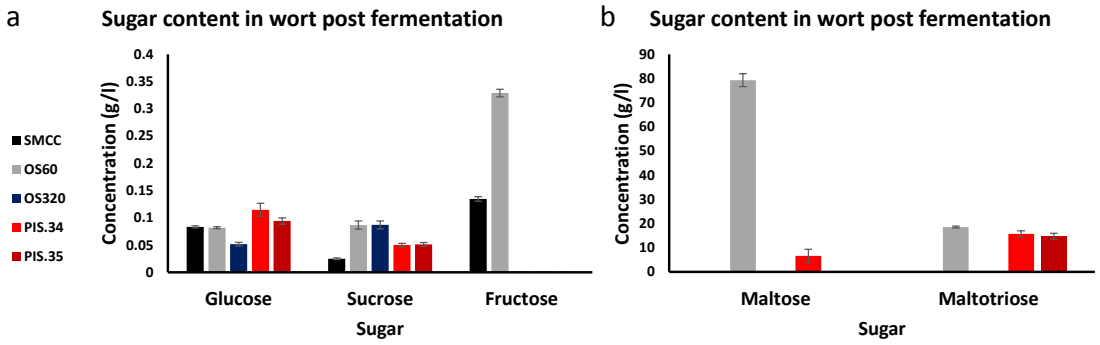


Figure 6.2.27 Sugar Utilisation of PIS Individuals

Comparison of the concentration (g/l) of key brewing sugars in the post fermentation wort. Glucose, sucrose and fructose (a). Maltose and maltotriose (b). The final wort produced by PIS individuals was compared to their parental strains and SMCC.

6.3 Discussion

6.3.1 Key Findings

It is clear from the Omnilog data that the F12 individuals show more variation within each set, compared with the F1 spores of the protoplast fusion isolate. This was an expected consequence of generating advanced intercross cell lines, with increasing genetic and phenotypic diversity as populations advanced through generations.

None of the individuals chosen for fermentation analysis performed as strongly as SMCC. However the majority compared favourably to their parental strains. The H179 and H188 individuals undertook fermentation at a faster rate than three of their parental strains, with only OS626 being competitive. Most of these individuals retained a cell viability remained higher than each of the parent strains. Their ability to utilise sugars compared favourably against their parentals, in particular displaying an improved ability to utilise fructose and maltotriose. Despite OS104 and OS626 displaying a very poor ability to consume maltose, each of the selected individuals were able to completely utilise the sugar. These findings suggest that it is vital to pre-screen parent strains to ensure that the critical brewing traits are present. Using more desirable parental strains, it will be possible to create brewing strains that are more competitive with the existing lager strains.

H51 individuals showed a broad range for rate of fermentation. Each individuals' final weight loss was very similar, however the time taken varied considerably. None of the individuals were as strong as the OS3 parent, and none were as poor as the OS104 parent. The cell viability for each of the individuals remained very high, suggesting these individuals would be suitable for re-pitching between fermentations. Their ability to utilise sugars was acceptable, with an improved ability to utilise maltotriose relative to their parents. However, in the final wort there was a small increase in the remaining glucose for each selected individual. It is probable that further variation would be found if, for a single hybrid, all 90 F12 individuals that were screened using the Omnilog were assessed for fermentation performance. Selecting the best performing individuals from the Omnilog, resulted in the diversity being cropped.

One aspect of the QTL analysis performed here earlier was to investigate the effect the mitochondrial origin had upon the hybrid. The two hybrid strains differing in mitotype were also compared here for phenotypic differences. Unfortunately streamlining the Omnilog process, between experiments, to enable more traits could be analysed on a single plate (both cost and time were saved), meant that the output for H179 and H188 were unable to be directly compared. Fewer and different concentrations were run for H179 compared to H188 and the other experimental runs. On the whole both sets

of individuals showed similar trends, including a broadly poor performance on maltotriose. It is difficult to identify major differences between the individuals picked for fermentation analysis. All six individuals displaying similar results including total weight loss, sugar utilisation, ethanol production, and cell viability.

6.3.2 Technical Application

Using the Omnilog system, it was apparent that individuals were able to be differentiated and ranked based on metabolic activity for a single trait. However, it was difficult to identify individuals that are capable of performing well under multiple conditions at once. Many individuals appeared to outperform SMCC for most traits, but when the fermentation performance of these were analysed in 100 ml mini fermentation vessels none were capable of matching the lager reference strain. An improved method of identifying individuals from the Omnilog data for fermentation analysis should be generated. The current method was somewhat crude. Individuals were manually selected from the inspection of the heat maps. This was suitable for picking strains that showed strong performance in individual traits, or those that showed good metabolic activity levels in most conditions. However, it was difficult to rank the full set of individuals for overall performance across all traits. One method could be to rank each individual by performance in each trait, and those with the highest overall score could be shortlisted for fermentation analysis. Some traits may be considered more important, like the ability to utilise maltose, in which case these traits could be weighted in the overall ranking of individuals.

One factor that may cause misleading results using the Omnilog, is that the yeasts are under aerobic conditions. During fermentation, after a brief period of aerobic conditions, yeast is in an anaerobic environment. The metabolic activity measured is of respiratory metabolism and not fermentative metabolism that would take place in a lager fermentation (Ishtar and Yde, 2007). It would be interesting to compare the data set presented here with another experimental run, where plates were sealed and individuals placed under anaerobic conditions. Based on the fermentation profiles analysed, and that lager strains have evolved under fermentative conditions, one would hypothesis that SMCC performs better under anaerobic conditions than

aerobic. Since this is not the case for the *de novo* hybrids, it is likely that they could fare worse in the absence of oxygen.

Since a carbohydrate source is required for yeast metabolism, measuring metabolic activity in the presence of each sugar gives a good indication of the ability of each individual to utilise each sugar. However environmental stresses can cause changes at the level of transcription (Strassburg et al., 2010). Here we have assumed that individuals resistant to the stress will have a higher metabolic activity than those that are sensitive to the condition. This would be true for those with very limited tolerance versus those particularly well adapted to the stress. One could however speculate that those tolerant to the stress will not undergo an adaptive response and instead will maintain a regular metabolic activity. Likewise those showing moderate sensitivity will undergo transcriptional changes to 'fight' against the environmental conditions, and therefore raise their metabolic activity. For future investigations, a comparison could be taken between strains known to be sensitive and strains known to be resistant to an environmental stress. The PHENOS platform (Barton et al., 2018) could be used in tandem with the Omnilog system for the same conditions, but on solid media. Rather than metabolic activity, PHENOS measures the growth of the individual colony. Individuals sensitive to an environmental stress, or unable to utilise the carbon source, will form small colonies.

The lower temperature limit for the Omnilog was 20 °C (**2.6.2**). The normal brewing temperature for lager is much lower than this (typically range between 6 – 15 °C (Boulton and Quain, 2007)). Individuals that have performed well at 20 °C, may not have the cold tolerance to maintain their performance at the standard brewing temperatures. Without the production company introducing a cooling unit to the equipment, assessment of individual traits at the relevant temperature is currently not possible at this level of throughput.

Improvement of the Omnilog screening process could include assessing individuals under multiple conditions alongside the current screen of single traits. Since during fermentation multiple stresses are influencing the yeast, it would be beneficial to identify isolates that can perform well, for example in the presence of ethanol and osmotic stress at once. Likewise, the ability of

individuals to utilise each sugar whilst under stress may be different to their ability under low stress conditions. Furthermore a system could be set up so that each plate is sealed creating anaerobic conditions, which would be more relevant to the requirements of lager strains.

6.3.3 Further Analysis

Alongside the analysis of fermentation performance above, it would be possible to investigate the production of flavour compounds during fermentation by gas chromatography mass spectrometry (GCMS). Ethanol and carbon dioxide are the main products of fermentation. However secondary metabolites are produced that contribute to the complex flavour profiles of lager. These metabolites can be clustered into four groups; higher alcohols, carbonyl compounds, esters, and VDKs (Olaniran et al., 2017). Below a threshold many of these are considered desirable, however at higher concentrations they contribute undesirable aroma and flavour. Individuals could be identified that produce low levels of off-flavours such as acetaldehyde, an intermediate in the conversion of glucose to ethanol, and give beers a cider-like taste (Carpenter, 2017). In tandem with the Omnilog screen, it would even be possible to select individuals based upon the profile of aroma compounds using GCMS. Previously (Gamero et al., 2016) used GCMS, on 96-well microtiter plates, to screen an abundance of non-conventional yeasts for potential use in food fermentation.

Individuals that are perceived to be of a high enough standard after fermentation assessment would be further investigated through the scaling up of fermentation vessel size. Scaling up is a key requirement for a yeast strain to go from microtiter plates to industrial size fermenters. As fermentations are scaled up, the conditions the yeast is under do not remain constant. In small fermentations a complete mixing of the wort can be assumed, however in industrial vessels gradients in ethanol, pH and sugar concentration occur (Formenti et al., 2014).

The *S. cerevisiae* x *S. uvarum* and *S. uvarum* x *S. eubayanus* hybrids are more similar to the *S. bayanus* hybrid used in wine and cider fermentations. It may be worth investigating their phenotype with traits that are more specific to these processes. The ethanol concentration at the end of these

fermentation is higher than in lager, and the juice has a naturally lower pH. The primary sugar in grape must and apple juice is fructose (Karadeniz and Ekşi, 2002). When selecting for candidates based upon sugar utilisation above, the focus was primarily upon their ability to utilise maltose and maltotriose; which are highly abundant in wort but not in grape and apple juice. Candidates of these new hybrids could be shortlisted with a focus on wine and cider specific traits over brewing traits, before being assessed for fermentation performance of grape must and apple juice.

6.3.4 Alternatives to the Omnilog

The Omnilog has been a useful tool for the crude screening of individuals, for a wide number of discrete traits. The redox dye is used to measure the level of metabolic activity. This does overcome common issues of measuring growth in liquid cultures; one key issue being differential flocculation levels. Cell clumping can disrupt observed density reads, as a measure of growth, using spectrophotometry techniques. However the Omnilog does have some limitations. A major concern, as discussed above, is that metabolic activity level does not necessarily indicate how an individual is performing under a stress condition. Another limitation is that it is difficult to assess an individual's response to multiple stresses at once, which is the case during fermentation.

The PHENOS system, used earlier in the QTL analysis (**Chapter 4**) of these hybrids, directly measures individual growth. However this alternative screening method is performed on solid media. The performance of individuals is likely to be different suspended in liquid cultures than fixed on a plate. PHENOS is much less labour and cost intensive than using the Omnilog. Running both methods in tandem would be possible to identify individuals with high metabolic activity due to being under stress, as well as individuals with high metabolic activity due to strong performance.

New pieces of equipment, such as the BioLector (m2p-labs) have recently become available, expanding options for screening individuals. The BioLector is capable of screening up to 48 individuals at once under highly controlled conditions. This can be used for individual traits in both aerobic and anaerobic conditions. The BioLector is also capable of functioning as a bioreactor, providing real-time measurements on a whole range of characteristics

including; pH, headspace O_2/CO_2, and concentration of biomass. This equipment would allow profiling of 32 simultaneous fermentations, enabling the selection of individuals based upon overall fermentation performance, rather than observing the ability of an individual to grow under stress conditions. M2p-labs claim that these small volume fermentations (800 – 2400 µl) are reflective of larger scale fermentations. Previously this could be an issue, since the fermentation environment changes with increased volume.

With the sheer number of new individuals created in this project, a major bottleneck has been the screening of individuals. For each new hybrid created there are millions of phenotypically distinct F12 individuals capable of being screened. This number brings into perspective what is typically considered high-throughput; the maximum capacity of the Omnilog is 50 96-well plates, the PHENOS system is up to 384 individuals per plate. One option to fast track this would be rounds of pooled selection prior to individual analysis. Much like in the pooled QTL analysis experiments, a population would be placed under a desired selectable trait with only a fraction of the population capable of growth. The Verstreppen group use a screening system that is capable of screening single cells, in picoliter volumes using microfluidics on microchips reviewed in (Vervoort et al., 2017). This screening is thus capable of analysing over 10^5 individuals in just hours (Huang et al., 2015, Sjostrom et al., 2014). This technique can be used to screen and isolate individuals for the secretion of a range of metabolites, including flavour compounds. Individuals would be isolated using this technique before secondary analysis at higher volumes, as not all individuals will be capable of the scaling up from picoliter volumes to standard lab and then industrial volumes (Crater and Lievense, 2018).

6.3.5 Continuation

As a consequence of finding individuals that outperformed their parents but were inadequate relative to the lager reference strain, any further creation of *de novo* hybrids should be determined by the qualities the parental strains possessed. Of the four *S. cerevisiae* strains analysed for fermentation performance, only OS3 should be considered of high enough standard to be used as a parental in new brewing hybrids. The other *S. cerevisiae* strains were unable to achieve a fast rate of fermentation, poorly utilising key brewing sugars. Excluding the *S. pastorianus* strain OS320, none of the parental

strains were capable of complete maltotriose utilisation, with most of maltotriose remaining in the wort. As maltotriose is a key component of lager wort, it is essential to identify new parental strains that can completely utilise maltotriose.

Another option would be to screen the complete set of 252 *de novo* hybrids created here (**Chapter 3**), prior to generating diversity through interbreeding. Individuals may possess some of the desired traits screened for, but lack others. If these hybrids possess the same two parental species, it would be possible to mate between them in an attempt to combine the desirable traits and possibly lose undesirable ones.

In the process of performing QTL analysis by pooled selection (**Chapter 4**), 384 individuals were arrayed and stored from each selection pool. These individuals were tolerant to brewing relevant stresses such as high ethanol concentration, osmotic stress (pooled selection was not analysed), and high maltose concentration. It would be worth analysing a selection of these individuals first using the Omnilog, and then by fermentation profiling to assess their candidacy as brewing strains. (Krogerus et al., 2018) improved the fermentation performance of their own *de novo* lager hybrids through adaption to high ethanol concentrations. Whereas Krogerus relied on the unstable nature of higher ploidy to allow their hybrids to adapt to the condition, the hybrid populations here had diversity generated in abundance through mating.

It is worth questioning whether SMCC was the ideal control for this analysis, with all strains the strains being normalised to. It is certainly necessary that an industrial lager strain should be assessed in comparison, and used as a benchmark for a desirable level of fermentation performance. However the *de novo* hybrids were primarily created to perform QTL analysis in hybrids. The parent strains were identified to maximise genetic diversity in each hybrid. The phenotypic profile of each parent with respect to lager fermentation was not considered prior to the creation of these hybrids. Therefore it was never expected that these hybrids would outperform the existing *S. pastorianus* strains. Instead the performance of the F12 individuals should be compared to their own parent strains: – Have they inherited desirable traits? Do they

show a stronger fermentation capacity? Due to variation between experimental runs of the Omnilog, it was difficult to directly compare the parents with the F12 individuals. It was possible however to compare the candidates identified in the initial Omnilog screen with their parental strains for the key metrics of fermentation performance. In regards to this the H179 and H188 individuals did outperform each of the parent strains, displaying a degree of heterosis. This observation supports the belief that creating new *S. pastorianus*-like hybrids is a viable means of improving yeast strain options for brewing. The selected H51 strains all performed better than the worst performing parent, OS104, but none completed fermentation as quickly as the OS3 parent. There was a broad observed range in rate of fermentation, showing that phenotypic diversity was able to be introduced through creation of hybrids. Unfortunately due to the limited time available on the project secondment, individuals of H230 were not compared to their parental strains. Despite the parent strains of these hybrids not being selected based upon brewing traits, the F12 individuals showed promise. Both diversity and improved phenotypes were observed. This is supportive of the view that creating new hybrids, with parents that display strong fermentative properties, would result in new individuals that are as strong, if not stronger than the existing lager strains. Arrays of candidate parental strains could be created, and assessed for desirable traits. A strong ability to utilise all of the key wort sugars, particularly maltose and maltotriose, would be the primary selection criteria, followed by good osmotic stress resistance and tolerance to ethanol and the other agents of environmental stress.

7 Concluding Remarks

7.1 Summary

In this project hybrid sterility has been overcome, with a large set of fertile tetraploid hybrids created. Each of these hybrids gives rise to viable offspring, which are capable of interbreeding due to our manipulation of the mating type mechanism. With each hybrid it is possible to generate a phenotypically diverse population, and perform detailed QTL analysis for a multitude of traits. There are many cases in plants where higher levels of ploidy have facilitated renewed fertility. Key crop plants, including within the *Triticum* genus (wheat plants), are polyploid. Durum wheat (*T. turgidum*) is a tetraploid hybrid species originating from two diploid grass species (Kubaláková et al., 2005). Using the principles we've applied here to yeast, it may be possible to create *de novo* wheat hybrids from the parental species. This may result in new strains with different traits to the existing durum wheats, as well create a diverse population that allows for QTL analysis to be performed.

QTL analysis was indeed performed upon a *S. cerevisiae* x *S. uvarum* hybrid (H51), as well as two *S. cerevisiae* x *S. eubayanus* hybrids (H179 and H188) that differed simply by mitotype. This analysis revealed a number of loci involved in industrially relevant traits. Selection on maltose rich media was particularly successful, identifying multiple loci in the DNA content of both parental species in H179 and H188. There were numerous intervals shared between the two mitotype variants, however there were also multiple that were unique to either strain. The proteins of the candidate genes within these intervals often were found to localise to, or interact with the mitochondria. These findings suggest that the species origin of the mitochondria is influencing the strains tolerance to these stresses.

Fertility was successfully introduced into *S. pastorianus* strains. A Saaz class strain was made fertile through protoplast fusion with a haploid *S. cerevisiae* strain, yielding an allotetraploid hybrid that can produce allodiploid F1 gametes. Frohberg strains are already allotetraploid, but still produce non-viable gametes. It was possible to isolate several rare viable spores, which were found to have a near diploid level of ploidy. Some of these spores were

homozygous at the mating type locus, meaning they were suitable for mating with other diploid maters. Mating these to *S. cerevisiae* diploid maters produced in chapter 3 produced near tetraploid isolates which had a low to moderate level of gamete viability. This tetraploid isolate has a 3:1 ratio of *S. cerevisiae* to *S. eubayanus* genetic content, therefore not all chromosomes will be capable of pairing with homologous chromosomes of the same species origin. This is most likely responsible for the lower than desired level of fertility. Options for future more successful breeding of these isolates is outlined below.

A phenotypic screen of individuals from both F12 populations of the *de novo* hybrids and F1 spores of the Saaz protoplast fusion isolate, found candidates that displayed a number of desirable phenotypes. Shortlisting these candidates, and assessing their fermentation performance found the F12 individuals out performed their parental strains. This provided a clear indication that there is huge potential for creation of new and improved strains through breeding. The Saaz fusion isolate spores appeared to inherit the inability to utilise maltose from the haploid *S. cerevisiae* parent and as a result performed poorly in fermentations.

7.2 Future QTL analysis

In this study, QTL analysis was successfully performed on *de novo* hybrids through the use of pooled selection. It is our contention that, upon receiving the sequencing data for the multipool analysis, it will be demonstrated that both techniques of QTL analysis were successful. Having assessed H179 and H188 by multipool for growth at temperature extremes, it is likely further loci will be identified that are mitotype specific. The major phenotypic difference between *S. eubayanus* and *S. cerevisiae* strains is their heat tolerance. *S. cerevisiae* strains are much more tolerant of high temperatures, which *S. eubayanus* are highly sensitive to. Conversely *S. eubayanus* displays stronger growth at lower temperatures.

Further techniques are available to study QTL analysis in these *de novo* hybrids. With lower costs of sequencing it is becoming more viable to perform individual segregant analysis. An array is created of individuals, in this case

F12 hybrid gametes, each is then sequenced prior to phenotypic analysis. Using the PHENOS platform it is possible to use this sequenced array to identify QTLs involved in many different traits. Using the R/QTL analysis package individuals' phenotypes are combined with their genotype data. Ranking individuals within the array by phenotype identifies genomic regions that are linked to the observed trait (Wilkening et al., 2013). PHENOS can also be used to investigate other factors than maximum growth. Using the full growth curve, produced using a plate reader taking OD measurements every 20 minutes, it is possible to rank individuals by max-slope which is equivalent to the growth rate (Barton et al., 2018). It is also possible to investigate the length of lag phase, the time taken for individuals to enter into the exponential growth phase. This is important to brewers, as a brief lag phase translates into a shorter overall fermentation time. Whilst there is an initial high monetary cost of ISA, due to sequencing every individual in an array, there is almost no limit to the variety of phenotypes to analyse. PHENOS is suited to rapid screening of variation, and has been shown to accurately reflect phenotypic profiles of traits that are typically observed in liquid cultures. It would also be possible to use ISA to assess characteristics that PHENOS can't analyse, for example the ability to utilise a specific sugar, or the production of aroma levels. Using HPLC and gas chromatography, it would be possible to rank individuals for these traits and identify contributing genetic loci.

After successfully performing QTL analysis on *de novo* hybrids, the focus of our analysis should switch to the naturally occurring hybrids. In unpublished data, this group has successfully performed QTL analysis after introducing fertility to a triploid wine hybrid of *S. kudriavzevii* x *S. cerevisiae*. This hybrid had two copies of the *S. cerevisiae* genome, and a single copy of the S. *kudriavzevii* genome. By mating with a haploid *S. kudriavzevii* strain, a tetraploid isolate was produced with two copies of each parental species. This tetraploid gave rise to viable diploid offspring which possessed one copy of each genome. Using the multipool technique, QTLs were identified for a variety of traits relevant to wine production and other and biotech processes. One trait studied was acetic acid tolerance, which is important in processes such as bioethanol production. Weak acids are used to prevent bacterial contamination (Tanaka et al., 2012). Having introduced fertility to the Saaz

strain through protoplast fusion, it is now possible to perform quantitative genetics with the fusion isolate spores. Initial phenotypic screening of the PIS individuals found a modest level of diversity (**Chapter 5 and 6**). The greatest variation was observed under stress from high levels of acetic acid. This phenotypic range observed demonstrates that these individuals are suitable for QTL analysis using either Bulk segregant analysis (BSA), via the multipool method, or by ISA, as outlined above.

There is also potential to perform QTL analysis using the rare spore isolates of the Frohberg strains. Mating these and isolating F1 gametes will allow for analysis by multipool and ISA. We have shown it is possible to mate these isolates to *S. cerevisiae* diploid maters and produce viable offspring. An ideal scenario, however as yet not attempted, would be to isolate rare viable spores of different Frohberg strains and mate between these. If the resulting isolates subsequently gave rise to viable gametes, QTL analysis in *S. pastorianus* strains would be possible without the introduction of genome content from a strain outside the species. The use of ISA may prove to be the most useful in these strains because the gametes are likely to be aneuploid. The level of aneuploidy in each individual would be observed through sequencing individuals (Mulla et al., 2014), but difficult to analyse by BSA techniques.

7.3 Future Possibilities for New Brewing Strains

The fermentation performance of the F12 isolates of the *de novo* hybrids was, in general, superior to their parental strains displaying evidence of heterosis. However the existing lager strain, SMCC used for comparison, outperformed all the individuals assessed. The most likely cause of this failure lies in the choice of parental strains. Strains were chosen to maximise genetic diversity within the hybrid, for the performance of QTL analysis. There was no pre-screening of these parental strains for brewing specific traits. It wasn't until the phenotypic analysis was performed that identified several parental strains, including OS60 and OS626, as being unable to utilised maltose and maltotriose for example. The future generation of hybrids intended for industrial use should begin with a comprehensive phenotypic screen of potential parental strains.

There is a rich wealth of diversity throughout the *Saccharomyces* clade from which to screen. Upon commencing this project there was limited availability of *S. eubayanus* strains, owing to its recent identification as a species (Libkind et al., 2011). Since this original isolate was found, using improved isolation protocols, a large strain set has been compiled with isolates from around the world (Peris et al., 2016). These geographic locations include North America (David et al., 2014), New Zealand (Gayevskiy and Goddard, 2016), China (Bing et al., 2014), and a multitude from the Patagonian region of Argentina and Chile (Rodriguez et al., 2014). There is also a vast genetic diversity of *S. cerevisiae*, going far beyond the five lineages used in this project (Liti et al., 2009). The diversity found in Chinese isolates alone is far greater than what is observed between the strains used here (Wang et al., 2012, Peter et al., 2018). Using the most desirable strains from these two species it is inevitable that new *S. pastorianus*-like hybrids can be synthesised that are superior to the existing lager hybrids. It would be possible to further expand the pool of diversity through including strains from other species. *S. uvarum* and *S. kudriavzevii* for example, both cold tolerant species, are highly suitable alternatives to the *S. eubayanus* portion of the hybrid (Peris et al., 2018).

It may be the case that parent strains are identified that have a range of desirable phenotypes, but also a few undesirable traits such as producing high levels of off-flavours. It can be easier to eliminate these negative traits prior to the synthesis of hybrids. This has already been achieved in *S. eubayanus* strains through a mutagenic screen. *S. eubayanus* strains are POF^+, producing undesirable levels of phenolic off flavours. Exposing a strain to UV radiation, and subsequently screening individuals for production of these phenolic compounds found isolates that were POF^- suitable for use in the creation of new hybrids (Diderich et al., 2018).

Persisting with the *de novo* hybrids created in this project, it would be possible to isolate more desirable individuals. Taking the most desirable F12 candidates identified (**Chapter 6**) it would be possible to cross these individuals and generate an F13 population. Continued rounds of selective breeding would over time produce individuals with more and more desirable traits. Most of the F12 individuals did however display a poor ability to utilise

maltotriose. It would also be possible to introduce alternative strains, which can use the sugar, to these hybrids.

Ultimately any new strain to be used in the brewing industry, for the foreseeable future, will need to be a non-GMO. The use of GM in this project has been to facilitate the rapid synthesis and selection of new hybrids. When creating such a large number of hybrids GM remains the most suitable technique. This large set of hybrids can be screened to identify isolates with the desired traits. Upon discovery of desired individuals, it is then possible to return to the parental strains and re-synthesise this hybrid using non-GM techniques. Individuals that have spontaneously lost a functional *HO* copy can be isolated. Mutagenesis can increase the frequency of these individuals occurring. Loss of function has the same effect on strains as deletion of the *HO* gene, leading to stable haploid isolates. Various auxotrophic mutations can be generated to facilitate selection of successful mating events. Individuals that have lost the ability to synthesise uracil for example can be selected on media containing 5-flouroorotic acid (FOA) (Boeke et al., 1984). Wild type strains convert FOA into the cytotoxic %-flourouracil (5-FU). Whereas individuals with a non-functional *URA3* gene cannot convert FOA to FU allowing colonies to grow. Isolates that are *ura3*$^{-}$ require uracil in the media. Auxotrophic haploids can be mated to generate diploids. Complementation of prototrophic markers used to select successful maters. The diploid population can be screened for individuals that have lost heterozygosity of the *MAT* locus. Crossing between diploid maters will generate tetraploid hybrids that have not undergone genetic modification.

7.4 Continued breeding of existing Lager strains

Our ability to breed with the *S. pastorianus* strains is an exciting prospect. Breeding is facilitating our aim of increasing phenotypic diversity within the hybrid species, as well as allowing us to introduce new traits not present in the existing collection of strains.

Introgressing *S. cerevisiae* genetic material into the Saaz strain has resulted in an increased phenotypic variation in its F1 spores. Unfortunately, fermentation analysis found that the haploid *S. cerevisiae* strain used here

was unable to utilise maltose and therefore not ideal for creating improved brewing strains. The principles proven here do however fill us with immense optimism that repeating these experiments with *S. cerevisiae* strains that have desirable properties will yield new and improved brewing isolates. Prior to a comprehensive parental screen, outlined above, using the fermentation analysis (chapter 6) one would say that OS3 would be a promising parental candidate. OS3 originates from European wine fermentations, and as a consequence has a strong fermentation profile and is tolerant to high ethanol concentrations. Here we found the strain was also highly capable of utilising maltose, but showed a weak ability to utilise maltotriose (**Figure 6.2.15**).

The near diploid Frohberg spores are suitable for crossing with other diploid maters. The resulting near tetraploids created in this way (**Figure 5.2.10**) had 3 copies of the *S. cerevisiae* genome and only one *S. eubayanus* copy. One option to increase gamete viability would be to cross with newly synthesised hybrids that have a single copy of each parental genome. The tetraploid isolate would inherit two copies of each species genome facilitating correct chromosomal segregation. The diploid hybrid to cross with the Frohberg spores could either be one created from the haploids, which were created in **Chapter 2**, or they could be taken from the F12 populations of the *de novo* hybrids. From the Omnilog and subsequent fermentation analysis, there were a number of H179 and H188 individuals that showed promise (**Figure 6.2.4** - **Figure 6.2.6**). The method of creating these tetraploid hybrids meant that 50 percent of the diploid progeny would be either *MATa* or *MATα*. A quick mating type test, by PCR, would identify which of these individuals would be capable of mating with the Frohberg spores.

Currently the spores isolated from Frohberg strains have the largest potential for use in industry since they have not undergone any genetic modifications. Crossing with *de novo* hybrid diploid maters would result in the progeny being labelled as GMOs. As briefly mentioned above whilst discussing options for QTL analysis, there is also the option of crossing between the Frohberg spores of different strains. This would not add new traits to the species, however it would lead to increased diversity as progeny would inherit different allele combinations which differentially contribute to desired brewing traits.

Since the copies of the HO gene in the Frohberg strains were not knocked out prior to isolate of rare viable spores, it was expected that the isolates would be heterozygous for the mating type locus. However in many instances we found individuals that were homozygous and therefore behaved as maters. The mating type of the protoplast fusion isolate spores has yet to be assessed. The case may be that some PIS are homozygous and are capable of mating. This would give the option of crossing isolates of the two classes of *S. pastorianus*. Both the PIS and rare spores are near diploid therefore would create new allotetraploid hybrids.

A common technique implemented to improve existing lager strains is adaptive evolution after mutagenesis (Ekberg et al., 2013, Yu et al., 2012, Blieck et al., 2007, Huuskonen et al., 2010). Mutagenesis creates variation in a strain, before being placed under a selectable condition such as high osmolarity. Repeated selection for the same trait overtime leads to the isolation of individuals that are more adapted to the condition than the original strain. Having developed phenotypically diverse interbreeding hybrid populations, it is possible for us to perform adaptive evolution. Maltotriose utilisation appears to be an issue for many of the individuals assessed in **Chapter 6**. A population can be generated from individuals that otherwise demonstrate strong fermentation performance. This population would be cultured on maltotriose media for generations until isolates are found that can completely utilise the sugar.

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8 Appendix

8.1 A.1 QTL Analysis

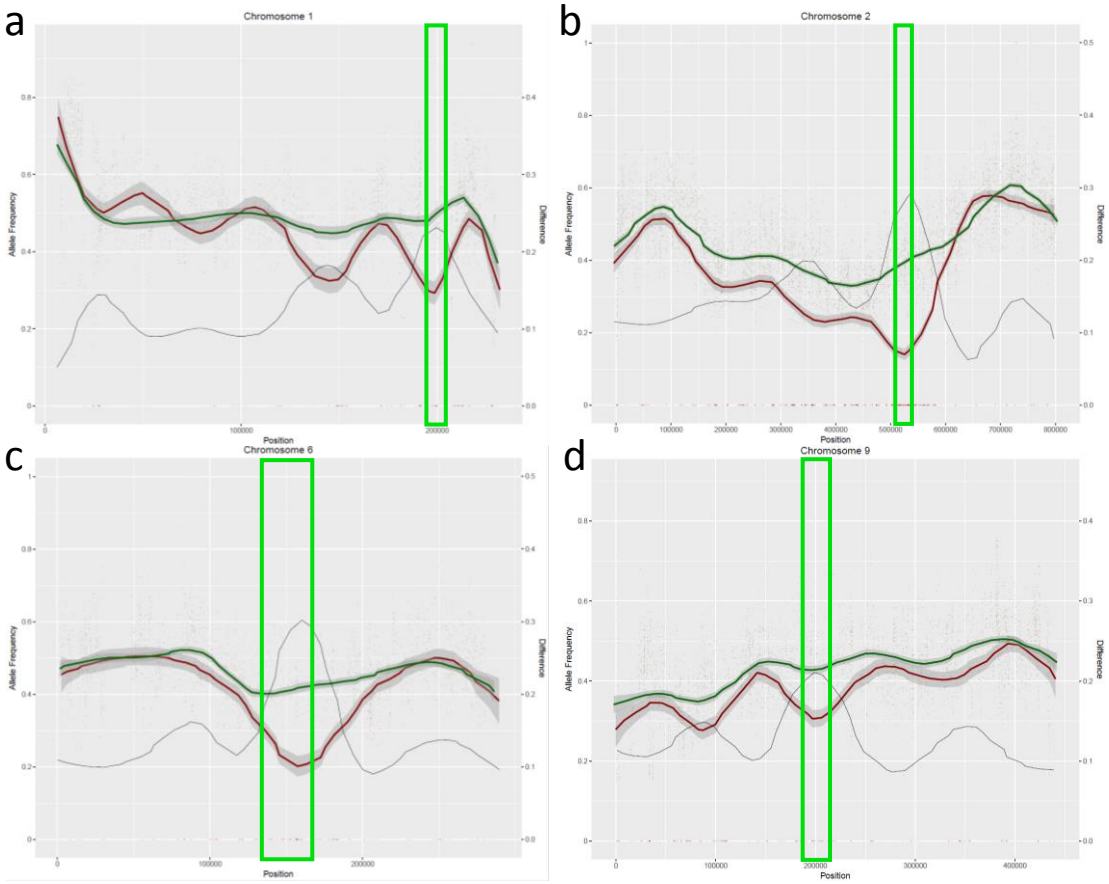


Figure 8.1.1 Intervals Identified within the *S. cerevisiae* Portion of H51

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-d) Four intervals that confer a selection advantage for growth at high temperature, on chromosome 1, 2, 6, and 13 respectively.

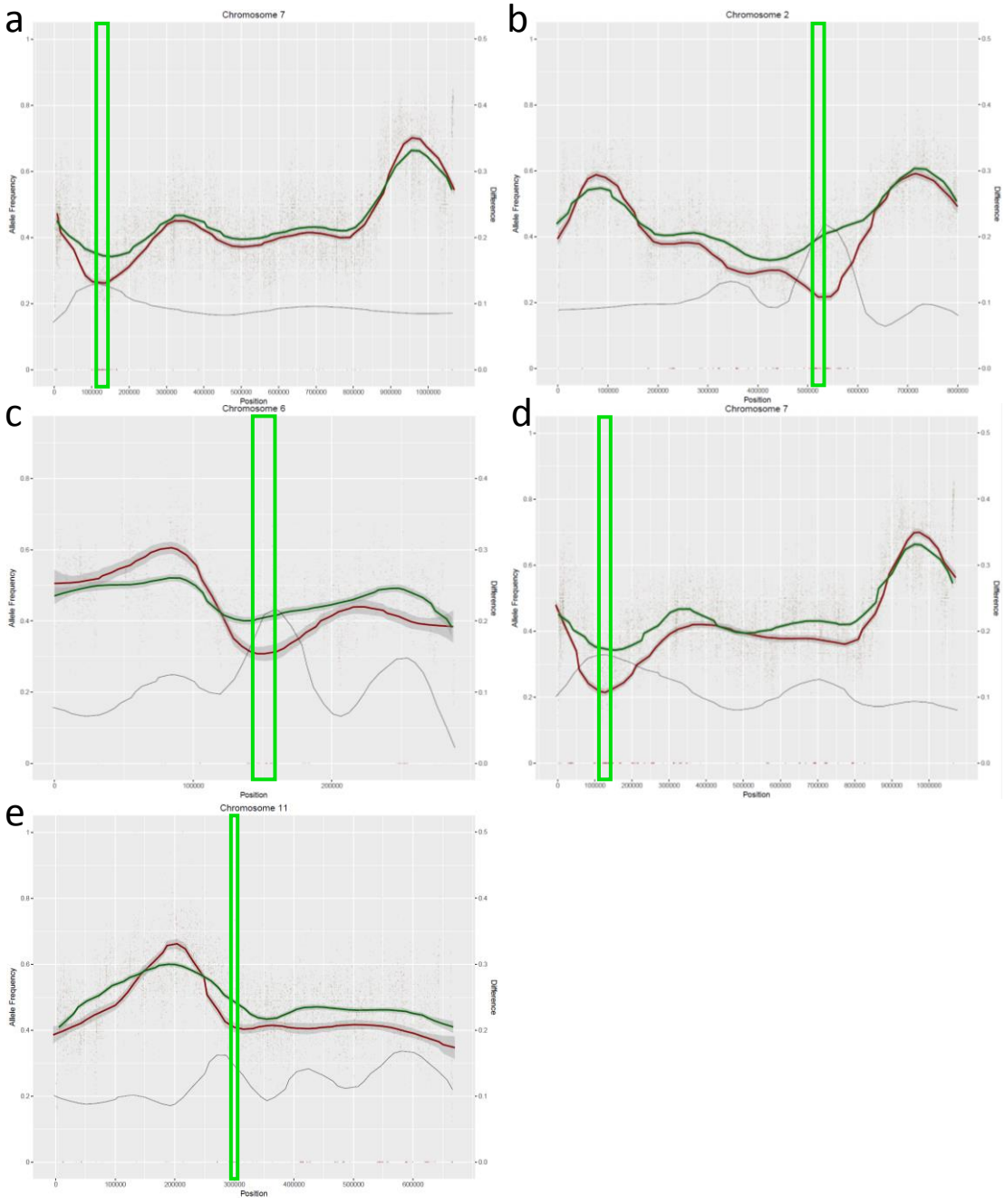


Figure 8.1.2 Intervals Identified within the *S. cerevisiae* Portion of H51

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a)(b-e) Four intervals that confer a selection advantage for growth in the presence of levulinic acid, on chromosome, 2, 6, 7 and 11 respectively.

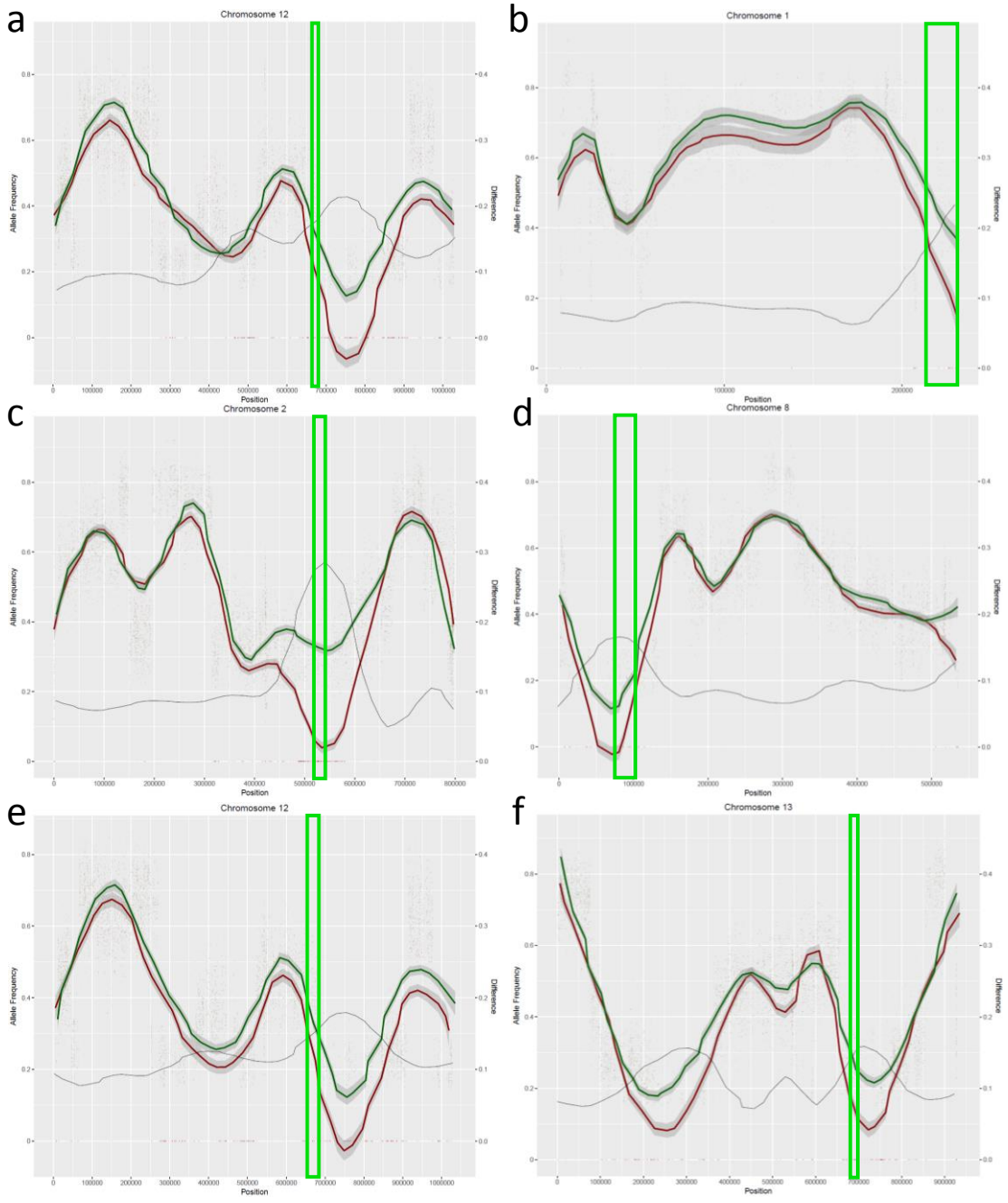


Figure 8.1.3 Intervals Identified within the *S. cerevisiae* Portion of H179

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a) Chromosome 12, with an interval conferring a selection advantage in the presence of H_2O_2. (b-f) five intervals that confer a selection advantage on maltose rich media, on chromosome 1, 2, 8, 12, and 13 respectively.

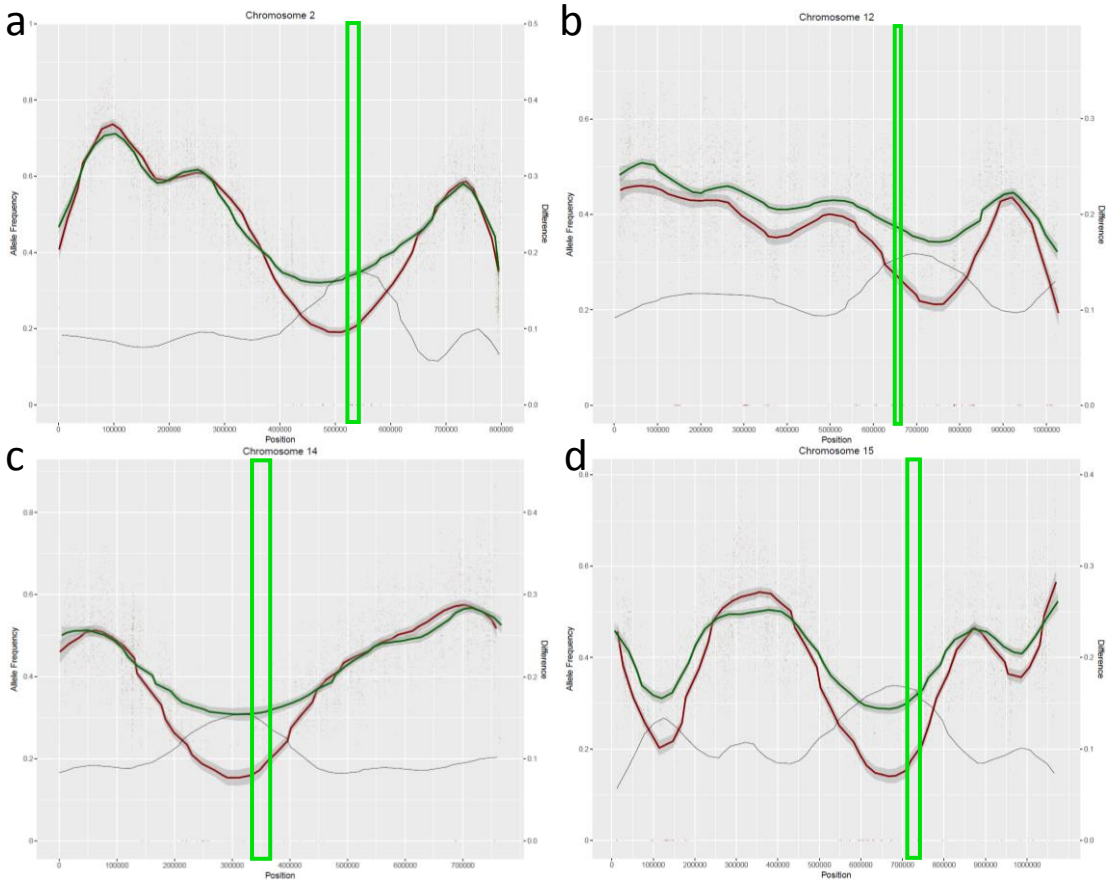


Figure 8.1.4 Intervals identified within the *S. cerevisiae* Portion of H188 Conferring an advantage on glucose rich media

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-d) Intervals that confer a selection advantage on glucose rich media, on chromosome 2, 12, 14, and 15 respectively.

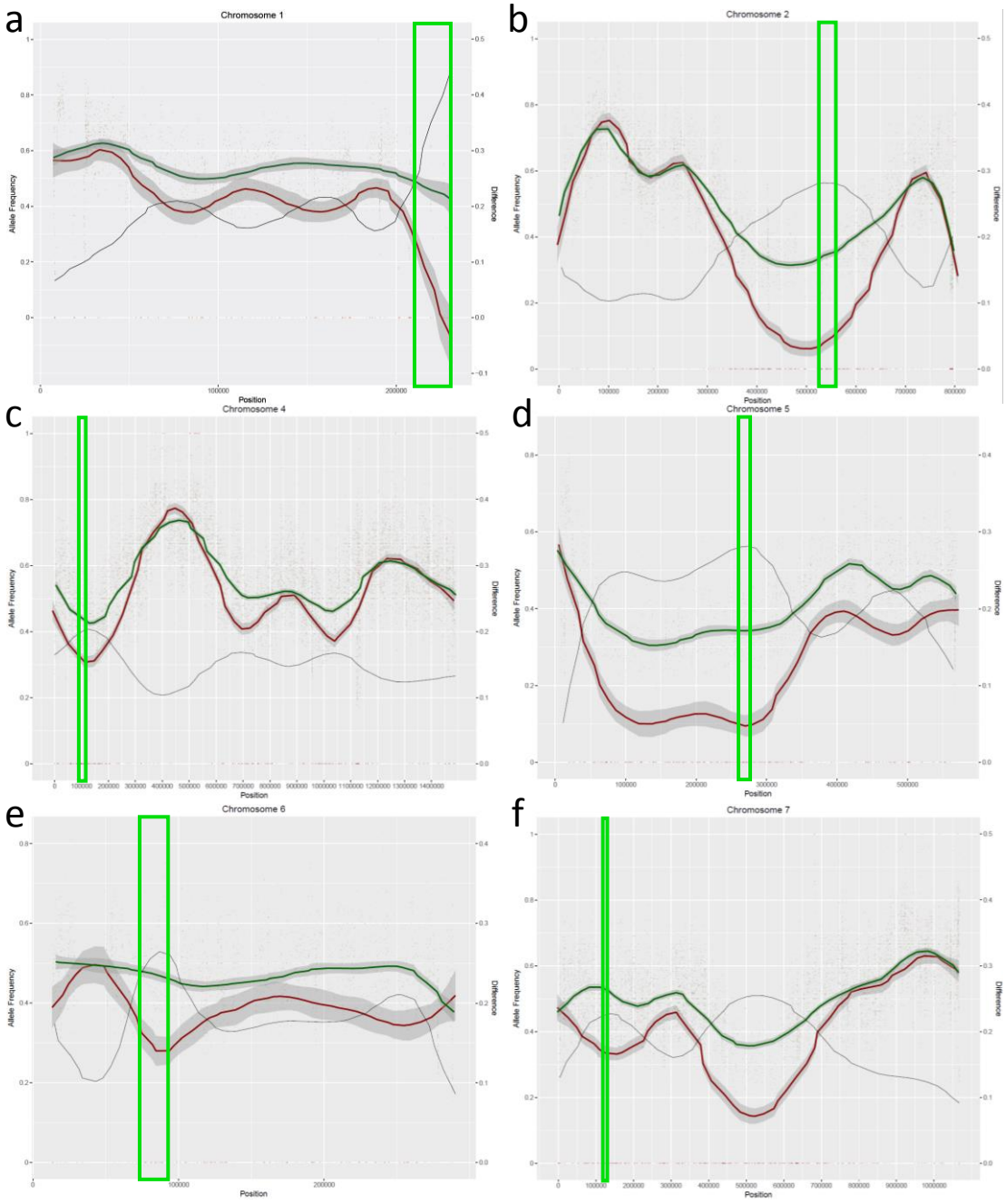


Figure 8.1.5 Intervals identified within the *S. cerevisiae* Portion of H188 Conferring an advantage on maltose rich media

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-f) Intervals that confer a selection advantage on maltose rich media, on chromosome 1, 2, 4, 5, 6, and 7 respectively.

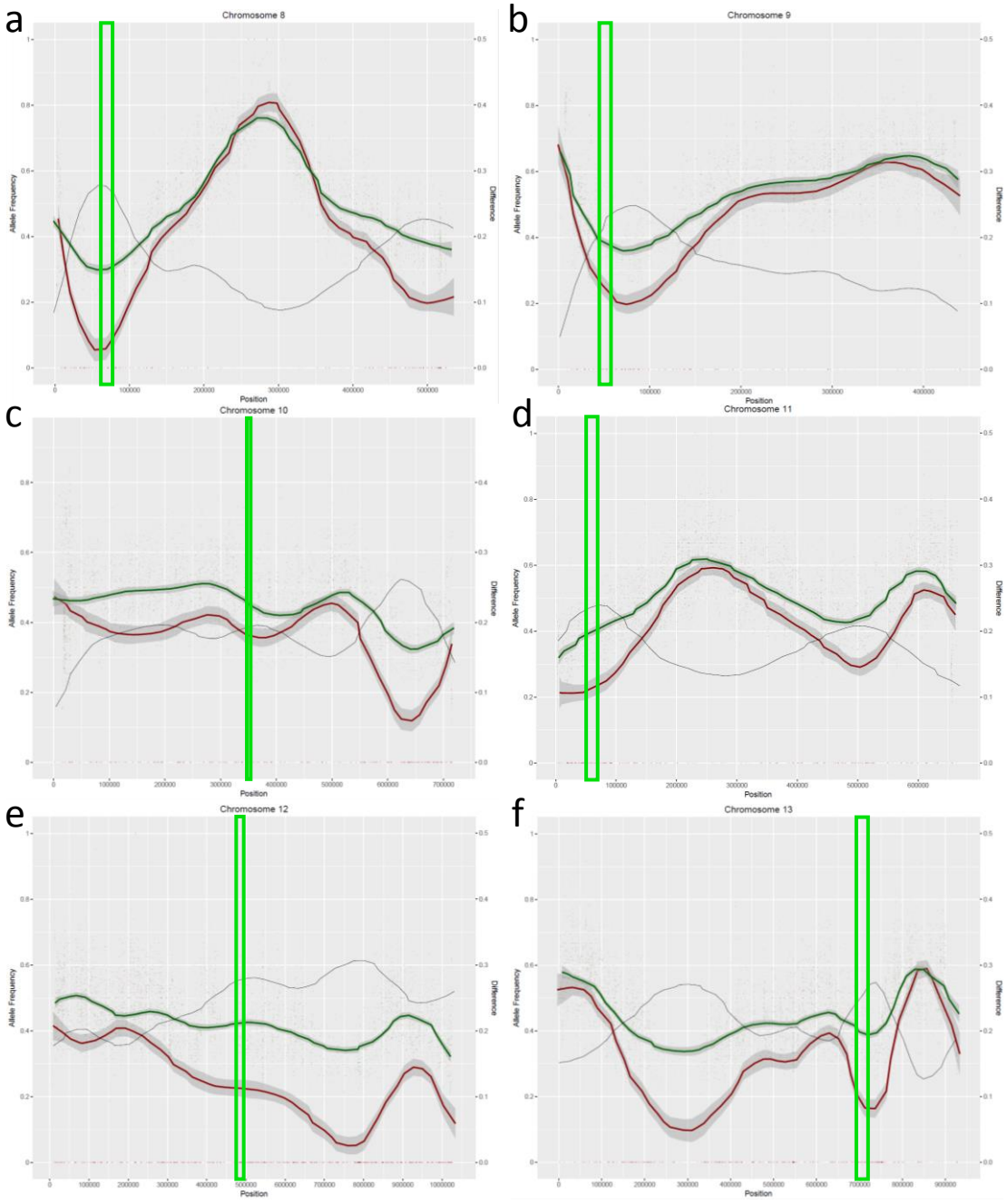


Figure 8.1.6 Intervals identified within the *S. cerevisiae* Portion of H188 Conferring an Advantage on Maltose Rich Media Continued

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-f) Intervals that confer a selection advantage on maltose rich media, on chromosome 8, 9, 10, 11, 12, and 13 respectively.

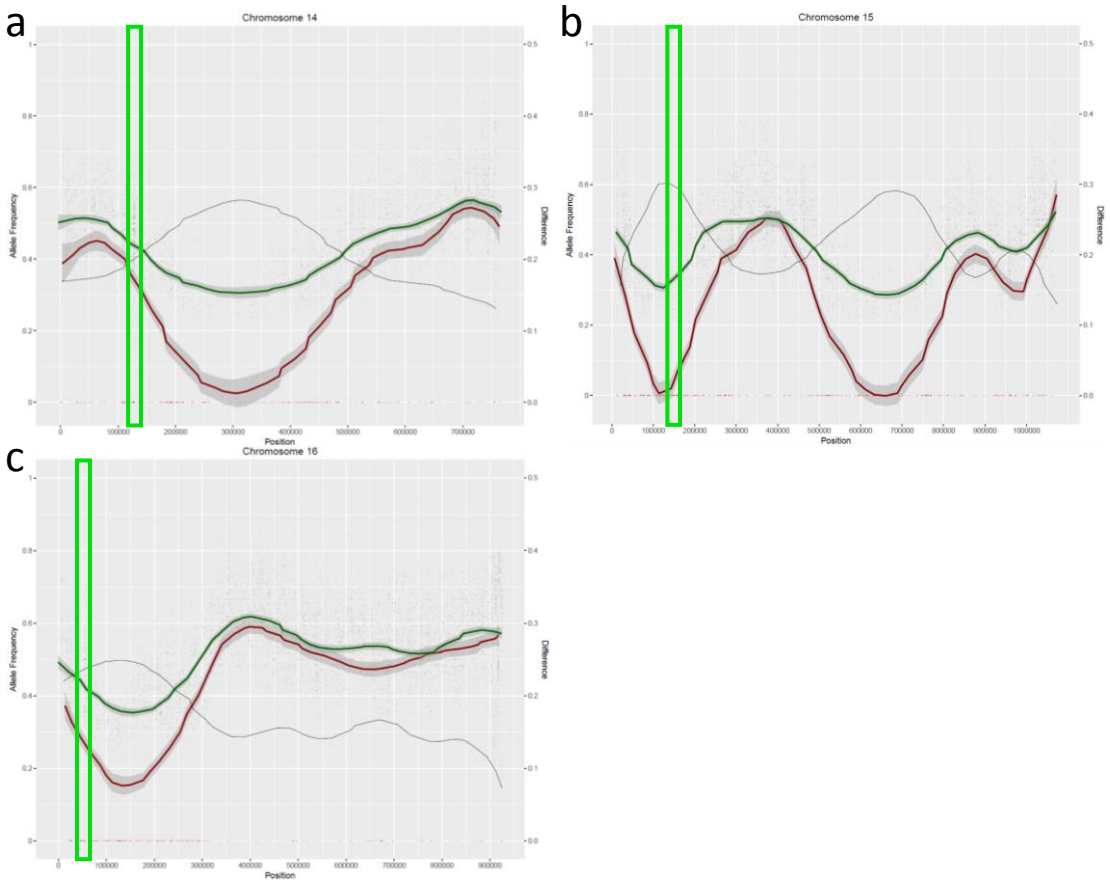


Figure 8.1.7 Intervals identified within the *S. cerevisiae* Portion of H188 Conferring an Advantage on Maltose Rich Media Continued.

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-c) Intervals that confer a selection advantage on maltose rich media, on chromosome 14, 15, and 16 respectively.

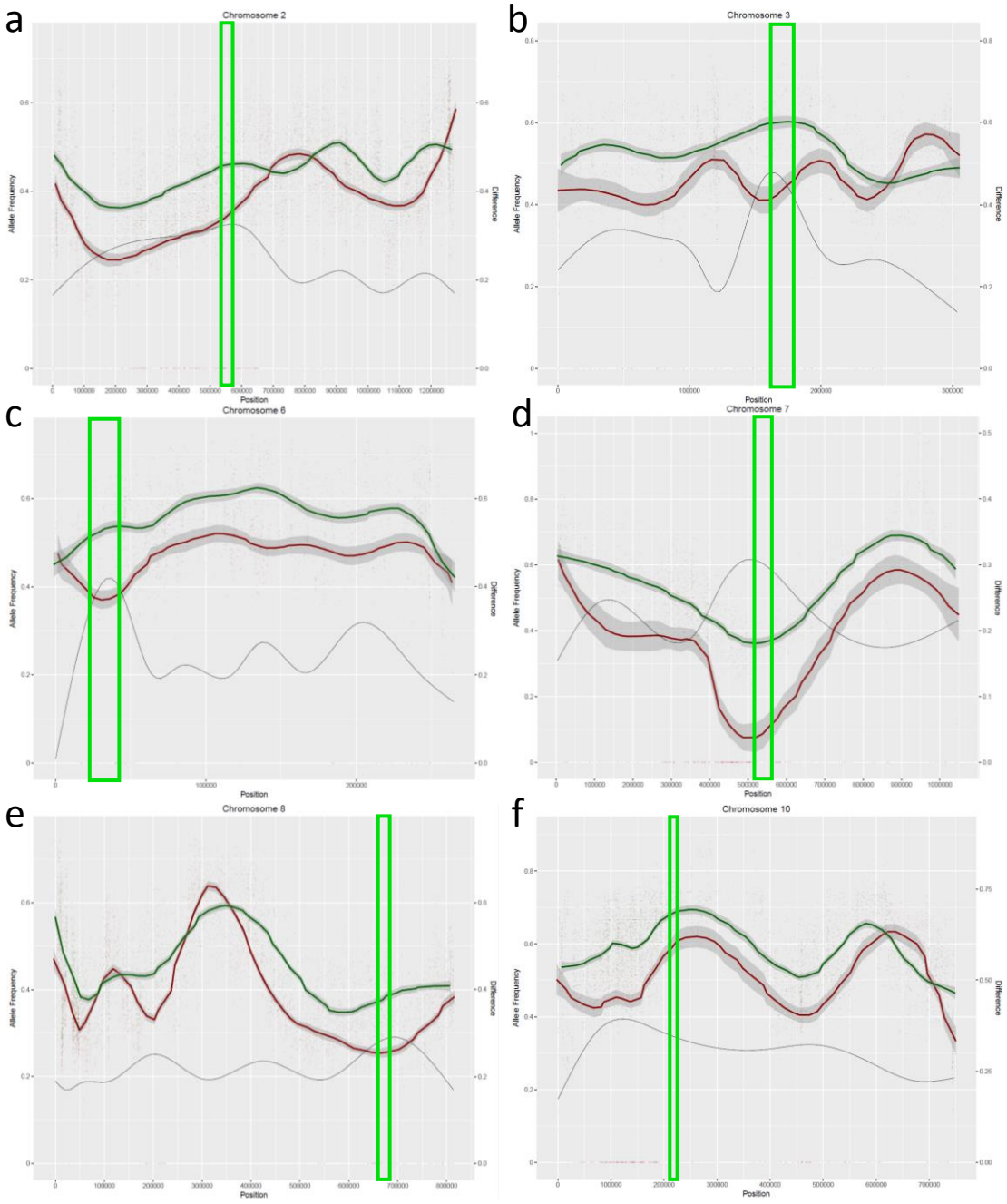


Figure 8.1.8 Intervals Identified within the *S. eubayanus* Portion of H179

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-f) Intervals that confer a selection advantage on maltose rich media, on chromosome 2, 3, 6, 7, 8, and 10 respectively.

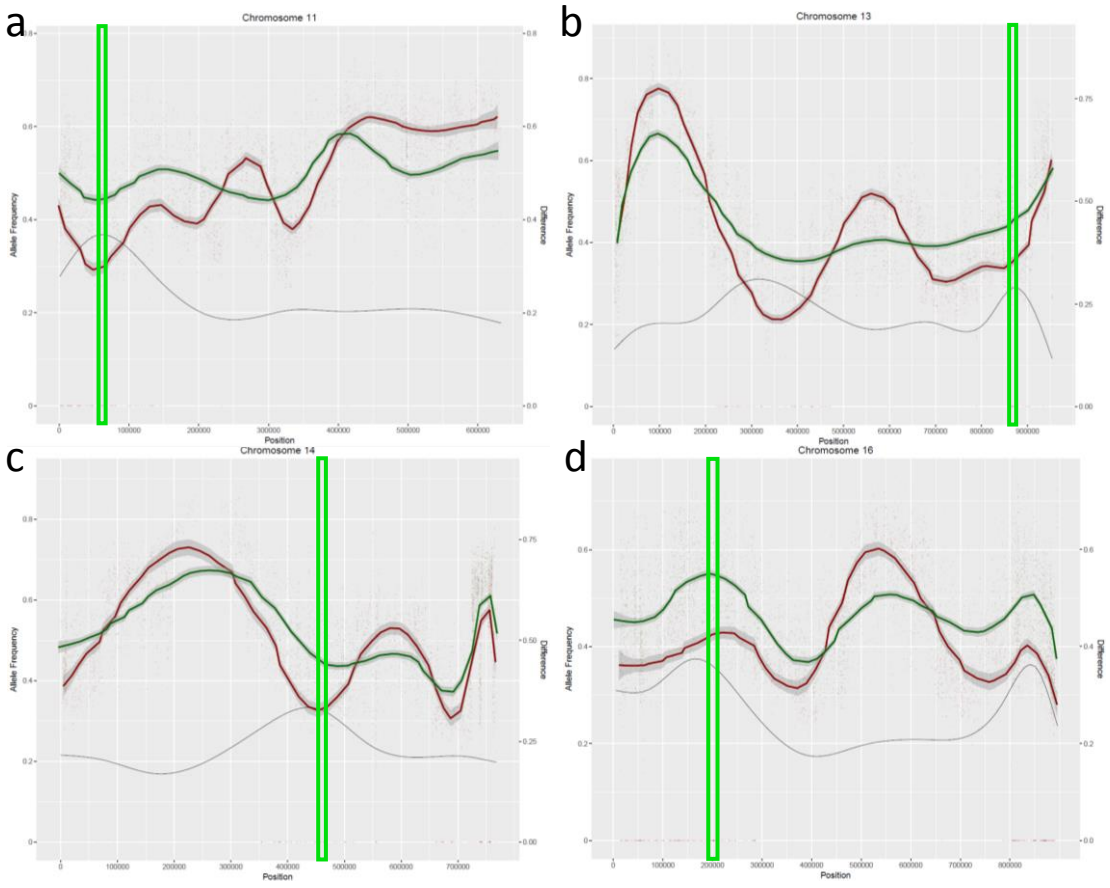


Figure 8.1.9 Intervals Identified within the *S. eubayanus* Portion of H179 Continued

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a-d) Intervals that confer a selection advantage on maltose rich media, on chromosome 11, 13, 14, and 16 respectively.

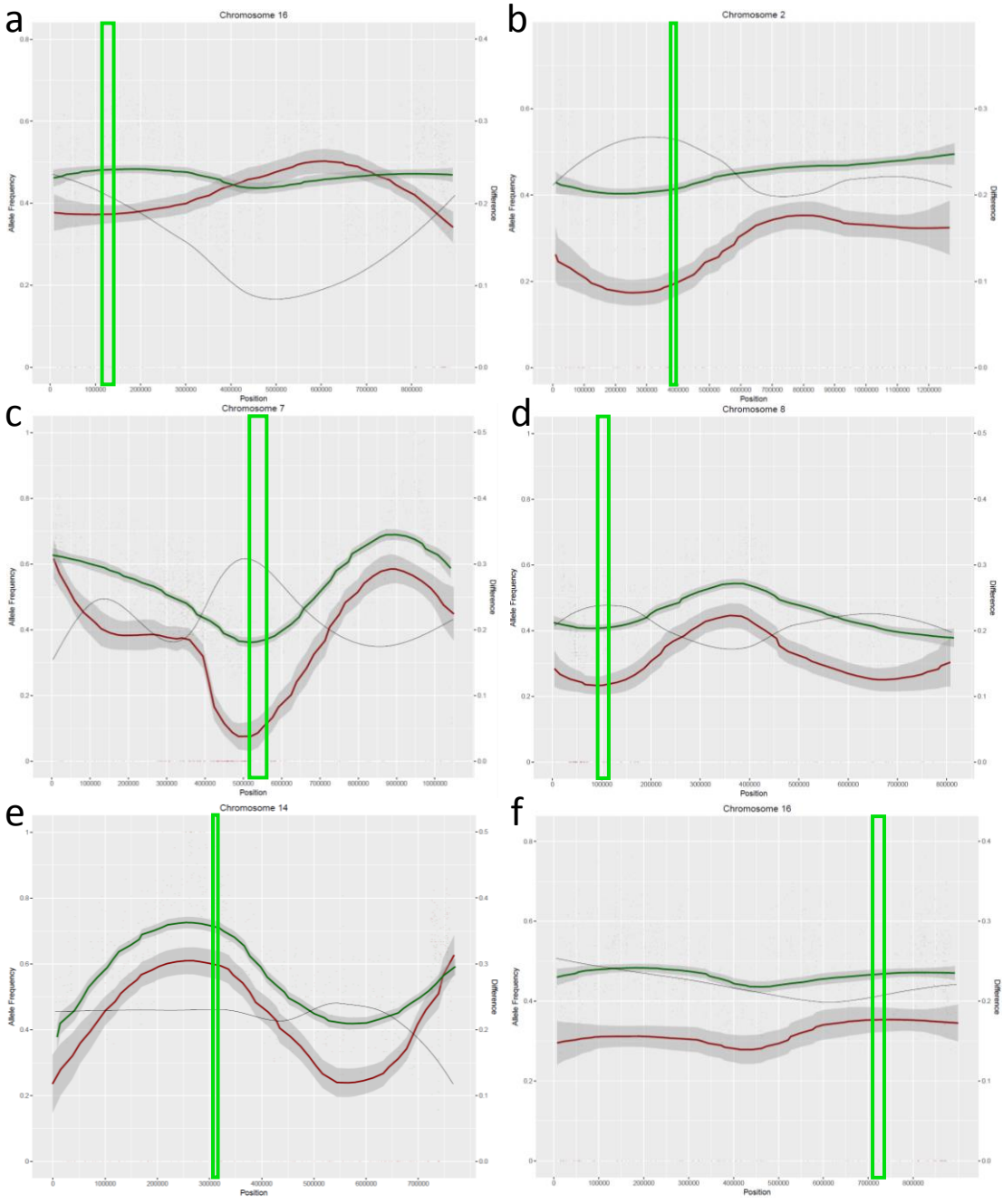


Figure 8.1.10 Intervals Identified within the *S. eubayanus* Portion of H188

QTL intervals were identified by the allele frequency difference (grey line) between the control pool (green line) and the selection pool (red line). Each interval is indicated by a green box. (a) Interval on chromosome 16 that confers a selection advantage at low temperature. (b-f) Intervals that confer a selection advantage on maltose rich media, on chromosome 2, 7, 8, 14, and 16 respectively.

8.2 B.1 Omnilog Phenotypic Analysis

Colour	Raw Values	Performance
	> 150	Very Strong
	130 – 150	Strong
	110 – 130	Moderate
	90 – 110	Weak
	60 – 90	Poor
	< 60	Very poor

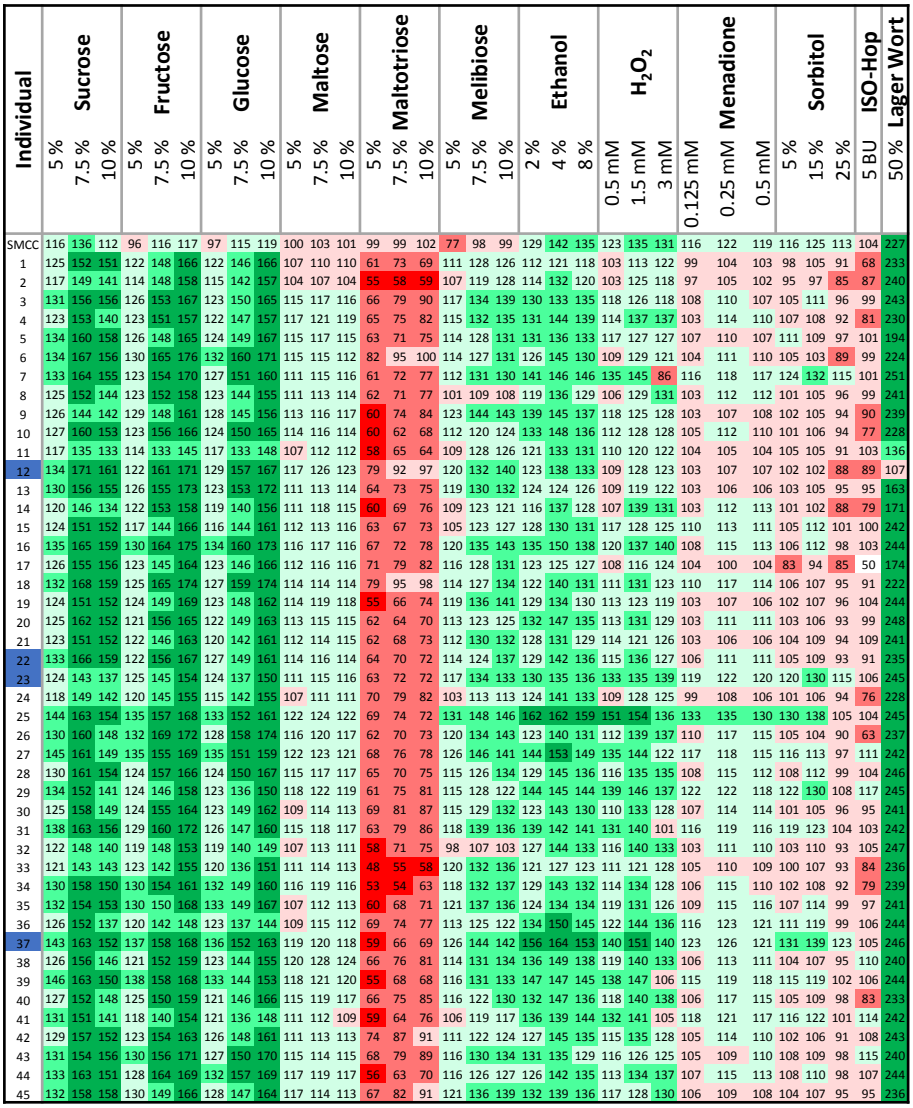
Figure 8.2.1 Colour Code for Heat Maps

The heat maps below are colour coded according to this reference table. The higher the value the stronger the metabolic activity. Raw values, which have been meaned, of over 150 are in dark green. Values of between 130 and 150 are light green. Values of between 110 and 130 are in pale green. Below 110 metabolic activity is considered less than desirable. Values between 90 and 110 are in pale red. Values between 60 and 90 are in light red. Finally values below 60 are in deep red.

Species	Individual	Sucrose			Fructose			Glucose			Maltose			Maltotriose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
		5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
S. pastorianus	SMCC	164	181	166	158	162	153	160	165	156	158	157	154	154	174	153	131	150	144	138	141	138	130	109	11	122	123	84	132	126	122	107	122
S. cerevisiae	OS3	166	187	178	147	160	165	146	161	161	144	145	145	74	126	115	139	148	138	133	139	98	122	134	126	114	120	53	121	121	106	107	129
	OS60	168	198	214	164	188	191	164	186	187	143	145	144	89	106	108	143	156	152	131	137	134	110	129	126	107	93	73	117	112	99	100	125
	OS104	169	199	200	157	183	188	159	183	187	141	144	145	86	103	107	141	151	148	129	137	133	104	129	125	99	37	84	119	114	101	99	115
	OS278	171	214	220	141	169	185	142	168	175	140	140	140	93	111	107	136	146	141	127	131	131	115	126	123	105	87	64	117	115	100	100	111
S. uvarum	OS274	168	182	187	162	198	206	166	200	208	149	156	149	133	154	144	57	62	59	144	143	147	129	141	136	111	59	97	126	110	101	102	140
	OS388	160	171	163	142	151	156	143	153	151	140	145	142	101	123	120	65	62	59	120	138	133	122	130	126	97	73	87	117	123	114	106	121
	OS449	179	206	223	173	203	215	167	206	214	148	153	145	93	104	102	156	172	172	129	134	132	119	129	125	107	105	63	116	109	101	94	114
S. eubayanus	OS578	160	190	195	145	164	164	153	169	170	143	142	141	90	109	113	135	148	144	133	138	138	117	126	123	102	40	90	119	114	106	104	125
	OS626	114	122	124	117	128	130	114	128	128	106	113	108	61	68	69	106	123	114	100	100	110	97	105	104	87	57	94	97	93	89	98	127
	OS627	191	199	203	162	183	187	165	186	187	143	145	144	113	132	132	143	157	151	135	143	140	133	135	133	117	52	99	136	130	111	117	137
S. pastorianus	OS320	163	171	164	143	153	159	143	157	159	142	138	140	76	90	88	54	53	48	131	137	132	125	129	123	108	93	45	123	121	116	93	112

Figure 8.2.2 Parental Strains

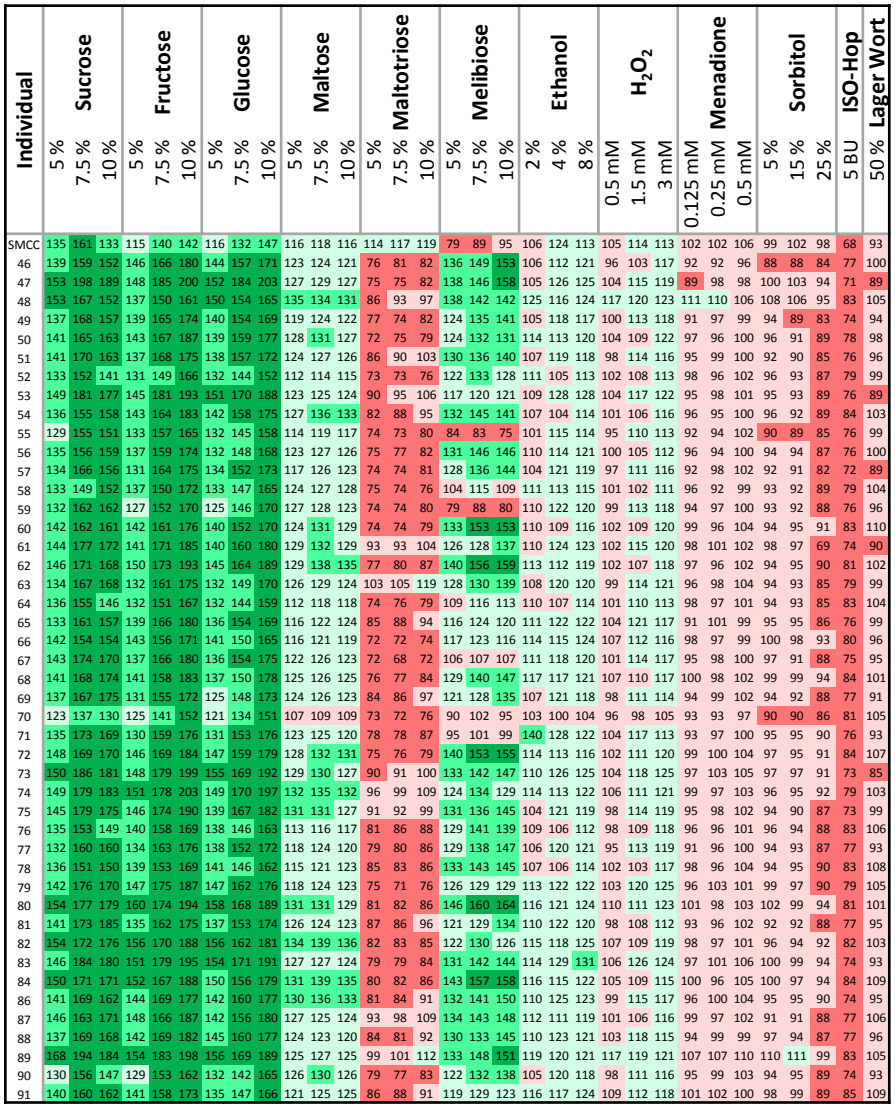
Heat map displaying the raw recorded values of the parental strains. Values for each strain under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Maltotriose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	116	136	112	96	116	117	97	115	119	100	103	101	99	99	102	77	98	99	129	142	135	123	135	131	116	122	119	116	125	113	104	227
1	125	152	151	122	148	166	122	146	166	107	110	110	61	73	69	111	128	126	112	121	118	103	113	122	99	104	103	98	105	91	68	233
2	117	149	141	114	148	158	115	142	157	104	107	104	55	58	59	107	119	128	114	132	120	103	125	118	97	105	102	95	97	85	87	240
3	131	156	156	126	153	167	123	150	165	115	117	116	66	79	90	117	134	139	130	133	135	118	126	118	108	110	107	105	111	96	99	243
4	123	153	140	123	151	157	122	147	157	117	121	119	65	75	82	115	132	135	131	144	139	114	137	137	103	114	110	107	108	92	81	230
5	134	160	158	126	148	165	124	149	167	115	117	115	63	71	75	114	128	131	131	136	133	117	127	127	107	110	107	111	109	97	101	194
6	134	167	156	130	165	176	132	160	171	115	115	112	82	95	100	114	127	131	126	145	130	109	129	121	104	111	110	105	103	89	99	224
7	133	164	155	123	154	170	127	151	160	111	115	116	61	72	77	112	131	130	141	146	146	135	145	86	116	118	117	124	132	115	101	251
8	125	152	144	123	152	158	123	144	155	111	113	114	62	71	77	101	109	108	119	136	129	106	129	131	103	112	112	101	105	96	99	241
9	126	144	142	129	148	161	128	145	156	113	116	117	60	74	84	123	144	143	139	145	137	118	125	128	103	107	108	102	105	94	90	239
10	127	160	153	123	156	166	124	150	165	114	116	114	60	62	68	112	120	124	133	148	136	112	128	128	105	112	110	101	106	94	77	228
11	117	135	133	114	133	145	117	133	148	107	112	112	58	65	64	109	128	126	121	133	131	110	120	122	104	105	104	105	105	91	103	136
12	134	171	161	122	161	171	129	157	167	117	126	123	79	92	97	120	132	140	123	138	133	109	128	123	103	107	107	102	102	88	89	107
13	130	156	155	126	155	173	123	153	172	111	113	114	64	73	75	119	130	132	124	124	126	109	119	122	103	106	106	103	105	95	95	163
14	120	146	134	122	153	158	119	140	156	111	118	115	60	69	76	109	123	121	116	137	128	107	139	131	103	112	113	101	102	88	79	171
15	124	151	152	117	144	166	116	144	161	112	113	116	63	67	73	105	123	127	128	130	131	117	128	125	110	113	111	105	112	101	100	242
16	135	165	159	130	164	175	134	160	173	116	117	116	67	72	78	120	135	143	135	150	138	120	137	140	108	115	113	106	112	98	103	244
17	126	155	156	123	145	164	123	146	166	112	116	116	71	79	82	116	128	131	123	125	127	108	116	124	104	100	104	83	94	85	50	174
18	132	168	159	125	165	174	127	159	174	114	114	114	79	95	98	114	127	134	122	140	131	111	131	123	110	117	114	106	107	95	91	222
19	124	151	152	124	149	169	123	148	162	114	119	118	55	66	74	119	136	141	129	134	130	113	123	119	103	107	106	102	107	96	104	244
20	125	162	152	121	156	165	122	149	163	113	115	115	62	64	70	113	123	125	132	147	135	113	131	129	103	111	111	103	106	93	99	248
21	123	151	152	122	146	163	120	142	161	112	114	115	62	68	73	112	130	132	128	131	129	114	121	126	103	106	106	104	109	94	109	241
22	133	166	159	122	156	167	127	149	161	114	116	114	64	70	72	114	124	137	129	142	136	115	136	127	106	111	111	105	109	93	91	235
23	124	143	137	125	145	154	124	137	150	111	115	116	63	72	72	117	134	133	130	135	136	133	135	139	119	122	120	120	130	115	106	245
24	118	149	142	120	145	155	115	142	155	107	111	111	70	79	82	103	113	113	124	141	133	109	128	125	99	108	106	101	106	94	76	228
25	144	163	154	135	157	168	133	152	161	122	124	122	69	74	72	131	148	146	162	162	159	151	154	136	133	135	130	130	138	105	104	245
26	130	160	148	132	169	172	128	158	174	116	120	117	62	70	73	120	134	143	123	140	131	112	139	137	110	117	115	105	104	90	63	237
27	145	161	149	135	155	169	135	151	159	122	123	121	68	76	78	126	146	141	144	153	149	135	144	122	117	118	115	116	113	97	111	242
28	130	161	154	124	157	166	124	150	167	115	117	117	65	70	75	115	126	134	129	145	136	116	135	135	108	115	112	108	112	99	104	246
29	134	152	141	124	146	158	123	136	150	118	122	119	61	75	81	115	128	122	144	145	144	139	146	137	122	122	118	122	130	108	117	245
30	125	158	149	124	155	164	123	149	162	109	114	113	69	81	87	115	129	132	123	143	130	110	133	128	107	114	114	101	105	96	95	241
31	138	163	156	129	160	172	126	147	160	115	118	117	63	79	86	118	139	136	139	142	141	131	140	101	116	119	116	119	123	104	103	242
32	122	148	140	119	148	153	119	140	149	107	113	111	58	71	75	98	107	103	127	144	133	116	140	133	103	111	110	103	110	93	105	247
33	121	143	143	123	142	155	120	136	151	111	114	113	48	55	58	120	132	136	121	127	123	111	121	128	105	110	109	100	107	93	84	236
34	130	158	150	130	154	161	132	149	160	116	119	116	53	54	63	118	132	137	129	143	132	114	134	128	106	115	110	102	108	92	79	239
35	132	154	153	130	150	168	133	149	167	107	112	113	60	68	71	121	137	136	124	134	134	119	131	126	109	115	116	107	114	99	97	241
36	126	152	137	120	142	148	123	137	144	109	115	112	69	74	77	113	125	122	134	150	145	122	144	136	116	123	121	111	119	99	106	244
37	143	163	152	137	158	168	136	152	163	119	120	118	59	66	69	126	144	142	156	164	153	140	151	140	123	126	121	131	139	123	105	246
38	126	156	146	121	152	159	123	144	155	120	128	124	66	76	81	114	131	134	136	149	138	119	140	133	106	113	111	104	107	95	110	240
39	146	163	150	138	158	168	133	144	153	118	121	120	55	68	68	116	131	133	147	147	145	138	147	106	115	119	118	115	119	102	106	244
40	127	152	148	125	150	159	121	146	166	115	119	117	66	75	85	116	122	130	132	147	136	118	140	138	106	117	115	105	109	98	83	233
41	131	151	141	118	140	154	121	136	148	111	112	109	59	64	76	106	119	117	136	139	144	132	141	105	118	121	117	116	122	101	114	242
42	129	157	152	123	154	163	126	148	161	111	113	113	74	87	91	111	122	124	127	145	135	115	135	128	105	114	110	102	106	91	108	243
43	131	154	156	130	156	171	127	150	170	115	114	115	68	79	89	116	130	134	131	135	129	116	126	125	105	109	110	108	109	98	115	240
44	133	163	151	128	164	169	132	157	169	117	119	117	56	63	70	116	126	127	126	142	135	113	134	137	107	115	113	108	110	98	107	244
45	132	158	158	130	149	166	128	147	164	117	114	113	67	82	91	121	136	139	132	139	136	117	128	130	106	109	108	104	107	95	95	236

Figure 8.2.3 H51.1 - H51.45

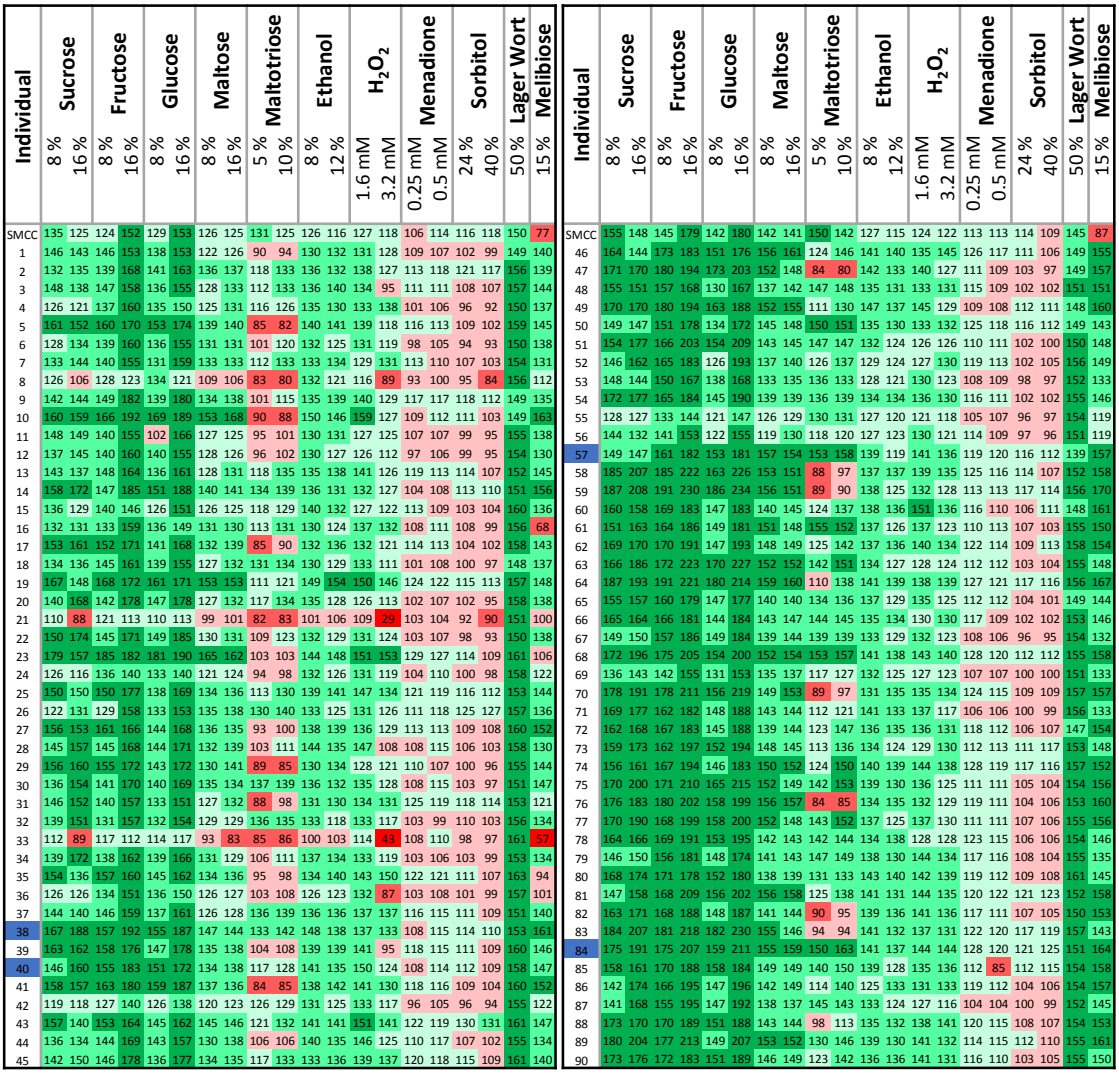
Heat map displaying the raw recorded values of the F12 individuals 1 to 45 of H51. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Maltotriose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	135	161	133	115	140	142	116	132	147	116	118	116	114	117	119	79	89	95	106	124	113	105	114	113	102	102	106	99	102	98	68	93
46	139	159	152	146	166	180	144	157	171	123	124	121	76	81	82	136	149	153	106	112	121	96	103	117	92	92	96	88	88	84	77	100
47	153	198	189	148	185	200	152	184	203	127	129	127	75	75	82	138	146	158	105	126	125	104	115	119	89	98	98	100	103	94	71	89
48	153	167	152	137	150	161	150	154	165	135	134	131	86	93	97	138	142	142	125	116	124	117	120	123	111	110	106	108	106	95	83	105
49	137	168	157	139	165	174	140	154	169	119	124	122	77	74	82	124	135	141	105	118	117	100	113	118	91	97	99	94	89	83	74	94
50	141	165	163	143	167	187	139	159	177	128	131	127	72	75	79	124	132	131	114	113	120	104	109	122	97	96	100	96	91	89	78	98
51	141	170	163	137	168	175	138	157	172	124	127	126	86	90	103	130	136	140	107	119	118	98	114	116	95	99	100	92	90	85	76	96
52	133	152	141	131	149	166	132	144	152	112	114	115	73	73	76	122	133	128	111	105	113	102	108	113	98	96	102	96	93	87	79	99
53	149	181	177	145	181	193	151	170	188	123	125	124	90	95	106	117	120	121	109	128	128	104	117	122	95	98	101	95	93	89	76	89
54	136	155	158	143	164	183	142	158	175	127	136	133	82	88	95	132	145	141	107	104	114	101	106	116	96	95	100	96	92	89	84	103
55	129	155	151	133	157	165	132	145	158	114	119	117	74	73	80	84	83	75	101	115	114	95	110	113	92	94	102	90	89	85	76	99
56	135	156	159	137	159	174	132	148	168	123	127	126	75	77	82	131	146	146	110	114	121	100	105	112	96	94	100	94	94	87	76	100
57	134	166	156	131	164	175	134	152	173	117	126	123	74	74	81	128	136	144	104	121	119	97	111	116	92	98	102	92	91	82	72	89
58	133	149	152	137	150	172	133	147	165	124	127	128	75	74	76	104	115	109	111	113	115	101	102	111	96	92	99	93	92	89	79	104
59	132	162	162	127	152	170	125	146	170	127	128	123	74	74	80	79	88	80	110	122	120	99	113	118	94	97	100	93	92	88	76	96
60	142	162	161	142	161	176	140	152	170	124	131	129	74	74	79	133	153	153	110	109	116	102	109	120	99	96	104	94	95	91	83	110
61	144	177	172	141	171	185	140	160	180	129	132	129	93	93	104	126	128	137	110	124	123	102	115	120	98	101	102	98	97	69	74	90
62	146	171	168	150	173	193	145	164	189	129	138	135	77	80	87	140	156	159	113	112	119	102	107	118	97	96	102	94	95	90	81	102
63	134	167	168	132	161	175	132	149	170	126	129	124	103	105	119	128	130	139	108	120	120	99	114	121	96	98	104	94	93	85	79	99
64	136	155	146	132	151	167	132	144	159	112	118	118	74	76	79	109	116	113	110	107	114	101	110	113	98	97	101	94	93	85	83	104
65	133	161	157	139	166	180	136	154	169	116	122	124	85	88	94	116	124	120	111	122	122	104	121	117	91	101	99	95	95	86	76	99
66	142	154	154	143	156	171	141	150	165	116	121	119	72	72	74	117	123	116	114	115	124	107	112	116	98	97	99	100	98	93	80	96
67	143	174	170	137	166	180	136	154	175	122	126	123	72	68	72	106	107	107	111	118	120	101	114	117	95	98	100	97	91	88	75	95
68	141	168	174	141	158	183	137	150	178	125	126	125	76	77	84	129	140	147	117	117	121	107	110	117	100	98	102	99	99	94	84	101
69	137	167	175	131	155	172	125	148	173	124	126	123	84	86	97	121	128	135	107	121	118	98	111	114	94	99	102	94	92	88	77	91
70	123	137	130	125	141	152	121	134	151	107	109	109	73	72	76	90	102	95	103	100	104	96	98	105	93	93	97	90	90	86	81	105
71	135	173	169	130	159	176	131	153	176	123	125	120	78	78	87	95	101	99	140	128	122	104	117	113	93	97	100	95	95	90	76	93
72	148	169	170	146	169	184	147	159	179	128	132	131	75	76	79	140	153	155	114	113	116	102	111	120	99	100	104	97	95	91	84	107
73	150	186	181	148	179	199	155	169	192	129	130	127	90	91	100	133	142	147	110	126	125	104	118	125	97	103	105	97	97	91	73	85
74	149	179	183	151	178	203	149	170	197	132	135	132	96	99	109	124	134	129	114	113	122	106	111	121	99	97	103	96	95	92	79	103
75	145	179	175	146	174	190	139	167	182	131	131	127	91	92	99	131	136	145	104	121	119	98	114	119	95	98	102	94	90	87	73	99
76	135	153	149	140	158	169	138	146	163	113	116	117	81	86	88	129	141	139	109	106	112	98	109	118	96	96	101	96	94	88	83	106
77	132	160	160	134	163	176	138	152	172	118	124	120	79	80	86	129	138	147	106	120	121	95	113	119	91	96	100	94	93	87	77	93
78	136	151	150	139	153	169	141	146	162	115	121	123	85	83	86	133	143	145	107	106	114	102	103	117	98	96	104	94	95	90	83	108
79	142	176	170	147	175	187	147	162	176	118	124	123	75	71	76	126	129	129	113	122	122	103	120	125	96	103	101	99	97	90	79	105
80	154	177	179	160	174	194	158	168	189	131	131	129	81	82	86	146	160	164	116	121	124	110	111	123	101	98	103	102	99	94	81	101
81	141	173	185	135	162	175	137	153	174	126	124	123	87	86	96	121	129	134	110	122	120	98	108	112	93	96	102	92	92	88	77	95
82	154	172	176	156	170	188	156	162	181	134	139	136	82	83	85	122	130	126	115	118	125	107	109	119	98	97	101	96	94	92	82	103
83	146	184	180	151	179	195	154	171	191	127	127	124	79	79	84	131	142	144	114	129	131	106	126	124	97	101	106	100	99	94	74	93
84	150	171	171	152	167	188	150	156	179	131	139	135	80	82	86	143	157	158	116	115	122	105	109	115	100	96	105	100	97	94	84	109
86	141	169	162	144	169	177	142	160	177	130	136	133	81	84	91	132	141	150	110	125	123	99	115	117	96	100	104	95	95	90	74	95
87	146	163	171	148	166	187	142	156	180	127	125	124	93	98	109	134	143	148	112	111	119	101	106	116	99	97	102	91	91	88	77	106
88	137	169	168	142	169	182	145	160	177	124	123	120	84	81	92	130	133	145	110	123	121	103	118	115	94	99	99	97	94	87	77	96
89	168	194	184	154	183	198	156	169	189	125	127	125	99	101	112	133	148	151	119	120	121	117	119	121	107	107	110	110	111	99	83	105
90	130	156	147	129	153	162	132	142	165	126	130	126	79	77	83	122	132	138	105	120	118	98	111	116	95	99	103	94	95	89	74	93
91	140	160	162	141	158	173	135	147	166	121	125	125	86	88	91	119	129	123	116	117	124	109	112	118	101	102	100	98	99	89	85	109

Figure 8.2.4 H51.1 - H51.45

Heat map displaying the raw recorded values of the F12 individuals 46 to 91 of H51. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).

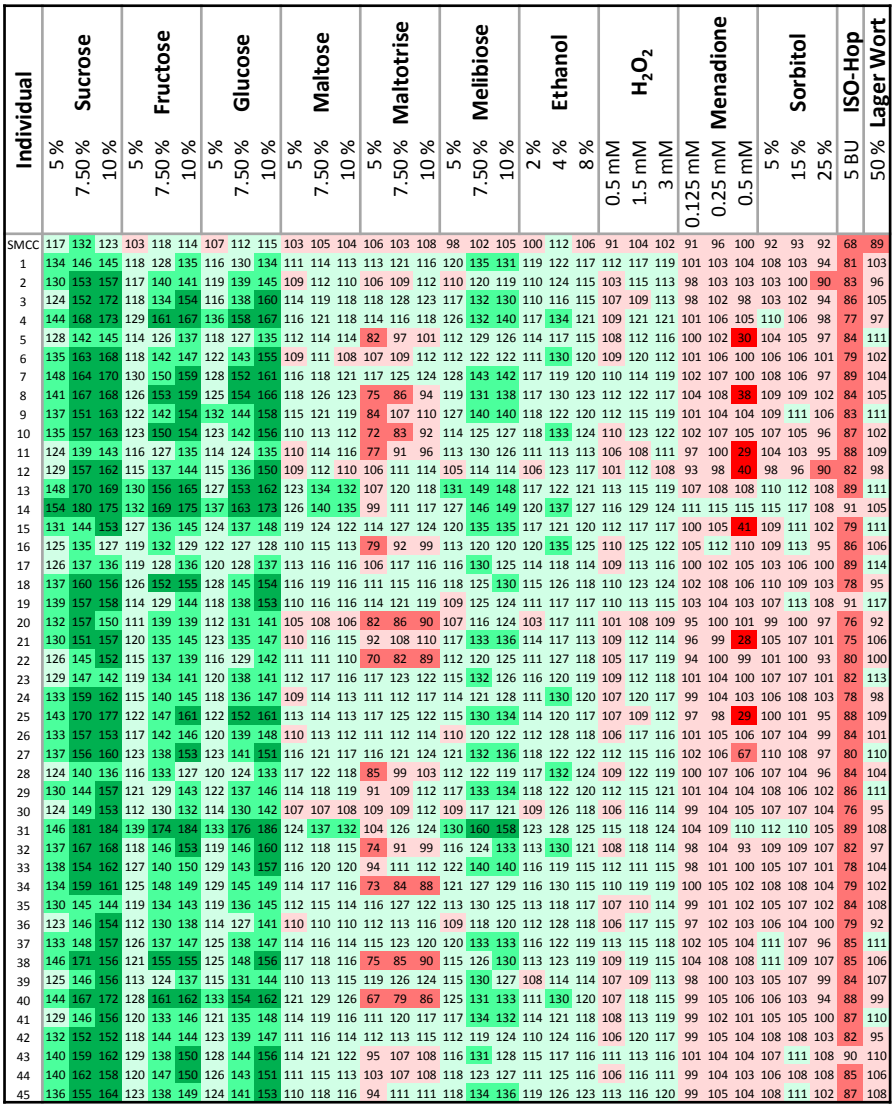


Individual	Sucrose		Fructose		Glucose		Maltose		Maltotriose		Ethanol		H_2O_2		Menadione		Sorbitol		Lager Wort	Melibiose
	8 %	16 %	8 %	16 %	8 %	16 %	8 %	16 %	5 %	10 %	8 %	12 %	1.6 mM	3.2 mM	0.25 mM	0.5 mM	24 %	40 %	50 %	15 %
SMCC	135	125	124	152	129	153	126	125	131	125	126	116	127	118	106	114	116	118	150	77
1	146	143	146	153	138	153	122	126	90	94	130	132	131	128	109	107	102	99	149	140
2	132	135	139	168	141	163	136	137	118	133	136	132	138	127	113	118	121	117	156	139
3	148	138	147	158	136	155	128	133	112	133	136	140	134	95	111	111	108	107	157	144
4	126	121	137	160	135	150	125	131	116	126	135	130	133	138	101	106	96	92	150	137
5	161	152	160	170	153	174	139	140	85	82	140	141	139	118	116	113	109	102	159	145
6	128	134	139	160	136	155	131	131	101	120	132	125	131	119	98	105	94	93	150	138
7	133	144	140	155	131	159	133	133	112	133	133	134	129	131	113	110	107	103	154	131
8	126	106	128	123	134	121	109	106	83	80	132	121	116	89	93	100	95	84	156	112
9	142	144	149	182	139	180	134	138	101	115	135	139	140	129	117	117	118	112	149	135
10	160	159	166	192	169	189	153	168	90	88	150	146	159	127	109	112	111	103	149	163
11	148	149	140	155	102	166	127	125	95	101	130	131	127	125	107	107	99	95	155	138
12	137	145	140	160	140	155	128	126	96	102	130	127	126	112	97	106	99	95	154	130
13	143	137	148	164	136	161	128	131	118	135	135	138	141	126	119	113	114	107	152	145
14	158	172	147	185	151	188	140	141	134	139	136	131	132	127	104	108	113	110	151	156
15	136	129	140	146	126	151	126	125	118	129	140	132	127	122	113	109	103	104	160	136
16	132	131	133	159	136	149	131	130	113	131	130	124	137	132	108	111	108	99	156	68
17	153	161	152	171	141	168	132	139	85	90	132	136	132	121	114	113	104	102	158	143
18	134	136	145	161	139	155	127	132	131	134	130	129	133	111	101	108	100	97	148	137
19	167	148	168	172	161	171	153	153	111	121	149	154	150	146	124	122	115	113	157	148
20	140	168	142	178	147	178	127	132	117	134	135	128	126	113	102	107	102	95	158	138
21	110	88	121	113	110	113	99	101	82	83	101	106	109	29	103	104	92	90	151	100
22	150	174	145	171	149	185	130	131	109	123	132	129	131	124	103	107	98	93	150	138
23	179	157	185	182	181	190	165	162	103	103	144	148	151	153	129	127	114	109	161	106
24	126	116	136	140	133	140	121	124	94	98	132	126	131	119	104	110	100	98	158	122
25	150	150	150	177	138	169	134	136	113	130	139	141	147	134	121	119	116	112	153	144
26	122	131	129	158	133	153	135	138	130	140	133	125	131	126	111	118	125	127	157	136
27	156	153	161	166	144	168	136	135	93	100	138	139	136	129	113	113	109	108	160	152
28	145	157	145	168	144	171	132	139	103	111	144	135	147	108	108	115	106	103	158	130
29	156	160	155	172	143	172	130	141	89	85	130	134	128	121	110	107	100	96	155	144
30	136	154	141	170	140	169	135	134	137	139	136	132	135	128	108	115	103	97	151	147
31	146	152	140	157	133	151	127	132	88	98	131	130	134	131	125	119	118	114	153	121
32	139	151	131	157	132	154	129	129	136	135	133	118	133	117	103	99	110	103	156	134
33	112	89	117	112	114	117	93	83	85	86	100	103	114	43	108	110	98	97	161	57
34	139	172	138	162	139	166	131	129	106	111	137	134	133	119	103	106	103	99	153	134
35	154	136	157	160	145	162	134	136	95	98	134	140	143	150	122	121	111	107	163	94
36	126	126	134	151	136	150	126	127	103	108	126	123	132	87	103	108	101	99	157	101
37	144	140	146	159	137	161	126	128	136	139	136	136	137	137	116	115	111	109	151	140
38	167	188	157	192	155	187	147	144	133	142	148	138	137	133	108	115	114	110	153	161
39	163	162	158	176	147	178	135	138	104	108	139	139	141	95	118	115	111	109	160	146
40	146	160	155	183	151	172	134	138	117	128	141	135	150	124	108	114	112	109	158	147
41	158	157	163	180	159	187	137	136	84	85	138	142	141	130	118	116	109	104	160	152
42	119	118	127	140	126	138	120	123	126	129	131	125	133	117	96	105	96	94	155	122
43	157	140	153	164	145	162	145	146	121	132	141	141	151	141	122	119	130	131	161	147
44	136	134	144	169	143	157	130	138	106	106	140	135	146	125	110	117	107	102	155	134
45	142	150	146	178	136	177	134	135	117	133	133	136	139	137	120	118	115	109	161	140

Individual	Sucrose		Fructose		Glucose		Maltose		Maltotriose		Ethanol		H_2O_2		Menadione		Sorbitol		Lager Wort	Melibiose
	8 %	16 %	8 %	16 %	8 %	16 %	8 %	16 %	5 %	10 %	8 %	12 %	1.6 mM	3.2 mM	0.25 mM	0.5 mM	24 %	40 %	50 %	15 %
SMCC	155	148	145	179	142	180	142	141	150	142	127	115	124	122	113	113	114	109	145	87
46	164	144	173	183	151	176	156	161	124	146	141	140	135	145	126	117	111	106	149	155
47	171	170	180	194	173	203	152	148	84	80	142	133	140	127	111	109	103	97	149	157
48	155	151	157	168	130	167	137	142	147	148	135	131	133	131	115	109	102	102	151	151
49	170	170	180	194	163	188	152	155	111	130	147	137	145	129	109	108	112	111	148	160
50	149	147	151	178	134	172	145	148	150	151	135	130	133	132	125	118	116	112	149	143
51	154	177	166	203	154	209	143	145	147	147	132	124	126	126	110	111	102	100	150	148
52	146	162	165	183	126	193	137	140	126	137	129	124	127	130	119	113	102	105	156	149
53	148	144	150	167	138	168	133	135	136	133	128	121	130	123	108	109	98	97	152	133
54	172	177	165	184	145	190	139	139	136	139	134	134	136	130	116	111	102	102	155	146
55	128	127	133	144	121	147	126	129	130	131	127	120	121	118	105	107	96	97	154	119
56	144	132	141	153	122	155	119	130	118	120	127	123	130	121	114	109	97	96	151	119
57	149	147	161	182	153	181	157	154	153	158	139	119	141	136	119	120	116	112	139	157
58	185	207	185	222	163	226	153	151	88	97	137	137	139	135	125	116	114	107	152	158
59	187	208	191	230	186	234	156	151	89	90	138	125	132	128	113	113	117	114	156	170
60	160	158	169	183	147	183	140	145	124	137	138	136	151	136	116	110	106	111	148	161
61	151	163	164	186	149	181	151	148	155	152	137	126	137	123	110	113	107	103	155	150
62	169	170	170	191	147	193	148	149	125	142	137	136	140	134	122	114	109	113	158	154
63	166	186	172	223	170	227	152	152	142	151	134	127	128	124	112	112	103	104	155	148
64	187	193	191	221	180	214	159	160	110	138	141	139	138	139	127	121	117	116	156	167
65	155	157	160	179	147	177	140	140	134	136	137	129	135	125	112	112	104	101	149	144
66	165	164	166	181	144	184	143	147	144	145	135	134	130	130	117	109	102	102	153	146
67	149	150	157	186	149	184	139	144	139	139	133	129	132	123	108	106	96	95	154	132
68	172	196	175	205	154	200	152	154	153	157	141	138	143	140	128	120	112	112	155	158
69	136	143	142	155	131	153	135	137	117	127	132	125	127	123	107	107	100	100	151	133
70	178	191	178	211	156	219	149	153	89	97	131	135	135	134	124	115	109	109	157	157
71	169	177	162	182	148	188	143	144	112	121	141	133	137	117	106	106	100	99	156	133
72	162	168	167	183	145	188	139	144	123	147	136	135	136	131	118	112	106	107	147	154
73	159	173	162	197	152	194	148	145	113	136	134	124	129	130	112	113	111	117	153	148
74	156	161	167	194	146	183	150	152	124	150	140	139	144	138	128	119	117	116	157	152
75	170	200	171	210	165	215	152	149	142	153	139	130	136	125	111	111	105	104	154	156
76	176	183	180	202	158	199	156	157	84	85	134	135	132	129	119	111	104	106	153	160
77	170	190	168	199	158	200	152	148	143	152	137	125	137	130	111	111	107	106	155	156
78	164	166	169	191	153	195	142	143	142	144	134	138	128	128	123	115	106	106	154	146
79	146	150	156	181	148	174	141	143	147	149	138	130	144	134	117	116	108	104	155	135
80	168	174	171	178	152	180	138	139	131	133	143	140	142	139	119	112	109	108	161	145
81	147	158	168	209	156	202	156	158	125	138	141	131	144	135	120	122	121	123	152	158
82	163	171	168	188	148	187	141	144	90	95	139	136	141	136	117	111	107	105	150	153
83	184	207	181	218	182	230	155	146	94	94	141	132	137	131	122	120	117	119	157	143
84	175	191	175	207	159	211	155	159	150	163	141	137	144	144	128	120	121	125	151	164
85	158	161	170	188	158	184	149	149	140	150	139	128	135	136	112	85	112	115	154	158
86	142	174	166	195	147	196	142	149	114	140	125	133	131	133	119	112	104	106	154	157
87	141	168	155	195	147	192	138	137	145	143	133	124	127	116	104	104	100	99	152	145
88	173	170	170	189	151	188	143	144	98	113	135	132	138	141	120	115	108	107	154	153
89	180	204	177	213	149	207	153	152	130	146	139	130	141	132	114	115	112	110	155	161
90	173	176	172	183	151	189	146	149	123	142	136	136	141	131	116	110	103	105	155	150

Figure 8.2.5 H179.1 – H179.90

Heat map displaying the raw recorded values of the F12 individuals of H179; 1 to 45 (left) and 46 to 90 (right). Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Maltotrise			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	117	132	123	103	118	114	107	112	115	103	105	104	106	103	108	98	102	105	100	112	106	91	104	102	91	96	100	92	93	92	68	89
1	134	146	145	118	128	135	116	130	134	111	114	113	113	121	116	120	135	131	119	122	117	112	117	119	101	103	104	108	103	94	81	103
2	130	153	157	117	140	141	119	139	145	109	112	110	106	109	112	110	120	119	110	124	115	103	115	113	98	103	103	103	100	90	83	96
3	124	152	172	118	134	154	116	138	160	114	119	118	118	128	123	117	132	130	110	116	115	107	109	113	98	102	98	103	102	94	86	105
4	144	168	173	129	161	167	136	158	167	116	121	118	114	116	118	126	132	140	117	134	121	109	121	121	101	106	105	110	106	98	77	97
5	128	142	145	114	126	137	118	127	135	112	114	114	82	97	101	112	129	126	114	117	115	108	112	116	100	102	30	104	105	97	84	111
6	135	163	168	118	142	147	122	143	155	109	111	108	107	109	112	112	122	122	111	130	120	109	120	112	101	106	100	106	106	101	79	102
7	148	164	170	130	150	159	128	152	161	116	118	121	117	125	124	128	143	142	117	119	120	110	114	119	102	107	100	108	106	97	89	104
8	141	167	168	126	153	159	125	154	166	118	126	123	75	86	94	119	131	138	117	130	123	112	122	117	104	108	38	109	109	102	84	105
9	137	151	163	122	142	154	132	144	158	115	121	119	84	107	110	127	140	140	118	122	120	112	115	119	101	104	104	109	111	106	83	111
10	135	157	163	123	150	154	123	142	156	110	113	112	72	83	92	114	125	127	118	133	124	110	123	122	102	107	105	107	105	96	87	102
11	124	139	143	116	127	135	114	124	135	110	114	116	77	91	96	113	130	126	111	113	113	106	108	111	97	100	29	104	103	95	88	109
12	129	157	162	115	137	144	115	136	150	109	112	110	106	111	114	105	114	114	106	123	117	101	112	108	93	98	40	98	96	90	82	98
13	148	170	169	130	156	165	127	153	162	123	134	132	107	120	118	131	149	148	117	122	121	113	115	119	107	108	108	110	112	108	89	111
14	154	180	175	132	169	175	137	163	173	126	140	135	99	111	117	127	146	149	120	137	127	116	129	124	111	115	115	115	117	108	91	105
15	131	144	153	127	136	145	124	137	148	119	124	122	114	127	124	120	135	135	117	121	120	112	117	117	100	105	41	109	111	102	79	111
16	125	135	127	119	132	129	122	127	128	110	115	113	79	92	99	113	120	120	120	135	125	110	125	122	105	112	110	109	113	95	86	106
17	126	137	136	119	128	136	120	128	137	113	116	116	106	117	116	116	130	125	114	118	114	109	113	116	100	102	105	103	106	100	89	114
18	137	160	156	126	152	155	128	145	154	116	119	116	111	115	116	118	125	130	115	126	118	110	123	124	102	108	106	110	109	103	78	95
19	139	157	158	114	129	144	118	138	153	110	116	116	114	121	119	109	125	124	111	117	117	110	113	115	103	104	103	107	113	108	91	117
20	132	157	150	111	139	139	112	131	141	105	108	106	82	86	90	107	116	124	103	117	111	101	108	109	95	100	101	99	100	97	76	92
21	130	151	157	120	135	145	123	135	147	110	116	115	92	108	110	117	133	136	114	117	113	109	112	114	96	99	28	105	107	101	75	106
22	126	145	152	115	137	139	116	129	142	111	111	110	70	82	89	112	120	125	111	127	118	105	117	119	94	100	99	101	100	93	80	100
23	129	147	142	119	134	141	120	138	141	112	117	116	117	123	122	115	132	126	116	120	119	109	112	118	101	104	100	107	107	101	82	113
24	133	159	162	115	140	145	118	136	147	109	114	113	111	112	117	114	121	128	111	130	120	107	120	117	99	104	103	106	108	103	78	98
25	143	170	177	122	147	161	122	152	161	113	114	113	117	125	122	115	130	134	114	120	117	107	109	112	97	98	29	100	101	95	88	109
26	133	157	153	117	142	146	120	139	148	110	113	112	111	112	114	110	120	122	112	128	118	106	117	116	101	105	106	107	104	99	84	101
27	137	156	160	123	138	153	123	141	151	116	121	117	116	121	124	121	132	136	118	122	122	112	115	116	102	106	67	110	108	97	80	110
28	124	140	136	116	133	127	120	124	133	117	122	118	85	99	103	112	122	119	117	132	124	109	122	119	100	107	106	107	104	96	84	104
29	130	144	157	121	129	143	122	137	146	114	118	119	91	109	112	117	133	134	118	122	120	112	115	121	101	104	104	108	106	102	86	111
30	124	149	153	112	130	132	114	130	142	107	107	108	109	109	112	109	117	121	109	126	118	106	116	114	99	104	105	107	107	104	76	95
31	146	181	184	139	174	184	133	176	186	124	137	132	104	126	124	130	160	158	123	128	125	115	118	124	104	109	110	112	110	105	89	108
32	137	167	168	118	146	153	119	146	160	112	118	115	74	91	99	116	124	133	113	130	121	108	118	114	98	104	93	109	109	107	82	97
33	138	154	162	127	140	150	129	143	157	116	120	120	94	111	112	122	140	140	116	119	115	112	111	115	98	101	100	105	107	101	78	104
34	134	159	161	125	148	149	129	145	149	114	117	116	73	84	88	121	127	129	116	130	115	110	119	119	100	105	102	108	108	104	79	102
35	130	145	144	119	134	143	119	136	145	112	115	114	116	127	122	113	130	125	113	118	117	107	110	114	99	101	102	105	107	102	84	108
36	123	146	154	112	130	138	114	127	141	110	110	110	112	113	116	109	118	120	112	128	118	106	117	115	97	102	103	106	104	100	79	92
37	133	148	157	126	137	147	125	138	147	114	116	114	115	123	120	120	133	133	116	122	119	113	115	118	102	105	104	111	107	96	85	111
38	146	171	156	121	155	155	125	148	156	117	118	116	75	85	90	115	126	130	113	123	119	109	119	115	104	108	108	111	109	107	85	106
39	125	146	156	113	124	137	115	131	144	110	113	115	119	126	124	115	130	127	108	114	114	107	109	113	98	100	103	105	107	99	84	107
40	144	167	172	128	161	162	133	154	162	121	129	126	67	79	86	125	131	133	111	130	120	107	118	115	99	105	106	106	103	94	88	99
41	129	146	156	120	133	146	121	135	148	114	119	116	111	120	117	117	134	132	114	121	118	108	113	119	99	102	101	105	105	100	87	110
42	132	152	152	118	144	144	123	139	147	111	116	114	112	113	115	112	119	124	110	124	116	106	120	117	99	105	104	108	108	103	82	95
43	140	159	162	129	138	150	128	144	156	114	121	122	95	107	108	116	131	128	115	117	116	111	113	116	101	104	104	107	111	108	90	110
44	140	162	158	120	147	150	126	143	151	111	115	113	103	107	108	118	123	127	111	125	116	106	116	111	99	104	103	106	108	108	85	106
45	136	155	164	123	138	149	124	141	153	110	118	116	94	111	111	118	134	136	119	126	123	113	116	120	99	105	104	108	111	102	87	108

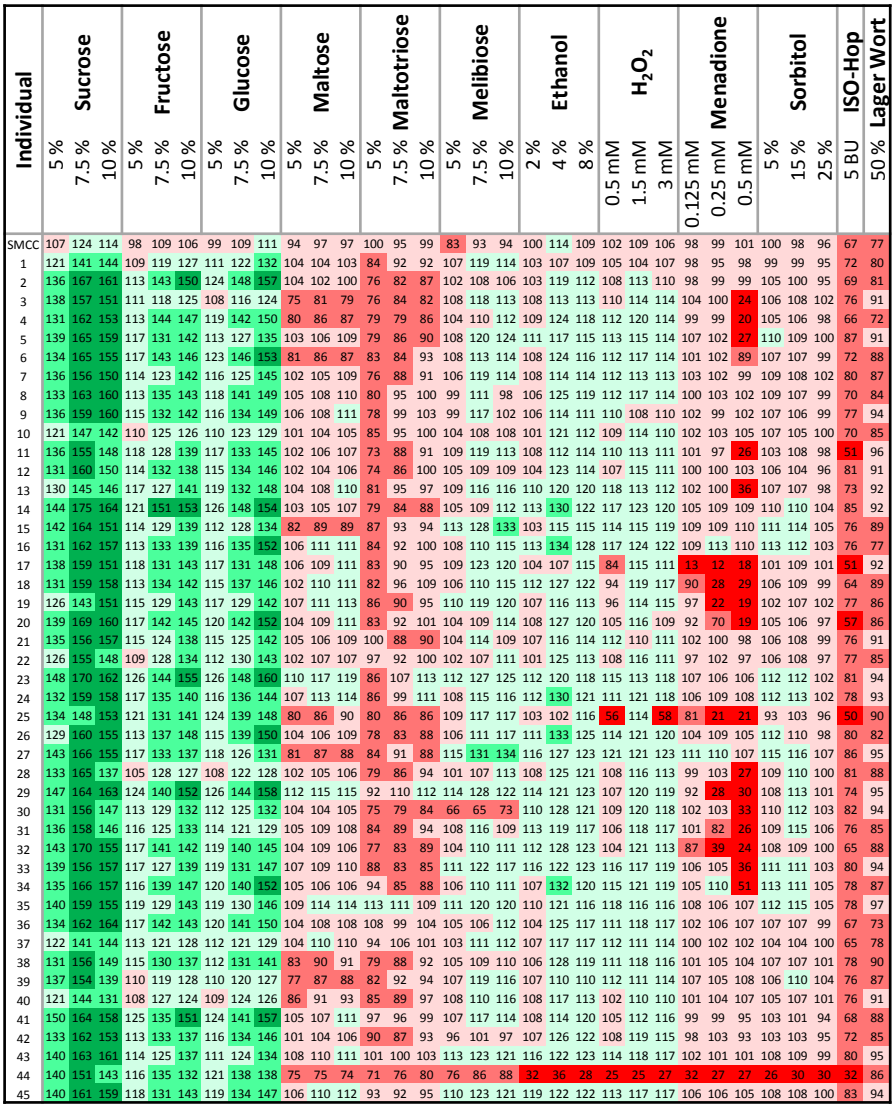
Figure 8.2.6 H188.1 – H188.45

Heat map displaying the raw recorded values of the F12 individuals of H188; 1 to 45. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).

Individual	Sucrose			Fructose			Glucose			Maltose			Maltotrise			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	5 %	7.50 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	99	109	106	96	102	100	93	97	98	96	94	95	95	95	99	57	65	71	104	115	107	98	105	102	93	93	38	95	93	95	69	80
46	116	119	122	112	115	120	111	116	120	103	106	108	57	72	76	107	117	113	114	118	116	110	113	116	97	100	103	103	105	102	78	95
47	112	130	122	104	117	115	104	126	126	99	103	101	51	62	72	100	101	104	108	121	112	104	111	110	96	102	98	98	90	92	77	79
48	122	134	133	117	126	132	117	131	136	104	109	110	91	104	104	111	119	114	111	117	116	108	111	117	97	99	103	102	100	96	77	90
49	127	143	134	121	142	137	118	133	135	111	114	111	73	87	92	106	109	111	117	130	120	108	120	117	101	107	40	103	99	89	68	86
50	113	121	116	112	115	116	110	119	121	101	104	107	92	102	101	104	114	111	118	117	118	105	111	116	97	100	26	102	102	95	81	90
51	113	131	122	109	121	116	113	125	125	83	102	74	83	90	70	97	100	103	111	125	115	107	113	116	96	101	102	102	101	97	76	85
52	124	132	132	118	126	130	115	128	133	106	113	110	98	110	109	112	122	120	121	120	123	111	117	123	104	106	109	108	105	95	82	95
53	131	152	144	118	136	135	120	137	138	105	111	109	97	105	111	105	111	115	113	127	119	110	117	112	101	104	38	105	106	98	77	84
54	136	150	143	122	134	137	117	134	139	106	108	109	59	76	80	107	117	118	112	114	115	107	108	109	103	103	106	104	108	105	82	97
55	121	144	138	111	132	131	111	127	130	103	106	105	48	64	75	101	108	110	116	131	123	112	123	117	104	107	97	109	108	104	77	96
56	124	133	133	117	126	129	119	133	132	100	110	110	75	90	95	77	82	74	115	118	118	108	110	114	100	99	88	103	102	95	83	107
57	125	144	135	117	140	139	118	141	143	103	110	108	98	107	110	105	108	112	111	128	118	108	116	116	98	102	103	102	101	98	77	81
58	124	131	134	119	129	132	120	129	136	106	109	111	86	103	107	109	121	117	117	122	120	114	112	119	102	104	94	105	107	104	76	91
59	111	138	109	117	132	129	122	135	134	107	112	111	71	87	91	105	110	112	117	129	119	111	118	119	99	105	101	104	103	97	71	84
60	119	124	121	118	116	120	113	115	119	104	109	112	87	102	104	112	119	117	117	121	121	111	115	120	103	105	102	106	107	100	81	103
61	123	145	138	115	131	129	114	131	136	109	110	110	80	92	99	103	106	111	117	133	122	111	121	119	104	106	110	109	109	101	74	88
62	122	130	126	116	123	124	118	129	129	107	109	111	103	115	112	95	103	102	118	117	122	107	110	85	106	95	96	107	108	102	73	99
63	121	129	120	115	122	118	115	118	116	93	93	92	52	60	69	95	92	93	121	132	124	112	124	122	105	110	108	108	105	97	78	94
64	114	131	130	107	114	124	107	117	126	104	105	108	70	90	91	103	110	111	110	113	114	106	107	108	99	101	105	102	104	105	85	95
65	122	143	135	112	136	133	115	134	140	103	105	105	90	98	95	101	104	108	111	126	117	106	114	112	101	103	103	102	101	97	79	87
66	134	141	139	123	133	138	124	141	142	109	116	115	99	113	111	109	120	116	113	116	114	108	109	113	100	102	103	103	102	94	83	95
67	131	143	133	125	140	134	126	138	134	114	119	115	47	62	71	82	84	85	116	129	118	110	123	122	101	108	103	106	104	97	77	97
68	118	127	126	110	115	123	111	118	126	104	105	110	59	75	81	87	95	95	111	115	113	109	109	114	102	102	48	103	107	103	84	108
69	135	153	151	123	144	144	124	147	149	109	111	110	100	107	113	107	113	116	116	132	119	113	122	119	103	106	107	110	106	99	80	83
70	123	130	136	119	128	134	117	132	139	107	111	112	88	106	106	112	122	118	119	122	121	112	116	120	101	102	91	108	109	106	75	94
71	119	140	138	114	133	133	116	133	140	106	108	107	67	88	95	105	111	115	115	131	121	110	118	118	99	105	102	102	104	101	77	83
72	119	135	133	113	125	132	111	128	132	102	109	111	52	63	66	112	118	116	115	116	116	112	113	118	104	106	107	106	110	108	87	105
73	117	132	123	115	132	127	113	129	128	111	114	111	62	74	84	104	109	110	116	129	122	109	119	118	99	105	104	103	101	98	72	85
74	122	134	135	117	133	137	115	135	142	106	107	108	98	113	110	106	118	115	114	117	116	110	111	119	103	105	107	103	102	95	84	93
75	115	129	123	113	121	115	109	116	115	101	100	97	65	78	85	95	97	102	119	129	122	108	121	120	103	107	67	104	107	99	73	92
76	118	134	139	110	126	135	112	130	137	103	107	108	106	113	111	106	116	113	109	114	113	104	107	116	100	101	106	98	102	103	79	87
77	117	129	123	111	127	123	111	123	121	100	109	105	46	64	74	99	104	105	116	128	122	107	121	122	100	105	106	106	108	101	82	101
78	133	143	144	118	128	133	117	130	136	103	108	111	51	63	66	116	121	118	114	120	115	109	113	117	102	104	106	104	104	98	85	99
79	138	149	136	135	148	138	140	148	141	118	119	113	77	92	102	115	118	118	126	137	126	116	129	129	117	119	116	114	107	97	76	84
80	116	122	119	109	112	116	109	115	120	102	108	111	57	70	73	107	118	114	112	115	115	108	110	116	103	103	105	105	108	105	88	100
81	130	148	149	124	142	143	121	138	146	110	112	112	85	100	110	108	114	116	117	131	120	110	119	122	103	107	109	107	108	101	78	88
82	124	137	140	118	129	135	119	136	141	110	111	114	91	108	105	112	121	118	116	121	117	112	112	120	99	104	105	103	108	105	78	93
83	121	141	139	115	131	132	115	131	134	108	108	108	68	88	96	106	111	113	117	131	120	111	120	120	101	104	107	107	105	102	77	85
84	124	138	137	118	123	133	116	133	138	102	108	110	49	65	72	106	117	116	115	117	118	108	110	121	103	102	107	104	105	103	83	96
85	128	142	133	122	134	127	124	131	129	113	112	112	95	106	112	109	116	117	121	135	126	116	124	125	108	111	113	111	112	107	80	95
86	117	126	127	112	122	122	107	121	124	100	105	105	99	110	108	75	82	81	117	116	116	107	111	116	100	103	107	101	103	96	88	104
87	118	143	136	110	130	128	109	129	132	103	105	103	93	99	103	99	104	108	113	125	118	107	118	117	99	105	105	104	102	98	77	82
88	122	136	140	117	128	134	116	135	140	103	108	108	53	68	72	110	121	120	124	127	126	118	119	126	105	107	110	111	113	110	92	103
89	115	126	121	111	120	117	112	116	116	100	102	102	96	103	108	102	104	108	120	126	125	102	122	112	112	95	97	112	110	101	47	97
90	123	133	139	117	129	135	114	132	139	103	109	113	90	106	103	108	119	117	127	126	130	116	121	123	102	108	107	109	107	102	86	103

Figure 8.2.7 H188.46 – H188.90

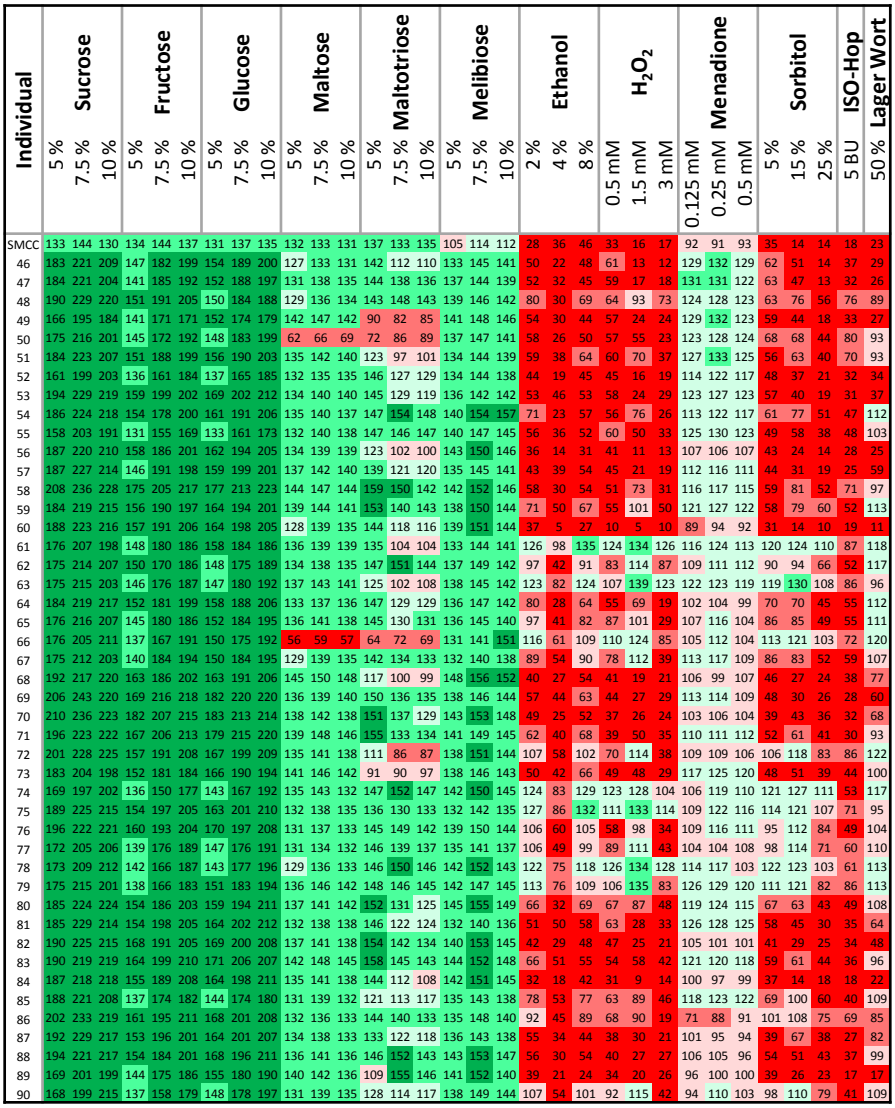
Heat map displaying the raw recorded values of the F12 individuals of H188; 46 to 90. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Maltotriose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	107	124	114	98	109	106	99	109	111	94	97	97	100	95	99	83	93	94	100	114	109	102	109	106	98	99	101	100	98	96	67	77
1	121	141	144	109	119	127	111	122	132	104	104	103	84	92	92	107	119	114	103	107	109	105	104	107	98	95	98	99	99	95	72	80
2	136	167	161	113	143	150	124	148	157	104	102	100	76	82	87	102	108	106	103	119	112	108	113	110	98	99	99	105	100	95	69	81
3	138	157	151	111	118	125	108	116	124	75	81	79	76	84	82	108	118	113	108	113	113	110	114	114	104	100	24	106	108	102	76	91
4	131	162	153	113	144	147	119	142	150	80	86	87	79	79	86	104	110	112	109	124	118	112	120	114	99	99	20	105	106	98	66	72
5	139	165	159	117	131	142	113	127	135	103	106	109	79	86	90	108	120	124	111	117	115	113	115	114	107	102	27	110	109	100	87	91
6	134	165	155	117	143	146	123	146	153	81	86	87	83	84	93	108	113	114	108	124	116	112	117	114	101	102	89	107	107	99	72	88
7	136	156	150	114	123	142	116	125	145	102	105	109	76	88	91	106	119	114	108	114	114	112	113	113	103	102	99	109	108	102	80	87
8	133	163	160	113	135	143	118	141	149	105	108	110	80	95	100	99	111	98	106	125	119	112	117	114	100	103	102	109	107	99	70	84
9	136	159	160	115	132	142	116	134	149	106	108	111	78	99	103	99	117	102	106	114	111	110	108	110	102	99	102	107	106	99	77	94
10	121	147	142	110	125	126	110	123	129	101	104	105	85	95	100	104	108	108	101	121	112	109	114	110	102	103	105	107	105	100	70	85
11	136	155	148	118	128	139	117	133	145	102	106	107	73	88	91	109	119	113	108	112	114	110	113	111	101	97	26	103	108	98	51	96
12	131	160	150	114	132	138	115	134	146	102	104	106	74	86	100	105	109	109	104	123	114	107	115	111	100	100	103	106	104	96	81	91
13	130	145	146	117	127	141	119	132	148	104	108	110	81	95	97	109	116	116	110	120	120	118	113	112	102	100	36	107	107	98	73	92
14	144	175	164	121	151	153	126	148	154	103	105	107	79	84	88	105	109	112	113	130	122	117	123	120	105	109	109	110	110	104	85	92
15	142	164	151	114	129	139	112	128	134	82	89	89	87	93	94	113	128	133	103	115	115	114	115	119	109	109	110	111	114	105	76	89
16	131	162	157	113	133	139	116	135	152	106	111	111	84	92	100	108	110	115	113	134	128	117	124	122	109	113	110	113	112	103	76	77
17	138	159	151	118	131	143	117	131	148	106	109	111	83	90	95	109	123	120	104	107	115	84	115	111	13	12	18	101	109	101	51	92
18	131	159	158	113	134	142	115	137	146	102	110	111	82	96	109	106	110	115	112	127	122	94	119	117	90	28	29	106	109	99	64	89
19	126	143	151	115	129	143	117	129	142	107	111	113	86	90	95	110	119	120	107	116	113	96	114	115	97	22	19	102	107	102	77	86
20	139	169	160	117	142	145	120	142	152	104	109	111	83	92	101	104	109	114	108	127	120	105	116	109	92	70	19	105	106	97	57	86
21	135	156	157	115	124	138	115	125	142	105	106	109	100	88	90	104	114	109	107	116	114	112	110	111	102	100	98	106	108	99	76	91
22	126	155	148	109	128	134	112	130	143	102	107	107	97	92	100	102	107	111	101	125	113	108	116	111	97	102	97	106	108	97	77	85
23	148	170	162	126	144	155	126	148	160	110	117	119	86	107	113	112	127	125	112	120	118	115	113	118	107	106	106	112	112	102	81	94
24	132	159	158	117	135	140	116	136	144	107	113	114	86	99	111	108	115	116	112	130	121	111	121	118	106	109	108	112	113	102	78	93
25	134	148	153	121	131	141	124	139	148	80	86	90	80	86	86	109	117	117	103	102	116	56	114	58	81	21	21	93	103	96	50	90
26	129	160	155	113	137	148	115	139	150	104	106	109	78	83	88	106	111	117	111	133	125	114	121	120	104	109	105	112	110	98	80	82
27	143	166	155	117	133	137	118	126	131	81	87	88	84	91	88	115	131	134	116	127	123	121	121	123	111	110	107	115	116	107	86	95
28	133	165	137	105	128	127	108	122	128	102	105	106	79	86	94	101	107	113	108	125	121	108	116	113	99	103	27	109	110	100	81	88
29	147	164	163	124	140	152	126	144	158	112	115	115	92	110	112	114	128	122	114	121	123	107	120	119	92	28	30	108	113	101	74	95
30	131	156	147	113	129	132	112	125	132	104	104	105	75	79	84	66	65	73	110	128	121	109	120	118	102	103	33	110	112	103	82	94
31	136	158	146	116	125	133	114	121	129	105	109	108	84	89	94	108	116	109	113	119	117	106	118	117	101	82	26	109	115	106	76	85
32	143	170	155	117	141	142	119	140	145	104	109	106	77	83	89	104	110	111	112	128	123	104	121	113	87	39	24	108	109	100	65	88
33	139	156	157	117	127	139	119	131	147	107	109	110	88	83	85	111	122	117	116	122	123	116	117	119	106	105	36	111	111	103	80	94
34	135	166	157	116	139	147	120	140	152	105	106	106	94	85	88	106	110	111	107	132	120	115	121	119	105	110	51	113	111	105	78	87
35	140	159	155	119	129	143	119	130	146	109	114	114	113	111	109	111	120	120	110	121	116	118	116	116	108	106	107	112	115	105	78	97
36	134	162	164	117	142	143	120	141	150	104	108	108	108	99	104	105	106	112	104	125	117	111	118	117	102	106	107	107	107	99	67	73
37	122	141	144	113	121	128	112	121	129	104	110	110	94	106	101	103	111	112	107	117	117	112	111	114	100	102	102	104	104	100	65	78
38	131	156	149	115	130	137	112	131	141	83	90	91	79	88	92	105	109	110	106	128	119	111	118	116	101	105	104	107	107	101	78	90
39	137	154	139	110	119	128	110	120	127	77	87	88	82	92	94	107	119	116	107	110	110	112	111	114	107	105	108	106	110	104	76	87
40	121	144	131	108	127	124	109	124	126	86	91	93	85	89	97	108	110	116	108	117	113	102	110	110	101	104	107	105	107	101	76	91
41	150	164	158	125	135	151	124	141	157	105	107	111	97	96	99	107	117	114	108	114	120	105	112	116	99	99	95	103	101	94	68	88
42	133	162	153	113	133	137	116	134	146	101	104	106	90	87	93	96	101	97	107	126	122	108	119	115	98	103	93	103	103	95	72	85
43	140	163	161	114	125	137	111	124	134	108	110	111	101	100	103	113	123	121	116	122	123	114	118	117	102	101	101	108	109	99	80	95
44	140	151	143	116	135	132	121	138	138	75	75	74	71	76	80	76	86	88	32	36	28	25	25	27	32	27	27	26	30	30	32	86
45	140	161	159	118	131	143	119	134	147	106	110	112	93	92	95	110	123	121	119	122	122	113	117	117	106	106	105	108	108	100	83	94

Figure 8.2.8 H230.1 -H230.45

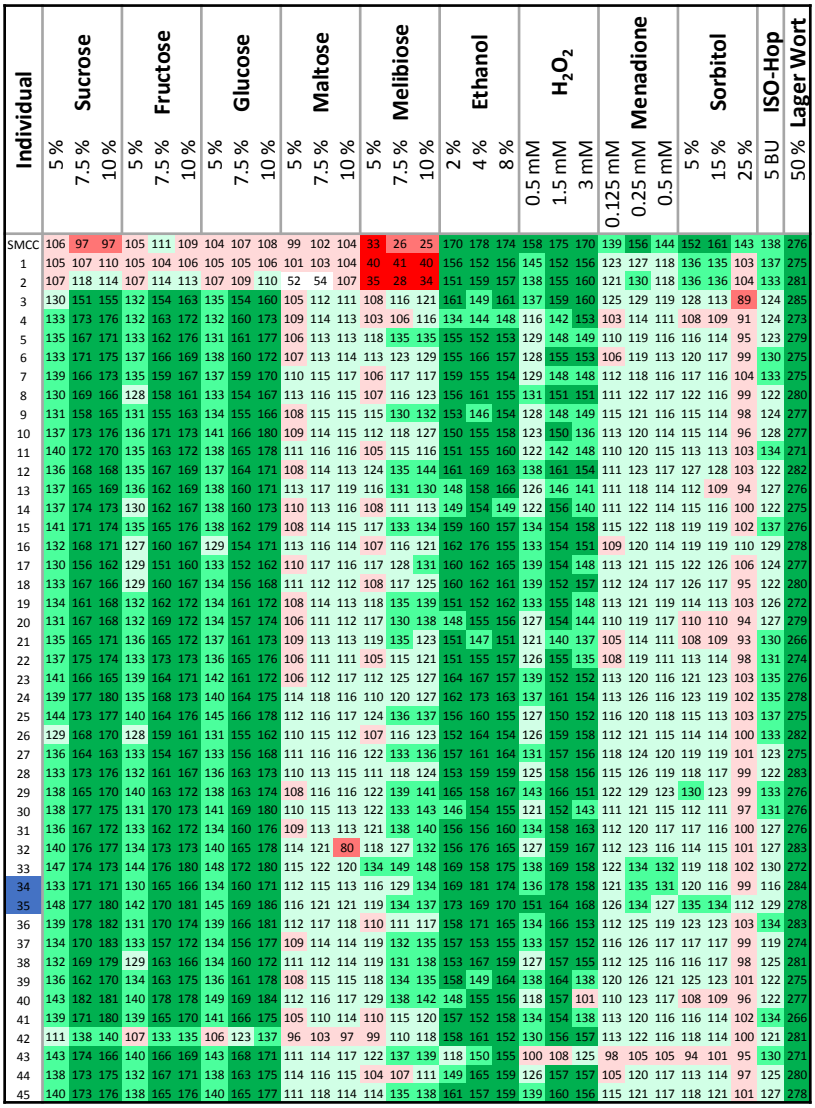
Heat map displaying the raw recorded values of the F12 individuals of H230; 1 to 45. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Maltotriose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	133	144	130	134	144	137	131	137	135	132	133	131	137	133	135	105	114	112	28	36	46	33	16	17	92	91	93	35	14	14	18	23
46	183	221	209	147	182	199	154	189	200	127	133	131	142	112	110	133	145	141	50	22	48	61	13	12	129	132	129	62	51	14	37	29
47	184	221	204	141	185	192	152	188	197	131	138	135	144	138	136	137	144	139	52	32	45	59	17	18	131	131	122	63	47	13	32	26
48	190	229	220	151	191	205	150	184	188	129	136	134	143	148	143	139	146	142	80	30	69	64	93	73	124	128	123	63	76	56	76	89
49	166	195	184	141	171	171	152	174	179	142	147	142	90	82	85	141	148	146	54	30	44	57	24	24	129	132	123	59	44	18	33	27
50	175	216	201	145	172	192	148	183	199	62	66	69	72	86	89	137	147	141	58	26	50	57	55	23	123	128	124	68	68	44	80	93
51	184	223	207	151	188	199	156	190	203	135	142	140	123	97	101	134	144	139	59	38	64	60	70	37	127	133	125	56	63	40	70	93
52	161	199	203	136	161	184	137	165	185	132	135	135	146	127	129	134	144	138	44	19	45	45	16	19	114	122	117	48	37	21	32	34
53	194	229	219	159	199	202	169	202	212	134	140	140	145	129	119	136	142	142	53	46	53	58	24	29	123	127	123	57	40	19	31	37
54	186	224	218	154	178	200	161	191	206	135	140	137	147	154	148	140	154	157	71	23	57	56	76	26	113	122	117	61	77	51	47	112
55	158	203	191	131	155	169	133	161	173	132	140	138	147	146	147	140	147	145	56	36	52	60	50	33	125	130	123	49	58	38	48	103
56	187	220	210	158	186	201	162	194	205	134	139	139	123	102	100	143	150	146	36	14	31	41	11	13	107	106	107	43	24	14	28	25
57	187	227	214	146	191	198	159	199	201	137	142	140	139	121	120	135	145	141	43	39	54	45	21	19	112	116	111	44	31	19	25	59
58	208	236	228	175	205	217	177	213	223	144	147	144	159	150	142	142	152	146	58	30	54	51	73	31	116	117	115	59	81	52	71	97
59	184	219	215	156	190	197	164	194	201	139	144	141	153	140	143	138	150	144	71	50	67	55	101	50	121	127	122	58	79	60	52	113
60	188	223	216	157	191	206	164	198	205	128	139	135	144	118	116	139	151	144	37	5	27	10	5	10	89	94	92	31	14	10	19	11
61	176	207	198	148	180	186	158	184	186	136	139	139	135	104	104	133	144	141	126	98	135	124	134	126	116	124	113	120	124	110	87	118
62	175	214	207	150	170	186	148	175	189	134	138	135	147	151	144	137	149	142	97	42	91	83	114	87	109	111	112	90	94	66	52	117
63	175	215	203	146	176	187	147	180	192	137	143	141	125	102	108	138	145	142	123	82	124	107	139	123	122	123	119	119	130	108	86	96
64	184	219	217	152	181	199	158	188	206	133	137	136	147	129	129	136	147	142	80	28	64	55	69	19	102	104	99	70	70	45	55	112
65	176	216	207	145	180	186	152	184	195	136	141	138	145	130	131	136	145	140	97	41	82	87	101	29	107	116	104	86	85	49	55	111
66	176	205	211	137	167	191	150	175	192	56	59	57	64	72	69	131	141	151	116	61	109	110	124	85	105	112	104	113	121	103	72	120
67	175	212	203	140	184	194	150	184	195	129	139	135	142	134	133	132	140	138	89	54	90	78	112	39	113	117	109	86	83	52	59	107
68	192	217	220	163	186	202	163	191	206	145	150	148	117	100	99	148	156	152	40	27	54	41	19	21	106	99	107	46	27	24	38	77
69	206	243	220	169	216	218	182	220	220	136	139	140	150	136	135	138	146	144	57	44	63	44	27	29	113	114	109	48	30	26	28	60
70	210	236	223	182	207	215	183	213	214	138	142	138	151	137	129	143	153	148	49	25	52	37	26	24	103	106	104	39	43	36	32	68
71	196	223	222	167	206	213	179	215	220	139	148	146	155	133	134	141	149	145	62	40	68	39	50	35	110	111	112	52	61	41	30	93
72	201	228	225	157	191	208	167	199	209	135	141	138	111	86	87	138	151	144	107	58	102	70	114	38	109	109	106	106	118	83	86	122
73	183	204	198	152	181	184	166	190	194	141	146	142	91	90	97	138	146	143	50	42	66	49	48	29	117	125	120	48	51	39	44	100
74	169	197	202	136	150	177	143	167	192	135	143	132	147	152	147	142	150	145	124	83	129	123	128	104	106	119	110	121	127	111	53	117
75	189	225	215	154	197	205	163	201	210	132	138	135	136	130	133	132	142	135	127	86	132	111	133	114	109	122	116	114	121	107	71	95
76	196	222	221	160	193	204	170	197	208	131	137	133	145	149	142	139	150	144	106	60	105	58	98	34	109	116	111	95	112	84	49	104
77	172	205	206	139	176	189	147	176	191	131	134	132	146	139	137	135	141	137	106	49	99	89	111	43	104	104	108	98	114	71	60	110
78	173	209	212	142	166	187	143	177	196	129	136	133	146	150	146	142	152	143	122	75	118	126	134	128	114	117	103	122	123	103	61	113
79	175	215	201	138	166	183	151	183	194	136	146	142	148	146	145	142	147	145	113	76	109	106	135	83	126	129	120	111	121	82	86	113
80	185	224	224	154	186	203	159	194	211	137	141	142	152	131	125	145	155	149	66	32	69	67	87	48	119	124	115	67	63	43	49	108
81	185	229	214	154	198	205	164	202	212	132	138	138	146	122	124	132	140	136	51	50	58	63	28	33	126	128	125	58	45	30	35	64
82	190	225	215	168	191	205	169	200	208	137	141	138	154	142	134	140	153	145	42	29	48	47	25	21	105	101	101	41	29	25	34	48
83	190	219	219	164	199	210	171	206	207	142	148	145	158	145	143	144	152	148	66	51	55	54	58	42	121	120	118	59	61	44	36	96
84	187	218	218	155	189	208	164	198	211	135	141	138	144	112	108	142	151	145	32	18	42	31	9	14	100	97	99	37	14	18	18	22
85	188	221	208	137	174	182	144	174	180	131	139	132	121	113	117	135	143	138	78	53	77	63	89	46	118	123	122	69	100	60	40	109
86	202	233	219	161	195	211	168	201	208	132	136	133	144	140	133	135	148	140	92	45	89	68	90	19	71	88	91	101	108	75	69	85
87	192	229	217	153	196	201	164	201	207	134	138	133	133	122	118	136	143	138	55	34	44	38	30	21	101	95	94	39	67	38	27	82
88	194	221	217	154	184	201	168	196	211	136	141	136	146	152	143	143	153	147	56	30	54	40	27	27	106	105	96	54	51	43	37	99
89	169	201	199	144	175	186	155	180	190	140	142	136	109	155	146	141	152	140	39	21	24	34	20	26	96	100	100	39	26	23	17	17
90	168	199	215	137	158	179	148	178	197	131	139	135	128	114	117	138	149	144	107	54	101	92	115	42	94	110	103	98	110	79	41	109

Figure 8.2.9 H230.46 -H230.90

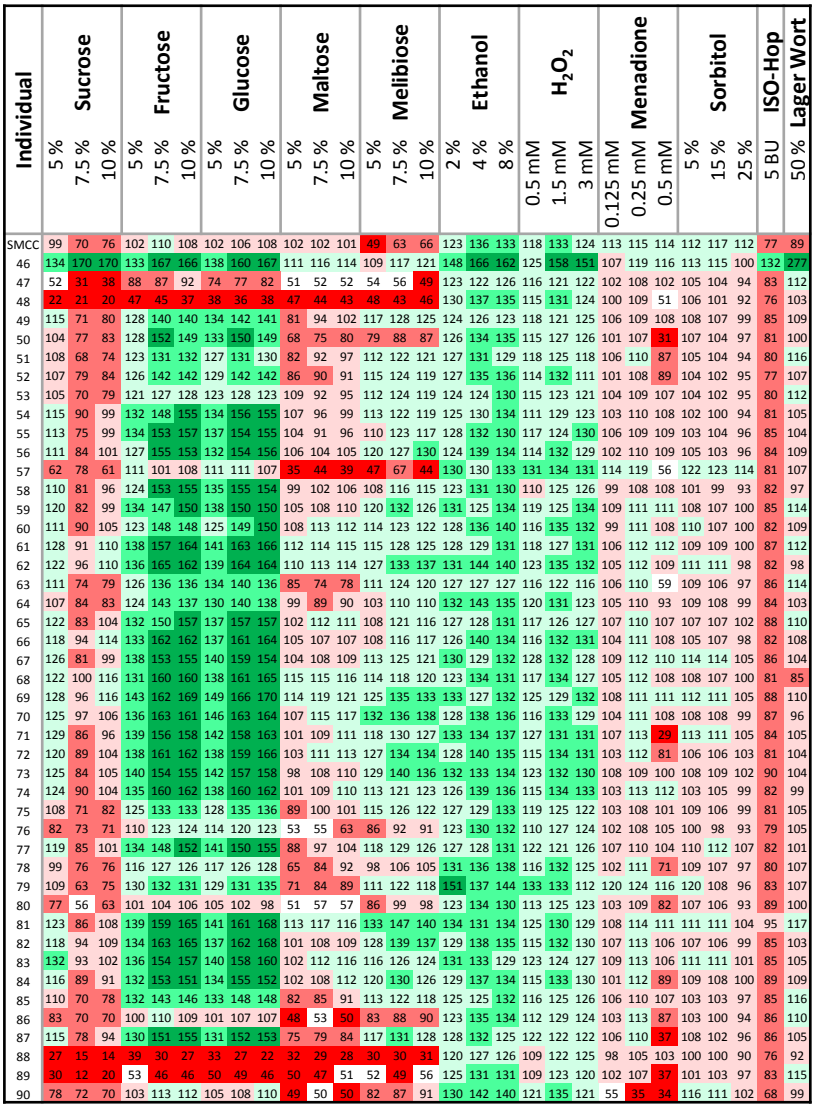
Heat map displaying the raw recorded values of the F12 individuals of H230; 46 to 90. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	106	97	97	105	111	109	104	107	108	99	102	104	33	26	25	170	178	174	158	175	170	139	156	144	152	161	143	138	276
1	105	107	110	105	104	106	105	105	106	101	103	104	40	41	40	156	152	156	145	152	156	123	127	118	136	135	103	137	275
2	107	118	114	107	114	113	107	109	110	52	54	107	35	28	34	151	159	157	138	155	160	121	130	118	136	136	104	133	281
3	130	151	155	132	154	163	135	154	160	105	112	111	108	116	121	161	149	161	137	159	160	125	129	119	128	113	89	124	285
4	133	173	176	132	163	172	132	160	173	109	114	113	103	106	116	134	144	148	116	142	153	103	114	111	108	109	91	124	273
5	135	167	171	133	162	176	131	161	177	106	113	113	118	135	135	155	152	153	129	148	149	110	119	116	116	114	95	123	279
6	133	171	175	137	166	169	138	160	172	107	113	114	113	123	129	155	166	157	128	155	153	106	119	113	120	117	99	130	275
7	139	166	173	135	159	167	137	159	170	110	115	117	106	117	117	159	155	154	129	148	148	112	118	116	117	116	104	133	275
8	130	169	166	128	158	161	133	154	167	113	116	115	107	116	123	156	161	155	131	151	151	111	122	117	122	116	99	122	280
9	131	158	165	131	155	163	134	155	166	108	115	115	115	130	132	153	146	154	128	148	149	115	121	116	115	114	98	124	277
10	137	173	176	136	171	173	141	166	180	109	114	115	112	118	127	150	155	158	123	150	136	113	120	114	115	114	96	128	277
11	140	172	170	135	163	172	138	165	178	111	116	116	105	115	116	151	155	160	122	142	148	110	120	115	113	113	103	134	271
12	136	168	168	135	167	169	137	164	171	108	114	113	124	135	144	161	169	163	138	161	154	111	123	117	127	128	103	122	282
13	137	165	169	136	162	169	138	160	171	113	117	119	116	131	130	148	158	166	126	146	141	111	118	114	112	109	94	127	276
14	137	174	173	130	162	167	138	160	173	110	113	116	108	111	113	149	154	149	122	156	140	111	122	114	115	116	100	122	275
15	141	171	174	135	165	176	138	162	179	108	114	115	117	133	134	159	160	157	134	154	158	115	122	118	119	119	102	137	276
16	132	168	171	127	160	167	129	154	171	113	116	114	107	116	121	162	176	155	133	154	151	109	120	114	119	119	110	129	278
17	130	156	162	129	151	160	133	152	162	110	117	116	117	128	131	160	162	165	139	154	148	113	121	115	122	126	106	124	277
18	133	167	166	129	160	167	134	156	168	111	112	112	108	117	125	160	162	161	139	152	157	112	124	117	126	117	95	122	280
19	134	161	168	132	162	172	134	161	172	108	114	113	118	135	139	151	152	162	133	155	148	113	121	119	114	113	103	126	272
20	131	167	168	132	169	172	134	157	174	106	111	112	117	130	138	148	155	156	127	154	144	110	119	117	110	110	94	127	279
21	135	165	171	136	165	172	137	161	173	109	113	113	119	135	123	151	147	151	121	140	137	105	114	111	108	109	93	130	266
22	137	175	174	133	173	173	136	165	176	106	111	111	105	115	121	151	155	157	126	155	135	108	119	111	113	114	98	131	274
23	141	166	165	139	164	171	142	161	172	106	112	117	112	125	127	164	167	157	139	152	152	113	120	116	121	123	103	135	276
24	139	177	180	135	168	173	140	164	175	114	118	116	110	120	127	162	173	163	137	161	154	113	126	116	123	119	102	135	278
25	144	173	177	140	164	176	145	166	178	112	116	117	124	136	137	156	160	155	127	150	152	116	120	118	115	113	103	137	275
26	129	168	170	128	159	161	131	155	162	110	115	112	107	116	123	152	164	154	126	159	158	112	121	115	114	114	100	133	282
27	136	164	163	133	154	167	133	156	168	111	116	116	122	133	136	157	161	164	131	157	156	118	124	120	119	119	101	123	275
28	133	173	176	132	161	167	136	163	173	110	113	115	111	118	124	153	159	159	125	158	156	115	126	119	118	117	99	122	283
29	138	165	170	140	163	172	138	163	174	108	116	116	122	139	141	165	158	167	143	166	151	122	129	123	130	123	99	133	276
30	138	177	175	131	170	173	141	169	180	110	115	113	122	133	143	146	154	155	121	152	143	111	121	115	112	111	97	131	276
31	136	167	172	133	162	172	134	160	176	109	113	113	121	138	140	156	156	160	134	158	163	112	120	117	117	116	100	127	276
32	140	176	177	134	173	173	140	165	178	114	121	80	118	127	132	156	176	165	127	159	167	112	123	116	114	115	101	127	283
33	147	174	173	144	176	180	148	172	180	115	122	120	134	149	148	169	158	175	138	169	158	122	134	132	119	118	102	130	272
34	133	171	171	130	165	166	134	160	171	112	115	113	116	129	134	169	181	174	136	178	158	121	135	131	120	116	99	116	284
35	148	177	180	142	170	181	145	169	186	116	121	121	119	134	137	173	169	170	151	164	168	126	134	127	135	134	112	129	278
36	139	178	182	131	170	174	139	166	181	112	117	118	110	111	117	158	171	165	134	166	153	112	125	119	123	123	103	134	283
37	134	170	183	133	157	172	134	156	177	109	114	114	119	132	135	157	153	155	133	157	152	116	126	117	117	117	99	119	274
38	132	169	179	129	163	166	134	160	172	111	112	114	119	131	138	153	167	159	127	157	155	112	125	116	116	117	98	125	281
39	136	162	170	134	163	175	136	161	178	108	115	115	118	134	135	158	149	164	138	164	138	120	126	121	125	123	101	122	275
40	143	182	181	140	178	178	149	169	184	112	116	117	129	138	142	148	155	156	118	157	101	110	123	117	108	109	96	122	277
41	139	171	180	139	165	170	141	166	175	105	110	114	110	115	120	157	152	158	134	154	138	113	120	116	116	114	102	134	266
42	111	138	140	107	133	135	106	123	137	96	103	97	99	110	118	158	161	152	130	156	157	113	122	116	118	114	100	121	281
43	143	174	166	140	166	169	143	168	171	111	114	117	122	137	139	118	150	155	100	108	125	98	105	105	94	101	95	130	271
44	138	173	175	132	167	171	138	163	175	114	116	115	104	107	111	149	165	159	126	157	157	105	120	117	113	114	97	125	280
45	140	173	176	138	165	176	140	165	177	111	118	114	114	135	138	161	157	159	139	160	156	115	121	117	118	121	101	127	278

Figure 8.2.10 Protoplast Fusion Isolate Spores 1 – 45

Heat map displaying the raw recorded values of the protoplast fusion isolate spores (PIS); 1 to 45. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).



Individual	Sucrose			Fructose			Glucose			Maltose			Melibiose			Ethanol			H_2O_2			Menadione			Sorbitol			ISO-Hop	Lager Wort
	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	5 %	7.5 %	10 %	2 %	4 %	8 %	0.5 mM	1.5 mM	3 mM	0.125 mM	0.25 mM	0.5 mM	5 %	15 %	25 %	5 BU	50 %
SMCC	99	70	76	102	110	108	102	106	108	102	102	101	49	63	66	123	136	133	118	133	124	113	115	114	112	117	112	77	89
46	134	170	170	133	167	166	138	160	167	111	116	114	109	117	121	148	166	162	125	158	151	107	119	116	113	115	100	132	277
47	52	31	38	88	87	92	74	77	82	51	52	52	54	56	49	123	122	126	116	121	122	102	108	102	105	104	94	83	112
48	22	21	20	47	45	37	38	36	38	47	44	43	48	43	46	130	137	135	115	131	124	100	109	51	106	101	92	76	103
49	115	71	80	128	140	140	134	142	141	81	94	102	117	128	125	124	126	123	118	121	125	106	109	108	108	107	99	85	109
50	104	77	83	128	152	149	133	150	149	68	75	80	79	88	87	126	134	135	115	127	126	101	107	31	107	104	97	81	100
51	108	68	74	123	131	132	127	131	130	82	92	97	112	122	121	127	131	129	118	125	118	106	110	87	105	104	94	80	116
52	107	79	84	126	142	142	129	142	142	86	90	91	115	124	119	127	135	136	114	132	111	101	108	89	104	102	95	77	107
53	105	70	79	121	127	128	123	128	123	109	92	95	112	124	119	124	124	130	115	123	121	104	109	107	104	102	95	80	112
54	115	90	99	132	148	155	134	156	155	107	96	99	113	122	119	125	130	134	111	129	123	103	110	108	102	100	94	81	105
55	113	75	99	134	153	157	137	154	155	104	91	96	110	123	117	128	132	130	117	124	130	106	109	109	103	104	96	85	104
56	111	84	101	127	155	153	132	154	156	106	104	105	120	127	130	124	139	134	114	132	129	102	110	109	105	103	96	84	109
57	62	78	61	111	101	108	111	111	107	35	44	39	47	67	44	130	130	133	131	134	131	114	119	56	122	123	114	81	107
58	110	81	96	124	153	155	135	155	154	99	102	106	108	116	115	123	131	130	110	125	126	99	108	108	101	99	93	82	97
59	120	82	99	134	147	150	138	150	150	105	108	110	120	132	126	131	125	134	119	125	134	109	111	111	108	107	100	85	114
60	111	90	105	123	148	148	125	149	150	108	113	112	114	123	122	128	136	140	116	135	132	99	111	108	110	107	100	82	109
61	128	91	110	138	157	164	141	163	166	112	114	115	115	128	125	128	129	131	118	127	131	106	112	112	109	109	100	87	112
62	122	96	110	136	165	162	139	164	164	110	113	114	127	133	137	131	144	140	123	135	132	105	112	109	111	111	98	82	98
63	111	74	79	126	136	136	134	140	136	85	74	78	111	124	120	127	127	127	116	122	116	106	110	59	109	106	97	86	114
64	107	84	83	124	143	137	130	140	138	99	89	90	103	110	110	132	143	135	120	131	123	105	110	93	109	108	99	84	103
65	122	83	104	132	150	157	137	157	157	102	112	111	108	121	116	127	128	131	117	126	127	107	110	107	107	107	102	88	110
66	118	94	114	133	162	162	137	161	164	105	107	107	108	116	117	126	140	134	116	132	131	104	111	108	105	107	98	82	108
67	126	81	99	138	153	155	140	159	154	104	108	109	113	125	121	130	129	132	128	132	128	109	112	110	114	114	105	86	104
68	122	100	116	131	160	160	138	161	165	115	115	116	114	118	120	123	134	131	117	134	127	105	112	108	108	107	100	81	85
69	128	96	116	143	162	169	149	166	170	114	119	121	125	135	133	133	127	132	125	129	132	108	111	111	112	111	105	88	110
70	125	97	106	136	163	161	146	163	164	107	115	117	132	136	138	128	138	136	116	133	129	104	111	108	108	108	99	87	96
71	129	86	96	139	156	158	142	158	163	101	109	111	118	130	127	133	134	137	127	131	131	107	113	29	113	111	105	84	105
72	120	89	104	138	161	162	138	159	166	103	111	113	127	134	134	128	140	135	115	134	131	103	112	81	106	106	103	81	104
73	125	84	105	140	154	155	142	157	158	98	108	110	129	140	136	132	133	134	123	132	130	108	109	100	108	109	102	90	104
74	124	90	104	135	160	162	138	160	162	101	109	110	113	121	123	126	139	136	115	134	133	103	113	112	103	105	99	82	99
75	108	71	82	125	133	133	128	135	136	89	100	101	115	126	122	127	129	133	119	125	122	103	108	101	109	106	99	81	105
76	82	73	71	110	123	124	114	120	123	53	55	63	86	92	91	123	130	132	110	127	124	102	108	105	100	98	93	79	105
77	119	85	101	134	148	152	141	150	155	88	97	104	118	129	126	127	128	131	122	121	126	107	110	104	110	112	107	82	101
78	99	76	76	116	127	126	117	126	128	65	84	92	98	106	105	131	136	138	116	132	125	102	111	71	109	107	97	80	107
79	109	63	75	130	132	131	129	131	135	71	84	89	111	122	118	151	137	144	133	133	112	120	124	116	120	108	96	83	107
80	77	56	63	101	104	106	105	102	98	51	57	57	86	99	98	123	134	130	113	125	123	103	109	82	107	106	93	89	100
81	123	86	108	139	159	165	141	161	168	113	117	116	133	147	140	134	131	134	125	130	129	108	114	111	111	111	104	95	117
82	118	94	109	134	163	165	137	162	168	101	108	109	128	139	137	129	138	135	115	132	130	107	113	106	107	106	99	85	103
83	132	93	102	136	154	157	140	158	160	102	112	116	116	126	124	131	133	129	123	124	127	109	113	106	111	111	101	85	105
84	116	89	91	132	153	151	134	155	152	102	108	112	120	130	126	129	137	134	115	133	130	101	112	89	109	108	100	89	109
85	110	70	78	132	143	146	133	148	148	82	85	91	113	122	118	125	125	132	116	125	126	106	110	107	103	103	97	85	116
86	83	70	70	100	110	109	101	107	107	48	53	50	83	88	90	123	135	134	112	129	124	103	113	87	103	100	94	86	110
87	115	78	94	130	151	155	131	152	153	75	79	84	117	131	128	128	132	125	122	122	122	106	110	37	108	102	96	86	105
88	27	15	14	39	30	27	33	27	22	32	29	28	30	30	31	120	127	126	109	122	125	98	105	103	100	100	90	76	92
89	30	12	20	53	46	46	50	49	46	50	47	51	52	49	56	125	131	131	109	123	120	102	107	37	101	103	97	83	115
90	78	72	70	103	113	112	105	108	110	49	50	50	82	87	91	130	142	140	121	135	121	55	35	34	116	111	102	68	99

Figure 8.2.11 Protoplast Fusion Isolate Spores 46 – 90

Heat map displaying the raw recorded values of the protoplast fusion isolate spores (PIS); 46 to 90. Values for each individual under each trait are given, and colour coded according to the above code (**Figure 8.2.1**).