NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF CHEMISTRY

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Bachelor in Biochemistry

INNOVATION AND QUALITY ASSESSMENT IN CRAFT BEER PRODUCTION

MASTER IN BIOTECHNOLOGY

NOVA University of Lisbon

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ABSTRACT

The yeast *Saccharomyces cerevisiae* has been used to produce fermented foods and beverages, such as beer, since the earliest civilizations. Nowadays, beer is one of the most popular drinks and the consumption of craft beer, i.e., artisanal beer prepared in small to medium-scale breweries, has increased. The constant search for innovative products has inspired brewers and scientists to experiment with new yeasts. In this study, eleven non-beer-brewing *S. cerevisiae* strains were used to investigate the use of unconventional cultures in beer production. By employing these yeasts in small-scale fermentations, we identified strains from wild and domesticated populations that revealed potential for beer production. As the industry grows and more breweries appear, it is relevant to implement good microbial practices for quality control and assessment. Here, we developed protocols to easily and accurately detect diastatic yeasts, a common contaminant in the craft beer industry. Due to the ability to break down complex sugars and consequently to attenuate beer and to produce CO_2 inside bottles and cans, diastatic yeasts represent a big threat to brewers, and therefore there is a strong demand for affordable detection methods. In this project, a PCR cycle was optimized to detect the gene responsible for the diastase ability, *STA1* and its promoter and a growth-based method with starch was developed. Analyses were performed in samples from a brewery to detect diastatic strains. Moreover, we aimed to study diastatic strains with respect to their ability to grow in beer and produce CO_2. We found that when compared to commercial beer and wine-brewing yeasts, the diastatic and beer-brewing yeasts were both able to grow and produce CO_2, whereas wine strains grew in beer without producing gas. Finally, we studied the resistance of a diastatic yeast to high temperatures, revealing that these strains are more resistant than beer or wine-brewing yeasts.

Keywords: *Saccharomyces cerevisiae,* craft beer, beer microbiology, diastatic yeasts, beer contamination, molecular detection methods

RESUMO

A levedura *Saccharomyces cerevisiae* tem sido utilizada para produzir alimentos e bebidas fermentados, como a cerveja, desde as primeiras civilizações. Hoje em dia, a cerveja é das bebidas alcoólicas mais populares estando em crescimento o consumo de cerveja artesanal, produzida em cervejarias de pequena e média dimensão tem aumentado. A busca contínua de produtos inovadores tem inspirado cervejeiros e cientistas a testarem novas leveduras. Neste estudo, onze estirpes de *S. cerevisiae* não-cervejeiras foram usadas para investigar o uso de culturas não-convencionais na produção de cerveja. Ao utilizar estas leveduras em fermentações de pequena escala, identificámos estirpes de populações selvagens e domesticadas que revelaram potencial cervejeiro. A par com o crescimento desta indústria torna-se relevante implementar boas práticas para controlo e avaliação da qualidade microbiológica. Nesse sentido, foram desenvolvidos protocolos para detetar com precisão leveduras diastáticas, um contaminante comum nesta indústria. Devido à capacidade de degradar oligossacáridos e, consequentemente, atenuar a cerveja e produzir CO_2 dentro de garrafas e latas, as leveduras diastáticas representam uma grande ameaça para os cervejeiros, havendo uma grande procura por métodos de deteção acessíveis. Neste projeto, foi otimizada uma reação de PCR para detetar o gene *STA1,* responsável pela capacidade diastática, e o seu promotor e também um método fenotípico. Foram realizadas análises em amostras de uma cervejaria para detetar estirpes diastáticas. Além disso, estudámos estirpes diastáticas no que diz respeito à sua capacidade de crescer em cerveja e produzir CO_2. Descobrimos que, quando comparadas com leveduras comerciais cervejeiras e vínicas, as leveduras diastáticas e cervejeiras são capazes de crescer e produzir CO2, enquanto as estirpes de vinho crescem em cerveja sem produzir gás. Por fim, estudámos a resistência de uma levedura diastática a altas temperaturas, observando que essas estirpes são mais resistentes do que as leveduras de cerveja ou vinho.

Palavras chave: *Saccharomyces cerevisiae*, cerveja artesanal, microbiologia da cerveja, leveduras diastáticas, contaminação de cerveja, métodos de deteção molecular

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LIST OF ABBREVIATIONS

ABV - Alcohol by volume

APA - American Pale Ale

CFU - Colony Forming Unit

CIP - Cleaning-In-Place

HPLC - High-Performance Liquid Chromatography

IBU - International Bitterness Units

IPA - Indian Pale Ale

LAB - Lactic Acid Bacteria

MO - Mediterranean Oaks

NA - North-America

OD - Optical Density

PCR - Polymerase Chain Reaction

POF - Phenolic Off Flavor

S. - *Saccharomyces*

YGL - Yeast Genomics Lab

YNB - Yeast Nitrogen Base

1. INTRODUCTION

1.1. *Saccharomyces cerevisiae* and domestication

The genus *Saccharomyces* includes eight natural species (*S. cerevisiae, S. paradoxus, S. mikatae, S. kudriavzevii, S. arboricola, S. jurei, S. eubayanus,* and *S. uvarum*) and a few hybrid species, associated with human-made environments, like *S. pastorianus* and *S. bayanus*. (Naseeb et al., 2017; Sampaio & Gonçalves, 2017). This genus is intimately related to anthropic fermentative environments (Sampaio & Gonçalves, 2017).

Amongst the various species in this genus, *S. cerevisiae* has been an essential tool to humankind due to its long history of use in food and beverage fermentation such as bread, beer, wine, sake and cachaça, with the earliest evidence of wine-like beverage production dating back to about 9000 years ago in China (McGovern et al., 2004).

In the traditional production of fermented products, some methods such as back slopping, which is the serial re-inoculation of new food or beverages with fermented material from previous batches, may have led to the genetic and phenotypic differentiation of yeast strains due to the implementation of such procedures over centuries. This adaption, also known as domestication, was most likely the result of humans unknowingly selecting the metabolic capabilities of microbes in an effort to control the characteristics of these foods and beverages (Gibbons & Rinker, 2015). Therefore, microbe domestication is defined as the unwittingly artificial selection and breeding of wild species to obtain fermented products with palatable properties (Gallone et al., 2016).

The wide use of *S. cerevisiae* in various industries has led to the emergence of genetically differentiated domesticated populations: Wine (with two clades), Bread, Sake, Beer (with two clades), and others (Pontes et al., 2020). Still, wild populations of this yeast persevere and can be found, for instance, associated with oak trees. *S. cerevisiae* found associated with oak trees in the Southern Region of Europe were shown, through population genomics studies, to belong to a distinct population, named the Mediterranean Oaks population (Almeida et al., 2015). Another wild population can be found in oak trees in Japan and North America and is therefore labeled as North America – Japan (Sampaio &

Gonçalves, 2017). Another population has been found in association with native trees in Brazil, consequently being labeled as the Wild Brazil population (Barbosa et al., 2016).

Amongst the strains associated with beer brewing environments, there can be found two domesticated clades, labeled as Beer 1 and Beer 2 (Pontes et al., 2020). Yeast strains from the Beer 1 population are distributed in three subpopulations based on their geographic locations: Belgium/Germany, Britain and United States (Gallone et al., 2016). Beer 2 clade contains isolates that originate from Eastern Europe, Belgium, the United Kingdom, the United States and Germany. Within this clade, there are also yeasts associated with beer contamination and deterioration, such as strains previously known as *Saccharomyces cerevisiae* var. *diastaticus*, and, thus, it has been recently renamed as Beer 2 – *Diastaticus* (Pontes et al., 2020).

Domesticated populations can be distinguished based on a variety of domestication signatures that differ from population to population. In the Beer 1 population, one of these signatures is the inactivation of *PAD1* and *FDC1*, two genes responsible for the decarboxylation of ferulic acid into 4- vinylguaiacol, whose presence results in a "phenolic-off-flavor" (POF) in many beers (Gonçalves et al., 2016). Another gene found consistently in the Beer population is *RTM1*, a gene belonging to the sucrose utilization locus and that provides resistance to toxic molasses (Gonçalves et al., 2016; Ness & Aigle, 1995). Moreover, much like the Wine population strains, yeasts from the Beer population show inactivation of aquaporin genes *AQY1* and *AQY2* (Gonçalves et al., 2016), a trait that increases fitness in sugar-rich environments (Will et al., 2010).

A relevant trait amongst the Beer 1 population is the ability to metabolize maltose and maltotriose, the most abundant sugars in beer wort, with concentrations of 50-60% and 15-20%, respectively. For the utilization of maltose, up to five *MAL* loci are present: *MAL1* to *MAL4* and *MAL6*. Each locus contains three genes encoding for a maltose permease (*MALx1*), a maltase (*MALx2*) and a positive regulatory protein (*MALx3*). *Malx1* transporters are repressed by the presence of glucose, thus maltose uptake can only commence once glucose decreases. Efficient consumption of maltotriose is linked with the presence of *AGT1,* a specific allele of the sugar transporter *MAL11*, known to show a high affinity for maltotriose. This allele is present in Beer 1 subpopulations and some mosaic strains (Alves et al., 2007).

1.2. History and characteristics of beer

The etymology of the word beer derives from the Latin word *bibere*, to drink, which provides little clue of how to define this beverage. The *Shorter Oxford Dictionary* describes beer as “alcoholic liquor obtained by the fermentation of malt (or other saccharin substances) flavored with hops or other bitters”. Although this is true, the word beer currently represents a large variety of beverages with broadly different appearances and flavors (Boulton & Quain, 2001). Beers can be produced from a variety of sources of fermentable sugars, may or may not contain hops and other bitters, and other flavors may be added with fruits, spices, or plant extracts (Boulton & Quain, 2001).

In a broad definition, modern beer is an alcoholic beverage made from four main ingredients: malted grain, water, hops and yeast. The origin of fermented beverages is unclear. Between 2000 and 4000 B.C., the Egyptians and Sumerians developed a process for brewing a beverage that closely resembles modern beer. Beer brewing remained mostly artisanal until the industrial revolution, with a few European countries, such as Germany, Belgium, and England taking the lead in grasping brewing practices (Rodhouse & Carbonero, 2019).

Based on the yeasts used and the conditions of brewing, there can be defined two main types of beer: ale and lager. Ales are brewed with top-fermenting yeasts, typically *S. cerevisiae*, that tend to remain in suspension in the fermentation container. As mentioned before, amongst domesticated *S. cerevisiae* yeasts, there are two clades of strains related to beer brewing: Beer 1 and Beer 2. Yeast strains from Beer 1 are used for fermentation of ale-type beers, whereas strains from the Beer 2 clade contain strains that are used to produce Saison-type beers. The fermentation of Ale beer is carried out at relatively high temperatures, from 18 to 25°C (Corran, 1975). Common Ale styles in craft breweries include American Pale Ale (APA), Wheat beers, Indian Pale Ale (IPA), American Brown Ale and Belgian Golden Ale (Rodhouse & Carbonero, 2019). On the other hand, Lager beers are brewed with bottom-fermenting yeasts belonging to *S. pastorianus* and typically have lighter and cleaner flavors when compared to Ale beers (Rodhouse & Carbonero, 2019). Lager fermentation occurs at lower temperatures, which range from 5 to 15°C (Corran, 1975). While ale-type beers represent the most ancient beer types, lagers were developed more recently and gained popularity and the largest market share since the XIX century (Meussdoerffer, 2009).

Currently, there are various styles of beer classified based on their properties, such as alcohol content, color, bitterness, clarity, flavor and ingredients. The alcohol content is typically measured in alcohol by volume (ABV) and it ranges from 3 to 14%, but the most commonly consumed styles don't surpass 6% (Rodhouse & Carbonero, 2019). Bitterness is measured in International Bitterness Units (IBU). IBU is influenced by the percentage of alpha acids in the hops, the volume produced and the boil time: a higher alpha acid hop and a longer boil will increase IBUs (Rodhouse & Carbonero, 2019).

1.3. **Beer production**

The beer production process may be separated into three main stages: wort manufacture, fermentation, and post-fermentation process, as illustrated in Figure 1.1. In each of these phases, there are several distinct steps, which can vary based on the style of beer desired (Boulton & Quain, 2001). The main raw materials for wort production are water, cereals (typically barley) and hops. Wort production starts at malting when the cereal undergoes germination, which is initiated by wetting the grains or steeping (Boulton & Quain, 2001). During germination, enzyme systems to transform starch reserves into fermentable sugars are activated. The final step of malting is kilning, which consists in drying the grains with a gradual application of heat. A variation of kilning is sometimes described as roasting, where higher temperatures are used.

The resulting product of the malting stage is milled and/or crushed, often on-site by craft brewers and mixed with hot water, in a process known as mashing. During mashing, the malt content is solubilized by the enzymes released during the malting stage. By manipulating the temperature of mashing, the brewer can influence the composition of the malt extract (Willaert, 2006). After the sugars are made available, the grains are separated from the sweet liquid, known as wort, through sparging. Often, hot water is sprayed on the grains to extract all the dissolved substances.

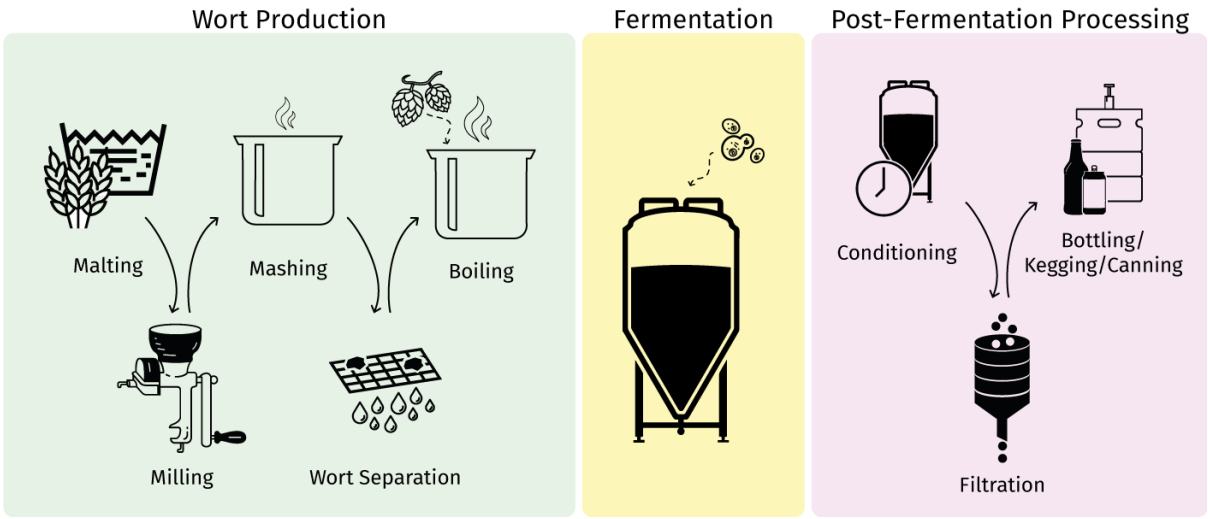

Figure 1.1. Schematic representation of the beer production process.

The wort is then boiled at temperatures between 103 and 110°C for approximately one hour (Rodhouse & Carbonero, 2019). The boiling stage occurs in order to isomerize the hops, clot proteins for easy removal, concentrate the liquid, enhance color and flavor and drive off sulfur compounds that lead to unwanted flavors and aromas (Willaert, 2006). Hops are conical flowers of *Humulus lupulus*, a member of the Cannabaceae family and can be used in beer brewing to confer both bitterness and floral character. The bitterness of the hops is due to the iso-α-acids. These compounds also confer antiseptic properties. The α-acid content varies from 2 to 15% of the hop weight (Boulton & Quain, 2001). Hops are added during the boil at different times, according to the type of hop used and the desired flavor and aroma.

For the fermentation stage, the cooled wort is transferred to fermentation tanks, where the yeast is added (pitching) to break down the sugars and polysaccharides and produce ethanol, CO_2 and other metabolites that contribute to the final flavor and/or aroma. Fermentation lasts approximately one week. Contrary to industrial production, craft brewers often re-pitch yeast, using the same yeast from one batch to ferment another. Re-pitching is usually limited to 10 times to avoid quality degradation (Rodhouse & Carbonero, 2019).

After fermentation, the beer goes through maturation or conditioning, where beer is stored at lower temperatures to promote stabilization. The beer is finally filtered, carbonated and bottled, kegged or canned (Rodhouse & Carbonero, 2019).

1.3.1. Recent advances in brewing innovation

Yeasts are an essential ingredient in beer brewing and have a direct impact on the quality and profile of the beverage, with different strains and species being used for distinct beer styles and types (Cubillos et al., 2019). In ale brewing, the most widely used yeasts belong to *S. cerevisiae*, but there has been an increase of interest in new strategies to create and explore new beer styles.

Due to their potential for flavor production, some non-*Saccharomyces* yeasts have been evaluated in beer fermentation (Bourbon-Melo et al., 2020; Cubillos et al., 2019; Gibson et al., 2017; Holt et al., 2018; Nikulin et al., 2020). These yeasts may not have the ability to ferment maltose and maltotriose, which can be seen as an advantage for the production of low-alcohol beer (Gibson et al., 2017). Nevertheless, mixed fermentation may be performed with both *S. cerevisiae* and non-*Saccharomyces* yeasts (Bourbon-Melo et al., 2020).

Besides the use of wild yeasts for fermentation, approaches like laboratory adaptation and artificial hybridization have been explored to increase maltose and maltotriose assimilation and flocculation (Gibson et al., 2020). There has even been an interest in repurposing strains from other industries, with strains isolated from the baking industry (Gibson et al., 2020).

1.3.2. Sources of microbiological contamination in the brewery

Breweries, especially craft breweries, are vulnerable to contaminations at numerous stages of beer manufacture, as described by Vaughan et al, 2005, which comes to show the need for the implementation of proper systems for quality assessment and control. Small breweries, due to their tendency to experiment with different strains, less strict quality control and rare beer pasteurization are most susceptible to contamination (Krogerus & Gibson, 2020).

The demand for newer and innovative products leads to the use of fermentation equipment for different beer styles and, consequently, different yeasts. This increases the risk of cross-contamination and the need for proper and strict cleaning systems (Davies et al., 2015).

Contaminants may be categorized as primary or secondary contaminants. Primary contaminants originate from the raw materials and the machinery used in the brewery, whereas secondary contaminants are introduced during bottling, kegging, or canning. About half of the documented contaminants are described as secondary contaminants. However, primary contaminants represent a bigger threat, with the potential loss of a complete batch (Vaughan et al., 2005).

The spoilage character of an organism depends on the stage at which it is found. For example, the yeast pitched for fermentation is seen as a contaminant if detected after filtration (Vaughan et al., 2005).

The first step in controlling contaminations in a brewery is to select and acquire raw materials that carry low or harmless microbial loads. During the beer production process, most raw materials are met with microbiological safeguards, such as elevated temperatures during mashing and wort boiling,

filtration of beer and storage of yeast and beer at low temperatures (Vaughan et al., 2005). It is also important to acquire brewing equipment designed to eliminate contaminations, with pipework that facilitates cleaning and materials without crevices and imperfection that promote the formation of biofilms (Davies et al., 2015). One of the most vital aspects of working in a brewery is maintaining all equipment in prime condition, with regular cleaning with approved detergents (Vaughan et al., 2005).

During wort manufacturing, the high temperatures of boiling should drastically reduce the amount of microorganisms present. However, after boiling, the wort is cooled and the high sugar content might lead to an increase of contaminants (Rodhouse & Carbonero, 2019). Contaminated wort can lead to a lowered fermentation rate, production of off-flavors/aromas, and haze (Hill, 2015). Fermentation conditions are perfect for the growth of contaminants, which increases the importance of wort and yeast analysis.

Hops, which are added during wort boiling, are used in beer brewing mainly for their antibacterial properties, which are due to the bitter and aromatic components that are transferred into the wort during boiling. Studies show that these hop compounds inhibit the growth of Gram-positive bacteria (Vaughan et al., 2005).

Secondary contamination usually occurs in the filling department with the introduction of airborne contaminants to the beer. This represents a great menace in breweries that do not perform pasteurization. Equipment used in this area is particularly prone to the formation of biofilms, which contributes to the persistence of contaminants (Vaughan et al., 2005). According to a 2017 survey, contaminations in the bottling area were the most common (Meier-Dörnberg et al, 2017).

Overall, the brewing process in itself is in some ways inhospitable to many microorganisms. The final product, beer, is considered microbiologically stable, due to the presence of ethanol, hop bitter compounds, CO_2, reduced O_2 and a pH ranging from 3.8 to 4.7. However, some microorganisms are able to withstand such conditions (Davies et al., 2015).

Breweries have adopted a Cleaning-In-Place (CIP) method, that allows for easy cleaning without dismantling the pipes and vessels (Davies et al., 2015). This system has three categories of procedures: mechanical, chemical and sanitization. The mechanical portion of cleaning can be done through turbulence or scouring. A CIP cycle usually includes a pre-rinse with water, usage of a hot caustic wash (2-3% caustic wash at 75-80°C), an acid wash and a final wash with deionized water to remove residual detergents (Davies et al., 2015).

1.4. **Diastatic strains of *S. cerevisiae* in the brewing industry**

Diastatic strains of *S. cerevisiae* are described as super-attenuating yeasts, due to their ability to hydrolyze residual carbohydrates in beer, such as soluble starch and dextrins, which are mixtures of D-glucose polymers derived from starch (Burns et al., 2020). Dextrins account for 10-20% of the total saccharides content in beer wort, an amount similar to glucose or maltotriose (Štulíková et al., 2021).

Although diastatic yeasts might be employed for the production of lighter beers, such as Saison (Krogerus & Gibson, 2020), Belgian Golden Strong and Biere de Garde (Burns et al., 2020), they can also be classified as a contaminant. These yeasts may be a primary contaminant and compete with the brewing yeast in the fermentation vessels or a secondary contaminant that occurs during the bottling process (Meier-Dörnberg, Kory, Jacob, Michel, & Hutzler, 2018).

Due to their ability to break down complex sugars, diastatic strains have a competitive advantage in dextrin and starch-rich environments where fermentable carbon sources concentrations are low when compared to other brewing yeasts. Fermented beer is a perfect example of such media and diastatic strains, unlike ale or lager strains, can grow in beer and produce CO_2 (Krogerus et al., 2019; Meier-Dörnberg et al., 2018). Therefore, contamination with diastatic strains leads to refermentation, resulting in undesired super-attenuated beer, an increase in CO_2 and ethanol production, drier mouthfeel and production of off-flavors. In more severe situations, the production of CO_2 in the bottle translates into gushing (overflowing of beer upon opening the bottle or can) or even into bottle explosion (Burns et al., 2020; Krogerus & Gibson, 2020). Thus, contamination with diastatic yeasts can translate into a financial burden and even expose the consumer to the risk of injury (Meier-Dörnberg et al., 2018). Depending on the diastatic yeast and its spoilage potential, it might take some time to detect the contamination visually or through sensory analysis (Meier-Dörnberg et al., 2018).

The ability of these yeasts to hydrolyze larger carbohydrates is due to the production of an extracellular glucoamylase, encoded by the *STA1* gene. This enzyme breaks dextrins down into individual glucose molecules, which can then be consumed by any yeast present (Burns et al., 2020).

Besides the hydrolysis of starch and dextrin, diastatic strains appear to also employ the Sta1p glucoamylase for maltotriose consumption, using this enzyme to hydrolyze the sugar in the extracellular environment. Strains from Beer 1 rely on the Agt1p permease to transport maltotriose inside the cell for hydrolysis (Burns et al., 2020).

Given that maltotriose is rarely present in other fermentations, maltotriose consumption is often seen as a signature of beer domestication (Gallone et al., 2016; Gonçalves et al., 2016). Likewise, the formation of *STA1* appears to be an alternate evolutionary mechanism for efficient usage of the sugars present in beer wort (Burns et al., 2020). Moreover, diastatic strains may have been unintentionally selected by brewers to produce beers with a drier mouthfeel, such as Saison.

STA1 is a chimeric gene resulting from the fusion of *FLO11* and *SGA1*, located on opposite ends of chromosome IX (Krogerus & Gibson, 2020). The 3' end of *STA1* is homologous to *SGA1*, which encodes an intracellular glucoamylase used during sporulation; whereas the 5' end and the upstream region are homologous to *FLO11*, a gene that encodes a membrane-bound flocculin. The peptide derived from *FLO11* enables the secretion of the *STA1* glucoamylase (Adam et al., 2004).

The *STA1* promoter contains at least two segments controlling expression: UAS1 and UAS2, both of which can be divided into an upstream activating sequence (UAS) and upstream repressing sequence (URS) (Kim et al., 2004; Krogerus & Gibson, 2020), as seen in Figure 1.2. Each of these four segments contains transcription-binding sites.

The repressors Nrg1 and Sfl1 bind to URS1-1 and URS2-2, respectively. On the other hand, activators Mss11 and Flo8 bind to UAS1-2, while activators Ste12 and Tec1 bind to UAS2-1 (Kim et al., 2004).

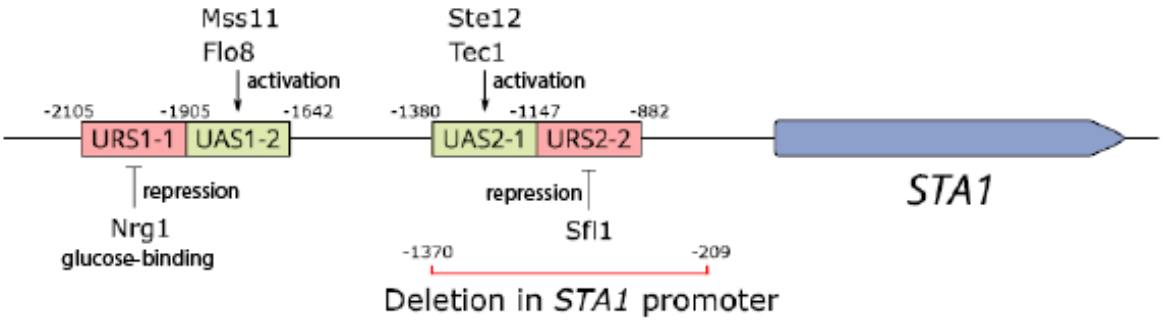

Figure 1.2. Structure of the *STA1* gene promoter. Adapted from Krogerus and Gibson 2020.

Kim et al., 2004 have shown that the presence of glucose represses the transcription of the *STA1* gene, which appears to be partially mediated by increased levels of the Ngr1 and Sfl1 repressors originated from growth on glucose (Kim et al., 2004). However, it has been shown in previous studies that glucose does not completely inhibit *STA1* expression (Burns et al., 2020). Moreover, other studies have shown that during fermentation with *STA1+* yeasts, a low concentration of glucose is always present, due to the breakdown of dextrin and starch (Krogerus et al., 2019). This continuous source of glucose may affect the overall fermentation, as many fermentation-related genes, such as the ones involved in the assimilation of maltose and maltotriose, are repressed by glucose (Day, Rogers, Dawes, & Higgins, 2002).

In 2019, Krogerus et al. showed, through screening of multiple *STA1+ S. cerevisiae* strains, that some of these strains may have an 1162-bp deletion within the *STA1* promoter (Krogerus, et al., 2019). The same study revealed that this deletion significantly decreased the expression of *STA1* and the ability to grow on beer and break down dextrin. Consequently, not all *STA1+* strains have the same spoilage potential.

1.5. **Objectives**

This project is divided into two themes: innovation of craft beer production and quality assessment, especially in what concerns diastatic strains of *S. cerevisiae*. With respect to the first topic, we aim to explore the utilization of non-conventional *S. cerevisiae* strains in beer production. As such, our objective was to: (i) perform laboratory-scale fermentations with wild yeasts previously selected at the Yeast Genomics Lab, (ii) compare the brewing potential of wild yeasts before and after being submitted to an adaptation experiment conducted at the YGL. In the context of the quality assessment part, we aimed at (iii) developing a method for the detection of diastatic yeasts, (iv) applying this method to a

real-life situation; and (v) comparing the beer deterioration potential of diastatic and non-diastatic yeasts.

2. METHODS

2.1. *Saccharomyces cerevisiae* strains

The strains used throughout this project belong to the Portuguese Yeast Culture Collection or the Yeast Genomics Lab@NOVA yeast collection and were conserved at -80°C. The strains were cultivated and kept in YMA plates (peptone 0.5% (w/v), yeast extract 0.3% (w/v), malt extract 0.3% (w/v), glucose 1% (w/v), agar 2% (w/v)).

Table 2.1. List of strains used in this project with their respective phylogenetic group, the substrate of isolation and geographic location. Highlighted in blue are the strains used in the adaptation to beer wort. The strains marked with  were employed in fermentations trials.

Phylogenetic Group	Strain	Substrate of isolation	Geographic Location
Wine 2 strains	PYCC 6726 	Jerez-wine	Spain
	EC 1118	Industrial strain isolated from champagne	France
Mediterranean Oaks 5 strains	ZP 560 	Q. pyrenaica	Castelo de Vide, Portugal
	ZP 541 	Fagus sylvatica	Adagoi, Portugal
	ZP 1008 	Soil underneath Q. cerris	Riserva Naturale Luccio-labella/Chianciano
	ZP 736 	Rotten figs	Caratão, Abrantes, Portugal
	ZP 742 	Rotten figs	Caratão, Abrantes, Portugal
Mosaic 1 strain	SON4c 	Ficus carica	Sonim, Portugal
	DBS 12 	Ficus carica	Halkidiki, Greece
NA/Japan 1 strain	ZP 779 	Q. acutissima	Hirusen highland, Okayma, Japan
Beer 1 3 strains	PYCC 4455	Brewer's Stock Yeast	Rotterdam
	Fosters B	Brewing (Ale)	Commercial
	US-05 	Brewing (Ale)	Commercial

Beer 2 **4 strains**	PYCC 2608	Spoiled Beer	-
	TUM PI-BA-31	Spoilage yeast isolated from brewery	Germany, State Rhineland Palatinate
	TUM-PI-BB-105	Spoilage yeast isolated from beer-mixed beverage	Unknown
	TUM 1-B-8	Spoilage yeast Isolated from a brewery	Bavaria, Germany

2.2. Craft beer production with non-conventional *S. cerevisiae* strains

The protocols in this section were adapted from Bourbon-Melo et al. 2021.

2.2.1. Preparation of malt extract beer wort

To carry out the beer production at a laboratory scale, a malt extract medium was prepared. To prepare this medium, a solution containing 100 g/L of light dried malt extract was added to a Brewferm electric brew kettle and boiled. After approximately 1 hour, 1 g/L of Lubelski 8,8% hops were added and the medium was boiled for another hour to promote the isomerization of alpha-acids. After production, the medium was distributed into the fermentation vessels and cooled before inoculation. The resulting wort had an IBU of 27.1 (Rager calculation), a pH of 6.03, and a °Brix of 11.

2.2.2. Pilot trials for beer production

All strains were precultured in 100 mL of YPD medium in 250 mL Erlenmeyer flasks and incubated overnight at 25°C with agitation (130 r.p.m.). The cells were then washed twice with sterile water and counted with a Neubauer counting-chamber. 2 x 10^6 cells/mL were inoculated in 1 liter of beer wort and incubated for 28 days at 18°C. Fermentation was monitored 3 times a week and samples were gathered for HPLC analysis every 7 days for sugar assimilation and glycerol and ethanol formation. This analysis was done using a 300x7,8 mm Aminex HPX-87H, BIO-RAD® column (300 x 7.8 mm Aminex HPX-87H, BIO-RAD®) and a differential refractometer (LKB 2142). The column was kept at 65 ºC, and 5 mM of H_2SO_4 was used as a mobile phase at 0.6 $mL.min^{-1}$.

pH was determined before and after fermentation. After 28 days of incubation, the resulting beer was kept at 4°C for 12 days, after which sensory trials were performed with a group of 3 experienced brewers and 2 brewery interns.

2.3. **Molecular and growth-based detection methods for diastatic strains of *S. cerevisiae***

2.3.1. Multiplex PCR

A multiplex polymerase chain reaction (PCR) cycle with Taq DNA Polymerase was developed to detect both the *STA1* gene and its promoter using the previously published primers *STA1*_UAS_Fw and *STA1*_UAS_Rv (Krogerus et al., 2019), which amplify a 599-bp fragment and the primers STA_RT_2_FW and STA_RT_2_RV, which amplify a 399-bp fragment. Previously studied strains were used as control.

The PCR cycle used was: 95°C 5 min, (95°C 20 sec, 55°C 30 sec, 72°C 20 sec) x 29 cycles, 72°C 2 min. PCR products were separated and visualized on 2% agarose gels.

Table 2.2. Primers used in molecular detection of diastatic strains of *S. cerevisiae*.

Name	Sequence (5' > 3')	Reference
STA_RT_2_FW	CTCCAACTTCATCAGTCACTACG	Ana Pontes, YGL
STA_RT_2_RV	GTCCCATTCATCAAGACTACATCC	
***STA1*_UAS_Fw**	CCTGGCTCAAATTAAACTTTCG	(Krogerus et al., 2019)
***STA1*_UAS_Rv**	ACCACCAATAGGCAATAGAAA	

2.3.2. Starch agar medium

In order to develop an easy-to-replicate method to detect diastatic strains of *S. cerevisiae*, a starch medium was produced with YNB (Formedium™) supplemented with 0,5% (w/v) of soluble starch and 2% (w/v) agar. The medium was distributed in small plates, cooled at room temperature overnight and stored at 4°C.

The plates were inoculated with 20 µL of a suspension of 1 x 10^7 cells/mL, sealed with parafilm and incubated at 25°C for 8 days. The plates were then revealed with a Lugol solution.

2.3.3. Liquid starch medium

The medium used in this experiment was created with YNB (Formedium™) and supplemented with 0,5% (w/v) of soluble starch. The medium was kept at 4°C.

To determine the phenotype of the yeasts, the medium was inoculated with 100 µL of a suspension of 1 x 10^6 cells/mL and incubated overnight at 25°C with agitation (70 r.p.m.) for 8 days. After this period, growth was analyzed optically and the medium was revealed with a Lugol solution.

2.4. Analysis of beer samples from a local brewery

The procedure for this analysis was adapted and developed throughout time, following the advances in the detection methods mentioned in 2.3.

Samples were analyzed at least one week after bottling/kegging to ensure the growth of yeasts, as the detection limits of the methods applied haven't been determined. When analyzing the samples, the bottles were opened in a sterile environment and observed for the occurrence of gushing. °Brix and pH. Cell count was determined through CFUs in a YMA plate. 10% of the colonies found were then separated and analyzed through PCR as described in 2.3.1. To confirm the phenotype of the sampled yeasts, a growth-based method was applied as described in 2.3.2.

Some of the batches were analyzed after 5 to 6 weeks and 9 to 10 weeks. These analyses were performed in case of an absence of diastatic yeasts in a first analysis, to confirm whether these yeasts were truly absent or below the detection limit of this protocol.

2.5. Comparative behavior of diastatic and non-diastatic strains of *S. cerevisiae*

2.5.1. Yeast growth in industrial lager beer

The yeasts were precultured overnight in 20 mL of beer wort acquired at a local brewery in a 50 mL Erlenmeyer flask at 25°C with agitation (150 r.p.m.). The cells were washed twice with sterile water and resuspended in industrial lager beer. The concentration of the suspension was determined with a cell-counting chamber and a 2 mL suspension was created with a concentration of 1×10^2 cells/mL.

Using this suspension, 0, 1, 2 and 5 cells were inoculated 5 times in closed Falcon tubes containing 45 mL of industrial lager beer. To ensure the accuracy of the inoculation, the same volumes were plated in 6 YMA plates (peptone 0.5% (w/v), yeast extract 0.3% (w/v), malt extract 0.3% (w/v), glucose 1% (w/v), agar 2% (w/v)). The inoculated beer was incubated at 20°C for 2 months.

The cell growth and °Brix were measured after 1, 2, 3, 4 and 8 weeks. For that, the sample was agitated and 100 mL were colected. Sugar assimilation and glycerol and ethanol production were analyzed after 4 weeks by HPLC, as described in 2.2.2.

2.5.2. Modified Durham tube test with industrial lager beer to determine gas-forming potential

This protocol was adapted from Meier-Dörnberg et al. 2018. The Durham tubes were mounted, filled with 2.7 and 3 mL of industrial lager beer and autoclaved at 121°C for 5 minutes. The yeasts were precultured overnight in 20 mL of the beer wort produced in 2.2.1. in a 50 mL Erlenmeyer flask at 25°C with agitation (150 r.p.m., Sartorius™ CERTOMAT™). The cells were washed twice with sterile water

and resuspended in sterile water. The concentration of the suspension was determined with a cell-counting chamber and a 1.5 mL suspension with a concentration of 3.0 x 10^7 cells/mL was created. To pitch a yeast cell amount of 3.0 x 10^6, 300 µL of the suspension were inoculated into the tubes.

A second suspension of 1,5 mL with a concentration of 1 x 10^2 cells/mL was made to pitch a cell amount of 2 and 5 in the tubes with 3 mL of lager beer.

The tubes were incubated at 20°C for 13 days and observed for the accumulation of gas after 2, 5, 6, 7, 8, 9, 12 and 13 days.

2.5.3. Temperature resistance in industrial lager beer

This protocol was adapted from Suiker et al. 2021. The yeasts were precultured in 20 mL of industrial lager beer in a 50 mL Erlenmeyer flask at 25°C with agitation (150 r.p.m.) overnight. The cell concentration was determined by hemocytometer and 3x10^3 cells/mL were inoculated in 20 mL of wort or industrial lager beer in a 100 mL Erlenmeyer flask.

The resulting culture was then incubated in a shaking water bath at 55°C. Samples were taken and inoculated in YMA plates to determine CFUs at 0, 1, 3, 5, 7, 10, 15 and 20 minutes. D_{55}-values, which is the time needed at 55°C to kill 90% of the population, were determined.

3. RESULTS AND DISCUSSION

3.1. Laboratory-scale fermentations: craft beer production with unconventional *S. cerevisiae* yeasts

Eleven non-conventional beer brewing strains were submitted to fermentation trials in 1L of beer wort. These strains were selected due to the potential revealed in preliminary tests performed at the Yeast Genomics Lab. Two of these strains had been previously submitted to adaptation to beer wort and the adapted yeasts were also employed. A beer brewing commercial strain, US-05, was used as control. The beer wort employed in these fermentations had a °Brix value of 11.0, with the sugar composition is depicted in Table 3.1.

Table 3.1. Composition of the beer wort used in laboratory-scale fermentations.

Sugar	Amount (g/L)
Maltotriose	12.37
Maltose	51.05
Glucose	8.03
Fructose	1.48

Fermentations were carried out for 28 days and sugar consumption was monitored through °Brix measurements and HPLC analyses. °Brix values at the end of the fermentations varied from 5.4 to 10.1 and pH values ranged from 3.88 to 4.85. The °Brix values after 28 days of fermentation can be grouped by yeast population, as shown in Figure 3.1. The domesticated strain PYCC 6726 and the control US-05, showed the highest sugar consumption, reaching °Brix values of 5.0 and 5.4, respectively. Despite both being a domesticated strain, PYCC 6726 is not adapted to beer-related environments. Wine must and beer wort are very distinct substrates, as wine must does not contain maltose or maltotriose and has much higher levels of glucose and fructose (Berthels et al., 2004). However, PYCC 6726 revealed a good ability to consume maltose and maltotriose, as shown in Figure 3.2. Although the final °Brix values of the beer produced by these two strains were similar, PYCC 6726 and US-05 have different

consumption rates. After 7 days of fermentation, PYCC 6726 had consumed 83% and 64% of maltotriose and maltose, respectively. On the other hand, US-05 consumed 59% and 81% of the same sugars.

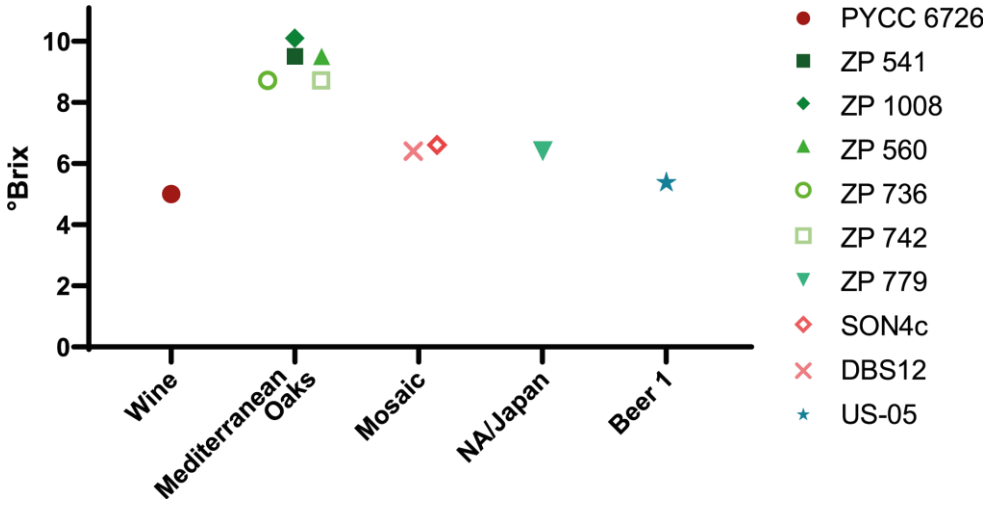

Figure 3.1. Brix values after 28 days of fermentation grouped by phylogenetic group. Adapted strains employed in these trials are not depicted.

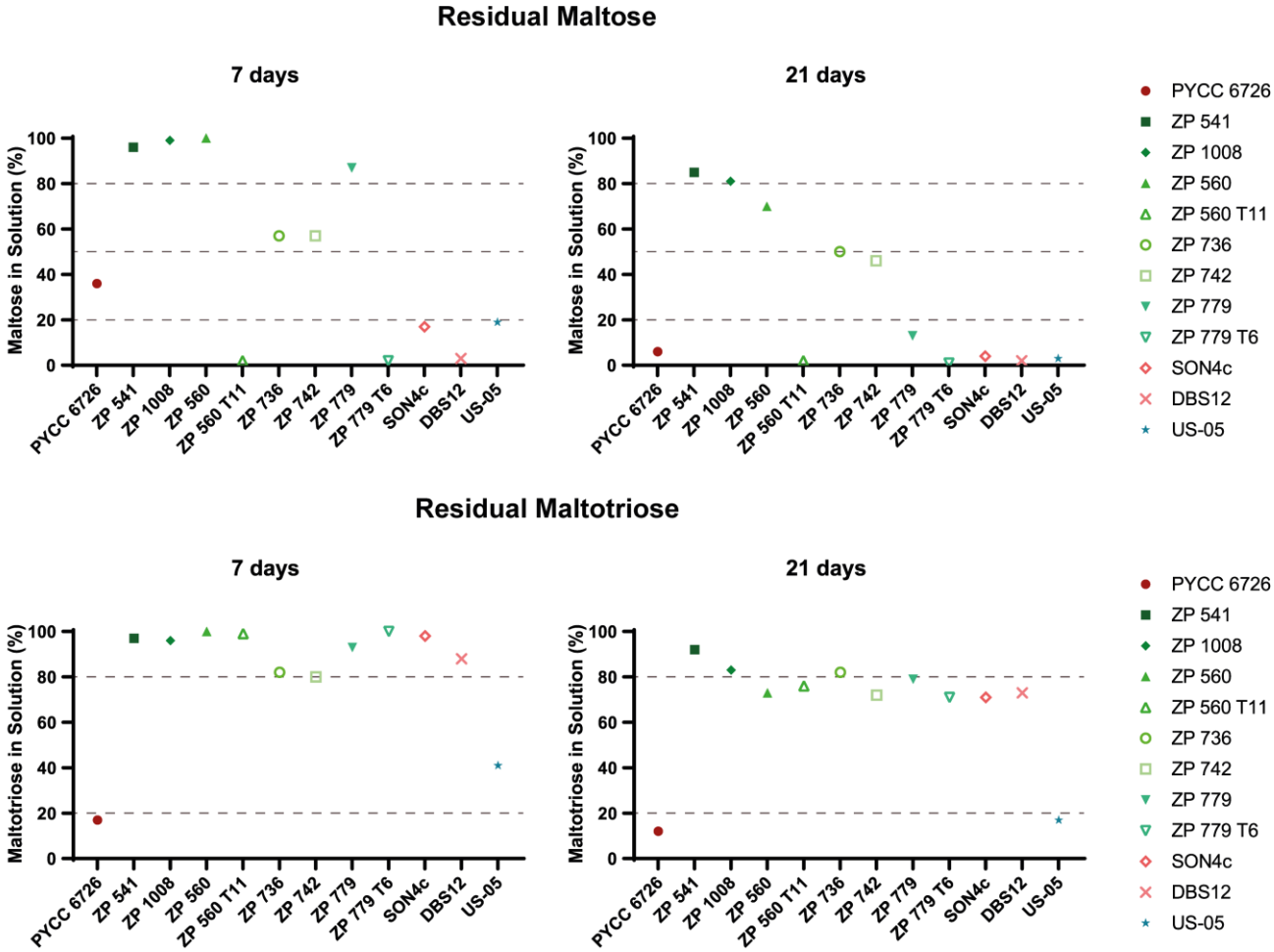

Figure 3.2. Residual maltose and maltotriose percentages present in the fermentation vials after 7 and 21 days of fermentation. These results were obtained through HPLC, with an initial amount of 51.05 and 12.37 g/L of maltose and maltotriose, respectively. ZP 760 T11 and ZP 779 T6 are the products of an adaptation to beer wort performed at the YGL with the strains ZP 560 and ZP 779, respectively.

Amongst the Mediterranean Oaks population (MO), the final °Brix values range from 8.7 to 10.1, with the two lowest values belonging to ZP 736 and ZP 742. These two strains were isolated from rotten figs, whereas the other three from this phylogeny were isolated from wild oaks and European beech, which might justify the different abilities displayed by these strains. In Figure 3.2, it is clear how distinct ZP 736 and ZP 742 are from the remaining MO strains, seeing they were able to consume approximately 50% of the maltose present.

The mosaic strains used in these fermentations, SON4c and DBS12 were capable of consuming maltose, but not maltotriose, as did ZP 779, a strain from the North-America/Japan (NA/Japan) population. However, ZP 779 requires more time to consume maltose, as seen in Figure 3.2, as 87% of the maltose was still present after 7 days.

As mentioned above, two of the strains selected for these laboratory-scale fermentations, ZP 560 and ZP 779, were adapted to beer wort through a series of re-inoculation of yeast in fresh wort every two to three months. This experiment has been occurring in the Yeast Genomics Lab since 2018 and has shown progress, with Francisca Paraíso reporting that the yeasts have improved their ability to assimilate maltose in her Master's dissertation. As such, adapted populations of these yeasts were added to this project to evaluate their beer-brewing potential. The adapted yeasts were named ZP 779 T6 and ZP 560 T11, where the 'T' represents the number of re-inoculations performed. In Figure 3.2 it is possible to observe the differences between the wild yeast and the adapted yeast in the consumption rates of maltose. After 7 days, both ZP 779 T6 and ZP 560 T11 had consumed 98% of the wort's maltose, whereas the wild yeasts had consumed 13% and 0%, respectively. When it comes to the consumption of maltotriose, neither the wild yeasts nor the adapted yeasts showed to be proficient.

Besides evaluating differences in these yeasts' ability to ferment the sugars present in beer wort, this project aimed at finding flavors and/or aromas that yeasts from different populations could add to beer. To allow for easier detection of the characteristics brought by the yeasts, the wort used in the fermentations was neutral and the commercial yeast US-05 was used as control.

The sensory trials were performed by three experienced brewers and two interns. The beer produced with US-05, which is not depicted in any figures related to the sensory analysis, was the first submitted to the trials to identify any flavors and/or aromas that originated from the beer wort or the fermentation conditions. This beer was very neutral in terms of sweetness or acidity, but it did contain a light aroma of acetaldehyde, an intermediate compound in the conversion of glucose to ethanol. Acetaldehyde is a volatile compound, thus its presence might be due to a poor maturation of the beer or excessive oxygen supply, which can increase levels of acetaldehyde (Olaniran et al., 2017). Due to the detection of this compound in the beer fermented with US-05, its presence can be attributed to an issue with the fermentation and not an intrinsic characteristic of the yeasts.

Similar to what was observed with the sugar consumption capabilities of these 11 strains, the sensory analysis of the beer produced by each of them was coherent within phylogenetic groups, as shown in Figure 3.3.

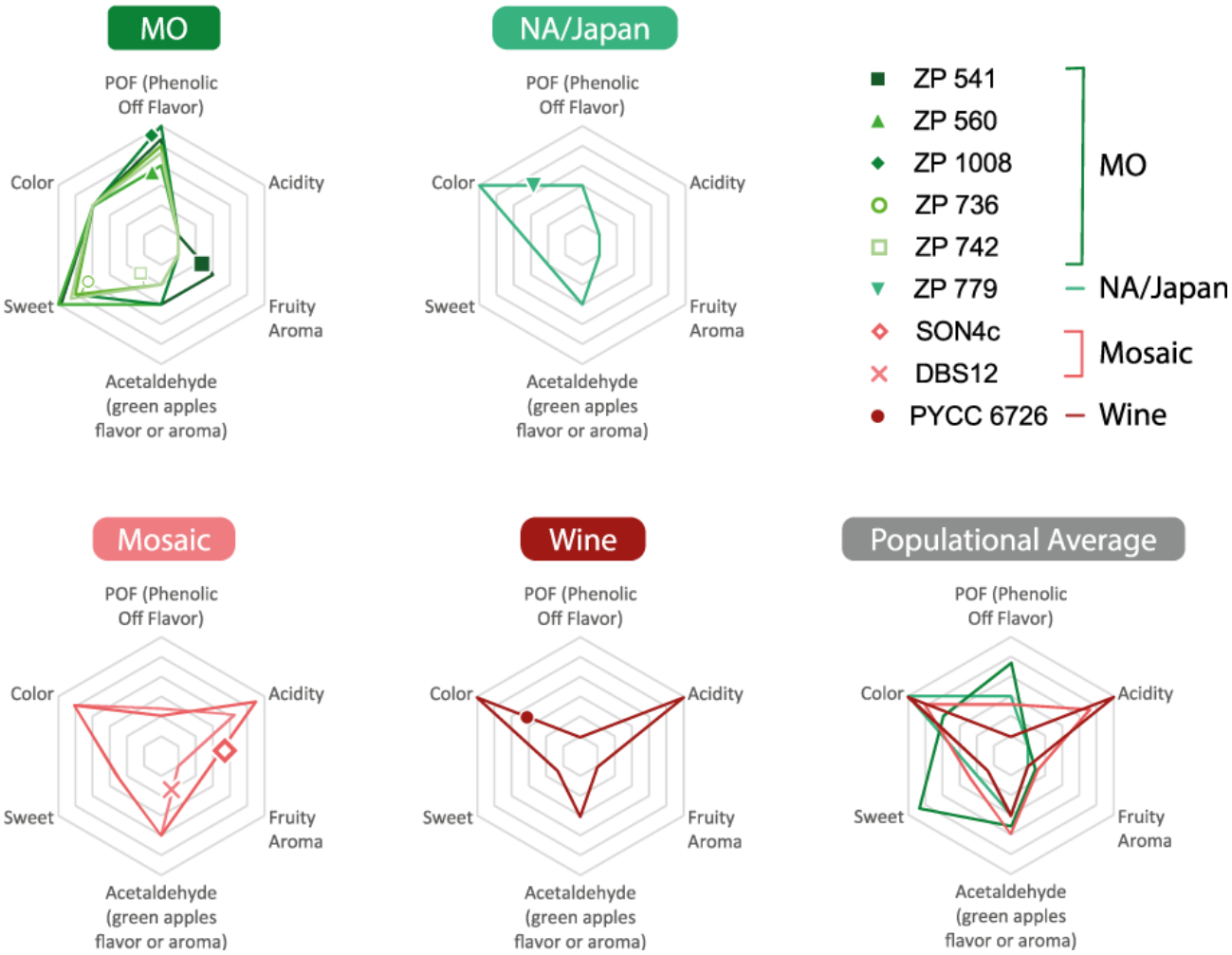

Figure 3.3. Sensory characteristics of the beers obtained in this project. The information depicted in this image was obtained with experienced brewers. Adapted strains employed in these trials are not depicted.

Strains from the MO population produced beers with similar characteristics. These 5 beers were described as sweet, as predicted through the °Brix measurements. The presence of phenolic off-flavors (POFs) was detected in all of these beers, although it was less prominent with the strain ZP 560. The production of 4- vinylguaiacol, the compound responsible for POFs, has been seen before in studies with wild yeasts (Nikulin et al., 2020). Amongst this population, ZP 541 was able to stand out due to the presence of a fruity aroma. However, due to the exaggerated sweetness and the POFs detected, none of the beers produced with these five strains was considered pleasant.

The other wild population included in this study was the North America/Japan population, represented by ZP 779. As seen when analyzing the wort's sugar consumption, this strain reached a lower °Brix value than the MO strains, resulting in a less sweet beer. The beer produced with this strain presented a good color, low acidity and the presence of POFs.

The mosaic strains, DBS12 and SON4c, produced acidic beers with lower POF levels when compared to the MO strains. The beer fermented with DBS12 presented a more neutral profile compared to the beer fermented with SON4c. Both beers were considered interesting with a potential to be used to produce sour beers, which are typically brewed in mixed fermentations with *S. cerevisiae* and

lactic acid bacteria (LAB) (Dysvik et al., 2019). Moreover, some non-*Saccharomyces* yeasts were studied as potential LAB alternatives (Osburn et al., 2018).

PYCC 6726, which showed an efficient consumption of the beer wort's sugars, produced a very distinct beer, with no POFs, pleasant color and very high acidity. Of all the beers submitted to sensory analysis, the beer produced with the Wine strain was the favorite, with a unique flavor and aroma.

Besides evaluating the characteristics of beer produced with yeasts from different populations, the sensory trials performed also aimed at finding differences and new characteristics that resulted from the adaptation to beer wort that two of the yeasts were submitted to. In Figure 3.4, it becomes clear that the adaptation to beer wort translates into more than an increase in sugar consumption rates.

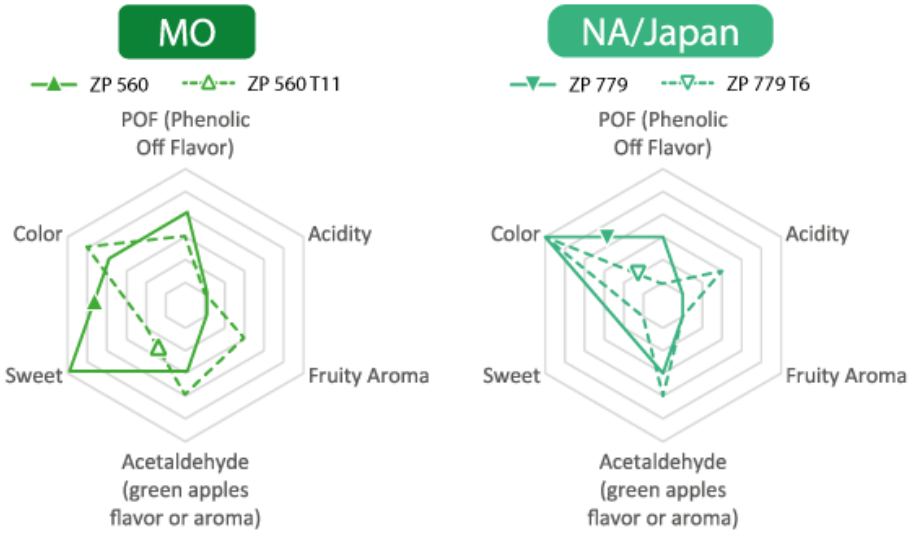

Figure 3.4. Sensory evaluation results for the fermentations performed with ZP 560, ZP 560 T11, ZP 779 and ZP 779 T6.

ZP 560 T11, besides losing the exaggerated sweetness that was identified with ZP 560, produced a fruity aroma and fewer POFs. However, the beer produced with ZP 560 T11 was oxidized, which resulted in a papery-like flavor. This issue usually appears when there's an excess of oxygen supply during the fermentation, thus this characteristic should not be assigned to the yeast.

ZP 779 T6 produced a beer with fewer POFs and more acidity than ZP 779. For this reason, ZP 779 T6 was considered a yeast with beer brewing potential.

3.2. Methods for efficient detection of *S. cerevisiae* var. *diastaticus*

Due to the negative impact diastatic yeasts can have on beer quality, it is vital for craft breweries to detect any contaminations rapidly and reliably (Krogerus & Gibson, 2020). The genetic and physiological resemblance of diastatic strains of *S. cerevisiae* to brewing strains makes this detection more challenging. Detection of yeasts with diastase (starch-degrading) ability has been explored, with both

growth-based and molecular methods. However, most of these methods are time-consuming and not applicable to the daily work of a craft brewery.

In this section of the dissertation, our goal was to develop easy-to-replicate methods for reliable detection of diastatic yeasts.

3.2.1. Molecular detection: multiplex PCR

Various molecular methods have been used for the detection of diastatic yeasts, such as quantitative PCR and even mass spectrometry (Krogerus & Gibson, 2020). The most widely used method is the detection of the *STA1* gene using polymerase chain reaction (PCR) and there are some commercial kits available for brewers (Krogerus & Gibson, 2020).

However, as seen by Krogerus et al., 2019 and Pontes et al., 2020, not all yeasts presenting the *STA1* gene have the diastase phenotype, due to an 1162-bp deletion in the gene's promoter. As such, the detection of merely the *STA1* gene may be misleading, as the yeasts might not be able to degrade starch as, consequently, might not represent problematic contamination.

In this project, we aimed at developing a multiplex PCR to detect both the *STA1* gene and its promoter in a short time to determine the spoilage potential of these yeasts. The PCR reaction was designed with two pairs of primers: one for the detection of the *STA1* gene, which amplify a 399-bp fragment, created by Ana Pontes and the other pair for the detection of the promoter, that amplify a 599-bp fragment, published by Krogerus et al., 2019.

To ensure the quality and reliability of the PCR reaction, previously studied strains were employed. The yeasts selected were PYCC 2608, TUM 3-D-2, TUM PI-BA-31, TUM PI-BB-105 and TUM 1-B-8, whose genomes were analyzed by Pontes et al., 2020. In Figure 3.5 we can see the result of the multiplex reaction using DNA extracted from these strains. As expected, amplification of the *STA1* gene and its promoter was observed for PYCC 2608 and TUM 3-D-2, whereas TUM PI-BA-31 and TUM PI-BB-105 only amplified the *STA1* gene, and no amplification was observed with TUM 1-B-8.

After testing the multiplex PCR with other *S. cerevisiae* strains and achieving accurate and consistent results, this reaction was applied to yeasts isolated from beer bottles, as will be explored in section 3.2.2.

As the PCR began being applied to a real-life situation, where speed was crucial, the reaction was tested with colony PCR, to eliminate the time spent in DNA extraction. Although this method was successful at times, it led to false-negative results due to a lack of a control gene, which could be any gene present in *S. cerevisiae*, to ensure that enough biomass was added to allow the reaction to occur. Thus, any negative results could be misleading and a consequence of human error. However, it was clear that the improvement of this method with the addition of a control gene would be greatly beneficial, allowing for faster identification of diastatic yeasts.

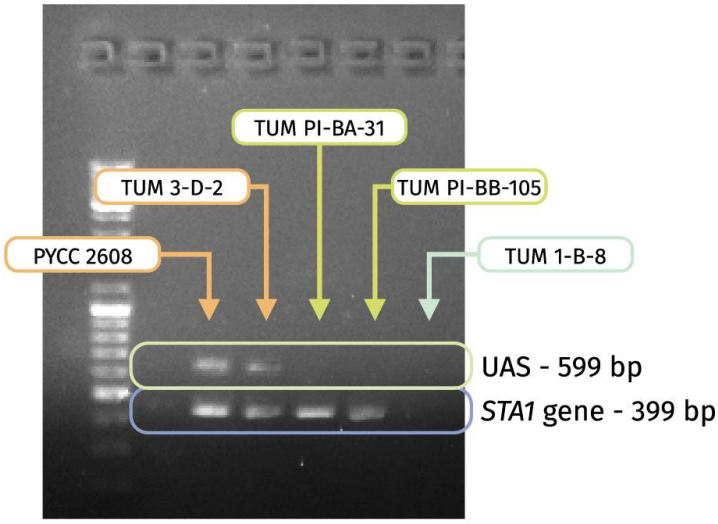

Figure 3.5. PCR products from the multiplex reaction with primers for the *STA1* gene and its promoter. The products were analyzed in a 2% agarose gel. The strains highlighted in orange are *STA1*+/UAS+, the strains highlighted in green are *STA1*+/UAS- and the strain in blue is *STA1*-.

Throughout this project, PCR was used for the detection of diastatic contaminants in a local brewery and, as the method was enhanced with the addition of the *STA1*_UAS_Fw/ *STA1*_UAS_Rv primer pair and the practice of colony-PCR, the time required to obtain a result was shortened from 1 week to 3 days.

3.2.2. Culture-based detection: diastase phenotype

Growth-based methods for the detection of contaminants are widely used in the brewing industry due to their simplicity and low cost. Krogerus & Gibson, 2020 recommend a combination of growth-based and PCR-based methods to accurately differentiate the spoilage potential of *STA1*+ strains, which requires more time than exclusively PCR-based methods.

For detection of diastatic *S. cerevisiae*, copper-containing media are often used, such as LCSM (Lin's Cupric Sulphate Medium) and MYGP + copper (malt extract, yeast extract, glucose and peptone media with copper) (Lin, 1981; Taylor & Marsh, 1984) due to the copper-tolerance these yeast possess, unlike non-diastatic yeasts. However, since these methods are based on copper tolerance and not on diastatic ability, they can lead to false positives (Krogerus & Gibson, 2020).

Burns et al., 2020 and Meier-Dörnberg et al., 2018 have developed media containing starch or dextrins as the only fermentable source. In this project, we aimed at creating a starch-agar media that could be revealed with an iodine (Lugol) solution. Krogerus et al., 2019 have shown a correlation between a deletion in the *STA1* gene promoter and a loss of the diastase phenotype, which piqued our interest in evaluating the starch degrading capabilities of the yeasts sequenced by Pontes et al., 2020: PYCC 2608, TUM 3-D-2, TUM PI-BA-31, TUM PI-BB-105 and TUM 1-B-8. These strains were applied in the pilot test for the starch medium. In Figure 3.6 the results obtained with these yeasts can be observed.

The iodine present in the Lugol solution reacts with starch creating a brown/dark red color. In the areas where the starch has been consumed, there should be a yellow color instead.

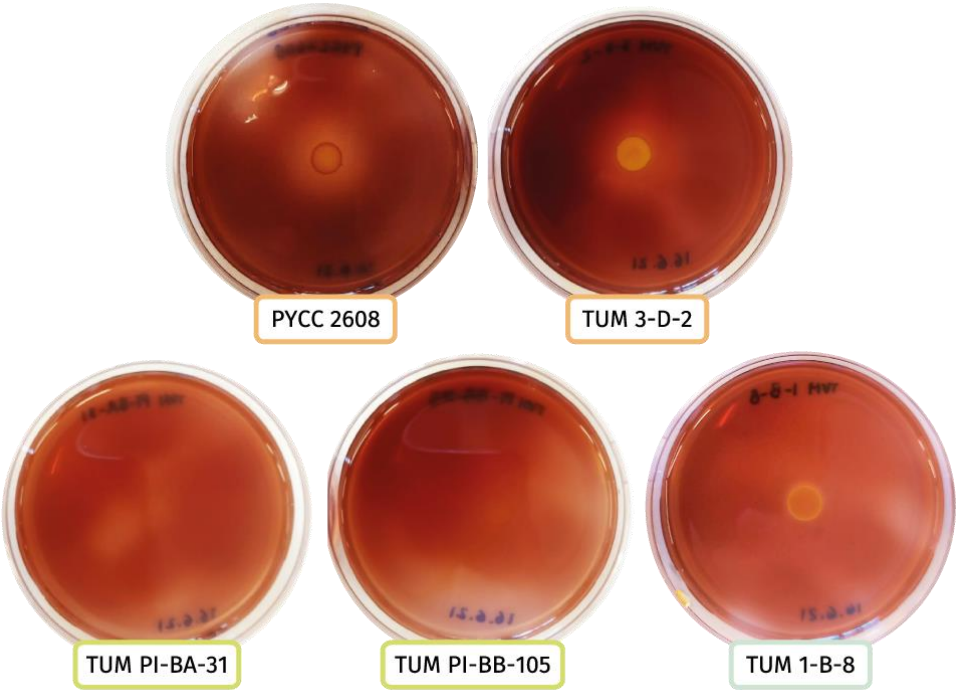

Figure 3.6. Starch-agar plates revealed with Lugol after 8 days of incubation at 25°C. The strains highlighted in orange are *STA1*+/UAS+, the strains highlighted in green are *STA1*+/UAS- and the strain in blue is *STA*1-.

The results obtained are coherent with the PCR results, as well as the information Pontes et al., 2020 published about the strains used. In the plates inoculated with PYCC 2608 and TUM 3-D-2, there is a yellow halo around the biomass. For the remaining strains, whether they are *STA1*+/UAS- or *STA1*, this halo did not form. However, growth was visible with every strain. This growth occurred to the presence of residual amounts of glucose, fructose, maltose and maltotriose. This did not interfere with the experiment because the Lugol solution reacted only with starch. The same experiment was performed with a liquid starch medium and the results were coherent. This method, both with solid or liquid media, requires incubation of 7-8 days, as plates incubated for shorter amounts of time did not produce any results. This might be due to the presence of simpler sugars, with can lead to a delay in starch consumption. To test this hypothesis and potentially reduce the time needed to observe results, a purer starch is required. Nevertheless, this method complements the molecular method discussed in 3.2.1 and has the potential to accurately identify yeast with a diastase ability.

3.2.3. Detection of diastatic *S. cerevisiae* in a craft brewery

In the context of ongoing diastatic contamination in a local brewery at the time of the beginning of this project, 30 batches of beer produced between January 2020 and June 2021 were analyzed. The

samples from January to July 2020 were analyzed by Francisca Paraíso, in the context of her dissertation in 2019/20. The contamination was first suspected during the first Covid-19 quarantine in Portugal, because of the decreasing rate of distribution of these products and the consequent extension of their storage time. The contamination was most likely caused by the production of a lighter beer, brewed with diastatic yeast. The same beer was last produced in July 2020.

In Figure 3.7 there is a schematic representation of all the beers analyzed and the results obtained. The numbers assigned to each beer were random and generally follow the chronological order of the analysis performed. The first number corresponds to the beer style and the second number corresponds to the number of the lot from that beer style. Throughout time, as an attempt of finding the source of the contamination, some batches were analyzed before and after the bottling of kegging. However, the results were always consistent within batches, which made the task of eliminating the contamination harder.

By following the protocol in place at the time of the beginning of this project, the samples received were analyzed 1 to 2 weeks after bottling/production, when possible. However, the lots with negative results were kept for more tests. On a lot of these lots, a positive result was achieved after a second or even third analysis. This might be justified with a low diastatic and non-diastatic yeasts ratio, which results in an increased difficulty in isolating diastatic contaminants. Throughout time, it is probable that diastatic yeasts will grow and outnumber the brewing yeast. To support this hypothesis, a higher cell count was obtained in the older samples of these beers. To avoid this issue without prolonging the wait time and consequently delaying the results, the analysis can be conducted with a larger number of colonies or with the entire population of yeasts in the bottle.

With the development of molecular and growth-based methods for the detection of *S. cerevisiae* var. *diastaticus*, the results were supplemented with an analysis of the *STA1+* promoter and phenotype confirmation. Most of the yeasts isolated presented either *STA1+*/UAS+ or a *STA1-*/UAS- profile with coherent phenotype, except for 7.1 and 11.1, which have the *STA1* gene with a promoter deletion and are unable to consume starch. The origin of these strains is still unknown, as they are distinct from the brewing yeasts used for these beers and the diastatic yeast used for the production of 5.1.

At the beginning of 2021, the brewery changed locations and acquired new material. The beers analyzed after this were only submitted to one analysis and the results were optimistic, with no diastatic yeasts detected. However, a second analysis is recommended, due to what was previously observed with samples 2.2, 9.1, 1.7, 10.2 and 13.1.

In what concerns the alterations that *S. cerevisiae* var. *diastaticus* can inflict in bottled beer, the results are insufficient. One of the most notable consequences of diastatic contaminants is the increase of CO_2 in the bottle, resulting in the overflowing of beer, otherwise known as gushing (Krogerus & Gibson, 2020). Despite the detection of diastatic yeasts in 19 lots, this phenomenon was only seen in samples 4.1 and 2.1. This might be explained by the bottled beer's age. It is reasonable to assume that a longer incubation period will lead to more cell growth, more sugar consumption and, consequently, more CO_2 production. However, there is not enough information to define the number of weeks or days

necessary to create a gushing phenomenon, seeing as variables like the initial cell amount at the time of bottling and the amount of available fermentable sources in the bottle vary in each bottle and beer style.

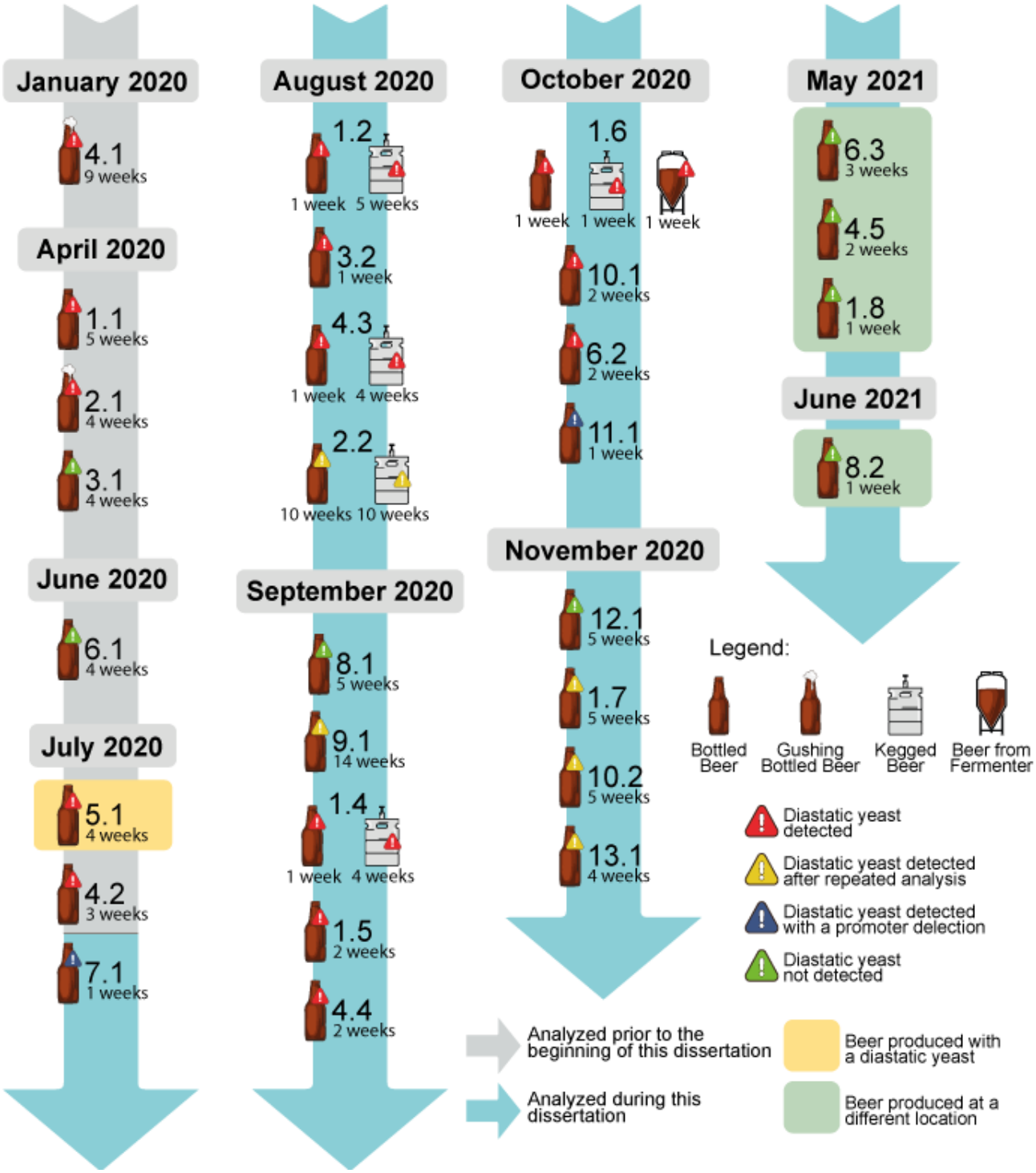

Figure 3.7. Schematic representation of the results obtained from the analysis of 30 lots of beer produced at the brewery organized by the date of bottling/production.

In a broader view of these analyses, it is quite worrisome to observe the perseverance of this contamination. For a year, this brewery, despite following the recommended cleaning cycles and procedures, battled with a diastatic yeast contaminant. The contamination appeared to be present everywhere in the brewery, with diastatic yeasts being found in bottles, kegs and fermenters. Although gushing was rarely observed in the laboratory, some consumers reported the occurrence of gushing and the brewery was forced to remove lots from the market, which, of course, has financial consequences. It is clear that diastatic contaminations are a very serious threat to brewers and there is a need to develop *S. cerevisiae* var. *diastaticus* detection methods that can be easily applied to the reality of a brewery and cater to their needs of accuracy and speed.

Although in this project accurate methods were perfected to require a maximum of a one-week wait to obtain results, these protocols are not optimized to be performed in the brewery. Moreover, issues such as the misleading results obtained due to a small concentration of diastatic yeasts in earlier analysis need to be resolved to respond to the brewery's need for immediate and trustworthy results.

3.3. Diastatic yeasts as contaminants: a comparative analysis of diastatic and non-diastatic yeasts

3.3.1. Growth in Beer

As an effort to further explore and understand contaminations of diastatic strains of *S. cerevisiae*, an experiment was designed to determine the spoilage potential of the smallest possible inoculation of a diastatic yeast. The yeast used in this experiment was isolated from sample 2.1 and was isolated in a local brewery in the context of section 3.2.3. For comparison with brewing yeasts, the strains Fosters B, from the Beer 1 population, and EC 1118, from the Wine population, were submitted to the same conditions. As described in 2.5.1, these three yeasts were precultured in YPD, diluted and then inoculated in industrial lager beer. The beer was previously analyzed with HPLC and its composition is described in Table 3.2.

Table 3.2. Composition of the industrial lager beer used in growth trials.

Sugar	Amount (g/L)
Maltotriose	2.45
Maltose	3.07
Glucose	0.08
Fructose	0.24

The number of cells inoculated at 0 weeks was approximately 0, 1, 2 and 5 per 45 mL and each condition was repeated five times. The inoculum with "0 cells" was made with a very small volume of the diluted yeast suspension. The cell amount was monitored for 8 weeks and the results are seen in Figure 3.8. The inoculums were kept in a closed recipient at 20°C with no agitation, to more accurately mimic the storage conditions of bottled beer.

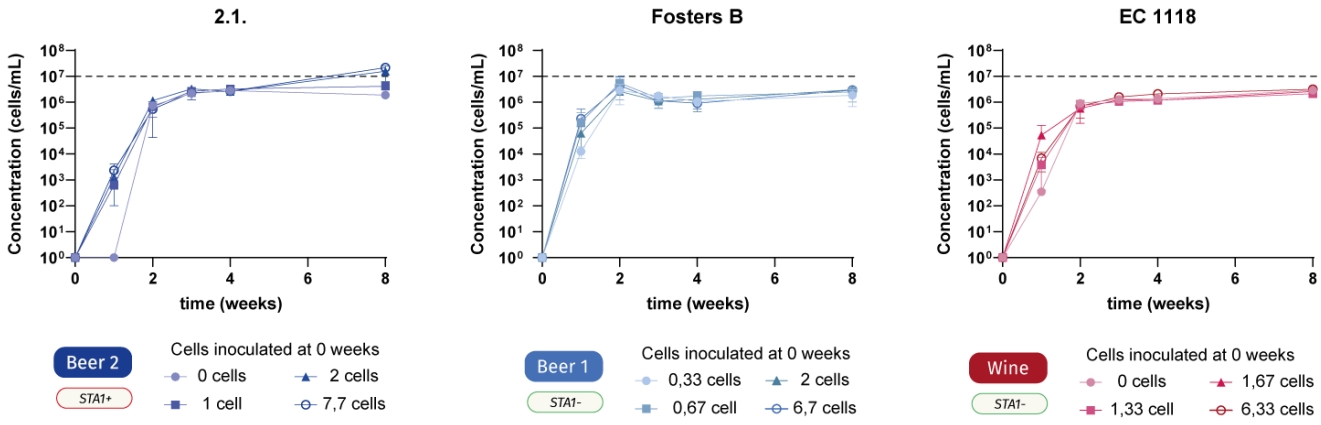

Figure 3.8. Side-by-side comparison of the concentration in 45 mL of lager beer of different yeasts throughout 8 weeks: 2.1 was isolated from a brewery sample and is *STA1+* and UAS+; Fosters B belongs to the Beer 1 population and is *STA-*; EC 1118 belongs to the Wine population and is *STA-*. The legend indicates the number of cells inoculated at t=0, determined with 6 replicates of CFUs.

All the strains proved to be capable of growing in beer, with concentrations as low as 1 cell/45 mL reaching over $1x10^6$ cells/mL. Surprisingly, the diastatic yeast 2.1 required a longer amount of time to be detected when inoculated with approximately zero cells. However, at 8 weeks, 2.1 reached concentrations of $1x10^7$ cells/mL, whereas Fosters B and EC 1118 maintained a concentration of $1x10^6$ cells/mL. These results corroborate the hypothesis created in 3.2.3, with diastatic yeasts requiring a longer time to grow and be detected in a beer bottle.

Fosters B presented a distinct growth profile, reaching a peak of nearly $1x10^7$ cells/mL at 2 weeks and then decreasing to $1x10^6$ cells/mL.

These results show that any brewing yeast, such as Fosters B, and even wine yeasts like EC 1118 are able to grow and survive in beer, even when the initial amount is as low as one cell.

3.3.2. Gas production

Despite having confirmed the ability of both diastatic and ale yeasts to grow in beer in low concentrations, the spoilage potential of yeasts comes down to their ability to ferment residual sugars and produce CO_2. Meier-Dörnberg et al., 2018 have shown, through a modified Durham tube test, the ability of *S. cerevisiae* var. *diastaticus* yeasts to form gas and, simultaneously, the inability of an *S. cerevisiae* strain and two *S. pastorianus* strains to do the same.

To supplement the results described in 3.3.1, a protocol adapted from Meier-Dörnberg et al., 2018 was created to determine the gas-forming potential of very low concentrations of a diastatic yeast, a brewer's yeast and a wine yeast. Concentrations of 0,6 cells/mL, 1,6 cell/mL and $3x10^6$ cells/mL were

tested with this protocol and the results observed are shown in Table 3.3. The formation of gas in the Durham tubes was observed in both the diastatic yeast, 2.1, and the brewing yeast, Fosters B when present at a concentration of $3x10^6$ cells/mL, whereas no gas was formed with EC 1118.

Table 3.3. Gas forming potential of the investigated strains using a modified Durham tube test.

Strain	Cell concentration at 0 days (cells/mL)	Time (days)						
		0	2	6	8	9	12	13
2.1	0.6	-	-	-	-	-	-	-
	1.6	-	-	-	-	-	-	+
	3.00E+06	-	-	+	++	+++	++++	++++
Fosters B	0.6	-	-	-	-	-	-	-
	1.6	-	-	-	-	-	-	-
	3.00E+06	-	-	+	++	++	+++	+++
EC 1118	0.6	-	-	-	-	-	-	-
	1.6	-	-	-	-	-	-	-
	3.00E+06	-	-	-	-	-	-	-

As for the tubes with low concentrations of yeast, this experiment has shown that a certain concentration is required to promote gas formation. In all of the tubes, cell growth is visible through increased turbidity, which supports the findings in 3.3.1. However, the growth is not accompanied by CO_2 production and gas formation is only visible after 2.1 reached a higher concentration.

It is reasonable to hypothesize that, if given more time, the tubes with 2 and 5 cells of 2.1 and Fosters B would have all begun forming gas in the Durham tubes. This hypothesis was not verified in this experiment due to signs of gas dissipation after 13 days, which threatened the accuracy of the experiment. It might be interesting to repeat this test resorting to more effective sealing methods.

3.3.3. Temperature resistance

Due to the persistence of diastatic contamination found in a local brewery seen in 3.2.3, it became relevant to study these contaminants and their resistance to typical cleaning processes in a brewery. Breweries use Cleaning-In-Plane (CIP) systems to clean and sanitize pipes and vessels. One of the methods used In CIP systems to disrupt biofilms is the application of high temperatures. As such, a temperature resistance experiment was conducted at 55°C because pasteurization of beer occurs at 55-60°C (Grossman, 2012). With a protocol adapted from Suiker et al., 2021, the D55 values, the number of times, in minutes, needed to kill 90% of the population, were determined for 2.1, Fosters B and EC 1118. The yeasts were grown in beer to mimic contamination in the bottling area, which is where most contaminations occur according to a 2017 survey (Meier-Dörnberg et al., 2017). As seen in

Table 3.4, the cultures showed different D_{55} values, although all of them are below 5 minutes. 2.1, the diastatic strain, was the most resistant strain, with a D55 of 4.58 minutes, followed by EC 1118, with 2.69 minutes.

Table 3.4. D_{55} values of 2.1, Fosters B and EC 1118 obtained in a temperature resistance test. The values displayed are the average result of three repetitions of this experiment.

	D_{55} (min)
2.1	4.58
Fosters B	0.93
EC 1118	2.69

As a direct consequence of diastatic yeasts being more resistant than non-diastatic beer brewing yeasts to high temperatures, these contaminants might be harder to remove in a brewery.

4. FINAL REMARKS AND FUTURE PERSPECTIVES

The production of craft beer is a growing industry with a high demand for continuous innovation. The search for new products has motivated brewers and scientists to find new yeasts and ingredients. Alongside innovation, there is a need to detect and control contaminations to ensure beer quality. In this project, these two concerns were addressed. The use of non-conventional *S. cerevisiae* cultures has become a subject of interest in both craft beer brewing and the scientific community. In this project, eleven non-beer brewing strains were tested for their maltose and maltotriose assimilation and the production of flavors or aromas in laboratory-scale fermentations. Moreover, strains submitted to beer wort adaptation through a series of re-inoculations were tested and compared to the wild ancestors from which they originated. With this experiment, we were able to find *S. cerevisiae* strains from the Wine, Mediterranean Oaks and NA/Japan populations with the potential to be applied in beer production. This shows that innovation in the craft beer industry can be achieved with yeasts from both domesticated and wild *S. cerevisiae* populations. However, in order to assess their real potential for the brewing industry, it is necessary to optimize the brewing process, experimenting with temperatures, wort composition and fermentation periods that may highlight the positive aspects found in this project and minimize issues such as oxidation and the presence of acetaldehyde. It is also important to keep in mind the need for scaling up to larger volume fermentations and the influence this might have in the final product. It would also be interesting to broaden the spectrum of yeasts put through these trials, focusing on wine yeast strains and adapted wild yeasts. Moreover, it might be relevant to identify and quantify the volatile components produced during wort fermentation.

As an effort to create simple and easy-to-replicate methods of diastatic yeasts detection, two protocols were developed and tested with beer samples from a brewery. Both the multiplex PCR with primers designed for the *STA1* gene and its promoter, and the starch media proved to be reliable and efficient. However, these methods do not provide immediate results, which would allow for a faster reaction and, consequently, lead to more effective elimination of the contaminant. The growth-based methods, despite proving to be effective and reliable, require a long waiting period. Moreover, since this method relies on growth in a starch-rich medium, which is susceptible to bacteria and mold contamination, it might not be ideal to be used in a brewery. On the other hand, the multiplex PCR, if improved with the addition of a control gene, can be performed as a colony-PCR and decrease the time

required for delivering results. Furthermore, with a change of methodology, the PCR reaction could be done with a group of colonies, removing the time needed for colony isolation. If this were to be successful, results could be obtained within 2 to 3 hours of starting the analysis. For this reason, we believe that the multiplex PCR can be a good methodology for diastatic yeasts detection in a brewery. However, it is a costly method, as it requires the acquisition of laboratory materials and reagents. Nevertheless, the results obtained in this dissertation can contribute to the development of simple and easy-to-replicate methods for the detection of diastatic yeasts throughout beer production and packaging.

The partnership with the local brewery allowed a deeper understanding of the importance of preventing contaminations through regular sanitization. However, the quality assessment work performed in the context of this partnership revealed that contaminations might be difficult to eliminate, especially when it concerns diastatic yeasts. There is a need in this brewery for more specific sanitation and hazard prevention protocols, which might begin with regular microbial detection in fermentation vessels, tubes and other equipment in the brewery. There seems to be a lack of affordable methods that can be applied by brewers to detect, prevent and eliminate contaminations quickly. This was observed through the persistence of diastatic yeast contamination despite the efforts to follow cleaning guidelines and even replace some components of the equipment. In the future, there seems to be an opportunity to study the sanitization guidelines in place in order to assess their effectiveness and, if needed, create new ones.

After witnessing the perseverance of diastatic yeast contamination in the brewery, there was an understandable interest to further study these yeasts, their abilities and their spoilage potential. We were able to conclude that diastatic yeasts, beer brewing yeasts and even wine yeasts have the ability to grow in bottled beer, even when present in low cell concentrations. However, diastatic strains appear to be more harmful due to their gas-forming potential. The beer-brewing yeasts also produced CO_2 at a slower pace, when in a high concentration. Additionally, when inoculated with 5 cells in 45 mL, the diastatic strain studied was able to begin production of CO_2 after 13 days, whereas no gas was produced during this time with other strains. These experiments highlight the importance of studying and understanding the behavior of diastatic yeasts to create better procedures to identify and eliminate them.

Besides revealing to have more spoilage potential, the diastatic yeast strain studied in this project, also showed higher tolerance to high temperatures, which highlights the threat diastatic yeasts represent to the brewing industry. It could be relevant to broaden the scope of these experiments to include sanitation products often used in a brewery, such as caustic soda and acids. Besides, it would be pertinent to introduce other diastatic and non-diastatic yeasts to these protocols.

REFERENCES

Adam, A. C., Latorre-García, L., & Polaina, J. (2004). Structural analysis of glucoamylase encoded by the *STA1* gene of *Saccharomyces cerevisiae* (var. *diastaticus*). *Yeast*, *21*(5), 379–388.

Almeida, P., Barbosa, R., Zalar, P., Imanishi, Y., Shimizu, K., Turchetti, B., … Sampaio, J. P. (2015). A population genomics insight into the Mediterranean origins of wine yeast domestication. *Molecular Ecology*, *24*(21), 5412–5427.

Alves, S. L., Herberts, R. A., Hollatz, C., Miletti, L. C., & Stambuk, B. U. (2007). Maltose and maltotriose active transport and fermentation by *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists*, *65*(2), 99–104.

Barbosa, R., Almeida, P., Safar, S. V. B., Santos, R. O., Morais, P. B., Nielly-Thibault, L., … Sampaio, J. P. (2016). Evidence of natural hybridization in Brazilian wild lineages of *Saccharomyces cerevisiae*. *Genome Biology and Evolution*, *8*(2), 317–329.

Berthels, N. J., Cordero Otero, R. R., Bauer, F. F., Thevelein, J. M., & Pretorius, I. S. (2004). Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. *FEMS Yeast Research*, *4*(7), 683–689.

Boulton, C., & Quain, D. (2001). The brewing process. In *Brewing Yeast and Fermentation* (pp. 19–68). Blackwell Science Ltd.

Bourbon-Melo, N., Palma, M., Rocha, M. P., Ferreira, A., Bronze, M. R., Elias, H., & Sá-Correia, I. (2020). Use of *Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae* to enhance the aromatic profile of beer in mixed-culture fermentation with *Saccharomyces cerevisiae*. *Food Microbiology*, *95*.

Burns, L. T., Sislak, C. D., Gibbon, N. L., Saylor, N. R., Seymour, M. R., Shaner, L. M., & Gibney, P. A. (2020). Improved Functional Assays and Risk Assessment for *STA1+* Strains of *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists*, *0*(0), 1–14.

Corran, H. S. (1975). *A History of Brewing*. David and Charles.

Cubillos, F. A., Gibson, B., Grijalva-Vallejos, N., Krogerus, K., & Nikulin, J. (2019). Bioprospecting for brewers: Exploiting natural diversity for naturally diverse beers. *Yeast*, *36*(6), 383–398.

Davies, S., Sykes, T., Philips, M., & Hancock, J. (2015). *Hygienic design and Cleaning-In-Place (CIP) systems in breweries*. *Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*. Elsevier Ltd.

Day, R. E., Rogers, P. J., Dawes, I. W., & Higgins, V. J. (2002). Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *68*(11), 5326–5335.

Dysvik, A., Liland, K. H., Myhrer, K. S., Westereng, B., Rukke, E. O., de Rouck, G., & Wicklund, T. (2019). Pre-fermentation with lactic acid bacteria in sour beer production. *Journal of the Institute of Brewing*, *125*(3), 342–356.

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*.

Gibbons, J. G., & Rinker, D. C. (2015). The genomics of microbial domestication in the fermented food environment. *Current Opinion in Genetics and Development*, *35*, 1–8.

Gibson, B., Dahabieh, M., Krogerus, K., Jouhten, P., Magalhães, F., Pereira, R., … Vidgren, V. (2020). Adaptive Laboratory Evolution of Ale and Lager Yeasts for Improved Brewing Efficiency and Beer Quality. *Annual Review of Food Science and Technology*, *11*, 23–44.

Gibson, B., Geertman, J. M. A., Hittinger, C. T., Krogerus, K., Libkind, D., Louis, E. J., … Sampaio, J. P. (2017). New yeasts-new brews: Modern approaches to brewing yeast design and development. *FEMS Yeast Research*, *17*(4), 1–13.

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*.

Grossman, K. (2012). *The Oxford Companion to Beer*. Oxford University Press.

Hill, A. E. (2015). Traditional methods of detection and identification of brewery spoilage organisms. In *Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste* (pp. 271–286). Elsevier Ltd.

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K. J., & Thevelein, J. M. (2018). Bioflavoring by non-conventional yeasts in sequential beer fermentations. *Food Microbiology*, *72*, 55–66.

Kim, T. S., Lee, S. B., & Kang, H. S. (2004). Glucose Repression of *STA1* Expression Is Mediated by the Nrg1 and Sfl1 Repressors and the Srb8-11 Complex . *Molecular and Cellular Biology*, *24*(17), 7695–7706.

Krogerus, K., & Gibson, B. (2020). A re-evaluation of diastatic *Saccharomyces cerevisiae* strains and their role in brewing. *Applied Microbiology and Biotechnology*, *104*(9), 3745–3756.

Krogerus, K., Magalhães, F., Kuivanen, J., & Gibson, B. (2019). A deletion in the *STA1* promoter determines maltotriose and starch utilization in *STA1+ Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology*, *103*(18), 7597–7615.

Lin, Y. (1981). Formulation and Testing of Cupric Sulphate Medium for Wild Yeast Detection. *Journal of the Institute of Brewing*, *87*(3), 151–154.

McGovern, P. E., Zhang, J., Tang, J., Zhang, Z., Hall, G. R., Moreau, R. A., … Wang, C. (2004). Fermented beverages of pre- and proto-historic China. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(51), 17593–17598.

Meier-Dörnberg, T., Jacob, F., Michel, M., & Hutzler, M. (2017). Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the Beverage Industry: Cases of Contamination, 2008–2017. *Technical Quarterly*.

Meier-Dörnberg, T., Kory, O. I., Jacob, F., Michel, M., & Hutzler, M. (2018). *Saccharomyces cerevisiae* variety *diastaticus* friend or foe?-spoilage potential and brewing ability of different *Saccharomyces cerevisiae* variety *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization. *FEMS Yeast Research*, *18*(4), 1–27.

Meussdoerffer, F. G. (2009). *A Comprehensive History of Beer Brewing*. *Handbook of Brewing: Processes, Technology, Markets*.

Naseeb, S., James, S. A., Alsammar, H., Michaels, C. J., Gini, B., Nueno-Palop, C., … Delneri, D. (2017). *Saccharomyces jurei* sp. Nov., isolation and genetic identification of a novel yeast species from *Quercus robur*. *International Journal of Systematic and Evolutionary Microbiology*, *67*(6), 2046–2052.

Ness, F., & Aigle, M. (1995). *RTM1*: A member of a new family of telomeric repeated genes in yeast. *Genetics*, *140*(3), 945–956.

Nikulin, J., Eerikäinen, R., Hutzler, M., & Gibson, B. (2020). Brewing characteristics of the maltotriose-positive yeast *Zygotorulaspora florentina* isolated from oak. *Beverages*, *6*(4), 1–19.

Olaniran, A. O., Hiralal, L., Mokoena, M. P., & Pillay, B. (2017). Flavour-active volatile compounds in beer: production, regulation and control. *Journal of the Institute of Brewing*.

Osburn, K., Amaral, J., Metcalf, S. R., Nickens, D. M., Rogers, C. M., Sausen, C., … Bochman, M. L.

(2018). Primary souring: A novel bacteria-free method for sour beer production. *Food Microbiology*.

Pontes, A., Hutzler, M., Brito, P. H., & Sampaio, J. P. (2020). Revisiting the taxonomic synonyms and populations of *Saccharomyces cerevisiae*—phylogeny, phenotypes, ecology and domestication. *Microorganisms*, *8*(6), 1–23.

Rodhouse, L., & Carbonero, F. (2019). Overview of craft brewing specificities and potentially associated microbiota. *Critical Reviews in Food Science and Nutrition*, *59*(3), 462–473.

Sampaio, J. P., & Gonçalves, P. (2017). Biogeography and ecology of the genus *Saccharomyces*. In *Yeasts in Natural Ecosystems: Ecology*.

Štulíková, K., Vrzal, T., Kubizniaková, P., Enge, J., Matoulková, D., & Brányik, T. (2021). Spoilage of bottled lager beer contaminated with *Saccharomyces cerevisiae* var. *diastaticus*. *Journal of the Institute of Brewing*, *127*(3), 256–261.

Suiker, I. M., Arkesteijn, G. J. A., Zeegers, P. J., & Wösten, H. A. B. (2021). Presence of *Saccharomyces cerevisiae* subsp. *diastaticus* in industry and nature and spoilage capacity of its vegetative cells and ascospores. *International Journal of Food Microbiology*, *347*(March).

Taylor, G. T., & Marsh, A. S. (1984). Mygp + Copper, a Medium That Detects Both *Saccharomyces* and Non-*Saccharomyces* Wild Yeast in the Presence of Culture Yeast. *Journal of the Institute of Brewing*, *90*(3), 134–145.

Vaughan, A., O'Sullivan, T., & Van Sinderen, D. (2005). Enhancing the microbiological stability of malt and beer - A review. *Journal of the Institute of Brewing*, *111*(4), 355–371.

Will, J. L., Kim, H. S., Clarke, J., Painter, J. C., Fay, J. C., & Gasch, A. P. (2010). Incipient balancing selection through adaptive loss of aquaporins in natural *Saccharomyces cerevisiae* populations. *PLoS Genetics*, *6*(4).

Willaert, R. (2006). *The Beer Brewing Process: Wort Production and Beer Fermentation*. *Handbook of Food Products Manufacturing* (Vol. 1).

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INNOVATION AND QUALITY ASSESSMENT IN CRAFT BEER PRODUCTION

NOVA SCHOOL OF SCIENCE & TECHNOLOGY