NTNU
Norwegian University of
Science and Technology

Genotypic and Phenotypic Characterization of Norwegian Farmhouse Ale Yeast Cultures: A Domestication-Driven Evolution

Iselin Renée Lægreid

Chemical Engineering and Biotechnology
Submission date: June 2017
Supervisor: Per Bruheim, IBT

Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Preface

This Master's thesis conclude my degree in Master of Science (M.Sc.) in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was written at the Department of Biotechnology and Food Science, and is a continuation of the specialization project carried out in the fall of 2016. All of the experimental work presented here was conducted during the spring of 2017 in the laboratories at the department.

Acknowledgements

I would first and foremost like to thank and express my gratitude to my supervisor, Per Bruheim, for his guidance, feedback and suggestions. He has been a great motivator, and I am very grateful for having the opportunity to work with such a wonderful and inspiring professor. I also want to thank Zdenka Bartosova, for her great help with the headspace GC-MS analysis and assisting me with the data acquisition. Thanks to Kåre A. Kristiansen for helping me with the LC-MS analysis.

I was lucky to have the opportunity to work with an Erasmus student, Miguel Capilla Lloris, and would therefore like to thank him for all the help he assisted me with in the laboratory. Thanks to Paula Izquierdo Sesa and Frederik Cruyt, for good company during long days in the laboratory.

Last but not least, I want to thank family and friends for their support, especially my wonderful sisters, Rine and Jeanette Lægreid. They have contributed with great feedback and comments, and correcting numerous spelling mistakes and sentences.

Declaration of Compliance

I declare that this is an independent work according to the exam regulations of the Norwegian University of Science and Technology (NTNU).

Trondheim, June 23, 2017
Iselin Renée Lægreid

Abstract

There is an immense gap in our knowledge regarding biodiversity of yeasts and the available untapped yeasts that can be found in nature. Studying both domesticated and wild species is important as it can enable us to better understand the natural history, and potentially reveal how selective pressures have affected and shaped its evolution. The yeast *Saccharomyces cerevisiae* has been used for thousands of years by mankind to produce the most consumed fermented beverage in the world; beer. Recently, the domestication and divergence of *S. cerevisiae* beer yeasts has been thoroughly studied. However, very little is known about the evolution and phenotypic behaviour of the Norwegian farmhouse ale yeast (NFAY) cultures that were used in traditional home-brewed beer.

In this work, 14 different NFAY cultures with a total of 24 yeast isolates, were characterized and compared with *S. cerevisiae* laboratory strains and commercially available beer yeasts. The yeasts were taxonomically classified at genus and species levels by sequencing of rRNA gene regions; internal transcribed spacer (ITS) region and partially sequencing of the large subunit (LSU), referred to as LSU1 and LSU2. Since incomplete fermentation of maltotriose is a common problem in the brewing industry and maltotriose utilization is highly associated with domestication, the presence and distribution of a gene encoding an α-glucoside transporter, AGT1, was investigated. This transporter has a wide substrate specificity for common sugars in the wort, including maltotriose. Moreover, the ploidy was investigated with flow cytometry, and a small-scale fermentation of the samples was carried out at 22 °C and 35 °C. The fermentation performance, as well as its flavor and aroma profile, was determined with chromatography and mass spectrometry instrumentation (headspace GC-MS, HPLC and LC-MS).

The species differentiation based on the ITS and LSU regions suggested that the NFAY cultures consist of at least three different species; *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and the less familiar non-*Saccharomyces* yeast *Meyerozyma caribbica*. The *Saccharomyces* species seems to be closely related, as it was generally observed low DNA sequence divergence among them. The AGT1 allele was present in all *Saccharomyces* species except two of the the laboratory strains. It was discovered that a particular AGT1 gene mutation producing a premature stop codon may not be limited to lager strains, as presumed before. Interestingly, the NFAY samples carried a variant of the AGT1 gene that is more similar to the domesticated ones rather than wild *S. cerevisiae* variants. This raises the suspicion of them originating from domesticated ancestors. Phenotypic variants among the NFAY samples were also observed, and were different from the performance of commercial yeasts. This diversity is believed to have emerged from the farmhouse brewing traditions, through high fermentation temperatures, unique storage techniques and repitching. However, the phenotypic traits were not conserved within the three species, nor did they carry geographical structure. This can indicate that the phenotypic characteristics are mainly strain dependent. This, together with high maltotriose utilization, low sequence divergence and wide geographical dispersal, further supports the theory of a domestication-driven evolution in the NFAY cultures.

Sammendrag

Det er et enormt gap mellom kunnskapen vår om det biologiske mangfoldet av gjær og de tilgjengelige ubenyttede gjærtypene som finnes i naturen. Studie av både domestiserte og ville arter er viktig, da det utvikler forståelsen av naturens historie og potensielt avslører hvordan selektive faktorer har påvirket og formet dens evolusjon. Gjæren *Saccharomyces cerevisiae* har blitt brukt i årtusener av menneskeheten til å produsere den mest konsumerte fermenterte drikken i verden; øl. I senere tid har domestiseringen og divergensen til *S. Cerevisiae* ølgjær blitt nøye studert. Imidlertid er det lite kunnskap om evolusjonen og den fenotypiske oppførselen til den norske gårdsgjæren (NFAY), som ble brukt i tradisjonelt hjemmbrygget øl.

I denne studien ble 14 forskjellige NFAY-kulturer med totalt 24 gjærisolater, karakterisert og sammenlignet med *S. cerevisiae* laboratoriestammer og kommersielt tilgjengelige ølgjærtyper. Gjærtypene ble taksonomisk klassifisert på slekts- og artnivå gjennom sekvensering av rRNA-genregioner; internt transkriberte spacer (ITS) region og delvis sekvensering av den store subenheten (LSU), referert til som LSU1 og LSU2. Siden ufullstendig fermentering av maltotriose er et utbredt problem i bryggeriindustrien, og maltotriose-utnyttelse er sterkt forbundet med domestisering, ble det undersøkt om et gen som koder for en α-glukosidtransportør, AGT1, var tilstede og en eventuell fordeling av denne. Denne transportøren har en bred substrat-spesifisitet for vanlige sukkertyper i vørteren, inkludert maltotriose. Videre ble ploiditeten undersøkt med flowcytometri, og en småskala fermentering av prøvene ble utført ved 22 °C og 35 °C. Fermenteringsytelsen, inkludert smak- og aromaprofil, ble bestemt ved kromatografi og massespektrometri instrumentering (headspace GC-MS, HPLC og LC-MS).

Artsdifferensieringen basert på ITS- og LSU regionene indikerte at NFAY-kulturene består av minst tre forskjellige arter; *Saccharomyces cerevisiae*, *Saccharomyces bayanus* og den mindre kjente ikke-*Saccharomyces* gjæren *Meyerozyma caribbica*. *Saccharomyces*-artene virker nært beslektet, da det generelt ble observert lav DNA-sekvensdivergens mellom dem. AGT1-allelen ble funnet i alle *Saccharomyces*-artene, med unntak av to av laboratoriestammer. Det ble oppdaget at en bestemt AGT1 genmutasjon som produserer et for tidlig stoppkodon, kanskje ikke er begrenset til lager-gjærstammer, som tidligere antatt. Et interessant funn var at NFAY-prøvene hadde en variant av AGT1-genet som mer ligner de domestiserte artene enn de ville *Saccharomyces* variantene. Dette gir grunn til mistanke om at de stammer fra domestiserte arter. Fenotypiske varianter blant NFAY-prøvene ble også observert, og hadde forskjelle egenskaper sammenlignet med de kommersielle gjærtypene. Dette mangfoldet antas å ha oppstått fra gårdenes bryggetradisjoner, gjennom høye fermenteringstemperaturer, forskjellige lagringmetoder og gjenbruk. De fenotypiske trekkene ble imidlertid ikke bevart innenfor de tre artene, og de hadde heller ikke geografisk sammenheng. Dette kan tyde på at fenotypiske egenskapene er hovedsakelig avhengig av gjærstamme. Dette, sammen med høy maltotrioseutnyttelse, lav sekvensdivergens og bred geografisk spredning, støtter videre teorien om at det er domestiseringen som driver utviklingen av NFAY-kulturene.

Contents

List of Tables

List of Figures

Abbreviations

AGT1 α-glucoside transporter 1

BLAST Basic Local Alignment Search Tool

CNV Copy number variation

DGGE Denaturing gradient gel electrophoresis

EDTA Ethylenediaminetetraacetic acid

ESTD External standard

EtOH Ethanol

FU Flavor unit

GC Gas chromatography

HEPES 4-(Hydroxymethyl)piperazine-1-ethanesulfonic acid

HPLC High-performance liquid chromatography

HS GC-MS Headspace gas chromatography-mass spectrometry

ISTD Internal standard

ITS Internal transcribed spacer

LAB Lactic acid bacteria

LC Liquid chromatography

LC-MS Liquid chromatography-mass spectrometry

LCSM Lin's copper sulfate medium

LOQ Limit of quantification

LSU Large subunit

LYS Lysine

m/z Mass-to-charge ratio

MAL Maltose

ME Malt extract

ML Maximum likelihood

MOPS 3-(N-morpholino) propanesulfonic acid

MQ Milli-Q

MS Mass spectrometry

MSA Multiple sequence alignment

MUSCLE MUltiple Sequence Comparison by Log-Expectation

NCYC National Collection of Yeast Cultures

NFAY Norwegian farmhouse ale yeast

OD Optical density

PCA Principal component analysis

PCR Polymerase chain reaction

ppm Parts per million

QC Quality control

RDP Ribosomal Database Project

RI Refractive index

RT Retention time

SG SYBR® Green I

SGD *Saccharomyces* genome database

SSU Small subunit

TE Tris-EDTA

Tris Tris-(hydroxymethyl)-aminomethane

UV Ultraviolet

WLD Wallerstein laboratory differential

WLN Wallerstein laboratory nutrient

Chapter 1

Introduction

Brewing yeasts are eukaryotic, unicellular and facultative anaerobic microorganisms. The cell normally has an ovoid shape, that measures around 5-10 μm in diameter when fully grown. The majority of brewing yeasts can be grouped into two; ale and lager strains. These two groups can be distinguished both phenotypically and genotypically, and carry very distinctive fermentation characteristics. Features such as flocculation behavior, fermentation time, stress tolerance, trehalose storage capacity and organoleptic impression may vary significantly between them, affecting the final beer product (Pires & Brányik, 2015). The fundamental differentiation is however based on the inability of ale yeasts to ferment the disaccharide melibiose, as they do not produce the enzyme α-galactosidase necessary to convert it into glucose and galactose (C. W. Bamforth, 2005).

The most important yeast species for beer fermentation technology belong to the genus *Saccharomyces*. The word '*Saccharomyces*' is Greek and translates into 'sugar fungus' (Saccharo = sugar and myces = fungus). As the name clearly suggests, yeasts from this genus are commonly found in sugary environments in nature. Ale-type beers are fermented by *Saccharomyces cerevisiae*. The species '*cerevisiae*' comes from the Latin language meaning 'of beer' and has been associated with the brewing process since ancient times. Traditionally, *S. cerevisiae* strains are referred to as 'top-fermenting' yeasts since they tend to accumulate in the foam during fermentation. Fermentation is carried out at relatively high temperatures (18-25 °C), resulting in fast fermentations and beers with fruity aromas (Pires & Brányik, 2015).

Lager-type beers are on the other hand fermented by *Saccharomyces pastorianus* (formerly named *S. carlsbergensis*), and are referred to as 'bottom-fermenting' yeasts based on their tendency to sink to the bottom. Recent studies have revealed that *S. pastorianus* is a hybrid of two species; *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (Pires & Brányik, 2015). While ale strains have been used for thousands of years by mankind, it has been suggested that lager strains originated only a few hundred years ago through selection during low temperature wort fermentations. Lager yeasts are cryotolerant as they perform well at low temperatures (6-14 °C), a feature that is believed to derive from the *S. eubayanus* partner (Vidgren, Multanen, Ruohonen, & Londesborough, 2010).

Norwegian farmhouse ale is home-brewed beer based on old Norwegian traditions (i.e. different from the ones used in modern home brewing) and is brewed with yeast cultures called 'kveik'. These traditional Norwegian farmhouse ale yeast (NFAY) cultures are prominent because they seem to have a very high fermentation temperature tolerance. They were harvested and reused for centuries, unlike the yeasts in today's beer production, and passed down from generation to generation. Moreover, the yeast was stored in many different ways; some kept the yeast bottled in a liquid mixture in between the batches, while others kept it dry. However, the latter was by far the most common practice. This was achieved using traditional equipment such as yeast rings and logs (Garshol, 2016).

It is not possible to pinpoint the exact time for the origin of the beer brewing traditions in Norway. However, according to the laws of 'Gulating' dating back to around year 1000, it was mandatory for free men to brew beer during Christmas. Since Norway is quite isolated from the rest of Europe, the tradition remained unaffected by modernization and industrialization, and survived much longer than elsewhere - until the 1880s. Long distances and shifting seasons made the availability of resources such as grain and hops unpredictable, and resulted in a great variety of beers. Today, the origin of the NFAYs used in this traditional beer still remains a mystery as it has not been investigated before (Garshol, 2016). Could they have been introduced through trade and import, perhaps from Germany or Great Britain? Or are they simply native?

1.1 Yeast Biodiversity

Ease of culture, simple life cycles and small genomes are some of the advantages that have made yeasts remarkable models for molecular genetics, biotechnology and evolutionary genomics. Sequencing of yeasts can provide useful functional and evolutionary hypotheses, and further increase our understanding of the diverse metabolisms and ecologies. The genome of *Saccharomyces cerevisiae*, a workhorse in beer production, was the first eukaryotic genome to be completely sequenced, and is arguably the most intensely studied eukaryotic organism besides human beings. Despite this, only a tiny fraction of yeast biodiversity has been explored by industry and science at the genomic level (Hittinger et al., 2015). Approximately 1500 yeast species are currently known. However, estimations suggests additional 669 000 existing yeast species that are yet to be described (Hill, 2015). This means that there is an immense gap in our knowledge regarding biodiversity and the available wild natural isolates of untapped yeasts. It is therefore of interest to conserve and exploit yeast biodiversity further (Walker, 2009).

There is far more diversity among ale strains than among lager strains, both genetically and aromatically. It has been suggested that this greater diversity of ale strains reflects their isolation in multiple locations, whereas the lager strains emerged from a very limited locality (Bokulich & Bamforth, 2013). This section will focus on the biodiversity that can arise from various yeast reproduction systems, hybridization and introgression events and domestication. The metabolic diversity and range of ecologies will not be elaborated extensively, though metabolic pathways will be described later on.

1.1.1 Yeast Growth

Yeast growth is related to how cells transport and assimilate nutrients, followed by the integration of numerous component functions that allow them to increase in mass and eventually divide. In fact, yeasts have proven to be invaluable in unraveling the major control elements of the eukaryotic cell cycle, especially through studies of the budding yeast, *S. cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe* (Herskowitz, 1988; Walker, 2009). There is a great diversity regarding yeast mating systems, as a multiplicity of forms exist. Yeasts may have asexual and sexual reproductive cycles, but also complex parasexual cycles being non-meiotic processes, involving mitotic recombination and chromosome loss (Hittinger et al., 2015).

Proliferation: The Mitotic Cell Cycle

Yeast cells double in number through the mitotic cell cycle. Budding, in which the 'mother' cell gives rise to an ellipsoidal daughter cell through a small outgrowth (Herskowitz, 1988), is by far the most common mode of vegetative reproduction in yeasts. This is predominant in ascomycetous yeasts such as *S. cerevisiae*, where the cell size at division is asymmetrical. The multilateral type of budding is especially common. During this process, daughter buds arise from different locations on the mother cell surface (Walker, 2009). The budding cell cycle is illustrated in Figure 1.1. Budding differs from the process of fission, where the initial cell enlarges and pitches off into two equal-size daughter cells. Yeast cells can abandon the proliferation mode under certain circumstances. For instance, when the nutrients are limited they may be arrested as unbudded cells in the G_1 phase until nutrients are available again. Cells can also be arrested in this phase when a mating partner (of different mating type) is nearby (Herskowitz, 1988).

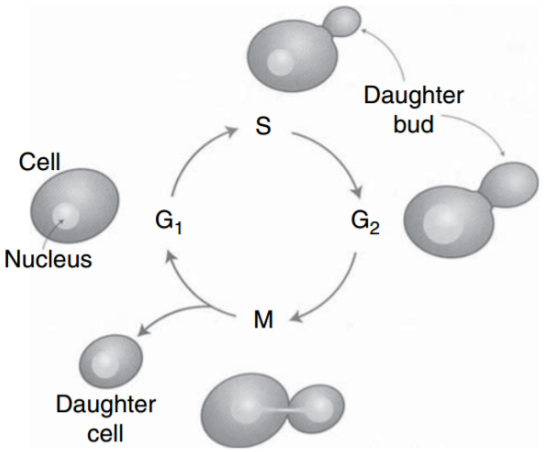


Figure 1.1: The budding cell cycle of *S. cerevisiae*. A small outgrowth, the daughter bud, grows from the surface of a mother cell that eventually separates to form a new cell. G_1, pre-DNA synthesis gap period; S, DNA synthesis period; G_2, post-DNA synthesis gap period; and M, mitosis. From Walker (2009).

Filamentous growth can also be considered as a mode of vegetative growth (alternative to budding and fission), and occurs in many yeast species. Yeasts typically undergo filamentous growth during unfavorable conditions, but can return to unicellular growth when more conductive growth conditions reoccur (Walker, 2009).

Transitions: Mating and Sporulation

S. cerevisiae is as previously denoted a unicellular organism. It can however exist in any of three specialized cell types, that all can undergo mitotic cell divisions. Only two of them, mating types a and α, can mate efficiently with each other by cell and nuclear fusion. The product of the mating, a stable zygote (see Figure 1.2), can give rise to daughter diploid cells by budding. This is the third specialized cell type; the diploid a/α. Ploidy refers to the number of copies of the set of chromosomes in a cell, e.g. a haploid has exactly one copy of each chromosome, while a diploid has two copies. The a/α cell is unable to mate with either a or α cells, though, it can undergo meiosis and form haploids (Herskowitz, 1988).

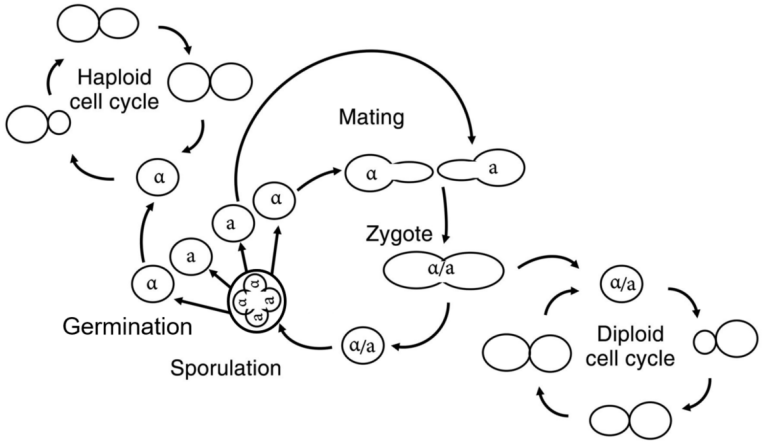


Figure 1.2: Yeast life cycle of *S. cerevisiae*. *S. cerevisiae* can grow by budding either as a haploid or diploid. Haploids exist as one of two mating types; a or α. These can mate with each other by events leading to the formation of a zygote. The zygote can enter the budding pathway as a diploid or undergo meiosis to form four spores that can germinate into haploid cells; two a and two α types. Modified after Bisson (2017).

There are two requirements in order for cells to initiate meiosis and spore formation (together referred to as sporulation). First, there must be an appropriate environmental stimulus with nutritional starvation of both nitrogen and carbon. The second requirement is that they have to be diploid and carry the appropriate genotype. This includes having *MAT*a/*MAT*α and a1-α2 activity. *MAT*a and *MAT*α are naturally occurring alleles of the mating-type locus, which is the key genetic regulator of the life cycle of *S. cerevisiae*. a1 and α2 are proteins that together form a regulatory unit, responsible for repression of mating genes and activation of the sporulation process (Herskowitz, 1988). The diploid

cells that fulfill these requirements can sporulate and later germinate in rich media to form four haploid spores. The resulting haploid budding cells can mate with each other to restore the diploid state again (Walker, 2009). Figure 1.2 shows mating and sporulation in *S. cerevisiae*.

Laboratory and Brewing Strains

Genome structure analysis has revealed significant differences between the laboratory and brewing strains. Most laboratory strains exists as haploids or diploids, whereas the majority of beer yeasts are poly- or alloploidal (containing two or more sets of chromosomes). Accordingly, the life cycle of laboratory strains may often include sexual reproduction by sporulation, with diploid cells undergoing meiosis leading to the formation of ascospores. These spores (a and α) can mate, hybridize and form diploidal cells (a/α) that are capable of budding or sporulating, as explained earlier. In contrast, brewing strains of *Saccharomyces* rarely sporulate, but if they do, the spores are usually non-viable and they are not able to mate. Brewing yeasts will generally reproduce by multilateral budding (Hutkins, 2006).

1.1.2 Hybridization and Introgression

The seven species of the genus *Saccharomyces* (*S. cerevisiae, S. paradoxus, S. mikatae, S. kudriavzevii, S. arboricola, S. uvarum and the newly identified S. eubayanus*) show a significant divergence at the nucleotide-sequence level. Nonetheless, there is a compatibility regarding the sexual reproduction as they all form viable diploids. This explains the rich reticulate evolution of the genus and industrially applicable species (Hebly et al., 2015). Hybridization events occasionally occur among the *Saccharomyces* species. Haploid cells from different *Saccharomyces* species can mate to form F1 hybrids (F1 indicating the first filial generation). These can grow normally by the mitotic cell cycle, but when meiosis is induced the chromosomes tend to fail to recombine and segregate efficiently. In fact, 99% or more of the ascospores produced are non-viable because they lack essential chromosomes. However, the few that do form viable spores that survive the F1 hybrid meiosis, normally contain a variable and aneuploid assimilation of chromosomes from both parental species. The ones that are indeed successful can furthermore mate and produce F2 hybrids (Boynton & Greig, 2014).

Many natural occurring hybrids have been established in the fermentation beverages wine, cider and beer. The most well-known include *S. pastorianus* and *S. bayanus* (Boynton & Greig, 2014). Furthermore, yeasts can also occur as double or even triple hybrids, e.g. *S. cerevisiae* $\times$ *S. bayanus*, *S. cerevisiae* $\times$ *S. kudriavzevii* and *S. cerevisiae* $\times$ *S. uvarum* $\times$ *S. kudriavzevii* (Hebly et al., 2015). A cladogram showing the phylogenetic relationships among *Saccharomyces* species and common hybrids is shown in Figure 1.3.

Portions of a chromosome can introgress from the genome of one *Saccharomyces* species to a different one, and is common within the genus. Introgression is most likely the result of a hybridization event (Boynton & Greig, 2014), where the gene flow is transferred between species through a process of successful mating and many backcrossing events (Hittinger, 2013). Introgressions into fermentation strains are commonly present in a subset of strains within a species, though, not fixed throughout all of them. Some of

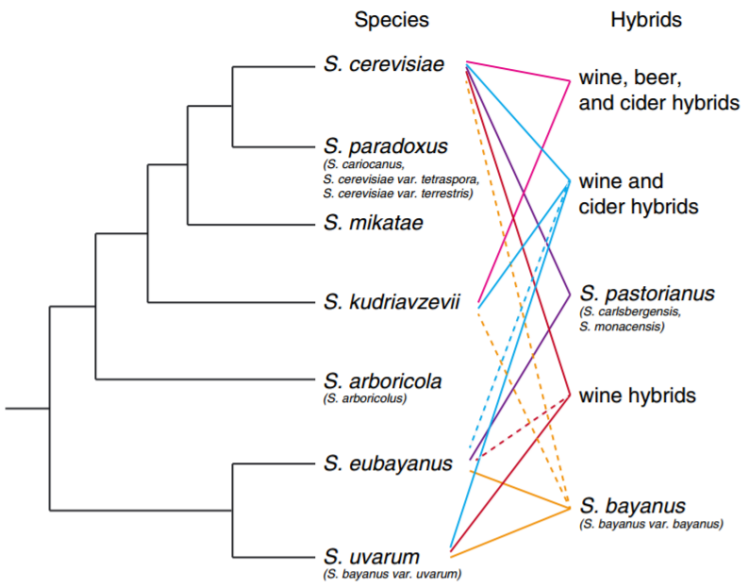


Figure 1.3: Cladogram showing the phylogenetic relationships among *Saccharomyces* species and common hybrids. Dashed lines represent introgressions from a third or fourth species into a hybrid, but most are not present in all hybrid strains. From Boynton and Greig (2014).

the reported introgressions into fermentation strains are shown in Figure 1.3, represented by dashed lines. However, naturally occurring strains have rarely been documented with such events. Most strains with introgressions are human-associated or closely related to human-associated strains (Boynton & Greig, 2014).

1.1.3 Domestication

Domestication can be considered as the human selection and breeding of wild species to obtain cultivated variants in manufactured environments, that can still behave adequately in nature. The domestication of livestock, pets and crops is well documented, but whether the beer yeast diversity is shaped by domestication or neutral divergence (e.g. due to geographic isolation and limited dispersal) has not been studied to the same extent (Gallone et al., 2016). In general, domestication (as opposed to local adaption) appears to have developed new niches that offers advantages that select for hybrid genome (Hebly et al., 2015). This is partly explained by the fact that laboratory-produced hybrids tend to have higher fitness than their parents in extremely stressful environments, suggesting that hybrids are more common in domesticated rather than wild environments. Moreover, a pattern of frequent hybridization and unfixed introgression events in domesticated species, further suggests that the selection in domesticated environments can be both extreme and utterly variable (Boynton & Greig, 2014).

Domestication usually increases the phenotypic diversity among species, e.g. the morphological diversity observed for dogs. This trend is also recognized for *S. cerevisiae*, a

species that has been largely affected by domestication. This model yeast has a greater phenotypic diversity, but a lower genome sequence diversity than *Saccharomyces paradoxus*; the first *Saccharomyces* yeast to be acknowledged as non-domesticated (Boynton & Greig, 2014). It is possible that the high phenotype diversity reflects variation in the source environment. For instance, rice wine, grape wine or beer, may select directly for different traits. However, observations made by Warringer et al. (2011) suggests that it is largely defined by population and influence of genetic drift rather than selection. In the case of independent domestication of different *S. cerevisiae* populations, domestication could relax stabilizing selection by allowing gain or loss of genes and functions by drift that usually would be maintained in the wild (Warringer et al., 2011). Bergström et al. (2014) also found that *S. cerevisiae* strains have a high variation in the gene content compared to *S. paradoxus*, in the form of presence or absence of genes as well as copy number variation (CNV). In *S. cerevisiae*, genes contained in these CNV regions were typically enriched for gene ontology terms related to sugar transport and metabolism, flocculation and ion and metal transport and metabolism (Bergström et al., 2014).

Domestication increases the biological dispersal and reduces geographical structure. In domesticated *S. cerevisiae*, most lineages are mosaics (i.e composed of more than one genotype) and the genetic structure tends to track human usage (Liti et al., 2009), unlike wild *S. cerevisiae*, where evidence for strong geographic structure has been found (Q. M. Wang, Liu, Liti, Wang, & Bai, 2012). When populations of domesticated and wild *Saccharomyces* species were compared in previous studies, it was established that high phenotypic diversity, low DNA sequence divergence, hybridization and introgression are all associated with domestication (Boynton & Greig, 2014). Other key hallmarks of domestication include polyploidy, aneuploidy and heterozygosity (Gallone et al., 2016). *In vitro* evolution experiments revealed that tetraploids undergo significantly faster adaption compared with haploids and diploids. This was supported by mathematical modelling, that suggested that rapid adaption of tetraploids is driven by higher rates of beneficial mutations having stronger fitness gains. This also applies to chromosome aneuploidy, concerted chromosome loss and point mutations, and may explain the role for polyploidization events during adaption to stressful environments (Selmecki et al., 2015), as often is the case for domestication.

Gallone et al. (2016) recently demonstrated that industrial yeasts were clearly subjected to domestication, both genetically and phenotypically. Interestingly, the domestication seems to be strongest in beer yeasts, and the present-day industrial yeasts originate from only a few domesticated ancestors as seen in Figure 1.4. They revealed a strong industry-specific selection for stress tolerance, sugar utilization and flavor production. Sexual cycle and other phenotypes related to survival in nature showed decay in beer yeast (Gallone et al., 2016). Similarly, Gonçalves et al. (2016) also concluded that current beer strains have distinctive genomic signatures of domestication. In addition, they observed clear differences between top-fermenting beer and wine yeast domestication; beer stains are polyphyletic and more diverse contrary to wine strains (Gonçalves et al., 2016).

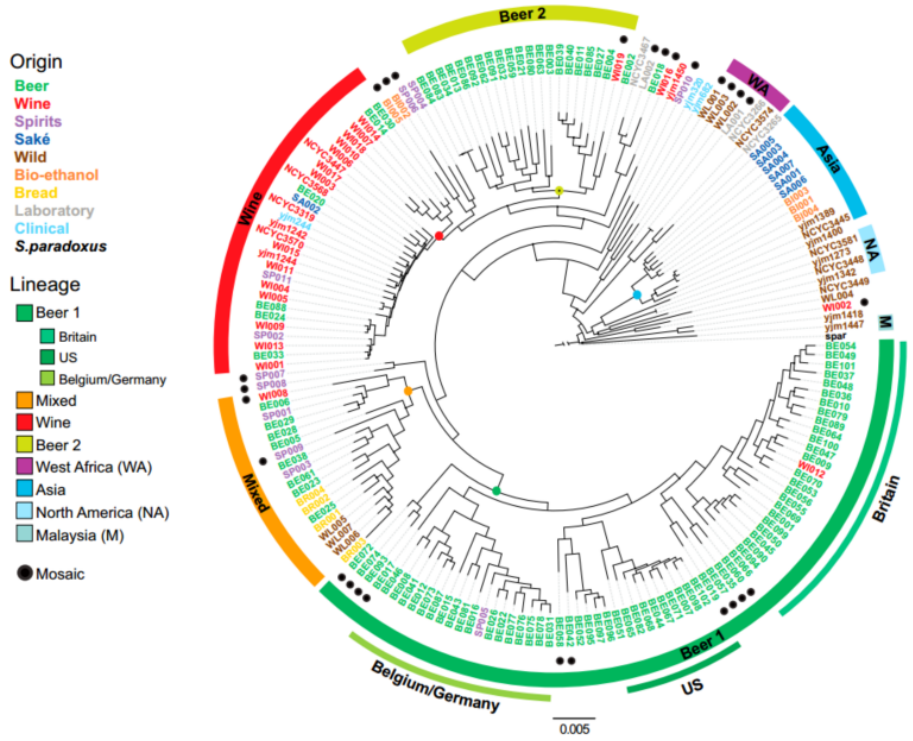


Figure 1.4: Maximum likelihood phylogenetic tree of *Saccharomyces cerevisiae* proposed by Gallone et al. (2016). They diversified the industrial *S. cerevisiae* strains into five sublineages separated from wild strains; Asian, wine, a mixed clade and two separate families of beer yeast.

1.1.4 Yeast-bacteria and Yeast-yeast Interactions

Yeast-bacteria or yeast-yeast interactions can form symbiotic relationships such as mutualism, commensalism, amensalism and predation. These interactions are commonly found and have successfully been applied to food and beverage products. There are plenty of examples describing symbiosis between *Saccharomyces cerevisiae* and a bacteria or another yeast species. Yeast interacting with lactic acid bacteria is prominent, and the main species associated with fermented products are *Pediococcus*, *Leuconostock*, *Lactobacillus*, *Lactococcus* and *Bacillus* (Viljoen, 2006).

Yeast-yeast associations are also frequently indicated in food technology, but few studies have reported the interactions in detail (as with bacteria) other than referring to the presence of them (Viljoen, 2006).

1.2 Phylogenetics and Taxonomy

1.2.1 Yeast Identification and Classification

The detection, identification and classification of yeasts was previously based on the interpretation of often inaccurate phenotypic tests (Kurtzman et al., 2015). This was especially challenging considering the fact that all *Saccharomyces* species have similar morphologies and biochemical phenotypes (Boynton & Greig, 2014). However, this has been replaced by a new approach involving phylogenetic analyses of DNA sequences, suggesting that yeast classification should be revised (Kurtzman et al., 2015).

One taxonomic method is DNA barcoding, which is based on the nucleotide sequence information of a target gene region. This approach can provide a rapid and accurate identification, making it possible to study the diversity (Toju, Tanabe, Yamamoto, & Sato, 2012). Gene sequence comparisons based on ribosomal rRNA genes offer the opportunity to distinguish between closely related species, as well as more distantly related ones (Kurtzman, Fell, & Boekhout, 2011). Multiple regions of the rRNA genes have been used to study fungal taxonomy and for diversity estimations of fungal isolates and uncultured taxa; these include the small subunit (SSU) and large subunit (LSU) rRNA genes and the widely used internal transcribed spacer (ITS) region (Liu, Porras-Alfaro, Kuske, Eichorst, & Xie, 2012). A map of the nuclear ribosomal RNA genes and their ITS regions is shown in Figure 1.5.

The use of the rRNA gene regions for fungal identification presents several advantages over the use of functional genes (i.e. protein-coding genes). A significant advantage is that ribosomes have a common evolutionary history. Within rRNA gene sequences there are generally highly conserved regions that can serve as primer sites for polymerase chain reaction (PCR) amplification and sequencing. In comparison, protein-coding genes tend to be variable across the entire gene (Kurtzman et al., 2011). Thus, these often have to face problems with primer design and be subject to variable third codon positions. The rRNA genes also have regions of high sequence variability throughout the majority of fungi, allowing identification at species level. Furthermore, rRNA genes have a high number of copies per cell, a feature that can be beneficial when little DNA is available. A rapidly growing number of sequences in public databases supports this type of sequence analysis as an integral part of fungal classification (Porras-Alfaro, Liu, Kuske, & Xiec, 2014).

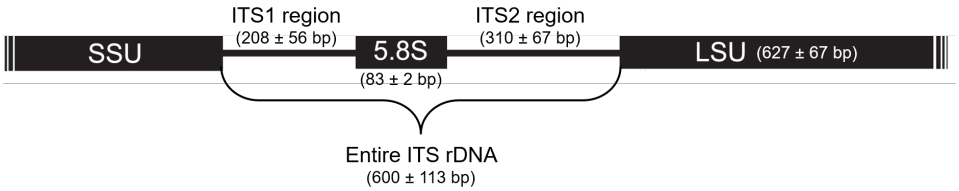


Figure 1.5: Map of nuclear ribosomal RNA genes and ITS regions. The value and rage enclosed by brackets represent the length (in base pairs) of each region. Modified after Toju, Tanabe, Yamamoto, and Sato (2012) and Porras-Alfaro, Liu, Kuske, and Xiec (2014).

The entire ITS rRNA region is approximately 600 bp in length, and is located between the SSU and LSU rRNA genes. It is composed of two hypervariable regions, ITS1 and ITS2, that are separated by the 5.8S rRNA gene (Porras-Alfaro et al., 2014). The 5.8S gene is highly conserved, and should therefore not be included when comparing substitutions in the ITS region (Kurtzman et al., 2015). The LSU rRNA region is located immediately downstream of the ITS region (Porras-Alfaro et al., 2014). This gene contains two hypervariable regions, referred to as the D1/D2 domain. The D1 and D2 regions stretch from position 127 to 264 and from 423 to 636 in *S. cerevisiae*, respectively, and are surrounded by relatively conserved sequence regions in most fungi (Liu et al., 2012). Kurtzman and Robnett (1998) demonstrated that most yeast species can be identified from the sequence divergence in the D1/D2 domain by a partial sequencing of the LSU rDNA.

The ITS and LSU regions each have their strengths and weaknesses regarding fungal identification. One of the limitations with the ITS region is low taxonomic resolution for some species. Other drawbacks for this region include difficulty in fungus-specific PCR primer design and high variability that ultimately can inhibit the use of alignments and tree-based methods. The LSU region on the other hand, is generally considered to be less variable than the ITS region, which may be an issue when the taxonomic resolution is expected to be at species level and concerning diversity analyses. Regardless, they both provide information-rich sequences that are amenable to alignment and phylogenetic identification altogether (Porras-Alfaro et al., 2014).

Many yeast species have been described almost exclusively from the divergence in the D1/D2 domain or from ITS sequences. However, it is apparent that when analyzing a single gene, it can lead to an incorrect interpretation. Consequently, it appears that in addition to the LSU and/or ITS regions, one or more protein coding gene sequences should be covered as well (Kurtzman et al., 2011).

1.2.2 The Ribosomal Database Project Classifier

The Ribosomal Database Project (RDP) Classifier is a naïve Bayesian classifier that is suitable for numerous rRNA sequences, providing satisfying overall accuracies. The term 'naïve' refers to the assumption that data attributes are independent. The naïve Bayesian classifier has been available for analysis of bacterial and archaeal sequences for many years (Q. Wang, Garrity, Tiedje, & Cole, 2007). Recently, Liu et al. (2012) demonstrated that the classifier can also be used for accurate classification of fungal sequences using a hand-curated LSU database. When the performance of the naïve Bayesian classifier was compared to that of a sequence similarity-based Basic Local Alignment Search Tool (BLAST), it was found that the classifier was computationally more rapid than the BLASTN approach. Moreover, it mostly provided results of equal or superior classification accuracy (Liu et al., 2012; Porras-Alfaro et al., 2014).

1.3 The Brewing Process

Presently, beer is the most consumed fermented beverage in the world (Gonçalves et al., 2016). Although only four main ingredients are required to make beer (water, malt, hops, and yeast), the process of making a quality beer is far more complicated. The general brewing process can be divided into four main stages; malting, mashing, fermentation and post-fermentation (Hutkins, 2006). A schematic representation of the process is shown in Figure 1.6.

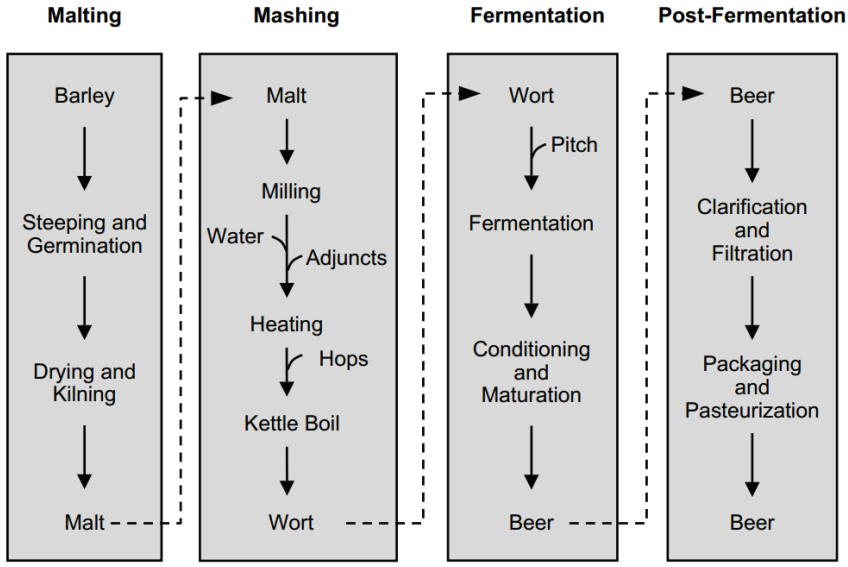


Figure 1.6: The general brewing process. The process can be divided into four stages; malting, mashing, fermentation and post-fermentation (Hutkins, 2006). Cooling steps are included between mashing and fermentation.

1.3.1 Malting

Cereal grain, usually barley, is converted into malt in the first stage. Malt is crucial because it serves as the source of enzymes, e.g. amylases and proteinases, that are necessary for the hydrolysis of macromolecules such as starch and protein. In addition, malt is also the primary determinant of color and body characteristics, and will have an impact on the flavor development of the beer (Hutkins, 2006).

1.3.2 Mashing

The malt is mixed with water, creating a mash. The mash is then gradually heated to allow for extraction and enzymatic reactions to take place, ultimately resulting in the formation of a nutrient-rich growth medium called wort. The main principle of this stage is to transform non-fermentable starch into sugars that yeast can ferment. As a result, the main

component of the wort are small sugars, typically maltose, glucose, fructose, sucrose, and maltotriose. Prior to fermentation, hops is added to the wort and brought to a boil. Boiling accomplishes several features; it kills nearly all of the microorganisms, inactivates most of the enzymes, and enhances extraction of oils and resins from the hops and accelerates isomerization of hop acids. Additionally, the color development is increased, and undesirable volatile components are removed (Hutkins, 2006).

1.3.3 Fermentation

The next step is to add yeast in order for the fermentation process to proceed. Fermentation is defined as the anaerobic catabolism in which an organic compound is both an electron donor and an electron acceptor, and ATP is produced by substrate-level phosphorylation. Respiration on the other hand, is the catabolism in which a compound is oxidized with O_2 (or O_2 substitutes) as the terminal electron acceptor, and is usually accompanied by ATP production by oxidative phosphorylation. Yeast can both ferment and respire, but respiration is the preferred choice since it can yield much more ATP. Yeast switch to fermentative metabolism using the glycolytic pathway *only* when conditions are anoxic and terminal electron acceptors are absent (Madigan et al., 2015).

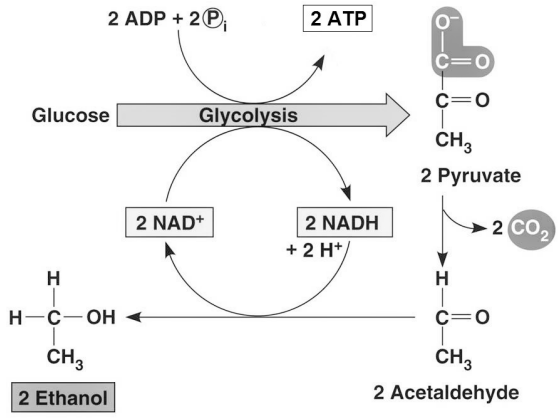


Figure 1.7: Production of ATP by substrate-level phosphorylation. Pyruvate, the end product of glycolysis, serves as an electron acceptor for oxidizing NADH back to NAD^+. The end products of alcohol fermentation are ethanol and CO_2 (Reece et al., 2011).

In the beginning of the process yeast grow primarily by respiration and consume the O_2 that is present. As soon as the O_2 is depleted fermentation begins (Madigan et al., 2015). In alcohol fermentation, see Figure 1.7, pyruvate is converted into acetaldehyde by the enzyme pyruvate decarboxylase and further reduced by NADH to ethanol (Reece et al., 2011). Only microorganisms with this enzyme can follow this pathway. Yeast being one of them explains why they ferment sugars to ethanol and CO_2 rather than to e.g. lactate (Nelson and Cox, 2013). However, not all microorganisms and yeasts feel as comfortable

as *Saccharomyces* species in an alcoholic environment (Pires & Brányik, 2015). *Saccharomyces* and some relatives have evolved an extreme preference for fermenting glucose into ethanol, even in the presence of oxygen. This event is known as 'Crabtree-Warburg Effect', and is a strategy that provides powerful ecological advantages as rich reserves of simple sugars (e.g. in sap and fruit) can be exploited (Hittinger et al., 2015).

Yeasts will generally produce ethanol and CO_2 from sugars under anaerobic conditions, but not all yeasts are necessarily suitable for brewing. For instance, an important feature of beer yeasts is that they can produce esters, acids, higher alcohols and ketones from wort sugars and proteins, in order to establish the desirable flavors in beer (Okafor, 2007). Beer yeasts have also shown a significantly higher capacity to metabolize maltotriose, a carbon source specifically found in beer medium (Gallone et al., 2016).

1.3.4 Post-fermentation

Post-fermentation includes additional measures such as removing the yeasts and other microorganisms or substances. When the fermentable sugars are depleted and the beer fully attenuated, flocculation of yeast cells occur. Flocculation is the ability of yeast cells to adhere to one another in the form of clumps, and high flocculation activity makes it easier to remove the remaining yeast (Hutkins, 2006). If flocculation takes place too early, it will produce a beer that is under attenuated and sweet. On the other hand, if the yeast fails to flocculate entirely, the result is a cloudy beer with a yeasty flavour (Hill, 2015). Consistent flocculation during fermentation is therefore also considered to be one of the most important traits of a good beer yeast (Hutkins, 2006).

Optional processing steps such as clarification, filtration, and pasteurization may also be applied. One of the most important steps is to provide carbonation in the final product. This can be done by adding more fermentable sugars for a secondary fermentation to occur. The containers must be able to withstand the accumulated pressure as a result of CO_2 formation. Post-fermentation measures can improve the shelf-life and quality, and make the beer more suitable for consumption (Hutkins, 2006).

1.4 Yeast Metabolism: The Impact of Yeast on Beer Appearance, Flavor and Aroma

Although raw materials such as hops contribute to the bitter taste and aroma to a certain extent, it is the yeast used that ultimately makes the significant impact on beer flavor and aroma. This occurs through yeast autolysis, catabolism of sugars, assimilable nitrogen, organic acids and other substances, as well as the generation of acids, alcohols, aldehydes, esters, ketones, volatile phenolic compounds, terpenoids and volatile sulphur compounds (Hill, 2015). Compared to lager yeasts, ale yeasts are considered to have a greater genetic diversity that is reflected in the diverse flavor and aroma profile of ale beer (Mertens et al., 2015). An overview of the main metabolic activities and pathways by *Saccharomyces* influencing beer quality and flavor is shown in Figure 1.8.

The level of each substance produced depends on yeast strain, but the fermentation conditions are also of importance. Parameters such as pitching rate, temperature, extent of oxygen addition, C:N ratio and the duration of fermentation and maturation will also affect the final product (Bokulich & Bamforth, 2013). Moreover, chemical reactions such as oxidation, degradation, condensation etc. during processing and storage can also give rise to certain compounds (Moreira, Meireles, Brandão, & De Pinho, 2013).

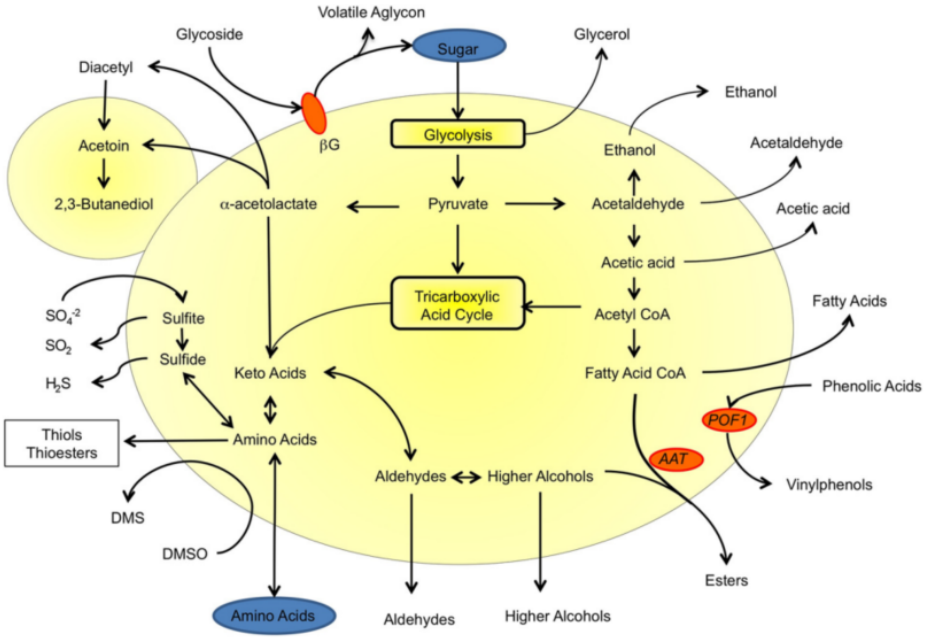


Figure 1.8: Simplified scheme of the main metabolic activities and pathways by *Saccharomyces* influencing beer quality and flavor. βG, β-glycosidase; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide. From Bokulich and Bamforth (2013).

Beer embodies around 30-40 g/L non-volatile compounds, such as sugars (80-85%), proteins, hops, metals, vitamins and color compounds. Yeast can utilize lower sugars like sucrose, glucose, fructose, maltose and maltotriose, but cannot ferment oligosaccharides of more than three glucose units. Thus, only small amounts of lower sugars remains when the fermentation is complete. The knowledge of how fermentable sugars and oligosaccharides are formed and assimilated is important for quality control of beer (Lethonen & Hurme, 1994). However, many of the constituents in beer are volatile compounds. The major classes of compounds include alcohols, acids, esters, carbonyl compounds (i.e. aldehydes and ketones), bases and sulfur compounds (Maarse, 1991). They can affect the taste significantly and contribute to the overall quality of the beer even at low concentrations (Moreira et al., 2013).

1.4.1 Carbohydrate Transport and Metabolism

The wort contains a variety of small sugar units such as sucrose, glucose, fructose, maltose and maltotriose, that brewing yeasts can assimilate and metabolize. A major determinant of a successful beer fermentation is the ability of the brewing yeast to transport the fermentable sugars from the wort efficiently into the cytoplasm. The sugar uptake in yeast fermentations is however an ordered, complex and highly regulated process (Pires & Brányik, 2015). In addition to contributing to the beer sweetness, residual carbohydrates also influence physical properties, such as viscosity, that might contribute to the overall body or mouthfeel (Hughes & Baxter, 2001; C. W. Bamforth, 2005). Slow and incomplete fermentations can also affect the productivity and cost efficiency and therefore represent a considerable economic loss in industry (Querol & Fleet, 2006).

Sucrose is hydrolyzed into glucose and fructose by invertases outside of the cell, while all the other sugars are transported into the cytoplasm where they are further processed. Inside the cell, maltose and maltotriose are hydrolyzed and broken down into glucose by an α-glucosidase (Pires & Brányik, 2015). However, glucose represses the maltose and maltotriose permeases responsible for the transportation into the cell (C. W. Bamforth, 2005). Glucose has in addition a higher affinity for the permeases, and will therefore hinder the passage of fructose (Pires & Brányik, 2015). As a consequence the sugars are ordinarily utilized according to the following order; sucrose, glucose, fructose, maltose and lastly maltotriose. Nevertheless, there might be some overlap (C. W. Bamforth, 2005). An overview of the sugar uptake in brewing yeast is shown in Figure 1.9.

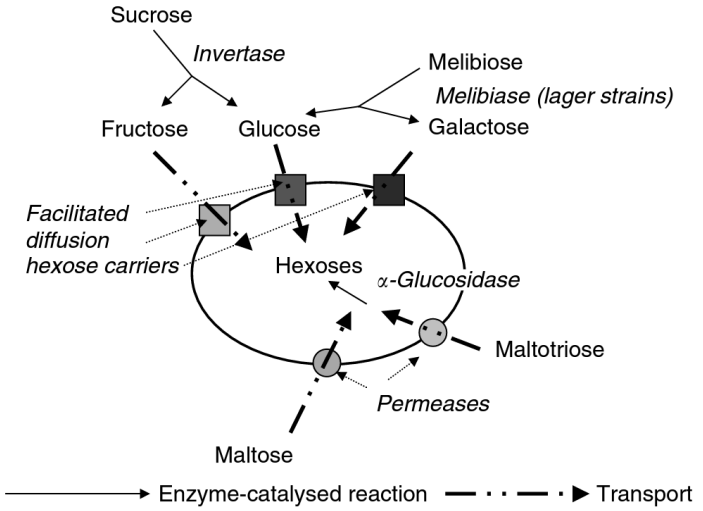


Figure 1.9: The uptake of sugars by brewing yeast. Enzyme-catalyzed reactions and transportation pathways are indicated with solid and dashed arrows, respectively. From C. W. Bamforth (2005).

Maltose and Maltotrisose Transporters

Maltose and maltotriose are the most abundant sugars in brewer's wort as they account for about 80 % of the fermentable sugars. So far, all of the α-glucoside transport systems studied have been found to be driven by the electrochemical proton gradient across the plasma membrane through H^+-symporters. The active transport of these two sugars is a major rate-limiting step of beer fermentation, and may therefore be of interest for the brewing industry in selecting suitable strains and predicting fermentation performance (Vidgren et al., 2010).

Several genes for α-glucoside transporters are present in *Saccharomyces* yeasts. The MALx1 genes (x= 1-4 and 6) occur in five unlinked maltose (MAL) loci. The α-glucoside transporter 1 (AGT1) encodes the transporter with the widest substrate specificity reported so far; it can carry trehalose, sucrose, maltose, maltotriose and α-methylglucoside (Vidgren et al., 2010). The ability of the AGT1 to efficiently transport maltotriose is of particular interest for the brewing industry as incomplete fermentation of maltotriose is a common problem (Jespersen, Cesar, & Meaden, 1999; Vidgren, Ruohonen, & Londesborough, 2005).

Vidgren et al. (2005) had previously discovered and described a frame shift in the AGT1 gene leading to a premature TGA stop codon (assumed to cause a non-functional protein product) found in lager strains. Amino acid changes between the proteins coded by the AGT1 genes have also been seen at various positions within both ale and lager strains (Vidgren et al., 2005). Vidgren, Viljanen, Mattinen, Rautio, and Londesborough (2014) have recently raised an interesting discussion about the temperature-dependent activity of AGT1. They investigated the capabilities of ale and lager strains in absorbing maltose under different temperature conditions, and proved that the activity of AGT1 was not only dependent on the temperature. Interestingly, the temperature dependence is not just related to the amino acid sequence of a particular AGT1 transporter, but also dependent on the genotype of the host yeast (mainly on the nature of plasma membrane) and on yeast-handling procedures. (Vidgren et al., 2014).

1.4.2 Alcohols

Ethanol is as mentioned one of the main fermentation products, and gives off an alcoholic odor. In addition to this, it also acts as a carrier of other odor-active volatile compounds. Besides ethanol, the major alcohols that impart sensory properties to beer are higher alcohols (also known as fusel alcohols or fusel oils), including n-propanol, isobutanol, active amyl alcohol, isoamyl alcohol and 2-phenylethyl alcohol. In fact, higher alcohols are also of great importance as they also serve as ester precursors, arguably the most important volatile compounds in beer (Hill, 2015).

Higher alcohols can be synthesized by yeasts from sugars and selected amino acids (normally branched-chain and aromatic amino acids) via the anabolic pathway and Ehrlich pathway, respectively. α-keto acids are generated from carbohydrates through de novo synthesis of amino acids in the anabolic pathway. The latter, Ehrlich pathway, forms α-keto acids from amino acid breakdown by transamination. The α-keto acids are decarboxylated with the formation of aldehydes, which are subsequently reduced to higher alcohols (Hill, 2015).

The compound glycerol is also classified as an alcohol, and can impact the taste impression because of its sweet taste and high viscosity. Glycerol is another product of yeast fermentation in the glycolytic pathway. The level of glycerol varies in different beer styles, and the production can increase in high-gravity wort fermentation as a result of osmotic stress that leads to redox imbalance and metabolic shift. In excessive levels it can affect the beer taste and mouth-feel (Hill, 2015).

1.4.3 Aldehydes

Not all aldehydes are of yeast origin, but acetaldehyde is produced as a by-product during alcoholic fermentation by brewing yeasts. In addition to being a precursor to ethanol (glycolytic pathway), it is also excreted in excess levels when abnormal physiological conditions occur, such as in high-gravity fermentation. Small amounts of branched-chain aldehydes and 2-phenylacetaldehyde might also be excreted from the catabolism of amino acids following the Ehrlich pathway described above. The contribution of aldehydes to the final beer is quantitatively rather limited because they are direct precursors to alcohols. Nonetheless, even low levels may have a great impact on the beer, and is reflected in their low detection thresholds (Hill, 2015).

1.4.4 Acids

There are both inorganic (primarily phosphoric acid) and organic acids (non-volatile and volatile) in beer that contribute to the total acidity. The acids can originate from the wort, whereas others are derived from yeast autolysis and metabolism. The main non-volatile acids include malic, citric, pyruvic, α-ketoglutaric, succinic and lactic acids, and are normally produced in small quantities. Some of the quantitatively most significant volatile acids are acetic, butyric, caproic, caprylic, capric and lauric acids (Hill, 2015).

During autolysis, brewing yeasts can give rise to longer-chain fatty acids due to membrane lipid breakdown. Especially the unsaturated fatty acids can potentially have a negative affect on the beer flavor upon oxidation. Production of non-volatile and volatile acids by yeasts is predominantly associated with glycolysis, the TCA cycle, amino acid metabolism and fatty acid metabolism (Hill, 2015).

1.4.5 Esters

Esters can have a great influence on beer aroma; possibly the most important volatile compounds. Generally, esters can be divided into two main categories; acetate esters and ethyl esters. The former typically includes ethyl acetate, isoamyl acetate and 2-phenylethyl acetate, while the latter mainly covers ethyl hexanoate and ethyl octanoate. Other esters include active amyl acetate, isobutyl acetate, ethyl butyrate, ethyl decanoate and ethyl dodecanoate, all of which may affect beer aroma, though are quantitatively minor. Esters normally impart fruity flavour notes and have a positive impact on the overall flavor in beer, but can lead to a very fruity, fermented off-flavor in excessive levels (Hill, 2015).

Brewing yeasts are undeniably the principal ester producers in beer, typically in the early phase of fermentation. Acetate esters can be produced via the reaction between an alcohol and acetyl CoA, that is catalyzed by alcohol acetyl transferase enzymes. Ethyl

esters are on the other hand formed through the reaction between ethanol and respective fatty acyl CoA. However, brewing yeasts produce predominantly ethyl esters of fatty acids (especially ethyl octanoate), with a limited formation of acetate esters (Hill, 2015).

1.5 Characteristics of Flavor and Aroma Compounds

Beer is a complex mixture with over 800 known substances detected. Not all compounds are flavor-active, and many act in synergy with other components. How much a particular constituent contribute in reality is decided by multiple factors. Its concentration, its odor and taste threshold values, and its interaction with both volatile and non-volatile constituents all play important roles. The occurrence of contaminants are also a variable capable of forming additional compounds (Maarse, 1991). The concentration of a substance where it can just be perceived is called the threshold value (Yonezawa & Fushiki, 2002). In order to develop meaningful information about flavor chemistry, it is necessary to relate the concentration of a compound to flavor thresholds to decide if it can be perceived or not. However, it is important to bear in mind that flavor threshold data varies with beer types, and are consequently not accurate for the whole range of beer types (Maarse, 1991). The typical concentration range in beer, flavor threshold and description for some of the carbohydrates, alcohols, acids and esters are listed in Table 1.1.

The flavor unit (FU) is defined as the concentration of a particular compound divided by its flavor threshold values (Maarse, 1991), as shown in Equation (1.1).

$$FU = \frac{Concentration\ compound}{Flavor\ threshold} \tag{1.1}$$

FU is a dimensionless number that can be very useful, as it provides an indication of the sensory level of a flavor attribute. FU-values of less than 1 means that the compound is below its threshold. Accordingly, the compound should in theory be detectable when it is at 1-2 FUs (at 1 FU a compound is at its threshold). When the level exceeds 2 FU it is likely to have major effect on the sensory properties of the product (Hughes & Baxter, 2001). Nonetheless, compounds present below 0.1 FU may still be flavor-active as a result of synergistic and potential effects when interacting with other compounds. In contrast, compounds may also become less flavor-active due to antagonistic effects (Maarse, 1991).

The sensory characteristics, for any food or beverage, are a vital parameter by which consumers evaluate a product (Hughes & Baxter, 2001). Recent years have brought a remarkable increase and interest in specialty beer, making product diversification crucial for success in order to fulfill the customer's demand (Mertens et al., 2015). Thus, analyzing the chemical composition and properties in beer is of great interest. The aim of performing a flavor analysis in beer is for quality control, but also quantitative descriptions to characterize the various beer types and styles (Maarse, 1991). The principal techniques for measuring non-volatile and volatile components are high-performance liquid chromatography (HPLC) and gas chromatography (GC) (C. Bamforth, 2016).

Table 1.1: Typical concentration range, flavor threshold values and flavor descriptions of common compounds in beer. Source: Hughes and Baxter (2001), Maarse (1991), Hough, Briggs, Stevens, and Young (1982), referred to as number 1, 2 and 3, respectively.

Carbohydrates		Concentration range (g/L)			Ref.
C_6	Fructose	0-0.19			1
	Glucose	0.04-1.1			1
C_{12}	Sucrose	0-3.3			1
	Maltose	0.7-3.0			1
C_{18}	Maltotriose	0.4-3.4			1
Alcohols		**Concentration range (mg/L)**	**Threshold (mg/L)**	**Flavor description**	**Ref.**
C_2	Ethanol	20 000-80 000	14 000	Alcoholic, strong	1
	Glycerol	1 200-2 000		Sweetish, viscous	1
C_3	1-Propanol	3-16	700	Alcoholic	1
C_4	2-Methyl-1-propanol; Isobutyl alcohol	4-56.6	100; 160; 200	Alcoholic	2
C_5	2-Methyl-1-butanol; Active amyl alcohol	7-33.9	65	Alcoholic, vinous, banana-like, iodoform-like	2
	3-Methyl-1-butanol; Isoamyl alcohol	25-122.5	70	Alcoholic, vinous, banana-like, sweet	2
Acids		**Concentration range (mg/L)**	**Threshold (mg/L)**	**Flavor description**	**Ref.**
C_2	Acetic acid	30-280	175; 200	Acetic, acidic, vinegar-like	2
C_4	Succinic acid	16-140		Acid, salt, forage	1
Esters		**Concentration range (mg/L)**	**Threshold (mg/L)**	**Flavor description**	**Ref.**
C_4	Ethyl acetate	10-60	30	Solvent-like, sweet	1
C_5	Ethyl propionate	0.03-0.2	1-10		2
	Propyl acetate		30	Pear	3
C_6	2-Methyl-1-propyl acetate; Isobutyl acetate	0.01-0.25	1.6	Banana-like, sweet fruity	2
C_7	2-Metyl-1-butyl acetate; 1-Butanol	0.6-4	1.2	Banana-like, estery, solvent-like, sweet	2
	3-Methyl-1-butanol acetate; Isoamyl acetate	0.5-5.0	1	Banana, ester, solvent	1
	Ethyl butanoate; Ethyl butyrate	0.04-0.2	0.4	Papaya-like, buttery, apple-like, perfumy	2
C_8	Ethyl hexanoate	0.1-1.5	0.2	Apple, fruity, sweet	1
C_{10}	Ethyl octanoate	0.1-1.5	0.5	Apple, tropical fruit, sweet	1
C_{12}	Ethyl decanoate	0.01-1.0	0.57; 1.5	Caprylic, fruity, apple-like, solvent-like	2
Acetals		**Concentration range (mg/L)**	**Threshold (mg/L)**	**Flavor description**	**Ref.**
C_6	1,1-Diethoxyethane; Acetal	1	4		2

1.6 Beer Spoilage Organisms

Any organism that has not intentionally been introduced to a beer is in principle considered a spoilage organism. For this reason, both *Saccharomyces* and non-*Saccharomyces* species can be recognized as spoilage organisms. For instance, they can spoil beer through the production of off-flavors (especially organic acids, esters and phenols), formation of haze, sediment or superattenuation, leading to overcarbonation and diminished body. Many non-*Saccharomyces* yeasts are capable of growing in beer, however, their spoilage potential is limited during optimal storage conditions. This is mainly due to oxygen limitation, ethanol toxicity and competition with *Saccharomyces* (Bokulich & Bamforth, 2013).

Considering all raw materials, the pitching of yeast is the most likely source of contamination as it is added after wort boiling. Overall, Gram-positive bacteria are inhibited by the hop constituents, and consequently they do not grow in beer. Yet some lactic acid bacteria (LAB) species (primarily lactobacilli and pediococci) are hop-resistant, and can grow in the harsh environment of beer (Hill, 2015). Studies suggest that these are specialized for growth in beer through resistance to oxidative and acid stress (Bokulich & Bamforth, 2013). The most common contaminants are those from the genera of *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Megasphaera*, besides the less frequent bacteria, acetic acid bacteria, some *Enterobacteria* and *Zymomonas* (Hill, 2015).

LAB can affect the beer appearance and flavor, and have both negative and positive impacts on beer quality. High turbidity, sedimentation, acidification and off-flavour typically through diacetyl production are common negative effects. In comparison, LAB play a beneficial role in terms of acidity. Acidification can for instance lower the risk of protein haze formation and other microbial contamination. Other benefits are finer foam bubbles and stable, longer-lasting foam, fresher mouth-feel, smoother bitterness and fuller flavour profile (Hill, 2015).

1.6.1 Detection of Brewery Spoilage Organisms

Many regularly test for the presence of beer spoilage bacteria, but do not test for wild yeast. Both categories can be detected by traditional plate checks with media containing inhibitors or stimulators (or in some cases both), using various incubation conditions. Lysine, copper sulfate or cycloheximide are commonly used chemicals. More than one medium is necessary to detect the broad range of possible contaminants, as no single method or medium reported so far can detect all members (Jespersen & Jakobsen, 1996; Hill, 2015).

Chapter 2

Materials and Methods

14 different NFAY cultures collected from Western Norway, with a total of 24 yeast isolates were investigated in this study. An overview of the samples as well as the origin of these is shown in Table 2.1. An additional 15 reference yeasts were also included in the research, and were divided into three categories; wild, laboratory and commercial. The reference yeasts are shown in Table 2.2. These were selected for several purposes. Comparing genetics and the fermentation performance of NFAY to wild and commercial yeasts could provide information regarding domestication. Laboratory yeasts were mainly introduced to be reference strains of known ploidy in the flow cytometer analysis.

Laboratory strains of the BY-series (S288C genetic background) were obtained from Euroscarf (http://www.euroscarf.de/), while the NCYC strains were retrieved from the National Collection of Yeast Cultures (NCYC) (http://www.ncyc.co.uk/). The commercial strains from the WLP-series were obtained from White Labs (http://www.whitelabs.com/).

2.1 Sequencing of the ITS, LSU and AGT1 Regions

The species differentiation of the samples was based on sequencing of the ITS region and partial sequencing of LSU. Two regions were chosen on the basis of being the most variable domains of LSU within the *Saccharomyces* genus. These will simply be referred to as LSU1 and LSU2 further on, and the regions of the LSU they span is shown in Figure 2.1. The presence of *S. cerevisiae*-type AGT1 genes was also investigated.

Figure 2.1: The LSU region was partially sequenced. The map shows the regions of the large subunit referred to as LSU1 and LSU2, where LSU1 covers the D1/D2 domain.

Table 2.1: Overview of the Norwegian Farmhouse Ale Yeast (NFAY) cultures studied. A few pure isolates from each culture were selected for further analysis. The name of the samples and the place of origin is listed in the table.

Yeast culture	Sample	Origin (place, county)
NFAY 1	NFAY 1_P1	Hornindal, Sogn og Fjordane
NFAY 2	NFAY 2_P1 NFAY 2_P2	Granvin, Hardanger
NFAY 3	NFAY 3.5 NFAY 3.7	Hornindal, Sogn og Fjordane
NFAY 4	NFAY 4.1	Olden, Sogn og Fjordane
NFAY 5	NFAY 5_P1	Hornindal, Sogn og Fjordane
NFAY 6	NFAY 6.2 NFAY 6.20	Voss, Hordaland
NFAY 7	NFAY 7.15 NFAY 7.24	Voss, Hordaland
NFAY 8	NFAY 8_P1	Stranda, Møre og Romsdal
NFAY 9	NFAY 9.8 NFAY 9.23 NFAY 9.24	Sykkylven, Møre og Romsdal
NFAY 10	NFAY 10_P1	Ljøsne, Sogn og Fjordane
NFAY 14	NFAY 14_P1 NFAY 14_P2	Skodje, Møre og Romsdal
NFAY 15	NFAY 15_P1 NFAY 15_P2	Hornindal, Sogn og Fjordane
NFAY 16	NFAY 16_P1 NFAY 16_P2	Årset, Møre og Romsdal
NFAY 20	NFAY 20_P1 NFAY 20_P2	Sandnes, Rogaland

Table 2.2: Overview of the wild, laboratory and commercial yeast samples included in the study. Sample name, description and source is listed in the table.

Category	Sample	Species	Origin (if known)
Wild	G518	*S. cerevisiae*	Rose hip from Stjørdalen, Nord-Trøndelag
	G561	*S. cerevisiae*	Rose hip from Stjørdalen, Nord-Trøndelag
	G562	*S. cerevisiae*	Rose hip from Stjørdalen, Nord-Trøndelag
Laboratory	BY4741	*S. cerevisiae*	S288C Isogenic yeast strain: *MATa*
	BY4743	*S. cerevisiae*	S288C Isogenic yeast strain: *MATa/*α
Commercial	NCYC361	*S. cerevisiae*	NCYC Collection
	NCYC456	*S. pastorianus*	NCYC Collection
Laboratory	NCYC660	*S. cerevisiae*	NCYC Collection
	NCYC661	*S. cerevisiae*	NCYC Collection
Commercial	WLP013	*S. cerevisiae*	WLP Collection
	WLP028	*S. cerevisiae*	WLP Collection
	WLP051	*S. cerevisiae*	WLP Collection
	WLP500	*S. cerevisiae*	WLP Collection
	WLP566	*S. cerevisiae*	WLP Collection
	Idun	*S. cerevisiae*	Idun Baker's yeast

Before carrying out any genetic analyses, the yeast samples were grown on 5% malt extract (ME) agar plates using the streak-plating technique with a three-phase streaking pattern to isolate a single colony. These were inoculated in 5% ME liquid media, and transferred to an incubator with shaking (150 rpm, 22 °C) until fully grown. The process of obtaining sequencing data from the ITS, LSU and AGT1 regions involve extracting DNA, running PCR, confirming and purifying PCR product. Each of these steps will be elaborated on in this section.

2.1.1 DNA Extraction

DNA from each sample was extracted by two methods; boiling and using a UltraClean Microbial DNA Isolation Kit. The boiling method is a rapid and inexpensive yeast DNA extraction that does not require any chemical reagents or any purification procedure. A brief experimentation was carried out with different combinations of cell concentration (in volumes of 20 and 50 µL), incubation time (5, 10 and 15 min) and temperature (95 °C and 99 °C), to decide which one gave the best DNA yield. This led to the the following protocol: A loopful (i.e. the amount held in a loop) of cell suspension to obtain roughly 10^{6-7} cells/mL was transferred to a 1.5 mL eppendorf tube with 50 µL of sterile water. The tube was put in a dry heat block (QBD series, Grant) at 95 °C for 10 min. The suspension with DNA was thereafter vigorously homogenized by vortex and cooled down. DNA samples obtained from this method were used in PCR reactions immediately.

The DNA extraction using the UltraClean Microbial DNA Isolation Kit was performed according to the protocol provided by the supplier, and is attached in Appendix A. The DNA concentration was determined with NanoDrop, and the samples were stored at -20 °C after use. This turned out to be the preferred method to obtain a high grade PCR product for the AGT1 region.

2.1.2 Polymerase Chain Reaction

A target sequence of DNA can be hugely amplified with an *in vitro* DNA replication method named PCR. The procedure requires a DNA template with the region of interest and a free 3'-OH (provided by site-specific oligonucleotide primers) to get the polymerase started. The primers are complementary to each of the ends of the target sequence. PCR can be divided into three major steps; denaturation, annealing of primers and primer extension. In the fist step, the DNA is heated to denature the template strands, that is, to obtain single-stranded DNA. During the next step, the DNA is cooled down to allow the primers to anneal, i.e. to bind the appropriate complementary strand. The annealing temperature varies depending on the size of the primer, the GC-content and its homology to the target DNA. At last, primer extension is executed by DNA polymerase on both strands from the 5' to 3' direction by its polymerase activity in presence of Mg^{2+} at 72 °C. The most common enzyme is *Taq* polymerase (from the thermophilic bacteria *Thermus aquaticus*). These three steps are normally repeated 28-35 times. More and more fragments are generated with each cycle, as they accumulate exponentially (Allison, 2007).

The primers used in this study to amplify the ITS, LSU and AGT1 regions, are listed in Table 2.3 along with their sequence and melting temperature, T_m. Initially, the universal primers ITS1-F_KYO2 and ITS4-R were used to amplify the ITS region. However, as some problems arose with the sequencing of this particular region for some of the samples, the forward primer (ITS1-F_KYO2) was eventually replaced by ITS5-F. ITS5-F and ITS4-R seemed to be a better match as primer pairs in terms of melting temperature. The LSU1 region was amplified with the primer pair LSU1-F and LSU1-R, while LSU2-F and LSU2-R were used for LSU2. The primers AGT1-F and AGT1-R were used to generate the *S. cerevisiae*-type AGT1 gene.

Table 2.3: PCR primers used to amplify the regions ITS, LSU1, LSU2 and AGT1. The primer sequence, melting temperature (T_m) and the source is stated for each primer.

Name[a]	Primer sequence	T_m [°C][b]	Reference[c]
ITS1-F_KYO2	5'-TAGAGGAAGTAAAAGTCGTAA-3'	52.6	1
ITS5-F	5'-GGAAGTAAAAGTCGTAACAAGG-3'	58.4	2
ITS4-R	5'-TCCTCCGCTTATTGATATGC-3'	61.5	2
LSU1-F	5'-TTGCCTTAGTAACGGCGA-3'	62.0	This study
LSU1-R	5'-TTGTGCACCTCTTGCGAG-3'	64.1	This study
LSU2-F	5'-GGGTTGATATGATGCCC-3'	58.6	This study
LSU2-R	5'-TTCCCCTTGTCCGTACC-3'	61.0	This study
AGT1-F	5'-TTGCTTTACAATGGATTTGGC-3'	63.5	3
AGT1-R	5'-CTCGCTGTTTTATGCTTGAGG-3'	63.7	3

[a] F = forward; R = reverse.

[b] The melting temperature, T_m, was calculated by the supplier Sigma-Aldrich using the nearest neighbor method (Rychlik, Spencer, & Rhoads, 1990).

[c] Source: Toju, Tanabe, Yamamoto, and Sato (2012), White, Bruns, Lee, and Taylor (1990) and Jespersen, Cesar, and Meaden (1999), referred to as number 1, 2 and 3, respectively.

PCR reactions were performed using standard procedures, with a 25 µL reaction volume in 0.2 mL PCR microtubes. The reaction volumes of the reagents used for one sample

is shown in Table 2.4. A master mix was created with everything listed in the table besides DNA template (upscaled to the number of samples in the run), adding Taq DNA polymerase at last. The mixture was then vortexed and divided equally by adding 24 µL to each of the microtubes. The tubes were placed in an Eppendorf® PCR Cooler to stop reactions during the preparation stage. 1 µL of DNA template (extracted by boiling or using the UltraClean Microbial DNA Isolation Kit) was added to the reaction mix in the tube. Negative controls with MQ-water instead of DNA-template were used in every experiment to test for the presence of contamination in the reagents. The microtubes were thereafter placed in a mini centrifuge (Galaxy Mini, VWR) and spinned down for a few seconds prior to running, to remove air bubbles and liquid on the sides of the tubes. The amplification conditions listed in Table 2.5 were programmed into Thermo Scientific Arktik Thermal Cycler PCR apparatus for the appropriate region (ITS, LSU1, LSU2 or AGT1), and the samples were placed in the block. The PCR products were put in the fridge at 4 °C for short-time storage until post-PCR steps were applied.

Table 2.4: PCR reaction volumes [µL] of reagents added to *one* microtube. The volumes in the master mix (containing all except DNA template) were upscaled by multiplying them with the number of samples planned for the run.

Name	Volume [µL]	Final concentration
10x Reaction buffer with $MgCl_2$	2.5	1x
10 mM dNTP	0.5	200 µM
BSA	0.75	
100 µM Forward primer	0.125	0.5 µM
100 µM Reverse primer	0.125	0.5 µM
Taq DNA polymerase	0.125	
DNA template	1.0	1 ng/µL
Filtered MQ-water	19.875	
Total	25.0	

Table 2.5: PCR amplification conditions for the different regions; ITS, LSU1, LSU2 and AGT1. The temperature and time duration for each step are listed in the tables, as well as the number of cycles. These were programmed into the thermal cycler apparatus.

			1 Cycle[a]								
	Initialization		Denaturation		Annealing		Elongation		Final elongation		No. of
Region	T [°C]	Time	T [°C]	Time	T [°C]	Time	T [°C]	Time	T [°C]	Time	cycles
ITS (1)[b]	95	10 min	94	20 sec	47	30 sec	72	40 sec	72	7 min	35
ITS (2)[b]	96	2 min	96	1 min	55	1 min	72	2 min	72	2 min	35
LSU1	94	10 min	94	45 sec	52	45 sec	72	1 min	72	10 min	35
LSU2	94	10 min	94	45 sec	52	45 sec	72	1 min	72	10 min	35
AGT1	94	3 min	94	1 min	58	2 min	72	2 min	72	10 min	30

[a] The conditions for the three main PCR steps (denaturation, annealing and elongation) is shown for 1 cycle. This was repeated for the number of cycles listed in the last column.

[b] The conditions used for (1) the primers ITS1-F_KYO2 and ITS4-R, that was later replaced by (2) ITS5-F and ITS4-R.

2.1.3 Post-PCR Steps

PCR products were examined by electrophoresis at 140 V for 45 minutes. They were separated on a 1% (w/v) agarose gel with GelRed nucleic acid gel stain (Biotium 41003) to confirm product before purification. The gel was prepared by dissolving agarose (Lonza, SeaKem LE) in 1x TAE buffer and adding 5 µL of GelRed per 100 mL of gel made. 5 µL PCR product with 1 µL 6x DNA loading buffer dye (Thermo scientific) were loaded into the wells. A 1 Kb Plus DNA ladder marker (Thermo scientific GeneRuler) served as the size standard. After electrophoresis, the gel was visualized and examined under UV light. G:box (Syngene) was used to capture images of the gel with the software GeneSnap. An image of one of the agarose gels after electrophoresis, including the ladder, is shown in Figure A.1 in Appendix A.

After PCR product had been confirmed, it was followed by a purification procedure with QIAquick PCR Purification Kit to remove fragments smaller than 40 bases. The purification was performed according to the protocol provided by the supplier, and is attached in Appendix A. However, a few extra measures that showed improvement were added to the steps in the protocol. This includes transferring the QIAquick column to a clean 1.5 mL microcentrifuge tube instead of placing it in the same tube (step 7), and heating the Buffer EB to 60 °C before eluting the DNA by adding 30 µL of buffer (step 9). NanoDrop was again used to check the DNA concentration after purification.

The samples were prepared for sequencing by adding 5 µL of purified sample with the concentration ranging from 20-80 ng/µL to two separate 1.5 mL eppendorf tubes. 5 µL of 5 µM forward- and reverse-primer of the target region was added to each tube, that were later marked with barcodes. Ultimately, the tubes were sent to GATC Biotech (https://www.gatc-biotech.com/en/index.html) for LIGHTrun Sanger sequencing. The process of extracting DNA and sending it for sequencing can be summarized in a simplified flowchart shown in Figure 2.2.

2.1.4 Analyzing the Sequencing Data

The software Chromas was used to manually inspect and quality control the sequencing chromatograms. An example of the chromatograms obtained is included in Figure A.2 in Appendix A.2. The FASTA files of the forward and reverse primers received from GATC Biotech were further processed in the software Clone Manager Professional 9. A consensus region was made by making a pairwise alignment of the forward primer sequence and reverse complement sequence of the reverse primer, by editing the alignment until a 100% match was obtained. Degenerate base symbols was used for incompletely specified bases as suggested by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) proposed by Comnish-Bowden (1985). The symbols with the associated description are listed in Appendix A.2 in Table A.1.

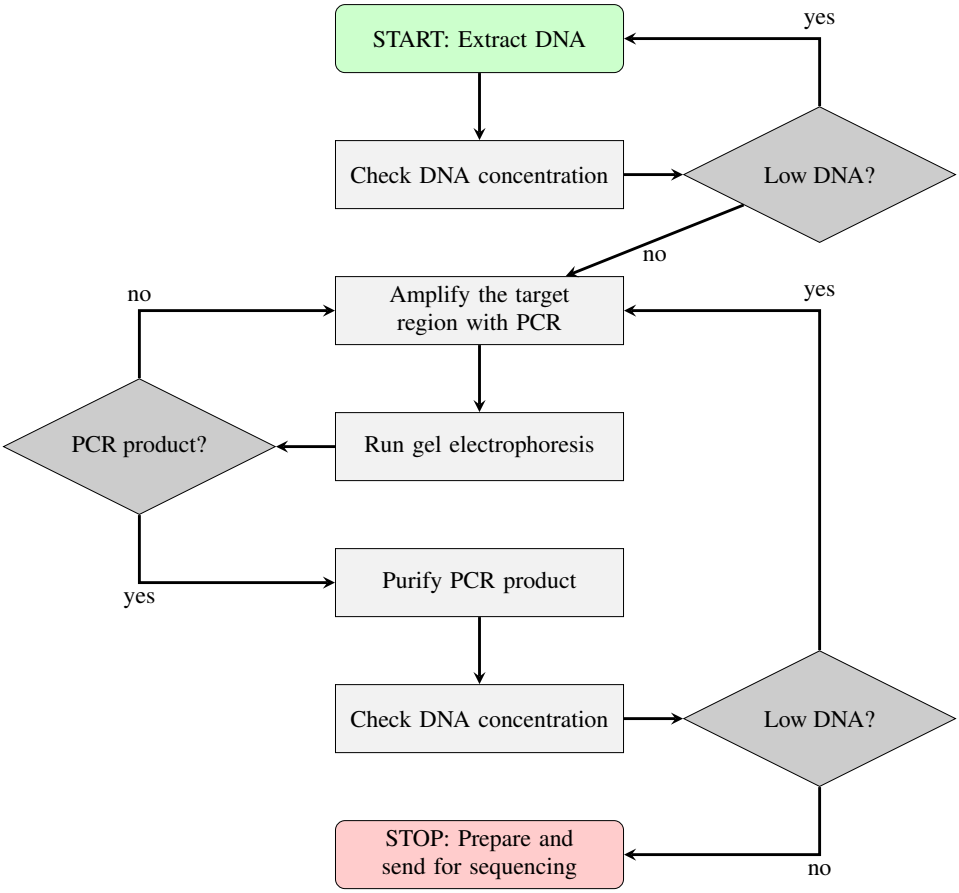


Figure 2.2: Flowchart of the process from isolating DNA to sending it for sequencing to the company GATC Biotech.

Identification and Classification

The reviewed sequences for the ITS and LSU regions were uploaded to the RDP (http://rdp.cme.msu.edu/classifier/classifier.jsp) for identification and classification. The 'Warcup Fungal ITS trainset 2' was used for the ITS region, while 'Fungal LSU training set 11' was applied to LSU1 and LSU2. All entries were run with the default confidence threshold of 80%. According to this set threshold, sequences with lower score are considered unclassified.

Phylogenetic Relationships

Evolutionary history can be recovered and represented as a graphical structure called a *phylogenetic tree* based on multiple alignment of sequence data. This rest on the general assumption that the more similar two sequences are, the more closely related they will be.

Methods for tree reconstruction can be divided into two broad groups based on distance or character. A tree based on distance is created by deriving a distance measure from each aligned pair of sequences, while the latter is obtained by evaluating all sequences at each alignment site separately. There are numerous methods and algorithms for tree construction, but in general non-distance methods are preferred as they produce results based on a stronger statistical foundation. A common character method is the maximum likelihood (ML) method. It estimates the likelihood of a given tree topology to have produced the observed data assuming a given model of evolution. This approach is considered to be more advanced, computationally intensive and accurate than distance-based methods or parsimony (Zvelebil & Baum, 2008).

A multiple sequence alignment (MSA) was applied to the collection of sequences obtained for ITS, LSU1, LSU2 and AGT1 using MUltiple Sequence Comparison by Log-Expectation (MUSCLE), and a phylogenetic tree was constructed using the ML method in the software MEGA7. The AGT1 region was run against the reference AGT1 sequence in the *Saccharomyces* genome database (SGD) (http://www.yeastgenome.org). AGT1 is entered as MAL11 in the database because it is the allele of MAL11 present in the S288C strain of the SGD.

2.2 DNA Content Determination by Flow Cytometry

The evaluation of ploidy was based on the protocol described by van den Broek et al. (2015). The total DNA content can be estimated by comparison with the total DNA content of *Saccharomyces cerevisiae* strains of known ploidy. The DNA content can be determined rapidly using flow cytometry. When samples are treated with a dye that form a fluorescent complex with DNA, the fluorescence intensity can be measured by the instrument. A linear correlation is expected between the fluorescence peaks corresponding to the original ploidy of the control strains and to its doubling. Thus, a relationship between fluorescence intensity and ploidy can be established (van den Broek et al., 2015). The control strains used in this experiment is listed in Table 2.6.

Table 2.6: *Saccharomyces cerevisiae* control strains with known ploidy used to establish a relationship between fluorescence intensity and DNA content.

Ploidy	Strain	Species	Source or reference(s)
Haploid (n)	BY4741	*S. cerevisiae*	Brachmann et al. (1998)
Diploid (2n)	BY4743	*S. cerevisiae*	Brachmann et al. (1998)
Tetraploid (4n)	NCYC660	*S. cerevisiae*	Mortimer (1958)
Hexaploid (6n)	NCYC661	*S. cerevisiae*	Mortimer (1958)

Cultivation and Fixation of Cells

Samples were grown as pure cultures in 5% ME media in shake flasks (150 rpm in a shaker; INFORS HT Minitron) at 22 °C. Samples were inoculated to make starter cultures, and grown overnight until the cells were in early stationary phase. An inoculum

that provided a final concentration of around 1×10^4 cells/mL was added to a larger culture. Approximately 10^7 cells were taken from mid-exponential phase. This was done by counting cells with a hemocytometer (example shown in Appendix B.1) and growing cells to 0.2-0.3 optical density $(OD)_{600}$. The cells were thereafter collected by centrifugation (5 min, 4,500 $\times$ g). The pellet was washed with cold filtered milli-Q (MQ) water, gently vortexed, centrifuged again (5 min, 4,500 $\times$ g), and suspended in 800 µL filtered 70% ethanol while vortexing. Then, another 800 µL of 70% ethanol was added using the same technique. Fixed cells were stored at 4 °C until further staining and analysis.

A time series of strain BY4743 was also made to see if the cell size distribution was affected at different growth stages (that might differ in physiological state) when running cell sorting on flow cytometry. Around 10^7 cells were taken after 0, 24, 48 and 72 hrs, and fixated using the same protocol as described above.

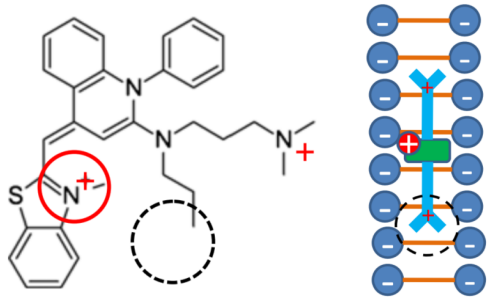
Dilution Agent

DNA flow cytometry requires suspensions of intact nuclei that are stained with a DNA-specific fluorochrome. Various buffer formulas have been developed in the interest of preserving the nuclear integrity, protect DNA from degradation and facilitate its stoichiometric staining. Common organic buffers used in nuclear isolation buffers include 3-(N-morpholino) propanesulfonic acid (MOPS), 4-(hydroxymethyl)piperazine-1-ethanesulfonic acid (HEPES) and Tris-(hydroxymethyl)-aminomethane (Tris). The organic buffering substances allows the stabilization of a pH between 7 and 8, which is the pH range compatible to most of the fluorochromes. When Loureiro, Rodriguez, Doležel, and Santos (2006) investigated the performance of buffers in plant DNA flow cytometry, they observed clear differences among buffers. Species were selected to cover a wide range of genome sizes, though none of the buffers worked best with all of these species (Loureiro et al., 2006). Bearing this in mind, it seems plausible that this may also be the case for yeast species. Hence, testing several buffers to find the best fit will give a greater experimental precision.

The performance of MOPS, HEPES and tris-EDTA (TE) buffer as the dilution agent was tested and compared to results obtained using simply filtered MQ-water. Solutions of 10 mM MOPS and 10 mM HEPES were used in the experiment. A 1x TE buffer was prepared with a final concentration of 10 mM Tris (adjusted to pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA). All buffer solutions were filtered through a 0.22 µm filter and stored at 4 °C.

Staining with SYBR® Green I

Delobel and Tesnière (2014) suggested that SYBR® Green I (SG) is the preferred fluorescent dye when accurate determination of cell DNA content is required, which is required for DNA ploidy application. Favorable photophysical properties, temperature stability, selectivity for dsDNA and high sensitivity are some of its excellent properties (Zipper, Brunner, Bernhagen, & Vitzthum, 2004). Figure 2.3 shows the chemical structure of SG and how it interacts with DNA.



(a) Structure of SG **(b)** SG/DNA complex

Figure 2.3: Chemical structure of SYBR® Green I (SG; left), and a model of the SG/DNA complex (right) showing the electro-static interaction with the DNA phosphate group. Modified after Dragan et al. (2012).

The fixed cells were diluted with respect to a cell count equal to <1000 cells/µL in TE-buffer. The DNA was stained with SG Gel Stain, 10 000x in DMSO (Life Technologies, Thermo Fisher Scientific Inc.). The stock solution was diluted 1:50 using the same diluting agent as before. 10 µL of this was added to 1 ml diluted sample, vortexed, covered with aluminium foil and incubated in the dark for 15 minutes. The solution was filtered with a 10 µm non-sterile syringe filcon (BD Biosciences) and transferred to a new tube. After being thoroughly vortexed again, samples were immediately analyzed in the cytometer.

The influence of dye concentration was also investigated briefly. This was to see if being exposed to increasing concentration of dye would further increase the fluorescence in the cells or if saturation occurs. Another motivation was to make sure that sufficient amounts of dye was added in respect to the amount of cells. The rations 1:10, 1:20 and 1:50 between SG stock solution and dilution agent were tested in the assay.

Flow Cytometry

Experiments were carried out using a BD Accuri C6™flow cytometer. The performance of the instrument was validated using 6- and 8-peak fluorescent bead mixtures provided by the manufacturer before every run. This ensures that lasers and detectors are working, and that there are no bubbles/clogs or contaminations in the system. A new sheet fluid solution was made every time the flow cytometer was used, by mixing 1 L of MQ water and 1 bottle of bacteriostatic solution. The cleaning solution was made new every 14th day, and a decontamination solution was created when empty.

Analysis was based on light-scatter and fluorescence signals produced from a 20 mW laser illumination at 488 nm. Signals corresponding to forward angle and 90°-side scatter (FSC and SSC, respectively) and fluorescence were accumulated. Fluorescence of cells stained with SG was recovered in the FL1 channel (533/30 nm). The run limit was set to 25,000 events and the flow speed was set to be slow to improve sensitivity. The detection was performed with a threshold value of 80,000 (default) without gating. Samples were run in the manually run mode so that they could be vortexed thoroughly right before being analyzed, to prevent cell sedimentation.

2.3 The Brewing Process and Fermentation

2.3.1 Making the Wort

A batch of an American style IPA was brewed in a 55 L Braumeister Speidel. First, the Braumeister was filled with cold tap water and $CaSO_4$, chalk and $MgSO_4$ was added. The mash and temperature profile showing a more detailed description of the brewing process can be found in Figure 2.4. The water was heated to 66.0 °C before the malt was stirred in. A pH measurement was done at this point to ensure that the mash was close to a pH-value of 5.20. After the mashing steps, the malt pipe was raised and 10 L of pre-heated water (80 °C) was carefully sparkled over the mixture. The wort was brought to a boil, and the zinc sulfate was added. The kettle was equipped with a hop filter, and the hops was added at the appropriate time during boiling as indicated in Figure 2.4. Two whirlfloc tablets that facilitates the precipitation of haze-causing materials were added 15 minutes before the end of the boiling process. The wort was cooled down to room temperature and frozen overnight.

2.3.2 Fermentation Conditions

To investigate the fermentation performance of NFAY, wild, laboratory and commercial yeasts, two fermentation temperatures were chosen; 22 °C and 35 °C. This was mainly due to the fact that some NFAY cultures are believed to have a high fermentation temperature tolerance. However, because of the comprehensive amount of yeast samples, the experiment was carried out in small-scale fermentation tubes. 50 mL Falcon tubes were filled with 30 mL wort and secured with fermentation lids designed for this setup. The lids were made by leading needles through plastic caps, creating a tight seal. The needle tip was preheated with a Bunsen burner to be able to pass through and melt the plastic. Fermentation lids and cotton balls were autoclaved and baked until dry. The cotton balls were placed inside of the needle dispenser. This way gas could escape the tubes during fermentation but not enter i.e. making the conditions anaerobic. A photograph of a fermentation tube is shown in Figure 2.5.

Figure 2.5: Fermentation tube with modified plastic lid.

Yeasts were transferred from a starter culture to new 5% ME media and grown overnight before added to the wort. 1×10^6 cells/mL of wort per degree Plato served as a guide for yeast pitching rate. The yeast cells were enumerated using a hemocytometer, in order to add approximately the same amount of cells (to create similar conditions). An example calculation showing how to determine the concentration for one yeast culture is shown in Appendix B.1. The fermentation tubes (with lids) were weighed both empty and after the addition of wort and yeast. The fermentation went on for 14 days. As part of the monitoring of this process, the tubes were weighed the last four days to make sure that the fermentation had finished. This is confirmed by small to no variation in weight.

MSc2017 IPA

American IPA (14 B)

Type: All Grain
Batch Size: 44,00 l
Boil Size: 55,34 l
Boil Time: 90 min
End of Boil Vol: 47,84 l
Final Bottling Vol: 43,00 l
Fermentation: Ale, Two Stage
Taste Notes:

Date: 13 Feb 2017
Brewer:
Asst Brewer:
Equipment: Braumeister 50L
Efficiency: 74,00 %
Est Mash Efficiency: 77,6 %
Taste Rating: 30,0

Ingredients

Amt	Name	Type	#	%/IBU
55,00 l	Jonsvatnet, Trondheim IPA	Water	1	-
22,00 g	Gypsum (Calcium Sulfate) (Mash 60,0 mins)	Water Agent	2	-
5,50 g	Baking Soda (Mash 60,0 mins)	Water Agent	3	-
5,50 g	Chalk (Mash 60,0 mins)	Water Agent	4	-
1,10 g	Epsom Salt (MgSO4) (Mash 60,0 mins)	Water Agent	5	-
9,00 kg	CHATEAU PALE ALE (8,5 EBC)	Grain	6	70,6 %
1,75 kg	Wheat (6,0 EBC)	Grain	7	13,7 %
1,50 kg	Caramel/Crystal Malt - 30L (59,1 EBC)	Grain	8	11,8 %
0,50 kg	Corn Sugar (Dextrose) (0,0 EBC)	Sugar	9	3,9 %
0,02 g	Zinc sulfate (ZnSO4 x 7H2O) (Boil 90,0 mins)	Water Agent	10	-
100,00 g	Target [9,00 %] - Boil 60,0 min	Hop	11	46,6 IBUs
50,00 g	Chinook [13,00 %] - Boil 15,0 min	Hop	12	16,7 IBUs
2,0 pkg	Safale American (DCL/Fermentis #US-05) [50,28 ml]	Yeast	13	-
150,00 g	Chinook [13,00 %] - Dry Hop 7,0 Days	Hop	14	0,0 IBUs

Gravity, Alcohol Content and Color

Est Original Gravity: 1,068 SG
Est Final Gravity: 1,011 SG
Estimated Alcohol by Vol: 7,5 %
Bitterness: 63,3 IBUs
Est Color: 19,8 EBC

Measured Original Gravity: 1,046 SG
Measured Final Gravity: 1,010 SG
Actual Alcohol by Vol: 4,7 %
Calories: 427,1 kcal/l

Mash Profile

Mash Name: Single Infusion, Light Body, No Mash Out
Sparge Water: 35,66 l
Sparge Temperature: 75,6 C
Adjust Temp for Equipment: TRUE

Total Grain Weight: 12,75 kg
Grain Temperature: 22,2 C
Tun Temperature: 22,2 C
Mash PH: 5,20

Mash Steps

Name	Description	Step Temperature	Step Time
Mash In	Add 32,45 l of water at 77,7 C	65,6 C	75 min

Sparge: Fly sparge with 35,66 l water at 75,6 C
Mash Notes: Simple single infusion mash for use with most modern well modified grains (about 95% of the time).

Carbonation and Storage

Carbonation Type: Bottle
Pressure/Weight: 252,93 g
Keg/Bottling Temperature: 21,1 C
Fermentation: Ale, Two Stage

Volumes of CO2: 2,3
Carbonation Used: Bottle with 252,93 g Corn Sugar
Age for: 30,00 days
Storage Temperature: 18,3 C

Notes

Created with BeerSmith

Figure 2.4: Mash and temperature profile of an American style IPA beer. The layout was made in the software BeerSmith.

2.3.3 Post-fermentation Steps

After the fermentation was done, the fermentation lids were replaced with regular ones. The tubes were centrifuged at maximum speed, 4500 g, for 5 min at 4 °C. The beer (supernatant) was poured carefully into new 50 mL Falcon tubes, making sure no cells from the pellet were transferred in the process. The pH value of the finished beer product was measured with a pH-meter. 1 mL and 100 µL of the beer was transferred to 1.5 mL eppendorf tubes for a HPLC and a liquid chromatography-mass spectrometry (LC-MS) analysis, respectively, later on. The rest of the beer was stored at -80 °C for a headspace gas chromatography-mass spectrometry (HS GC-MS) analysis.

The biomass produced during the fermentation was measured using the cell pellet left in the tubes. Distilled water was added until the level reached 40 mL. Next, samples were diluted until an OD of 0.2-0.5 was obtained using a spectrophotometer at 600 nm. The biomass production was only used for relative comparison between the samples in the study.

2.4 Analysis of Volatile and Non-volatile Compounds

The term 'chromatography' designates several techniques that allow the separation of different analytes in a mixture. In chromatographic separation, the sample is introduced in a flowing *mobile phase* that passes a *stationary phase*. When the mobile phase is a gas, it is indicated as GC, and liquid chromatography (LC) when it is a liquid. The separated molecules can be recognized by various detectors, and are eluted at different times known as retention time (RT). The graphic output of the electrical signals detected are represented as peaks in a chromatogram (Moldoveanu & David, 2013).

2.4.1 Headspace Gas Chromatography-Mass Spectrometry

The concentration of volatile compounds can be determined quantitatively with headspace GC techniques. The terminology 'headspace' in GC refers to the vapor phase within a sealed container with a liquid or solid, as the vapor phase is on top of it. A typical headspace extraction involves a sample placed in a sealed vial that is heated to a preset temperature. The system reaches equilibrium before the vapor is sampled, providing a constant gas composition. A defined amount of the equilibrium vapor is taken from the container and transferred to a gas chromatograph for separation and analysis of the components (Sithersingh & Snow, 2012). Headspace gas chromatography can also be coupled to mass spectrometry (MS) and provide the mass-to-charge ratio (m/z) of the ion used (Grayson, 2016).

The internal standard (ISTD) method is considered to give the most accurate quantification when using GC-MS, and is therefore the preferred method. The ISTD corrects for losses during subsequent separation and concentration steps, in addition to the variation in the amount of injected sample. However, this requires that the concentration of the compound used as internal standard is known in the sample analyzed (Sparkman, Penton, & Kitson, 2011).

Instrument Operating Conditions and Method Parameters

A HS GC-MS analysis was performed to quantify the volatile compounds in the end-point fermentation samples using a 7890 A GC System coupled with the 7000 Series Triple Quadrupole GC-MS system (Agilent Technologies), with a Teledyne Tekmar HT3^{TM} Static and Dynamic Headspace System autosampler. The experimental conditions for the static headspace system and the chromatographic conditions for the GC-MS are listed in Table 2.7 and Table 2.8. The software MassHunter was used for instrument control, data acquisition and processing, while HT3 TekLink was the autosampler control software.

Table 2.7: Experimental conditions for the static headspace system.

Parameter	Condition
Constant heat time	On
GC cycle time [a]	53.00 min
Valve oven temp.	105 °C
Transfer line temp.	110 °C
Standby flow rate	25 mL/min
Platen/Sample temp.	60 °C
Platen temp. equil. time	0.50 min
Sample equil. time	20.00 min
Pressurize	9 psig
Pressurize time	2.00 min
Pressurize equil. time	0.20 min
Loop Fill pressure	7 psig
Loop Fill time	2.00 min
Inject time	0.50 min

[a] The GC cycle time can vary;
= Time of GC separation (31.95 min) + time needed to cool down the column (15-25 min).

Table 2.8: Chromatographic conditions for GC-MS.

Parameter	Condition		
Column	Agilent J&W DB-624 UI, 30 m × 0.25 mm, 1.4 μm		
Carrier	Helium, constant flow, 1.8 mL/min		
Oven	Temperature	Hold time	Rate
	35 °C	5.66 min	8.8 °C/min
	100 °C	1.7 min	13.3 °C/min
	220 °C	3.39 min	22.1 °C/min
	250 °C	3.43 min	End
Inlet	220 °C, Split ratio 5:1		
MSD	Scan 30-400 amu, scan time 70 (ms/cycle)		
	Source temp. 230 °C		
	Quad temp. 150 °C		
	Solvent delay 4.5 min		

Preparation of Internal Standard

4-methyl-2-pentanol was used as ISTD in all samples. This compound was considered a good candidate for ISTD as it is chemically similar to the ones measured, but elutes in an empty space in the chromatogram. A stock solution of ISTD with a concentration ranging from 1.0-1.5 mg/mL was prepared by adding 3-5 mL of ethanol (EtOH) to a 20 mL volumetric flask and weighing accurately 20-30 mg of ISTD to the flask. The volumetric flask was filled with MQ water until 20 mL and thoroughly mixed after. A working solution of ISTD with a final concentration of 100 µg/mL was created by diluting the stock solution, and divided into aliquots after. The aliquots were stored at -80 °C.

Preparation of Calibration Series

A calibration series including the compounds 1-propanol, ethyl acetate, isobutyl alcohol, isoamyl alcohol, active amyl alcohol, 1-butanol, ethyl propionate, propyl acetate, acetal, isobutyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, was prepared for the HS GC-MS analysis. Three mixtures, Mix A, Mix B and Mix C, were made to include the various compounds, as described in Table 2.9. The compounds were accurately weighed in 20 mL volumetric flasks, aiming to be close to the preset range listed in Table 2.9. The exact weights are listed in Appendix C in Table C.1. The flasks were filled with EtOH and distilled water in the specified ratios.

A stock solution of 40 parts per million (ppm) was made by adding 5 mL of Mix A, 2 mL of Mix B, 2 mL of Mix C, and filled with water until 100 mL. The stock solution was the base for a serial dilution made in 50 mL volumetric flasks ranging from 0.2-40 ppm, as shown in Table 2.10. 5 mL of each prepared calibration solution was pipetted into HS vials, and 100 µL of ISTD was added. The vials were promptly capped with butyl-rubber/PTFE septa caps using a crimper. The remaining calibration solutions were transferred to 50 mL Falcon tubes, sealed with parafilm, and stored at -80 °C. A few of the calibration solutions were included at the beginning and end of each HS GC-MS run.

Sample Preparation

The beer samples were sonicated for 10 seconds for CO_2 removal. 5 mL of the sample was carefully and slowly pipetted to a 22 mL headspace vials, and 100 µL of working solution of ISTD was added. The vials were closed promptly in the same manner as with the calibration solutions, and analyzed immediately.

Quantification of Compounds

Identification and quantification of compounds found in beer samples was based upon retention characteristics recovered from the standards. A method was created in the Agilent MassHunter Quantitative Analysis (QQQ) software to determine the concentration in the various compounds. This was based on the relative response of the compounds and ISTD. The calibration curve fit was set to quadratic. Samples that exceeded the calibration curve were set to be greater than the highest point of the calibration range (131,440 µg/mL). The

Table 2.9: Composition of Mix A, Mix B and Mix C, used to create a stock solution for the calibration series.

Mix A	Weight range [mg]	Volume [mL]
1-propanol	45-50	
Ethyl acetate	45-55	
Isobutyl alcohol	35-45	20 (EtOH/H_2O, 50:50 v/v)
Isoamyl alcohol	65-75	
Active amyl alcohol	40-50	

Mix B	Weight range [mg]	Volume [mL]
1-butanol	35-45	
Ethyl propionate	30-40	
Propyl acetate	20-30	20 (EtOH/H_2O, 80:20 v/v)
Acetal	20-30	
Isobutyl acetate	20-30	

Mix C	Weight range [mg]	Volume [mL]
Ethyl butyrate	20-30	
Isoamyl acetate	18-25	
Ethyl hexanoate	18-25	20 (EtOH/H_2O, 80:20 v/v)
Ethyl octanoate	20-30	
Ethyl decanoate	40-50	

Table 2.10: Serial dilution of the stock solution containing Mix A, Mix B and Mix C. The resulting concentrations have a range of 0.2-40 ppm.

Calibration series			
Serial dilution		Volume [mL]	dH_2O [mL]
1	40 ppm	-	-
2	$\hookrightarrow$ 20 ppm	25	25
3	$\hookrightarrow$ 10 ppm	25	25
4	$\hookrightarrow$ 5 ppm	25	25
5	$\hookrightarrow$ 2.5 ppm	25	25
6	$\hookrightarrow$ 1 ppm	20	30
7	$\hookrightarrow$ 0.5 ppm	25	25
8	$\hookrightarrow$ 0.2 ppm	20	30

various compounds, RT, the equation and coefficient of determination (R^2) of the calibration curves, and lastly, the limit of quantification (LOQ) used to quantify the compounds in the samples are listed in Table 2.11.

Table 2.11: The retention time (RT), equation, linearity (expressed as the coefficient of determination, R^2) and the LOQ (given in response) for the standard calibration curves for the various compounds in the headspace GC-MS analysis.

		Calibration curve		
Compound	RT	Equation[a]	R^2	LOQ
1-Propanol	5.160	y = 0.045493 x + 0.005486	0.9996	560
Ethyl acetate	6.360	y = -0.003170 x^2 + 1.088717 x + 0.221282	0.9997	525
Isobutyl alcohol	7.881	y = 0.143510 x + 0.014781	0.9998	400
1-Butanol	9.120	y = 0.172782 x - 0.012790	0.9998	430
Ethyl propionate	9.769	y = 1.976947 x + 0.044473	0.9991	158
Propyl acetate	9.916	y = 1.726330 x + 0.005336	0.9994	115
Acetal	10.062	y = 2.206446 x - 0.104293	0.9995	238
Isoamyl alcohol	11.260	y = -4.561667E-004 x^2 + 0.253266 x - 0.041800	0.9999	1600
Active amyl alcohol	11.370	y = -0.001301 x^2 + 0.321937 x - 0.010304	0.9999	430
Isobutyl acetate	11.687	y = 2.807216 x - 0.048650	0.9970	102
Ethyl butyrate	12.342	y = 2.621633 x + 0.045144	0.9996	102
Isoamyl acetate	14.497	y = 2.047766 x + 0.049071	0.9992	87
Ethyl hexanoate	17.524	y = -0.056060 x^2 + 2.500487 x + 0.119507	0.9997	120
Ethyl octanoate	20.674	y = 0.846870 x - 0.244264	0.9989	1280
Ethyl decanoate	23.017	y = 0.012858 x^2 + 0.132817 x - 0.247608	0.9966	4800

[a] y = Relative response between compound and ISTD;
x = Relative concentration between compound and ISTD.

The results were analyzed with principal component analysis (PCA) in The Unscrambler® X software. PCA is a model that performs a linear transformation on data in order to reduce the multidimensional data for a simplified and forthright analysis. In other words, it allows for interpretations based on multiple variables simultaneously rather than just looking at individual ones, and can therefore reveal nontrivial correlations between them (Esbensen & Geladi, 2009). Concentrations were 4th root transformed prior to the PCA and averaged by site because preliminary tests indicated that this transformation improved overall data visualization and reduced the impact of large scaling differences between proportions.

Heat maps were made with the Morpheus software (https://software.broadinstitute.org/morpheus/). The concentrations (mg/L) were normalized by conversion to Z-scores (i.e. by subtracting the mean and dividing by standard deviation). The samples were also hierarchically clustered based on the phenotypic behavior and the compounds were clustered by temperature, using one minus Pearson correlation and average linkage mapping.

2.4.2 Sugar, Alcohol and Organic Acid Quantification with High-Performance Liquid Chromatography

In HPLC (also referred to as high-pressure liquid chromatography), the liquid mobile phase is moved through the stationary phase (typically a column packed with small porous particles) by a pump at elevated pressure, with the result of a 'high-performance' separation (Moldoveanu & David, 2013). Carbohydrates have traditionally been analyzed by using refractive index (RI) detection (Lethonen & Hurme, 1994), as the ultraviolet (UV) absorption is very low (except for very low wavelengths). Moreover, the compounds are not fluorescent, which also makes them less suitable for UV detection (Moldoveanu & David, 2013). Even though RI is the most popular detection method and it accommodates a wide linear range, it also has some drawbacks. Lack of sensitivity, baseline sensitivity, temperature and flow-rate dependency and incompatibility with gradient elution are some of the disadvantages (Lethonen & Hurme, 1994; Ferreira, 2009).

A HPLC analysis was performed using Shimadzu HPLC systems coupled to both a UV and RI detector. The experimental and chromatographic conditions for the analysis is shown in Table 2.12.

Table 2.12: The experimental and chromatographic conditions for the HPLC analysis.

Parameter	Condition
HPLC system	Shimadzu HPLC chromatographic system in combination with RI and UV (210 nm) detection
Column type	Bio-Rad Aminex HPX-87H column (300 $\times$ 7.8 mm)
Column oven temperature	45 °C
Mobile phase	5 mM H_2SO_4
Flow rate	0.6 mL/min
Shimadzu system:	
Autoinjector	Shimadzu SIL-9A
Pump	LC9A
Oven	CTI6A
RI-detector	RID6A
UV-detector	UV SPD6A

The carbohydrates glucose and fructose, glycerol and ethanol were analyzed with a RI detector, while the carboxylic acids, acetic- and succinic acid, were analyzed with an UV detector. The calibration was performed with an external single point standard (near the highest expected concentration of analyte). The method using an external standard (ESTD) assumes a linear detector response and that samples do not contain a wide range of analyte concentrations. The standard solution was run twice before running samples, thereafter it was run every 10^{th} sample, and at last, repeated two more times. An example calculation of how the amount of sample was calculated based on the single point standard is shown in Appendix D.

Preparation of Standard

A set of six stock solutions (glucose, fructose, acetic acid, glycerol, EtOH and succinic acid) was prepared in order to make a standard mix for the experiment. The concentrations of the stock solutions are listed in Table 2.13. Volumes of each compound (specified in Table 2.13) were mixed in a 10 mL volumetric flask, and MQ-water was added until the volume reached 10 mL. The final concentration obtained for each compound is listed in the same table.

Table 2.13: Overview of the compounds included in the standard solution used for the HPLC analysis. The volume of stock solution added to a 10 mL volumetric flask, and the final concentration in the mixture is listed.

Compound	$C_{\text{Stock solution}}$ [g/L]	Volume [mL]	Dilution factor	C_{final} [g/L]
Glucose	50	1	10	5
Fructose	50	1	10	5
Acetic acid	16	0.4	25	0.64
Glycerol	25	1	10	2.5
EtOH	50	2	5	10
Succinic acid	50	0.5	20	2.5

Sample Preparation

1 mL of beer sample was filtered using a 0.2 µm syringe filter and a 1 mL syringe. 400 µL of filtered beer sample and 800 µL of filtered MQ-water were transferred to a 1.5 mL eppendorf tube with safe-lock, and mixed together making it a 1:3 dilution. Finally, parafilm was wrapped around the tubes to cover the opening, hence, preventing it from evaporating. The samples were stored at -20 °C until the analysis was performed.

2.4.3 Liquid Chromatography-Mass Spectrometry

Gradient elution is necessary when the mixtures analyzed contain a wide range of sugar types (Lethonen & Hurme, 1994). A more extensive analysis of compounds was therefore carried out with LC-MS, in order to inspect the metabolites (mainly sugars) left in the end-point fermentation samples. A gradient elution was performed with a mixture of two solvents; solvent A and solvent B.

Standard Operating Protocol for Beer Sugar Analysis

A high resolution LC-MS method for analysis of sugars (mono to oligomers, up to 7mer) was carried out using the Acquity™ Ultra Performance LC systems coupled with a Waters Synapt™ High Definition MS Q-Tof mass spectrometer, controlled with the Waters MassLynx™ software. The method was developed by Kåre A. Kristiansen, IBT. The experimental and chromatographic conditions for the LC-MS analysis is specified in Table 2.14. Table 2.15 shows the settings for the gradient elution, where the hold time, flow rate, along with the ratio between A and B are given.

A pooled quality control (QC) sample was prepared by adding 5 µL of each sample to the same vial. This way, the QC was a representative of the entire collection of samples studied (qualitatively and quantitatively), providing an average of all of the metabolites studied. The QC was injected every 10^{th} sample analyzed, for the duration of the assay. This measure increases the confidence and can ensure that the data are not affected by technical biases or differences in the operating conditions within the course of the experiment. A blank sample was also included during the run.

Table 2.14: The experimental and chromatographic conditions for the LC-MS analysis.

Parameter	Condition
Column type	ACQUITY UPLC® BEH Amide 1.7 µm Column
Column temperature	35.0 °C
Solvent A	H_2O/ACN (70/30) + 0.1 % NH_4OH
Solvent B	ACN/H_2O (80/20) + 0.1 % NH_4OH
MS method	Can use both MS scan and MSE, in negative mode, Scan 50-2000 m/z, 4 scans/second
Source	Capillary 2.80 kV, sampling cone 25, source offset 50
Temperatures	Source 120 °C, desolvation 350 °C
Gas flows	Cone gas 50 L/h, desolvation gas 500 L/h, Nebulizer 6 bar

Table 2.15: Gradient table. The hold time, flow rate, ratio between the solvents A and B, as well as the curve is listed in the table.

Time [min]	Flow Rate [mL/min]	A [%]	B [%]	Curve
Initial	0.170	0.0	100.0	Initial
7.00	0.170	98.0	2.0	6
9.00	0.170	98.0	2.0	6
9.10	0.170	0.0	100.0	6
11.00	0.170	0.0	100.0	6

Sugar Standard

A standard with the sugars maltotriose, maltotetraose, maltopentose, maltohexose and meltoheptose, was included in the assay to identify these compounds in the beer samples. This was based on based on RT and mass characteristics. Table 2.16 shows the monoisotopic mass and $[M\text{-}H]^-$ in negative ionization mode for the sugars named, verified and determined in a previous run by Kåre A. Kristiansen. The concentration of each sugar in the standard solution was 100 µM.

Table 2.16: Sugar standard. A mixture of the sugars maltotriose, maltotetraose, maltopentose, maltohexose and meltoheptose, was used for identification of compounds in beer samples based on RT and mass characteristics.

Compound	Molar mass [g/mol]	Monoisotopic mass	$[M-H]^-$
Maltotriose	504.40	504.1690	503.1612
Maltotetraose	666.60	666.2219	665.2141
Maltopentose	828.70	828.2747	827.2669
Maltohexose	990.90	990.3275	989.3197
Meltoheptose	1153.00	1152.3803	1151.3725

Sample Preparation

100 µL of beer sample was added to 900 µL of pure acetonitrile (ACN) (1:10 dilution) in 1.5 mL eppendorf tubes for protein removal. The mixture was vortexed thoroughly, and then incubated for 30 min on ice. The tubes were centrifuged (2 min, 4,500 $\times$ g) at 4 °C in order to remove the protein precipitate. 600 µL of supernatant (without touching the pellet) was transferred to a LC-MS vial. The vials were stored at -20 °C until the analysis was run.

The standards had a concentration of 100 µM, which is suitable for 1 µL injections. However, the samples turned out to be too dilute. Consequently, the injection volume was adjusted to 10 µL (as opposed to the ideal 1 µL). As this is too high for optimal chromatography, it is suggested to use 200 µL sample and 1800 µL acetonitrile in future events. It can then be beneficial to concentrate the samples even more by transferring 1500 µL to a eppendorf vial, freezedry, and thereafter reconstitute it in 150 µL mobile phase B before injecting 1 µL.

2.5 Methods for Detection and Identification of Brewery Spoilage Organisms

The yeast samples were plated on four different media used for bacteria and wild yeast detection; Wallerstein laboratory nutrient (WLN), Wallerstein laboratory differential (WLD), Lin's copper sulfate medium (LCSM) and lysine (LYS) medium. The purpose of the media, compositions and procedures are explained in detail under. Finally, the target microorganism(s) and incubation conditions for all four media are summarized in Table 3.1.

2.5.1 Wallerstein Laboratory Nutrient Agar

WLN agar is based on a color reaction determined by the ability to reduce bromocresol green, and is useful in analyzing contamination with lager yeast and varieties of wild yeast in ale yeast cultures. *Saccharomyces cerevisiae* form dark green colonies on the media as they are not able to utilize it, which permits a rapid screening of ale strains. Lager strains, *Saccharomyces* and non-*Saccharomyces* wild yeast can, in contrast, reduce bromocresol green and will therefore form pale green, blueish, or white colonies (Jespersen & Jakobsen, 1996; Hill, 2015).

Table 2.17: Composition of the Wallerstein laboratory nutrient agar, provided by the supplier Sigma-Aldrich. The medium is based on the ability to reduce bromocresol green.

Compound	Formula	Composition [g/L]
Agar		20
Bromocresol green		0.022
Calcium chloride	$CaCl_2$	0.125
Casein enzymic hydrolysate		5
Dextrose		50
Ferric chloride	$FeCl_3$	0.0025
Magnesium sulfate	$MgSO_4$	0.125
Manganese sulfate	$MnSO_4$	0.0025
Monopotassium phosphate	KH_2PO_4	0.55
Potassium chloride	KCl	0.425
Yeast extract		4

The composition of the WLN agar is shown in Table 2.17. 80.25 g of WL Nutrient Agar (Sigma-Aldrich, Lot: BCBR9042V) was suspended in 1 litre of distilled water, and stirred on a magnetic stir plate until all the solids had dissolved. The solution was sterilized by autoclavation at 121 °C for 20 minutes, and poured into plates. 100 µL of yeast culture was spread-plated using aseptic techniques.

2.5.2 Wallerstein Laboratory Differential Agar

The WLD agar has the same composition as the WLN agar (see Table 2.17) except for the addition of 0.004 g/L actidione (cycloheximide) to inhibit yeast growth. WLD is for this reason considered a selective and differential general purpose medium for bacteria.

80.26 g of WL Differential Agar (Sigma-Aldrich, Lot: BCBR0945V) was suspended in 1 litre of distilled water, and stirred on a magnetic stir plate until all the solids had dissolved. The solution was sterilized by autoclavation at 121 °C for 20 minutes, and poured into plates. 100 µL of yeast culture was spread-plated using aseptic techniques.

2.5.3 Lin's Copper Sulfate Medium

The use of copper sulfate, $CuSO_4$, to detect primarily non-*Saccharomyces* wild yeast was introduced by Lin (1981). Copper will in very small quantities increase the growth of yeast. However, at relatively higher concentrations, the opposite effect is observed; copper inhibits yeast growth. This phenomena is exploited in LCSM, by generating a copper sulfate concentration optimal for wild yeast growth (some *Saccharomyces* wild yeast growth might be inhibited) whilst suppressing culture yeast. Distinct colonies developing on the medium may therefore be considered wild yeasts (Lin, 1981).

Table 2.18: Composition of Lin's copper sulfate medium. The concentration of copper sulfate allows wild yeasts to grow, while suppressing culture yeast.

Compound	Formula	Composition [g/L]
Agar		20
Ammonium chloride	NH_4Cl	0.5
Copper(II) sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.86
Dextrose		10
Malt extract		2
Monopotassium phosphate	KH_2PO_4	1.1
Peptone		2
Yeast extract		4

The composition of the medium is shown in Table 2.18. All the listed compounds, except the copper sulfate, were weighed accurately and added to a bottle and filled with distilled water to a final volume of 1 L. The solution was stirred on a magnetic stir plate until all the solids had dissolved, and thereafter sterilized by autoclavation at 121 °C for 20 minutes. A copper sulfate stock solution was prepared with MQ-water and sterilized by filtration using a syringe filter with a 0.2 µm membrane, in respect to the desired final concentration in the medium. The filtered copper sulfate stock solution was then added to the autoclaved bottle, and thoroughly mixed on a magnetic stir plate to obtain an uniform distribution.

The finished LCSM was poured into plates and used within 3 days of preparation. A suspension of 100 µL containing approximately 1×10^6 of yeast cells was spread-plated using aseptic techniques.

2.5.4 Lysine Medium

Another medium used to investigate the presence of beer spoilage organisms is a LYS medium, using L-(+)-lysine as the sole nitrogen source for growth. The formula was first developed as a liquid medium by Walters and Thiselton (1953), but later modified to a solid media by Morris and Eddy (1957). The medium is primarily used to detect non-*Saccharomyces* wild yeasts. Neither lager and ale yeasts nor most other yeasts belonging to the genus *Saccharomyces* can grow on this medium, because they are not capable of lysine utilization (Walters & Thiselton, 1953).

The composition of the LYS medium is shown in Table 2.19. The listed compounds were weighed accurately as indicated in the table. All compounds, except lysine and the vitamins- and oligo-elements solutions, were transferred to a bottle where distilled water was added until a final volume of 1 L. The solution was stirred on a magnetic stir plate until all the solids had dissolved, and thereafter sterilized by autoclavation at 121 °C for 20 minutes. A lysine stock solution was prepared with MQ-water and sterilized by filtration using a syringe filter with a 0.2 µm membrane, in respect to the desired final concentration in the medium. The filtered lysine solution, 1 mL of 100x vitamins solution (see Table 2.20) and 100 µL of 1000x oligo-elements solution (see Table 2.21) were added to the autoclaved bottle. The solution was thoroughly mixed on a magnetic stir plate to obtain an uniform distribution, and at last poured into plates.

Table 2.19: Composition of the lysine medium. L-(+)-lysine is used as the only nitrogen source for growth, that mainly non-*Saccharomyces* wild yeasts can utilize.

Compound	Formula	Composition [g/L]
Agar		20
Calcium chloride	$CaCl_2$	0.178
Dextrose		44.5
L-(+)-lysine		1
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	0.5
Monopotassium phosphate	KH_2PO_4	3
Sodium chloride	NaCl	0.089
100x Vitamins solution		
1000x Oligo-elements solution		

Table 2.20: The composition of the 100x vitamins solution. The solution had been sterilized by filtration and stored at -20 °C.

Compound	Composition [g/L]
Biotine	0.0003
Myo-inositol	2
Nicotinic acid	0.2
Pantothenate calcium	0.15
Pyridoxine	0.025
Thiamine hydrocloride	0.025

Table 2.21: The composition of the 1000x oligo-elements solution. The solution had been sterilized by filtration and stored at 4 °C.

Compound	Formula	Composition [g/L]
Ammonium heptamolybdate	$(NH_4)_6Mo_7O_{24}$	1
Boric acid	H_3BO_3	1
Cobalt(II) chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	0.4
Copper(II) sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	1
Manganese(II) sulfate monohydrate	$MnSO_4 \cdot H_2O$	4
Potassium iodide	KI	1
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	4

The yeast cultures were washed with distilled water before inoculating, in order to remove extraneous nutrients (with possible trace nitrogen) which may support the growth of culture yeast. A suspension of 100 µL containing approximately 1×10^6 of yeast cells was spread-plated using aseptic techniques.

Incubation Conditions

The target mircroorganism(s) and incubation conditions with respect to temperature and incubation period, for the various media are listed in Table 3.1.

Table 2.22: The target mircroorganism(s) and incubation conditions for Wallerstein Laboratory Nutrient (WLN) agar, Wallerstein Laboratory Differential (WLD) agar, Lin's copper sulfate medium (LCSM) and lysine (LYS) medium.

Medium	Target microorganism(s)	Incubation conditions
WLN	Enteric, acetic, and lactic bacteria, yeast	25-30 °C, 3-16 days
WLD	General purpose medium for bacteria	25-30 °C, 3-16 days
LCSM	Wild yeast	28 °C, 2-6 days
LYS	Enteric, acetic and lactic bacteria, wild yeast	25 °C, 5 days

Chapter 3

Results

The NFAY cultures collected span a great area of Western Norway; from Sandnes in the south to Skodje in the north, as seen in Table 2.1 and Figure 3.1. The NFAY cultures has not been intensely studied, and very little is therefore known about their evolution. To address this, the yeast cultures were characterized both genotypically and phenotypically to explore signatures of domestication.

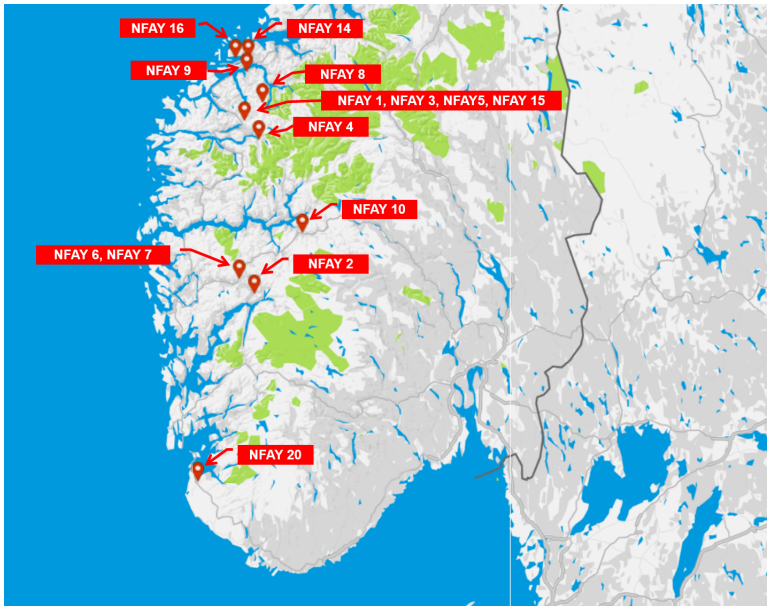


Figure 3.1: Map of Western Norway, showing the place of origin each Norwegian farmhouse ale yeast (NFAY) culture was collected from.

The genotypic characterization included a taxonomically classification, the presence and distribution of the AGT1 gene (encoding a α-glucoside transporter) and investigation of the ploidy. A small-scale fermentation of the samples at 22 °C and 35 °C provided characterization of phenotypic diversity and behaviour through chromatography and mass spectrometry instrumentation (headspace GC-MS, HPLC and LC-MS).

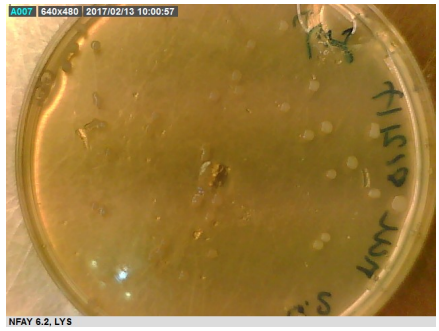
3.1 Preliminary Phenotypic Classification

The yeast samples were plated on four different kinds of agar media, WLN, WLD, LCSM and LYS media, and this procedure was included as an alternative phenotypic characterization. However, in the study it was established that several of the assays are not as selective for *S. cerevisiae* versus wild yeasts as reported in literature. The results were therefore used as a classification tool for growth characteristics, rather than determination of genus or species. Figure 3.2 shows examples of what was considered a positive outcome in the various tests. The results were recorded as positive (+) or negative (-) depending on the outcome, and are presented in Table 3.1.

(a) Wallerstein Laboratory Nutrient Agar

(b) Wallerstein Laboratory Differential Agar

(c) Lin's Copper Sulfate Medium



(d) Lysine Medium

Figure 3.2: Examples of positive results of samples on the various selective and differential media, showing agar plates of WLN, WLD, LCSM and LYS media, respectively.

Table 3.1: Results of the media used for the preliminary phenotypic classification. Positives and negative results from the tests are indicated as + and -, respectively.

	Medium			
Sample	WLN	WLD	LCSM	LYS
NFAY 1_P1	+	+	-	-
NFAY 2_P1	-	-	-	-
NFAY 2_P2	-	+	-	-
NFAY 3.5	+	-	-	-
NFAY 3.7	-	-	-	-
NFAY 4.1	+	-	-	-
NFAY 5_P1	-	+	-	-
NFAY 6.2	+	-	-	+
NFAY 6.20	-	-	-	-
NFAY 7.15	-	-	-	-
NFAY 7.24	-	-	-	-
NFAY 8_P1	-	-	-	-
NFAY 9.8	-	-	-	-
NFAY 9.23	-	-	-	-
NFAY 9.24	-	-	-	-
NFAY 10_P1	-	-	-	-
NFAY 14_P1	+	+	-	-
NFAY 14_P2	-	+	-	-
NFAY 15_P1	-	+	-	-
NFAY 15_P2	-	+	-	+
NFAY 16_P1	-	+	-	-
NFAY 16_P2	-	+	-	-
NFAY 20_P1	-	+	-	-
NFAY 20_P2	-	+	+	-
G518	+	-	-	-
G561	+	-	-	-
G562	+	-	-	-
BY4741	-	-	-	-
BY4743	-	-	-	-
NCYC361	-	-	-	-
NCYC456	-	-	-	-
NCYC660	-	-	-	-
NCYC661	-	-	-	-
WLP013	+	+	-	-
WLP028	+	-	-	-
WLP051	-	-	-	-
WLP500	-	+	-	-
WLP566	-	-	-	-
Idun	-	-	-	-

Samples NFAY 1_P1, NFAY 3.5, NFAY 6.2, NFAY 14_P1, G518, G561, G562, WLP013 and WLP028 formed dark green colonies on the WLN media. That is, these samples were not able to utilize bromocresol green. Growth was observed for samples NFAY 1_P1, NFAY 2_P2, NFAY 5_P1, both samples (P1 and P2) from cultures NFAY 14, NFAY 15, NFAY 16 and NFAY 20, and the commercial samples WLP013 and WLP500. This suggests that the named samples can grow on WLD agar with 0.004 g/L cycloheximide. NFAY 20_P2 was able to grow on the LCSM agar plate, containing a copper(II) sulfate pentahydrate concentration of 0.86 g/L. At last, it was observed that NFAY 6.2 and NFAY 15_P2 are L-(+)-lysine positive, meaning they can grow with this as the sole nitrogen source.

An observation of a slightly dissimilar morphology in sample NFAY 15_P2 led to the discovery of two different species. The sample was therefore subdivided into two; NFAY 15_P2.1 and NFAY 15_P2.2. Unfortunately, this finding was discovered after the fermentation had been carried out. Consequently, this segmentation of sample NFAY 15_P2 was only introduced to the genetic analyses.

3.2 Genotypic Characterization

3.2.1 Identification, Classification and Phylogenetic Relationships

First, the entire ITS region was amplified with PCR using the primers ITS1-F KYO2 and ITS4. Due to poor sequence quality for many of the samples when ITS1-F KYO2 was paired with ITS4, they were replaced by ITS5 and ITS4. These are universal primers that have gained a wide acceptance for work with fungal ITS region and are generally well supported in literature (White, Bruns, Lee, & Taylor, 1990; Toju et al., 2012). Regardless, they seemed to be a better primer pair match in terms of melting temperature, as the difference between them was only 3.1 °C. Despite this, it was only possible to obtain good sequencing results for approximately half of the samples of the ITS region. In contrast, good sequencing data of the LSU1 and LSU2 regions were retrieved for nearly all samples.

The consensus sequences were uploaded to the RDP Classifier. An overview of the species and genera identified in the NFAY samples using the classifier (including the score percentage), as well as the presence of the AGT1 gene, is given in Table 3.2. The classification results of the wild, laboratory and commercial yeast samples are given in Table 3.3. All sequence obtained had scores above 80%, hence, all can be considered classified according to the set threshold.

Phylogenetic trees were created for the ITS, LSU1, LSU2 and AGT1 regions. A MSA was computed using MUSCLE, and a ML tree was constructed by applying the Tamura-Nei model (Tamura & Nei, 1993). The bootstrap method with 1000 replicates was used to evaluate the reliability of each branching in the trees.

Table 3.2: Sequencing results for NFAY samples. Species suggested on the basis of the ITS region, and genus based on the LSU1 and LSU2 regions, with the corresponding scores [%] are listed for each sample. The presence of the AGT1 gene is also listed.

Sample	ITS, score (%)[a]		LSU1, score (%)[b]		LSU2, score (%)[b]		AGT1[c]
NFAY 1_P1			*Saccharomyces*	100	*Saccharomyces*	100	D
NFAY 2_P1			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 2_P2			*Saccharomyces*	100	*Saccharomyces*	100	D
NFAY 3.5			*Saccharomyces*	100	*Saccharomyces*	100	D
NFAY 3.7	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 4.1	*Saccharomyces bayanus*	100	*Saccharomyces*	98	*Saccharomyces*	100	P
NFAY 5_P1			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 6.2	*Saccharomyces bayanus*	100	*Saccharomyces*	98	*Saccharomyces*	100	P
NFAY 6.20			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 7.15			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 7.24			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 8_P1	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	D
NFAY 9.8	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 9.23	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 9.24	*Saccharomyces cerevisiae*	98	*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 10_P1			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 14_P1	*Meyerozyma caribbica*	100					M
NFAY 14_P2	*Meyerozyma caribbica*	99					M
NFAY 15_P1	*Meyerozyma caribbica*	100					M
NFAY 15_P2.1	*Meyerozyma caribbica*	99					M
NFAY 15_P2.2			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 16_P1	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	
NFAY 16_P2			*Saccharomyces*	100	*Saccharomyces*	100	D
NFAY 20_P1			*Saccharomyces*	100	*Saccharomyces*	100	P
NFAY 20_P2			*Saccharomyces*	100	*Saccharomyces*	100	P

[a] Species suggested using the 'Warcup Fungal ITS trainset 2' with a 80% confidence threshold in the RPD classifier.
[b] Genus suggested using the 'Fungal LSU training set 11' with a 80% confidence threshold in the RPD classifier.
[c] D, defective, frame shift mutation; P, present; M, missing.

Table 3.3: Sequencing results for wild, laboratory and commercial yeast samples. Species suggested on the basis of the ITS region, and genus based on the LSU1 and LSU2 regions, with the corresponding scores [%] are listed for each sample. The presence of the AGT1 gene is also listed.

Sample	ITS, score (%)[a]		LSU1, score (%)[b]		LSU2, score (%)[b]		AGT1[c]
G518			*Saccharomyces*	100	*Saccharomyces*	100	P
G561			*Saccharomyces*	100	*Saccharomyces*	100	P
G562			*Saccharomyces*	100	*Saccharomyces*	100	P
BY4741	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
BY4743	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
NCYC361			*Saccharomyces*	100	*Saccharomyces*	100	P
NCYC456			*Saccharomyces*	100	*Saccharomyces*	100	D
NCYC660	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	M
NCYC661	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	M
WLP013	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
WLP028	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	D
WLP051			*Saccharomyces*	100	*Saccharomyces*	100	D
WLP500	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
WLP566	*Saccharomyces cerevisiae*	100	*Saccharomyces*	100	*Saccharomyces*	100	P
Idun			*Saccharomyces*	100	*Saccharomyces*	100	P

[a] Species suggested using the 'Warcup Fungal ITS trainset 2' with a 80% confidence threshold in the RPD classifier.
[b] Genus suggested using the 'Fungal LSU training set 11' with a 80% confidence threshold in the RPD classifier.
[c] D, defective, frame shift mutation; P, present; M, missing.

Internal Transcribed Spacer Region

To reconstruct rooted phylogeny, an outgroup was included to establish the direction of evolution. *Saccharomyces* species and *Meyerozym caribbica* belong to subphylum Saccharomycotina. A phylogenetic tree recovered by Hittinger et al. (2015) was used to determine the appropriate outgroup for rooting the tree. The species *Yarrowia lipolytica* (GenBank: DQ680671.1) was considered as a good candidate for an outgroup, as it shares a common ancestor with the ingroup.

The phylogenetic tree including the bootstrap values and outgroup for the ITS region is presented in Figure 3.3. The samples classified as *M. caribbica* were clustered together with a bootstrap value of 100, and had the greatest branch length. Branch lengths are measured in number of substitutions per site, thus it can indicate the evolutionary distance. The samples classified as *S. bayanus* were also grouped together with a high bootstrapping value (97), which increases the confidence that the branching is correct. The remaining samples classified as *S. cerevisiae*, had lower bootstrapping values where branches had been collapsed. The same relationship was obtained when the outgroup was removed.

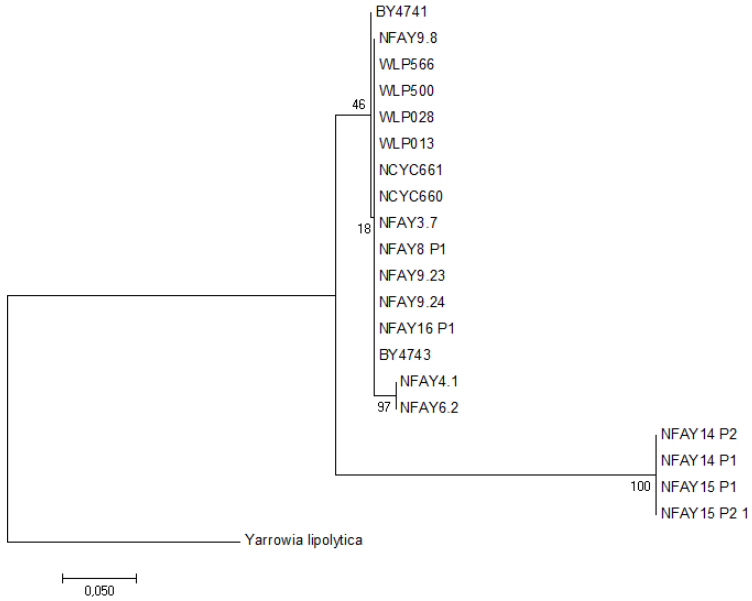
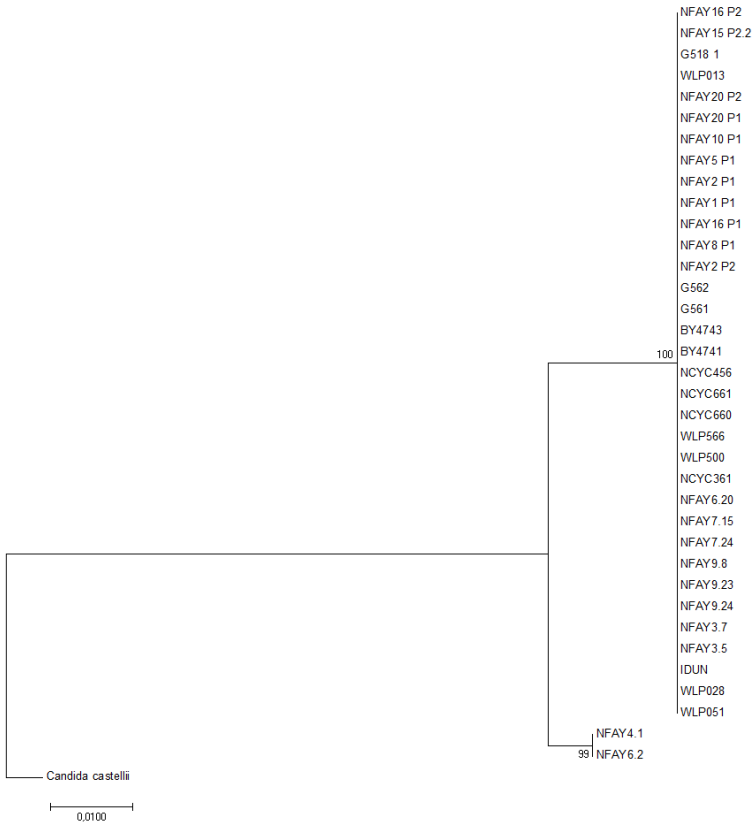


Figure 3.3: Phylogenetic tree of ITS region where *Yarrowia lipolytica* served as the outgroup. The tree was created by the Maximum Likelihood method, using the Tamura-Nei model (Tamura & Nei, 1993). The tree inferred from 1000 replicates to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985), where branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, and there were a total of 477 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

LSU1 and LSU2 Regions

The LSU1 region includes the D1/D2 domain, while LSU2 spans a section in the middle of the LSU region (see Figure 2.1). The classifier proposed that all LSU1 and LSU2 sequences obtained belonged to the genus *Saccharomyces*. This time, an outgroup more closely related to the ingroup was chosen; *Candida castellii*. The GenBank accessions KY106389.1 and AF399794.1 were included, and the constructed trees for LSU1 and LSU2 are shown in Figure 3.4a and Figure 3.4b, respectively.

The phylogenetic tree of the LSU1 region (Figure 3.4a) does not include the samples classified as *M. caribbica*. However, the same relationship between the *S. bayanus* and *S. cerevisiae* samples is established, and high bootstrapping scores suggests that this branching can be trusted. The remaining samples belonging to the *Saccharomyces* genus were all clustered in one group. In contrast, another grouping emerge in the phylogenetic tree of the LSU2 region in Figure 3.4b. In this case, only sample NFAY 4.1, is distinguished from the rest with a slightly longer branch length. Similarly, the same relationships were obtained for the LSU1 and LSU2 trees when removing the outgroup.



(a) ML phylogenetic tree of LSU1 region.

(b) ML phylogenetic tree of LSU2 region.

Figure 3.4: Phylogenetic trees of the **(a)** LSU1 and **(b)** LSU2 regions, where *Candida castellii* served as the outgroup. The tree was created by the Maximum Likelihood method, using the Tamura-Nei model (Tamura & Nei, 1993). The tree inferred from 1000 replicates to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985), where branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, and there were a total of 577 positions for LSU1 and 683 for LSU2 in the final datasets. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

The α-glucoside Transporter AGT1

The *S. cerevisiae*-type AGT1 gene was not present in samples NFAY 14_P1, NFAY 14_P2, NFAY 15_P1, NFAY 15_P2.1 and in the laboratory strains NCYC660 and NCYC661. These samples had, as seen in Figure 3.5 (lane 3, 4, 11, 14, 19 and 20), no visible bands when PCR-products were run on an agarose gel. Three independent PCRs were run to verify this result, and the same result was obtained each time. A band was observed for NFAY 16_P1. However, it was not possible to obtain good sequencing data from this sample.

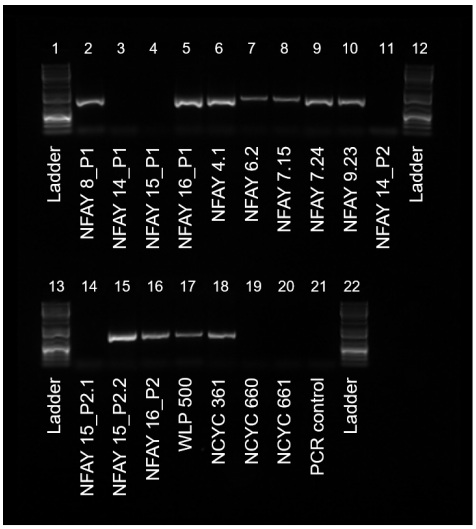


Figure 3.5: PCR products of the AGT1 gene electrophoresed in a 1.0% agarose gel at 140 V for 45 minutes. A 1 Kb Plus DNA ladder marker served as the size standard (lane 1, 12, 13 and 22), and a negative PCR control (lane 21) with MQ-water instead of DNA-template was included in each run. Visible bands of PCR products for some of the samples are shown, while no band was observed in lane 3, 4, 11, 14, 19 and 20.

The remaining samples were aligned against the reference AGT1 sequence retrieved from the SGD. A homologue of the AGT1 gene was not found outside of the *Saccharomyces* genus when conducting a BLAST search with the SGD sequence as entry. The resulting unrooted ML tree of the AGT1 gene is shown in Figure 3.6. The samples were distributed into three main clades, where only the laboratory strains BY4741 and BY4743 were grouped together with the reference AGT1 sequence. A second group includes the majority of the NFAY samples, but also the wild type *S. cerevisiae*, G518, G561 and G562, and commercial samples Idun and WLP013. The last group that emerged, contains the remaining commercial samples and the NFAY samples NFAY 4.1, NFAY 6.2, NFAY 1_P1, NFAY 2_P2, NFAY 3.5, NFAY 8_P1 and NFAY 16_P2. The two samples identified as *S. bayanus* (i.e. NFAY 4.1 and NFAY 6.2) share similar AGT1 sequences, as they are in the same group. Regardless, there does not seem to be a correlation between the groupings and the geographical location the samples were collected from.

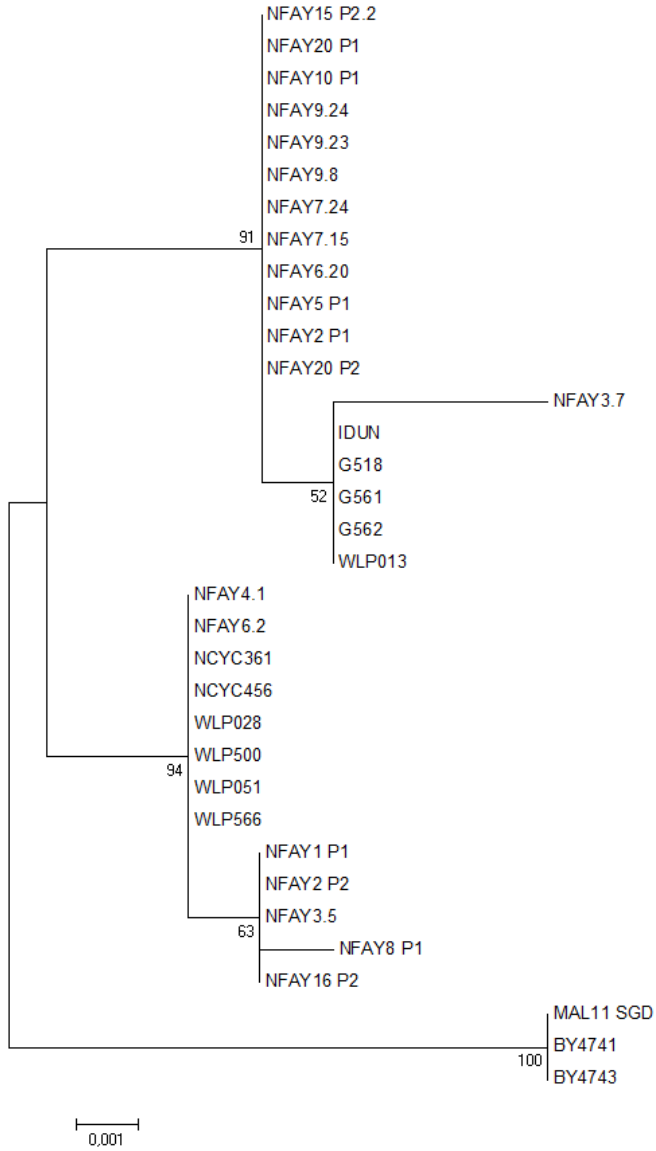


Figure 3.6: Phylogenetic analysis of the AGT1 gene by Maximum Likelihood method, using the Tamura-Nei model (Tamura & Nei, 1993). The tree inferred from 1000 replicates to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985), where branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, and there were a total of 861 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

3.2.2 Expanded AGT1 Phylogenetic Tree

A BLAST search within the Gallone et al. (2016) sequence data was performed. The reference sequence for AGT1 (i.e. MAL11 sequence form the SGD) was used as the query sequence, and the search was limited to the particular dataset by using the BioProject code PRJNA323691 (that contain only *S. cervisiae* strains) in the whole-genome shotgun contigs (wgs) database. 136 sequences with the full-length AGT1 allele were obtained. Combining these sequences with the AGT1 sequences from this study, another ML phylogenetic tree was created. The tree is shown in Figure 3.7, while Figure 3.8 shows extended labels.

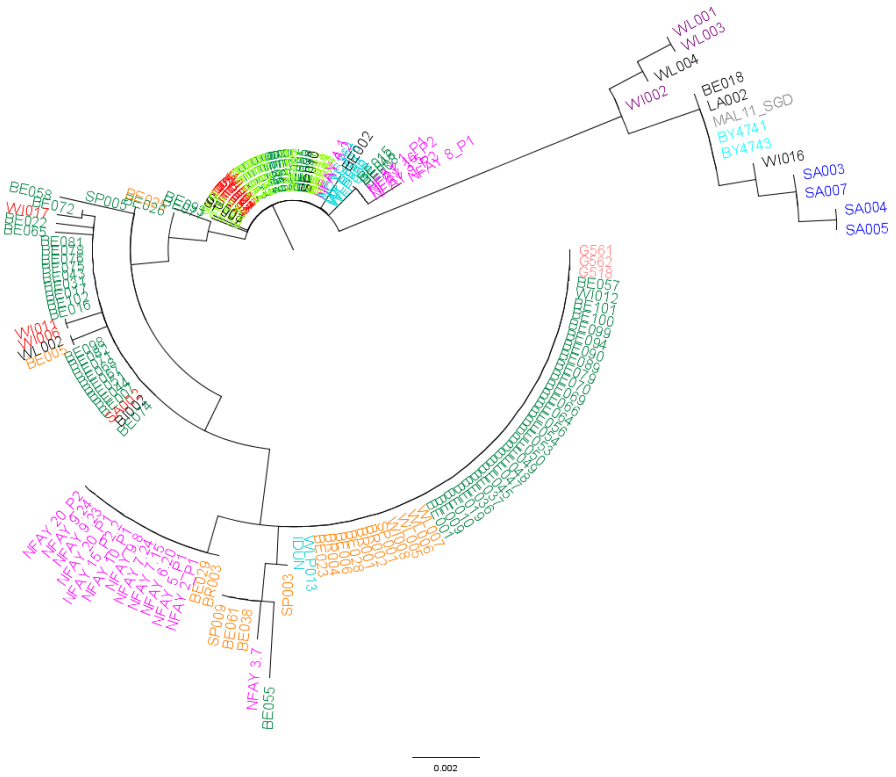


Figure 3.7: Phylogenetic analysis of the AGT1 gene by Maximum Likelihood method, using the Tamura-Nei model (Tamura & Nei, 1993). The analysis involved 170 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 892 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016), but the tree was represented using FigTree. The categories in this study are colored as following; NFAY ■, Wild ■, Laboratory ■ and Commercial ■. The previously sequenced strains by Gallone et al. (2016) are colored by the associated linage; Asia ■, West Africa ■, Beer 2 ■, Wine ■, Mixed ■ and Beer 1 ■.

Figure 3.8: ML phylogenetic tree showing extended labels from Figure 3.7. The categories in this study are colored as following; NFAY ■, Wild ■, Laboratory ■ and Commercial ■. The previously sequenced strains by Gallone et al. (2016) are colored by the associated linage; Asia ■, West Africa ■, Beer 2 ■, Wine ■, Mixed ■ and Beer 1 ■.

The phylogenetic tree of the AGT1 in Figure 3.7 shows that the reference AGT1 sequence and the laboratory strains (BY4741 and BY4743) are grouped between sequences belonging to the lineages Asia and West Africa. The remaining samples are distributed among the other lineages; Beer 2, Wine, Mixed and Beer 1. Nearly the same clustering appears, though, they are generally dispersed into three groups. NFAY 8_P1 to NFAY 1_P1 and WLP028 to NFAY 4.1 are located close to where the majority of Beer 2 samples are, NFAY 20_P2 to NFAY 2_P1, NFAY 3.7, Idun and WLP013 are close to Mixed and Beer 1, while the wild type G518 to G561 are located with Beer 1.

3.2.3 Mutation in AGT1 Gene

The insertion of an extra T possibly leads to a frameshift, and thereupon a stop codon. This results in a stretch of eight consecutive T's instead of seven. The TGA stop codon range from positions 1183 to 1185 relative to the reference sequence in SGD. A region of the AGT1 gene sequencing data showing this stretch is illustrated in Figure 3.9. Eight consecutive T's were observed for samples NFAY 1_P1, NFAY 2_P2, NFAY 3.5, NFAY 8_P1, NFAY 16_P2, NCYC456, WLP028 and WLP051.

Vidgren et al. (2005, 2010) found that the AGT1 sequences from the ale strains encoded full-length (616 amino acid) polypeptides, while the lager strains encoded truncated (394 amino acid) polypeptides. The authors concluded that this particular AGT1 gene mutation producing a premature stop codon, is a characteristic of lager strains (Vidgren et al., 2010). This matches the result for the control strain NCYC456, that is a *S. pastorianus* strain, as the extra T was established. The majority of the other samples that carried the presumably defective gene, had only been classified to the genus level *Saccharomyces*, and it is therefore not possible to draw the same conclusion from these. Interestingly, two of the samples, NFAY 8_P1 and WLP028, also carried the particular gene mutation and were classified as *S. cerevisiae*.

This trait was further investigated by inspecting the Gallone et al. (2016) sequence data, by executing a MSA of the data. Out of the 136 sequences with the full-length AGT1 allele, 33 had the insertion of the extra T at the named position.

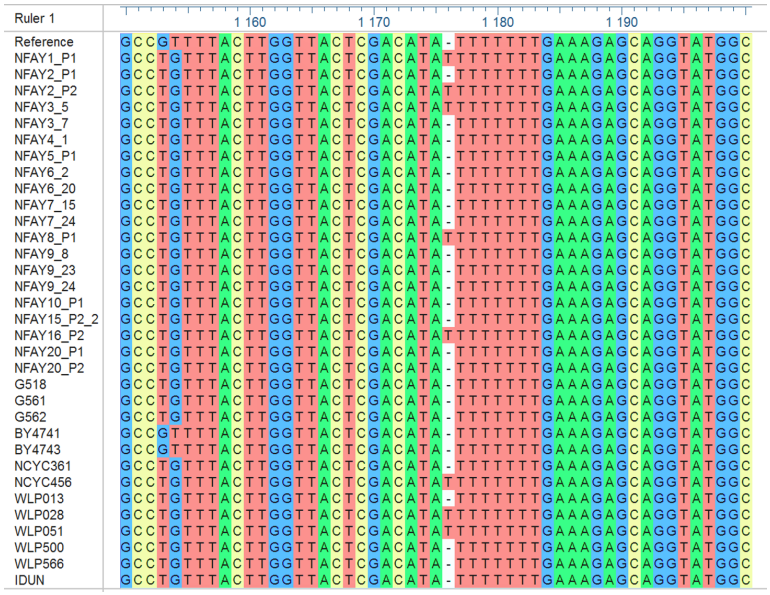
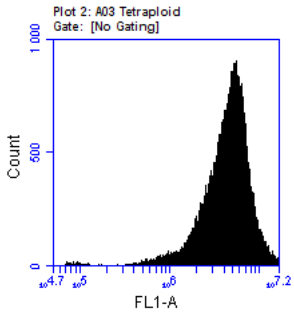


Figure 3.9: AGT1 region that spans position 1150 to 1200 of the SGD reference sequence of the AGT1 gene. The frameshift and premature stop codon TGA, starting at nucleotide 1183, is included in the region. A gap is inserted for the samples that do not carry this extra T (i.e. that only have seven consecutive T's).

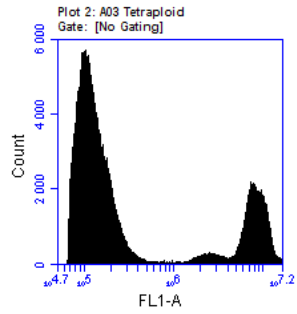
3.2.4 DNA Content Determination by Flow Cytometry

When evaluating the best suitable dilution agent among MOPS-, HEPES- and TE-buffers, only minor differences between them were observed. However, TE-buffer showed more consistency regarding the cell size distribution, and was therefore considered to be the best candidate. TE-buffer is comprised of both Tris and EDTA, where the latter is a chelator agent (meaning that it is capable of forming complexes with metal ions) (Loureiro et al., 2006). Ca^{2+} ions have repeatedly been reported to lead to cell clumping and promote flocculation in yeasts. Removal of calcium ions by chelating agents such as EDTA can therefore encourage deflocculation (Stratford, 1989). The properties and effect of EDTA can justify why the TE-buffer performed better than the others, causing less cell clumping. The cell size distribution only showed subtle changes when cells were harvested after 0, 24, 48 and 72 hours. Similarly, the effect of adding various staining ratios (1:10, 1:20 and 1:50), did not seem to affect the distribution of the fluorescence recovered. Hence, the ratio that required the least amount of dye (i.e. 1:50) was chosen for the assay.

Clumping of cells led to increased intensity of fluorescence, as seen in Figure 3.10a, and was evident when inspecting the samples in a microscope. A linear relationship between fluorescence intensity and ploidy can not be established in this case. Several steps were added to prevent the clumping of cells. During the fixation, an extra washing step with TE-buffer was executed. This extra step with TE-buffer was admitted for the same reasons as explained above. Moreover, the fixation was prepared in 15 mL Falcon tubes to allow better mixing when adding ethanol while vortexing. Before being analyzed on the flow cytometer the sample was also sonicated for approximately 10 seconds. Finally, the solution was filtered with 10 µm non-sterile syringe filcon (BD Biosciences) to remove the cells who were already clumped together. An improvement was observed when these additional steps were applied, both in the flow cytometer results and recognized in the microscope. This measure is therefore encouraged to use in future studies when flow cytometry is practiced. Figure 3.10b shows the appearance of a peak with lower fluorescence intensity, as one would expect with single cell suspensions.



(a) Before applying additional steps.



(b) After applying additional steps.

Figure 3.10: Flow cytometry of tetraploid strain stained with SYBR® Green I, before and after additional measures to improve the protocol was applied. The plot shows the fluorescence intensity of the stained cells recovered in the FL1-A channel on the x-axis (logarithmic scale), while the count is displayed on the y-axis. The run-limit was set to 25,000 events.

Despite all this effort, it was not possible to estimate the ploidy based on the DNA content due to problems with clumping of cells in some of the control strains. High quality and reproducible flow cytometry depends on a single cell suspension, thus, the ensuing data was not good enough for this analysis. However, a lot of effort was made to optimize the protocol for the analysis. Other treatments that could be of interest to explore are the addition of DNase prior to analysis and increasing the concentration of EDTA in the buffer to avoid cell clumping. Non-ionic detergents such as Triton X-100 or Tween 20 could also be included in the buffer formula, or one could also get hold of other reference strains with minimal flocculation ability characteristics.

3.3 Characterization of Phenotypic Properties

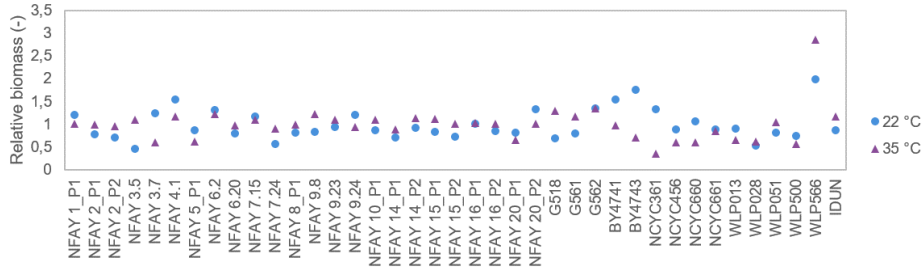
As part of the monitoring of the fermentation process the tubes were weighed the last four days to make sure the fermentation had finished, confirmed by small to no variation in weight. The weights from this monitoring are listed in Table B.3 and Table B.4 for 22 °C, and Table B.5 and Table B.6 for 35 °C in Appendix B.

The biomass yield produced during the fermentation was measured by diluting the cell pellet in 40 mL distilled water, and measuring the OD at 600 nm. The average of all OD measurements was used to calculate the relative biomass for each sample. Graphs showing the relative biomass and pH-value for the samples are given in Figure 3.11. Raw data and calculated relative biomass are given in Table B.7 in Appendix B.

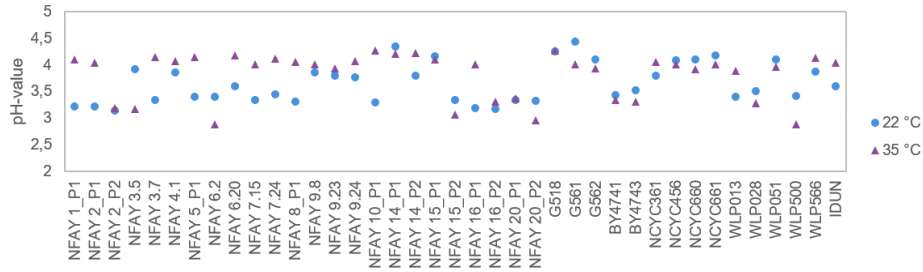
Figure 3.11a shows that sample WLP566 had a very high biomass yield compared to the rest. Samples NFAY 3.7, NFAY 4.1, BY4741, BY4743 and NCYC361 appeared to have a much greater yield at 22 °C than at 35 °C. The opposite trend was observed for samples NFAY 3.5, G518 and WLP566.

The pH-values in Figure 3.11b range from pH 2.9 to pH 4.4. The differences observed in pH-values between the two temperatures were greater than those observed for biomass yield. Especially samples NFAY 1_P1 (22 °C), NFAY 2_P1 (22 °C), NFAY 1_P1 (22 °C and 35 °C), NFAY 3.5 (35 °C), NFAY 6.2 (35 °C), NFAY 15_P2 (22 °C and 35 °C), NFAY 16_P1 (22 °C), NFAY 20_P1 (22 °C and 35 °C), NFAY 20_P2 (22 °C and 35 °C) and WLP500 (35 °C) had low pH-levels. NFAY 10_P1 had the greatest difference in pH between the two temperatures; 0.97.

Three analyses were performed to quantify and inspect volatile and non-volatile compounds in beer. HS GC-MS was run to analyze volatile flavor and aroma compounds, while HPLC with UV and RI detection was performed to quantify sugars, alcohols and organic acids. LC-MS was run to analyze remaining sugar in the beer samples, especially focusing on the carbohydrate maltotriose.



(a) Relative biomass



(b) pH-values

Figure 3.11: OD values of resuspended biomass relative to an average biomass concentration and pH-value for samples at 22 °C and 35 °C.

3.3.1 Quantification of Volatile Flavor and Aroma Compounds by Headspace GC-MS

15 compounds were investigated in the HS GC-MS analysis; 1-propanol, ethyl acetate, isobutyl alcohol, 1-butanol, ethyl propionate, propyl acetate, acetal, isoamyl alcohol, active amyl alcohol, isobutyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate. The concentrations determined for the samples at 22 °C are given in Table 3.4 and Table 3.5, while the concentrations determined for the samples at 35 °C are given in Table 3.6 and Table 3.7. Some of the samples had very high levels of the compound ethyl acetate. It was evident that the column was overloaded, as the peak shape was shifted to the right and thus not representative. However, since this issue only occurred for this particular compound, the samples were not diluted and ran again. Instead, the samples with this problem were set to be greater than the highest point of the calibration range; 131,440 µg/mL. The samples that were below the LOQ were set to be zero. The compounds ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate had levels below the LOQ in all samples.

As an alternative presentation of the results, the data was analyzed with PCA. The concentrations of the compounds ethyl propionate, propyl acetate and isobutyl acetate were not included in the PCA, as too much data was missing for these compounds in order for PC2 to converge. Samples BY4741 and BY4743 at 35 °C were also excluded for the same reasons. The PCA on 4^{th} root transformed profiles is shown in Figure 3.12 and Figure 3.13. The former show both the distribution between the categories (i.e. NFAY, wild, laboratory and commercial) at 22 °C and 35 °C, whereas the latter only show the distribution only according to temperature. The plots shows the first two principal components, PC1 and PC2, which together account for 79% of the data set variation.

The two scores plots in Figure 3.12 and Figure 3.13 reveal that the samples are generally divided into two groupings. The first plot shows that the NFAY samples are distributed in both groups (along with commercial samples), while the second PCA plot shows that there is only a slight discrimination between the two fermentation temperatures. Sample NFAY 6.2 (35 °C) and NCYC456 (35 °C) can be considered as outliers according to Hotelling T^2 statistics with a 95% confidence. The loadings plot in Figure 3.14 suggests that the group furthest to the left seems to be largely affected by the variance in the acetate esters ethyl acetate and isoamyl acetate. The group to the right is associated with higher alcohols.

Table 3.4: Quantification of volatile compounds by headspace GC-MS for NFAY samples at 22 °C. The compounds are numbered as following: 1, 1-Propanol; 2, Ethyl acetate; 3, Isobutyl alcohol; 4, 1-Butanol; 5, Ethyl propionate; 6, Propyl acetate; 7, Acetal; 8, Isoamyl alcohol; 9, Active amyl alcohol; 10, Isobutyl acetate and 11, Isoamyl acetate.

	Concentration [mg/L]										
Sample	1	2	3	4	5	6	7	8	9	10	11
NFAY 1_P1	19.02	131.44	34.27	0.61	0.00	0.56	0.85	108.61	25.04	0.73	2.57
NFAY 2_P1	16.65	131.44	17.22	0.73	0.00	0.36	0.00	68.23	14.10	0.33	1.10
NFAY 2_P2	15.06	49.75	13.73	3.16	0.00	0.00	2.13	67.42	13.85	0.00	0.17
NFAY 3.5	35.78	22.72	34.71	0.89	0.00	0.00	1.49	118.87	27.53	0.00	0.36
NFAY 3.7	37.32	131.44	31.02	0.51	0.00	0.52	0.56	109.87	24.05	0.38	1.27
NFAY 4.1	30.52	19.28	69.43	1.33	0.25	0.00	1.68	139.88	41.07	0.00	0.19
NFAY 5_P1	22.49	131.44	33.29	0.61	0.00	0.27	0.44	120.74	24.78	0.37	1.12
NFAY 6.2	32.87	131.44	34.17	1.20	0.20	0.41	0.58	109.74	26.15	0.36	1.14
NFAY 6.20	24.54	30.37	35.74	0.69	0.00	0.00	2.94	125.39	27.45	0.00	0.19
NFAY 7.15	21.21	131.44	44.62	0.59	0.00	0.57	0.51	110.17	30.08	0.99	2.62
NFAY 7.24	22.46	131.44	50.26	0.64	0.00	0.30	0.50	141.89	34.36	0.52	1.45
NFAY 8_P1	42.14	131.44	24.71	0.55	0.22	0.62	0.56	89.91	17.45	0.38	1.21
NFAY 9.8	26.13	28.01	28.05	0.69	0.00	0.00	1.21	117.65	22.87	0.00	0.34
NFAY 9.23	29.54	19.02	39.47	1.24	0.00	0.00	0.40	123.08	29.00	0.00	0.09
NFAY 9.24	32.29	26.91	37.69	0.73	0.00	0.00	0.26	131.86	28.16	0.00	0.43
NFAY 10_P1	22.14	27.32	32.71	0.79	0.00	0.00	0.83	116.23	26.86	0.11	0.25
NFAY 14_P1	36.94	24.84	40.47	0.96	0.20	0.00	2.11	99.39	29.38	0.12	0.74
NFAY 14_P2	43.78	131.44	49.23	0.84	0.17	0.18	0.74	127.63	38.32	0.20	0.42
NFAY 15_P1	49.62	22.13	56.72	0.98	0.26	0.00	2.31	127.53	37.19	0.00	0.56
NFAY 15_P2	33.89	131.44	51.53	0.60	0.00	0.95	0.32	100.02	31.80	1.11	2.16
NFAY 16_P1	16.13	131.44	28.39	0.55	0.00	0.59	0.54	84.87	19.90	0.74	2.18
NFAY 16_P2	15.50	131.44	19.65	0.76	0.00	0.46	0.65	62.02	13.81	0.49	1.34
NFAY 20_P1	26.43	131.44	24.56	1.13	0.17	0.41	0.56	114.05	21.95	0.35	1.68
NFAY 20_P2	23.65	131.44	22.85	0.90	0.18	0.49	0.62	85.47	18.22	0.46	1.67

Table 3.5: Quantification of volatile compounds by headspace GC-MS for wild, laboratory and commercial samples at 22 °C. The compounds are numbered as following: 1, 1-Propanol; 2, Ethyl acetate; 3, Isobutyl alcohol; 4, 1-Butanol; 5, Ethyl propionate; 6, Propyl acetate; 7, Acetal; 8, Isoamyl alcohol; 9, Active amyl alcohol; 10, Isobutyl acetate and 11, Isoamyl acetate.

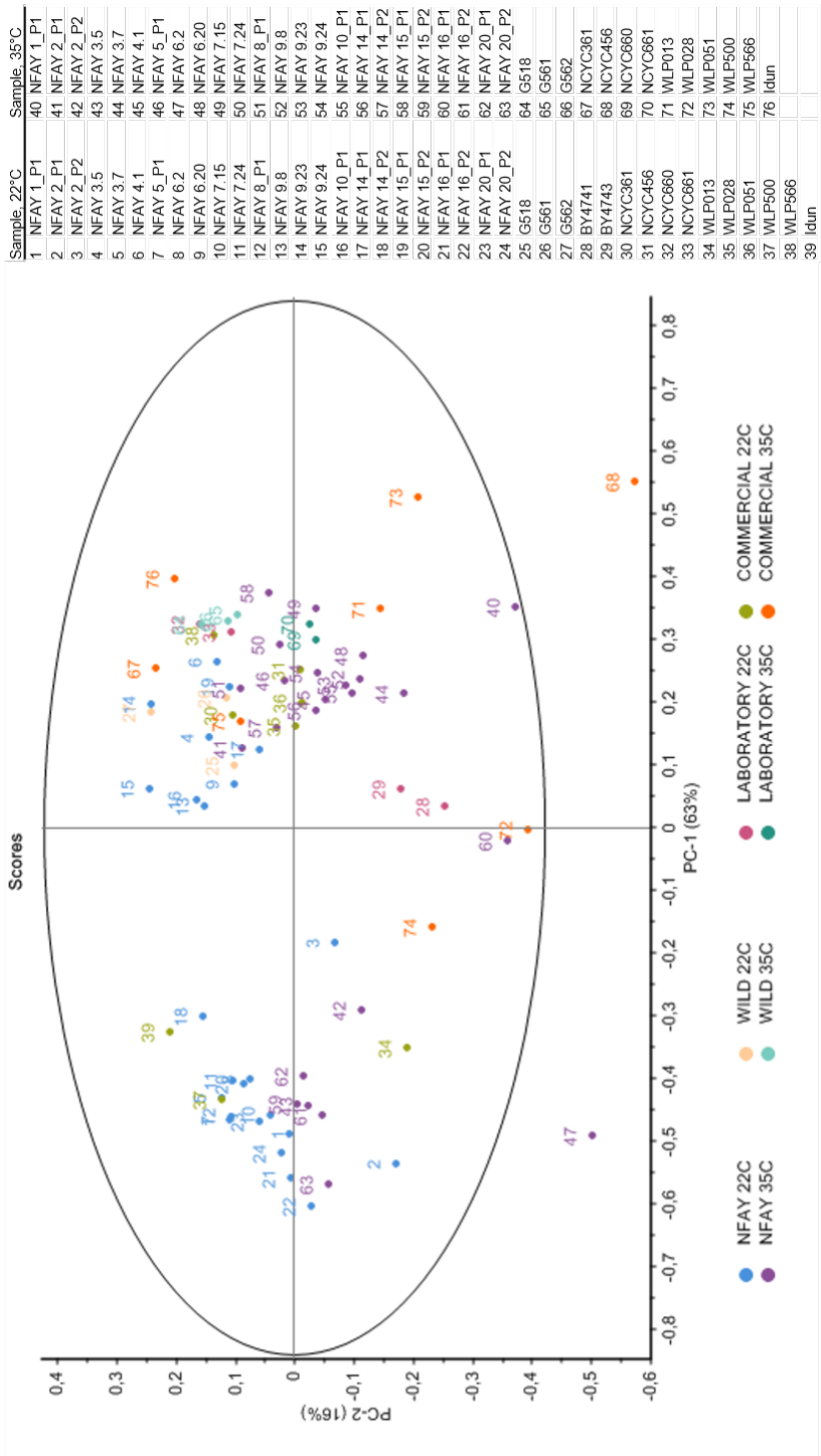
	Concentration [mg/L]										
Sample	1	2	3	4	5	6	7	8	9	10	11
G518	39.29	27.41	51.41	0.74	0.00	0.00	1.19	120.52	40.68	0.22	1.51
G561	45.50	23.85	69.28	1.13	0.45	0.00	1.46	138.23	49.55	0.17	0.89
G562	80.07	40.26	85.97	0.61	0.40	0.00	1.30	145.06	66.70	0.00	0.17
BY4741	16.26	25.04	30.00	0.67	0.00	0.00	3.53	55.51	16.44	0.00	0.00
BY4743	14.18	20.13	31.27	0.51	0.00	0.00	1.85	61.93	17.25	0.00	0.00
NCYC361	51.42	31.02	66.03	1.11	0.20	0.00	3.03	163.92	45.21	0.12	0.64
NCYC456	39.83	21.59	63.74	1.10	0.00	0.00	0.00	144.51	48.19	0.00	0.31
NCYC660	53.21	10.67	31.24	1.58	0.00	0.00	0.52	67.42	17.70	0.00	0.24
NCYC661	76.83	14.36	26.30	2.88	0.28	0.00	0.98	63.67	21.95	0.00	0.28
WLP013	24.48	131.44	60.26	0.00	0.00	0.56	0.75	72.86	24.67	0.94	1.19
WLP028	32.13	21.15	77.04	0.53	0.00	0.00	0.19	94.86	24.19	0.00	0.00
WLP051	29.98	13.25	18.09	1.58	0.00	0.00	0.27	81.25	22.93	0.00	0.00
WLP500	40.50	131.44	31.73	0.66	0.17	0.79	0.36	116.25	22.89	0.51	1.90
WLP566	33.70	16.30	60.43	1.38	0.00	0.00	1.95	135.97	39.51	0.00	0.15
Idun	44.50	131.44	47.48	0.80	0.00	0.27	0.26	141.91	37.92	0.24	0.56

Table 3.6: Quantification of volatile compounds by headspace GC-MS for NFAY samples at 35 °C. The compounds are numbered as following: 1, 1-Propanol; 2, Ethyl acetate; 3, Isobutyl alcohol; 4, 1-Butanol; 5, Ethyl propionate; 6, Propyl acetate; 7, Acetal; 8, Isoamyl alcohol; 9, Active amyl alcohol; 10, Isobutyl acetate and 11, Isoamyl acetate.

	Concentration [mg/L]										
Sample	1	2	3	4	5	6	7	8	9	10	11
NFAY 1_P1	39.22	12.88	56.61	3.73	0.00	0.00	0.00	92.83	28.85	0.00	0.00
NFAY 2_P1	32.06	27.71	47.19	1.89	0.00	0.00	1.13	100.62	27.45	0.00	0.25
NFAY 2_P2	12.04	53.43	20.56	0.75	0.00	0.00	0.33	63.20	17.20	0.00	0.00
NFAY 3.5	27.69	131.44	43.04	0.64	0.22	1.16	1.00	83.07	25.16	1.35	2.85
NFAY 3.7	39.95	17.72	32.24	1.66	0.00	0.00	2.34	67.74	16.71	0.00	0.00
NFAY 4.1	23.65	16.64	52.02	0.55	0.00	0.00	0.00	64.28	25.34	0.00	0.16
NFAY 5_P1	35.01	21.51	42.46	4.80	0.00	0.00	1.82	102.78	26.78	0.00	0.19
NFAY 6.2	5.77	131.44	35.83	0.00	0.00	0.38	0.00	45.58	18.30	1.42	1.52
NFAY 6.20	38.13	14.07	35.04	4.02	0.00	0.00	0.40	78.37	21.87	0.00	0.00
NFAY 7.15	34.01	14.69	44.76	4.25	0.00	0.00	4.72	96.32	26.86	0.00	0.11
NFAY 7.24	32.48	16.31	51.09	3.09	0.00	0.00	2.02	92.04	27.84	0.00	0.17
NFAY 8_P1	39.90	17.54	24.68	1.88	0.00	0.00	1.90	83.17	16.99	0.00	0.11
NFAY 9.8	21.62	17.69	40.44	3.68	0.00	0.00	0.00	97.47	25.56	0.00	0.11
NFAY 9.23	30.26	17.10	44.36	3.59	0.00	0.00	0.24	98.70	27.85	0.00	0.00
NFAY 9.24	32.97	20.88	44.70	2.97	0.00	0.00	4.78	92.84	26.71	0.00	0.17
NFAY 10_P1	37.69	23.44	43.57	3.43	0.00	0.00	0.00	106.35	29.17	0.00	0.23
NFAY 14_P1	45.57	26.70	48.61	3.73	0.00	0.00	2.87	92.85	29.89	0.14	0.64
NFAY 14_P2	40.97	23.15	41.70	1.63	0.00	0.00	1.86	83.12	26.36	0.13	0.60
NFAY 15_P1	47.86	15.37	60.54	3.74	0.00	0.00	2.73	107.36	34.44	0.00	0.13
NFAY 15_P2	28.57	131.44	50.85	0.67	0.00	1.38	0.53	87.55	27.76	2.06	3.85
NFAY 16_P1	36.69	50.57	40.78	1.70	0.00	0.00	0.00	81.69	23.21	0.00	0.00
NFAY 16_P2	24.89	131.44	33.44	0.76	0.28	0.58	1.68	70.88	19.21	0.60	1.32
NFAY 20_P1	27.26	131.44	41.79	1.03	0.19	0.48	1.49	99.74	25.63	0.63	1.59
NFAY 20_P2	12.18	131.44	35.49	0.61	0.19	0.76	0.61	71.16	20.01	1.38	2.53

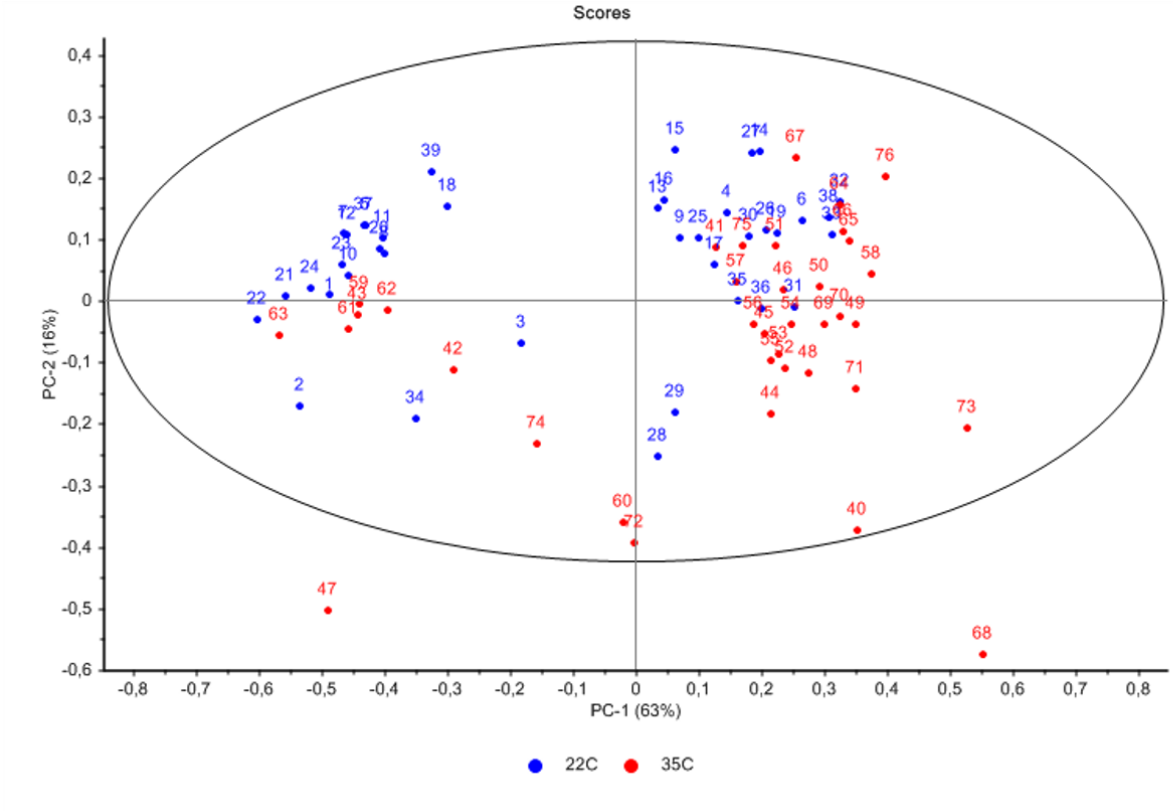
Table 3.7: Quantification of volatile compounds by headspace GC-MS for wild, laboratory and commercial samples at 35 °C. The compounds are numbered as following: 1, 1-Propanol; 2, Ethyl acetate; 3, Isobutyl alcohol; 4, 1-Butanol; 5, Ethyl propionate; 6, Propyl acetate; 7, Acetal; 8, Isoamyl alcohol; 9, Active amyl alcohol; 10, Isobutyl acetate and 11, Isoamyl acetate.

	Concentration [mg/L]										
Sample	1	2	3	4	5	6	7	8	9	10	11
G518	74.43	20.31	78.42	1.95	0.19	0.00	1.04	111.22	40.38	0.00	0.23
G561	58.70	18.40	76.14	2.06	0.16	0.00	2.39	125.50	43.61	0.10	0.28
G562	63.33	20.46	76.82	2.09	0.19	0.00	2.37	131.04	43.29	0.00	0.22
BY4741	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BY4743	0.00	4.29	2.89	0.00	0.00	0.00	0.00	0.00	5.98	0.00	0.00
NCYC361	46.88	18.48	60.25	1.39	0.00	0.00	0.25	107.73	32.47	0.00	0.18
NCYC456	6.78	1.04	25.51	0.00	0.00	0.00	0.00	23.35	5.55	0.00	0.00
NCYC660	62.30	16.78	63.17	1.47	1.98	0.00	0.45	88.27	33.63	0.00	0.00
NCYC661	84.36	16.29	56.63	1.91	0.00	0.00	0.30	73.63	32.27	0.00	0.00
WLP013	59.54	13.69	64.59	1.37	0.00	0.00	1.79	67.89	24.16	0.00	0.00
WLP028	26.37	42.85	63.77	0.00	0.00	0.00	0.91	50.33	18.89	0.00	0.00
WLP051	29.81	3.30	32.17	1.18	0.00	0.00	2.23	40.98	12.70	0.00	0.00
WLP500	1.38	20.24	32.29	0.00	0.00	0.00	0.31	54.55	15.83	0.90	1.07
WLP566	34.85	35.64	114.82	1.63	0.00	0.00	1.57	150.00	60.20	0.15	0.41
Idun	44.00	10.10	48.94	2.88	0.00	0.00	0.30	102.63	27.85	0.00	0.10



Sample, 22°C		Sample, 35°C	
1	NFAY 1_P1	40	NFAY 1_P1
2	NFAY 2_P1	41	NFAY 2_P1
3	NFAY 2_P2	42	NFAY 2_P2
4	NFAY 3.5	43	NFAY 3.5
5	NFAY 3.7	44	NFAY 3.7
6	NFAY 4.1	45	NFAY 4.1
7	NFAY 5_P1	46	NFAY 5_P1
8	NFAY 6.2	47	NFAY 6.2
9	NFAY 6.20	48	NFAY 6.20
10	NFAY 7.15	49	NFAY 7.15
11	NFAY 7.24	50	NFAY 7.24
12	NFAY 8_P1	51	NFAY 8_P1
13	NFAY 9.8	52	NFAY 9.8
14	NFAY 9.23	53	NFAY 9.23
15	NFAY 9.24	54	NFAY 9.24
16	NFAY 10_P1	55	NFAY 10_P1
17	NFAY 14_P1	56	NFAY 14_P1
18	NFAY 14_P2	57	NFAY 14_P2
19	NFAY 15_P1	58	NFAY 15_P1
20	NFAY 15_P2	59	NFAY 15_P2
21	NFAY 16_P1	60	NFAY 16_P1
22	NFAY 16_P2	61	NFAY 16_P2
23	NFAY 20_P1	62	NFAY 20_P1
24	NFAY 20_P2	63	NFAY 20_P2
25	G518	64	G518
26	G561	65	G561
27	G562	66	G562
28	BY4741	67	NCYC361
29	BY4743	68	NCYC456
30	NCYC361	69	NCYC660
31	NCYC456	70	NCYC861
32	NCYC660	71	WLP013
33	NCYC661	72	WLP028
34	WLP013	73	WLP051
35	WLP028	74	WLP500
36	WLP051	75	WLP566
37	WLP500	76	Idun
38	WLP566		
39	Idun		

Figure 3.12: PCA of volatile compounds data obtained by headspace GC-MS. The scores plot shows the distribution of the categories, NFAY, wild, laboratory and commercial samples, at 22 °C and 35 °C. The ellipses represent Hotelling T^2 statistics with a 95% confidence, to reveal potential outliers.



Sample, 22°C		Sample, 35°C	
1	NFAY 1_P1	40	NFAY 1_P1
2	NFAY 2_P1	41	NFAY 2_P1
3	NFAY 2_P2	42	NFAY 2_P2
4	NFAY 3.5	43	NFAY 3.5
5	NFAY 3.7	44	NFAY 3.7
6	NFAY 4.1	45	NFAY 4.1
7	NFAY 5_P1	46	NFAY 5_P1
8	NFAY 6.2	47	NFAY 6.2
9	NFAY 6.20	48	NFAY 6.20
10	NFAY 7.15	49	NFAY 7.15
11	NFAY 7.24	50	NFAY 7.24
12	NFAY 8_P1	51	NFAY 8_P1
13	NFAY 9.8	52	NFAY 9.8
14	NFAY 9.23	53	NFAY 9.23
15	NFAY 9.24	54	NFAY 9.24
16	NFAY 10_P1	55	NFAY 10_P1
17	NFAY 14_P1	56	NFAY 14_P1
18	NFAY 14_P2	57	NFAY 14_P2
19	NFAY 15_P1	58	NFAY 15_P1
20	NFAY 15_P2	59	NFAY 15_P2
21	NFAY 16_P1	60	NFAY 16_P1
22	NFAY 16_P2	61	NFAY 16_P2
23	NFAY 20_P1	62	NFAY 20_P1
24	NFAY 20_P2	63	NFAY 20_P2
25	G518	64	G518
26	G561	65	G561
27	G562	66	G562
28	BY4741	67	NCYC361
29	BY4743	68	NCYC456
30	NCYC361	69	NCYC660
31	NCYC456	70	NCYC661
32	NCYC660	71	WLP013
33	NCYC661	72	WLP028
34	WLP013	73	WLP051
35	WLP028	74	WLP500
36	WLP051	75	WLP566
37	WLP500	76	Idun
38	WLP566		
39	Idun		

Figure 3.13: PCA of volatile compounds data obtained by headspace GC-MS. The scores plot shows the distribution according to temperature; 22 °C and 35 °C. The ellipses represent Hotelling T^2 statistics with a 95% confidence, to reveal potential outliers.

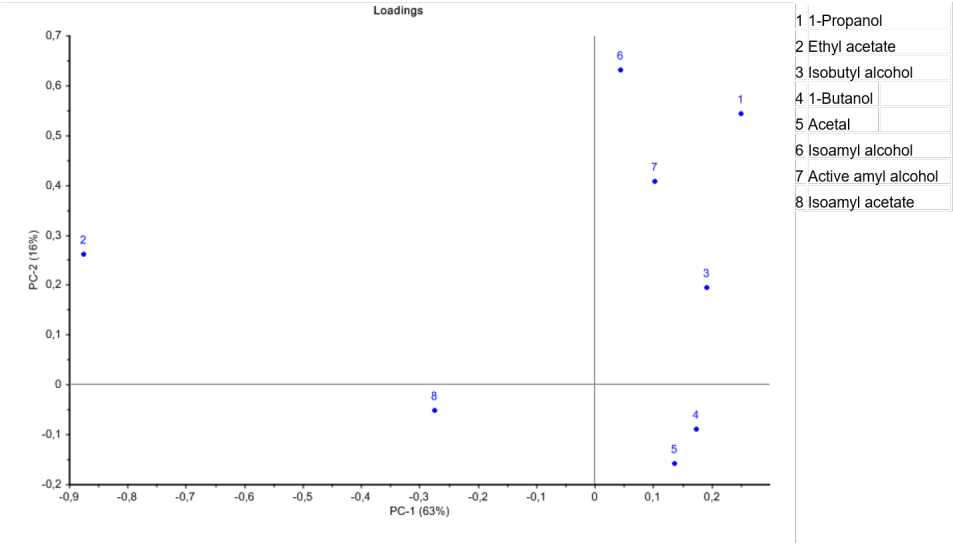


Figure 3.14: PCA of volatile compounds data obtained by headspace GC-MS. The loadings plot shows the distribution of the compounds.

Heat Map According to Flavor Thresholds

The absolute amount of flavor-active compounds is not really relevant by itself for beer flavor, as it may not be perceived at this concentration. Maarse (1991) established that more important is the relationship between the concentration and the sensory flavor threshold of the individual compounds, to enable a proper estimation of their contribution. The FU-value was therefore calculated for the compounds that were quantified with HS GC-MS, using the flavor thresholds in Table 1.1 under Section 1.5. The calculated FU-values are given in Table C.2 and Table C.3 in Appendix C for 22 °C and 35 °C, respectively. A heat map representation of the FU-values according to flavor threshold is shown in Figure 3.15. The compounds isoamyl alcohol, 1-butanol, ethyl aceate and isoamyl acetate seems to be especially flavor-active at both 22 and 35 °C, when inspecting the coloring scheme. It is also evident that the first group (i.e. NFAY 1_P1 through NFAY 15_P2) has extensive values of ethyl acetate at 22 °C. Furthermore, there is an increase in 1-butanol from 22 °C to 35 °C.

The heat map in Figure 3.15 shows that the compounds isoamyl alcohol, 1-butanol, ethyl acetate and isoamyl acetate are prominent in the beer samples. Isoamyl alcohol contributes with alcoholic, vinous, banana-like, sweet flavors, while 1-butanol is described as banana-like, estery, solvent-like and sweet. Similarly, the two last flavors are also a characteristic of ethyl acetate. At last, isoamyl acetate is described as having a banana, ester and solvent-like flavor. In other words, many of the compounds are repetitious. It is therefore fair to assume that the finished beer product will be strongly affected by sweet, banana- and solvent-like flavors.

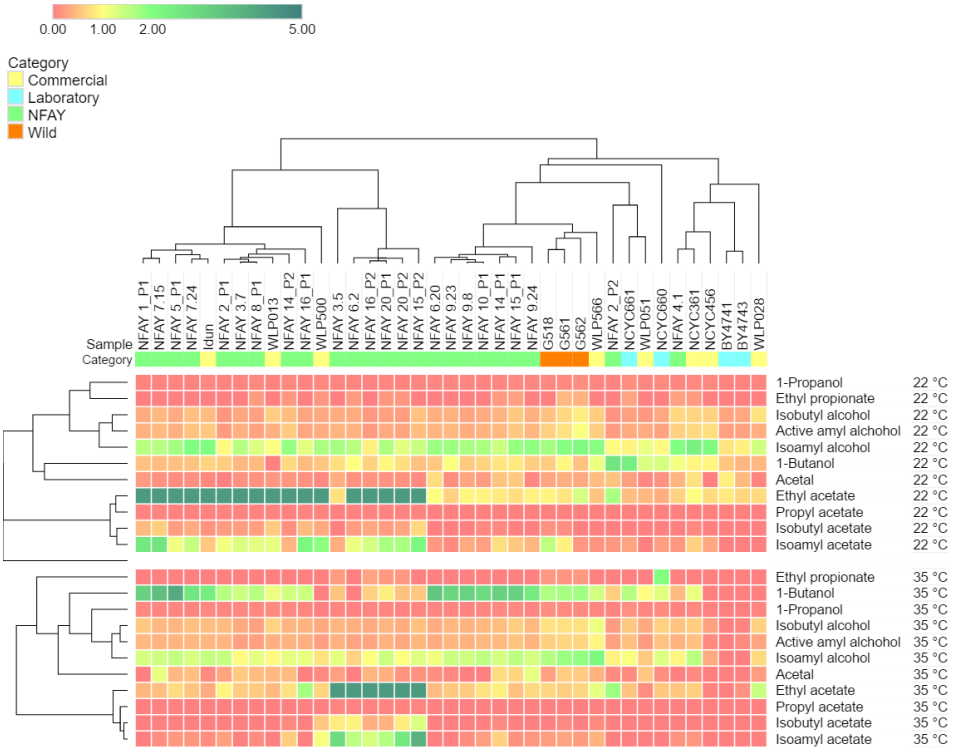


Figure 3.15: Heat map representation of the flavor unit (FU). The FU-values are colored according to the scale on top, in order to assess if they can be perceived. The samples are hierarchically clustered based on phenotypic behavior and according to compound. The samples are also colored to show which category (NFAY, wild, laboratory and commercial) it belongs to.

3.3.2 Sugar, Alcohol and Organic Acid Quantification with HPLC

The concentrations determined for samples at 22 °C and 35 °C are given in Table 3.8 and Table 3.9, respectively. The raw data and calculations for the external single point calibration for the various compounds (along with an example calculation), are given in Appendix D. Similarly to the headspace GC-MS results, the concentrations were analyzed with PCA. Glucose and fructose had been completely consumed in nearly all the samples, and were not included in the analysis. The PCA on 4^{th} root transformed profiles are shown in Figure 3.16 and Figure 3.17. The former show both the distribution between the categories (i.e. NFAY, wild, laboratory and commercial) at 22 °C and 35 °C, whereas the latter only show the distribution only according to temperature. The plots shows the first two principal components, PC1 and PC2, which together account for 98% of the data set variation.

Table 3.8: Sugar, alcohol and organic acid quantification with HPLC for samples at 22 °C. The concentrations of glucose, fructose, glycerol, ethanol, acetic acid and succinic acid are given in g/L.

	Concentration [g/L]					
Sample	Glucose	Fructose	Glycerol	Ethanol	Acetic acid	Succinic acid
NFAY 1_P1	0.00	0.00	0.61	29.24	17.83	5.59
NFAY 2_P1	0.00	0.00	1.53	28.86	14.85	5.93
NFAY 2_P2	2.33	0.42	0.81	25.30	10.76	5.20
NFAY 3.5	0.00	0.00	1.45	40.76	1.32	6.47
NFAY 3.7	0.00	0.00	1.37	34.94	10.65	5.62
NFAY 4.1	0.00	0.00	0.00	35.92	0.05	6.03
NFAY 5_P1	0.00	0.00	1.62	37.46	9.92	6.37
NFAY 6.2	0.00	0.00	1.40	40.66	0.10	5.79
NFAY 6.20	0.00	0.00	1.89	38.85	7.77	6.45
NFAY 7.15	0.00	0.00	1.28	28.89	18.25	6.80
NFAY 7.24	0.00	0.00	1.57	35.27	10.09	4.98
NFAY 8_P1	0.00	0.00	0.94	34.38	11.06	4.74
NFAY 9.8	0.00	0.00	1.68	41.30	3.47	5.94
NFAY 9.23	0.00	0.00	1.54	39.16	3.98	6.17
NFAY 9.24	0.00	0.00	1.77	40.07	4.05	5.19
NFAY 10_P1	0.00	0.00	1.13	31.63	13.64	6.58
NFAY 14_P1	0.00	0.00	1.69	40.74	0.05	6.93
NFAY 14_P2	0.00	0.00	1.88	38.82	6.53	7.73
NFAY 15_P1	0.00	0.00	1.38	41.21	3.01	6.97
NFAY 15_P2	0.00	0.00	0.66	25.36	20.03	8.64
NFAY 16_P1	0.00	0.00	0.67	20.87	20.55	4.64
NFAY 16_P2	0.00	0.00	0.55	27.73	18.36	5.82
NFAY 20_P1	0.00	0.00	1.29	34.17	12.04	6.01
NFAY 20_P2	0.00	0.00	1.00	31.71	13.87	6.56
G518	0.00	0.00	1.97	43.05	0.05	6.30
G561	0.00	0.00	1.86	42.43	0.05	5.86
G562	0.00	0.00	0.53	36.26	0.05	4.14
BY4741	0.59	0.00	1.91	43.88	11.25	4.42
BY4743	1.20	0.00	0.45	41.08	8.44	5.11
NCYC361	0.00	0.00	1.33	47.44	4.77	5.16
NCYC456	0.00	0.00	1.55	41.91	0.05	6.01
NCYC660	0.00	0.00	0.00	29.59	3.15	4.30
NCYC661	0.00	0.00	0.00	33.07	0.05	4.83
WLP013	0.00	0.00	0.96	31.51	16.59	9.09
WLP028	0.00	0.00	1.81	33.37	12.13	7.96
WLP051	0.00	0.00	0.58	41.32	0.05	6.22
WLP500	0.00	0.00	1.55	31.37	14.79	5.46
WLP566	0.00	0.00	0.66	43.63	3.92	2.77
Idun	0.00	0.00	1.61	37.84	7.79	7.30

Table 3.9: Sugar, alcohol and organic acid quantification with HPLC for samples at 35 °C. The concentrations of glucose, fructose, glycerol, ethanol, acetic acid and succinic acid are given in g/L.

	Concentration [g/L]					
Sample	Glucose	Fructose	Glycerol	Ethanol	Acetic acid	Succinic acid
NFAY 1_P1	0.00	0.00	0.89	37.63	3.78	7.78
NFAY 2_P1	0.00	0.00	2.00	38.69	4.33	6.48
NFAY 2_P2	1.27	0.00	1.33	22.77	18.23	7.22
NFAY 3.5	0.00	0.74	1.05	23.04	20.55	7.57
NFAY 3.7	0.00	0.00	1.75	40.56	0.05	7.92
NFAY 4.1	0.00	0.00	1.65	48.04	0.05	6.97
NFAY 5_P1	0.00	0.00	2.00	39.34	3.46	7.64
NFAY 6.2	0.92	0.86	0.00	0.00	20.55	7.63
NFAY 6.20	0.00	0.00	1.95	35.84	3.38	9.26
NFAY 7.15	0.00	0.00	1.95	38.48	4.10	7.65
NFAY 7.24	0.00	0.00	1.72	38.89	3.97	7.39
NFAY 8_P1	0.00	0.00	1.77	40.76	3.27	7.43
NFAY 9.8	0.00	0.00	1.76	38.93	3.56	6.91
NFAY 9.23	0.00	0.00	1.69	38.48	4.08	7.55
NFAY 9.24	0.00	0.00	1.72	40.11	3.91	8.36
NFAY 10_P1	0.00	0.00	1.87	37.47	0.05	8.25
NFAY 14_P1	0.00	0.00	1.72	41.22	1.33	8.67
NFAY 14_P2	0.00	0.00	1.76	41.66	0.05	8.38
NFAY 15_P1	0.00	0.80	1.64	40.05	3.69	8.30
NFAY 15_P2	0.00	0.79	1.09	18.45	20.55	10.26
NFAY 16_P1	0.00	0.00	1.36	39.57	3.56	4.75
NFAY 16_P2	0.00	0.66	1.14	30.87	15.37	4.82
NFAY 20_P1	0.00	0.00	1.31	30.08	14.39	8.06
NFAY 20_P2	0.00	0.72	0.00	8.02	20.55	7.10
G518	0.00	0.00	1.38	38.39	0.05	6.86
G561	0.00	0.00	1.73	38.91	3.48	5.03
G562	0.00	0.00	1.76	38.64	3.59	5.05
BY4741	0.00	0.00	0.00	0.00	14.76	6.26
BY4743	0.00	0.00	0.00	0.00	16.10	6.95
NCYC361	0.00	0.00	1.90	47.63	3.62	4.38
NCYC456	0.00	0.41	1.43	13.38	0.05	7.25
NCYC660	0.00	0.43	0.00	25.41	0.05	7.86
NCYC661	0.00	0.38	0.00	26.90	0.05	7.12
WLP013	0.00	0.00	1.96	35.59	4.25	9.01
WLP028	0.00	0.94	1.88	24.39	19.25	8.50
WLP051	0.00	0.00	0.97	35.70	1.37	7.57
WLP500	0.00	1.19	0.93	0.00	20.55	7.02
WLP566	0.00	0.00	2.51	43.69	3.64	8.71
Idun	0.00	0.00	1.63	37.73	3.73	7.87

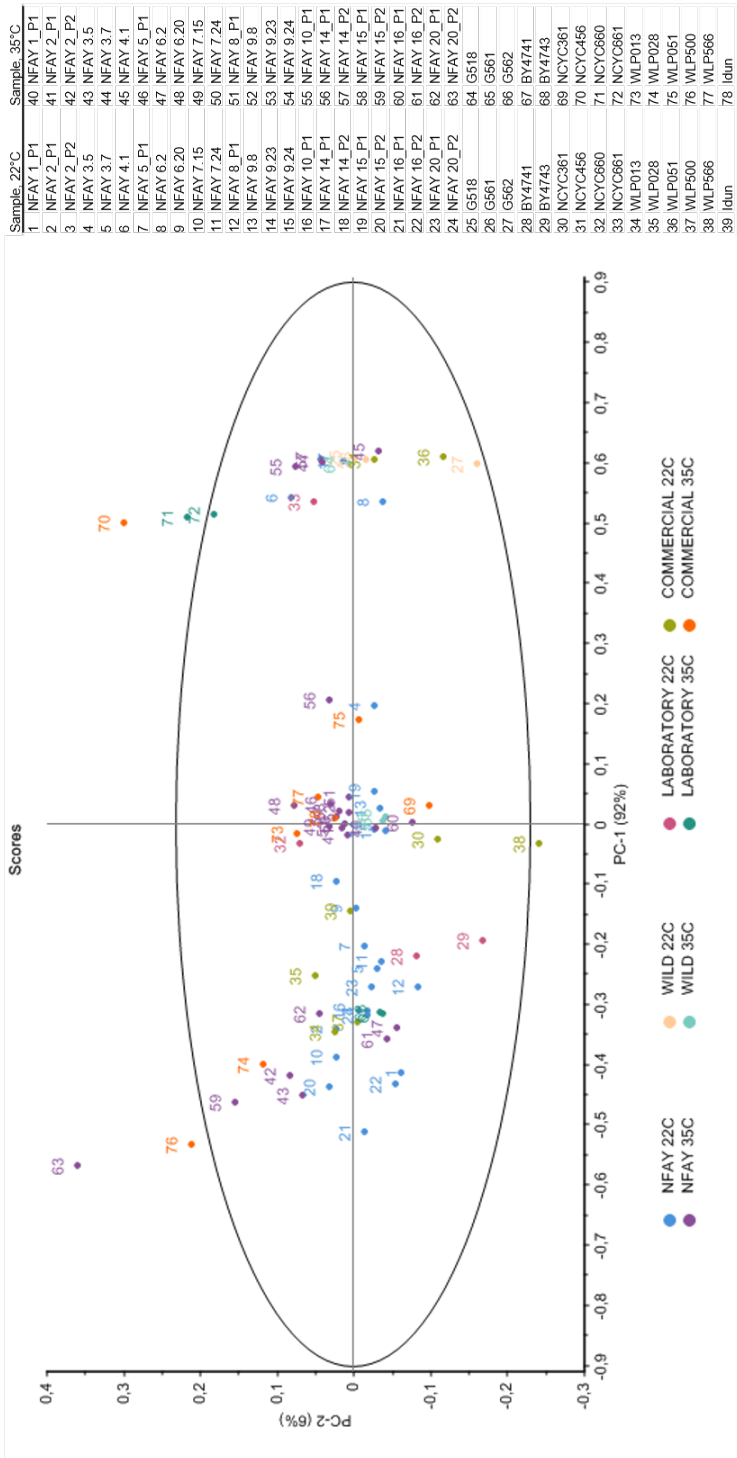
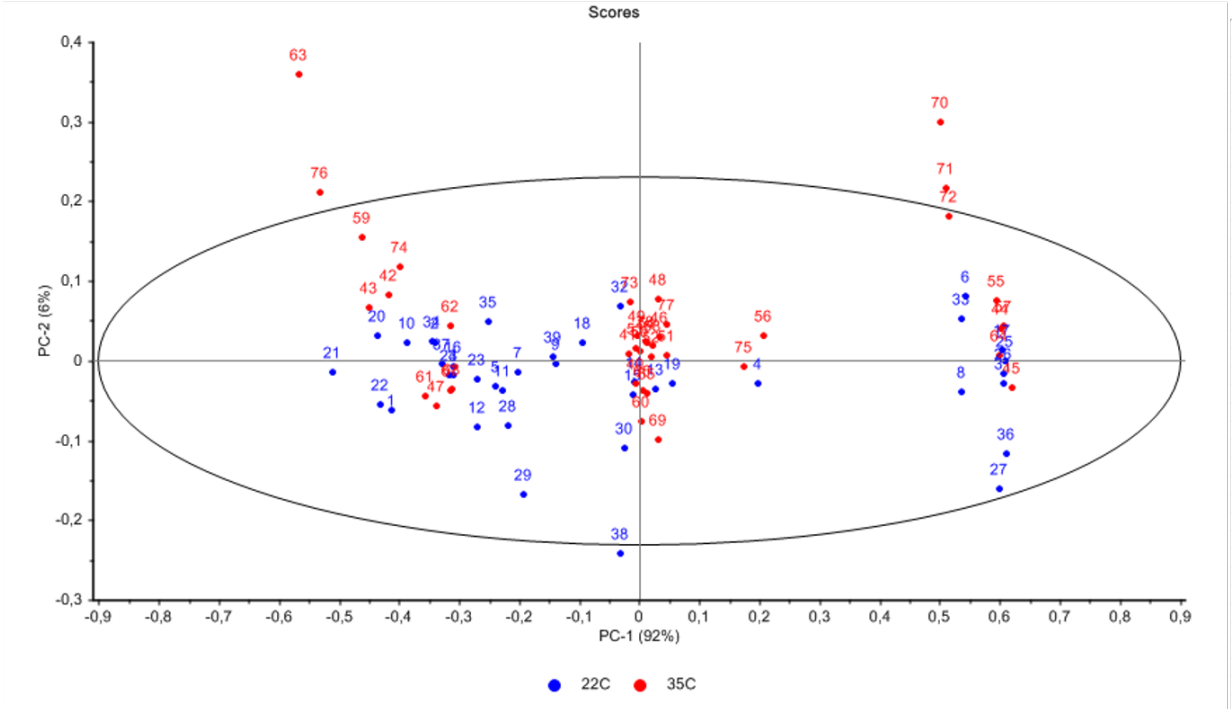


Figure 3.16: PCA of data obtained by HPLC. The scores plot shows the distribution of the categories, NFAY, wild, laboratory and commercial samples, at 22 °C and 35 °C. The ellipses represent Hotelling T^2 statistics with a 95% confidence, to reveal potential outliers.



Sample, 22°C		Sample, 35°C	
1	NFAY 1_P1	40	NFAY 1_P1
2	NFAY 2_P1	41	NFAY 2_P1
3	NFAY 2_P2	42	NFAY 2_P2
4	NFAY 3.5	43	NFAY 3.5
5	NFAY 3.7	44	NFAY 3.7
6	NFAY 4.1	45	NFAY 4.1
7	NFAY 5_P1	46	NFAY 5_P1
8	NFAY 6.2	47	NFAY 6.2
9	NFAY 6.20	48	NFAY 6.20
10	NFAY 7.15	49	NFAY 7.15
11	NFAY 7.24	50	NFAY 7.24
12	NFAY 8_P1	51	NFAY 8_P1
13	NFAY 9.8	52	NFAY 9.8
14	NFAY 9.23	53	NFAY 9.23
15	NFAY 9.24	54	NFAY 9.24
16	NFAY 10_P1	55	NFAY 10_P1
17	NFAY 14_P1	56	NFAY 14_P1
18	NFAY 14_P2	57	NFAY 14_P2
19	NFAY 15_P1	58	NFAY 15_P1
20	NFAY 15_P2	59	NFAY 15_P2
21	NFAY 16_P1	60	NFAY 16_P1
22	NFAY 16_P2	61	NFAY 16_P2
23	NFAY 20_P1	62	NFAY 20_P1
24	NFAY 20_P2	63	NFAY 20_P2
25	G518	64	G518
26	G561	65	G561
27	G562	66	G562
28	BY4741	67	BY4741
29	BY4743	68	BY4743
30	NCYC361	69	NCYC361
31	NCYC456	70	NCYC456
32	NCYC660	71	NCYC660
33	NCYC661	72	NCYC661
34	WLP013	73	WLP013
35	WLP028	74	WLP028
36	WLP051	75	WLP051
37	WLP500	76	WLP500
38	WLP566	77	WLP566
39	Idun	78	Idun

Figure 3.17: PCA of data obtained by HPLC. The scores plot shows the distribution of the categories, NFAY, wild, laboratory and commercial samples, at 22 °C and 35 °C. The ellipses represent Hotelling T^2 statistics with a 95% confidence, to reveal potential outliers.

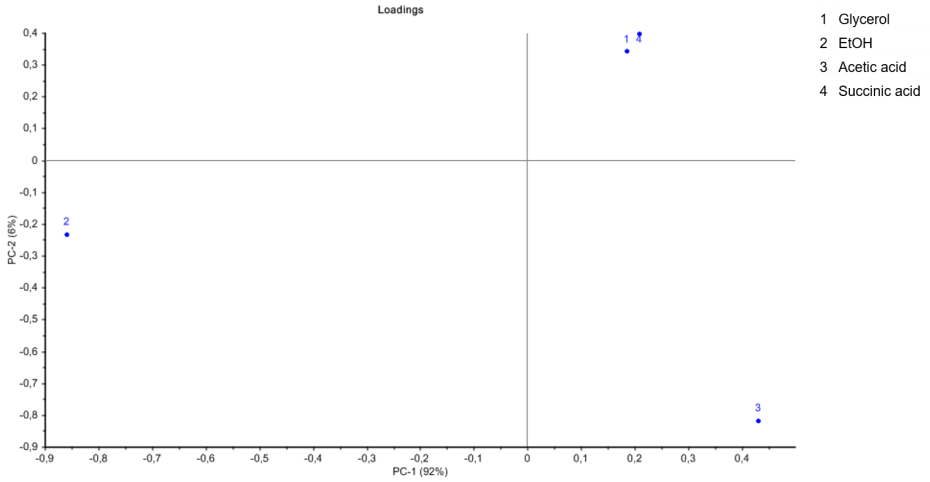


Figure 3.18: PCA of data obtained by HPLC. The loadings plot shows the distribution of the compounds.

No detectable amounts of glucose and fructose were found in the majority of the samples, as seen in Table 3.8 and Table 3.9. This is consistent with what is expected of an end-point fermentation analysis, as these sugars are consumed before higher carbohydrates like maltose and maltotriose. Residual sugars were found in only a few samples (NFAY 2_P2, BY4741 and BY4743) at 22 °C. However, at 35 °C a much greater fraction contained residual fructose and/or glucose. This result suggests that fermentation is more favorable at 22 °C.

The two scores plots in Figure 3.16 and Figure 3.17 display that the NFAY samples are distributed along with commercial, wild and laboratory yeast samples. There is not a clear discrimination between the two fermentation temperatures, though, the majority of the outliers were fermented at 35 °C. According to Hotelling T^2 statistics with a 95% confidence, samples WLP566 (22 °C), NFAY 20_P2 (35 °C), NCYC456 (35 °C), NCYC660 (35 °C) and WLP500 (35 °C) are considered outliers. The loadings plot suggests that the variation is especially influenced by the compounds acetic acid and ethanol.

However, since the PCA plots of the HS GC-MS and HPLC data did not cover all compounds, the visualization by heat map is considered to be a more fitting way of representing the overall data from the analyses of volatile and non-volatile compounds. The phenotypic diversity within the yeast samples is shown in Figure 3.19. The figure displays the Z-scores of the compounds, from the HS GC-MS and HPLC results. The samples are roughly divided into two clades, ranging from NFAY 3.7 to BY4743 and from NFAY6.20 to WLP051, as seen in the figure. Whereas the first group mainly consist of NFAY samples, the second group can be divided into two sub-groups; one with NFAY samples and a second including essentially wild, laboratory and commercial samples. There does not seem to be a distinct correlation between the groupings and the geographical location the samples were collected from.

The laboratory samples immediately stand out by having large blue regions, corresponding to low concentrations. The first group (apart from NFAY 3.5, NFAY 2_P2, BY4741 and BY4743) has high levels of ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate and acetic acid at 22 °C. The opposite is observed for the remaining samples, which have relatively low levels of these compounds in comparison. The second group has generally higher levels of 1-propanol, ethyl propionate, isobutyl alcohol, acetal, ethanol and 1-butanol. However, at 35 °C the levels of the compounds ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate and acetic acid are prominent only for samples NFAY 3.5 to WLP500 in the former group. The first half of the group (NFAY 3.7 to WLP013), resembles the trend observed in the second group.

3.3.3 Sugar Analysis with LC-MS

The data from the LC-MS analysis was imported to the Progenesis QI software and thereafter aligned for comparative abundance profiling. The peak picking was based on approximately 860 compounds, and the total ion count normalization method was applied. The quality control samples (QCs) had a remarkably good overlap and were located close to the mean, indicating that the data provided can be trusted. Thus, it is fair to assume that it has not been affected by technical biases. The blanks were outliers located far away from the samples, which is also a satisfying assurance. The chromatogram of sample NFAY 3.5 at 22°C revealed that something went wrong most likely during the injection. The sample is therefore not included in the analysis.

The samples were studied by exploring the 100 most abundant metabolites and the compound maltotriose (deprotonated molecule and dimer). Figure 3.20 displays that there is a clear separation regarding temperature when the 100 most abundant compounds are considered. This may be due to fact that the 100 most abundant compounds were included, that may contain water-soluble metabolites other than those of interest. The distribution of the various categories does not have a consistent pattern, as NFAY, wild, laboratory and commercial samples seems to be equally spread out. Two distinct outliers are seen for laboratory samples at 35 °C.

A sugar-mix including maltotriose and higher sugars made it possible to identify this compound based on the m/z and the RT. In the negative ionization mode, the deprotonated molecule was detected at m/z 503.160 $[M\text{-}H]^-$ and its dimer at m/z 1007.613 $[2M\text{-}H]^-$. The retention time (min) was 5.523 and 5.518, respectively. Figure 3.21a shows the distribution of the samples when colored by category and temperature, while 3.21b shows the abundance profile. Samples NFAY 2_P2, NFAY 16_P1 and NFAY 20_P2 had higher abundance of maltotriose at 22 °C, while the same was observed for samples NFAY 2_P2, NFAY 6.2 and NFAY 20_P2 at 35 °C. In the commercial samples there is a more distinct separation between the two temperatures; with a better performance at 22 °C, whereas samples WLP028, WLP051 and WLP500 had high abundance profiles at 35 °C. While the samples categorized as wild have low abundance profiles, the opposite is observed for two of the laboratory strains NCYC660 and NCYC661, both at 22 °C and 35 °C.

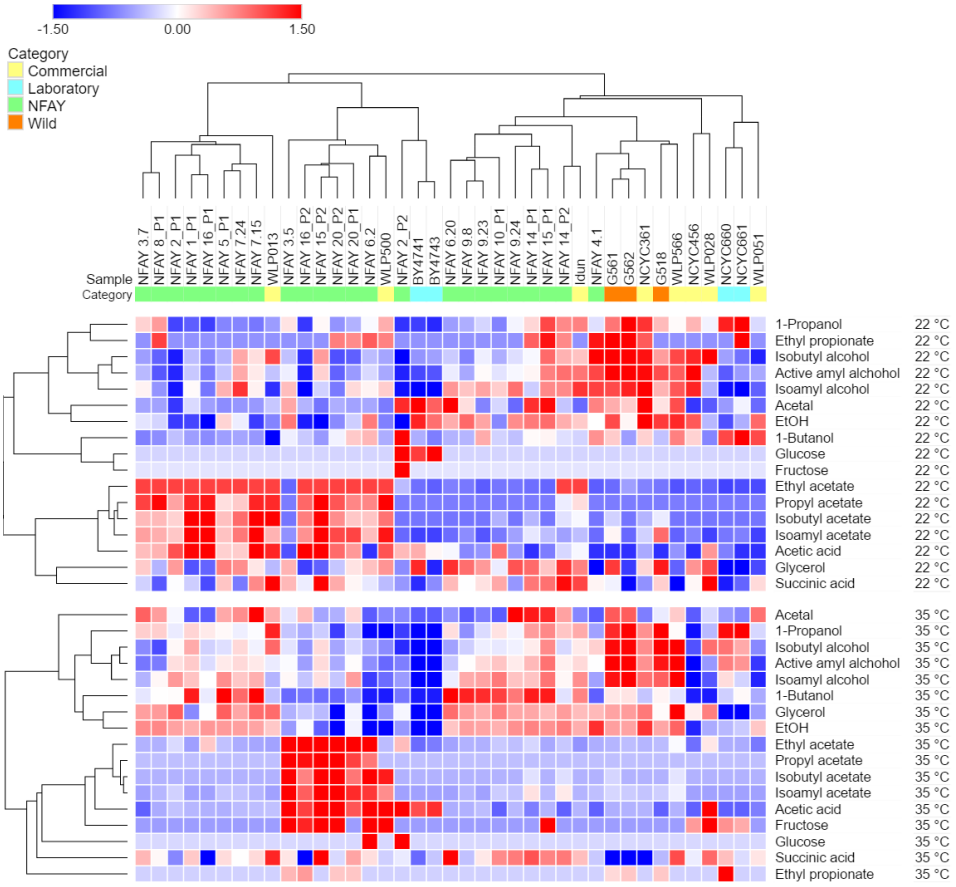
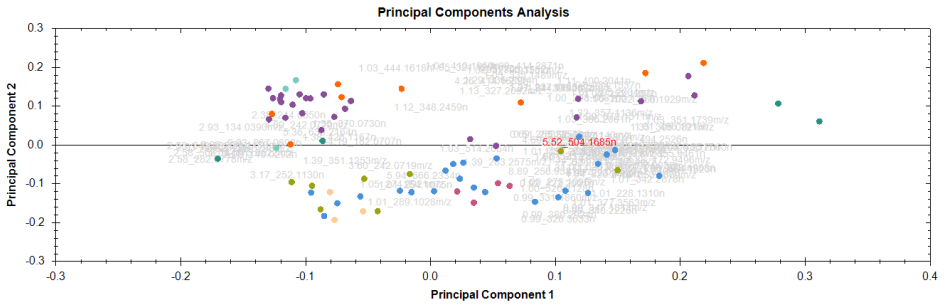
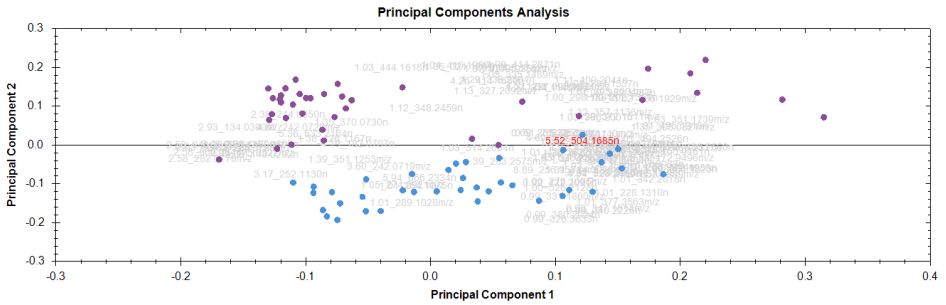


Figure 3.19: Heat map representation of the phenotypic diversity within the yeast samples. Phenotypic values are calculated as Z-scores (normalized values) and colored according to the scale on top. The samples are hierarchically clustered based on phenotypic behavior and according to compound. The samples are also colored to show which category (NFAY, wild, laboratory and commercial) it belongs to.

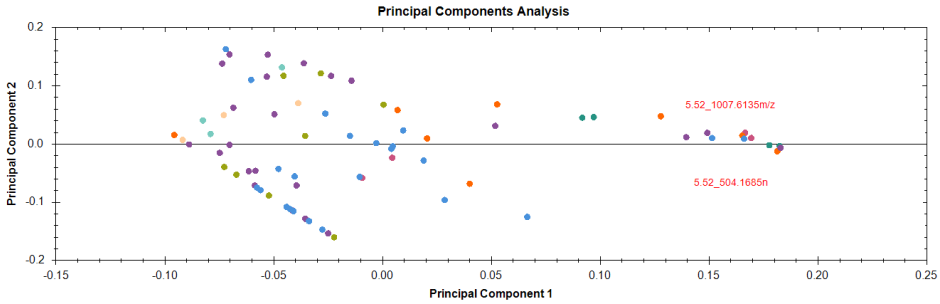


(a) PCA plot of all samples colored by category and temperature.

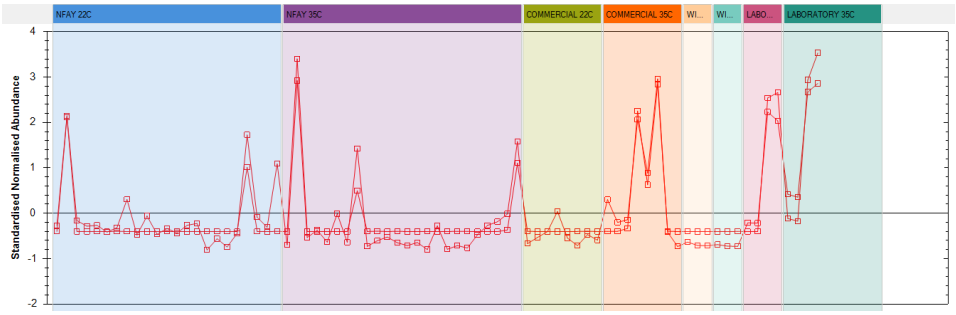


(b) PCA plot of all samples colored by temperature.

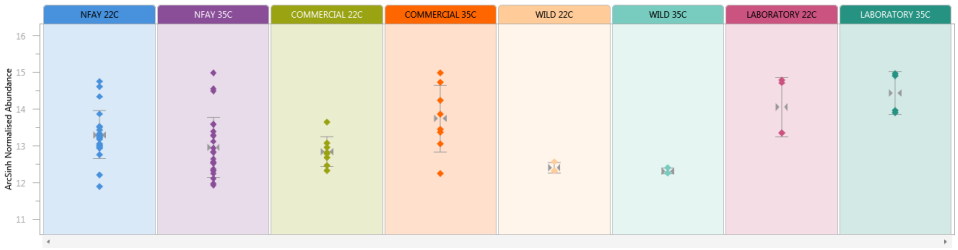
Figure 3.20: PCA plot of the distribution of all samples when the 100 most abundant compounds were considered. Top: The samples are divided into NFAY 22 °C (●), NFAY 35 °C (●), Wild 22 °C (●), Wild 35 °C (●), Laboratory 22 °C (●), Laboratory 35 °C (●), Commercial 22 °C (●) and Commercial 35 °C (●). Bottom: The samples are divided into 22°C (●) and 35 °C (●)



(a) PCA plot of all samples colored by category and temperature.



(b) Standardized and normalized abundance profile of maltotriose.



(c) Normalized abundance profile with mean values included (indicated by arrows).

Figure 3.21: PCA plot of the distribution of all samples when the compound maltotriose (deprotonated molecule and dimer) was considered, with associated abundance profiles. The samples are divided into NFAY 22 °C (●), NFAY 35 °C (●), Wild 22 °C (●), Wild 35 °C (●), Laboratory 22 °C (●), Laboratory 35 °C (●), Commercial 22 °C (●) and Commercial 35 °C (●).

Chapter 4

Discussion

4.1 Species Differentiation Based on the ITS, LSU1 and LSU2 Regions

The species differentiation based on the ITS and LSU regions implied that the NFAY samples consists of at least three different species; *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and the non-*Saccharomyces* yeast *Meyerozyma caribbica* (see Table 3.2). However, many of the samples were unsuccessfully sequenced and not identified at species level by using the ITS region, which limits and makes it more difficult to attain the complete picture of the diversity. Regardless, the LSU regions suggests that all of the samples, except the ones classified as *Meyerozyma caribbica*, belongs to the *Saccharomyces* genus. The primers used for the LSU1 and LSU2 regions were designed using sequences closely related to the *Saccharomyces* genus, which may be the reason why these regions were not obtained for the four samples identified as *M. caribbica*.

Identification of *Saccharomyces* and Non-*Saccharomyces* Yeasts

Ale-type beers are by definition fermented by *S. cerevisiae* in the brewing industry, thus, it is expected that the majority of the samples would be classified as the named species. *S. bayanus* is also a common species in the fermentation industry, but has traditionally been used in wine fermentations (Querol & Fleet, 2006). *S. bayanus* is accepted to be the result of multiple hybridization processes, that is believed to involve events between a *S. bayanus var. uvarum* and an unknown European *S. eubayanus*-like strain. However, there is a complex diversity within this particular species; it contains both pure or hybrid lineages with different levels of homozygosity, hybridization and introgression. This makes species definition difficult and classification unclear (Pérez-Través, Lopes, Querol, & Barrio, 2014). It also raises questions to whereas this classification can be trusted, as it contradicts what WhiteLabs previously determined NFAY 4 to be; *S. pastorianus*. This can be reasonable as it was only recently that the *Saccharomyces* species *S. eubayanus* (one parent of the hybrid *S. pastorianus*) was discovered.

Interestingly, the RDP Classifier suggested that four of the samples, NFAY 14_P1, NFAY 14_P2, NFAY 15_P1 and NFAY 15_P2.1, can be classified as *Meyerozyma caribbica*. Even though this species is less familiar in beer brewing industry than the dominating *Saccharomyces cerevisiae*, it is a known yeast (together with several other non-*Saccharomyces* yeasts) involved in traditional beers enjoyed throughout Africa. These beers are fermented by backslopping flocculent yeast slurry from a previous batch; the exact same inoculation process that was traditionally used for European beer (Bokulich & Bamforth, 2013). N'guessan, Brou, Jacques, Casaregola, and Dje (2011) also reported that *M. caribbica* was identified in the traditional African sorghum beer, where it survived throughout the alcoholic fermentation. *M. caribbica* has also been isolated from various locations in the Western Hemisphere, which suggests that the species is widely distributed geographically (Kurtzman, 2011). In other words, the identification of *M. caribbica* in some of the NFAY cultures might be unexpected, yet, not necessarily a strange affair.

Poor Sequencing Data for ITS Region

It is still unknown why none of the ITS primer pairs used did not work optimally for all samples. Several PCR conditions were tested, DNA purity (by inspecting Ab 260/230 and Ab 260/280 ratios), concentrations were verified with NanoDrop and possible contaminations in reagents were ruled out by including a PCR control in each run. Moreover, the PCR-products observed were estimated to be of the correct size regarding the region of interest. In some cases, multiple or poorly defined peaks in the chromatograms can indicate that multiple products are present. If the products are of similar sequence length, it could have been unnoticed when evaluating the agarose gel as the bands might have overlapped. Poor sequence data can also be caused by degraded DNA from nucleases, repeated freeze-thaw or excessive UV light exposure.

Nevertheless, the issues with the ITS region may also be related to the nature of the region. As mentioned in Section 1.2, some of the limitations with the ITS region is low taxonomic resolution for some species and difficulty in fungus-specific PCR primer design. In future studies in can be desirable to design primers more specific to the relevant target yeasts. This can be accomplished either by looking for conserved regions in a set of closely related taxa or by creating species-specific primers. The use of species-specific primers can be effective when the identification involves a small number of species, or when a particular species is the subject of interest. Other methods for species identification that could be applied in the future to map the diversity of the original cultures include denaturing gradient gel electrophoresis (DGGE). DGGE is a technique based on separation of DNA fragments of differing nucleotide sequences (e.g., species-specific), using the decreased electrophoretic mobility of partially melted double-stranded DNA amplicons in a polyacrylamide gel containing a linear gradient of DNA denaturants (a mixture of urea and formamide). This technique has been used for species identification and quantitation of yeast populations in foods and beverages (Kurtzman et al., 2011), and could therefore also be applied to NFAY cultures.

Phylogenetic Relationships

The classification at genus and species level is generally mirrored in the phylogenetic trees. As seen in the phylogenetic tree of the ITS region in Figure 3.3, the samples classified as *M. caribbica* were clustered together with a high bootstrap, suggesting that this branching is correct. The branch length indicate that it is distanced far from the rest of the samples in terms of evolutionary history. Not surprisingly, the two sequences that were classified as *S. bayanus*, NFAY 4.1 and NFAY 6.2, were grouped together with a high bootstrapping value as they share a similar DNA. This group is evolutionary closer to the *S. cerevisiae* samples, expressed with a shorter branch length.

The phylogenetic tree of the LSU1 region (Figure 3.4a) does not include the samples classified as *M. caribbica*. However, the same relationship between the *S. bayanus* and *S. cerevisiae* samples is established. Similarly, high bootstrapping scores suggests that this relationship can be trusted. In contrast, another grouping emerge in the phylogenetic tree of the LSU2 stretch in Figure 3.4b. In this case only sample NFAY 4.1 is distinguished from the rest. This result can imply that this region does not contain enough differences in substitution sites to distinguish between closely related species. To conclude, the correlation between classification and phylogenetic relationships suggests that only the ITS and LSU1 regions should be used for species differentiation, as the nucleotide substitution rate in LSU2 is not adequate for recognition of closely related species. When considering both the ITS and LSU1 trees, it is also fair to assume that the rest of the samples (see Figure 3.4a) are *S. cerevisiae*, or at least very closely related to the species.

It was generally observed low DNA sequence divergence among *Saccharomyces* yeasts when inspecting the sequences. The close relation is also portrayed in the phylogenetic trees, indicated by short branch lengths. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed, and can also be an implication that these are closely related. The ITS and LSU1 regions can offer species differentiation at genus and species level. However, sequencing of these two regions might not be suitable for sub-species classification. This might be due to the fact that they contain regions that are highly conserved between the yeasts studied. Sequencing of other genes or possibly the whole genome can hopefully reveal additional differences that seemingly exist among the NFAY samples.

Presence and Distribution of *S. cerevisiae*-type AGT1 Genes

The *S. cerevisiae*-type AGT1 gene was present in all samples, apart from the following six; NFAY 14_P1, NFAY 14_P2, NFAY 15_P1, NFAY 15_P2.1 and in the laboratory strains NCYC660 and NCYC661 (see Figure 3.5). The fact that this specific gene was not present in these samples is not surprising. The first four samples were classified as *Meyerozyma caribbica*, a species that belongs to the CTG clade, whereas *S. cerevisiae* belongs to the WGD clade in the subphylum Saccharomycotina (Hittinger et al., 2015). The two species are in other words relatively far distanced from each other in the subphylum, and the genome may therefore vary accordingly.

Nonetheless, the absence of the gene does not necessarily mean that they do not carry a homologous gene to AGT1. For instance, Nakao et al. (2009) found a gene (LBYG13187) in a lager strain that had a 79% identity to the *S. cerevisiae*-type AGT1 gene (Nakao et

al., 2009; Vidgren et al., 2010). Vidgren and Londesborough (2012) discovered that this gene, that is believed to be a *Saccharomyces bayanus* counterpart of the AGT1, encodes a functional α-glucoside transporter with a wide-substrate range, including maltose and maltotriose. The absence of the *S. cerevisiae*-type AGT1 gene has been observed for several distiller's yeasts and laboratory strains (Vidgren et al., 2010), and therefore it seems reasonable for this to be the case with NCYC660 and NCYC661 as well.

The phylogenetic tree in Figure 3.6 displays that there are several variants of the AGT1 gene. The sequences are roughly divided into three groups based on these variants of the gene, supported by high bootstrap values. Multiple nucleotide changes were observed when inspecting the DNA sequences. These differences can potentially lead to amino acid changes between the proteins coded by the AGT1 genes. However, it has not been conducted comprehensive studies on how such small sequence changes may affect the structure of the protein, activity and substrate specificity of the encoded α-glucoside transporter. The possible amino acid changes and the kinetics of the transport could be of interest to explore in future studies.

Furthermore, the distribution of the various samples in the phylogenetic tree (Figure 3.6) is not congruent with the rRNA gene trees, meaning that the same phylogenetic relationship is not recovered as for the ITS and LSU regions. That is, this gene should not be used for separation of species. However, a gene tree can represent the evolutionary history of the AGT1 gene, and provide evidence for gene duplication events as well as speciation events (Zvelebil & Baum, 2008).

The expanded AGT1 tree in Figure 3.7 (extended labels shown in Figure 3.8) includes the previously sequenced strains by Gallone et al. (2016), and can possibly provide additional evolutionary information. As seen in the figure, some of the ancestry relationships are conserved when comparing it to the phylogenetic tree presented by Gallone et al. (2016) in Figure 1.4. The AGT1 sequences from the Asian and West African lineages, that are estimated to be of early origin and considered wild, are distanced far from the rest. The relationships of the clade Beer 2 being grouped together with Wine (considered to have limited domestication) and Mixed grouped with Beer 2 (considered to have strong domestication), are also maintained to some extent. This can indicate that this gene also evolved from the variant in the wild yeasts (in the Asia and West Africa clades), perhaps through duplication and speciation events, and led to the different variants of the gene found in the more recent lineages. Moreover, it can signify that mutations in the AGT1 gene can be associated with domestication, and might have selected for a more efficient fermentation in terms of beer-specific carbon sources (e.g. maltose and maltotriose).

As the NFAY, commercial and wild samples are spread among these four clades that exhibit clear and profound hallmarks of domestication as reported by Gallone et al. (2016), one could hypothesize that these also originate from domesticated ancestors. This is because they carry a variant of the AGT1 gene that is more similar to the domesticated ones, than the wild *S. cerevisiae* variants. However, since the NFAY samples were distributed in different clades, one can also speculate that the degree of domestication varies in the samples. Samples NFAY 8_P1 to NFAY 1_P1 and NFAY 6.2 to NFAY 4.1 (Figure 3.8) might be less domesticated, as they are placed together with mainly the Beer 2 and Wine lineages. The rest of the NFAY samples might be more affected by domestication traits, since they are placed with the Mixed and Beer 1 lineages.

Mutation in AGT1 Gene Not Limited to Lager Strains?

As pointed out before, Vidgren et al. (2005, 2010) concluded that a specific AGT1 gene mutation producing a premature stop codon is a characteristic of lager strains. Contradicting results led to a further investigation by inspecting the AGT1 gene sequences that were present in the data retrieved by Gallone et al. (2016). This attribution revealed that 33 out of 136 obtained sequences carried this extra T. This accounts for 24.3% of the sequences in the collection, and is a rather immense share. Because of this great fraction, it seems that this is quite a frequent appearance and not a random matter among *S. cerevisiae* species. Indeed, this discovery may suggest that the mutation is not limited to lager strains as presumed before.

However, before this statement can be concluded, it requires additional research. For instance, the functionality of the gene should also be tested and determined. One way of doing so would be to carry out a sugar transport assay using a maltose or maltotriose medium, by transforming plasmids with the gene into a maltose-negative (or maltotriose-negative) strain. Transformants with empty plasmids should also be included as a negative control, whereas a maltose-positive strain can be used as a positive control. The functionality can be estimated by measuring the rate of uptake of [^{14}C]-maltose through counting the radioactivity at a set of times (Vidgren et al., 2010).

4.2 DNA Content Determination by Flow Cytometry

There are several causes that could explain why the flow cytometry analysis still did not work optimally. For instance, it might be linked to the fixation of cells. Ethanol can induce serious cell shrinkage, an observation that was also made in the microscope during the assay. A propose to address this issue can be to try another fixation protocol, e.g. use aldehyde fixation instead. Furthermore, it is possible that the vigorous vortexing damaged fragile cells. Dead cells can release DNA into the medium due to loss of membrane integrity, which can be notoriously sticky and lead to an increase in cell clumping.

4.3 Phenotypic Behavior and Diversity

It was established that several of the assays in the preliminary phenotypic classification were not as selective as reported in literature. For instance, *Saccharomyces* yeasts has been proclaimed to need a concentration of 10 mg/L cycloheximide for inhibition of *Saccharomyces* yeasts (M. E. Greig, Walk, & Gibbons, 1958; Fugelsang & Edwards, 2007), which is past the 4 mg/L many suppliers use in the WLD media. The tests did however unfold some growth characteristics. The samples classified as *Meyerozyma caribbica* (i.e. samples NFAY 14_P1, NFAY 14_P2, NFAY 15_P1 and NFAY 15_P2) all had growth on the media. This is in compliance with what has been reported for *M. caribbica* in literature, as some strains have the ability to grow in environments with 1000 mg/L cycloheximide (Kurtzman, 2011).

Other samples in Table 3.1 that were recorded as positives for WLD, might possibly be contaminated with acid producing bacteria or other organisms. This concern can be correlated to Figure 3.11b, where the pH value seems to be fairly low for these samples.

The presence of microbial interactions involving yeast, bacteria or fungi is a plausible scenario, as explained in Subsection 1.1.4. Yeast-bacteria or yeast-yeast interactions can form symbiotic relationships, and are commonly found in fermentation products. Section 1.6 also explains the effect of some common beer spoilage organisms. They can often spoil beer through acidification and production of off-flavors, especially organic acids, esters and phenols. Considering this, it might very well explain the immense ester production and high concentrations of acetic acid at 35 °C for NFAY 3.5, NFAY 16_P2, NFAY 15_P2, NFAY 20_P2, NFAY 20_P1, NFAY 6.2 and WLP500, as seen in Figure 3.19. Regardless, contaminants like LAB might also carry several benefits, as acidification can lower the risk of protein haze formation and other contamination, fresher mouth-feel, smoother bitterness and fuller flavor profile.

The copper sulfate concentration in LCSM plates is optimal for wild yeast growth, though, some *Saccharomyces* wild yeasts might still be inhibited. Thus, this may explain why the wild type *S. cerevisiae* isolated from a rose hip (i.e. samples G518, G561 and G562) did not grow on this media. The result of NFAY 20_P2 being able to grow on this medium should be further investigated. The results that derive from the LYS media suggest that NFAY 6.2 and NFAY 15_P2 are L-(+)-lysine positive, a feature that is typically associated with non-*Saccharomyces* wild yeasts. Yet, the reliability in the test is weakened by the possibility of other trace nitrogen sources.

Carbohydrate Metabolism and Fermentation Performance

As seen in Figure 3.19, the laboratory strains seemed to have high levels of residual sugars. The relatively poor fermentation of the laboratory strains BY4741 and BY4743 derive from the *S. cerevisiae* S288C genetic background. Harsch, Lee, Goddard, and Gardner (2010) addressed the basis for the slow fermentation of these, and discovered that it is not due to mutations that have accumulated because of relaxed selection for fermentation, but rather it seems to be an inherent property of the parent S288C strain (Harsch et al., 2010). Haploids are known to ferment and sometimes grow slightly faster than diploids. This feature is most likely due to their fundamental differences in the ratio of cell size and volume (Salmon, 1997; Marullo et al., 2006; Harsch et al., 2010). This was also observed for BY4741 (haploid) and BY4743 (diploid), where more glucose was left unfermented by BY4743 than BY4741 at 22 °C.

The samples that had low ability to utilize maltotriose (high abundance profile in Figure 3.21) in the end-point fermentation samples were not clustered together in the phylogenetic tree of the entire AGT1 gene (Figure 3.6). Moreover, out of all the samples with a high maltotriose abundance profile (at either 22 or 35 °C), only WLP028 and WLP051 had the insertion of an extra T at position 1183 that is presumed to produce a non-functional protein. Nonetheless, this may be explained by the fact that AGT1 in not the single gene involved in maltotriose utilization. Though AGT1 encodes the transporter with the widest substrate specificity reported, there are also several other genes for α-glucoside transporters that might be active. Maltotriose transport have complex kinetics that involves both high- and low-affinity transporters (Piddocke, Kreisz, Heldt-Hansen, Nielsen, & Olsson, 2009). I.e. having a defective AGT1 gene, does not necessarily mean that the yeast cannot transport maltotriose at all. Consequently, it was not possible to observe a consistent correlation between the maltotriose utilization to the DNA-sequence of AGT1 in this

experimental setup. As mentioned in detail before, this could be resolved by transforming only the gene of interest and thereafter performing a maltotriose transportation assay to test for its functionality.

However, an interesting observation was seen for the laboratory strains NCYC660 and NCYC661. As seen in Figure 3.5, the AGT1 gene was not present in either one of them. This can indicate that they are missing some or possibly all genes responsible for transporting maltotriose, and may be the reason why these did not utilize maltotriose well. A possible explanation is that they underwent gene loss when there was a change in the ploidy level, as NCYC660 and NCYC661 are tetraploid and hexaploid, respectively.

Differences in Alcohol, Acid and Ester Production

In this experiment, similar fermentation conditions were created for all samples by using the same wort composition and the same pitching rate. The apparent metabolic differences will therefore be largely affected by the yeast strain and the variation in the two fermentation temperatures used. However, the first grouping that emerged at 22 °C can be an effect of the wort composition itself, as the type of assimilable sugars can play a role in determining ester levels. Piddocke et al. (2009) observed that an increase in gravity led to an increase in the concentrations of ethyl acetate and isoamyl acetate in the final beer. Correspondingly, the levels of higher alcohols such as 1-propanol, isobutyl alcohol and isoamyl alcohol decreased. The same observation was made for the majority of the samples in the first clustering group in Figure 3.19 at 22 °C. During the course of high-gravity beer fermentation, the brewing yeast is exposed to a number of stressful conditions. At the beginning of fermentation, they are exposed to high osmotic pressure caused by the high glucose concentrations, whereas toward the end of the fermentation they might face ethanol stress imposed by the elevated ethanol concentrations. How the yeasts responded to this particular stress may differ within the samples at both temperatures.

Temperature is an important fermentation variable. An increase in temperature raises the fermentation rate and can heighten the final concentration of both higher alcohols and esters (Saerens, Delvaux, et al., 2008; Saerens, Verbelen, Vanbeneden, Thevelein, & Delvaux, 2008). The temperature dependency can be related to the availability of higher alcohols, which serve as ester precursors and are necessary for ester formation. The second group including most commercial samples (Figure 3.19) did not seem to be largely affected in the production of esters and higher alcohols when the temperature was raised from 22 °C to 35 °C. However, an interesting trend is observed for the first group when increasing the temperature. The first half (sample NFAY 3.7 to WLP013) seems to experience a shift in the metabolic fluxes in the cell, leading to a boost in higher alcohols as opposed to esters at 35 °C. Meanwhile, the ethanol level is enhanced and the amount of acetic acid is reduced. This tendency was not recognized for the second half of group one (sample NFAY 3.5 to WLP500). In fact, this group seemed to produce even more of acetate esters at 35 °C, which is a familiar case when the the fermentation is executed at such a high temperature. In this case, elevated levels of acetic acid were observed in addition to low levels of ethanol. This may be due to the conditions it is exposed to, as stressed cells at a high sugar concentration can lead to an increased production of acetic acid (Remize, Barnavon, & Dequin, 2001). However, as specified before, it can also be related to possible contamination of acid producing organisms (low pH values and growth on WLD media).

Phenotypic Variants due to Unique Brewing Traditions?

Why do these differences in phenotypic behaviour occur, and why does this metabolic shift take place when the temperature is raised from 22 °C to 35 °C for only some of the samples? Even though it was not observed a clear correlation between the grouping and origin, another remark was observed. Nearly all NFAY samples placed together with the Beer 2 and Wine lineages in the expanded AGT1 tree (samples NFAY 8_P1 to NFAY 1_P1 and NFAY 6.2 in Figure 3.8), that are suspected to be less domesticated are placed in this first group. Non-domesticated yeasts are know to have variable and less consistent fermentations, and so this can maybe be part of the explanation. Another aspect is the traditions associated with the NFAY brewings. The fermentations were traditionally carried out at very high temperatures, and certain properties that are favorable at 35 °C could therefore have been developed. The differences in the metabolic flux in the cell can possibly also be linked to different temperature optimums for the enzymes involved in the pathways, e.g. alcohol acyltransferase (AAT) that is essential for ester production (see Figure 1.8).

The farmhouse brewing traditions could also have affected the phenotypic diversity in other ways. The NFAY cultures were harvested and reused for centuries, being exposed to repitching over a substantial period of time. It is generally accepted that serial repitching can have negative impacts on yeast quality. It will typically not cause loss of prominent physiological characteristics, but genetic mutations or metabolic drifts may alter specific stress responses. Perhaps did a variant with a specific stress response accumulate in the NFAY cultures. These may have caused certain features to linger on generations later on. Evidence that phenotypic heterogeneity (i.e. that is nonuniform) regularly emerges from within microbial population has been documented, also for populations in industrial bioprocesses (Pires & Brányik, 2015). Thus, it is also a probability for the populations in the NFAY cultures.

Flavor and Aroma Profile

Each yeast strain has a genome-associated phenotypic character that is unique and will impact the final flavor and aroma profile of the product (Pires & Brányik, 2015). Variations within the different samples is observed to some extent, though, one component seems prominent in a particular set of samples. Evaluating the coloring scheme in Figure 3.15, reveals that ethyl acetate is abnormally high. This applies to the samples NFAY 1_P1 through NFAY 15_P2 at 22 °C and NFAY 3.5 to NFAY 15_P2 at 35 °C. Since these have levels that exceeds 2 FU, ethyl acetate is likely to have a major effect on the sensory properties of the product. Overproduction of a compounds is not favorable in terms of flavor balance. In general, when flavor and aroma compounds are present in excessive concentrations, their flavor influence is most often negatively accepted by the consumer. Disproportionate higher levels of esters is common in beer brewing, particularly ethyl acetate and isoamyl acetate (Piddocke et al., 2009). Nonetheless, how the flavor and aroma profile of each beer is ultimately accepted by the consumer should be determined by a sensory analysis.

Generally, *Saccharomyces* is the sole microbial component in beer, and any deviation is considered a flaw or spoilage. However, there are also some beer types that uses non-*Saccharomyces* starter cultures intentionally, such as including *M. caribbica*. These are

in fact gaining increased popularity worldwide, and can lead to very unique beer products (Bokulich & Bamforth, 2013). In other words, other yeasts such as *M. caribbica* could potentially increase the diversity of the flavor and aroma profile. However, it was not observed a unique profile of fermentation properties for the samples classified as this particular species. Nor did the samples classified as *S. bayanus* contribute to a noticeably different profile. In fact, samples of the same species often differed substantially from one another, which suggests that the phenotypic properties often are strain dependent.

4.4 Domestication or Neutral Divergence?

Some of the domestication hallmarks Gallone et al. (2016) highlighted in their study were an increase in maltotriose utilization, genome decay, aneuploidy and CNV. Meanwhile, the production of off-flavors, the ability to sexual reproduce and survive in nature were reduced. Are the NFAY cultures affected by domestication, and if so, to what extent? The majority of the NFAY samples in this study were able to utilize maltotriose well, only with the exception of a few. The NFAY and commercial samples had mutations in the AGT1 gene relative to the reference gene retrieved from the SGD, possibly encoding different amino acids in the protein product. The reference AGT1 gene was grouped together with sequences from Asia and West Africa (Figure 3.7), which are believed to be of earlier origin. This can signify that mutations in the AGT1 gene can have selected for a more efficient fermentation in terms of beer-specific carbon sources.

A noteworthy observation is that there seems to be a limited correlation between the genotypic and phenotypic properties in terms of geographical origin for the NFAY samples. This was observed for the AGT1 gene in Figure 3.6, where the distribution of samples seemed to be independent of the yeast culture. That is, the groups contained samples collected from various counties, and samples that originated from the same yeast culture were sometimes divided into different clades. The heat map that displayed the phenotypic behaviour (Figure 3.19), also lacked a consistency regarding grouping of samples and its origin, nor did the samples classified as the same species automatically share the same phenotype. This might be explained by the fact that domestication reduces the geographical structure. Instead, the genetic structure tends to track human usage, which can explain why this correlation was not persistent. In addition, as described before, phenotypic traits and stress responses can often be strain dependent, which seems to be the case here.

Moreover, the NFAY samples do not seem to have been geographically isolated. For instance, the two samples classified as *S. bayanus*, NFAY 4.1 and NFAY 6.2, were collected from different counties. This is also the case for the samples classified as *M. caribbica*, that derive from cultures NFAY 14 and NFAY 15. This can imply that the dispersal has been unrestricted. Perhaps it was common to exchange yeast cultures and beer through trade among the various farmhouses? This can indicate that the diversity in the NFAY cultures is shaped by selection and niche adaption rather than neutral divergence caused by geographic isolation and limited dispersal.

The evolution of *S. cerevisiae* has been largely affected by domestication, and can be dated back to several hundred years ago. The traditions associated with the NFAY cultures also support the theory of them being domesticated. They were part of a man-made environment where backslopping of yeast slurry was a fundamental part of the tradition. As

elaborated before, phenotypic variants and stress responses may have become prominent in certain populations due to the practice of repitching. By losing contact to their niches, the perfect setting for domestication can emerge. However, one can argue that the farmhouse traditions may have created a different selection regime than what is common in the brewing industry. Gonçalves et al. (2016) demonstrated that domestication differed between top-fermenting beer and wine yeast. For instance, the higher diversity of beer strains was partly considered to be an effect of the environment it was exposed to. Whereas wine is produced seasonally, beer is normally produced throughout the whole year; which provides beer yeast with a predictable and stable growth environment. As storing the NFAY cultures dry in between batches was the most common practice, it is plausible that this has made them especially adapted to surviving such harsh conditions. Consequently, there might also be differences in domestication of industrial beer yeast and NFAY cultures.

There are several observations that suggest that a great part of the NFAY samples are affected by domestication. However, it is hard to draw any substantial conclusions without investigating a greater scope of the domestication characteristics. This should be done through expanding both the genotypic and phenotypic features considered. Namely, a greater part of the genome should be studied, preferably through whole genome sequencing, to determine the ploidy, investigating the sexual reproduction and examine the CNV. A more thoroughly phenotypic characterization can be obtained by exposing the yeast for other environmental and nutrient stresses, to test for its tolerances and response.

Chapter 5

Concluding Remarks

The species differentiation based on the ITS and LSU regions implied that the NFAY samples consist of at least three different species; *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and the non-*Saccharomyces* yeast *Meyerozyma caribbica*. It was generally observed low DNA sequence divergence among the *Saccharomyces* species, suggesting they are closely related. Considering the correlation between the classification and phylogenetic relationships, it can be concluded that only the ITS and LSU1 regions should be used for species differentiation. This is because the substitution rate in LSU2 did not appear suitable for closely related species, as it might be too conserved.

The AGT1 allele was present in all *Saccharomyces* species except two of the the laboratory strains. It was observed three main variants of the AGT1 gene, encoding different amino acids in the protein product. Interestingly, a particular AGT1 gene mutation producing a premature stop codon was found in several *Saccharomyces cerevisiae* strains - a mutation believed to be a characteristic of lager strains. This can imply that the mutation is not limited to lager strains as presumed before. The distribution of the samples in the AGT1 phylogenetic tree was not congruent with the rRNA gene trees. Consequently, this gene should not be used for separation of species. However, important evolutionary information might still be recovered from the tree. An extended AGT1 tree including sequencing data from Gallone et al. (2016), revealed that the NFAY samples carry a variant of the AGT1 gene that is more similar to the domesticated ones rather than wild *S. cerevisiae* variants. This raises the suspicion of them originating from domesticated ancestors.

Whereas the commercial samples did not seem to be largely affected in the ester and alcohol production when the temperature was raised from 22 °C to 35 °C, two groups of NFAY samples had apparent trends. One group underwent a metabolic shift, going from high levels of esters to an enhanced production of higher alcohols at 35 °C. The second group produced elevated levels of acetate esters, especially the compound ethyl acetate, contributing to solvent-like and sweet sensory characteristics. The phenotypic variants may have emerged from the farmhouse brewing traditions, as common practices included high fermentation temperatures, unique storage techniques and repitching. The latter can impact the yeast through genetic mutations or metabolic drifts, that may alter specific

stress responses and phenotypic trait that can accumulate and linger on later generations. The phenotypic profiles observed were not conserved within a certain species nor was it related to the geographical origin of the culture, which can suggest that the phenotypic behaviors are mainly strain dependent.

Domestication can have dramatic consequences for evolution, and the NFAY samples seems to carry some of the signatures. For instance, the majority of the samples showed abilities to utilize maltotriose well. This can signify that mutations in the AGT1 gene found in domesticated species can have selected for fermentations with beer-specific carbon sources such as maltose and maltotriose. Moreover, the fact that the genotype and the associated phenotypic characteristics seems to lack geographical structure and isolation, are also signs of domestication. That being said, the results also suggests that there might be individual differences as to what extent they could have been affected by domestication; some NFAY samples may have limited domestication, while others might exhibit strong hallmarks of domestication. This was reflected in both the AGT1 gene and differences in phenotypic behaviour.

In future studies, a greater part of the genome should surely be considered in the genetic analyses. This can hopefully provide sub-species classification, and make it possible to further investigate domestication hallmarks regarding ploidy, sexual reproduction and CNV. A more thoroughly phenotypic characterization can be obtained by including other environmental and nutrient stresses, e.g. temperature-, ethanol- and acid tolerance, and test the abilities of flocculation and spore viability. Transportation assays in maltose or maltotriose media is of particular interest to test the functionality of the AGT1 gene.

Bibliography

Allison, L. A. (2007). Recombinant DNA Technology and Molecular Cloning. In *Fundamental molecular biology* (Chap. 8, pp. 180–231). Blackwell Publishing.

Bamforth, C. W. (2005). Beer. In *Food, fermentation and micro-organisms* (Chap. 2, pp. 40–88). Wiley-Blackwell.

Bamforth, C. (2016). Flavorsome Components of Beer. In C. W. Bamforth (Ed.), *Brewing materials and processes: a practical approach to beer excellence* (Chap. 8, pp. 151–155). San Diego: Academic Press. doi:10.1016/B978-0-12-799954-8.00008-3

Bergström, A., Simpson, J. T., Salinas, F., Barré, B., Parts, L., Zia, A., … Liti, G. (2014). A High-Definition View of Functional Genetic Variation from Natural Yeast Genomes. *Molecular Biology and Evolution*, *31*(4), 872–888. doi:10.1093/molbev/msu037

Bisson, L. F. (2017). Yeast Hybrids in Winemaking. *Catalyst: Discovery into Practice*, *1*(1), 27–34. doi:10.5344/catalyst.2016.16001

Bokulich, N. A. & Bamforth, C. W. (2013). The Microbiology of Malting and Brewing. *Microbiology and Molecular Biology Reviews*, *77*(2), 157–172. doi:10.1128/MMBR.00060-12

Boynton, P. J. & Greig, D. (2014). The ecology and evolution of non-domesticated Saccharomyces species. *Yeast*, *31*(12), 449–462. doi:10.1002/yea.3040

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., & Boeke, J. D. (1998). Designer Deletion Strains Derived from Saccharomyces cerevisiae S288C: A Useful set of Strains and Plasmids for PCR-mediated Gene Disruption and Other Applications. *Yeast*, *14*(2), 115–132.

Comnish-Bowden, A. (1985). Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. *Nucleic Acids Research*, *13*(9), 3021–3030.

Delobel, P. & Tesnière, C. (2014). A simple FCM method to avoid misinterpretation in Saccharomyces cerevisiae cell cycle assessment between G0 and sub-G1. *PLoS ONE*, *9*(1), 1–8. doi:10.1371/journal.pone.0084645

Dragan, A. I., Pavlovic, R., McGivney, J. B., Casas-Finet, J. R., Bishop, E. S., Strouse, R. J., … Geddes, C. D. (2012). SYBR Green I: Fluorescence properties and interaction with DNA. *Journal of Fluorescence*, *22*(4), 1189–1199. doi:10.1007/s10895-012-1059-8

Esbensen, K. & Geladi, P. (2009). Principal Component Analysis: Concept, Geometrical Interpretation, Mathematical Background, Algorithms, History, Practice. In S. D. Brown, R. Tauler, & B. Walczak (Eds.), *Comprehensive chemometrics* (Chap. 2.13, pp. 211–226). Oxford: Elsevier. doi:10.1016/B978-044452701-1.00043-0

Felsenstein, J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, *39*(4), 783–791.

Ferreira, I. M. (2009). Quantification of Beer Carbohydrates by HPLC. In V. R. Preedy (Ed.), *Beer in health and disease prevention* (Chap. 103, pp. 1067–1073). San Diego: Academic Press. doi:10.1016/B978-0-12-373891-2.00103-6

Fugelsang, K. C. & Edwards, C. G. (2007). Media Preparation and Culture Techniques. In *Wine microbiology: practical applications and procedures* (2nd ed., Chap. 13, pp. 194–223). Springer US. doi:10.1007/978-0-387-33349-6

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., … Verstrepen, K. J. (2016). Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. *Cell*, *166*(6), 1397–1410. doi:10.1016/j.cell.2016.08.020

Garshol, L. M. (2016). *Gårdsøl: Det norske ølet*. Latvia: Cappelen Damm.

Gonçalves, M., Pontes, A., Almeida, P., Barbosa, R., Serra, M., Libkind, D., … Sampaio, J. P. (2016). Distinct Domestication Trajectories in Top-Fermenting Beer Yeasts and Wine Yeasts. *Current Biology*, *26*(20), 2750–2761. doi:10.1016/j.cub.2016.08.040

Grayson, M. A. (2016). A History of Gas Chromatography Mass Spectrometry (GC/MS). In *The encyclopedia of mass spectrometry* (pp. 152–158). Boston: Elsevier. doi:10.1016/B978-0-08-043848-1.00020-1

Greig, M. E., Walk, R. E., & Gibbons, A. J. (1958). Effect of actidione (cycloheximide) on yeast fermentation. *Journal of bacteriology*, *75*(4), 489–491.

Harsch, M. J., Lee, S. A., Goddard, M. R., & Gardner, R. C. (2010). Optimized fermentation of grape juice by laboratory strains of Saccharomyces cerevisiae. *FEMS Yeast Research*, *10*(1), 72–82.

Hebly, M., Brickwedde, A., Bolat, I., Driessen, M. R. M., de Hulster, E. A. F., van den Broek, M., … Daran-Lapujade, P. (2015). S. cerevisiae x S. eubayanus interspecific hybrid, the best of both worlds and beyond. *FEMS Yeast Research*, *15*(3), 1–14. doi:10.1093/femsyr/fov005

Herskowitz, I. (1988). Life cycle of the budding yeast Saccharomyces cerevisiae. *Microbiological reviews*, *52*(4), 536–553. doi:10.1016/0168-1656(95)00139-5

Hill, A. E. (2015). *Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*. Oxford: Woodhead Publishing.

Hittinger, C. T. (2013). Saccharomyces diversity and evolution: a budding model genus. *Trends in Genetics*, *29*(5), 309–317. doi:10.1016/j.tig.2013.01.002

Hittinger, C. T., Rokas, A., Bai, F. Y., Boekhout, T., Gonçalves, P., Jeffries, T. W., … Kurtzman, C. P. (2015). Genomics and the making of yeast biodiversity. *Current Opinion in Genetics and Development*, *35*, 100–109. doi:10.1016/j.gde.2015.10.008

Hough, J. S., Briggs, D. E., Stevens, R., & Young, T. W. (1982). Chemical and Physical Properties of Beer. In *Malting and brewing science: hopped wort and beer* (2nd ed., Chap. 22, pp. 776–838). Gaithersburg, MD: Springer. doi:10.1007/978-1-4615-1799-3

Hughes, P. S. & Baxter, E. D. (2001). Flavour Determinants of Beer Quality. In *Beer: quality, safety and nutritional aspects* (pp. 40–73). Royal Society of Chemistry.

Hutkins, R. W. (2006). Beer Fermentation. In *Microbiology and technology of fermented foods* (Chap. 9, pp. 301–348). Blackwell Publishing. doi:10.1002/9780470277515

Jespersen, L., Cesar, L. B., & Meaden, P. G. (1999). Multiple α-Glucoside Transporter Genes in Brewer's Yeast. *Society*, *65*(2), 450–456.

Jespersen, L. & Jakobsen, M. (1996). Specific spoilage organisms in breweries and laboratory media for their detection. *International Journal of Food Microbiology*, *33*(1), 139–155. doi:10.1016/0168-1605(96)01154-3

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874.

Kurtzman, C. P. (2011). Meyerozyma Kurtzman & M. Suzuki (2010). In C. P. Kurtzman, J. W. Fell, & T. Boekhout (Eds.), *The yeasts* (5th ed., Chap. 47, pp. 621–624). London: Elsevier B.V. doi:10.1016/B978-0-444-52149-1.00065-3

Kurtzman, C. P., Fell, J. W., & Boekhout, T. (2011). Gene Sequence Analyses and Other DNA-Based Methods for Yeast Species Recognition. In *The yeasts: a taxonomic study* (5th ed., Chap. 10, Vol. 1, pp. 137–144). London: Elsevier B.V. doi:10.1016/B978-0-444-52149-1.00010-0

Kurtzman, C. P., Mateo, R. Q., Kolecka, A., Theelen, B., Robert, V., & Boekhout, T. (2015). Advances in yeast systematics and phylogeny and their use as predictors of biotechnologically important metabolic pathways. *FEMS Yeast Research*, *15*(6), 1–17. doi:10.1093/femsyr/fov050

Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *73*(4), 331–371. doi:10.1023/A:1001761008817

Lethonen, P. & Hurme, R. (1994). Liquid Chromatographic Determination of Sugars in Beer By Evaporative Light Scattering Detection. *Journal of the Institute of Brewing*, *100*, 343–346.

Lin, Y. (1981). Formulation and Testing of Cupric Sulphate Medium for Wild Yeast Detection. *Journal of the Institute of Brewing*, *87*(3), 151–154. doi:10.1002/j.2050-0416.1981.tb04005.x

Liti, G., Carter, D. M., Moses, A. M., Warringer, J., Parts, L., James, S. A., … Louis, E. J. (2009). Population genomics of domestic and wild yeasts. *Nature*, *458*(7236), 337–341. doi:10.1038/nature07743

Liu, K. L., Porras-Alfaro, A., Kuske, C. R., Eichorst, S. A., & Xie, G. (2012). Accurate, Rapid Taxonomic Classification of Fungal Large-Subunit rRNA Genes. *Applied and Environmental Microbiology*, *78*(5), 1523–1533. doi:10.1128/AEM.06826-11

Loureiro, J., Rodriguez, E., Doležel, J., & Santos, C. (2006). Comparison of four nuclear isolation buffers for plant DNA flow cytometry. *Annals of Botany*, *98*(3), 679–689. doi:10.1093/aob/mcl141

Maarse, H. (1991). *Volatile compounds in foods and beverages*. New York: Marcel Dekker.

Marullo, P., Bely, M., Masneuf-Pomarède, I., Pons, M., Aigle, M., & Dubourdieu, D. (2006). Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. *FEMS Yeast Research*, *6*(2), 268–279.

Mertens, S., Steensels, J., Saels, V., De Rouck, G., Aerts, G., & Verstrepen, K. J. (2015). A large set of newly created interspecific Saccharomyces hybrids increases aromatic diversity in lager beers. *Applied and Environmental Microbiology*, *81*(23), 8202–8214. doi:10.1128/AEM.02464-15

Moldoveanu, S. C. & David, V. (2013). Basic Information about HPLC. In *Essentials in modern hplc separations* (Chap. 1, pp. 1–51). Elsevier. doi:10.1016/B978-0-12-385013-3.00001-X

Moreira, N., Meireles, S., Brandão, T., & De Pinho, P. G. (2013). Optimization of the HS-SPME-GC-IT/MS method using a central composite design for volatile carbonyl compounds determination in beers. *Talanta*, *117*, 523–531. doi:10.1016/j.talanta.2013.09.027

Morris, E. O. & Eddy, A. A. (1957). Brewing Industry Foundation Method for the Measurement of Wild Yeast. *Journal of the Institute of Brewing*, *63*, 34–35.

Mortimer, R. K. (1958). Radiobiological and genetic studies on a polyploid series (haploid to hexaploid) of Saccharomyces cerevisiae. *Radiation research*, *9*(3), 312–326.

Nakao, Y., Kanamori, T., Itoh, T., Kodama, Y., Rainieri, S., Nakamura, N., … Ashikari, T. (2009). Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Research*, *16*(2), 115–129. doi:10.1093/dnares/dsp003

N'guessan, K. F., Brou, K., Jacques, N., Casaregola, S., & Dje, K. M. (2011). Identification of yeasts during alcoholic fermentation of tchapalo, a traditional sorghum beer from Côte d'Ivoire. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *99*(4), 855–864. doi:10.1007/s10482-011-9560-7

Okafor, N. (2007). Production of Beer. In *Modern industrial microbiology and biotechnology* (Chap. 12, pp. 237–260). Enfield, NH: Science Publishers. doi:10.1021/ie50036a053

Pérez-Través, L., Lopes, C. A., Querol, A., & Barrio, E. (2014). On the complexity of the Saccharomyces bayanus taxon: Hybridization and potential hybrid speciation. *PLoS ONE*, *9*(4). doi:10.1371/journal.pone.0093729

Piddocke, M. P., Kreisz, S., Heldt-Hansen, H. P., Nielsen, K. F., & Olsson, L. (2009). Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts. *Applied Microbiology and Biotechnology*, *84*(3), 453–464. doi:10.1007/s00253-009-1930-y

Pires, E. & Brányik, T. (2015). *Biochemistry of Beer Fermentation*. SpringerBriefs in Biochemistry and Molecular Biology. Cham: Springer International Publishing. doi:10.1007/978-3-319-15189-2

Porras-Alfaro, A., Liu, K. L., Kuske, C. R., & Xiec, G. (2014). From genus to phylum: Large-Subunit and Internal Transcribed Spacer rRNA Operon Regions Show Similar Classification Accuracies Influenced by Database Composition. *Applied and Environmental Microbiology*, *80*(3), 829–840. doi:10.1128/AEM.02894-13

Querol, A. & Fleet, G. H. (2006). *Yeast in Food and Beverages*. Springer-Verlag.

Remize, F., Barnavon, L., & Dequin, S. (2001). Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in Saccharomyces cerevisiae. *Metab Eng. 3*(4), 301–312.

Rychlik, W., Spencer, W., & Rhoads, R. (1990). These values have been found to be the most appropriate for PCR. *Nucl. Acids Res. 18*(21), 6409–6412.

Saerens, S. M. G., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., & Delvaux, F. R. (2008). Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. *Applied and Environmental Microbiology*, *74*(2), 454–461. doi:10.1128/AEM.01616-07

Saerens, S. M. G., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., & Delvaux, F. R. (2008). Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. *Applied Microbiology and Biotechnology*, *80*(6), 1039–1051. doi:10.1007/s00253-008-1645-5

Salmon, J. M. (1997). Enological fermentation kinetics of an isogenic ploidy series derived from an industrial Saccharomyces cerevisiae strain. *Journal of Fermentation and Bioengineering*, *83*(3), 253–260.

Selmecki, A. M., Maruvka, Y. E., Richmond, P. A., Guillet, M., Shoresh, N., Sorenson, A. L., … Pellman, D. (2015). Polyploidy can drive rapid adaptation in yeast. *Nature*, *519*(7543), 349–352. doi:10.1038/nature14187

Sithersingh, M. J. & Snow, N. H. (2012). Headspace-Gas Chromatography. In C. Poole (Ed.), *Gas chromatography* (Chap. 9, pp. 221–233). Elsevier Inc. doi:10.1016/B978-0-12-385540-4.00030-4

Sparkman, O. D., Penton, Z. E., & Kitson, F. G. (2011). Quantitation with GC/MS. In *Gas chromatography and mass spectrometry* (2nd ed., Chap. 6, pp. 207–218). Amsterdam: Academic Press. doi:10.1016/B978-0-12-373628-4.00006-X

Stratford, M. (1989). Yeast flocculation: Calcium specificity. *Yeast*, *5*(6), 487–496. doi:10.1002/yea.320050608

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular biology and evolution*, *10*(3), 512–26.

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE*, *7*(7). doi:10.1371/journal.pone.0040863

van den Broek, M., Bolat, I., Nijkamp, J. F., Ramos, E., Luttik, M. A. H., Koopman, F., … Daran, J. M. (2015). Chromosomal copy number variation in Saccharomyces pastorianus is evidence for extensive genome dynamics in industrial lager brewing strains. *Applied and Environmental Microbiology*, *81*(18), 6253–6267. doi:10.1128/AEM.01263-15

Vidgren, V. & Londesborough, J. (2012). Characterization of the saccharomycesbayanus-type Agt1 transporter of lager yeast. *Journal of the Institute of Brewing*, *118*(2), 148–151. doi:10.1002/jib.22

Vidgren, V., Multanen, J. P., Ruohonen, L., & Londesborough, J. (2010). The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. *FEMS Yeast Research*, *10*(4), 402–411. doi:10.1111/j.1567-1364.2010.00627.x

Vidgren, V., Ruohonen, L., & Londesborough, J. (2005). Characterization and Functional Analysis of the MAL and MPH Loci for Maltose Utilization in Some Ale and Lager Yeast Strains. *Applied and Environmental Microbiology*, *71*(12), 7846–7857. doi:10.1128/AEM.71.12.7846-7857.2005

Vidgren, V., Viljanen, K., Mattinen, L., Rautio, J., & Londesborough, J. (2014). Three Agt1 transporters from brewer's yeasts exhibit different temperature dependencies for maltose transport over the range of brewery temperatures (0-20 °C). *FEMS Yeast Research*, *14*(4), 601–613. doi:10.1111/1567-1364.12147

Viljoen, B. C. (2006). Yeast Ecological Interactions. Yeast-Yeast, Yeast-Bacteria, Yeast-Fungi Interactions and Yeasts as Biocontrol Agents. In A. Querol & G. Fleet (Eds.), *Yeasts in food and beverages* (Chap. 4, pp. 83–110). Springer Berlin Heidelberg. doi:10.1007/978-3-540-28398-0

Walker, G. M. (2009). Yeasts. In M. Schaechter (Ed.), *Encyclopedia of microbiology* (3rd ed., pp. 478–491). Oxford: Academic Press. doi:10.1016/B978-012373944-5.00335-7

Walters, L. S. & Thiselton, M. R. (1953). Utilization of Lysine by Yeasts. *Journal of the Institute of Brewing*, *59*(5), 401–404.

Wang, Q. M., Liu, W. Q., Liti, G., Wang, S. A., & Bai, F. Y. (2012). Surprisingly diverged populations of Saccharomyces cerevisiae in natural environments remote from human activity. *Molecular Ecology*, *21*(22), 5404–5417. doi:10.1111/j.1365-294X.2012.05732.x

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267.

Warringer, J., Zörgö, E., Cubillos, F. A., Zia, A., Gjuvsland, A., Simpson, J. T., ... Blomberg, A. (2011). Trait variation in yeast is defined by population history. *PLoS Genetics*, *7*(6).

White, T., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *Pcr protocols: a guide to methods and applications* (Chap. 38, pp. 315–322). San Diego: Academic Press. doi:10.1016/B978-0-12-372180-8.50042-1

Yonezawa, T. & Fushiki, T. (2002). Testing for Taste and Flavour of Beer. In J. F. Jackson & H. F. Linskens (Eds.), *Analysis of taste and aroma* (Chap. 3, Vol. 21, pp. 29–45). Springer Berlin Heidelberg. doi:10.1007/978-3-662-04857-3

Zipper, H., Brunner, H., Bernhagen, J., & Vitzthum, F. (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Research*, *32*(12). doi:10.1093/nar/gnh101

Zvelebil, M. J. & Baum, J. O. (2008). Part 3: Evolutionary Processes. In *Understanding bioinformatics* (Chap. 7-8, pp. 223–410). New York: Garland Science.

Appendix A

Genetic Analyses

A.1 DNA Extraction and Post-PCR Steps

The protocol provided by the supplier was followed when extracting DNA using the UltraClean Microbial DNA Isolation Kit. The DNA extraction was performed without any deviations. The PCR-products were examined by electrophoresis to verify product. Figure A.1 shows an image of one of the agarose gels after electrophoresis. The DNA ladder included made it possible estimate the size of the product, as seen in the figure.

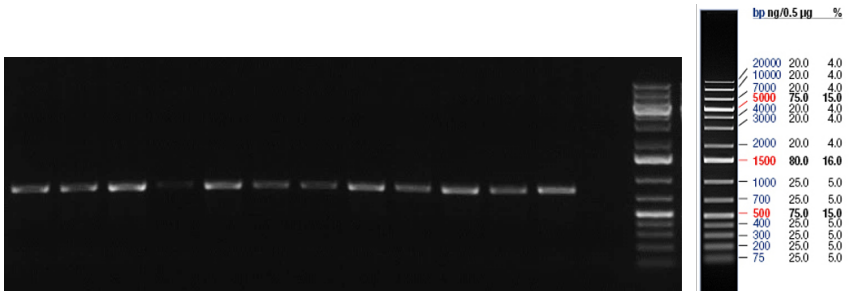


Figure A.1: PCR products were examined by electrophoresis at 140 V for 45 minutes, on a 1% (w/v) agarose gel to confirm product. A 1 Kb Plus DNA ladder (Thermo scientific GeneRuler) served as the size standard, shown to the right.

After PCR product had been confirmed, it was followed by a purification procedure to remove fragments than 40 bases. The QIAquick PCR Purification Kit was used for this objective. A few modifications was applied to the protocol to improve the DNA yield and purity. These includes transferring the QIAquick column to a clean 1.5 mL microcentrifuge tube instead of placing it in the same tube (step 7), and heating the Buffer EB to 60 °C before eluting the DNA by adding 30 µL of buffer (step 9).

The protocols for the UltraClean Microbial DNA Isolation Kit and QIAquick PCR Purification Kit are included in the next few pages.

DNA Isolation Protocol

Experienced User Protocol

(If this is your first time using this kit please read the Detailed Protocol on the following page)
Please wear gloves at all times

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a **2 ml Collection Tube** (provided) and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Decant the supernatant and spin the tubes at 10,000 x *g* for 30 seconds at room temperature and completely remove the media supernatant with a pipette tip. **NOTE**: Based on the type of microbial culture, it may be necessary to centrifuge longer than 30 seconds.
2. Resuspend the cell pellet in 300 µl of **MicroBead Solution** and gently vortex to mix. Transfer resuspended cells to **MicroBead Tube**.
3. Check **Solution MD1.** If **Solution MD1** is precipitated, heat the solution at 60°C until the precipitate has dissolved. Add 50 µl of **Solution MD1** to the **MicroBead Tube**.
4. **Optional:** To increase yields, to minimize DNA shearing, or for difficult cells, see Alternative lysis methods in the "Hints & Troubleshooting Guide" section before continuing.
5. Secure **MicroBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. (See "Hints & Troubleshooting Guide" for less DNA shearing).
6. Make sure the 2 ml **MicroBead Tubes** rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
8. **NOTE**: Expect 300 to 350 µl of supernatant.
9. Add 100 µl of **Solution MD2**, to the supernatant. Vortex for 5 seconds. Then incubate at 4°C for 5 minutes.
10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
11. Avoiding the pellet, transfer the entire volume of supernatant to a clean **2 ml Collection Tube** (provided). Expect approximately 450 µl in volume.
12. Shake to mix Solution MD3 before use. Add 900 µl of **Solution MD3** to the supernatant and vortex for 5 seconds.
13. Load about 700 µl into the **Spin Filter** and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the **Spin Filter**, and centrifuge at 10,000 x *g* for 30 seconds at room temperature. **NOTE**: A total of 2 to 3 loads for each sample processed are required. Discard all flow through liquid.
14. Add 300 µl of **Solution MD4** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
15. Discard the flow through.
16. Centrifuge at room temperature for 1 minute at 10,000 x *g*.
17. Being careful not to splash liquid on the spin filter basket, place **Spin Filter** in a new **2 ml Collection Tube** (provided).
18. Add 50 µl of **Solution MD5** to the center of the white filter membrane.
19. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
20. Discard **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20°C). **Solution MD5** contains no EDTA.

Thank you for choosing the UltraClean® Microbial DNA Isolation Kit.

PCR Purification Protocol

Protocol: QIAquick PCR Purification Kit using a Microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 7). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb can be purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature (15–25°C).
- Add 1:250 volume pH Indicator I to Buffer PB (i.e., add 120 µl pH Indicator I to 30 ml Buffer PB or add 600 µl pH Indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH Indicator I indicates a pH of ≤ 7.5.
- Add pH Indicator I to entire buffer contents. Do not add pH Indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH Indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH Indicator I has been added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

PCR Purification Protocol

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

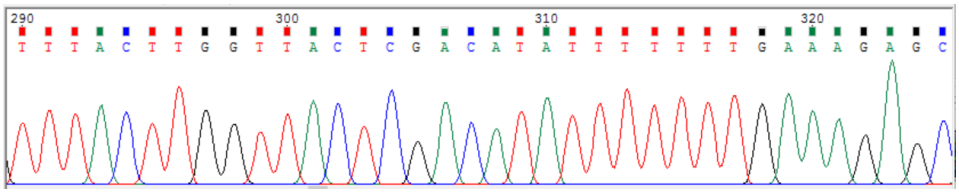
   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

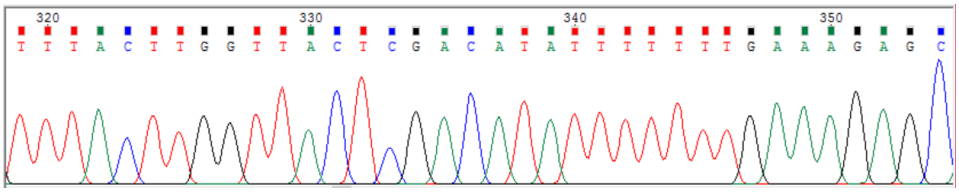
    Loading Dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 14) to identify the dyes according to migration distance and agarose gel percentage and type.

A.2 Analyzing the Sequence Data

The software Chromas was used to manually inspect and quality control the sequencing chromatograms, before further processed in the software Clone Manager Professional 9. Figure A.2 shows how the chromatograms were used for the control. The reverse sequence data was transformed to the reverse complement sequence, in order to compare the two. A consensus region was made by making a pairwise alignment of the forward primer sequence and reverse complement sequence of the reverse primer, by editing the alignment until a 100% match was obtained.



(a) NFAY 4.1 Forward sequence



(b) NFAY 4.1 Reverse complement of reverse sequence

Figure A.2: Chromatograms of forward and reverse sequence data, showing a stretch of sample NFAY 4.1 of the AGT1 region.

Degenerate base symbols was used for incompletely specified bases (as suggested by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) proposed by Comnish-Bowden (1985)). The symbols with the associated description are listed in Table A.1.

Table A.1: Degenerate base symbols. The symbols are used to encode the consensus sequence of aligned sequences. The fundamental bases in DNA are represented by the first letters of their chemical names; Guanine (G), Cytosine (C), Adenine (A) and Thymine (T).

Symbol	Description	Bases represented				
A	**A**denine	A				
C	**C**ytosine		C			
G	**G**uanine			G		1
T	**T**hymine				T	
U	**U**racil				U	
W	**W**eak	A			T	
S	**S**trong		C	G		
M	a**M**ino	A	C			2
K	**K**eto			G	T	
R	pu**R**ine	A		G		
Y	p**Y**rimidine		C		T	
B	not A (**B** comes after A)		C	G	T	
D	not C (**D** comes after C)	A		G	T	3
H	not G (**H** comes after G)	A	C		T	
V	not T (**V** comes after T and U)	A	C	G		
N or -	any **N**ucleotide (not a gap)	A	C	G	T	4
Z	**Z**ero					0

Appendix B

The Brewing Process and Fermentation

B.1 Cell Counting by a Hemocytometer

The depth of Bürker counting chamber is 0.1 mm. The counting grids are subdivided by triple lines that give rise to nine large squares, as seen in the middle part of Figure B.1. Each of these nine squares are again divided into 16 smaller squares. The dimensions of the counting chamber is indicated in the figure.

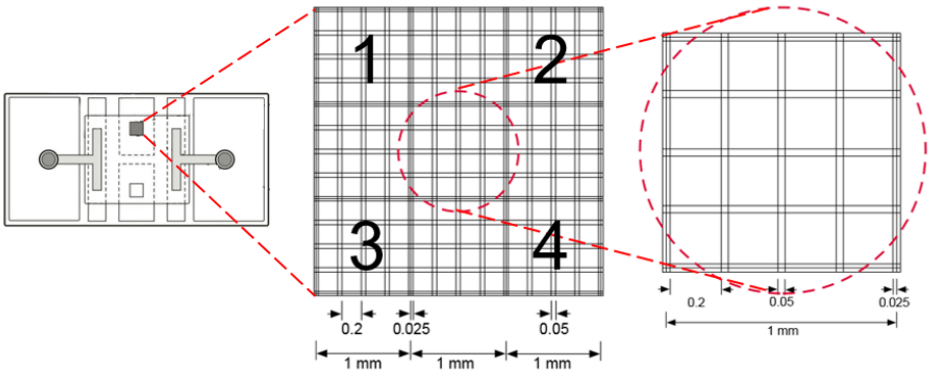


Figure B.1: Bürker counting chamber. The dimensions of the counting chamber is indicated on the figure.

The cells in the four outer corners of the nine large squares were counted, shown in Figure B.1. The average of the counted squares was used to calculate the concentration of cells, using Equation (B.1). An example calculation is shown under.

$$\begin{aligned} \textit{Concentration (cells/mL)} &= \frac{\textit{Average cells per square} \cdot \textit{Dilution factor}}{\textit{Volume of square [mL]}} \\ \textit{Concentration (cells/mL)} &= \frac{\textit{Average cells per square} \cdot \textit{Dilution factor}}{1\ mm \cdot 1\ mm \cdot 0.1\ mm \cdot \frac{1\ mL}{1000\ mm^3}} \end{aligned} \tag{B.1}$$

In one case the four squares were counted, the number of cells in each square was determined to be: 72 + 74 + 69 + 89 = 304. The average number of cells per square is therefore 76. Meanwhile, the dilution factor was 100. The calculation proceeds as following:

$$\begin{aligned} \textit{Concentration} &= \frac{\textit{Average cells per square} \cdot \textit{Dilution factor}}{1\ mm \cdot 1\ mm \cdot 0.1\ mm \cdot \frac{1\ mL}{1000\ mm^3}} \\ &= \frac{76 \cdot 100}{1\ mm \cdot 1\ mm \cdot 0.1\ mm \cdot \frac{1\ mL}{1000\ mm^3}} \\ &= \underline{\underline{7.6 \cdot 10^7\ cells/mL}} \end{aligned}$$

B.2 Monitoring of the Fermentation Process

As part of the monitoring of the fermentation process the tubes were weighed the last days to make sure the fermentation had finished, confirmed by small to no variation in weight. The weights of empty and full tubes, as well as the sample for the first day of fermentation is listed in Table B.1 and Table B.2 for 22 °C and 35 °C, respectively. These weight of the empty tubes was subtracted from the weight of the full tubes in order to get the actual weight of the sample.

The weights from the monitoring period the last days of the fermentation process are listed in Table B.3 and Table B.4 for 22 °C, and Table B.5 and Table B.6 for 35 °C.

The pH was measured when the fermentation had finished, and the cell pellet (i.e. biomass) was diluted in 40 mL distilled water. The OD was measured for this solution was measured at 600 nm, and the relative biomass was calculated by dividing the OD with the average of all samples. The pH values, OD measurements at 600 nm of diluted biomass and relative biomass to the average of the beer samples are listed in Table B.7 for both 22 °C and 35 °C.

Table B.1: Weight of tubes day 1, 22 °C. The weight of the empty tube with lid, full tube with addition of wort and yeast and the sample itself (full tube - empty tube) is listed in the table.

	Day 1		
Sample	Empty tube [g]	Full tube [g]	Sample [g]
NFAY 1_P1	14.778	46.105	31.327
NFAY 2_P1	14.609	46.701	32.092
NFAY 2_P2	14.542	46.551	32.009
NFAY 3.5	13.467	45.220	31.753
NFAY 3.7	13.348	45.728	32.380
NFAY 4.1	13.360	45.321	31.961
NFAY 5_P1	14.676	46.510	31.834
NFAY 6.2	13.410	45.699	32.289
NFAY 6.20	13.363	45.363	32.000
NFAY 7.15	13.337	45.505	32.168
NFAY 7.24	13.335	44.789	31.454
NFAY 8_P1	14.671	45.519	30.848
NFAY 9.8	13.387	45.121	31.734
NFAY 9.23	13.360	44.915	31.555
NFAY 9.24	13.394	45.370	31.976
NFAY 10_P1	14.681	46.375	31.694
NFAY 14_P1	14.655	46.320	31.665
NFAY 14_P2	13.453	44.735	31.282
NFAY 15_P1	14.693	46.096	31.403
NFAY 15_P2	13.406	45.266	31.860
NFAY 16_P1	13.297	45.273	31.976
NFAY 16_P2	13.353	45.289	31.936
NFAY 20_P1	13.498	45.440	31.942
NFAY 20_P2	13.455	45.064	31.609
G518	13.428	45.144	31.716
G561	13.324	45.193	31.869
G562	13.312	45.398	32.086
BY4741	13.355	45.268	31.913
BY4743	13.445	45.472	32.027
NCYC361	14.642	46.919	32.277
NCYC456	14.680	46.442	31.762
NCYC660	13.235	45.199	31.964
NCYC661	14.832	47.015	32.183
WLP013	13.445	45.796	32.351
WLP028	13.476	45.654	32.178
WLP051	13.138	45.271	32.133
WLP500	13.478	45.193	31.715
WLP566	13.258	45.565	32.307
Idun	13.352	45.364	32.012

Table B.2: Weight of tubes day 1, 35 °C. The weight of the empty tube with lid, full tube with addition of wort and yeast and the sample itself (full tube - empty tube) is listed in the table.

	Day 1		
Sample	Empty tube [g]	Full tube [g]	Sample [g]
NFAY 1_P1	14.698	46.424	31.726
NFAY 2_P1	14.810	46.178	31.368
NFAY 2_P2	14.803	46.466	31.663
NFAY 3.5	14.718	46.662	31.944
NFAY 3.7	14.646	46.613	31.967
NFAY 4.1	14.657	46.317	31.660
NFAY 5_P1	14.658	46.446	31.788
NFAY 6.2	14.661	45.843	31.182
NFAY 6.20	14.832	46.241	31.409
NFAY 7.15	14.623	46.457	31.834
NFAY 7.24	14.685	45.971	31.286
NFAY 8_P1	14.678	46.275	31.597
NFAY 9.8	14.654	46.452	31.798
NFAY 9.23	14.694	46.445	31.751
NFAY 9.24	14.631	46.354	31.723
NFAY 10_P1	14.743	46.488	31.745
NFAY 14_P1	14.672	46.655	31.983
NFAY 14_P2	14.718	46.006	31.288
NFAY 15_P1	14.826	46.457	31.631
NFAY 15_P2	14.833	46.553	31.720
NFAY 16_P1	14.691	46.553	31.862
NFAY 16_P2	14.595	46.397	31.802
NFAY 20_P1	14.646	46.532	31.886
NFAY 20_P2	14.696	46.484	31.788
G518	14.711	46.335	31.624
G561	14.805	46.350	31.545
G562	14.788	46.792	32.004
BY4741	14.639	45.409	30.770
BY4743	14.787	46.734	31.947
NCYC361	14.791	46.689	31.898
NCYC456	14.737	46.378	31.641
NCYC660	14.822	46.855	32.033
NCYC661	14.809	46.508	31.699
WLP013	14.777	46.400	31.623
WLP028	14.825	46.271	31.446
WLP051	14.676	46.591	31.915
WLP500	14.831	46.539	31.708
WLP566	14.675	46.465	31.790
Idun	14.663	46.477	31.814

Table B.3: Weights of NFAY samples at day 9, 12, 13 and 14 of the fermentation process at 22 °C. The standard deviation (SD) in percentage is also included.

Weights	Day 9		Day 12		Day 13		Day 14		
Sample	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	SD [%]
NFAY 1_P1	44.973	30.195	44.972	30.194	44.991	30.213	45.014	30.236	2.103
NFAY 2_P1	45.571	30.962	45.597	30.988	45.608	30.999	45.615	31.006	0.907
NFAY 2_P2	45.556	31.014	45.559	31.017	45.559	31.017	45.553	31.011	0.346
NFAY 3.5	43.961	30.494	43.949	30.482	43.942	30.475	43.938	30.471	0.557
NFAY 3.7	44.487	31.139	44.511	31.163	44.520	31.172	44.531	31.183	1.002
NFAY 4.1	43.928	30.568	43.847	30.487	43.826	30.466	43.804	30.444	2.150
NFAY 5_P1	45.263	30.587	45.280	30.604	45.287	30.611	45.293	30.617	0.651
NFAY 6.2	44.277	30.867	44.279	30.869	44.282	30.872	44.291	30.881	0.624
NFAY 6.20	44.104	30.741	44.105	30.742	44.105	30.742	44.109	30.746	0.231
NFAY 7.15	44.269	30.932	44.326	30.989	44.348	31.011	44.373	31.036	2.352
NFAY 7.24	43.522	30.187	43.533	30.198	43.540	30.205	43.547	30.212	0.700
NFAY 8_P1	44.333	29.662	44.364	29.693	44.373	29.702	44.385	29.714	1.054
NFAY 9.8	43.864	30.477	43.851	30.464	43.848	30.461	43.843	30.456	0.404
NFAY 9.23	43.598	30.238	43.574	30.214	43.554	30.194	43.541	30.181	1.662
NFAY 9.24	44.047	30.653	44.024	30.630	44.017	30.623	44.016	30.622	0.436
NFAY 10_P1	45.177	30.496	45.211	30.530	45.219	30.538	45.232	30.551	1.060
NFAY 14_P1	45.000	30.345	44.988	30.333	44.986	30.331	44.977	30.322	0.586
NFAY 14_P2	43.399	29.946	43.394	29.941	43.399	29.946	43.409	29.956	0.764
NFAY 15_P1	44.787	30.094	44.771	30.078	44.766	30.073	44.757	30.064	0.709
NFAY 15_P2	43.812	30.406	43.845	30.439	43.848	30.442	43.856	30.450	0.569
NFAY 16_P1	44.282	30.985	44.338	31.041	44.364	31.067	44.393	31.096	2.751
NFAY 16_P2	44.144	30.791	44.171	30.818	44.174	30.821	44.183	30.830	0.624
NFAY 20_P1	44.237	30.739	44.243	30.745	44.247	30.749	44.252	30.754	0.451
NFAY 20_P2	43.925	30.470	43.944	30.489	43.954	30.499	43.970	30.515	1.311

Table B.4: Weights of wild, laboratory and commercial samples at day 9, 12, 13 and 14 of the fermentation process at 22 °C. The standard deviation (SD) in percentage is also included.

Weights	Day 9		Day 12		Day 13		Day 14		
Sample	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	SD [%]
G518	43.716	30.288	43.703	30.275	43.700	30.272	43.698	30.270	0.252
G561	43.844	30.520	43.834	30.510	43.831	30.507	43.826	30.502	0.404
G562	43.675	30.363	43.644	30.332	43.628	30.316	43.605	30.293	1.960
BY4741	43.994	30.639	43.881	30.526	43.875	30.520	43.870	30.515	0.551
BY4743	44.800	31.355	44.173	30.728	44.117	30.672	44.090	30.645	4.234
NCYC361	45.359	30.717	45.324	30.682	45.311	30.669	45.294	30.652	1.504
NCYC456	45.098	30.418	45.078	30.398	45.072	30.392	45.067	30.387	0.551
NCYC660	44.251	31.016	44.186	30.951	44.166	30.931	44.145	30.910	2.050
NCYC661	45.999	31.167	45.889	31.057	45.883	31.051	45.876	31.044	0.651
WLP013	44.475	31.030	44.524	31.079	44.541	31.096	44.564	31.119	2.007
WLP028	44.396	30.920	44.441	30.965	44.453	30.977	44.473	30.997	1.617
WLP051	43.883	30.745	43.845	30.707	43.823	30.685	43.811	30.673	1.724
WLP500	43.900	30.422	43.960	30.482	43.974	30.496	43.994	30.516	1.709
WLP566	43.994	30.736	43.951	30.693	43.937	30.679	43.924	30.666	1.350
Idun	44.042	30.690	44.070	30.718	44.078	30.726	44.093	30.741	1.168

Table B.5: Weights of NFAY samples at day 9, 12, 13 and 14 of the fermentation process at 35 °C. The standard deviation (SD) in percentage is also included.

Weights	Day 9		Day 12		Day 13		Day 14		
Sample	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	SD [%]
NFAY 1_P1	44.989	30.291	44.916	30.218	44.897	30.199	44.871	30.173	2.259
NFAY 2_P1	44.899	30.089	44.795	29.985	44.780	29.970	44.769	29.959	1.305
NFAY 2_P2	45.406	30.603	45.460	30.657	45.477	30.674	45.494	30.691	1.700
NFAY 3.5	45.318	30.600	45.494	30.776	45.448	30.730	45.477	30.759	2.326
NFAY 3.7	45.164	30.518	45.125	30.479	45.107	30.461	45.090	30.444	1.750
NFAY 4.1	44.735	30.078	44.689	30.032	44.677	30.020	44.664	30.007	1.250
NFAY 5_P1	45.069	30.411	45.050	30.392	45.041	30.383	45.030	30.372	1.002
NFAY 6.2	44.834	30.173	44.914	30.253	44.929	30.268	44.974	30.313	3.122
NFAY 6.20	44.559	29.727	44.430	29.598	44.405	29.573	44.386	29.554	2.207
NFAY 7.15	45.015	30.392	44.949	30.326	44.931	30.308	44.912	30.289	1.850
NFAY 7.24	44.579	29.894	44.537	29.852	44.519	29.834	44.502	29.817	1.750
NFAY 8_P1	44.895	30.217	44.865	30.187	44.852	30.174	44.840	30.162	1.250
NFAY 9.8	45.089	30.435	45.070	30.416	45.062	30.408	45.054	30.400	0.800
NFAY 9.23	45.051	30.357	45.018	30.324	45.007	30.313	44.995	30.301	1.150
NFAY 9.24	44.939	30.308	44.901	30.270	44.889	30.258	44.874	30.243	1.353
NFAY 10_P1	45.109	30.366	45.053	30.310	45.024	30.281	45.006	30.263	2.371
NFAY 14_P1	45.188	30.516	45.150	30.478	45.136	30.464	45.121	30.449	1.450
NFAY 14_P2	44.566	29.848	44.520	29.802	44.504	29.786	44.450	29.732	3.668
NFAY 15_P1	45.024	30.198	44.977	30.151	44.962	30.136	44.946	30.120	1.550
NFAY 15_P2	45.324	30.491	45.427	30.594	45.457	30.624	45.468	30.635	2.122
NFAY 16_P1	45.194	30.503	45.151	30.460	45.141	30.450	45.130	30.439	1.050
NFAY 16_P2	45.090	30.495	45.128	30.533	45.137	30.542	45.150	30.555	1.106
NFAY 20_P1	45.965	31.319	44.980	30.334	45.286	30.640	45.293	30.647	17.872
NFAY 20_P2	45.442	30.746	45.517	30.821	45.538	30.842	45.564	30.868	2.354

Table B.6: Weights of wild, laboratory and commercial samples samples at day 9, 12, 13 and 14 of the fermentation process at 35 °C. The standard deviation (SD) in percentage is also included.

Weights	Day 9		Day 12		Day 13		Day 14		
Sample	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	Tube [g]	Sample [g]	SD [%]
G518	44.803	30.092	44.757	30.046	44.729	30.018	44.713	30.002	2.227
G561	44.914	30.109	44.868	30.063	44.849	30.044	44.832	30.027	1.801
G562	45.413	30.625	45.389	30.601	45.379	30.591	45.369	30.581	1.000
BY4741	46.128	31.489	46.081	31.442	46.055	31.416	46.020	31.381	3.061
BY4743	46.432	31.645	46.454	31.667	46.464	31.677	46.450	31.663	0.721
NCYC361	44.960	30.169	44.831	30.040	44.812	30.021	44.787	29.996	2.207
NCYC456	45.792	31.055	45.743	31.006	45.722	30.985	45.699	30.962	2.201
NCYC660	45.530	30.708	45.481	30.659	45.464	30.642	45.445	30.623	1.801
NCYC661	45.358	30.549	45.290	30.481	45.273	30.464	45.244	30.435	2.326
WLP013	44.933	30.156	44.839	30.062	44.812	30.035	44.791	30.014	2.406
WLP028	45.077	30.252	45.154	30.329	45.172	30.347	45.191	30.366	1.850
WLP051	45.906	31.230	45.132	30.456	45.113	30.437	45.092	30.416	2.001
WLP500	45.448	30.617	45.496	30.665	45.496	30.665	45.494	30.663	0.115
WLP566	44.954	30.279	44.910	30.235	44.897	30.222	44.883	30.208	1.350
Idun	44.908	30.245	44.793	30.130	44.763	30.100	44.714	30.051	3.988

Table B.7: pH values, OD measurements at 600 nm of diluted biomass and relative biomass to the average of the beer samples at both 22 °C and 35 °C.

	pH measurement		OD measurement		Relative biomass	
Sample	22 °C	35 °C	22 °C	35 °C	22 °C	35 °C
NFAY 1_P1	3.22	4.10	17.05	6.92	1.22	1.01
NFAY 2_P1	3.23	4.04	11.00	6.88	0.78	1.01
NFAY 2_P2	3.14	3.20	10.15	6.60	0.72	0.97
NFAY 3.5	3.93	3.17	6.63	7.52	0.47	1.10
NFAY 3.7	3.34	4.16	17.40	4.20	1.24	0.61
NFAY 4.1	3.87	4.08	21.80	8.00	1.56	1.17
NFAY 5_P1	3.41	4.16	12.40	4.30	0.88	0.63
NFAY 6.2	3.41	2.89	18.40	8.44	1.31	1.23
NFAY 6.20	3.60	4.19	11.20	6.74	0.80	0.99
NFAY 7.15	3.34	4.02	16.55	7.56	1.18	1.11
NFAY 7.24	3.45	4.12	8.00	6.20	0.57	0.91
NFAY 8_P1	3.32	4.06	11.55	6.82	0.82	1.00
NFAY 9.8	3.87	4.02	11.85	8.44	0.85	1.23
NFAY 9.23	3.80	3.94	13.30	7.62	0.95	1.11
NFAY 9.24	3.77	4.07	17.00	6.52	1.21	0.95
NFAY 10_P1	3.30	4.27	12.25	7.58	0.87	1.11
NFAY 14_P1	4.35	4.22	10.05	6.12	0.72	0.90
NFAY 14_P2	3.80	4.23	12.95	7.84	0.92	1.15
NFAY 15_P1	4.17	4.10	11.85	7.74	0.85	1.13
NFAY 15_P2	3.35	3.07	10.35	7.02	0.74	1.03
NFAY 16_P1	3.20	4.01	14.30	7.12	1.02	1.04
NFAY 16_P2	3.17	3.32	12.05	6.94	0.86	1.02
NFAY 20_P1	3.34	3.38	11.60	4.58	0.83	0.67
NFAY 20_P2	3.33	2.97	18.65	7.02	1.33	1.03
G518	4.26	4.28	9.90	8.95	0.71	1.31
G561	4.44	4.01	11.30	8.04	0.81	1.18
G562	4.10	3.94	18.95	9.30	1.35	1.36
BY4741	3.43	3.35	21.80	6.70	1.56	0.98
BY4743	3.53	3.32	24.75	4.88	1.77	0.71
NCYC361	3.80	4.06	18.75	2.44	1.34	0.36
NCYC456	4.09	4.01	12.65	4.14	0.90	0.61
NCYC660	4.10	3.92	15.15	4.14	1.08	0.61
NCYC661	4.18	4.02	12.55	5.88	0.90	0.86
WLP013	3.41	3.90	12.75	4.50	0.91	0.66
WLP028	3.51	3.28	7.44	4.26	0.53	0.62
WLP051	4.10	3.97	11.50	7.16	0.82	1.05
WLP500	3.42	2.89	10.45	3.90	0.75	0.57
WLP566	3.88	4.13	28.05	19.55	2.00	2.86
Idun	3.61	4.05	12.40	8.00	0.88	1.17
Average	3.66	3.82	14.02	6.83	1.00	1.00

Appendix C

Headspace Gas Chromatography Mass Spectroscopy

C.1 Preparation of Calibration Series

A calibration series including the compounds 1-propanol, ethyl acetate, isobutyl alcohol, isoamyl alcohol, active amyl alcohol, 1-butanol, ethyl propionate, propyl acetate, acetal, isobutyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, was prepared for the HS GC-MS analysis.

The compounds were accurately weighed in 20 mL volumetric flasks, aiming to be close to the preset range. The exact weights are listed in Table C.1. The flasks were filled with EtOH and distilled water in the specified ratios.

C.2 Calculation of Flavor Unit

The FU value of the samples was calculated by dividing the concentration of each compound by its flavor threshold values using Equation (1.1). An example calculation for sample NFAY 1_P1 for 1-propanol at 22 °C is included under:

$$FU = \frac{Concentration\ compound}{Flavor\ threshold} = \frac{19.02\ mg/L}{700\ mg/L} = \underline{0.03}$$

The calculated FU-values are given in Table C.2 and Table C.3 for 22 °C and 35 °C, respectively.

Table C.1: Composition of Mix A, Mix B and Mix C, used to create a stock solution for the calibration series with accurate weights [mg].

Mix A	Weight range [mg]	Weight [mg]	Volume [mL]
1-propanol	45-50	55.95	
Ethyl acetate	45-55	52.84	
Isobutyl alcohol	35-45	43.12	20 (EtOH/H_2O, 50:50 v/v)
Isoamyl alcohol	65-75	68.46	
Active amyl alcohol	40-50	43.68	

Mix B	Weight range [mg]	Weight [mg]	Volume [mL]
1-butanol	35-45	43.40	
Ethyl propionate	30-40	39.63	
Propyl acetate	20-30	28.93	20 (EtOH/H_2O, 80:20 v/v)
Acetal	20-30	24.11	
Isobutyl acetate	20-30	25.70	

Mix C	Weight range [mg]	Weight [mg]	Volume [mL]
Ethyl butyrate	20-30	25.95	
Isoamyl acetate	18-25	21.86	
Ethyl hexanoate	18-25	30.23	20 (EtOH/H_2O, 80:20 v/v)
Ethyl octanoate	20-30	25.87	
Ethyl decanoate	40-50	48.95	

Table C.2: Calculated flavor unit (FU) values for samples at 22 °C. The compounds are numbered as following: 1, 1-Propanol; 2, Ethyl acetate; 3, Isobutyl alcohol; 4, 1-Butanol; 5, Ethyl propionate; 6, Propyl acetate; 7, Acetal; 8, Isoamyl alcohol; 9, Active amyl alchohol; 10, Isobutyl acetate and 11, Isoamyl acetate.

	Flavor unit (FU)										
Sample	1	2	3	4	5	6	7	8	9	10	11
NFAY 1_P1	0.03	4.38	0.34	0.51	0.00	0.02	0.21	1.55	0.39	0.46	2.57
NFAY 2_P1	0.02	4.38	0.17	0.61	0.00	0.01	0.00	0.97	0.22	0.21	1.10
NFAY 2_P2	0.02	1.66	0.14	2.64	0.00	0.00	0.53	0.96	0.21	0.00	0.17
NFAY 3.5	0.05	0.76	0.35	0.74	0.00	0.00	0.37	1.70	0.42	0.00	0.36
NFAY 3.7	0.05	4.38	0.31	0.43	0.00	0.02	0.14	1.57	0.37	0.24	1.27
NFAY 4.1	0.04	0.64	0.69	1.10	0.25	0.00	0.42	2.00	0.63	0.00	0.19
NFAY 5_P1	0.03	4.38	0.33	0.51	0.00	0.01	0.11	1.72	0.38	0.23	1.12
NFAY 6.2	0.05	4.38	0.34	1.00	0.20	0.01	0.15	1.57	0.40	0.22	1.14
NFAY 6.20	0.04	1.01	0.36	0.57	0.00	0.00	0.73	1.79	0.42	0.00	0.19
NFAY 7.15	0.03	4.38	0.45	0.49	0.00	0.02	0.13	1.57	0.46	0.62	2.62
NFAY 7.24	0.03	4.38	0.50	0.53	0.00	0.01	0.12	2.03	0.53	0.33	1.45
NFAY 8_P1	0.06	4.38	0.25	0.46	0.22	0.02	0.14	1.28	0.27	0.24	1.21
NFAY 9.8	0.04	0.93	0.28	0.58	0.00	0.00	0.30	1.68	0.35	0.00	0.34
NFAY 9.23	0.04	0.63	0.39	1.03	0.00	0.00	0.10	1.76	0.45	0.00	0.09
NFAY 9.24	0.05	0.90	0.38	0.61	0.00	0.00	0.07	1.88	0.43	0.00	0.43
NFAY 10_P1	0.03	0.91	0.33	0.66	0.00	0.00	0.21	1.66	0.41	0.07	0.25
NFAY 14_P1	0.05	0.83	0.40	0.80	0.20	0.00	0.53	1.42	0.45	0.07	0.74
NFAY 14_P2	0.06	4.38	0.49	0.70	0.17	0.01	0.18	1.82	0.59	0.12	0.42
NFAY 15_P1	0.07	0.74	0.57	0.82	0.26	0.00	0.58	1.82	0.57	0.00	0.56
NFAY 15_P2	0.05	4.38	0.52	0.50	0.00	0.03	0.08	1.43	0.49	0.69	2.16
NFAY 16_P1	0.02	4.38	0.28	0.46	0.00	0.02	0.14	1.21	0.31	0.46	2.18
NFAY 16_P2	0.02	4.38	0.20	0.64	0.00	0.02	0.16	0.89	0.21	0.31	1.34
NFAY 20_P1	0.04	4.38	0.25	0.94	0.17	0.01	0.14	1.63	0.34	0.22	1.68
NFAY 20_P2	0.03	4.38	0.23	0.75	0.18	0.02	0.15	1.22	0.28	0.29	1.67
G518	0.06	0.91	0.51	0.61	0.00	0.00	0.30	1.72	0.63	0.13	1.51
G561	0.07	0.79	0.69	0.94	0.45	0.00	0.36	1.97	0.76	0.10	0.89
G562	0.11	1.34	0.86	0.51	0.40	0.00	0.32	2.07	1.03	0.00	0.17
BY4741	0.02	0.83	0.30	0.56	0.00	0.00	0.88	0.79	0.25	0.00	0.00
BY4743	0.02	0.67	0.31	0.42	0.00	0.00	0.46	0.88	0.27	0.00	0.00
NCYC361	0.07	1.03	0.66	0.92	0.20	0.00	0.76	2.34	0.70	0.07	0.64
NCYC456	0.06	0.72	0.64	0.92	0.00	0.00	0.00	2.06	0.74	0.00	0.31
NCYC660	0.08	0.36	0.31	1.32	0.00	0.00	0.13	0.96	0.27	0.00	0.24
NCYC661	0.11	0.48	0.26	2.40	0.28	0.00	0.24	0.91	0.34	0.00	0.28
WLP013	0.03	4.38	0.60	0.00	0.00	0.02	0.19	1.04	0.38	0.59	1.19
WLP028	0.05	0.71	0.77	0.44	0.00	0.00	0.05	1.36	0.37	0.00	0.00
WLP051	0.04	0.44	0.18	1.31	0.00	0.00	0.07	1.16	0.35	0.00	0.00
WLP500	0.06	4.38	0.32	0.55	0.17	0.03	0.09	1.66	0.35	0.32	1.90
WLP566	0.05	0.54	0.60	1.15	0.00	0.00	0.49	1.94	0.61	0.00	0.15
Idun	0.06	4.38	0.47	0.66	0.00	0.01	0.07	2.03	0.58	0.15	0.56

Table C.3: Calculated flavor unit (FU) values for samples at 35 °C. The compounds are numbered as following: 1, 1-Propanol; 2, Ethyl acetate; 3, Isobutyl alcohol; 4, 1-Butanol; 5, Ethyl propionate; 6, Propyl acetate; 7, Acetal; 8, Isoamyl alcohol; 9, Active amyl alchohol; 10, Isobutyl acetate and 11, Isoamyl acetate.

	Flavor unit (FU)										
Sample	1	2	3	4	5	6	7	8	9	10	11
NFAY 1_P1	0.06	0.43	0.57	3.11	0.00	0.00	0.00	1.33	0.44	0.00	0.00
NFAY 2_P1	0.05	0.92	0.47	1.57	0.00	0.00	0.28	1.44	0.42	0.00	0.25
NFAY 2_P2	0.02	1.78	0.21	0.62	0.00	0.00	0.08	0.90	0.26	0.00	0.00
NFAY 3.5	0.04	4.38	0.43	0.53	0.22	0.04	0.25	1.19	0.39	0.85	2.85
NFAY 3.7	0.06	0.59	0.32	1.39	0.00	0.00	0.58	0.97	0.26	0.00	0.00
NFAY 4.1	0.03	0.55	0.52	0.46	0.00	0.00	0.00	0.92	0.39	0.00	0.16
NFAY 5_P1	0.05	0.72	0.42	4.00	0.00	0.00	0.45	1.47	0.41	0.00	0.19
NFAY 6.2	0.01	4.38	0.36	0.00	0.00	0.01	0.00	0.65	0.28	0.89	1.52
NFAY 6.20	0.05	0.47	0.35	3.35	0.00	0.00	0.10	1.12	0.34	0.00	0.00
NFAY 7.15	0.05	0.49	0.45	3.54	0.00	0.00	1.18	1.38	0.41	0.00	0.11
NFAY 7.24	0.05	0.54	0.51	2.58	0.00	0.00	0.50	1.31	0.43	0.00	0.17
NFAY 8_P1	0.06	0.58	0.25	1.56	0.00	0.00	0.47	1.19	0.26	0.00	0.11
NFAY 9.8	0.03	0.59	0.40	3.07	0.00	0.00	0.00	1.39	0.39	0.00	0.11
NFAY 9.23	0.04	0.57	0.44	2.99	0.00	0.00	0.06	1.41	0.43	0.00	0.00
NFAY 9.24	0.05	0.70	0.45	2.48	0.00	0.00	1.20	1.33	0.41	0.00	0.17
NFAY 10_P1	0.05	0.78	0.44	2.86	0.00	0.00	0.00	1.52	0.45	0.00	0.23
NFAY 14_P1	0.07	0.89	0.49	3.11	0.00	0.00	0.72	1.33	0.46	0.09	0.64
NFAY 14_P2	0.06	0.77	0.42	1.36	0.00	0.00	0.47	1.19	0.41	0.08	0.60
NFAY 15_P1	0.07	0.51	0.61	3.12	0.00	0.00	0.68	1.53	0.53	0.00	0.13
NFAY 15_P2	0.04	4.38	0.51	0.56	0.00	0.05	0.13	1.25	0.43	1.28	3.85
NFAY 16_P1	0.05	1.69	0.41	1.42	0.00	0.00	0.00	1.17	0.36	0.00	0.00
NFAY 16_P2	0.04	4.38	0.33	0.63	0.28	0.02	0.42	1.01	0.30	0.37	1.32
NFAY 20_P1	0.04	4.38	0.42	0.86	0.19	0.02	0.37	1.42	0.39	0.40	1.59
NFAY 20_P2	0.02	4.38	0.35	0.51	0.19	0.03	0.15	1.02	0.31	0.86	2.53
G518	0.11	0.68	0.78	1.63	0.19	0.00	0.26	1.59	0.62	0.00	0.23
G561	0.08	0.61	0.76	1.72	0.16	0.00	0.60	1.79	0.67	0.07	0.28
G562	0.09	0.68	0.77	1.74	0.19	0.00	0.59	1.87	0.67	0.00	0.22
BY4741	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BY4743	0.00	0.14	0.03	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00
NCYC361	0.07	0.62	0.60	1.16	0.00	0.00	0.06	1.54	0.50	0.00	0.18
NCYC456	0.01	0.03	0.26	0.00	0.00	0.00	0.00	0.33	0.09	0.00	0.00
NCYC660	0.09	0.56	0.63	1.22	1.98	0.00	0.11	1.26	0.52	0.00	0.00
NCYC661	0.12	0.54	0.57	1.59	0.00	0.00	0.08	1.05	0.50	0.00	0.00
WLP013	0.09	0.46	0.65	1.14	0.00	0.00	0.45	0.97	0.37	0.00	0.00
WLP028	0.04	1.43	0.64	0.00	0.00	0.00	0.23	0.72	0.29	0.00	0.00
WLP051	0.04	0.11	0.32	0.98	0.00	0.00	0.56	0.59	0.20	0.00	0.00
WLP500	0.00	0.67	0.32	0.00	0.00	0.00	0.08	0.78	0.24	0.56	1.07
WLP566	0.05	1.19	1.15	1.36	0.00	0.00	0.39	2.14	0.93	0.09	0.41
Idun	0.06	0.34	0.49	2.40	0.00	0.00	0.08	1.47	0.43	0.00	0.10

Appendix D

High-Performance Liquid Chromatography

The final concentrations of the compounds in the standard mix after purity corrections, are listed in Table D.1. The area of the peaks in the chromatograms obtained by the HPLC analysis are listed in Table D.2 for the standard mix. The calculated average peak area and the final concentration of the standards were used to calculate the response factor by applying it to Equation (D.1). The amount of analyte (i.e. samples) was calculated by dividing the peak area with the response factor, as seen in Equation (D.2). The final concentration was multiplied with 3 to obtain the correct one, as the samples were prepared with a 1:3 dilution.

$$\textit{Response Factor} = \frac{\textit{Peak Area}}{\textit{Standard Amount}} \tag{D.1}$$

$$\textit{Amount of Analyte (Sample)} = \frac{\textit{Peak Area}}{\textit{Response Factor}} \tag{D.2}$$

Table D.1: Concentration of compounds included in the standard mix in the HPLC analysis. The concentrations of the stock solutions [g/L], the volume added [L] and the final concentration (corrected for purity) [g/L] are specified in the table.

Standard	C_{stock} [g/L]	Volume [L]	C [g/L]	Purity [%]	C_{final} [g/L]
Glucose	50.0500	0.0010	5.0050	100.60	5.035
Fructose	50.0250	0.0010	5.0025	100.60	5.033
Glycerol	25.0293	0.0010	2.5029	99.50	2.490
Acetic acid	16.1900	0.0004	0.6476	99.70	0.646
EtOH	50.3840	0.0020	10.0768	99.80	10.057
Succinic acid	50.2520	0.0005	2.5126	99.50	2.500

Table D.2: Area of the compounds in the standard mix detected in the chromatograms from the HPLC analysis. The average for each compound, the standard deviation (SD) and the relative standard deviation (RSD) were also calculated.

	Area Compound					
Standard	Glucose	Fructose	Glycerol	Acetic acid	EtOH	Succinic acid
Std1	619.83	615.67	261.46	603.03[a]	548.07	2079.03[a]
Std2	621.42	616.61	260.28	531.18	545.97	2042.00
Std3	614.25	611.57	255.88	520.22	541.57	2048.43
Std4	608.42	603.65	255.20	512.68	534.57	2018.60
Std5	606.68	603.43	253.24	517.14	529.66	2008.49
Std6	604.46	602.53	252.64	518.28	527.78	2037.57
Std7	608.30	605.26	253.60	520.57	531.85	2024.30
Std8	603.87	601.88	252.99	511.12	511.12	2015.74
Std9	596.91	592.88	253.39	510.79	527.05	2014.45
Std10	610.14	606.04	255.69	515.30	535.90	2001.81
Std11	596.00	594.70	250.63	780.33[a]	524.14	2162.39[a]
Average	608.21	604.93	255.00	517.48	532.52	2023.49
SD	8.14	7.52	3.28	6.31	10.53	15.91
RSD (%)	1.34	1.24	1.28	1.22	1.98	0.79

[a] The value was excluded in the calculation of average, SD and RSD.

The areas of the compounds detected by HPLC for samples at 22 °C and 35 °C are listed in Table D.3 and Table D.4, respectively. An example calculation to quantify glucose for sample NFAY 2_P2 at 22 °C is conducted under:

$$\textit{Response Factor} = \frac{\textit{Peak Area}}{\textit{Standard Amount}} = \frac{608.21}{5.035\ g/L} = 120.80\ L/g$$

$$\textit{Amount of Analyte (Sample)} = \frac{\textit{Peak Area}}{\textit{Response Factor}} = \frac{93.79}{120.80\ L/g} = 0.78\ g/L$$

$$Concentration\ = 0.78\ g/L \cdot 3 = 2.33\ g/L$$

Table D.3: Area of compounds detected in the chromatograms by HPLC for samples at 22 °C.

	Area Compound					
Sample	Glucose	Fructose	Glycerol	Acetic acid	EtOH	Succinic acid
NFAY 1_P1	0.00	0.00	20.86	5202.18	516.03	1508.51
NFAY 2_P1	0.00	0.00	52.12	4331.73	509.45	1600.33
NFAY 2_P2	93.79	17.02	27.49	3135.03	446.53	1401.60
NFAY 3.5	0.00	0.00	49.42	371.75	719.41	1744.36
NFAY 3.7	0.00	0.00	46.63	3100.84	616.78	1515.18
NFAY 4.1	0.00	0.00	0.00	0.00	634.09	1626.00
NFAY 5_P1	0.00	0.00	55.41	2887.12	661.23	1718.52
NFAY 6.2	0.00	0.00	47.80	15.09	717.68	1561.21
NFAY 6.20	0.00	0.00	64.62	2259.02	685.80	1739.43
NFAY 7.15	0.00	0.00	43.54	5327.07	509.89	1835.67
NFAY 7.24	0.00	0.00	53.66	2937.15	622.53	1343.18
NFAY 8_P1	0.00	0.00	32.03	3221.27	5606.86	1279.07
NFAY 9.8	0.00	0.00	57.28	1001.73	729.01	1602.23
NFAY 9.23	0.00	0.00	52.43	1149.80	691.23	1664.24
NFAY 9.24	0.00	0.00	60.42	1171.23	707.33	1400.58
NFAY 10_P1	0.00	0.00	38.47	3977.71	558.35	1775.42
NFAY 14_P1	0.00	0.00	57.78	0.00	719.14	1869.90
NFAY 14_P2	0.00	0.00	64.18	1894.73	685.28	2085.79
NFAY 15_P1	0.00	0.00	47.27	866.03	727.39	1881.36
NFAY 15_P2	0.00	0.00	22.47	5847.13	447.65	2330.52
NFAY 16_P1	0.00	0.00	22.85	6774.22	368.31	1251.02
NFAY 16_P2	0.00	0.00	18.62	5358.40	489.50	1569.63
NFAY 20_P1	0.00	0.00	43.87	3508.34	603.12	1622.19
NFAY 20_P2	0.00	0.00	34.11	4043.48	559.64	1770.02
G518	0.00	0.00	67.09	0.00	759.90	1698.84
G561	0.00	0.00	63.32	0.00	748.95	1580.00
G562	0.00	0.00	18.17	0.00	639.97	1117.49
BY4741	23.74	0.00	65.33	3278.68	774.48	1191.77
BY4743	48.38	0.00	15.41	2456.20	725.17	1379.92
NCYC361	0.00	0.00	45.40	1380.80	837.30	1391.32
NCYC456	0.00	0.00	52.90	0.00	739.69	1620.85
NCYC660	0.00	0.00	0.00	906.37	522.28	1158.82
NCYC661	0.00	0.00	0.00	0.00	583.62	1302.40
WLP013	0.00	0.00	32.75	4841.20	556.25	2451.17
WLP028	0.00	0.00	61.62	3534.03	589.08	2147.72
WLP051	0.00	0.00	19.68	0.00	729.38	1676.79
WLP500	0.00	0.00	52.90	4313.56	553.73	1472.29
WLP566	0.00	0.00	22.57	1131.51	770.11	746.01
Idun	0.00	0.00	55.00	2265.52	667.96	1969.57

Table D.4: Area of compounds detected in the chromatograms by HPLC for samples at 35 °C.

	Area Compound					
Sample	Glucose	Fructose	Glycerol	Acetic acid	EtOH	Succinic acid
NFAY 1_P1	0.00	0.00	30.52	1091.81	664.25	2100.11
NFAY 2_P1	0.00	0.00	68.42	1251.36	682.89	1749.37
NFAY 2_P2	51.13	0.00	45.41	5320.29	401.84	1948.63
NFAY 3.5	0.00	29.85	35.99	7355.92	406.66	2043.04
NFAY 3.7	0.00	0.00	59.77	0.00	715.88	2135.81
NFAY 4.1	0.00	0.00	56.21	0.00	847.99	1881.76
NFAY 5_P1	0.00	0.00	68.43	997.20	694.40	2061.20
NFAY 6.2	37.24	34.41	0.00	13506.87	0.00	2058.09
NFAY 6.20	0.00	0.00	66.59	974.29	632.54	2498.60
NFAY 7.15	0.00	0.00	66.67	1185.14	679.19	2064.57
NFAY 7.24	0.00	0.00	58.72	1145.80	686.46	1993.07
NFAY 8_P1	0.00	0.00	60.40	943.15	719.41	2004.26
NFAY 9.8	0.00	0.00	60.16	1025.66	687.08	1865.38
NFAY 9.23	0.00	0.00	57.77	1180.37	679.14	2036.40
NFAY 9.24	0.00	0.00	58.65	1130.11	708.03	2255.87
NFAY 10_P1	0.00	0.00	63.95	0.00	661.41	2225.66
NFAY 14_P1	0.00	0.00	58.68	373.19	727.58	2338.24
NFAY 14_P2	0.00	0.00	60.06	0.00	735.37	2260.03
NFAY 15_P1	0.00	32.14	55.93	1063.70	706.88	2239.97
NFAY 15_P2	0.00	31.68	37.23	8663.69	325.57	2767.82
NFAY 16_P1	0.00	0.00	46.52	1027.56	698.44	1281.73
NFAY 16_P2	0.00	26.41	39.02	4481.81	544.80	1301.30
NFAY 20_P1	0.00	0.00	44.81	4196.51	530.97	2173.65
NFAY 20_P2	0.00	29.01	0.00	11514.55	141.63	1914.69
G518	0.00	0.00	47.17	0.00	677.55	1850.64
G561	0.00	0.00	59.17	1002.46	686.73	1356.34
G562	0.00	0.00	60.16	1034.73	681.94	1363.40
BY4741	0.00	0.00	0.00	4305.25	0.00	1688.80
BY4743	0.00	0.00	0.00	4697.28	0.00	1876.36
NCYC361	0.00	0.00	64.95	1045.60	840.73	1180.92
NCYC456	0.00	16.51	48.94	0.00	236.25	1955.45
NCYC660	0.00	17.24	0.00	0.00	448.46	2119.80
NCYC661	0.00	15.06	0.00	0.00	474.83	1919.86
WLP013	0.00	0.00	66.88	1229.71	628.19	2431.56
WLP028	0.00	37.57	64.05	5619.05	430.46	2292.62
WLP051	0.00	0.00	33.15	386.83	630.06	2043.16
WLP500	0.00	47.60	31.63	13147.14	0.00	1895.12
WLP566	0.00	0.00	85.61	1050.76	771.24	2351.24
Idun	0.00	0.00	55.76	1075.55	665.96	2121.96