INVESTIGATING YEAST AND HOP INTERACTIONS DURING THE FERMENTATION OF BEER

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Abstract

The brewing of beer requires four main ingredients: malt, water, hops, and yeast. During the brewing process, the brewer creates a malt-derived sugar water, called wort, that is inoculated with yeast, causing fermentation to create carbon dioxide and alcohol from the sugars. Traditionally hops are added to the wort during the boiling process in order to balance the sweetness with isomerized alpha-acids that create bitterness. Hops also contain many other flavor-active compounds that brewers are attempting to harness for the creation of aromatic and complex beers. These flavor-active compounds can become even more desirable when hops are added to active fermentation in the presence of *Saccharomyces* yeast, a process known as dry-hopping. During this dry-hopping process, aromatic compounds from the hops can undergo biotransformation with glycosides of the yeast to create unique flavors, but these dry-hops also contain enzymes that are biochemically changing more than just aromatic compounds. The enzymes in the hops are breaking down residual oligosaccharides in the beer that are meant to create a fuller bodied beverage, known as dextrins, and converting them into simple, more fermentable sugars that the yeast can easily assimilate. The yeast ferments the newly hydrolyzed sugars, adding excess alcohol and possibly yeast derived off-flavors to the beer, a process termed "hop creep".

In this series of studies, the interactions between yeast and hops during the dry-hopping and fermentation process of beer was examined, as well as factors contributing to the hop creep phenomenon. Hop creep has been extensively studied from the back end of the brewing process, the hop perspective; this work intends to look at the phenomenon from the front end of fermentation, the yeast perspective. In the first part of this study, a bench top analysis was repeated to determine contribution to dry-hop creep from different hop cultivars. Previous

research had determined a statistical difference amongst cultivars, but was unable to be confirmed here, so research set out to develop a bench method for hop creep analysis that brewers could perform easily with little laboratory skills. Following method development, a screening of *Saccharomyces* yeasts from the UC Davis Phaff Yeast Culture Collection commenced in order to identify yeasts with properties advantageous to brewing. Yeasts were screened for their ethanol tolerance, carbohydrate metabolism, as well as nitrogen and amino acid assimilation, all of which are important qualities for the brewer. The screening was successful and expanded to full pilot scale at Anheuser-Busch InBev Research Pilot Brewery at the Robert Mondavi Institute. Six non-*cerevisiae* and non-*pastorianus Saccharomyces* yeasts that had not previously been used to create ales were used in fermentations in duplicate, with one fermenter in each set receiving 10 g/L of dry-hops during fermentation. Beers were measure for alcohol, real degree of fermentation (RDF), gravity, calories, pH, and yeast viability during fermentation, and sensory analysis was performed on finished beer. All yeasts displayed unique characteristics that may offer great potential to a complexly evolving and consumer driven beer market.

In addition to the six yeasts described above, twenty-four more *Saccharomyces* strains chosen for their typical use in the production of alcoholic beverages were also used in fermentation at 40 L pilot scale, totaling thirty unique yeasts used in this study. Yeasts from the Phaff Collection, as well as three commercial suppliers, were aseptically propagated from single cultures with the necessary cell volume for these pilot scale fermentations. Again, they were fermented in duplicate, with one fermenter receiving a dry-hop of 10 g/L, and fermentation was monitored until gravity was deemed terminal and the beer had fully attenuated. RDF, alcohol, and pH were tracked as fermentation progressed; the previously determined bench method was

also utilized to determine the amount of hop creep expected. No *Saccharomyces* yeasts in this study presented themselves for the effective mitigation of hop creep, but analysis of this manner has never been performed before, so much insight was gained. More research remains on a correlation of flocculation and dry-hop creep, amino acid and diacetyl analysis on dry-hopped in comparison to non-hopped fermentations, and secondary metabolites from the fermentation of beer with these unique yeasts, especially in the presence of hops.

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Acknowledgements

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Covid grad school was rough. Let’s have some beers about it once everyone is vaccinated.

Chapter 1

Literature Review

Portions published in *Fermentation*.

Bruner, J.; Fox, G. Novel Non-*cerevisiae Saccharomyces* Yeast Species Used in Beer and Alcoholic Beverage Fermentations. *Fermentation* **2020**, *6*, 116, doi:10.3390/fermentation6040116.

A. Introduction to Brewing History

Fermented beverages have played an important and unique role over the course of human history due to their economic and cultural importance, perhaps even lending to the beginning of modern civilizations [1,2]. Archaeological evidence places the oldest fermented beverage in the fertile crescent as far back as 11000 BCE [3,4], and based on the agricultural evidence of the time and region, that beverage was likely beer. While beer may have originally been an accidental beverage, it progressed into one of the most artfully crafted beverages known to man. No longer thought of as just an art, the science of beer has led to several very important discoveries in scientific history (*Table 1.1*). As scientific discoveries keep developing, there have been some amazing innovations that have led to advances in the quality and stability of beer over the past 40 years [5]. However, minimal advancement has been made when considering the raw ingredients used in the brewing process.

Table 1.1. Significant discoveries of the 150 years from 1760-1910 that came from scientists working at breweries or specifically studying beer and its adjacent ingredients.

Year	Scientist	Employment	Discovery
1762	Michael Combrune	*Brewer's Company Middlesex*	using a thermometer for analysis [2]
1769	James Baverstock	*family brewery*	using a hydrometer for analysis [2]
1833	Anselme Payen and Jean-François Perzoz	*École Centrale Paris*	*d*iscovered diastase enzyme and cellulose while working with barley [2]
1843	Karl J.N. Balling	*Polytechnic in Prague*	invents the balling saccharimeter [6]
1843	James Joule and Lord Kelvin	*family brewery*	create temperature scale and first law of thermodynamics [7]
1857	Louis Pasteur	*University of Lille*	microbes are responsible for fermentation [8]
1860	P.E. Marcellin Berthelot	*Collège de France*	discovered invertase in *Saccharomyces* [2]
1873	Carl von Linde	*Spaten Brewery*	invented the refrigeration cycle [2]
1883	Johan Kjeldahl	*Carlsberg Brewery*	develops method for protein quantification [9]
1888	Emil Christian Hansen	*Carlsberg Brewery*	first isolation of pure yeast strain [9]
1908	William Sealy Gosset	*Guinness Brewery*	invents the statistical t-test for students [10]
1909	Søren Sørenson	*Carlsberg Brewery*	creates pH scale based on H^+ ion concentration [11]

B. Brewing Process Overview

On a base level, beer consists of four main ingredients: malted cereal grains, water, hops, and yeast, and the brewing process can be separated into a hot side and a cold side. In the most basic overview of the brewing process, the hot side begins when cereal grains from the malting process are crushed and combined with warm water so the maltose sugars are hydrolyzed from starch, the resultant liquid is then boiled with hops to add bitterness and flavor; this liquid, called wort, provides the nutrients for yeast. Moving from the hot side to the cold side, wort is subsequently chilled for fermentation (*Fig. 1.1*); yeast is added, metabolizing 50 to 80 percent of the sugar and nutrients to fermentation products, leaving behind non metabolized proteins, oligosaccharides, and other compounds [12–14].

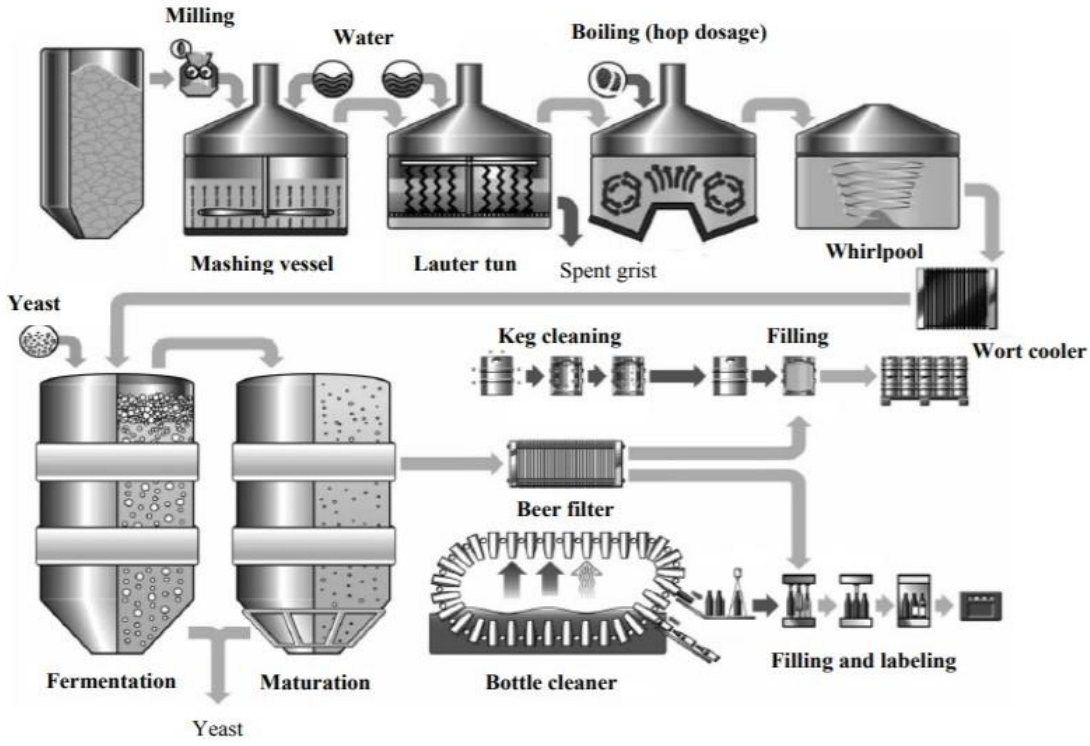

Figure 1.1. Schematic diagram of the brewing process, as presented in Magalhães et al. 2009 [14].

1. Malting

The brewing process begins in the malthouse, where raw cereal grains go through a controlled germination process to allow endogenous enzymes to break down cell walls in the aleurone and proteins in the endosperm while maintaining starch content to be utilized later in the brewing process. Without the malting process, these grains cannot be used in the brewing process. Barley (*Hordeum vulgare*) is the preferred cereal grain for malting and is the primary raw ingredient in the brewing process, however, other grains (termed "adjuncts"), such as wheat (*Triticum aestivum*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*), millet (*Panicum miliaceum*), rice (*Oryza sativa*), corn (*Zea mays*), or oats (*Avena sativa*), may be malted and subsequently used. Generally, the 2-row barley is preferred over the 6-row in modern malting due to its symmetrical and plump kernels, higher starch, and lower protein content [9].

Barley is received from the farmer, then analyzed for protein and starch content, germinative energy and capacity, moisture content, and size, before being cleaned to remove and stems, chaff, or rocks. Grains are then steeped in variable cycles dependent on parameters set forth by maltsters, with full immersion in water then draining for an air rest and repeating for 24 to 48 hours. Moisture content is raised from about 12% to about 45% as the barley begins to chit, with oxygen being pumped through the wet grain bed in order to induce germination and drive off carbon dioxide. The wet barley is then fully drained and allowed to germinate, where metabolic processes ramp up and enzymes move from the scutellum and aleurone layers in barley. In the 4 to 6 days of germination at 14 to 20 ºC, beta-glucanase (EC 3.2.1.39) and xylanase (EC 3.2.1.8) enzymes begin to hydrolyze the beta-glucan and arabinoxylan in the endosperm of barley. Proteases then move into the cell wall to break down the hordein protein surrounding the starch

and release beta-amylase (EC 3.2.1.2). Amylase and limit dextranase (EC 3.2.1.41) enzymes will start to hydrolyze the newly exposed starch, but to a small extent [15].

The controlled germination and amylase activity is halted with a kilning process, both drying the malt and contributing to Maillard reactions that promote color and flavor in beer. Water is driven off slowly at first, with convective heat of 45 to 50 ºC; once the moisture content drops from 45% down to 30%, the temperature is incrementally raised over a period of time in order to not denature the enzymes needed for brewing. Final moisture content for standard barley malt is around 4.5%, but can vary based on the style of malt desired by both maltster and brewer. The time and temperature of kilning or roasting also leads to a spectrum of flavors and colors in malt that translate into the finished beer product, whether pale light lagers or dark stout ales. The entire malting process takes about a week and can see the barley move between a series of vessels, from steeping tank, to germination bed, to kiln or roaster, or can take place in a modern Saladin box, which is a combination of all vessels in one [16].

2. Wort Production

The brewer receives the grain from the malthouse and inspects for a myriad of parameters that affect the brewing process both positively and negatively, including size, color, moisture content, starch and protein content, enzyme activity and diastatic power, among others. The brewer then initiates a process called milling, where the grain kernels are coarsely ground in order to expose the internal starch granules of the endosperm while maintaining the husk for downstream filtration [9]. The crushed malt is combined with warm water, temperature dependent on what enzymes the brewer wishes to utilize (35°C to 70°C), in a vessel called a mash tun. Here, the enzymes from the forced germination in malting are reactivated and begin to break down the starches into fermentable simple sugars (*Fig 1*.2). After a recipe dependent length of

time, the oatmeal-like mixture is transferred to a vessel called the lauter tun, where the resulting liquid, now called wort, is separated from the solid malt - now termed “spent grain”. This process is aided by increasing temperature to 78°C in order to promote viscosity, as well as the intact husks in the mixture from the milling process creating channels for liquid to flow. The wort is separated into a kettle while the spent grain is removed and usually sent to farmers as a livestock feed high in protein and fiber, although there is increasing research on novel uses for this byproduct of the brewing process [17,18].

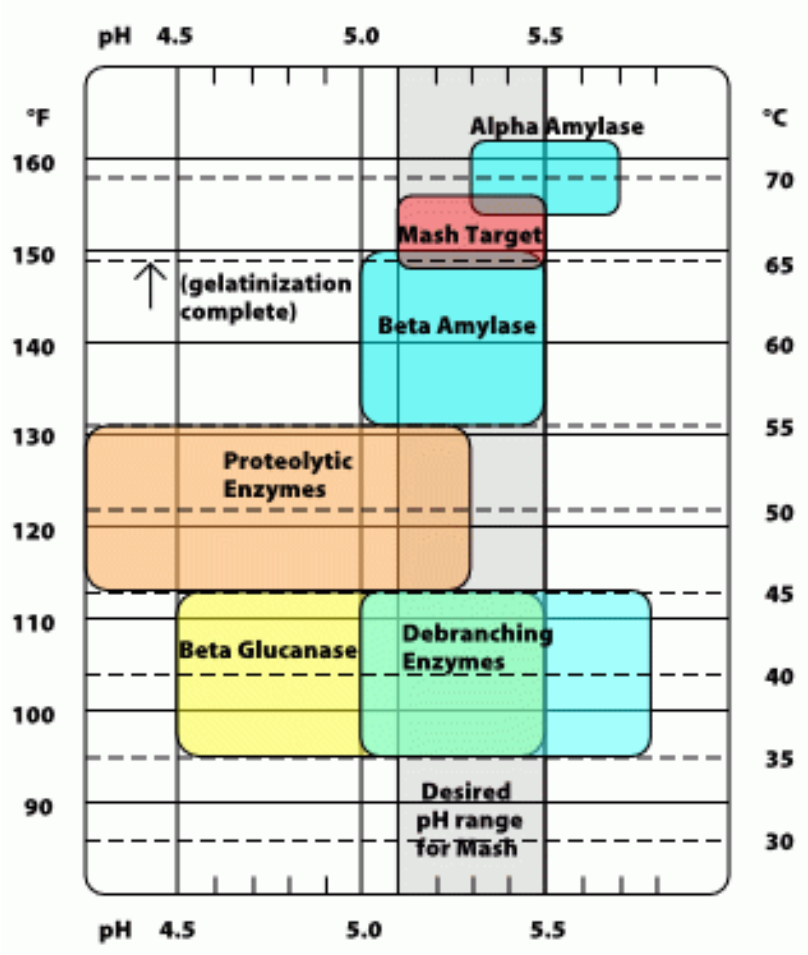

Figure 1.2. Optimal temperature and pH for typical enzymes in the brewer’s mash [19].

In the kettle, wort is boiled at temperatures around 100°C in order to sanitize the liquid, concentrate sugars for the fermentation process, denature the enzymes from the mashing process, volatize unwanted compounds such as dimethyl sulfide, and flocculate any high molecular weight proteins not wanted in the finished product. Hops are also added to the boil at various times in order to impart flavor and balance the sugar with bitterness; the longer the hops are boiled, the more the alpha acids are isomerized, which results in varying degrees of bitterness [20]. The wort is then sent to a vessel called a whirlpool through a tangential inlet, where the force of the vortex creates an eddy that causes solids (termed "trub") to settle in the middle of the vessel, allowing clear wort to be cooled and drawn off for fermentation [21,22]. The entire process outlined above takes place in six to ten hours, depending on recipe and style parameters set forth by the brewer.

3. Fermentation

The wort made in the brewing process outlined above is then cooled to a temperature range of 10 to 30°C depending on the strain of yeast utilized or style of beer desired by the brewer. Brewers' yeast is introduced into a fermentation bioreactor: *Saccharomyces cerevisiae* for the production of ales in the 18 to 30°C range, and *S. pastorianus* for the production of lagers in the 10 to 16°C range [23]. Traditionally, the fermentation of beer takes anywhere from 1 to 6 weeks, and is one of the longest phases in the brewing and malting process. Lagers require longer cold conditioning times that extend the fermentation times to the upper end of that timeframe, while ales can be fully fermented in a week or less [9]. Commercial production of beer utilizes mostly cylindroconical stainless steel fermenters, insulated and surrounded by a double-walled jacket that has glycol or ammonia cooling liquids circulating to keep temperatures in check as fermentation is exothermic [24].

For most of the scientific history of beer, *Saccharomyces cerevisiae* was the yeast used to produce alcohol [25–27] although the first pure culture isolate of brewing yeast was *S. carlsbergensis* (later renamed *S. pastorianus*) [28]. For alcoholic fermentation, the general rule of thumb for the amount of yeast to use, known as the pitching rate, is one million cells per milliliter per percent of sugar in solution [9,12,29]. *S. cerevisiae*, when used at the proper pitching rate, takes the maltose and other sugars produced from the hot side of the brewing process [30], and anaerobically converts the disaccharides into carbon dioxide (CO_2) and ethanol. More than 600 flavor active compounds can also be produced during the alcoholic fermentation process, depending on type of beverage produced (*Fig. 1.3*) [31–33]. Yeast works via an anaerobic pathway of glycolysis; if oxygen is present it performs respiration and cell reproduction [34].

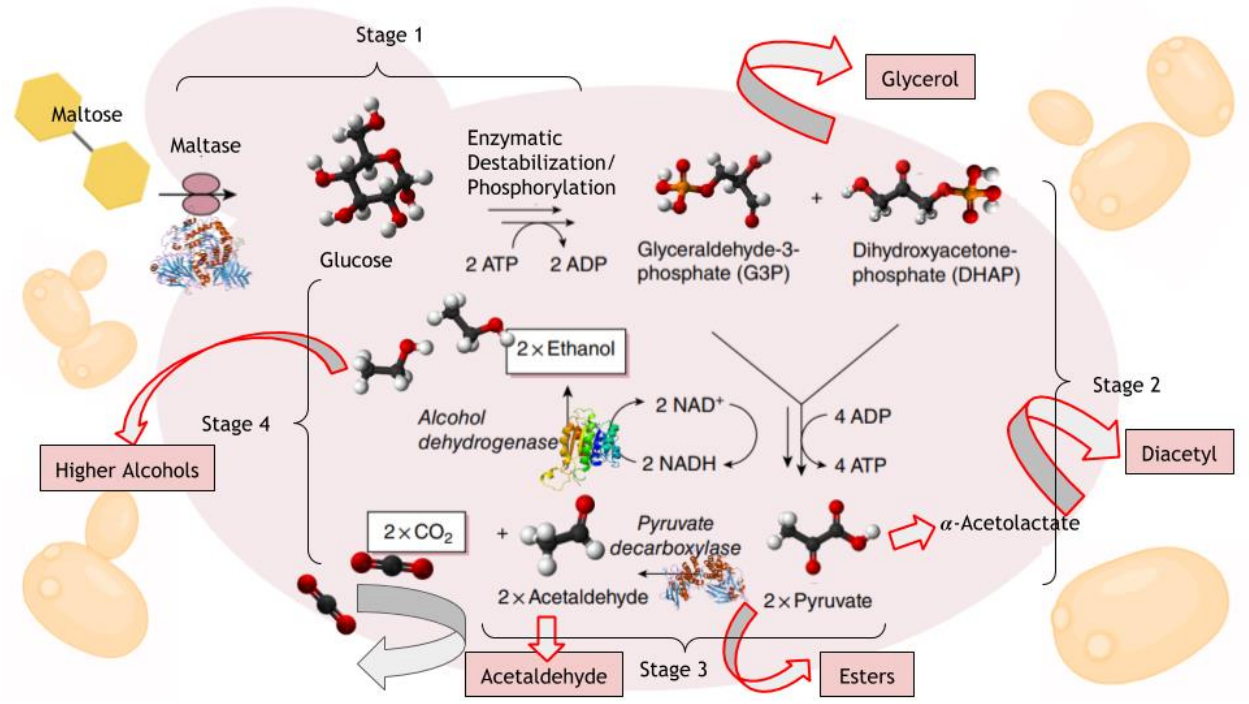

Figure 1.3. The metabolic role of *Saccharomyces* yeast in the development of flavor for fermented alcoholic beverages. The sole products of yeast fermentation are not just ethanol and CO_2, this schematic representation shows the derivation and synthesis of flavor active compounds from sugar, amino acids, and sulfur metabolism, delineated

by the arrows on the diagram. Alcoholic fermentation of beer by *Saccharomyces* is the substrate level phosphorylation anaerobic pathway of glycolysis, which converts maltose sugar into ethanol and carbon dioxide.

At a rudimentary level, sugars are being offered as substrate for yeast to metabolize and release ethanol and carbon dioxide as products via the anaerobic fermentation pathway of glycolysis [35]. The main fermentable sugars extracted in the brewing process include maltose and maltotriose, which are respectively disaccharide and trisaccharide units of glucose bound by α-1,4 linkages. Glucose is also produced in lower quantities than maltose, as well as small amounts of both sucrose and fructose, which *S. cerevisiae* can also utilize in fermentation [23]. The wort sugar profile depends on the raw ingredients and wort preparation procedures, and also relies on the type and the quantity of adjuncts employed. Glucose is preferentially fermented, then maltose is broken down by maltase to form glucose and fermented [36], followed by the assimilation of maltotriose [23].

In Stage 1 of alcoholic fermentation, free glucose is assimilated first, followed by the hydrolysis of maltose or other disaccharides into two glucose, by the enzyme alpha glucosidase (a.k.a maltase, EC 3.2.1.20). Several other enzymatic destabilization and phosphorylation reactions then happen in Stage 1, which turns the substrate into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). Stage 2 oxidizes G3P and DHAP, as well as the ADP generated previously, to create ATP as energy for the cell and pyruvate. Stage 3 enzymatically decarboxylates pyruvate to acetaldehyde and CO_2 that leaves the cell, before the alcohol dehydrogenase (EC 1.1.1.1) converts the acetaldehyde to ethanol in Stage 4 (*Fig. 1.3*).

Sugar from wort is taken directly across the plasma membrane of the yeast, in the case of glucose, maltose, or maltotriose. In the case of sucrose or longer chain dextrins, the sugars must be hydrolyzed prior to uptake, with invertase (EC 3.2.1.26) and glucoamylase (a.k.a. amyloglucosidase, EC 3.2.1.3) respectively. The assimilation of monosaccharides like glucose or

fructose have no energy requirement, whereas disaccharides like maltose or the trisaccharide maltotriose require the energy from ATP to active transport the molecules into the cell [37]. Amino acids are essential for yeast to assimilate during fermentation in order to create new cell wall and membranes for budding or for the synthesis of new structural or enzymatic proteins. These nitrogen compounds can also have a direct relationship to the flavor of the resultant beer, contributing to the formation of higher alcohols, esters, and carbonyls [38]. In brewing, amino acids and nitrogen are measured as free amino nitrogen (FAN); optimum values are dependent on wort sugar levels and yeast strain, but typical levels of FAN are 150 to 400 mg/L [39].

C. Hops for Brewing

1. Botany, Morphology, and Physiology

Humulus lupulus is commonly referred to as the hop plant; it is a viniferous, dioecious, flowering, perennial of the *Cannabaceae* family [15]. Hops are native to Europe and western Asia, but subspecies such as *Humulus lupulus* var. *neomexicanus*, var. *lupuloides*, and var. *pubescens* have been found growing wild in the United States [40]. They are now cultivated on every continent besides Antarctica, with Germany and the United States commanding the largest share of that production [41]. Greater than 95% of hops cultivated worldwide are used for brewing, but some niche uses have presented themselves over the years [42].

Hops grow on rising bines (vines without tendrils) and produce flowers, or cones, but it is the female flower that is most important to the brewer. Males produce pollen, and due to wind pollination the males are generally discouraged in commercial hop fields to lessen the occurrence of seeded hops [43]. The cones of the useful female hop plant consist of a central strig as the stalk of the flower, with attached bracts, or modified leaves, that contain bracteoles, smaller sub-leaves. In the spaces between these bracts and the base of the bracteoles are the lupulin glands,

containing powdery yellow substance called lupulin (*Fig 1.4*). The lupulin glands are small, ovoid, glandular trichomes that secrete hard and soft resins that contain terpenophenolics (alpha and beta acids, oils, terpenes, polyphenols) and other secondary metabolites.

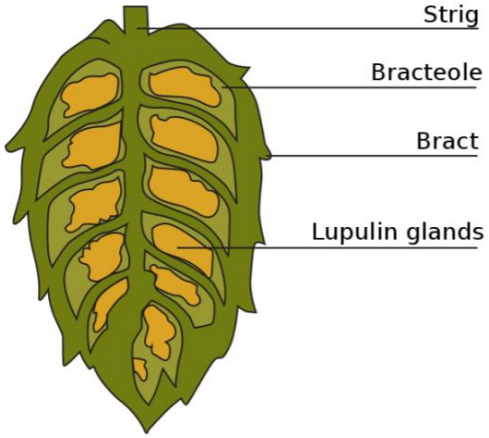

Figure 1.4. Structure of the female hop cone; figure adapted from Hieronymous 2012 [44].

2. Agronomy and Breeding

a. Hop Farming

Hops require an average day length of sixteen to eighteen hours for optimal growth throughout the summer growing season, and prefer temperatures ranging between 15 °C and 18 °C, but can tolerate 5 °C to 25 °C. For these reasons, they are commonly cultivated between the 35° and 55° parallels in both the northern and southern hemispheres [41]. In the United States, hops are primarily grown in the Pacific Northwest states of Oregon, Washington, and Idaho, but recently an emergence of cultivation in the Midwest and Northeast, even small pockets in Texas and California, have supplied local markets [41]. In 2020, just 4.1% of hops in the United States were cultivated outside of the three states in the Pacific Northwest [45].

Hops are grown from rhizomes of underground rootstock in spring, whether planted annually for new cultivars or perennially left in the ground from previous years. They are a climbing bine and grow in a clockwise, helical manner around a twine or wire guide line

attached to a trellis at heights of 5 to 10 meters. The flower of the female hop plant is a strobile, or hop cone, which develops lupulin glands that contain the majority of the chemicals important to brewers [46]. Lupulin glands containing these secondary metabolites are developed during the late stages of female hop bine growth. Hops are eventually harvested in late summer to early fall, when cones have ripened and lupulin content is at its highest concentration [44]. Bracts and bracteoles are fully elongated at the time of harvest, with mass increasing until the final stage of development and accumulation of terpenophenolics (alpha acids, beta acids, and polyphenols), signaling full lupulin gland development [47].

b. Breeding to Trends

The breeding of hops has two main objectives for farmers, to ensure disease and pest resistance from infections like the hop mosaic virus, powdery mildew, or aphids and weevils, as well as to harvest as much as possible, with good yields being greater than 2000 kg per hectare [44]. In addition to growth vigor and disease resistance considerations, hops are bred for their organoleptic properties derived from oils and other terpenophenolics [43]. Hops were traditionally grown for lupulin content and subsequent bittering contributions to the brewing process [25]. With the emergence of craft beer and the rise of American India Pale Ale (IPA), hops were increasingly cultivated for aroma purposes [48].

As with all plant breeding for agronomic purposes, hop breeding is a long and expensive process, taking between 10 and 15 years from genetic cross to commercial production. Take as a case example, one of the most desired hop cultivars in the brewing industry, Nelson Sauvin™ from New Zealand: first selected from seedling population in 1987, it wasn't released for commercial production until 2000 [49]. This cultivar, while now prized for it's unique "grape-like" aroma, didn't reach commercial success until well over a decade later.

c. Hop Cultivars

Many breeds become proprietary cultivars, like Nelson Sauvin™ or more famously Citra®, which are owned by hop suppliers and growing groups, requiring farmers to pay licensee rights in order to produce them. More than 100 named cultivars (varieties) of hops exist [50,51], with five subspecies based upon morphology and geographical location [52]. The most popular cultivars for growers have changed over the last six years, following the trends of brewers in the craft beer industry and the changing consumer demands. Citra®, an aroma hop used heavily in modern IPAs, has held the top spot in terms of acreage grown in the United States for the last three years, prior to that it was Cascade, famous for its use in Sierra Nevada Pale Ale. (*Table 1.2*). Centennial, Willamette, Mosaic®, and Amarillo® cultivars were all used in various parts of the research herein.

Table 1.2. Top ten hop cultivars in the Pacific Northwest of the United States for the years 2015 to 2020, with total hectare listed in parenthesis for each, as well as the total hectares of the top ten and all other cultivars grown in the states Oregon, Washington, and Idaho. Figure adapted from Hop Growers of America 2020 Statistical Report [45].

Rank	2015	2016	2017	2018	2019	2020
1	Cascade (2748)	Cascade (3068)	Cascade (2944)	Citra® (2692)	Citra® (3656)	Citra® (4450)
2	CTZ (2154)	Centennial (2057)	Centennial (2240)	CTZ (2469)	CTZ (2646)	CTZ (2544)
3	Centennial (1781)	CTZ (1820)	Citra® (2138)	Cascade (2432)	Cascade (2137)	Mosaic® (2224)
4	Simcoe® (1338)	Citra® (1819)	CTZ (2004)	Centennial (1968)	Simcoe® (1766)	Simcoe® (1665)
5	Citra® (1211)	Simcoe® (1753)	Simcoe® (1861)	Simcoe® (1585)	Mosaic® (1710)	Cascade (1634)
6	Mosaic® (728)	Mosaic® (1022)	Mosaic® (1122)	Chinook (1149)	Centennial (1489)	Centennial (1187)
7	Chinook (723)	Chinook (785)	Chinook (983)	Mosaic® (1120)	Amarillo® (959)	Pahto® (894)
8	Summit (656)	Summit (716)	Willamette (671)	Amarillo® (1106)	Chinook (958)	Amarillo® (870)
9	Willamette (550)	Willamette (632)	Summit (654)	Pahto® (671)	Pahto® (870)	Chinook (766)
10	Apollo™ (402)	Apollo™ (393)	Apollo™ (369)	Summit (637)	Summit (434)	El Dorado® (641)
Top 10 Total	12,292	14,063	14,727	15,830	16,626	16,874
Other Hectare	5,883	7,370	7,555	6,442	6,257	6,857
Total Hectare	18,174	21,433	22,282	22,272	22,883	23,731
Top Ten %	67.63%	65.61%	66.09%	71.08%	72.66%	71.11%

3. Processing and Hop Products

Upon harvesting, hop cones contain 75% to 80% moisture and must be processed quickly in order to prevent deterioration and microbial growth. Hops are mechanically picked from bines

and transferred to kilns where moisture is reduced to about 10% in a kiln at temperatures between 50 °C and 75 °C over the course of twenty-four hours with convective heat. Traditionally, drying methods focused on preserving the bittering quality of hops and not necessarily the volatile aromatics desired in craft brewing [44]. Over the course of the last three decades, kiln temperatures have slowly dropped in order to preserve these aromatics [53,54], but this lowered temperature no longer denatures the amylolytic enzymes that are meant to aid the hop plant during growth [55]. These enzymes can have detrimental effects on the quality of dry-hopped beer in a phenomenon known as hop creep, discussed further in the following section. After drying, hop cones are conditioned at room temperature for an additional twenty-four hours prior to being compressed into 100 kg bales that are 120 cm long by 60 cm tall and wide.

From bales, hops can either go straight to the brewer to use as whole cone hops, or be processed in one of several different forms that allow several unique advantages. Due to its value in brewing, lupulin is concentrated and recovered from hop cones through optimized processes such as mechanical sieving or CO_2 extraction. The most common form of processing is into pellets, which are hammer-milled hop cones that are extruded through a die. These pellets come in two types, T-45 or T-90, based on the percentage of mass that remains from the original hop cones; T-45 hop pellets have been cryogenically processed to shatter the cones and remove the bract and strig, leaving behind lupulin powder, so pellets of this type are more concentrated. Lupulin powder, known as T-35, has had even more of the vegetative matter removed and is idealized for aroma purposes [44].

There also exist a vast array of advanced hop products that involve even greater processing from their start as hop bales. Kettle extracts are the most common; a solvent, either ethanol or supercritical CO_2, is used to extract the resinous alpha and beta acids, along with some

polyphenol material and can add bitterness without the vegetative hop material of pellets or whole cone [43]. Isomerized Kettle Extracts (IKE) are more sophisticated than the previously mentioned hop extract, as the alpha-acids used for bittering have all been extracted from the hops and converted into its isomeric form via magnesium sulfate catalysis [56]. There are light stable extracts that do not degrade in UV light called *rho*- or *tetra*-hops; these contain reduced isomerized alpha-acids and are used either in the kettle or post fermentation for bittering adjustments and can also protect against mercaptan off-flavors [43]. *Hexa*-hops, produced from a combination of both borohydride reduction and hydrogenation using a palladium/carbon catalyst, are used for the production of a very stable foam on the beer [15]. Last but not least are thin film evaporated or steam-distilled hop oils; these products contain the complete range of essential oils found in the hops, offering extra aroma that can be added at any time during the fermentation process [43].

4. *Dry Hopping and Hop Creep*

Occasionally, hops are added to green beer during fermentation, while yeast is active or once it has finished fermenting, a process known as dry-hopping. This was historically performed to provide packaging and transport stability for ales in the transport of casks from brewer to publican. In fact, the term "dry-hopping" likely originates from observations of British brewers noting that adding hops to cask beer caused it to referment and become drier, reducing the levels of residual dextrins that contribute to mouthfeel [57]. More recently, dry-hopping is performed in order to add intense aroma and flavor to finished beer, particularly the India Pale Ale (IPA) style, as the hops are not boiled and therefore volatile aromatics are retained [58,59]. The Brewers Association, the largest trade group of craft brewers in the United States, has continually recorded IPA to be the most purchased beer style in the country, and with historic

numbers of these small breweries operating there is more IPA, and subsequently more dry-hopping, than ever before [60].

With more dry-hopping in modern beer production, brewers pushed for increase aromatics from their hop suppliers and farmers, which has led to lower kilning temperatures (50-55 ºC) at the farm in order to preserve volatile compounds for brewing [53]. With the decrease in hop kiln temperatures, comes an increase in diastatic enzymes, meant to break down starch reserves for plant growth, in the hops that would have previously been denatured at the higher kiln temperatures (75 ºC) [55]. With more of these residual enzymes in hops, and more dry-hopping than ever before, brewers have anecdotally mentioned recipe deviations from this addition of large amounts of hops into the fermenter. The observed deviations can result in higher alcohol and lower residual sugar contents, which not only effect the mouthfeel of the beer, but can come with consequences from the regulatory bodies in the United States and elsewhere [61].

In an industry that also touts consistency even as crops change, this deviation can lead to more inconsistent quality in the finished beer. If dry-hopped beer is not pasteurized prior to packaging, the enzymes from the hops will continue to hydrolyze residual dextrins, resulting in a different sugar profile. Concurrently, if this dry-hopped beer is not sterile filtered to remove yeast, any remaining yeast can begin to referment the newly hydrolyzed sugars. Any yeast that remains in the beer to this point has already gone through primary fermentation, and likely a cold conditioning cycle of -2 ºC to 0 ºC for an extended period of time, meaning it is highly stressed and vitality is low. This stressed yeast is far more likely to produce yeast derived off-flavors, such as acetaldehyde [62], diacetyl [63], and mercaptan [64]. Craft brewers, in large part, do not have the technology or resources necessary to pasteurize or sterile filter their beers, so this stands as a large quality issue. Most concerning though, is the production of CO_2 from extra fermentation in

a packaged product, as this can become a consumer safety risk when bottles or cans start to explode from over-pressurization.

D. Yeast in Brewing

The flavor of beer is largely determined by the yeast strain utilized, together with the wort composition and hops employed. Yeast properties including flocculation, fermentation ability (including the uptake of wort sugars and amino acids), ethanol tolerance, and oxygen requirements have a crucial impact on fermentation and the resultant aroma and taste of beer. Additionally, the environment that brewers' yeasts are routinely subjected to can be deemed stressful, if not harsh. Yeast experience fluctuations in oxygen at the start of fermentation, and a subsequent accumulation of carbon dioxide as fermentation progresses. pH falls drastically during fermentation, which can be made even more extreme if acid washing is practiced between consecutive fermentations. Brewing yeasts are under hyperosmotic stress dependent on concentration of sugar in the wort they are fermenting. They experience accumulation of metabolites that can become toxic in large quantities, including acetaldehyde, ethanol, and organic acids. An average brewery fermentation can easily see temperature shifts from around 25 °C to -2 °C, dependent on recipe parameters.

Even under these stressful conditions, the brewing industry is the only fermented alcoholic beverage that reuses its yeast from batch to batch [65]. In brewing, yeast is typically reused (repitched) for ten generations or more [9], while in wine, the yeast is generally used far lesser times, due to the prominence of other microorganisms and the higher mortality from more stressful conditions of osmotic pressure and higher ethanol concentrations [66]. In most cases, serial repitching can cause genetic mutation within the cells and the desired flavor profile might no longer be attainable [67–70].

1. *Morphology and Physiology*

Brewers' yeasts commonly come from the genus *Saccharomyces*, and are unicellular fungi that have ultrastructure features similar to that of higher order eukaryotic cells. They contain a cell wall, plasma membrane, mitochondria, Golgi apparatus, endoplasmic reticulum (ER), vacuoles and microbodies, secretory vesicles and a nucleus (*Fig. 1.5a*). The cell wall is mostly composed of carbohydrates surrounding the cell and is a semi-rigid structure, approximately 250 nm thick and comprises 25% of the dry weight of the cell [23].

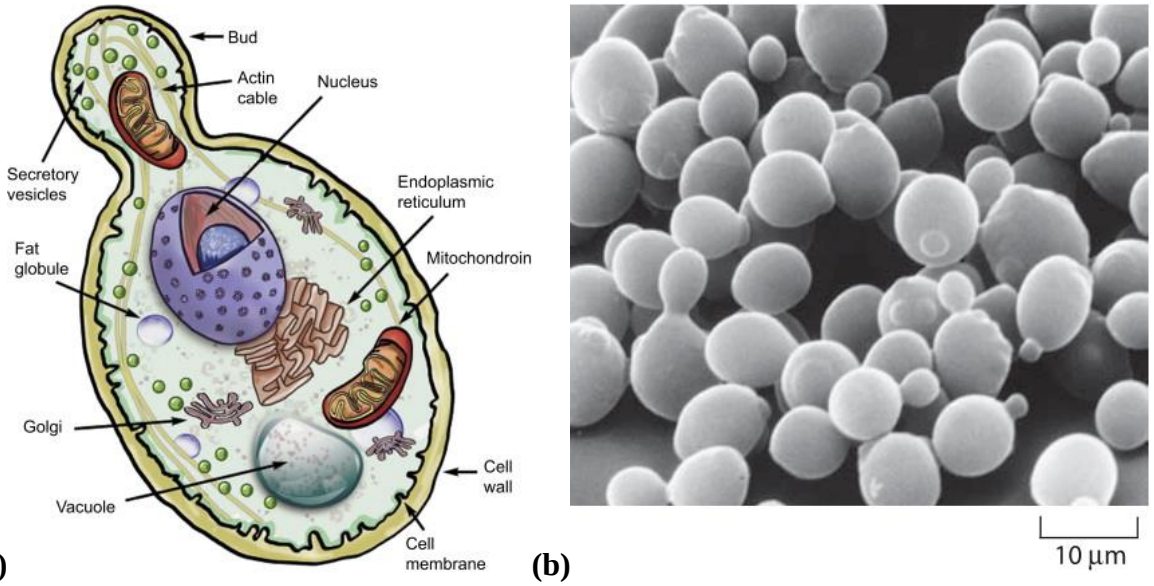

Figure 1.5. **(a)** Diagram of a yeast cell taken from Russell and Stewart 1998 [71], and **(b)** electron micrograph of yeast with visible budding cells and bud scars taken from Bokulich and Bamforth 2017 [65].

Saccharomyces yeast are generally ellipsoid in shape, but can also be spherical or cylindrical, with a diameter ranging from 5 µm to 20 µm. *Saccharomyces* generally occur singly or in pairs, but can occasionally occur in short chains or clusters. Nearly all brewers' yeasts reproduce asexually by budding, creating bud scars composed of chitin as the cells separate (*Fig. 1.5b*), but they can also reproduce sexually by the conjugation of cells of opposite mating types.

As bud scars accumulate, the yeast runs out of room for asexual reproduction, and therefore does not clone itself more than about twenty times over the course of its lifespan [65].

2. Common Brewing Yeasts

Brewing yeasts are most commonly of the genus *Saccharomyces*, and consist of either the ale fermenting species of *Saccharomyces cerevisiae* or the lager fermenting species of *Saccharomyces pastorianus*. *S. cerevisiae* is a widely studied model organism as it is easily propagated in the lab, and was the first eukaryote to have its genome sequenced completely back in 1996 [23]. The yeast is used in the production of bread and several fermented beverages, including beer, wine, and the pre-distillation washes for whiskey, rum, gin, vodka, and brandy. In beer, ale fermentations with *S. cerevisiae* occur at 16 ºC to 25 ºC, and are referred to as top-fermenting because of the yeast's propensity to flocculate and rise to the top of the fermentation vessel with the production of CO_2 [1]. This type of yeast is far more popular in craft beer, as the total fermentation time can be two weeks or less, allowing for quick turnaround and higher production output with limited space and resources, while also producing higher amounts of flavorful secondary metabolites. *S. cerevisiae* has the ability to assimilate glucose, fructose, sucrose, maltose, maltotriose, and with var. *diastaticus*, even larger oligosaccharides containing α-1,4 linked glucose molecules [23].

The most important scientific factor differentiating *S. pastorianus* strains are their ability to utilize the disaccharide melibiose (an α-1,6 linked glucose and galactose) in addition to the sugars assimilated by *S. cerevisiae.* This property can be used in diagnostic tests to distinguish between ale and lager yeast strains in the brewing industry [65]. *S. pastorianus* is the most widely used brewing yeast in the world, used for lager production at temperatures of 7 ºC to 15 ºC. It is referred to as bottom-fermenting because of its propensity to flocculate and fall to the bottom of

the fermentation vessel, where it can be easily removed from the tank for use in further fermentations [23]. Fermentations with lagers proceed slower at the colder temperatures, finishing in four to eight weeks, requiring conditioning time to produce the clean and delicate flavors of lager beer. While these beers require more time and space for production, they have become pervasive worldwide, as consumers appreciate the refreshing characteristics and lighter body of lagers [72].

3. Hybridization

Interspecies hybrids occur when two or more species of the same genus contribute genetic material to offspring. *S. pastorianus* is itself the most famous and successful in the *Saccharomyces* genus, resulting from a hybridization event of *S. cerevisiae* and the cold-tolerant species *S. eubayanus* [73]. Many of the hybrids within the Saccharomyces genus are not the result of laboratory manipulation or genetic modification, but rather have occurred naturally in industry, as yeasts with desired characteristics were selected and propagated for commercial beer [74]. While hybrid species are generally selected for their optimal manufacturing characteristics in the brewing industry, they can also be selected for their organoleptic properties and increased yield of desired aromatic compounds [75]. One of these such selected interspecies hybrids was chosen for its increased production of terpene compounds common in wine grape varieties [76]; these compounds are more increasingly beneficial in hop focused beers [77].

4. Non-Conventional Yeasts in Alcoholic Beverage Fermentation

a. Non-Saccharomyces

A vast amount of research exists on non-*Saccharomyces* yeast strains, such as *Brettanomyces, Pichia, Hanseniaspora, Metschnikowia,* and *Torulaspora* in the brewing industry [78–80]. *Brettanomyces* spp. are still considered a spoilage organism responsible for off-flavor

production in wine, cider and dairy fermentations, but can add desirable organoleptic characteristics to lambic and gueuze beers [81], contribute interesting aromas to hoppy beers [82], and have even been explored for their valuable properties like high ethanol yield and tolerance to low pH [83]. Like *Brettanomyces*, the genus *Hanseniaspora* can add desirable aromatic qualities in mixed-culture fermentations such as lambic, but ferment slowly and are not ideal at lower temperatures [84]. *Pichia* spp. are also considered spoilage organisms in beer, as they contribute to gushing in package product [85], but can be desirable in some mixed fermentations and indigenous beer [86]. Beers produced with *Metschnikowia* spp. have promise for low and non-alcoholic products, but consumer preference requires their inclusion in a mixed-culture fermentation [87]. *Torulaspora* spp. in the production of beer produced increased levels of citronellol and linalool [88], which are terpenes of great interest in the fermentation of hop forward beers [89].

b. Non-Conventional Saccharomyces spp.

The search for unique flavors and aromas, and a desire to invoke new technologies and techniques for making alcoholic beverages led to the use of non-*cerevisiae Saccharomyces* spp. in the alcoholic fermentation process [90,91]. While the most widely used non-*cerevisiae* species is *S. pastorianus*, traditionally used in the production of lager beer around the world [92–94], this section focuses on some of the more distinct species. The focus is on five species of *Saccharomyces sensu stricto* (*Sss*) yeasts, *S. kudriavzevii*, *S. paradoxus*, *S. mikatae*, *S. uvarum*, and *S. bayanus*, as well as other non-conventional species not currently in the *Sss*, such as *S. abulensis* and *S. florentinus*. When selecting yeast strains for fermentation, brewers consider its attenuation (the amount of sugar consumed by the yeast), flocculation (the yeast's ability to clump together and fall out of solution), fermentation temperature range, effects on flavor profile, capacity for reuse, and supply chain availability [95]. These facets, as well as a

yeast's ability to ferment various carbon sources, morphological characteristics, and genetic hybridization can all assist brewers, when adopting a new strain.

i. *Saccharomyces* Species Diversity

Since Louis Pasteur's groundbreaking and historic report that fermentation was caused by a microorganism instead of a spontaneous mystery [8], the *Saccharomyces* genus was continuously studied, with several distinct species identified [96]. This diversity was termed the *Saccharomyces sensu stricto* (*Sss*) complex and is currently composed of ten genetically distinct species, all of which are capable of metabolizing glucose to produce ethanol (*Fig. 1.6*). Each of these species was perceptibly delineated from other *Saccharomyces* species, through studies of reproductive isolation and application of the biological species concept [69,97–99]. All non-conventional *Sss* species were isolated from unique sources in nature, including tree bark, flowers, fruit, and insects, while *S. cerevisiae* and *S. pastorianus* were isolated from environments associated with bread or alcoholic fermentations, demonstrating their lineage from wild type to the cultured stock of *Saccharomyces* spp[100]. While all members of the *Sss* were proven to produce energy with fermentation, and many of these species are non-conventional, some were used and studied for their potential use in commercial alcohol production for human consumption. The distribution of *S. cerevisiae* and *S. pastorianus* were long linked to alcoholic beverage production, along with minor mentions of other species in the *Sss* complex. Cultured species, specific to beer production, were shown to have evolved from European wine and Asian sake fermentations [27,101], therefore, its relation to wine production proliferates much of the research.

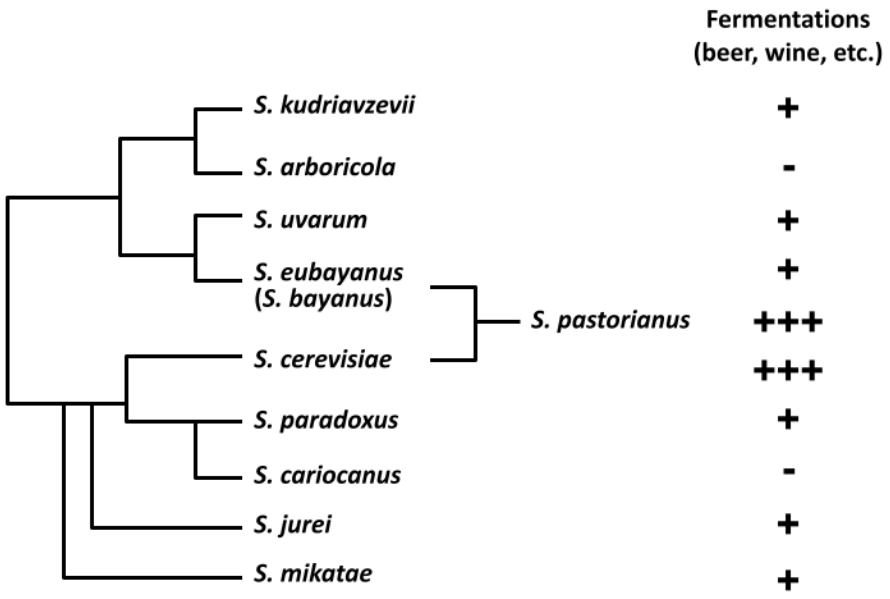

Figure 1.6. *Sss* phylogeny and extent of use in alcoholic beverage fermentations. *Saccharomyces bayanus* is listed in parenthesis to indicate it was derived from multiple hybridization events [73]. *S. pastorianus* is shown as a genetic hybrid of *S. eubayanus* and *S. cerevisiae* [93]. Use in fermented beverages is indicated with plus signs (+) for current commercial use, with *S. cerevisiae* and *S. pastorianus* exhibiting the most ubiquitous use in beer, and negative signs (−) for no known use. *S. cariocanus* is known to be harboring just four translocated chromosomes different than *S. paradoxus* [97]. *S. jurei* has very recently been proven to have brewing potential [102].

ii. *Saccharomyces kudriavzevii*

S. kudriavzevii was first isolated from a decaying leaf and has since been isolated repeatedly from the bark of oak trees in Portugal and France [103,104]. The yeast is a multipolar budding species, with a size of 5–10 µm, and an oval to slightly elongated shape [97]. It was shown to ferment glucose, sucrose, and maltose, but it did not ferment lactose, melibiose, or starch, which are common characteristics of *Sss* yeast (*Table 1.3*). *S. kudriavzevii* is a naturally occurring *S. cerevisiae* hybrid that might constitute 23–100% of the genome for some yeast [105,106], including Belgian trappist ale strains, such as Chimay, Westmalle, and Orval, and was also genetically isolated in draft beer from the United Kingdom, Germany, and New Zealand [107].

This implies that the attenuation, flocculation, and flavor profiles of *S. kudriavzevii* might be similar to that of most Belgian strains. This meant low flocculation, high attenuation, and phenolic off-flavor positive (POF+) [95,108], though there is research in the wine industry that suggests *S. kudriavzevii* ferments slowly and produces less ethanol when used on grape juice [109]. Other research suggests it has high flocculation, as in overnight liquid culture, it grew into spherical 2–3 mm pellets [69].

Table 1.3. Physiological characteristics that distinguish each species of the *Saccharomyces sensu stricto* complex are discussed. Growth ability scored as positive (+), negative (−), evidence of both positive and negative (−,+), and unknown (u). Ethanol tolerance is defined as being able to grow in the presence of 2.5% *v/v* EtOH, the low-end strength of standard beer. Attenuation and flocculation scored on a relative basis scale, ranging from low, to moderate, to high. Type strain as defined in MycoBank (mycobank.org), origin, isolation, and commercial availability, as defined in the cited literature.

	S. kudriavzevii	***S. paradoxus***	***S. uvarum***	***S. mikatae***	***S. bayanus***
Fermentation:					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Melibiose	-	-	+	+	-,+
Dextrins (STA1)	-,+	-	-,+	-	-
Ethanol Tolerant	+	+	+	+	+
Characteristics:					
Attenuation	moderate	low-moderate	moderate	moderate	moderate
Flocculation	moderate-high	moderate	high	moderate	moderate
Growth at 10ºC	+	+	+	+	+
Growth at 25ºC	+	+	+	+	+
Growth at 37ºC	-	-	+	-	+
POF	+	u	-	u	+
Region of Origin	Western Europe	Northeastern Europe	Scandinavia	Japan	Europe

	S. kudriavzevii	*S. paradoxus*	*S. uvarum*	*S. mikatae*	*S. bayanus*
Isolated From	Oak tree bark	Oak sap	Fruit / Seeds	Soil / Leaves	Insects / Leaves
Type Strain	NCYC 2889T	DBVPG 6411	DBVPG 6173	NCYC 2888T	CBS 380
Commercial Availability	Anchor Vin7	Anchor Exotics SPH	AWRI 1176 & 1375	AB Biotek / AWRI 2526	Lalvin S6U

It is advised to ferment *S. kudriavzevii* in tandem with a traditional *Saccharomyces* [74] and it was shown to form a triple hybrid complex with *S. cerevisiae* and *S. uvarum*, as it was isolated as such from farmhouse ciders made in France [110,111]. *S. kudriavzevii* is a cryophilic strain in the *Sss* that prefers fermentation temperatures in the 10–15 °C range [103,112,113], and is currently used to ferment lower temperature pinot noir and lager beer in Europe [105]. The only current commercially available example is Anchor Oenology's Vin7 strain, developed in Stellenbosch, South Africa, for enhancing thiol aromas in white wine [114,115], but it stands to reason that it can be isolated from previously noted commercial beer examples. Due to its cryophilic tendencies and aromatic potential, *S. kudriavzevii* has potential for use in the production of hoppy lager beers in the brewing industry. Further research remains to be done on this species, considering it is POF+ and it is likely also diastaticus (*STA1*) positive, meaning it could ferment residual maltodextrins. Additionally, minimal commercial production was done with the direct intention of using *S. kudriavzevii*, as most fermentations did not take place with the intention of the use of this species.

iii. *Saccharomyces paradoxus*

S. paradoxus is one of the first isolates of the *Sss* [116], a wild-type strain commonly isolated from the bark of deciduous trees and occasionally from fruit and insects in North America and Eastern Europe [117–119]. Even though genetically *S. cerevisiae* and *S. paradoxus* were proven to be distinct species [120], phylogenetically the two were the closest relatives in

the *Sss* (*Fig. 1.6*) and were 90% genetically similar [106]. They share the same morphological and phenotypic characteristics, such as being spherical or ellipsoid in shape, with a diameter of 1–5 µm [121]. Previous research indicates mixed results of the fermentative capacity of *S. paradoxus*, but it has the ability to convert glucose into ethanol and a relatively high alcohol tolerance [97,122,123]. It is a positive fermenter for glucose, sucrose, and maltose, but it does not ferment lactose, melibiose, or starch (*Table 1.3*). Little evidence exists for the domestication and commercial use of *S. paradoxus* in alcohol fermentation, but it was found to be naturally co-fermenting with *S. cerevisiae* in Eastern European wine fermentations [123,124], as well as with *S. cerevisiae*, *S. bayanus*, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, and *S. pastorianus* in indigenous African sorghum beer [125].

In laboratory fermentations, the optimal growth temperature for *S. paradoxus* falls 7 °C lower than *S. cerevisiae*, and is likely cryophilic, due to the climates in which it is found, but *S. paradoxus* is yet to be trialed extensively in a production environment [126,127]. Unfortunately, no information exists on the attenuation or flocculation characteristics of *S. paradoxus*, nor are there any commercially produced examples of the purely isolated species, but it does seem to have positive sensory attributes in white wine fermentations [123,124]. There is a commercially available hybrid of *S. paradoxus* and *S. cerevisiae* produced by Anchor Oenology, which when used for Syrah and Merlot wine fermentations, shows increased aromas of cherries, strawberries, cocoa, and floral notes, and the wine is described as full-bodied, well-balanced, complex and intense [128]. Much work remains to be done on the versatility of this species for the brewing industry, but it might have potential for unique and novel flavor characteristics if a pure culture from a culture collection is obtained for further experimentation.

iv. *Saccharomyces mikatae*

S. mikatae is a wild yeast that has been found as part of the genome of a natural genetic hybrid resulting from introgression events with *S. cerevisiae* and *S. paradoxus* [106,129]. Current *S. mikatae* hybrids described for use in industrial wine fermentation were deliberately created in a lab with the intent of adding complexity in resultant wines, akin to those that are spontaneously fermented, but more easily controlled due to the inclusion of typical *S. cerevisiae* yeast [130,131]. *S. mikatae* was first isolated from decaying leaves and soil in Japan [97,110]. It is ovoid in shape and approximately 5–9 µm in diameter; it reproduces by multipolar budding, and generally appears in pairs or short chains. *S. mikatae* was also shown to form a pellicle after 25 days at 20 °C, similar to *Brettanomyces* and other wild-type yeasts [97]. The inclusion of *S. mikatae* in the *Sss* means it is capable of alcoholic fermentation and assimilation of glucose, it is also capable of fermenting maltose, sucrose, and melibiose, but not lactose or starch (*Table 1.3*). However, *S. mikatae* might have a lower attenuation, due to genetic diversion from *S. cerevisiae*, while still exhibiting similar levels of flocculence [69].

S. mikatae readily creates hybrids with *S. cerevisiae*, and these hybrids were shown to produce higher concentrations of multiple compounds that yield fruity, banana, floral, and sweet perfume aromas in the fermentation of white wine. Although no information on beer fermentation with either the type strain or any hybrids exist, the additional amounts of certain volatile compounds in the research by Bellon et al. [130,131] might show signs of this yeast's production of phenolic off flavors. *S. mikatae* grows readily in temperatures from 4–30 °C, with expected slower growth in the range limits and no growth outside the range, making it a cryotolerant fermenter [97,113]. Commercial availability is limited, but yeast manufacturer AB Biotek commenced exploratory production of an *S. mikatae* and *S. cerevisiae* hybrid, AWRI

2526; brewers and winemakers can expect the hybrid as an active dried yeast product that is expected to be available for trials, by the fall of 2020 [131].

v. *Saccharomyces uvarum*

S. uvarum is a fairly well-known member of the *Sss*, originally believed to be identical to *S. bayanus* and often referred to as *S. bayanus* var. *uvarum*, it was shown to be a genetically distinct *Saccharomyces* species [132–134]. *S. uvarum* is also similar in size and shape to *S. bayanus*, being spherical or ellipsoid in shape, with a diameter of 1–5 µm, and reproducing by multipolar budding. *S. uvarum* was isolated in natural European wine and cider fermentations [135–137], as well as in South American chicha fermentations [138,139], but was first isolated in 1894 and described in 1898 by M.W. Beijerinck, from spontaneous wine fermentation [140]. *S. uvarum* is known to hybridize with *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* [135,141,142], and thus can show signs of being POF+ and possibly might have the *STA1* gene for diastaticus [141,143]. *S. uvarum* showed the capacity to ferment glucose, sucrose, melibiose, and maltose, but it does not ferment lactose. *S. uvarum* is a known bottom-fermenting yeast, meaning it acts similar to a *S. bayanus* or *S. pastorianus* when not in hybrid form, offering cryotolerance [144], moderate attenuation, and high flocculation (*Table 1.3*).

Research with wine showed that *S. uvarum* produces comparatively higher amounts of volatile aromatics when fermented cold [145], implying potential use as a lager strain. In Chardonnay winemaking trials, wines were described as showing apricot, cooked orange peel, citrus, lime, honey, and nutty aromas with some tasters, and estery, pineapple, peach, melon, and floral aromas with others [130,146]. While *S. uvarum* continues to predominate in spontaneous European wine fermentations [136,137,147] and is a known species in some Norwegian kveik hybrid strains [141], its commercial availability is limited to the Australian Wine Research Institute at this

time [148]. Cryotolerance and increased aromatic potential means *S. uvarum* could be used in the production of some complex and eccentric lagers in the brewing industry, but currently, it has only been isolated as part of a hybrid culture in the aforementioned kveik beer. Further research remains to be done on the brewing potential of *S. uvarum*, but brewers should be aware of the increased aromatic character that might come from the POF+ genes.

vi. *Saccharomyces bayanus*

S. bayanus is a well-studied species in the *Sss* and is often used as a model organism for comparative functional genomics studies of yeast, based on introgression and interspecific hybridization [149]. It is genetically similar to *S. cerevisiae*, but evolved to be a distinct member of the *Saccharomyces sensu stricto* complex [106,150], and is now referred to as *S. bayanus* in order to delineate it from *S. eubayanus* and *S. uvarum*, of which it is a hybrid [73,93]. *S. bayanus* was previously thought to be the parent of the lager strain, *S. pastorianus* [93,110,135], but the hybridization event that produced lager brewing yeast is now proven to have occurred between *S. cerevisiae* and *S. eubayanus* [27,101,151,152]. While *S. bayanus* might not be the true hybrid parent of the most used brewing yeast in the world, it still forms natural hybrids with other members of the *Sss* and was identified in these complexes in the fermentation of wine [112,135,153]. While it was first isolated from turbid beer in 1927 [72], *S. bayanus* was also isolated from beer, wine, fruit, and even soda [97]. *S. bayanus* is ellipsoid to elongate in shape, with a diameter of 1–5 µm, and reproduces by multipolar budding. Research showed it to be a positive fermenter for glucose, sucrose, and maltose. Other studies reported *S. bayanus* to show both positive and negative fermentation of melibiose, but it does not ferment lactose or starch (*Table 1.3*).

S. bayanus is well-known as a fermenter of beer and cider [72,93,154], but is most commonly used in wine [64,146,151]. It can be purchased from several commercial suppliers, but unfortunately

several commercially available strains were genetically identified as *S. cerevisiae*, including the famous Lalvin EC-1118 strain that was originally typed *S. bayanus* [155,156]. *S. bayanus* ferments best in the upper end of the lager strain temperature range of 10 to 21 °C [73,127], is moderately flocculant [69], and has a fairly standard attenuation [157], as expected, given its genetic similarity to *S. pastorianus* lager yeast. The commercially available hybrid of *S. bayanus* and *S. cerevisiae* available from Lallemand, Lalvin S6U, is known to increase the varietal characteristics in white wine, and might produce elevated levels of POF [158,159]. More research needs to be carried out with regards to the flavor profile of beers made with *S. bayanus*, but the research on wine and its history as a potential lager strain means, it is capable of fermenting remarkable lager style beers.

vii. Other *Saccharomyces* spp. Used in Alcoholic Fermentation

Several other non-conventional species of *Saccharomyces* used in alcoholic fermentation were determined to be genetically distinct by current research but might not yet be included in the *Saccharomyces sensu stricto* complex. *S. abulensis* is a novel species dubbed the "Santa Maria strain" and was isolated from yeast originating from breweries in Madrid and Sevilla, Spain [142]. *S. florentinus*, formerly known as *S. pyriformis*, is a species of yeast isolated from the scoby of traditionally fermented ginger beer, known as "bees wine," but is yet to be used in commercial production of beer [160,161]. Three other strains included in the phylogenetic tree of the *Sss* (*Figure 1.6*) exist—*S. arboricola*, *S. jurei*, and *S. cariocanus*—but research is limited on their fermentation capacity. *S. arboricola* is a wild-type hybrid of *S. bayanus* and *S. kudriavzevii*, which was isolated from oak and beechwood bark in China [162,163], and is currently being used in sake production [164]. *S. jurei* is closely related to *S. mikatae* and *S. paradoxus* and was isolated from a high-altitude tree bark in France; little is known of its fermentative capacity [165]. *S.*

cariocanus, isolated from insects in South America [166], is a wild-type hybrid of *S. paradoxus*, which is capable of fermenting sucrose and shows ethanol tolerance [167].

These yeasts are not members of the *Sss*, but is likely to be included, as the complex underwent many changes over the years, in accordance with the system employed in classifying yeast cultures. Very little information exists on these yeasts' ability to ferment beer or their use in a commercial setting, but by contacting yeast culture collections directly, the strain could be obtained for further experimentation. There also exists multiple variants of *S. cerevisiae* that have not distinguished themselves genetically from the species, such as var. *boulardii*, which is known to produce higher levels of polyphenols, and can thus be used in functional probiotic beer [168–173]. Another variant, var. *diastaticus*, can cause over-attenuation [174–176], which was discussed earlier as having the *STA1* gene. *S. cerevisiae* var. *chevalieri* may present benefits in low or no-alcohol fermentations of beer as it does not have the ability to assimilate maltose [177–179].

Chapter 2

Hop Creep Confirmation and Bench Method Development

Published in the Master Brewer's Association of the Americas Technical Quarterly

Bruner, J. R.; Williams, J.; Fox, G. P. Further Exploration of Hop Creep Variability with *Humulus lupulus* Cultivars and Proposed Method for Determination of Secondary Fermentation. *TQ* 2020, *57* (3), doi:10.1094/TQ-57-3-1002-01.

Poster, "Confirmation of Endogenous Enzymes in *Humulus lupulus* and Proposed Method for Determination of Secondary Refermentation," presented at World Brewing Congress Connect 2020 (*Appendix A.1*).

1. Abstract

Dry hopping beer to add aroma is a long-standing practice, and recently brewers have been adding greater quantities in an effort to get even more hop aroma. This has led to a seemingly new phenomenon termed “hop creep,” in which the dry hop addition leads to additional attenuation and lower final gravity than expected. Studies have shown this to be caused by endogenous starch-degrading enzymes in hops hydrolyzing unfermentable dextrins into fermentable sugars. This study describes two experiments: first, a study to confirm refermentation in finished beer was possible with added hops, and second, developing a forced fermentation method to speed up the process for assessing the degree of refermentation. In our study, laboratory-scale dry hopping was carried out by adding 10 g/L of pelletized T-90 hops to both a commercially available ale and lager, in the presence of yeast, over the course of 30 days. Extract and alcohol measurements were made using an Alcolyzer at 1, 2, 4, 10, and 30 days during the trial in order to confirm refermentation. Four different hop cultivars were used, using one variety from each of the four categories established previously by an Oregon State study, ranking hops based on starch-degrading potential. Refermentation due to dry hopping in the presence of yeast was confirmed, with alcohol increasing as much as 1.06% v/v for the lager and 0.88% v/v for the ale over the course of the 30 days. However, the verification of the hierarchical classification by cultivar was inconclusive, because observed results in this study showed similar potential across all hop varieties. Further studies should be done to address these observations, the enzymatic potential of other advanced hop products, and the implications this information has on production brewing. Additionally, five different experimental brews with variable malt profiles and yeast selections were performed and forced fermentations done with and without hops. Each forced fermentation with hops showed a significant reduction in residual sugar and

increase in alcohol when compared with the non-hopped sample. A simple method is proposed to determine the effect of dry hopping on secondary fermentation.

Keywords: dry hopping, hops, enzymes, hop creep, laboratory method, forced fermentation

2. Introduction

Hops (*Humulus lupulus*) have historically been used in beer production during the boil, adding bitterness, flavor, and aroma to the finished beer, foam and microbiological stability, and clarity on the hot side [25,180]. Hops are also added to beer once fermentation is active or finished, historically providing packaging and transport stability [57] and more recently to add intense aroma and flavor in a process called dry hopping [58]. Dry hopping has become the standard procedure for adding hop aroma and flavor to many styles of beer, but mostly to India pale ale (IPA). The Brewers Association has repeatedly found IPA to be the most purchased beer style from craft brewers in the United States [181,182], and with historic numbers of these small breweries operating [183] there is more dry hopping than ever before. With more dry hopping in modern beer production, brewers have anecdotally mentioned recipe deviations from the addition of large amounts of hops. These have been known to result in higher alcohol and lower residual sugar contents, which can come with consequences from the regulatory bodies in the United States and elsewhere [61].

Brewers and researchers have noticed a recurrence of this phenomenon, known colloquially as “hop creep” or the “freshening power of hops,” for more than a century, with publications dating as far back as 1893 [57] and 1941 [184]. Hop creep causes yeast to reactivate after a finished fermentation, which can also yield higher amounts of yeast-related off-flavors such as diacetyl and acetaldehyde [62,63,185,186]. These off-flavors can create a deviation in quality and consistency for breweries, and perhaps the most concerning consequence of hop creep is when non-filtered

beer is packaged with residual yeast. Over carbonation in the container can also occur, causing hazardous conditions for the consumers of bottles or cans of this beer. Industry standard bottles (ISBs) suggest no greater than three volumes of CO_2 (6 g/L). A typical carbonation level in beer is around 5 g/L, which means only 0.004 specific gravity of additional fermentable sugar is sufficient to approach the pressure limit of an ISB when calculating CO_2 produced with the ideal gas equation [9,187].

More recently, studies have been carried out to scientifically explain this occurrence, with both commercially available finished products [188,189] and production beer before it has left the brewery [190]. These studies found that it may not just be a "diastase" [184] enzyme that is causing hop creep, but some of the same starch-degrading enzymes found in barley and model organisms such as *Arabidopsis* spp. [191,192] that are breaking down residual dextrins left from the brewing process. The yeast is then metabolizing the newly hydrolyzed fermentable sugars and producing more alcohol as well as the previously mentioned side effects. While those more recent studies focus on two specific varieties of hops, Cascade [188] and Centennial [190], respectively, there was another study that attempted to categorize the potential enzyme content of hops of different cultivars [51]. Kirkpatrick and Shellhammer chose 30 different hop cultivars and placed them into four classes of enzymatic activity after analysis using agglomerative hierarchical clustering. It was reported that five class 1 cultivars represent those having the highest production of fermentable sugars in beer, ten class 3 and five class 4 hops are those cultivars having moderate enzymatic activity, and eleven class 2 hops are categorized as low sugar producing. The first part of this study plans to assess the validity of this classification system and the variability of hop creep potential among the different hop cultivars by selecting one representative from each class.

A forced fermentation test is a rapid bench-scale fermentation of wort that is helpful in determining the final gravity of an unknown beer. Although the American Society of Brewing Chemists (ASBC) explains two fairly detailed and sophisticated methods to perform something similar [193,194], the benefit of the forced fermentation method is that any brewer can perform one with limited laboratory skills or supplies [195]. White Labs posits that as long as a brewer has a way to aseptically sample wort, a collection jar to put the wort in, a small excess of yeast, a way to agitate the collection jar during a 48-hr. period, and a way to measure density, then a good approximation of where the fermentation will finish can be achieved. When compared with the beer in the fermenter, this simple method can reveal many important things about the brewing process. If the main fermentation does not reach the same level as the forced fermentation, a problem lies in the fermenter, whether it be pitch rate, temperature, oxygenation, nutrient levels, or something else. If both the main fermentation and the forced fermentation finish out of desired specifications—too high or too low— there is a problem on the brewing side with either mash temperature or times, crush of the malt, or ingredient selection.

Most brewers use hydrometers or saccharometers to measure the density of their beer as it ferments; in this and most studies a more quantitative way to measure, such as an Anton Paar Alcolyzer, is used to obtain more precise results. The density of beer in relation to a given standard substance, in this case distilled water, is represented as specific gravity and recorded as a ratio [196]. This density, measured in g/mL, contains the total of all dissolved solids in the beer, including carbohydrates, lipids, proteins, and minerals. Carbohydrates in the order of 4 to 7 degrees of polymerization make up the large majority of the potential residual density, with 75–80% consisting of unfermentable dextrins, β-glucans, and a minute amount of pentose sugars [9].

Typical mashes in brewing consist of 65–70% fermentable sugars, leaving some residual dextrins that contribute to mouthfeel and body of the finished beer [197].

The study herein utilizes a systematic laboratory-scale dry hopping of two commercially available beers with one cultivar of each hop classification from the previously mentioned publication. Alcohol and extract of the resultant beer were measured to confirm the ability of each hop to alter the fermentability of finished beer in conjunction with the presence of yeast during the dry hopping stage. The attempt is to both confirm the phenomenon of hop creep and corroborate the hierarchical classification seen in previous research [51,188,190]. To address the time delay to carry out a refermentation test on finished beer, this study proposes a simple laboratory method that may be used to determine the effect that dry hopping can have on secondary fermentation. The hope is that any brewer that is capable of a forced fermentation will be able to presuppose the amount of additional fermentation caused by dry hopping.

3. Materials and Methods

A. Hops

All hops for this study were in the form of T-90 pellets and were provided by BSG Hops (Yakima Valley, WA). All were from the U.S. crop year of 2018, stored in a –2°C freezer prior to use, and were freshly opened 230 g packages that had been sealed with nitrogen cover gas in mylar packages. In the referenced study [51], hops were organized into four classification groups based on enzymatic potential. For this experiment, one hop from each class was chosen to create a representative example that covered all previous research. From class 1, Amarillo was chosen, from class 2, Centennial, from class 3, Mosaic, and from class 4, Willamette, with respective α-acid contents of 10.3, 9.3, 10.8, and 6.0%. Each hop chosen was an American cultivar to limit any detrimental effect that may come from poor handling during transit and is used in high

amounts in the U.S. brewing industry. No additional analytical information was provided on the packaging or through BSG's supply network. For the forced fermentation study, Willamette was the sole dry hop used for all trials due to its low level of hop creep potential in Kirkpatrick and Shellhammer's work [51], but relatively high potential was observed in the work here.

B. Chemicals

All chemicals used were reagent grade and were purchased from Fisher Scientific (Hampton, NH). 1-Octanol (CAS 111- 87-5) was used during sampling to prevent foaming in the pipettes. Sodium azide (CAS 26628-22-8) was diluted down to a 1.0% w/v solution and dosed at 0.02% w/v into control samples to prevent microbiological growth. Alconox Tergazyme was used as a 1.0% w/v solution for cleaning the machine between sample days on the Anton Paar Alcolyzer.

C. Yeast

Yeast used for secondary refermentation testing was provided by Fermentis (Milwaukee, WI). The strain selected was SafAle US-05, American ale yeast (*Saccharomyces cerevisiae*), with normal packaged quantities of 10^{10} cells/g, normal fermentation temperatures of 18–28°C, and alcohol tolerance to 11% v/v. The yeast was mixed into a 10% w/v solution with sterile water and dosed into beer at 10^{6} cells/mL for refermentation trials. Previous studies have used American Ale yeast strains from other suppliers [51,188,190].

D. Beer

Beer was a commercially available finished product purchased in the typical consumer market. A lager, Coors Original Banquet (Golden, CO, USA), and an ale, Lagunitas IPA (Petaluma, CA, USA), were used to represent a mixed set, as well as for their wide availability, consistent physicochemical properties, and relatively low residual sugar contents. All cans from

the subset of lager were packaged within a day of each other and held at 4°C prior to testing. All bottles from the subset of ale were packaged within 3 h of each other and held at 4°C prior to testing.

E. Laboratory-Scale Dry Hopping Setup

To assess refermentation capabilities of each beer and hop combination, 710 mL of each beer was placed into sterilized 1 L glass bottles and decarbonated via successive agitation and depressurization. The beer was then dry hopped aseptically with 10 g/L of each of the four experimental hops and yeast added to the solution, as was found to be an appropriate level by the Kirkpatrick and Shellhammer study [188]. Controls were also assessed using beer with sodium azide dosed at 0.02% w/v to prevent microbiological growth, beer with yeast and no hops, and beer with each of the chosen hop cultivars and no yeast, respectively, in the bottles. Biological replicates of each sample were completed in triplicate for a total of 60 sample bottles (*Table 2.1*). Bottles were held at 21°C for 30 days and sampled periodically to determine the amount of fermentation that had taken place.

Table 2.1. Total number of experimental and control samples used in part 1 of this study[a].

Beer	Hops	Yeast	No yeast	Total samples
Lager	Willamette	3	3	6
	Mosaic	3	3	6
	Centennial	3	3	6
	Amarillo	3	3	6
	None	3	3	6
Ale	Willamette	3	3	6
	Mosaic	3	3	6
	Centennial	3	3	6
	Amarillo	3	3	6
	None	3	3	6
Total		30	30	60

[a]*Lager* is Coors Original, and *Ale* is Lagunitas IPA. All hops are 2018 crop year from BSG. Yeast is SafAle US-05. Samples with no hops or yeast had sodium azide dosed at 0.02% w/v into control samples to prevent microbiological growth.

F. Sample Collection

Each bottle was sampled five times, in a laminar flow hood, at 24, 48, 96, 240, and 720 h after initial dry hop. The tips of sterile pipettes were quickly dipped in 1-octanol to prevent foaming while sampling. A sample (13 mL) was then drawn into the pipette from a randomly selected bottle; randomization was done using an online random number generator (Random.org). The sample was then evacuated into the appropriately labeled sterile 15 mL conical tube and stored in a –2°C freezer to arrest fermentation and lyse the yeast. The samples were moved to 4°C storage 48 h before measurement to allow the liquid to thaw.

G. Sample Measurement

The thawed samples were spun in a benchtop temperature-controlled centrifuge from ThermoFisher Scientific (Waltham, MA, USA) at 23°C and 3,500 rpm for 3 min. This separation, in conjunction with freezing the conical tubes, was enough to decarbonate the samples sufficiently for analysis. Samples were then measured for extract and alcohol (1) using a benchtop Anton Paar (Graz, Austria) density meter (DMA 4100 M) and Alcolyzer (Alcolyzer Plus, Beer). The DMA 4100 M has a repeatability within 0.00001 g/mL, and the Alcolyzer Plus has a repeatability within 0.01% v/v alcohol.

H. Statistical Analysis

Refermentation data for commercial beers were plotted in Excel (Microsoft). Mean and standard deviation were determined for all alcohol and specific gravity subsets using Google Sheets. Two-way analysis of variance (ANOVA) was performed using Google Sheets spreadsheet with XLMiner Analysis Tool-Pak.

I. Experimental Beers

Five experimental brews were performed on pilot systems in the University of California Davis Anheuser-Busch Brewing laboratory in order to test variety in malt and yeast profiles and their determination on hop creep results. All mashes targeted a standard 3:1 ratio of liters of water to kilograms of grist [198] and were lautered and sparged to set kettle full volumes. Wort was boiled for 60 min targeting 10.0% evaporation with 30–35 IBUs, cooled with groundwater on counter-flow heat exchangers, and knocked out to glycol-jacketed and temperature-controlled fermenters.

Three of the brews were performed on a 180 L, five-vessel brew system: Brew 1A consisted of 85.7% pale two-row malt, 5.7% Caramalt 15L, 5.7% Carastan malt, and 2.9% Munich II malt; using mash profile B (*Fig. 2.1*) yielded 11.7°P original extract, and it was pitched with Fermentis S-04 English ale yeast to represent a typical English pale ale. Brew 2A was 57.1% pale two-row, 37.1% pilsner malt, 2.9% Caramalt 15L, and 2.9% Munich II; using mash profile C (*Fig. 2.1*) yielded a 12.0°P original extract, and it was pitched with Fermentis K-97 German ale yeast to represent a Kölsch-style ale. And Brew 3A was 88.2% pale two-row, 5.9% Caramalt 20L, and 5.9% Caramel 20 malt; using mash profile C (*Fig. 2.1*) yielded a 11.3°P original extract, and it was pitched with Fermentis US-05 American ale yeast to represent an American-style pale ale.

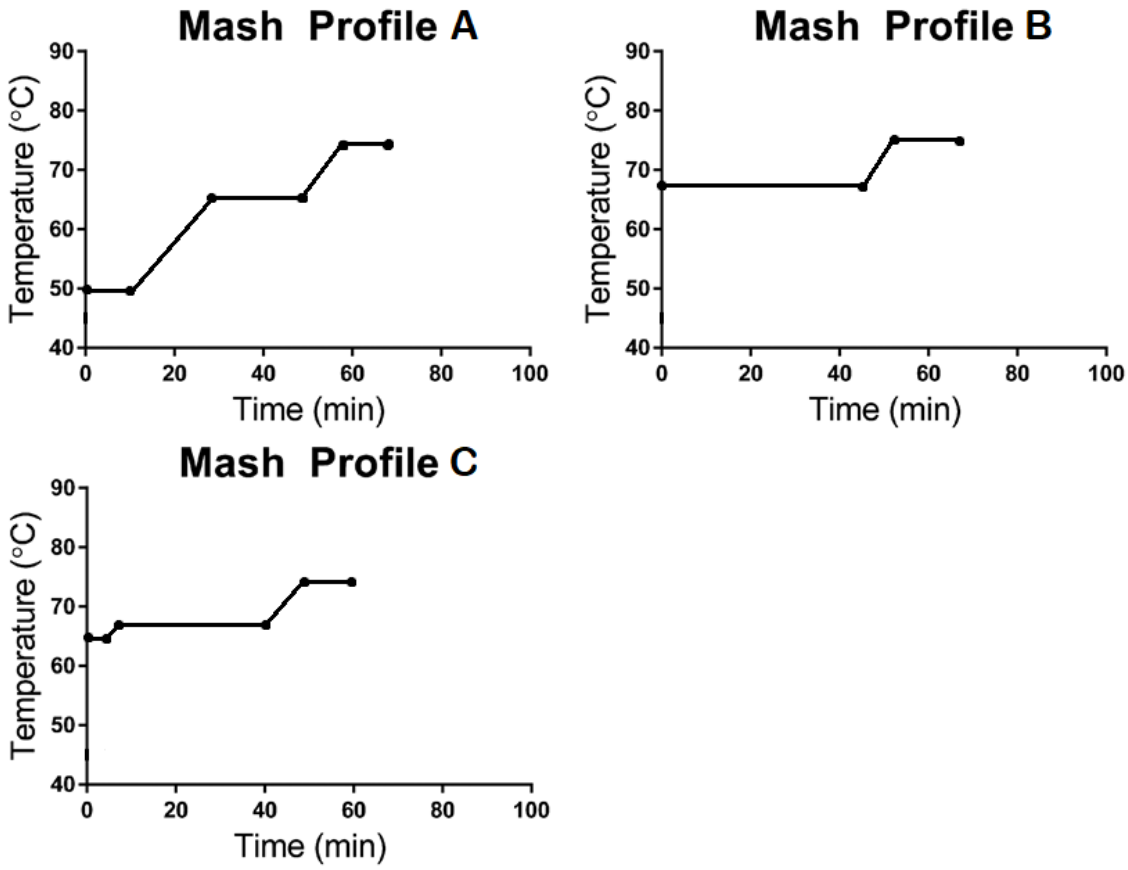

Figure 2.1. Mash profiles of the experimental beers performed for lab method. Mash Profile A is mashed at 50°C and held for 10 minutes, heated to 65°C and held for 25 minutes, then heated to 74°C and held for 10 minutes to mimic a German multi-step infusion mash for lagers. Mash Profile B is mashed at 68°C and held for 45 minutes, then heated to 74°C and held for 10 minutes to mimic the typical single-step infusion mash most American craft brewers perform. Mash Profile C is mashed at 65°C and held for 5 minutes, heated to 68°C and held for 30 minutes, then heated to 74°C and held for 5 minutes to mimic a typical multi-step ale mash.

Two additional brews were performed on a 38 L three-vessel brew system: Brew 1B was 89.5% pilsner, 8.4% dextrin malt, and 2.1% acidulated malt; using mash profile A (*Fig. 2.1*) yielded a 12.7°P original extract, and it was pitched with White Labs WLP830 German lager yeast to represent a pilsner lager. Brew 2B was 69.4% pale two-row, 15.3% flaked oats, 7.6% malted white wheat, and 7.6% dextrin malt; using mash profile B (*Fig. 2.1*) yielded a 16.0°P original extract, and it was pitched with White Labs WLP013 London ale yeast to represent a

hazy IPA. Mash profiles can be seen in *Figure 1.1*, and the yeast used is referenced in *Table 2.2*. All ingredients were sourced through the Northern California branch of Brewers Supply Group (San Leandro, CA, USA).

Table 2.2 Specific gravity and alcohol by volume data from forced fermentation samples on five experimental beers, with and without 20g/L of Willamette hops[a].

Parameter	Treatment	Brew 1A	Brew 2A	Brew 3A	Brew 1B	Brew 2B
SG	*No Hops*	1.0135	1.0111	1.0112	1.0115	1.0161
	With Hops	1.0111	1.0081	1.0081	1.0092	1.0121
ABV	*No Hops*	4.37	4.84	4.41	5.05	5.55
	With Hops	4.91	5.35	4.90	5.55	6.27
Yeast Pitched		S-04	K-97	US-05	WLP830	WLP013
Mash Profile		B	C	C	A	B

[a] Tested after 48 hours on a 150 rpm stir plate at room temperature. Yeast pitched: S-04 (English Ale), K-97 (German Kolsch) and US-05 (American Ale) from Fermentis, and WLP830 (German Lager) and WLP013 (English Ale) from White Labs. Mash profiles as outlined in Figure 1 used for each experimental brew reported.

J. Forced Fermentations

Bench-top fermentations were performed using a combination of the White Labs forced fermentation and the ASBC Beer-16 and Wort-5 methods [193–195]. Two hours after initial yeast pitch to fermenter, two 100 mL amounts were aseptically sampled into sterilized 250 mL Erlenmeyer flasks with magnetic stir bars inside. One sample was capped with an air lock, and the other had 20 g/L of Willamette hops added before being capped with an air lock. The 20 g/L was chosen to best simulate double the normal hopping rate established in the previous half of this experiment—the method developed will instruct brewers to use double their typical dry hop amount in this benchtop determination to ensure significant hop creep potential. Both flasks were placed on a stir plate set to 150 rpm for 48 h at room temperature before being analyzed.

An Anton Paar Alcolyzer was used to measure density and alcohol: 35 mL of each sample was placed in a 50 mL conical centrifuge tube and spun for 3 min at 5,000 rpm. The sample was decanted to fill a syringe designed for sampling on the Anton Paar Alcolyzer and decarbonated

in the syringe as needed. This was done by plugging the tip of the syringe with a finger and pulling the plunger out to create a vacuum inside the syringe. The syringe was then shaken to force gas out of the sample, and then the resulting foam and air were expressed out into a towel. This was repeated until a satisfactory amount of gas had been removed from the sample while still having at least 10 mL of sample, such that there were no bubbles in the oscillation tube on the Anton Paar.

4. Results and Discussion

A. Secondary Refermentation of Commercial Beer

The commercial beers in this experiment fell within typical ranges [9,198], with real degrees of fermentation (RDF) in the 67– 70% range (measured via Anton Paar Alcolyzer). RDF is the extent to which fermentation has genuinely occurred and is calculated using the original and real extract numbers. This means it is a quantifiable data point that represents the fermentable sugars and as a result the residual dextrins left in a fermented beer. These residual dextrins are what can cause the additional attenuation in the presence of dry hops and yeast. The parameter that most brewers are interested in, as well as regulatory authorities, is more commonly the final gravity of the beer, which directly correlates to RDF and alcohol by volume (% v/v). Alcohol by volume, recorded as percentage volume per volume, is a measurement of the concentration of ethanol in the finished beer.

In this experiment a subset of lager samples were initially analyzed to an average of 4.84% v/v alcohol (±0.05) and specific gravity of 1.0064 (±0.0003), whereas the ale samples were measured to an average of 6.07% v/v alcohol (±0.04) and specific gravity of 1.0102 (±0.0008) at base value. The beers were dosed according to experimental protocol previously outlined, and after a 30-day exposure to both hops and yeast the results of previous experiments were

corroborated (12,13,20). The experimental ale saw an average increase of 0.82% v/v alcohol and an average decrease of 0.0051 specific gravity, whereas the experimental lager saw an average increase of 1.04% v/v alcohol and an average decrease of 0.0064 specific gravity (*Fig. 2.2*). Interestingly, the experimental lager samples finished at an average specific gravity of 1.0000 (±0.0003), suggesting that the beer fermented down completely, leaving no residual sugar. The controls with just beer and beer with hops showed no real change in alcohol, but there was an average increase in density for all hopped beer of 0.0010 g/mL. This increase was to be expected because there is inherent carbohydrate content in all plants, including *H. lupulus* [199].

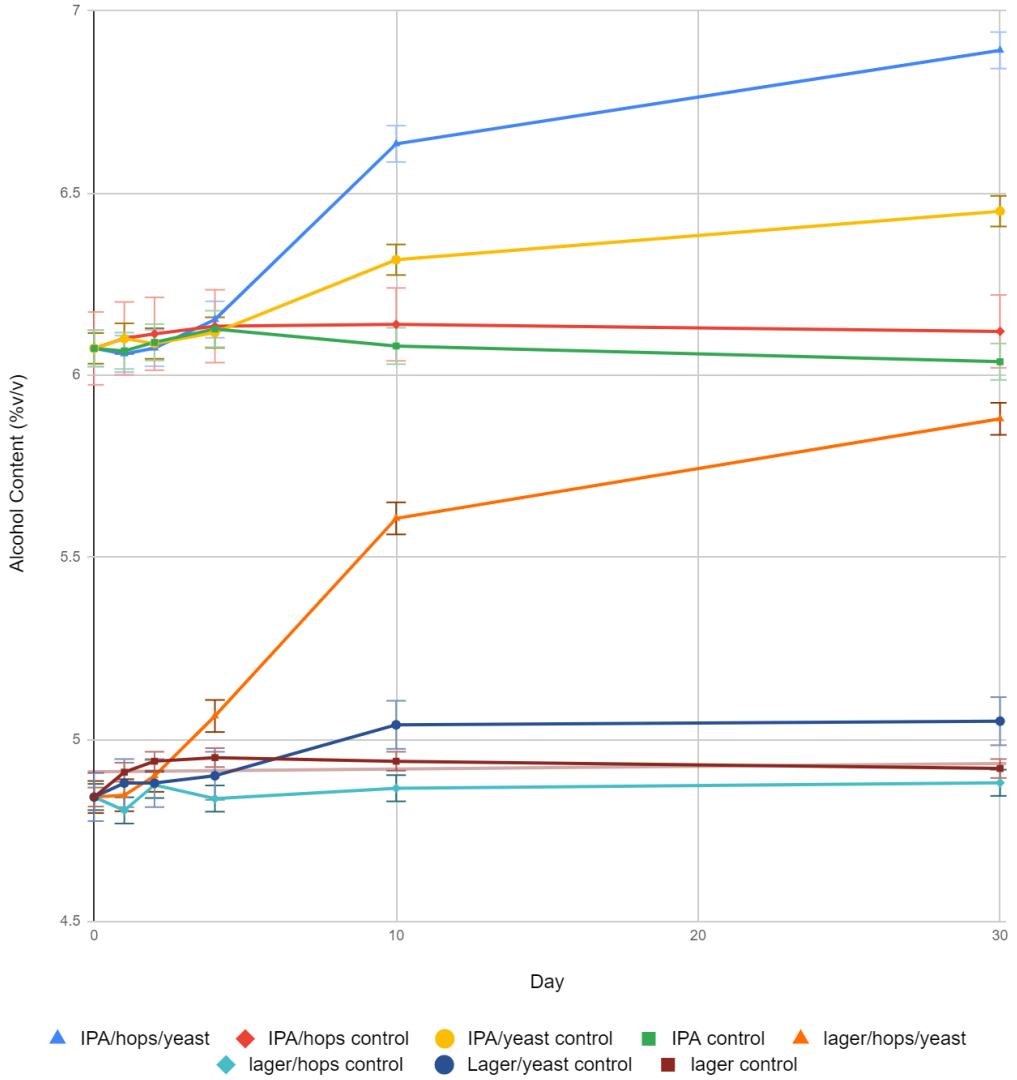

Figure 2.2 Average of the alcohol content measured as percent volume per volume at 1, 2-, 4-, 10- and 30-day time points after the addition of hops to the experimental samples, with error bars representing one standard deviaiton. The lower four curves are for the lager, as the upper four curves are for the ale. The bottom two curves on both ale and lager represent the controls with nothing added to the beer and with only hops added. The middle curve on each represents the samples with beer and yeast. The top curve on each beer shows the average across all cultivars of the experimental trials, with hops, yeast, and the beer.

The yeast and beer samples saw an average alcohol content increase of 0.38% v/v for the ale and 0.20% v/v for the lager. Of note, both of these increases kept the beers used in this part of

the study within the Tax and Trade Bureau's (TTB's) tolerance levels of 0.3% above or below the label stated alcohol percentage of 6.2% v/v and 5.0% v/v for Lagunitas IPA and Coors Original, respectively [61]. This is possibly explained by the experimental yeast used having a higher relative attenuation than the strains used by both Lagunitas and Coors but could also be a recipe choice of each brewer, allowing for a sweeter finish of the beer by arresting fermentation. The combination of the change in density from solely yeast and the added sugar content from the hops is still less than the averages when you combine both yeast and hops in the finished beers. This means there was additional attenuation, likely from the enzymatic activity of the hops producing more fermentable sugars (i.e., breaking down the residual dextrins left in the finished beer into more simple sugars), allowing the yeast to referment the beer.

These results did not corroborate previous research by Kirkpatrick and Shellhammer [51], that being the classification of the hops into enzymatic categories. It was reported that class 1 hops represent those having the highest production of fermentable sugars in beer, and in this study, those are represented by Amarillo. Classes 3 and 4 are those hops having moderate sugar production, which are represented by Mosaic and Willamette, respectively. They are all followed by class 2, which may be categorized as low sugar producing hops, represented by Centennial in this study. Class 4 in this case actually showed the most additional attenuation, as both ale and lager produced more alcohol and had the greatest specific gravity change in the presence of Willamette and yeast (*Table 2.3*). Class 2 was not the lowest either, falling in the middle of the data set on this study, but statistical analysis did not provide enough evidence to rank the experimental cultivars. ANOVA provided insufficient evidence to claim that the averages were not the same for each style of beer, regardless of hop cultivar. Of note is that Centennial saw the

least variance across each experimental subset, with the lowest standard deviation in both lager and ale trials.

Table 2.3. Average decrease in specific gravity (SG) and increase in alcohol content %v/v (ABV) for all experimental varieties of hops over 30 days. The data are presented as means ± standard deviation.

Hops	Lager SG	Lager ABV	Ale SG	Ale ABV
Amarillo	0.0066 ± 0.00035	1.02 ± 0.06	0.0053 ± 0.00011	0.84 ± 0.04
Centennial	0.0064 ± 0.00015	1.05 ± 0.02	0.0051 ± 0.00005	0.78 ± 0.05
Mosaic	0.0061 ± 0.00035	1.03 ± 0.05	0.0046 ± 0.00032	0.78 ± 0.06
Willamette	0.0064 ± 0.00031	1.06 ± 0.04	0.0053 ± 0.00052	0.88 ± 0.09

That being said, this experiment did not go through as extensive of an enzymatic or sugar analysis as the cited study, so classification by alcohol content and density may be different, although this method was used in other studies [188–190]. Also, their analysis was done on sugar concentration alone, and only between day 0 and 1; after 1 day all samples had equivalent sugar concentration increases (within 0.0001 g/mL as measured on the Anton Paar Alcolyzer) in comparison with day 0 for the study outlined here. Additionally, all the data for alcohol change were fairly precise, falling within two standard deviations of the error for the Alcolyzer for alcohol values, so classification may be negligible. The Kirkpatrick and Shellhammer classification study also fully noted that hops from different crop years fell into different categories, hinting that the classification system itself may be flawed [51]. As with other published studies that have addressed hop creep, and conference proceedings from an ASBC meeting in 2019, endogenous enzyme activity may differ based on a multitude of variables, including hop cultivar, harvest maturity, kilning temperatures, and storage conditions of the hops [200], as well as recipe conditions such as dry hop procedure [201–203], yeast selection [204], temperature of fermentation, and contact time with the hops [189]. Further experiments could be done to address these particular parameters, the enzymatic potential of other advanced hop products, and the

implications this information has on production brewing for the myriad brewers in the United States and the rest of the world.

B. Proposed Laboratory Method for Hop Creep Detection

Upon analysis of the experimentally brewed samples, it was found that all samples showed an increase of alcohol and decrease in specific gravity when the forced fermentation was performed with hops added (*Table 2.2*). This proves that this method could be used to determine the extent to which the hops used in dry hopping by a brewer will contribute to hop creep. It may not show a hard quantitative number for the difference the brewer will experience, but it can give a general idea of the difference when done in comparison with and without dry hops. It is also known that a laboratory-scale dry hop may not be the most accurate aggregate for a production situation [203], but when used in comparison to non-hopped forced fermentation the data are useful for informing the brewer the extent to which their chosen hops contribute to hop creep.

The alcohol increase seen here from hop creep was fairly consistent at an amount of 0.50% ABV, with the exception of Brew 2B, which was an even greater change with an increase of 0.72% ABV. Brew 2B was designed to mimic the current trend of hazy/juicy IPAs in the craft beer market, with a high percentage of high-protein flaked oats and wheat, as well as a large amount of residual dextrins from the grist bill and mash profile B [197]. The dry hop schedule was also chosen to reflect current trends of heavily dry hopped beer [58], with 20 g/L being double what would be considered a high rate (normally more than 2.5 lbs/BBL, which equates to 9.7 g/L), but not outside of the realm of possibility for new IPAs. The 20 g/L was also chosen to best simulate double the normal dry hopping rate as established in the previous half of this experiment, and the method developed instructs brewers to use double their dry hop amount in this benchtop determination to ensure significant hop creep potential. Although the existing science states that

these levels of dry hop may be excessive, your procedure can be adapted [201,202], and it does not seem that the craft beer community will change the way they use hops in the foreseeable future.

There was no observable difference in the amount of change in comparison to the yeast strain selected. German ale and lager both showed the same change within the margin of error for the Anton Paar Alcolyzer; German, English, and American ale yeasts all showed similar change when comparing the wort samples, with English showing a slight bit more. Additional trials need to be performed in order to solidify the data and confirm no observable difference outside of that expected from yeast or malt selection, and a larger variety of hops should be confirmed to contribute in this expedited method. Future trials will be done with local breweries in order to ensure the ease of performing the method as well as the ability to interpret results. The method proposed is scalable upward if you do not have flasks this small or are using hydrometers to measure your specific gravity, but scaling down beyond one-half may be a challenge, because there will not be enough liquid to sample. As previously stated, be sure to use double your intended dry hop rate to ensure significant hop creep potential, and similar effects to this study should be observed. The hope is that this simple laboratory method can help brewers when they are attempting to quantify the difference in fermentation and attenuation values that can be observed from the enzymatic activity of their dry hopping level.

C. Method

Standard Operating Procedure (SOP) for Dry Hop Creep Forced Fermentation Sampling

1. Purpose
- 1.1. This SOP is to explain the process for testing wort samples in order to determine hop creep potential of dry hopped beers.

2. Scope
- 2.1. This document applies to all persons, practices, and procedures involved in the brewing and sampling of beer at a given brewery.
- 2.2. This document details the minimum necessary materials and competencies to be completed by the trained analyst, as outlined by the technical expert.

3. Definitions
- 3.1. Forced fermentation: benchtop fermentation at room temperature with agitation to get to maximum attenuation of a particular brew and yeast combination
- 3.2. Dry hop creep: additional attenuation due to the endogenous enzymes in hops, added to fermenting beer, a.k.a. dry hopping

4. References
- 4.1. ASBC Method Wort-5
- 4.2. ASBC Method Beer-16
- 4.3. White Labs Forced Fermentation Lab Method

5. Equipment/Materials
- 5.1. Sterilized Erlenmeyer flasks with stir bars
- 5.2. Stir plate with variable speed settings
- 5.3. Double the amount of dry hops added to normal beer
  - 5.3.1. One pound per barrel (1 lb/BBL) = 3.867 g/L, for reference
- 5.4. Tool to analyze initial and final gravity of wort and beer

6. Procedure
- 6.1. Perform original gravity readings on wort at knockout.
- 6.2. Aseptically sample 1–4 h following final knockout and yeast pitch.
  - 6.2.1. For Hydrometer Readings
    - 6.2.1.1. Fill two 250 mL flasks with 100 mL of wort with yeast each.
      - 6.2.1.1.1. If more wort is needed for the hydrometer tube, use 200 mL in a 500 mL flask, 400 mL in a 1,000 mL flask, etc.
  - 6.2.2. For Alcolyzer or Densitometer Readings
    - 6.2.2.1. Fill two 250 mL flasks with 100 mL of wort with yeast each.
- 6.3. Add double the amount of typical dry hop for given beer to one flask.
  - 6.3.1. Cap each flask with an air lock.
- 6.4. Place on stir plate set to approximately 150 rpm for 48 h.
- 6.5. At 48 h, take gravities of both samples.
  - 6.5.1. Filter through coffee filter or spin with centrifuge to remove solids.
  - 6.5.2. Compare for change in gravity due to dry hop creep.

5. Conclusions

Verification of the hierarchical classification by hop cultivar in previous research was inconclusive, as observed results in this study showed a similar drop in gravity and gain in alcohol independent of hop variety. This may be a result of the enzyme activity variability due to different procedures in growing, processing, and storing hops by different farms, because hops, after all, are an agricultural product and can be variable year to year, farm to farm, and even lot to lot. Different parameters of the experimental beers were measured than in the original

hierarchical classification study, so additional analysis remains to verify the classification of different hop cultivars. The molecular structures of the unfermentable dextrins of each test beer in this or previous studies are also unknown at this time, which could elucidate more information of the true enzymatic potential of hops. The method proposed is designed to be used by craft breweries to determine the risk of hop creep. This is pertinent when cellar operations and dry hopping techniques also vary between production brewing and current bench-scale dry hop procedures with a number of varying parameters such as temperature, yeast strain, or osmotic pressure. Further studies should be done to address the influence of these parameters, as well as the enzymic potential of other hop products, the interaction between hop cultivars and yeast strains, and how best to use this information within the brewery.

Acknowledgements

Many thanks to the UC Davis Department of Food Science and Technology for funding in the form of the Food Science Graduate Fellowship. Additional funding came from the H.A. Jastro Shields Fellowship, the Margrit Mondavi Fellowship, the George Stewart Memorial Award, and the Michael J. Lewis Endowment. Thank you to Kelly Scott and Andrew Marcus for helping with some of the brews and Lagunitas for providing beer.

Chapter 3

Saccharomyces Yeast Screening

Poster, “Diversity of Properties Related to Brewing in *Saccharomyces* Species and Strains,” presented at the World Brewing Congess Connect 2020 (*Appendix A.2*).

1. Abstract

The Phaff Yeast Culture Collection at University of California, Davis, allows researchers a unique opportunity to use yeasts that may be unavailable in any other case. The collection is one of the largest public collections of wild yeasts in the world, with over 7,500 strains belonging to 1,000 different species, including upwards of 200 novel species not found anywhere else. This study used the opportunity of another research project that was reviving 46 different yeast strains to assess the fermentation capabilities of these various cultures. Species of *Saccharomyces* were mainly *cerevisiae* or *pastorianus*, but there were also novel examples of *bayanus*, *paradoxus*, and *mikatae*. The yeast cultures were grown in several media, including Yeast Carbon Broth (YCB) assessing nitrogen assimilation, Yeast Nitrogen Base (YNB) determining sugar source digestion, YNB with glucose evaluating ethanol tolerance, and wort media with and without hop extract to determine brewing growth capacity. Each medium was put into 96 well plates with the 46 different yeast strains in biological duplicates and grown on microplate shakers for five days. Optical density readings at 600 nm were performed every 6 hours and the concentration plotted against a control; MATLAB was used to create heat maps for growth. There was uneven growth in some plates and unexpected growth in the YCB with proline, but most yeast grew well, with wort media having the most vigorous growth. There were a number of yeasts that survived well under the higher ethanol concentrations. Further work will be carried out to explore the proline growth, as well as the higher ethanol treatments. The Phaff yeast collection is an untapped resource for non-conventional yeast strains for research with yeast in brewing.

2. Introduction

The brewing industry is constantly looking for new and innovative ways to make beer [205,206], and a great deal of that innovation is coming from the brewers' selection of yeast [29,80,207], as it is

one of the four main ingredients in beer. The brewing industry is searching for increased novelty and flavor from yeast, but to be used for brewing they must have specific physiological properties in order to withstand the fermentation environment in beer. Ethanol tolerance is of great importance, as not only are yeasts used for beer cultured for their alcohol production [208], brewers are constantly pushing the limits of high-gravity brewing [209]. Amino acid assimilation is also of great importance to commercial brewers, as free amino nitrogen (FAN) is one of the most important metabolite measurements used as a predictor of yeast health during fermentation [210]. FAN is defined as the measurement of individual amino acids, ammonium ions, and small peptides present in the brewers' wort. Typically, glutamine is the most commonly assimilated amino acid by *Saccharomyces* yeast, while proline is the only amino acid that is not readily absorbed by yeast during fermentation [23,39].

A yeast's ability to metabolize certain carbohydrates is also of great importance to brewers, as wort is a complex matrix of variable sugar amounts composed of glucose, maltose, and longer chain dextrins [23]. Typical lager yeast *Saccharomyces pastorianus* is known to ferment melibiose, while *S. cerevisiae* ale yeast does not [211]. *S. cerevisiae* strains formerly classified as *S. diastaticus* have a glucoamylase *STA1* gene that can break down maltodextrins for fermentation while they remain in resultant beer with most other *Saccharomyces* yeasts [174,212]. *S. cerevisiae* strains formerly classified as *S. chevalieri* do not have the ability to ferment maltose, as other *Saccharomyces* yeasts do [178]. These are just three examples among a myriad of *Saccharomyces* species that have varying sugar metabolisms.

The Phaff Yeast Culture Collection at University of California, Davis, allows academic, industry, and government agency researchers the opportunity to use yeast for basic and applied studies. The collection offers a unique opportunity to use yeasts that may be unavailable in many

other situations. According to a 2012 review [213], the Phaff Collection the fourth largest public collection of wild yeasts in the world, with over 7,500 strains belonging to 1,000 different species. This includes upwards of 200 novel species in the collection that cannot be found anywhere else in the world. The collection holds the type strains of many members of the *Saccharomyces* genus, some of which have never been used in the production of alcoholic beverages. The Phaff Collection holds many yeasts that may be of interest for the commercial production of beer, but also many strains that have been isolated from other fermented beverages. This screening hopes to elucidate some of the potential of the Phaff Collection's yeast for use in the commercial brewing industry by screening for ethanol tolerance, amino acid assimilation, and sugar metabolism.

3. Materials and Methods

a. Yeast

Forty-six different *Saccharomyces* yeast strains (*Table 3.1*) selected for their ability to produce ethanol were revived from the Phaff Collection to assess the fermentation capabilities of these various cultures. For this assessment, nine commercially available brewing yeasts were used as baselines for analysis, with Danish, German, English, Irish, and American ale and lager strains chosen. An additional ten strains isolated from commercial or home brewed beers in the Netherlands, Denmark, the United Kingdom, West Africa, Italy, Germany, Mexico, and the United States. Eight strains were isolated from wineries located all over the world, five were cultured from fruit, four from distillery fermentations, and three were cultured from tree sap or bark. Four yeasts were lab strains of unknown origin, one was isolated from a kafir fermentation, one from a cherry soda in the United States, and one from Japanese soil. Species of

Saccharomyces were mainly *S. cerevisiae* or *S. pastorianus*, but there were also examples of *S. bayanus*, *S. paradoxus*, and *S. mikatae* included in the screening.

Table 3.1. Yeasts used in Saccharomyces screening. "Phaff Strain ID" each starts with UCDFST prior to the numerical strain listed; "Isolated From" is the habitat of original isolate; "Geographic Origin" as determined by source or original isolate; "Date of Isolation" is record in Phaff Collection. ***signifies information is unknown

Phaff Strain ID	Genus Species	Other Collection Numbers	Isolated From	Geographic Origin	Date of Isolation
96-13	*S. cerevisiae*	Wyeast 1084	Irish Ale Yeast	Ireland	<1996
96-11	*S. cerevisiae*	Wyeast 1028	London Ale Yeast	London, England	<1996
96-15	*S. cerevisiae*	Wyeast 1338	European Ale Yeast	Europe	<1996
96-16	*S. cerevisiae*	Wyeast 1728	Scottish Ale Yeast	Scotland	<1996
96-17	*S. cerevisiae*	Wyeast 1968	Special London Ale Yeast	London, England	<1996
96-14	*S. cerevisiae*	Wyeast 1098	British Ale Yeast.	England	<1996
96-12	*S. cerevisiae*	Wyeast 1056	American Ale Yeast.	USA	<1996
02-124	*S. cerevisiae*	Wyeast 3068	Weihenstephan Wheat	Germany	2002
96-20	*S. cerevisiae*	Wyeast 2042	Danish Lager Yeast	Denmark	<1996
01-157	*S. pastorianus*	DBVPG 6047; ATCC 12752; CBS 1538	Hansen's culture of 23 Jan 1888	Denmark	2001-12
69-53	*S. cerevisiae*	***	brewing yeast, used commercially in APV tower fermenters	Nutfield, UK	<1969
01-158	*S. cerevisiae*	DBVPG 6173; ATCC 18824; CBS 1171	*S. cerevisiae* neotype strain. Orangeboom brewery	Rotterdam, Netherlands	<2001
11-131	*S. cerevisiae*	YJM271; CBS 1782	Brewery	North Carolina, USA	<2011
40-419 wrinkly	*S. cerevisiae*	UCD VEN 1419	Brewery top yeast originally from Tuborg Brewery in Copenhagen	Denmark	<1940
82-15.2	*S. pastorianus*	NRRL Y-48765	Lager beer yeast obtained from Maynard Dimond	Germany	<1982
82-805	*S. cerevisiae*	ATCC 42082	Tesguino (indigenous beer)	Mexico	<1982
01-135	*S. bayanus*	DVBPG 6171	turbid beer	Italy	<2001
15-388	*S. cerevisiae*	***	beer	Missouri, USA	<2015
15-380	*S. cerevisiae*	***	beer	West Africa	<2015
70-12	*S. cerevisiae*	ATCC 26108; CBS 8803	lab strain	USA	<1970
40-80	*S. cerevisiae*	***	***	***	<1946
72-58	*S. cerevisiae*	***	***	***	<1972
01-141	*S. cerevisiae*	***	***	Italy	<2001
84-09	*S. cerevisiae*	***	cherry soda	USA	<1984
15-397	*S. cerevisiae*	***	wine	Europe	<2015
15-357	*S. cerevisiae*	***	wine	Italy	<2015
15-367	*S. cerevisiae*	***	wine	Japan	<2015
15-302	*S. cerevisiae*	***	wine	Nigeria	<1973
15-405	*S. cerevisiae*	***	wine	Slovenia	2014
06-152	*S. cerevisiae*	***	wine	Tokaj, Hungary	<2006
01-122	*S. cerevisiae*	ATCC 42940	Riesling strain	***	<2001

Phaff Strain ID	Genus Species	Other Collection Numbers	Isolated From	Geographic Origin	Date of Isolation
15-389	*S. cerevisiae*	***	kefir	***	<2015
15-392	*S. cerevisiae*	***	fruit	Winters, CA, USA	2005
75-04	*S. cerevisiae*	***	pineapple concentrate	California, USA	1975
15-371	*S. cerevisiae*	***	guava	Philipines	<2015
15-358	*S. cerevisiae*	***	fig	Maryland, USA	<2015
15-343	*S. cerevisiae*	***	fruit	Illinois, USA	<2015
15-370	*S. cerevisiae*	***	wild cherry tree gum	***	<2015
15-348	*S. cerevisiae*	***	oak tree	Pennsylvania, USA	<2015
01-161	*S. paradoxus*	DBVPG 6411; CBS 432; NRRL Y-17217	tree exudate	Europe	2001-12
11-510	*S. mikatae*	NCYC 2888; NRRL Y-27341	Soil	Japan	<2011
15-300	*S. cerevisiae*	McCusker (Duke U) YJM189	Distillery	***	<1955 or (2014?)
15-363	*S. cerevisiae*	***	rum fermentation	Trinidad	<2015
15-361	*S. cerevisiae*	***	molasses	Illinois, USA	<2015
15-366	*S. cerevisiae*	***	sugar cane	Jamaica	<2015

Yeast was revived from cryogenic storage and streaked on Potato Dextrose Agar (PDA) plates to be grown at room temperature for at least one week prior to suspension. Single yeast colonies were transferred from the PDA plates with sterile pipette tips to be suspended in 400 µL of saline/Tween buffer in 46 of the wells on a 96-well plate. 200 µL of the suspended yeast solution was transferred to the 46 wells remaining for biological duplicates on each screening plate. All wells on the microplate were then filled with 200 µL more of selected media so that each had 400 µL total. 4 wells of the 96-well plate were filled with just saline/Tween buffer as a negative control and to served as the blank for optical density readings at 600 nm wavelength (OD_{600}) readings.

b. Media

All media was made aseptically in biological safety cabinets. Sixty PDA plates were made for cryogenic revival of yeasts from the Phaff Collection. The yeast cultures were grown in a total of seventeen different media (*Table 3.2*) This included Yeast Carbon Broth (YCB)

assessing amino acid assimilation with either proline or glutamine amino acids added, Yeast Nitrogen Base (YNB) determining sugar source digestion, YNB with glucose evaluating ethanol tolerance (0%, 6%, 12% and 16%), and wort media (10% w/v Briess CBW® Pilsen Light malt extract) with and without hop extract (25 mg/L calculated iso-alpha acids) to determine brewing growth capacity. 200 µL of each medium was added into 96-well plates with the 46 different yeast strains in biological replicates and grown on microplate shakers set to 200 rpm and 30 ºC for five days; one plate was placed in a 4 ºC for the five-day period. Plates were sealed with a polyurethane membrane with acrylic adhesive called Brethe-Easy® (Sigma-Aldrich; Saint Louis, MO, USA) because it was both breathable and transparent for absorbance readings.

Table 3.2. Screening media used; each medium was on its own 96 well plate with all 46 yeasts screened. Medium base of Yeast Carbon Broth (YCB), Yeast Nutrient Base (YNB), or Wort with additional ingredients for screening of brewing potential, reason for addition, and temperature of microplate shaker during screening. (***) signifies no addition to the media.

Media	Addition	Purpose	Temperature
YNB	0.5% glucose	Positive Control	30˚ C
YNB	0.5% glucose	Cold Tolerance	4˚ C
YNB	0.5% glucose and 2% Ethanol	Ethanol Tolerance	30˚ C
YNB	0.5% glucose and 5% Ethanol	Ethanol Tolerance	30˚ C
YNB	0.5% glucose and 8% Ethanol	Ethanol Tolerance	30˚ C
YNB	0.5% glucose and 12% Ethanol	Ethanol Tolerance	30˚ C
YNB	0.5% glucose and 16% Ethanol	Ethanol Tolerance	30˚ C
YNB	***	Negative Control	30˚ C
YNB	Mineral oil overlay	Negative Control	30˚ C
YNB	0.5% maltose	Sugar Assimilation	30˚ C
YNB	0.5% maltodextrin	Sugar Assimilation	30˚ C
YCB	25 mM Ammonium Sulfate	Positive Control	30˚ C
YCB	25 mM Proline	Amino Acid Assimilation	30˚ C
YCB	25 mM Glutamine	Amino Acid Assimilation	30˚ C
Wort	***	Brewing Potential	30˚ C
Wort	With Hops	Brewing Potential	30˚ C
***	Mineral oil overlay	Negative Control	30˚ C

c. Optical Density Reading

Optical density readings by absorbance for yeast concentration were performed at 600 nm wavelength (OD_{600}) on a VersaMax™ tunable microplate reader (Molecular Devices, LLC; San Jose, CA, USA) every six hours for five days. 600 nm wavelength is chosen because it is not harmful to the culture growing in the 96-well microplate and because there are no known yeast compounds that absorb in this wavelength that could interfere with measurements. Readings were done in technical replicate and automatically saved via a shared spreadsheet and plotted against a control. OD_{600} readings were recorded as the value minus the absorbance at 600 nm wavelength for the blank.

d. MATLAB Heat Mapping

Heat Maps were created by utilizing the highest absorbance reading over the course of 102 hours for each 96-well microplate. Optical densities were converted to a color scale and MATLAB Simulink R2020 Software was used to create the resultant figures.

4. Results and Discussion

a. Yeast Growth

Wort media created a high amount of CO_2 bubbles in the microplates during fermentation, making it impossible to read absorbance at the earlier hours of the study. There was uneven growth in some plates, as yeast shifted to the corner of the plates from the centripetal force of the microplate shaker. Some timepoints had to be excluded due to reading mistakes, including uncorrected mist and droplets on the parafilm sealant. Other readings were excluded because of missing media due to undergraduate researcher error when loading or possible evaporation if the wells weren't sealed completely. Most yeast grew well, with wort media having the most vigorous growth. All growth curves showed deviations in optical density following the 102-hour

reading, so data past this time was deleted as outliers and the highest absorbance value before this used as peak growth for the Heat Map creation (*Fig. 3.1*).

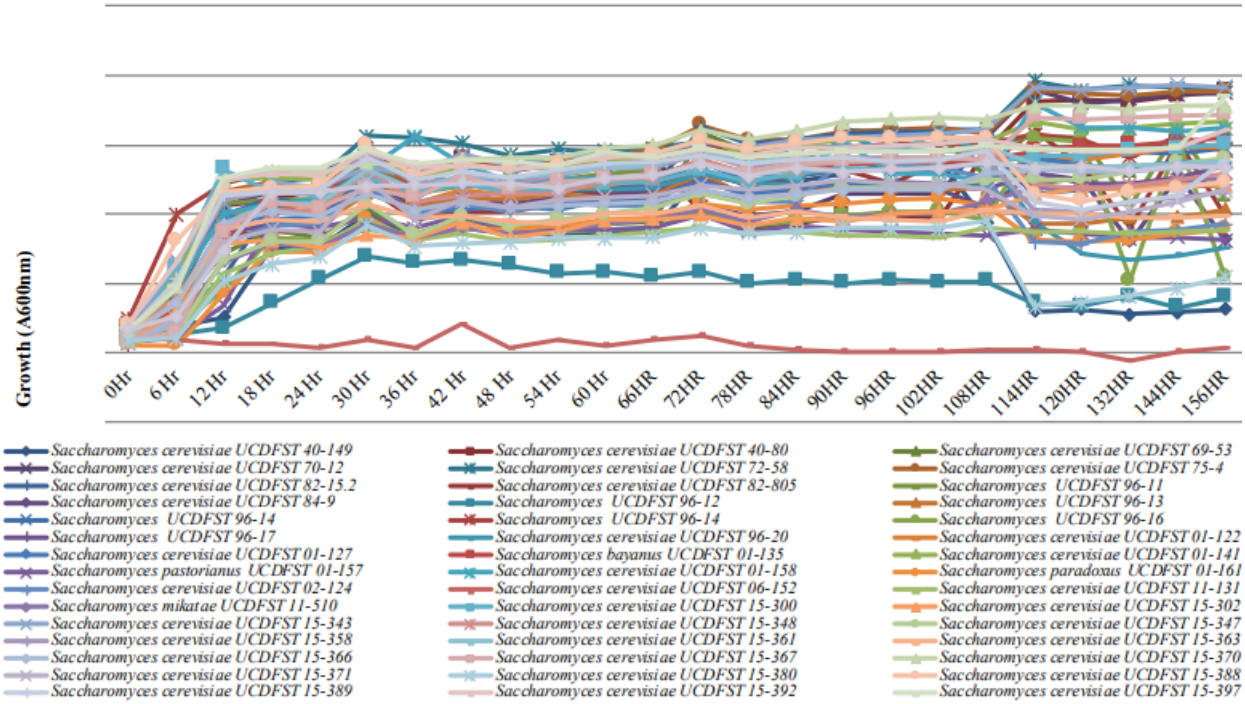

Figure 3.1. Example of growth curves for all yeasts used in this screening, with optical densities at 600 nm wavelength in the YNB with glucose and mineral oil overlay positive control.

b. Amino Acid Metabolism

Standard growth media for the culture of *Saccharomyces cerevisiae* yeast includes ammonium sulfate for the source of nitrogen and amino acids [214], and was therefore included as a positive control in this study. Growth of the studied cultures was moderate to high with ammonium sulfate as the sole nitrogen source (*Fig. 3.2*). L-Glutamine was included as another expectedly positive control from a brewing perspective, as it is the most commonly assimilated amino acid produced from an all-malt wort [23] and is necessary for the growth of *S. cerevisiae* [215] and production of ATP in glycolysis [216]. The most vigorous growth of all yeasts on an amino selective medium in this study was observed with the combination of YCB and L-glutamine. L-Proline was selected as an amino acid source due to its known problem with assimilation into

Saccharomyces yeast in the anaerobic conditions of fermentation [217]. Growth on YCB media with proline as the sole amino acid source was unanticipated in this screening as it is not readily assimilated when other amino acids are present in wort. This may be due to the age of the anhydrous L-proline powder used in this experiment; proline is known to be a metal chelator in plants [218] and degrades to glutamate in vivo [219], suggesting it may have decomposed to a more readily assumable amino acid due to exposure to atmospheric conditions for some time. Additionally, proline is not readily assimilated by *Saccharomyces* yeast in wort fermentation, but that does not mean it is incapable of using that amino acid if no others are present [23,217].

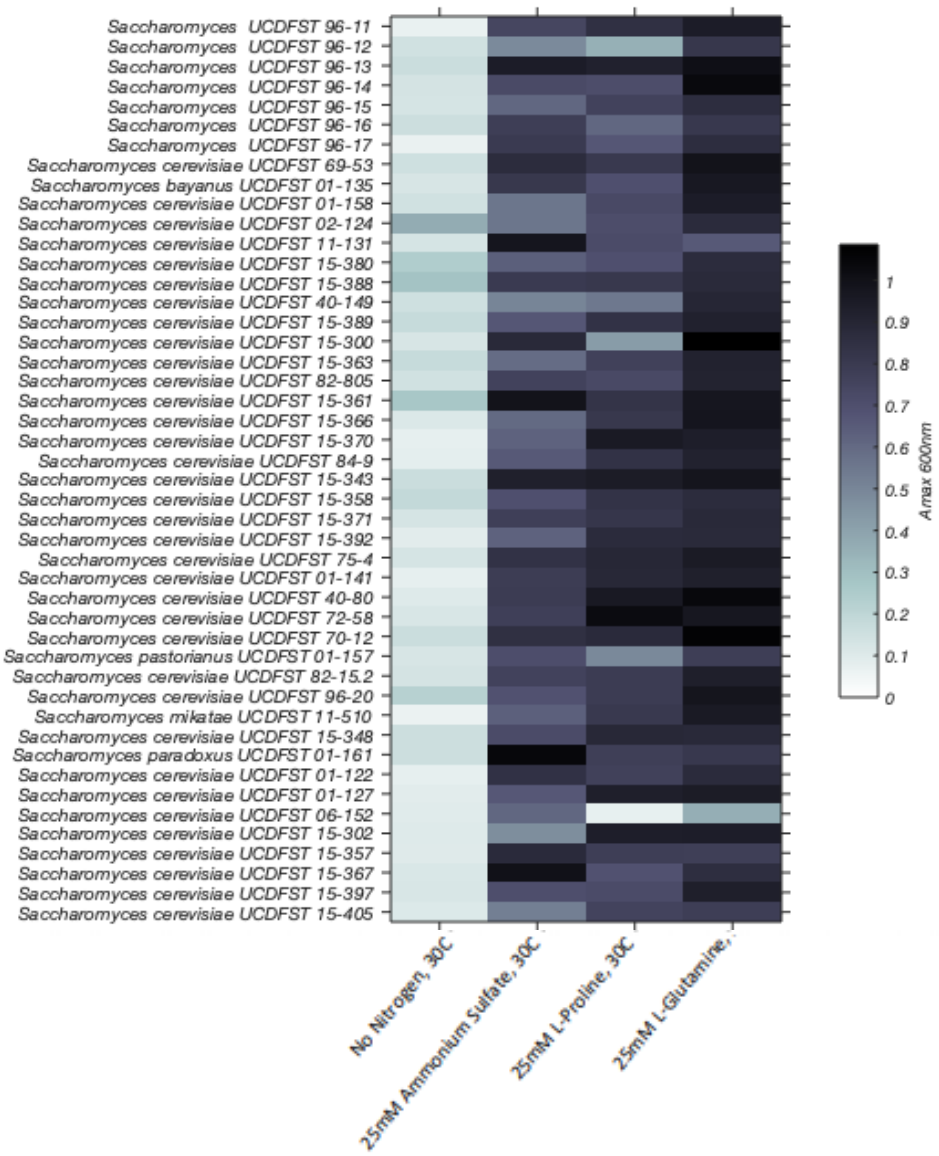

Figure 3.2. Heat Map of all yeasts when screening for amino acid assimilation. All wells with YCB no nitrogen as a negative control, ammonium sulfate added as a positive control, and either L-proline or L-glutamine for brewing potential screening. Figure created in MATLAB.

c. Ethanol Tolerance

YNB with 0.5% glucose added was used to evaluate ethanol tolerance of yeasts in this study. Media was made with either 0%, 6%, 12% or 16% ethanol concentrations and then overlayed with mineral oil so that the ethanol did not evaporate. There were a number of yeasts that survived well under the 12% ethanol concentrations, which curiously had more growth than

the 8% ethanol (*Fig. 3.3*). Just three *S. cerevisiae* yeasts, UCDFST 15-300 from a distillery fermentation, UCDFST 15-348 isolated from an oak tree in Pennsylvania, USA, and UCDFST 15-397 from a European wine, showed ethanol tolerance to concentrations as high as 16%. Craft beers can reach very high alcohol levels as brewers push new limits for curious consumers, so potential for ethanol tolerance can be useful in production brewing.

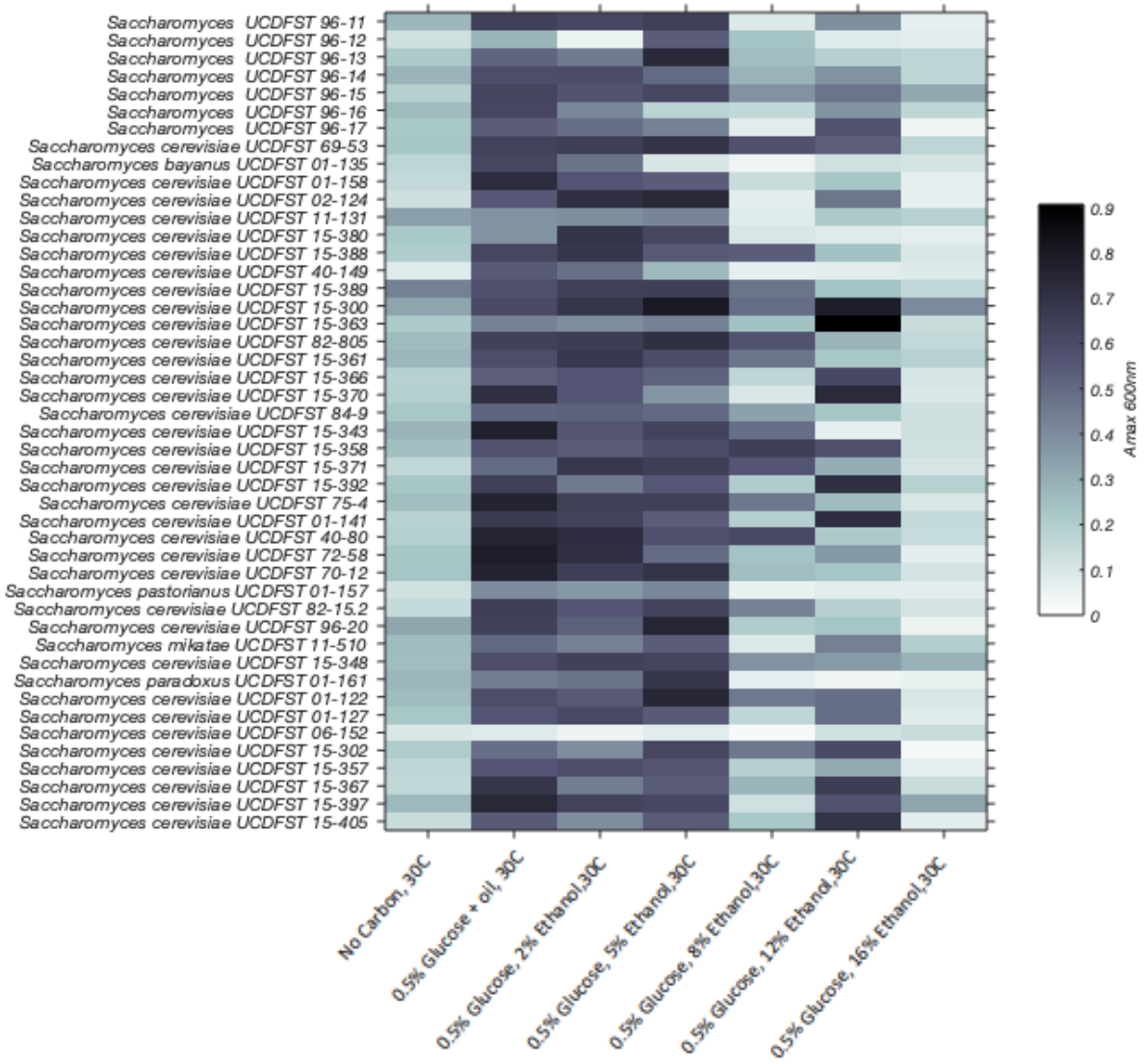

Figure 3.3. Heat Map of all yeasts when screening ethanol tolerance. All wells with YNB and no carbon as a negative control, 0.5% glucose added as a positive control, and 0.5% glucose with either 2, 5, 8, 12, or 16% ethanol for brewing potential screening. All wells overlayed with mineral oil so that ethanol did not evaporate. Figure created in MATLAB.

d. Brewing Characteristics

Potential brewing characteristics and environments were studied using YNB as a base, with 0.5% glucose added at 4 ºC to simulate lager brewing conditions, 0.5% maltose added because it is the most abundant sugar in wort, 0.5% maltodextrin added to screen for potential *STA1* gene for *diastaticus*. Lab made wort media was also used to simulate real world brewing conditions, with 25 calculated IBUSs worth of hops added to one of the screening media. The positive control with 0.5% glucose showed more growth than 0.5% maltose (*Fig. 3.4*), perhaps because of yeasts' preference for glucose [23], or the screening media being outside of the pH optimum for maltase activity [36]. Little growth was observed at 4 ºC as it is outside the optimal growth range for most brewing yeast, even *S. pastorianus*, though the *S. bayanus* strain UCDFST 01-135 recorded the highest optical density. Significant growth on maltodextrin was only observed with UCDFST 74-4, a *S. cerevisiae* isolated from pineapple concentrate used in industry, and a *S. cerevisiae* of unknown origin in UCDFST 72-58. Both of these *S. cerevisiae* strains should be genetically screened for the glucoamylase *STA1* gene for var. *diastaticus* prior to use in the brewing industry. Wort media had the most vigorous growth for all yeasts in the study, showing just how much nutrient potential it has and the evolution of *Saccharomyces* to prefer it as a growth medium.

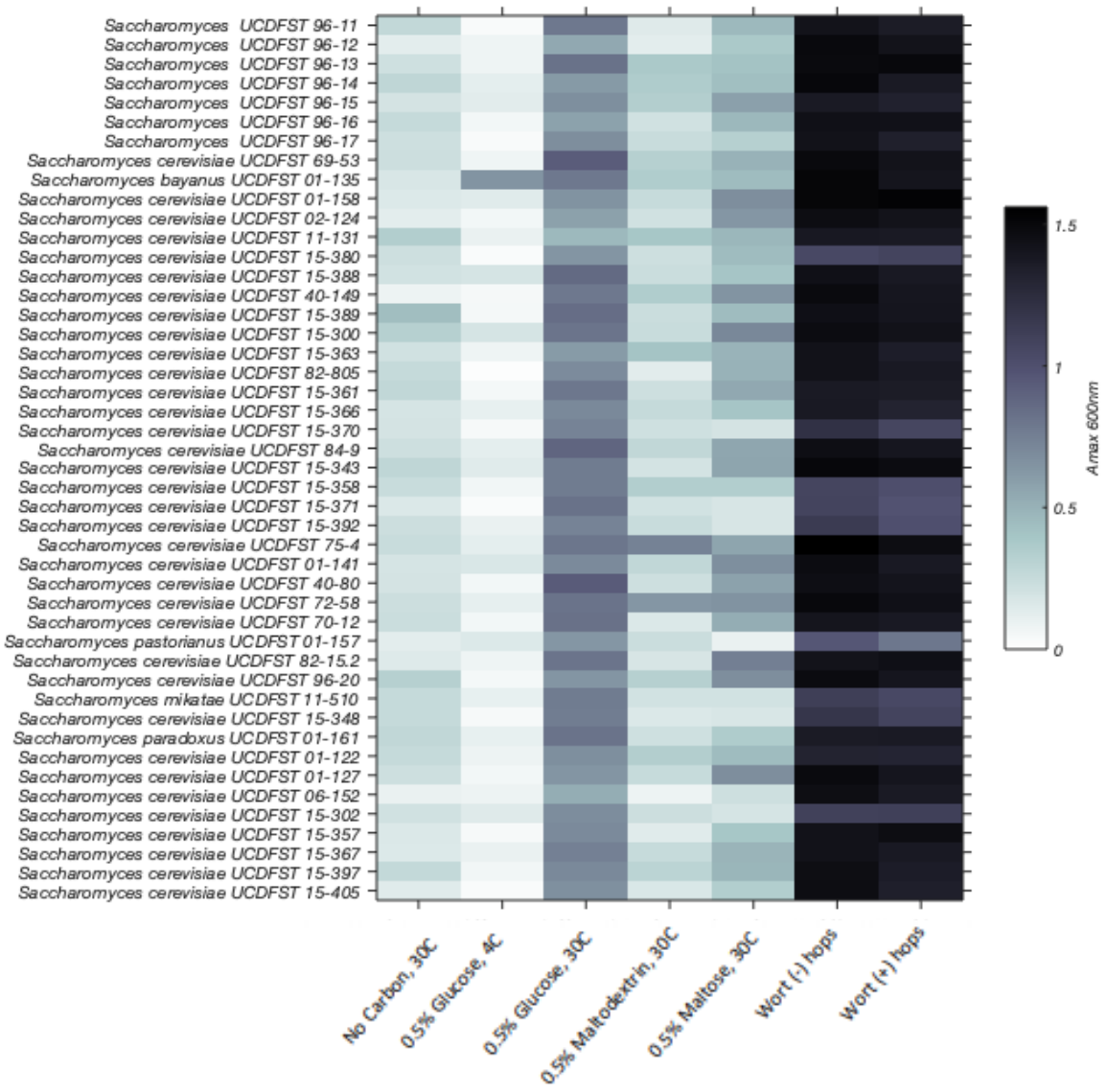

Figure 3.4. Heat Map of all yeasts when screening brewing potential. All wells with YNB and no carbon as a negative control, 0.5% glucose added as a positive control at 30 ºC, 0.5% glucose added at 4 ºC to simulate lager brewing conditions, and 0.5% maltose or maltodextrin added for brewing potential screening. Lab wort was also used to screen potential brewing use, with (+) and without (-) hops added. Figure created in MATLAB.

e. Non-Conventional Yeasts

Five different species of *Saccharomyces* were used in this study, including the typical S. cerevisiae and S. pastorianus used the fermentation of beer around the world. There were also non-conventional species of *S. bayanus*, *S. paradoxus*, and *S. mikatae* included in the screening.

All three of these yeasts grew similarly to the majority of the other strains screened in this study, showing their potential for use in the brewing of beer.

5. Conclusions

Most yeast in this screening grew well, with wort media having the most vigorous growth of all media used. The highest optical density from an amino selective medium was observed with the combination of YCB and L-glutamine, but unexpected growth was also recorded with the addition of L-proline. Further work should be carried out to explore the growth with proline as the sole nitrogen source, but the most obvious answer is to perform the screening again with newly acquired crystalline L-proline. All yeasts screened seemed to tolerate up to 12% ethanol, but three *S. cerevisiae* yeasts, UCDFST 15-300, UCDFST 15-348, and UCDFST 15-397 showed ethanol tolerance to concentrations as high as 16%. Further research remains to the higher ethanol treatments, as many craft brewers are pushing the limits of alcohol by volume in their beers. Wort media had the most vigorous growth for all yeasts when comparing potential brewing conditions, displaying its excellent potential as a growth medium for *Saccharomyces* spp.

Successful strains, including the non-conventional species, will be used for pilot scale fermentations of beer in the Anheuser Busch InBev UC Davis Research Pilot Brewery. Wells missing media need to be addressed, as this was a researcher error and is most likely a pipetting problem or evaporation issue from unsealed plates. Optical density readings could also be optimized by resuspending the yeast in each well by pipetting at the end of the growth, this would give a better understanding of the overall ability to grow. Yeasts should also be genetically confirmed with PCR and sequencing at the end of the screening to ensure purity in each well, and to validate the species identity as many strains bear the species name assigned by

Dr. Herman Phaff over 20 years ago. Additionally, future studies are needed to gauge whether the best performing yeasts in the screening study show statistically significant improvement over conventional strains with regards to the responses measured here. The Phaff yeast collection is a valuable resource for non-conventional yeast strains for the brewing industry.

Chapter 4

Brewing Efficacy of Non-Conventional *Saccharomyces* non-*cerevisiae* Yeasts

Finalizing submission for American Society of Brewing Chemists Journal

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Abstract

Consumer demands for new sensory experiences have driven the research of unconventional yeasts in beer. While much research exists on the use of various common *Saccharomyces cerevisiae* strains as well as non-*Saccharomyces* yeasts, there exists a gap in knowledge regarding other non-*cerevisiae Saccharomyces* species in the fermentation of beer, outside that of *S. pastorianus*. Here, five distinct species of *Saccharomyces* from the UC Davis Phaff Yeast Culture Collection, as well as one interspecies hybrid from Fermentis, were chosen to ferment 40 L pilot scale beers. *S. kudriavzevii*, *S. mikatae, S. paradoxus, S. bayanus,* and *S. uvarum* yeasts were fermented in duplicate, with one fermenter in each pair receiving 10 g/L dry-hop during fermentation. Analytical measurements were made each day of fermentation and compared to controls of SafAle US-05 and SafLager W 34/70 for commercial brewing parameters of interest. Finished beers were also analyzed for aroma, taste, and mouthfeel to determine the flavor of each yeast as it pertains to brewing potential. All beers exhibited spicy characteristics, likely from the presence of phenols; dry-hopping increased fruit notes while also increasing perceived bitterness and astringency. All of the species in this study displayed great brewing potential, and might be an ideal addition to beer depending on a brewery's desire to experiment with flavor and willingness to bring a new yeast into their production environment.

Keywords: non-conventional yeasts, *Saccharomyces*, fermentation, beer, dry-hopping, brewing potential

A. Introduction

Increasingly, changing demands by beer drinkers in search of new sensory experiences are driving research into novel fermentations [182,186,205,220]. Much of this research has utilized non-*Saccharomyces* yeast strains [78,80,89,207,221–224], which can be attributed to the rise in popularity of

mixed-fermentation beers [169,225,226]. This pursuit of distinctive aromas and flavors has similarly driven the increased use of non-*cerevisiae Saccharomyces* species in the alcoholic fermentation of all beverages [92,93,105,141,170,227,228]. While much of this work has been focused on wine fermentations, the most widely used non-*cerevisiae* species is *S. pastorianus*, which has been used the world over in the production of lager beers for centuries [72,92,94,101,157].

In addition to novel yeast-derived flavors, brewers are increasingly turning to dry-hopping to enhance their consumers' sensory experience. Historically this procedure of adding hops (*Humulus lupulus*) cones to beer when fermentation is active or finished was performed to provide packaging and transport stability [25,57]. Relatively more recently with the rise of Craft Brewers, dry-hopping with pellets or advanced hop products [58] has become a common tactic used by brewers desiring to add interesting flavors and aromas to their beer [59].

All *Saccharomyces* yeast species that have been found to produce ethanol from carbohydrate sugar sources have been classified as part of the *Saccharomyces sensu stricto* (*Sss*) complex [96,98,154]. While the *Sss* currently contains ten distinct species, only eight have been linked to alcoholic beverage fermentation (*Fig. 1.6*). Use of *S. cerevisiae* and *S. pastorianus* have long been known for their use in alcoholic beverage production, but the *Sss* contains several non-conventional species. *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, *S. bayanus*, and *S. uvarum* that have already shown potential for alcoholic beverages, and have been identified in fermentations of wine, tepache, cider, chicha, palm wine, umqombothi, and other beverages [105,125,136,138,151,158,179]. Many of these fermented beverages, however, contain mixed cultures of yeasts and sometimes bacteria, in addition to naturally formed interspecies hybrids between two or more different *Saccharomyces* species [157,229]. To date, none of these species have been evaluated in monoculture fermentations in a beer brewing context.

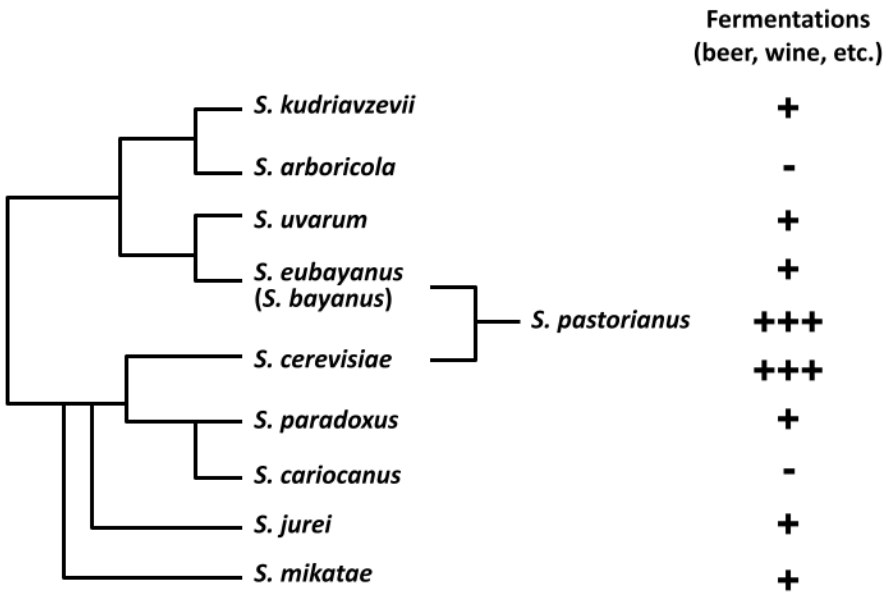

Figure 1.6. *Sss* phylogeny and extent of use in alcoholic beverage fermentations. *Saccharomyces bayanus* is listed in parenthesis to indicate it was derived from multiple hybridization events [73]. *S. pastorianus* is shown as a genetic hybrid of *S. eubayanus* and *S. cerevisiae* [93]. Use in fermented beverages is indicated with plus signs (+) for current commercial use, with *S. cerevisiae* and *S. pastorianus* exhibiting the most ubiquitous use in beer, and negative signs (−) for no known use. *S. cariocanus* is known to be harboring just four translocated chromosomes different than *S. paradoxus* [97]. *S. jurei* has very recently been proven to have brewing potential [102].

First isolated from oak trees of western Europe, *S. kudriavzevii* is a wild-type yeast that has been sequenced to contribute 23-96% of its genome to hybrids with *S. cerevisiae* [104,105,227]. While no commercial examples of its use in beer fermentation exist, *S. kudriavzevii* has been isolated from mixed-cultures of farmhouse ciders in France and draft beer systems in Germany to New Zealand [110,111]. Due to its propensity to hybridize, this yeast has even been found as part of the genetic makeup in Belgian Trappist ale strains from Chimay, Westmalle, and Orval [107]. *S. kudriavzevii* is a cryophilic species and is currently used to ferment wines at lower temperatures (10 °C to15 °C) in Europe and Australia [103,105]. Because it thrives at low temperatures and may have aromas similar to Belgian beers, *S. kudriavzevii* has potential for use in the production of hoppy lager beers in the brewing industry.

S. paradoxus has been found in African umqombothi [125] and white wine fermentations previously [123], but has only been studied for its beer brewing potential (at 15 °C) very recently, since the inception of this research [228]. *S. paradoxus* was one of the first species isolated as a member of the *Sss* outside of *S. pastorianus* and *S. cerevisiae* and is typically found in tree sap of Northeastern Europe [116]. Being a wild-type yeast species suggests *S. paradoxus* may produce interesting volatile aroma compounds at warmer (18 °C to 24 °C) ale temperatures [124]. *Saccharomyces mikatae* is a wild yeast that contributes to genetic hybrids from interspecies hybridization events with *S. cerevisiae* and *S. paradoxus* [129], and was first isolated from soil and decaying leaves in Japan [97]. *S. mikatae* was shown to form a biofilm on the surface of liquid media (pellicle) after twenty-five days at 20 °C, similar to wild-type strains [97]. It produced fruity, banana, floral, and sweet perfume aromas in white wine, and ferment slowly, perhaps all due to its diversion from the *S. cerevisiae* parent genome [130,131]. Both *S. paradoxus* and *S. mikatae* offer unique characteristics that might be of interest to craft brewers creating beer at ale fermentation temperatures.

Saccharomyces bayanus was previously thought to be the parent of the lager strain, *S. pastorianus* [93,110,135], but the hybridization event that produced lager brewing yeast is now proven to have occurred between *S. cerevisiae* and *S. eubayanus* [27,101,151,152]. *S. bayanus* has been characterized as its own species within the *Sss*, but in order delineate it from *S. eubayanus* and *S. uvarum*, it is commonly referred to as *S. bayanus* var. *bayanus* [73,93]. Genetic analysis of organisms in beer fermentations have identified *S. bayanus* as part of blended cultures due to its chromosomal similarity to *S. pastorianus* [72], but it is most common as a solitary species in wine fermentations [152]. A close relative, *Saccharomyces uvarum*, was once was thought to be a variant of *S. bayanus*, but has since been confirmed as a distinct species [133]. *S. uvarum* has been found to

be part of the mixed culture of spontaneously fermented wines [136], as well as an interspecies hybrid known in some Norwegian kveik strains [141]. Both *S. bayanus* and *S. uvarum* exhibit increased levels of isoamyl acetate in wine and brandy [230,231], and might contribute similar flavor to beer.

Some yeast suppliers are leveraging the power of interspecies hybrids to create distinctive sensory experiences, including a *S. cerevisiae* x *S. bayanus* hybrid produced by Fermentis-LeSaffre (Marcq-en-Baroeul, France, EU; fermentis.com/en/) known as SafŒno HDT18 [76]. This interspecies hybrid has been created through a LeSaffre R&D program to select a yeast strain that exhibits increased expression of aromatic terpenes. New research has identified these terpene compounds as some of the most impactful on dry-hopped beer aroma [59,77] through biotransformation with glycosides and alcohols to produce unique aroma characteristics [232]. While this yeast was developed for wine fermentations, it may be of great interest to brewers making dry-hopped beers, and was therefore selected for this study.

While there is much research regarding the use of some of these species in a laboratory scale or wine fermentation, work remains for their efficacy and commercial use in the production of beer. Additionally, little to no sensorial analysis exists on the use of any of these *Saccharomyces* spp. in the fermentation of beer, most notably at ale fermentation temperatures (18-20ºC) or in dry-hopped beers. The aim of this study is to assess the brewing potential of the non-conventional non-*cerevisiae Saccharomyces* species outlined above by assessing fermentation kinetics and performance, yeast abundance and viability post-fermentation for serial re-pitching, as well as the flavor characteristics of the resultant beer. Beers in this study will be run as both dry-hopped and standard fermentations due to the pervasiveness of dry-hopping in the American craft brewing industry. While the most widely used non-*cerevisiae*

Saccharomyces species is *S. pastorianus*, it will not be discussed here as much research already exists on its brewing potential.

B. Materials and Methods

a. Experimental Beers

A total of eight all-malt pilot scale brews were performed on the 1.8 hL Anheuser-Busch Research Pilot Brewery at the University of California, Davis. Brewing parameters, as well as the malt, hops, water chemistry, mashing regime, pH, boiling parameters, and knockout temperatures followed the same method as outlined in previous research[233]. The experimental beer recipe was similar to an American Pale Ale or Session IPA, with a target original gravity of 10 ºP, to yield a 4.2% (v/v) alcohol beer under standard ale fermentation conditions. Wort from each of the eight brews was split evenly by volume between four 56 L fermenters, to fill each with approximately 40 L of cooled wort.

b. Yeasts

Saccharomyces yeasts sourced from the University of California, Davis, Phaff Yeast Culture Collection (phaffcollection.ucdavis.edu) included the type strains of *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, *S. bayanus*, and *S. uvarum*. Additionally, the control *S. cerevisiae* and *S. pastorianus* species and one *S. cerevisiae* x *S. bayanus* hybrid were provided by Fermentis (*Table 4.1*). Yeasts from the Phaff Collection were revived from cryogenic storage and streaked onto potato dextrose agar (PDA) plates and incubated for 2 days at 30 ºC before being moved to room temperature storage until propagation. Yeasts from Fermentis were provided as an active dry yeast with the emulsifier E491 (sorbitan monostearate) and stored at 4 ºC until propagation.

Table 4.1. Non-conventional non-*cerevisiae Saccharomyces* and control yeasts used in the fermentations of the experimental beer. Yeasts were sourced from either the Phaff Yeast Culture Collection at the University of California, Davis (UCD), or from Fermentis LeSaffre of Marcq-en-Baroeul, France (Saf). Type strain as defined in MycoBank (mycobank.org), origin, isolation, flocculation, and attenuation, as defined in the scientific or product literature. SafAle US-05 and SafLager W 34/70 are included as controls.

Scientific Name	**Yeast Name**	**Type Strain**	**Isolated From**	**Geographic Origin**	**Flocculation**	**Attenuation**
Saccharomyces kudriavzevii	UCDFST 11-515	NCYC 2889T	oak tree bark	Western Europe	Medium High	Moderate
Saccharomyces paradoxus	UCDFST 01-161	DBVPG 6411	tree exudate	Northeast Europe	Medium	Moderate
Saccharomyces mikatae	UCDFST 11-510	NCYC 2888T	soil	Japan	Medium	Moderate Low
Saccharomyces bayanus	UCDFST 01-135	CBS 380	turbid beer	Italy	Medium	Moderate
Saccharomyces uvarum	UCDFST 11-512	CBS 395	fruit and seeds	Scandinavia	High	Moderate
Saccharomyces cerevisiae x *Saccharomyces bayanus*	SafŒno HD T18	(R&D)*	LeSaffre R&D	France	Medium	High
Saccharomyces cerevisiae	SafAle US-05**	*	*	USA	Medium	78-82%
Saccharomyces pastorianus	SafLager W 34/70	W 34/70	Weihenstephan	Germany	High	80-84%

* unknown **SafAle US-05 fermentations were done in biological triplicate.

All yeast were propagated according to the same procedure to ensure consistency throughout this study. Due to time constraints with research brewing, only one yeast was chosen on which to perform three biological replicates to ferment from three separate brews: *S. cerevisiae* SafAle US-05. Yeasts were propagated in wort consisting of 10.0% w/v (10.0 °P, 1.040 Specific Gravity) dried pilsner malt extract (Briess CBW® Pilsen Light; Chilton, WI, USA) in deionized water with 20 ppm $CaCl_2$ salts, targeting 5.2 pH, and 0.10% w/v yeast nutrient (Kerry Yeastex® 82; Beloit, WI, USA). Wort was boiled for ten minutes and sterilized via autoclave before being sterile filtered to remove protein and trub particulate. All transfers of yeast and wort were done in a laminar flow hood or positive pressure room. Yeast colonies were transferred from PDA plate or package of active dry yeast via sterile inoculation loop to propagation wort and propagated stepwise over the course of 11 days following the methods outlined in previous research[233] and *Figure 4.2*. All propagations were performed at room temperature on a platform orbital shaker (Innova™ 2000, New Brunswick Scientific; Edison, NJ, USA) set to 150 rpm. Yeast cell counts and viability testing with methylene blue were performed on all propagations and fermentations according to standard methods [234].

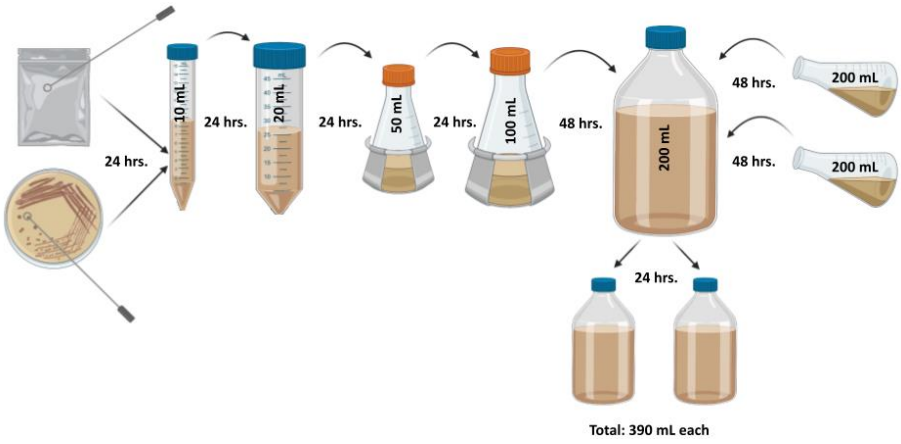

Figure 4.2. Yeast propagation schematic following previous methods[233]. Yeasts were propagated to a final approximate total of 40.0 x 10^{10} cells in each bottle with a total of 390 mL of propagation wort, equivalent to the

standard ale pitch rate of 1.0 x 10^6 cells per mL per ºP [9] for each 40 L, 10 ºP pilot fermentation. Figure created on BioRender.com, not to scale.

c. Pilot Scale Fermentations

Pilot fermentations were performed in 56.0 L glycol-cooled cylindroconical fermenters (JV Northwest; Canby, OR, USA) filled to 40.0 L and set to a standard ale temperature of 20.0 ºC. Each unique *Saccharomyces* species (*Table 4.1*) was pitched to its own fermenter in duplicate, with the control *S. cerevisiae* US-05 duplicates fermented in biological triplicate for quality assurance, totaling twenty distinct fermentations. One fermenter in each yeast pair received 10.0 g/L Centennial (8.3% AA, Hopsteiner, New York, NY) T-90 hop pellets as a dry-hop when the measured gravity decreased to below 4.0 ºP or at seven days into fermentation, whichever occurred first. This amount of dry-hopping has become standard practice among craft breweries today, with many brewers far exceeding this amount at times [58,59,201,202]. End of fermentation or "terminal gravity" [23] was defined here as a change of less than 0.10 ºP gravity for two simultaneous days following dry-hop.

After fermentation was completed, all beer in all fermenters except the *S. cerevisiae* and *S. pastorianus* controls were cold conditioned at 0.0 ºC for two days to allow for natural clarification. Yeast and hops were removed from the bottom of the cylindroconical fermenter before the beer was transferred to a 19.6 L Sankey keg for carbonation. All were packaged from the kegs into CO_2-purged 0.95 L (32 oz.) "Crowler" cans (Ball Corporation; Westminster, CO, USA) and stored below 4.0 ºC until sensory analysis and shipping.

d. Sample Collection and Preparation

Fermenting beers were aseptically sampled daily within a two-hour window of the time of knockout transfer of wort to fermenter. 50 mL conical tubes of each sample were centrifuged (ThermoFisher Scientific; Waltham, MA, USA) at 20 °C and 3000 x g RCF for five minutes. The clarified supernatant was then degassed for five minutes using the degas setting on a VWR B1500A-DTH 1.90 L ultrasonic cleaner (Radnor, PA, USA). Degassed samples were then decanted into the sample tubes of the Anton Paar (Graz, Austria, EU) auto-sampling carousel for immediate analysis. Samples were then measured for extract, gravity, alcohol [196], real degrees of fermentation (RDF), and calories using an Anton Paar Density Meter (DMA 5000 M) and alcolyzer (Alcolyzer Beer M). The DMA 5000 M has a repeatability within 0.000001 g/mL and the Alcolyzer Beer M has a repeatability within 0.03 ºP and 0.01 % v/v alcohol. pH was measured on a ThermoFisher Scientific benchtop pH meter that received a weekly three-point calibration.

e. Sensory Analysis

Each set of packaged beer from an individual fermentation was assigned a randomly selected three-digit code in order to ensure blind analysis of experimental samples. The willing members of the UC Davis Brewing and Malting Science laboratory team (n = 7) used a modified consensus method [235] with check-all-that-apply (CATA) [236] in two tastings to choose appropriate aroma descriptors from the DraughtLab Beer Flavor Map© (*Fig. 4.3*) for the twelve beers being analyzed. *S. cerevisiae* and *S. pastorianus* controls fermentations were not included. The lab members assessed beers served in 60 mL volumes in clear straight sided glasses, after being removed from cold storage (4.0 ºC), under white light. Consensus panelists were instructed to cleanse their palates with water and unsalted crackers between each sample. The common aroma

descriptors were parsed down to the twelve most recurrent amongst the experimental beers. Each of these twelve descriptors, the five accepted taste modalities, and three recurrent mouthfeel descriptors from the consensus panel were placed on a 9-point intensity scale for scoring by the local brewery panelists (*Table 4.2*).

Figure 4.3. Beer Flavor Map©, as provided by DraughtLab, that outlines the flavor descriptors common to beer and was used to determine terms for consensus method and subsequent descriptive analysis.

Beers were cold transferred to local breweries within three weeks of packaging for sensory analysis with the descriptors previously determined via consensus. Trained beer sensory taste panels at Lagunitas Brewing Company (Petaluma, CA, USA), Deschutes Brewery (Bend, OR, USA), Russian River Brewing Company (Windsor, CA, USA), Sierra Nevada Brewing Company (Chico, CA, USA), Budweiser Brewery (Fairfield, CA, USA), and Sudwerk Brewing

Company (Davis, CA, USA) used the descriptors determined previously by consensus method and rated each on a 9-point intensity scale from "none" to "extremely strong" [237,238]. Training, methods and frequency of sensory panels varied from brewery to brewery, however it was minimally required that the panelists were able to accurately distinguish dry-hopped from non-hopped beer and identify German, Belgian, and American ale strain characteristics. The total sample group to perform sensory analysis on the experimental beers consisted of 51 panelists (36 male and 15 female), ranging in age from 24 to 61. No panelists had medical reasons for not consuming alcohol.

Table 4.2. Sample ballot given to brewery taste panels accompanying the beer for sensory analysis. Aroma attributes determined from consensus method with CATA performed by UC Davis Brewing Lab members.

Beer: XXX				Sex: M / F				Age:	
Score each attribute by circling a number, with 0 = none to 9 = extremely strong									
Aroma:									
Cereal: Grainy, Biscuit, Cracker, Wort	0	1	2	3	4	5	6	7	8	9
Earthy: Musty, Barnyard, Mushroom	0	1	2	3	4	5	6	7	8	9
Spicy: Clove, Black Pepper, Ginger	0	1	2	3	4	5	6	7	8	9
Grassy: Fresh Cut, Dry Leaves, Green, Hay	0	1	2	3	4	5	6	7	8	9
Citrus: Grapefruit, Orange, Lemon, Lime	0	1	2	3	4	5	6	7	8	9
Tropical: Mango, Papaya, Guava, Banana	0	1	2	3	4	5	6	7	8	9
Stone Fruit: Apricot, Nectarine, Peach	0	1	2	3	4	5	6	7	8	9
Stale: Cardboard, Goat Hair, Oxidation, Meaty	0	1	2	3	4	5	6	7	8	9
Vegetal: Cooked Vegetable, Onion, Celery	0	1	2	3	4	5	6	7	8	9
Solvent: Chemical, Paint Thinner, Nail Polish Remover	0	1	2	3	4	5	6	7	8	9
Rotten: Baby Vomit, Sweat, Boiled Egg	0	1	2	3	4	5	6	7	8	9
Metallic	0	1	2	3	4	5	6	7	8	9
Other: (Write In)	0	1	2	3	4	5	6	7	8	9
Taste:									
Sweet	0	1	2	3	4	5	6	7	8	9
Bitter	0	1	2	3	4	5	6	7	8	9
Sour	0	1	2	3	4	5	6	7	8	9
Salty	0	1	2	3	4	5	6	7	8	9
Umami	0	1	2	3	4	5	6	7	8	9
Mouthfeel:									
Body	0	1	2	3	4	5	6	7	8	9
Alcohol	0	1	2	3	4	5	6	7	8	9
Astringency	0	1	2	3	4	5	6	7	8	9

f. Statistical Analysis

Standard deviation values, two-tailed statistical analysis (*t*-test) of fermentation data with corresponding *p*-values, as well as two-way analysis of variance (ANOVA) and coefficients of variance for sensory data were performed in Microsoft® Excel 2019, Version 2102 (Build 13801.20360).

C. Results and Discussion

a. Pilot Fermentations

Ten total brews were performed for the twenty fermentations, with a mean original gravity (O.G.) of 10.2º Plato (±0.36), and a higher brewhouse efficiency than expected for the recipe designed at 10.0º P (*Fig. 4.4*). Fermentations were carried out at 20.0ºC, standard ale temperatures, and analytical parameters were measured on each day of fermentation. Results were compared with the two control strains, *S. cerevisiae* US-05 and *S. pastorianus* W 34/70. Vigorous fermentations of the control species suggest an adequate yeast pitching rate, nutrients levels, and wort aeration were utilized. All fermentations reached terminal gravity within two weeks, with the exception of *S. uvarum* UCDFST 11-512, which took fifteen days for the non-hopped fermentation but only eleven days for the dry-hopped fermentation (*Table 4.3*). However, all average fermentation lengths were not shown to be statistically different between dry-hopped and non-hopped fermentations ($p > 0.05$). These fermentation lengths indicate all the yeasts studied here are viable candidates for production breweries that normally ferment lagers, but perhaps too long for breweries that normally produce ales. Conditioning time was not accounted for in this study, as all fermentations were deemed terminal based on gravity instead of from the presence of secondary metabolites, such as diacetyl or acetaldehyde concentrations.

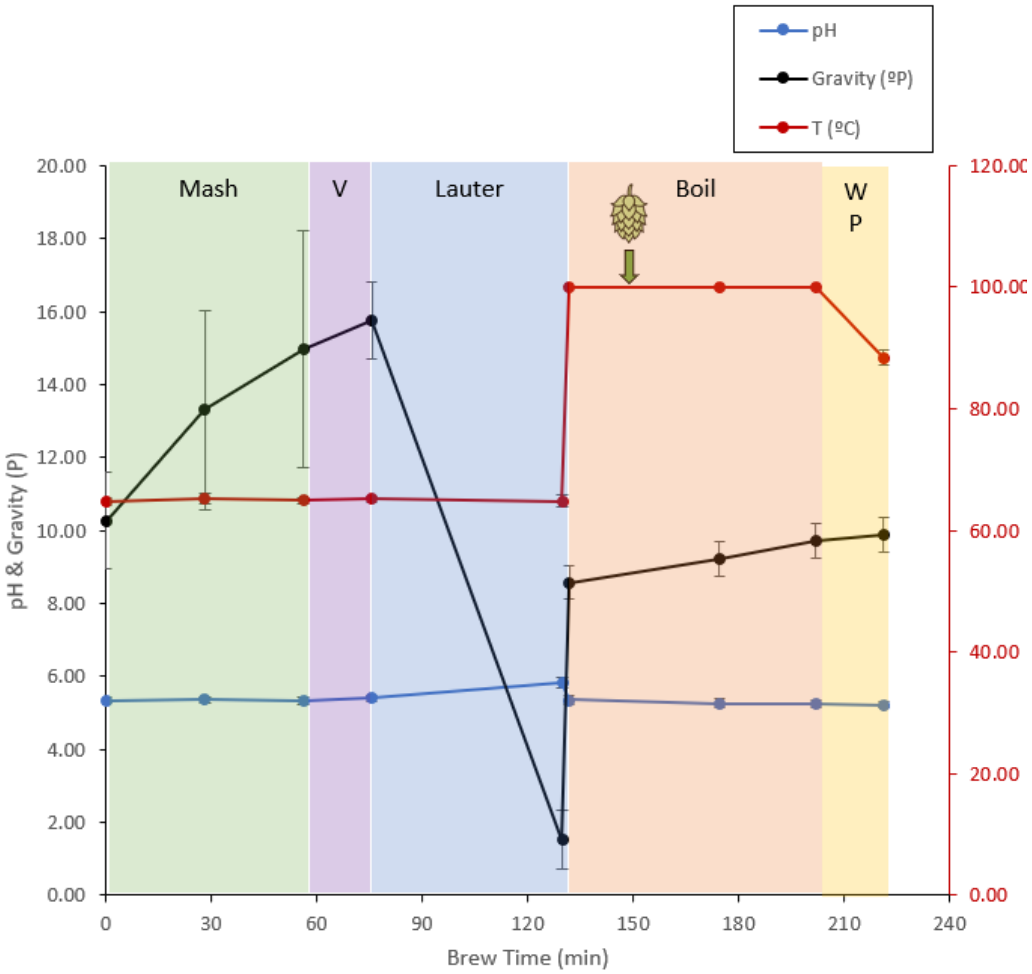

Figure 4.4. Average of important brew day analytical parameters, with error bars representing standard deviation.

Table 4.3. Terminal fermentation characteristics of *Saccharomyces* species and reference strains used to ferment all-malt wort at 40.0 L pilot scale under two different conditions: non-hopped or dry-hopped during fermentation. Measurements of original gravity (O.G.), final gravity (F.G.), alcohol by volume (ABV), real degree of fermentation (RDF), and calories (Cal) performed on Anton Paar Alcolyzer Beer M. Viability was performed from cells in suspension on non-hopped beers on day of terminal gravity, stained with methylene blue, as per standard procedure (69). Viability was not performed on dry-hopped beers due to interference from hops in suspension. Fermentation length as given in days to achieve final gravity. Strain listed as "Hybrid" is interspecies hybrid of *S. bayanus* x *S. cerevisiae* from LeSaffre R&D.

	S. kudriavzevii	***S. paradoxus***	***S. mikatae***	***S. bayanus***	***S. uvarum***	**Hybrid**	***S. cerevisiae***	***S. pastorianus***
	11-515	01-161	11-510	01-135	11-512	HD T18	US-05*	W 34/70
				Non-Hopped				
O.G. (°P)	10.0	10.1	10.1	10.3	10.4	9.80	10.3 ± 0.6	10.4
F.G. (°P)	1.93	3.43	8.75	2.10	3.33	3.32	1.99 ± 0.36	1.84
ABV (%v/v)	4.26	3.78	0.95	4.51	3.74	3.56	4.44 ± 0.19	4.45
RDF (%)	66.4	55.9	14.2	66.1	56.2	55.4	66.6 ± 1.63	67.5
Cal (kJ/100 mL)	150	160	163	160	157	152	156 ± 10.6	154
pH	4.24	4.45	4.60	4.31	4.48	4.16	4.36 ± 0.06	4.42
Viability (%)	80.7 ± 2.4	97.1 ± 0.8	99.0 ± 0.5	83.7 ± 1.9	81.6 ± 4.5	**	**	**
Ferm. Length (days)	13	10	8	6	15	9	8.33 ± 0.58	6
				Dry-Hopped				
Original Gravity (°P)	10.0	10.1	10.1	10.3	10.4	9.80	10.3 ± 0.6	10.4
Final Gravity (°P)	1.79	3.44	8.96	1.64	3.39	3.09	1.69 ± 0.42	1.57
Alcohol (% v/v)	4.43	3.91	0.92	4.85	3.86	3.91	4.69 ± 0.20	4.76
RDF (%)	67.8	56.5	13.5	69.8	56.5	58.2	69.2 ± 2.19	70.0
Calories (kJ/100 mL)	153	163	165	162	161	158	159 ± 11.5	159
pH	4.47	4.68	4.75	4.47	4.55	4.45	4.64 ± 0.02	4.70
Ferm. Length (days)	13	11	9	8	11	10	10.0 ± 1.0	8

* indicates the mean of the three biological replicates **data not recorded

All yeasts measured for viability showed greater than 80.0 % living cells at the end of fermentation, signifying a potential for serial re-pitching in a commercial setting. Viability was not measured on the two control strains, US-05 and W 34/70, as their ability for propagation and serial re-pitching has been extensively studied [70,239,240]. Viability data for *S. bayanus* x *S. cerevisiae* HD T18 was not available and should be further evaluated as it is not standard practice to re-pitch wine yeasts due to ethanol toxicity [241].

When comparing the dry-hopped and non-hopped fermentations, average differences for alcohol, calorie, and pH measurements between the two treatments when comparing each yeast species were highly significant ($p < 0.01$), and less so when measuring RDF ($p < 0.05$). Dry-hopping has been shown to biochemically change the composition of wort during fermentation, allowing yeast access to a greater amount of fermentable sugars and subsequent additional fermentative capacity, a phenomenon known as hop creep [51,189,190,242,243]. Most of the novel yeasts shown here show no ability to mitigate the hop creep phenomenon in an effective manner, as all yeasts, with the exception of *S. mikatae* UCDFST 11-510, showed increases in RDF (*Table 4.3*) and alcohol (*Fig. 4.5*) from the addition of dry-hops during fermentation.

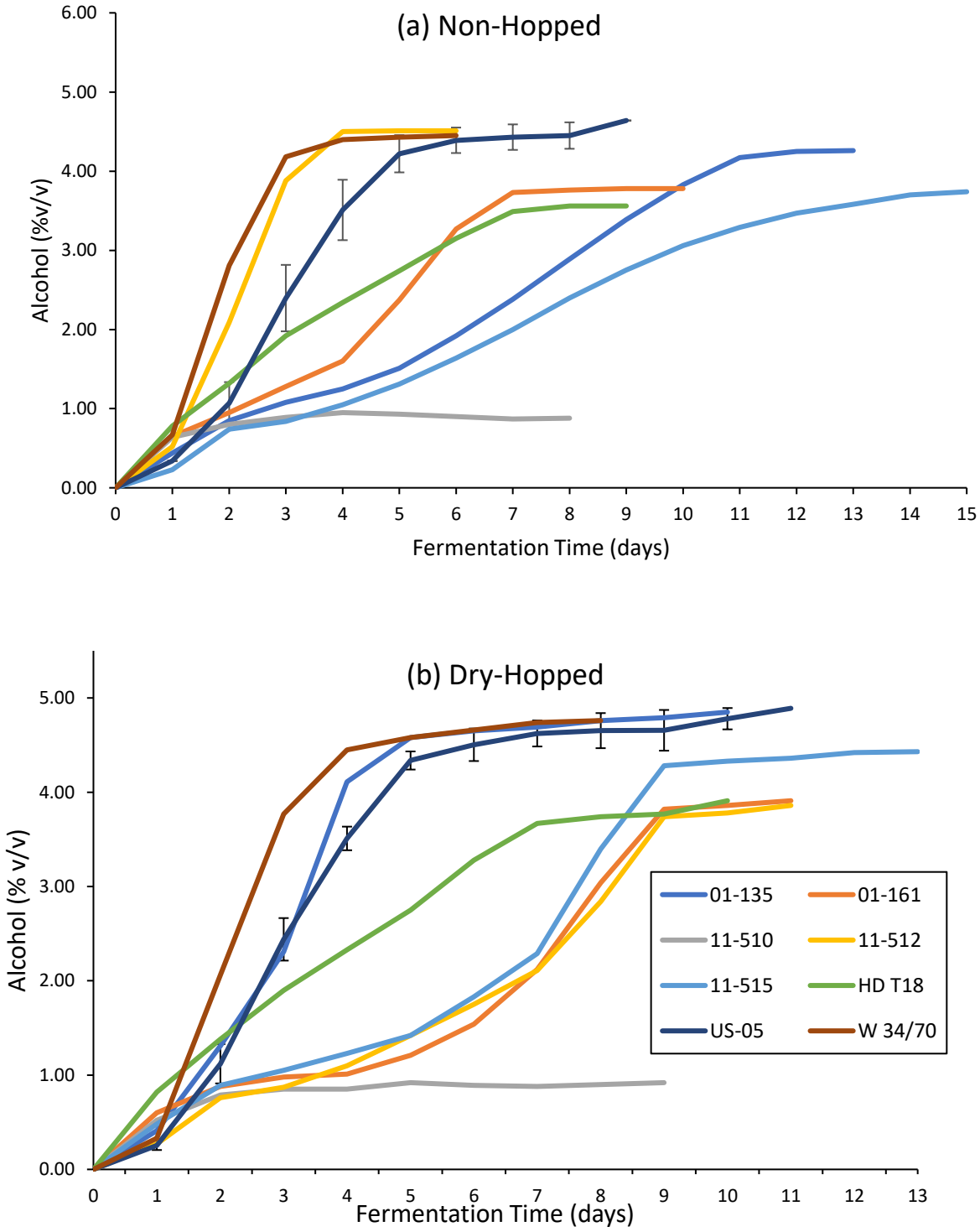

Figure 4.5. Alcohol content by volume measured daily on the Anton Paar Alcolyzer Beer M, as reported for both **(a)** non-hopped and **(b)** dry-hopped fermentations of all yeasts in this study. Results for US-05 are reported as the mean of three biological replicates with error bars for standard deviation at each day of fermentation.

Fermentation kinetics were grouped more closely in the dry-hopped fermentations compared to the non-hopped treatment (*Fig. 4.5*), with *S. bayanus* UCDFST 01-135 showing the most similar fermentation profile to both of the control strains, and *S. kudriavzevii* UCDFST 11-515, *S. paradoxus* UCDFST 01-161, and *S. uvarum* UCDFST 11-512 showing slower, yet steady fermentation. *S. paradoxus* UCDFST 01-161 showed decreased kinetics with the addition of dry-hops (*Fig. 4.5b*), but was still a slower fermenter than the control strains in both treatments. The *S. cerevisiae* x *S. bayanus* hybrid HD T18 showed no change in kinetics with the addition of dry-hops, showing moderate and steady fermentative capacity, with a terminal RDF similar to *S. paradoxus* UCDFST 01-161 and *S. uvarum* UCDFST 11-512. The *S. cerevisiae* x *S. bayanus* hybrid HD T18 fermented to a lower relative alcohol content than these other strains due to it starting from a brew with the lowest O.G.

Of note, is the strain UCDFST 11-510, *S. mikatae*, as it was an outlier from the group with the lowest RDF (*Table 4.3*) and final amount of alcohol produced, whether dry-hopped or not (*Fig. 4.5*). UCDFST 11-510 recorded 99.0 ± 0.5% yeast viability in suspension at the end of fermentation, yet only 14.2% RDF in the non-hopped treatment. This indicates the strain is a potential candidate for low or no alcohol beer fermentations if brewing parameters are adjusted to get the final alcohol below 0.5% (v/v) and considerations are taken for microbial stability. Analysis of the sugars remaining in this beer may aid in determining which carbohydrates this *S. mikatae* strain was able to assimilate during fermentation. Additionally, this species has been shown to form a pellicle on top of fermenting beer after twenty-five days at 20 ºC [97], suggesting it may ferment comparably slowly as wild-type yeasts, such as *Brettanomyces* or *Hanseniaspora* spp. Further research regarding *S. mikatae* in fermentation for the production of low and no alcohol beers should be performed.

b. Sensory Analysis

The flavor of the beers from these fermentations was investigated for aroma, taste, and mouthfeel in order to further qualify the brewing potential of these novel *Saccharomyces* yeasts. Modified consensus method from the UC Davis Brewing and Malting Science lab members yielded twelve aroma and three mouthfeel descriptors that were deemed most discriminant and non-redundant from the Beer Flavor Map© as provided by DraughtLab. The most commonly agreed upon descriptors included Cereal, Earthy, Spicy, Grassy, Citrus, Tropical, Stone Fruit, Stale, Vegetal, Solvent, Rotten, and Metallic for aroma, with additional descriptors within each aroma category outlined above (*Table 4.2*). DraughtLab software was used to confirm that statistically significant differences were observed for all of the consensus CATA terms after accounting for both panelist and replication effects. Body, Alcohol, and Astringency were selected as the most common mouthfeel descriptors.

From the panelists at participating breweries, all beers showed increases in bitterness and astringency from the high level of dry-hopping (*Fig 4.6*), suggesting beer clarification prior to packaging may have been necessary to fully distinguish the effects of the hops without particulates in suspension effecting flavor. The base beer was also of low alcohol and IBU content, which could contribute to perceived bitterness from the increase of humulinones from dry-hopping a low IBU beer [244], or perceived astringency from the increase of polyphenol content [189]. Dry-hopping increased the fruit (Citrus, Tropical, and Stone Fruit) perception on all beers as expected from the Centennial cultivar used here [245], with the exception of Stone Fruit in UCDFST 01-161 *S. paradoxus*.

All experimental beers displayed Spicy aromas, likely from the expression of phenols, but genetic testing for the POF phenotype should be performed to confirm [246]. Interestingly, these

Spicy aromas were perceived lower in the dry-hopped beers, in contrast to expectations, as resinous and spicy characteristics are also noted as aroma characteristics of Centennial hops. On average, many of the unique attributes perceived in the beers fermented with these yeasts can be generally considered as off-flavors in beer (Solvent, Metallic, Vegetal, Rotten, or Stale). Trained panelists perceived these descriptors in very low amounts, with no off-flavor characteristic achieving an average greater than 2 on the 9-point intensity scale.

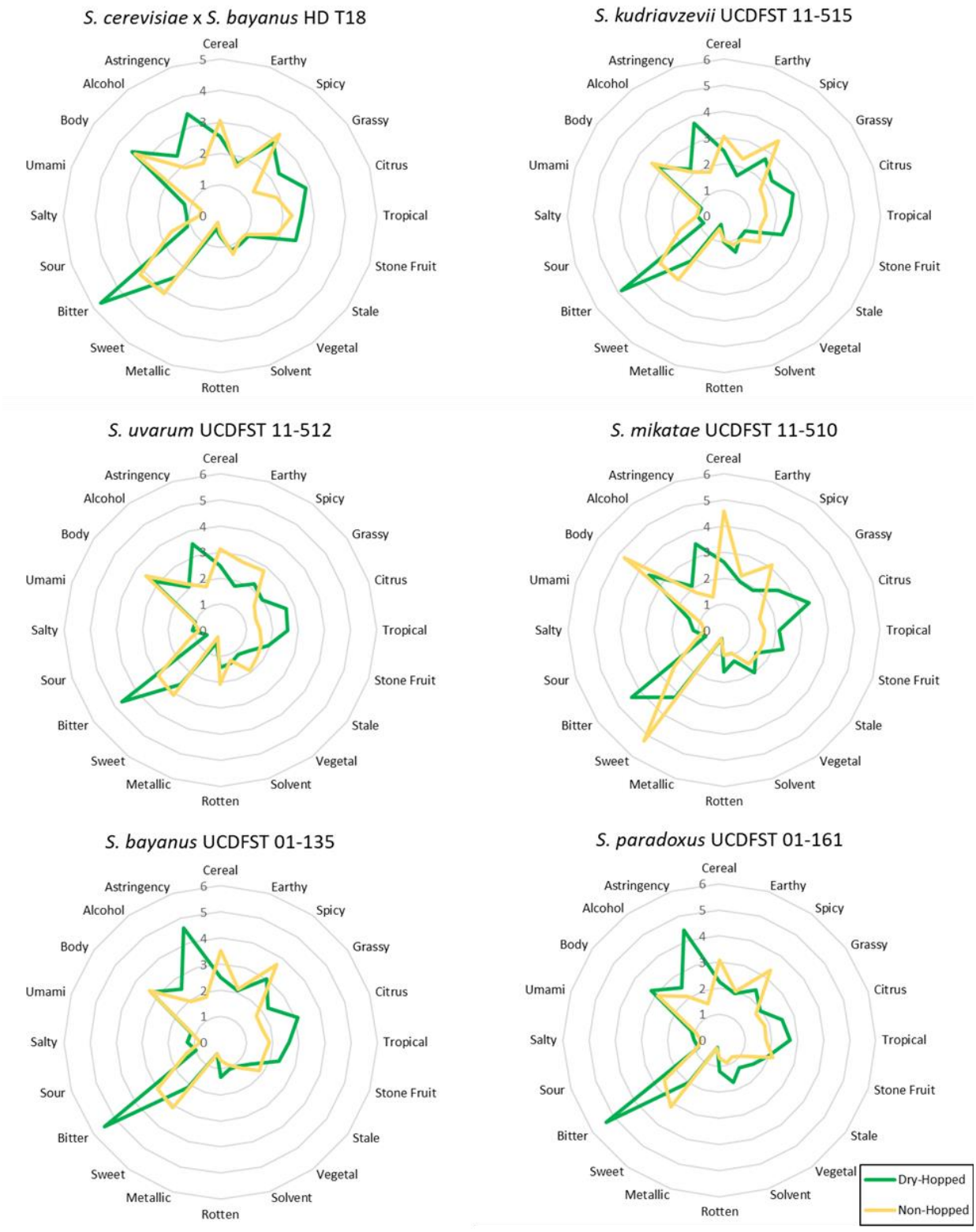

Figure 4.6. Radar charts of attributes for each experimental yeast fermentation in this study. Dry-hopped treatments are shown in green, while non-hopped are shown in yellow. (n = 51, with 36 male and 15 female)

Other descriptors were written in on the ballot (*Table 4.2*) by the trained panelists at breweries. Beers made with *S. uvarum* UCDFST 11-512 commonly had notes of diacetyl in the dry-hopped treatment and sulfur in the non-hopped fermentation. Beers made with *S. kudriavzevii* UCDFST 11-515 were described as having distinct phenolic and sulfur characteristics in the non-hopped treatment. The non-hopped beer fermented with *S. mikatae* UCDFST 11-510 was perceived as being wort-like, likely due to its low attenuation. Descriptors are given only if more than 10% of panelists (n = 5) reported a given characteristic.

D. Conclusion

Fermentation kinetics and yeast viabilities here suggest appropriate pitching rate, adequate nutrients, and proper aeration from the brewhouse were achieved on all brews and fermentations. All yeasts reached terminal gravity in under two weeks, with the exception of *S. uvarum* UCDFST 11-512, which took fifteen days for the non-hopped fermentation. These kinetics makes all the yeasts studied viable candidates for production breweries, but conditioning time should be accounted for but were not studied here. All fermentations in this study were deemed terminal based on gravity as opposed to metabolite production, so further analysis and brewer-specific standards are required. All yeasts displayed high potential for re-pitching in a commercial setting with high viabilities at the end of fermentation in the non-hopped fermentations. These high numbers are promising, but viability should be assessed during fermentation and prior to re-pitch in order to ensure adequate cell count for vigorous growth in a commercial setting.

With the exception of *S. mikatae* UCDFST 11-510, all yeasts displayed increased RDF and alcohol with the addition of dry-hops during fermentation, as was expected due to hop creep. Further research should be pursued in the use of *S. mikatae* UCDFST 11-510 and other strains of

this species for the production of low and no alcohol beers and its possible resistance to hop creep. Strong phenolic characteristics were perceived in the flavor of beers fermented with all yeasts, but dry-hopping, in this case with Centennial, decreased this aroma while increasing all fruit aromas, as well as bitterness and astringency. No flavors that are generally associated with poor fermentation scored high among trained sensory panels. Comparisons to standard beer yeast fermentations should have been performed in sensory analysis as well, but experimental design mistakes and time constraints did not allow. Previous research has shown these yeasts' ability to co-ferment with standard *S. cerevisiae*, and flavor analysis should also be performed on these potential combinations. All of these species in the *Sss* displayed great brewing potential given a brewery's desire to experiment with flavor and willingness to bring in a new yeast.

Acknowledgements

Much gratitude to Anne Flesch and Kevin Lane of Fermentis, as well as Kyria Boundy-Mills and Irnayuli Sitepu from the UC Davis Phaff Collection for advice in yeast selection and subsequent revival of cryogenically stored yeast. Much appreciation to Lindsey Barr and Victoria Chaplin at DraughtLab for providing access to their amazing sensory software. Thanks to Jean-Xavier Guinard and Lindsey Barr for helping formulate the way to run this sensory analysis. Thank you to Joy Wilson of Lagunitas Brewing, Amanda Benson of Deschutes Brewery, Vinnie Cilurzo of Russian River Brewing, Katrina Kettler of Budweiser Fairfield, Andrew Reyes of Sierra Nevada Brewing, and Alex Patil of Sudwerk Brewing for helping coordinate sensory panels for these beers. Thanks to the UC Davis Food Science and Technology Department for their financial support, as well as funds from the H.A. Jastro-Shields Research Award, Margrit Mondavi Graduate Fellowship, and Michael J. Lewis Endowment.

Chapter 5

Dry-Hop Creep Potential of Various *Saccharomyces* Yeast Species and Strains

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Abstract

Previous research has shown that hops contain enzymes able to hydrolyze unfermentable dextrins into fermentable sugars when added during the dry-hopping process. In the presence of live yeast, these additional fermentable sugars can lead to an over-attenuation of the beer; a phenomenon known as “hop creep”. This study attempts to analyze the effect of different Saccharomyces yeast species and strains on hop creep, with the intent to find an ability to mitigate the effects of dry-hop creep by using a specific yeast. Thirty different yeast species and strains were chosen from commercial and academic collections and propagated for pilot fermentations. Brews were performed at the Anheuser-Busch Research Brewery (1.8 hL, 10ºP, 20 IBU) at UC Davis and split to 40.0 L cylindroconical fermenters, with one fermenter in each yeast pair receiving 10.0 g/L Centennial hop pellets towards the end of fermentation. Standard analytical measurements were performed over the course of fermentation, with real degrees of fermentation (RDF) and extract measured on an Anton Paar alcolyzer. In order to preemptively determine the amount of hop creep to be experienced with each unknown fermentation, bench-top fermentations with 20.0 g/L dry-hops were performed concurrently and compared to the pilot scale fermentations. RDF was significantly higher ($p < 0.01$) on dry-hopped and non-dry-hopped fermentations beginning two days post dry-hopping to the end of fermentation, with the exceptions of SafAle BE-134, a *S. cerevisiae* var. *diastaticus*, and UCD 11-510, a S. mikatae. No apparent correlation between flocculation and increased RDF was shown in dry-hopped treatments. pH was significantly different between the dry-hopped and non-hopped fermentations ($p < 0.05$ one day post dry-hop, $p < 0.01$ for all subsequent days); this may have impacted on additional attenuation. No yeasts in this study indicated their use for mitigation of

dry-hop creep, but this is a first look at beer fermentation for some of the chosen yeasts. The results also present a new perspective on how hop creep varies in fermentation.

Keywords: yeast, *Saccharomyces*, fermentation, beer, dry-hopping, hop creep

A. Introduction

Traditionally in the production of beer, hops (*Humulus lupulus*) are added in the brewhouse in relatively small quantities, adding bitterness to balance the sugary wort, flavor and aroma to the finished beer, foam and microbiological stability, and clarity in the brewhouse [15,25,180]. Hops (cones or pellets) are also added to beer when fermentation is active or finished, a process called dry-hopping, historically to provide packaging and transport stability [25,57] and more recently to add intense hop aroma and flavor [58,59]. In craft breweries, dry-hopping has become the standard procedure for adding hop flavor and aroma without the resultant bitterness of the alpha acid content to many styles, but most frequently to the India Pale Ale (IPA) style of beer [59,201]. The Brewers' Association, trade group for craft brewers in the USA, has reported IPA as the most purchased beer style from their members for more than a decade, and as more of these small breweries are operating than ever before in history, dry-hopping is more increasingly frequent [182,183]. Dry-hopping has become so ubiquitous that even global brands like Budweiser [247] and Guinness [248] are producing and advertising dry-hopped beers.

Typical wort produced with malted barley has a carbohydrate content composed of 60% maltose, 15–20% maltotriose, 5-10% glucose, and less than 5% each of sucrose and fructose. The 5-10% remaining is composed of longer chain oligosaccharides, or "dextrins", that are typically unfermentable by *Saccharomyces* yeasts [23,30,249]. Standard brewing yeasts assimilate the glucose, fructose, and sucrose within the first twenty four hours, before moving to the fermentation of maltose, and finally maltotriose after the other sugars have been used, leaving

behind the unfermentable dextrins [23]. These residual sugars generally remain stable through to the consumer, providing body and mouthfeel to create a balanced beer. Yeast also produces alcohol, carbon dioxide, and other desirable fermentation byproducts that contribute to the overall flavor of beer [9], and can create even more unique flavors in the presence of dry-hops [232]. There is evidence that hops contain enzymes that enzymatically alter the composition of beer during dry-hopping, contributing to the hydrolysis of the aforementioned unfermentable dextrins into fermentable sugars [57,184,188–190]. In the presence of live yeast, the newly present fermentable sugars can lead to an over-attenuation of the beer, which is commonly referred to by brewers as "hop creep."

This over-attenuation can result in higher alcohol and lower residual sugar contents in packaged product if not mitigated with pasteurization or filtration, which can come with consequences from the regulatory bodies in the United States and elsewhere [61]. If beers are dry-hopped after fermentation is complete, hop creep can cause yeast to leave dormancy, yielding higher amounts of yeast-related off-flavors such as diacetyl and acetaldehyde [62,63,186]. These off-flavors create a variation in consistency and quality for breweries and their consumers. Perhaps the most frightening effect of hop creep is when unfiltered beer is packaged with residual yeast, as is common at most craft breweries. With the residual dextrins being hydrolyzed by hop enzymes and the resultant over-attenuation, over carbonation in the container can also occur, causing safety concerns for the consumers of bottles or cans of this beer. Industry standard bottles (ISBs) suggest no greater than three volumes of CO_2 (6 g/L). With a typical carbonation level in beer being 5 g/L, only 0.1ºP of additional fermentable sugar is adequate to encroach on the pressure limit of an ISB when calculating CO_2 produced with the ideal gas equation [23].

The fermentation of beer is typically carried out using either one of two species of *Saccharomyces* yeast. *S. pastorianus* is a bottom-fermenting lager yeast and is the most commonly utilized fermentative in the world of beer. *S. cerevisiae* is a top-fermenting ale yeast, which is more common in craft beer and traditionally used for IPAs [9]. The ability of these yeasts to ferment the wort sugars into alcohol is commonly referred to as attenuation or degree of fermentation, expressing the relative remaining extract in the beer with considerations for the comparative destiny of ethanol produced and any other solids in suspension contributing to density. It can be measured a multitude of ways, but the most effective method given the resources is using near-infrared (NIR) detection on a density meter equipped with these capabilities [196]. Using this instrument, a researcher can accurately determine the real degree of fermentation (RDF), a direct correlation of the attenuation of a certain yeast taking into account variability in the gravity of the starting wort [250,251].

Previous research has attempted to quantify the enzymatic power of multiple hop cultivars (varieties) and relate this to their hop creep potential using only one *S. cerevisiae* yeast strain [51]. Thus, to shift focus from hops to how yeasts deal with potential hop creep, the aims of this research are to analyze the effect of different *Saccharomyces* yeast species and strains on the hop creep phenomenon as well as relate yeast flocculation and hop creep, while holding all other variables, including the hop variety, constant. Yeast strain or species potential for dry-hop creep has not yet been investigated, and research into this area has been at the top of mind for craft brewers [252]. Due to the lack of previous beer brewing potential of a number of the yeasts used in this study, a previously developed method will be used as a way to anticipate the amount of hop creep to be experienced with each pilot fermentation [242,243]. In addition, some brewers hold the belief that hop creep yeast variability is tied to a specific strain's flocculation, or the tendency of

yeast cells to aggregate together; this research will investigate a correlation. The outcomes of this research should identify specific *Saccharomyces* species and strains that may have an ability to mitigate the effects of dry-hop creep, as this would be of incredible value to the commercial brewing industry.

B. Materials and Methods

1. Experimental Beers

Sixteen pilot scale brews were performed on a 1.8 hL brewhouse in the Anheuser-Busch Research Pilot Brewery at University of California, Davis over the course of three months. The experimental beer attempted to emulate an American Pale Ale or Session IPA, with a target of 10.0 ºP original gravity. Mash water consisted of deionized water was adjusted using $CaCl_2$ and $CaSO_4$ salts to 85.0 ppm calcium, 95.0 ppm sulfate, and 80.0 ppm chloride and a target mash pH of 5.30. This was added to the grist at a liquor-to-grist ratio of 3:1 (L:kg) and held at 65ºC for sixty minutes, then heated to 72ºC for five minutes for mash out (*Fig. 5.1*). The grain bed was sparged with fresh deionized water using a lauter tun and the wort was extracted at an average of 4.00 liters per minute until a kettle full volume was achieved. Wort was boiled for seventy-five minutes with an evaporation rate of 10.0% per hour on a kettle with steam-powered internal calandria. Pelletized hops were added at 0.8 g/L with sixty minutes remaining in the boil to target 20.0 IBUs, and 0.08 g/L of yeast nutrient (Kerry Yeastex® 82; Beloit, WI, USA) added at the end of boil. Wort was then whirlpooled and allowed ten minutes for trub to settle before being knocked out on a dual stage plate and frame heat exchanger to a target fermentation temperature of 20.0 ºC. Each brew was split evenly by volume into four, 40.0 L glycol-cooled cylindroconical fermenters (JV Northwest; Canby, OR, USA).

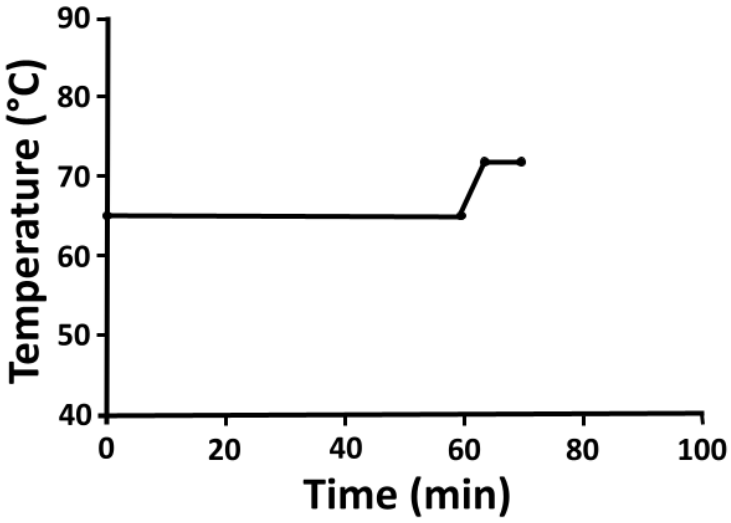

Figure 5.1. Target mash profile for the experimental beers performed. Malt and pH-adjusted water was held at 65 °C for 60 minutes, then heated to 72.0 °C and held for 5 minutes to mimic the typical single-step infusion mash used by many American craft brewers.

2. Malt

All malt was supplied by Admiral Maltings (Alameda, CA, USA; admiralmaltings.com) and milled fresh for each brew at the pilot brewhouse on a Seeger two-roller dry mill, type ZSM-0 mini (Schmidt-Seeger AG; Beilngries, Germany, EU) to standard crush: 70% retained above the 75 mm sieve, with 25% above and 5% below the 45 mm sieve. The grist consisted of 25.0 kg of pilsner malt (84.2%, equal blend of the two batches) made with Butta 12 barley [253] grown in Esparto, CA, 3.1 kg (10.4%) of chit malt made with organic Copeland barley grown in Tulelake, CA, and 1.6 kg (5.4%) of kilned caramel malt made with UC Davis Experimental barley grown in Esparto, CA. Malt specifications available below with values reported from supplier (*Table 5.1*).

Table 5.1. Malt analysis provided by Admiral Maltings. Moisture content, friability, extract, and protein contents are reported as percentages (%), color is reported as SRM, beta-glucan and FAN are reported as mg/L, diastatic power is reported in degrees Linter (ºL), and alpha-amylase is reported in dextrinizing units (D.U.). Some values were note reported by supplier for all malts (---).

Malt/Batch No.	**Pils**		**It's the Chit**	**Kilnsmith**
Batch No.	**20-099**	**20-106**	**20-085**	**20-095**
Moisture Content	4.6	4.2	4.0	3.2
Friability	89.3	91.2	---	---
Extract (FGDB)	81.6	82.1	78.5	77.0
Color	1.66	1.75	1.25	90.0
Beta-Glucan	242	164	1100	---
Soluble Protein	4.49	4.53	27.0	---
Total Protein	10.7	10.6	10.8	---
FAN	186	189	110	---
Diastatic Power	127	143	50	---
Alpha Amylase	44.6	52.5	5.0	---

3. Hops

Pelletized T-90 hops of the Centennial cultivar were provided by Hopsteiner (S.S. Steiner; New York, NY, USA). They were used both in the boil and for dry-hop due to their use as a dual-purpose hop, common in American craft brewing as both aroma and bittering uses. The hops were of 2020 crop year and reported to contain 8.3% alpha acid, 3.7% beta acid, 8.3% moisture, a hop storage index (HSI) of 0.502, and delivered in 5.0 kg packages sealed with nitrogen cover gas in mylar packages. Upon receiving, hops were sorted into separate vacuum-sealed packages for each bittering and dry-hop addition for all brews, then stored in a refrigerated room at 1.0 °C until use.

4. Yeast

Yeasts (*Table 5.2*) were chosen based on either their widespread commercial use in dry-hopped beer, historical significance, or unique characteristics. Yeast from Berkeley Yeast (Oakland, CA, USA; berkeleyyeast.com) was provided on a yeast peptone dextrose agar (YPD) plate and stored at room temperature until propagation. Yeasts from White Labs (San Diego, CA, USA; www.whitelabs.com) were provided in 35 mL PurePitch™ packages and stored at 4.00 ºC until propagation. Yeasts from Fermentis (Marcq-en-Baroeul, France, EU; fermentis.com/en/) were provided as active dry yeast in mylar sachets with an emulsifier (E491, sorbitan monostearate) and stored at 4.00 ºC until use. Non-conventional yeasts were supplied by the UC Davis Phaff Yeast Culture Collection (phaffcollection.ucdavis.edu), and were revived from cryogenic storage and streaked onto potato dextrose agar (PDA) plates, then incubated for two days at 30.0 ºC before being moved to room temperature storage. Due to time constraints with research brewing, only one yeast was chosen on which to perform three biological replicates to ferment from three separate brews: SafAle US-05.

Table 5.2. Thirty *Saccharomyces* yeast species used in the fermentations of the experimental beer, reported in alphabetical order. Yeasts were sourced from either the Phaff Yeast Culture Collection at University of California, Davis (**UCD**), White Labs of San Diego, CA (**WLP**), Berkeley Yeast of Oakland, CA (**BY**), or from Fermentis LeSaffre of Marcq-en-Baroeul, France (**Saf**), signified in the "Yeast Name" column. "Other collection names" determined as best estimate by the researcher, or genetic sequence for UCD yeasts. "Origin" as defined by original source or colloquial name. "Attenuation" and "Flocculation" is defined in yeast supplier literature or research as previously known values, designated as percent values when possible [254].

Yeast Name	Scientific Name	Other Collection Names	Origin	Flocculation	Attenuation
BY881	*S. cerevisiae*	(R&D)**	Oakland, CA, USA	Medium	75-85%
SafAle BE-134	*S. cerevisiae* var. *diastaticus*	WLP566	Wallonia, Belgium	Low	89-93%
SafAle BE-256	*S. cerevisiae*	WLP530; WY3787	Westmalle, Belgium	High	82-86%
SafAle K-97	*S. cerevisiae*	WLP029	Koln, Germany	Medium High	80-84%
SafAle S-33	*S. cerevisiae*	WLP005	Bedford, England	Medium Low	68-72%
SafAle T-58	*S. cerevisiae*	WLP565	Saison - Belgium	Medium Low	72-78%
SafAle US-05*	*S. cerevisiae*	WLP001; UCDFST 96-12	Chico, CA, USA	Medium	78-82%
SafLager W 34/70	*S. pastorianus*	WLP830	Germany	High	80-84%
SafŒno BC S103	*S. bayanus*	Red Star Premier Blanc	White Wine - France	High	High
SafŒno CK S102	*S. cerevisiae*	**	Val de Loire, France	High	High
SafŒno HD T18	*S. cerevisiae* x *S. bayanus*	(R&D)**	Marcq-en-Baroeul, France	Medium	High
SafSpirit USW-6	*S. cerevisiae*	**	Bourbon, KY, USA	Medium Low	High
UCDFST 01-135	*S. bayanus*	CBS 380; DVBPG 6171	Turbid Beer - Italy	Medium	Moderate
UCDFST 01-157	*S. pastorianus*	CBS 1538; DBVPG 6047	Carlsberg - Denmark	Medium High	72-78%
UCDFST 01-161	*S. paradoxus*	CBS 432; DBVPG 6411	Northeast Europe	Medium	Moderate
UCDFST 11-510	*S. mikatae*	CBS 8839; NCYC 2888	Soil - Japan	Medium	Moderate Low

Yeast Name	Scientific Name	Other Collection Names	Origin	Flocculation	Attenuation
UCDFST 11-512	*S. uvarum*	CBS 395; DBVPG 6179	Fruit - Scandinavia	High	Moderate
UCDFST 11-515	*S. kudriavzevii*	CBS 8840, NCYC 2889	Western Europe	Medium High	Moderate
UCDFST 21-101	*S. cerevisiae* var. *chevalieri*	CBS 400; DBVPG 6174	Ivory Coast	Medium Low	15%
UCDFST 77-65	*S. cerevisiae*	WLP076	Santa Rosa, CA, USA	Medium	70-74%
UCDFST 96-12	*S. cerevisiae*	WLP001; US-05	Chico, CA, USA	Medium Low	73-78%
WLP001	*S. cerevisiae*	US-05; UCDFST 96-12	Chico, CA, USA	Medium	73-80%
WLP002	*S. cerevisiae*	WY1968	London, England	Very High	63-70%
WLP013	*S. cerevisiae*	UCDFST 96-11	London, England	Medium	67-75%
WLP030	*S. cerevisiae*	WY1275	Trent, England	High	72-78%
WLP066	*S. cerevisiae*	A38; OYL011	London, England	Medium Low	75-82%
WLP090	*S. cerevisiae*	OYL043	San Diego, CA, USA	Medium High	76-83%
WLP095	*S. cerevisiae*	OYL052; GY054	Burlington, Vermont, USA	Medium High	75-80%
WLP351	*S. bayanus*	UCDFST 02-124	Weiss - Germany	Low	75-82%
WLP518	*S. cerevisiae*	NCYC 4285	Kveik - Norway	High	70-80%

*SafAle US-05 fermentations were done in biological triplicate. ** no known other collection

5. Yeast Propagation

Propagation wort consisted of 10.0% w/v (10.0 °P, 1.040 Specific Gravity) dried pilsner malt extract (Briess CBW® Pilsen Light; Chilton, WI, USA) in deionized water with 20 ppm $CaCl_2$ salts and 0.10% w/v yeast nutrient, targeting 5.2 pH. Wort was boiled for ten minutes then sterilized via autoclave before being sterile filtered to remove protein and trub particulate. All transfers of yeast and wort were done in a laminar flow hood or positive pressure room. Yeast cell counts and viability were performed on all propagations and fermentations according to standard methods [234].

Yeast colonies were transferred from plate or package via sterile inoculation loop to 10.0 mL of propagation wort in a 15.0 mL conical tube and placed on an orbital shaker table (Innova™ 2000, New Brunswick Scientific; Edison, NJ, USA) set to 150 rpm for twenty-four hours at room temperature. The contents of that tube were then vortexed and transferred to a 50.0 mL conical tube containing 20.0 mL of fresh sterile propagation wort and placed on the orbital shaker table as above for an additional twenty-four hours at room temperature. The tube was then vortexed and transferred to a 125 mL Erlenmeyer flask containing 50.0 mL of fresh sterile propagation wort and placed on the orbital shaker table as above for twenty-four hours at room temperature. The contents of that flask were then homogenized and transferred to a 250 mL Erlenmeyer flask containing 100 mL of fresh sterile propagation wort and placed the same shaker table for twenty-four hours. The contents of that flask were then homogenized and transferred to a 1.0 L glass bottle containing an additional 200 mL fresh sterile propagation wort and placed on the shaker table for forty-eight hours at room temperature. This final step was repeated two additional times, after which an additional 200 mL of sterile propagation wort was added and the total volume was split between two sterile bottles before both were placed back on

the same shaker for twenty-four hours at room temperature. The propagation for each yeast was completed over 11 days (*Fig. 5.2*).

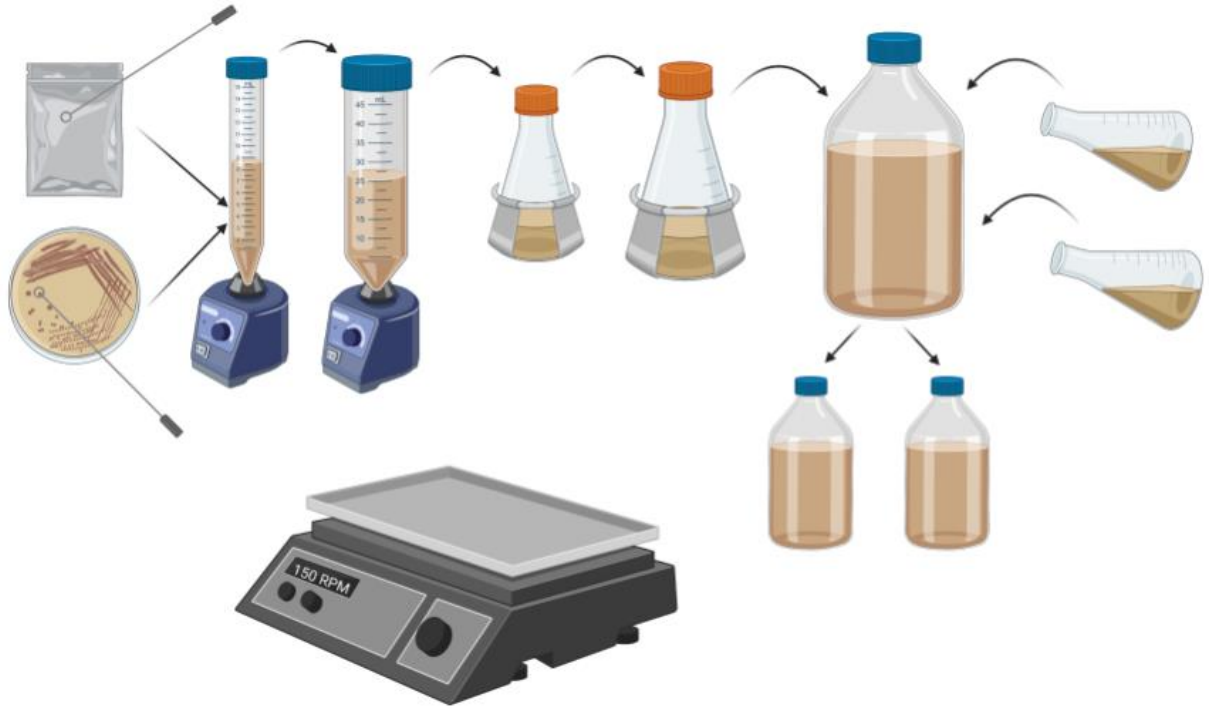

Figure 5.2. Yeast was transferred for propagation as shown in this schematic diagram. Yeasts were propagated to a desired total amount of 40.0 x 10^{10} billion cells in each bottle with 390 mL of propagation wort, equivalent to the standard ale pitch rate of 10.0 x 10^{5} cells per mL per ºP [9] for the 40.0 liter pilot fermentation. Figure created on BioRender.com, not to scale.

6. Sample Collection and Preparation

Beers were aseptically sampled daily within a two-hour window of knockout time. 50.0 mL conical tubes of each sample were centrifuged (ThermoFisher Scientific; Waltham, MA, USA) at 20.0 °C and 3000 x g RCF for five minutes. The clarified supernatant was then degassed for five minutes using the degas setting on a VWR B1500A-DTH 1.90 L ultrasonic cleaner (Radnor, PA, USA). Degassed samples were then decanted into the sample tubes of the Anton Paar (Graz, Austria, EU) auto-sampling carousel for immediate analysis.

7. Pilot Fermentations

Pilot fermentations of 40.0 L were set to 20.0 ºC with each unique *Saccharomyces* species or strain (*Table 5.2*) being transferred to its own fermenter in duplicate, totaling sixty-four fermentations. One fermenter in each yeast pair received 10.0 g/L (equivalent to 2.59 lbs./BBL) as a dry-hop when the fermentation reached between 3.00 and 4.00 ºP gravity, or at seven days into fermentation, whichever occurred first. This amount of dry-hopping has become standard practice among craft breweries today, with many brewers far exceeding this amount at times [58,59,201,202]. End of fermentation at a commercial brewery is delineated as "terminal gravity" and defines when the yeast has assimilated all the available fermentable sugars [23]. Terminal gravity in this study was defined a change of less than 0.10 ºP gravity for two simultaneous days following dry-hop, similar to methods utilized in commercial breweries.

8. Bench Top Fermentations

Bench-top fermentations were performed to preemptively determine the amount of dry-hop creep to be experienced with each pilot scale fermentation [242,243]. Forty-eight hours after initial yeast pitch to fermenter, 100 mL of high krausen green beer was aseptically sampled into two sterilized 250 mL Erlenmeyer flasks with magnetic stir bars. Hops were added to one of the flasks at double the dry-hopping rate of the pilot brews, equivalent to 20.0 g/L, and the openings for all flasks were covered with aluminum foil. Flasks were placed on a stir plate set to 225 rpm in a laminar flow hood for two days at room temperature, after which the samples were clarified as above. Degassing was deemed unnecessary as these samples were under constant agitation at room temperature and not under pressure. Centrifuged supernatant was transferred to the Anton-Paar sample tubes as above.

9. Analytical Measurements

Samples were then measured for extract, gravity, alcohol [196], and RDF using an Anton Paar Density Meter (DMA 5000 M) and alcolyzer (Alcolyzer Beer M) with an auto sampling carousel. The DMA 5000M instrument measures density by means of the built-in oscillating density meter and the Alcolyzer Beer M separately measures absorbance at NIR wavelengths (750 nm to 2500 nm) to calculate the alcohol content of the sample. From these two values, alcohol (% v/v and w/w), apparent and real extract, original extract, specific gravity, and RDF are calculated and reported from each sample. The DMA 5000 M has a repeatability within 0.000001 g/mL and the Alcolyzer Beer M has a repeatability within 0.03 ºP and 0.01 % v/v alcohol. pH was measured on a ThermoFisher Scientific benchtop pH meter that received three-point calibration weekly.

10. Biochemical Analysis (not included in *Fermentation* publication)

Amino acid content was determined using an L-8800a (Hitachi High-Tech America; Santa Clara, CA, USA) amino acid analyzer at the Molecular Structure Facility at UC Davis. 200 µL of centrifuged beer was diluted with 50 µL of 10% (w/v) aqueous solution of salicylic acid, frozen overnight, thawed, vortexed then centrifuged, and diluted with a norleucine standard before injection into the analyzer. Malt and hop samples were hydrolyzed in both base (4.5N NaOH at 110 ºC) then acid (6N HCl at 110 ºC) for twenty-four hours, then diluted in a lithium citrate buffer before injection. The analyzer utilizes ion-exchange chromatography to separate amino acids followed by a post-column ninhydrin reaction detection system [255].

Diacetyl (vicinal diketones) content was determined in beer samples using an Agilent 6890N Network Gas Chromatograph (GC) coupled to a 5973N transmission (single quadrupole) Mass Selective (MS) detector with electron ionization (Agilent Scientific; Santa Clara, CA,

USA) at Berkeley Yeast. Samples were incubated at 80 ºC for ten minutes, then cooled to room temperature and derivatized using 0.5 mL 20 mM 4,5-Dichloro-o-phenylenediamine (DOP) in 1 M HCL (35.4mg/10mL). They were then incubated again at 80 ºC for five minutes, cooled to room temperature and extracted in 1.5 mL toluene. Samples were then injected into the GC/MS and vicinal diketones quantified against known standards of diacetyl and pentanedione [256,257]. 1.0 mL samples of beer were stored in 1M potassium phosphate buffer at pH 7.5 and -20 ºC until delivery to Berkeley Yeast.

11. Statistical Analysis

In order to correlate the two concepts of flocculation and attenuation for this study, a numerical value was assigned to these (type converting): a "very low" value equating to a number of 0, "low" to a value of 1, "medium-low" to 2, "medium" to 3, "medium-high" 4, "high" to 5, and "very high" to a value of 6 (*Table 5.2*). Standard deviation for RDF and pH values, correlation (R^2 and Pearson's) values for flocculation and RDF, as well as one-tailed statistical analysis (*t*-test) and corresponding *p*-value were performed in Microsoft® Excel 2019, Version 2102 (Build 13801.20360).

C. Results and Discussion

1. Pilot Fermentations

Real degree of fermentation (RDF) is a measure expressing the degree to which the available extract was fermented and reported as a percentage calculated from the ethanol content and gravity of the remaining extract. RDF was chosen as the basis of comparison for these yeasts due to its ability to relate all fermentations, regardless of the variability in starting gravities. The potential limitation of the RDF calculation is that the enzymatic degradation of the dextrins to fermentable sugars in hop creep can only be quantified if those sugars are fermented by yeast to

produce more ethanol. The effect on attenuation, or the degree to which the yeast metabolized fermentable sugars, cannot be measured without a change in density and alcohol, but this study's main focus was on the fermentation parameters of variable yeasts, therefore the RDF calculation is appropriate.

With two exceptions, all fermentations in this study showed an increased attenuation when comparing dry-hopped to non-dry-hopped beers, ranging from 0.24 % to 12.5% increased RDF with dry-hops added (*Table 5.3*). All yeasts, with one exception, reached terminal gravity within two weeks. These kinetics indicated all yeasts were appropriately pitched with an adequate cell count to complete fermentations within a typical ale production schedule. The exception was UCDFST 01-157, a *S. pastorianus* strain isolated originally by Emil Christian Hansen from Carlsberg Brewery in 1888 [9]. The long lag phase of UCDFST 01-157 may be from the relative age of the culture, as most production breweries have switched from the type I Saaz strains to type II Frohberg strains due to its increased fermentation kinetics and cleaner profile [211]. The sluggish fermentation may also be due to its increased adaptation to ferment at colder lager fermentation temperatures, as has been shown previously [211,258].

Table 5.3. Heat Map of the Real Degrees of Fermentation (RDF), expressed as a percentage based on the color values associated to the scale at right, for all paired dry-hopped and non-dry-hopped 40 L pilot fermentations. The difference in RDF between the samples that were dry-hopped and those that were not is also reported. A negative difference value represents a lower RDF in the dry-hopped treatment when compared to the non-hopped and vice versa.

Yeast Name	Non-Hopped	Dry-Hopped	Difference		RDF (%)
BY881			2.26		
SafAle BE-134			-0.94		
SafAle BE-256			1.45		
SafAle K-97			4.55		
SafAle S-33			3.04		
SafAle T-58			7.14	75%	
SafAle US-05*			2.56		
SafLager W-34/70			2.48		
SafŒno BC S103			12.49		
SafŒno CK S102			0.67		
SafŒno HD T18			2.88		
SafSpirit USW-6			2.95		
UCDFST 01-135			3.67		
UCDFST 01-157			2.02		
UCDFST 01-161			0.54		
UCDFST 11-510			-0.66	65%	
UCDFST 11-512			0.24		
UCDFST 11-515			1.42		
UCDFST 21-101			10.70		
UCDFST 77-65			9.18		
UCDFST 96-12			4.78		
WLP001			2.34		
WLP002			4.21		
WLP013			5.59		
WLP030			0.99		
WLP066			3.94	55%	
WLP090			1.50		
WLP095			3.00		
WLP351			3.20		
WLP518			7.87		

*indicates this value as the mean of the three biological replicates using that yeast, with a standard deviation in the difference among the three replicates of ± 0.57.

The two exceptions from the average in this study that showed negative difference in RDF between the paired dry-hopped and non-hopped fermentations are SafAle BE-134 and UCDFST 11-510. SafAle BE-134 is a Belgian yeast strain known to be *S. cerevisiae* var. *diastaticus*, a variant of brewing yeast that contains the extracellular glucoamylase *STA1* gene, capable of hydrolyzing dextrins without the presence of the enzymes from dry-hopping [174,259].

The var. *diastaticus* strain used in this study fermented rapidly, reaching predetermined dry-hop conditions in less than two days, and completing fermentation in just one week, with 76.3 % and 75.3% RDF for the non-hopped and dry-hopped beers, respectively. Both of these RDFs were the highest values reported in this study, as expected from the manufacturer's specifications for attenuation. The other exception, UCDFST 11-510, is a *S. mikatae* species of yeast, which has only been used in prior alcoholic beverage fermentations, specifically wine, as a hybrid with *S. cerevisiae* [130,131]. On its own, UCDFST 11-510 *S. mikatae* was a poor fermenter, attenuating only 14.2 % and 13.5% RDF between the non-hopped and dry-hopped beers in this study, respectively. Neither of these yeasts presented themselves as appropriate yeasts to use in order to reduce the effects of hop creep. Due to fermentation characteristics, both present other challenges in commercial production, but are both unique yeasts that can offer desirable beer profiles if used appropriately.

On average, fermentations with all yeasts experienced an increased attenuation from the addition of dry-hops 3.54 % ± 3.19 (*Fig. 5.3*). Excluding the slow fermenter of UCDFST 01-157 and the low attenuator UCDFST 11-510 discussed above, a *p*-value of < 0.01 was calculated for day two to terminal gravity following dry-hop; even with those two yeasts included, there were significant changes for days two through five following dry-hop ($p < 0.01$). This means there is a significantly different value for the average RDF in the dry-hopped versus non-hopped fermentations. UCDFST 21-101 *S. cerevisiae* var *chevalieri* and SafŒno BC S103 *S. bayanus* yeasts had a z-score greater than 2 for the difference values, indicating these yeasts that had remarkably greater RDF values as a result of dry-hopping (*Table 5.3*).

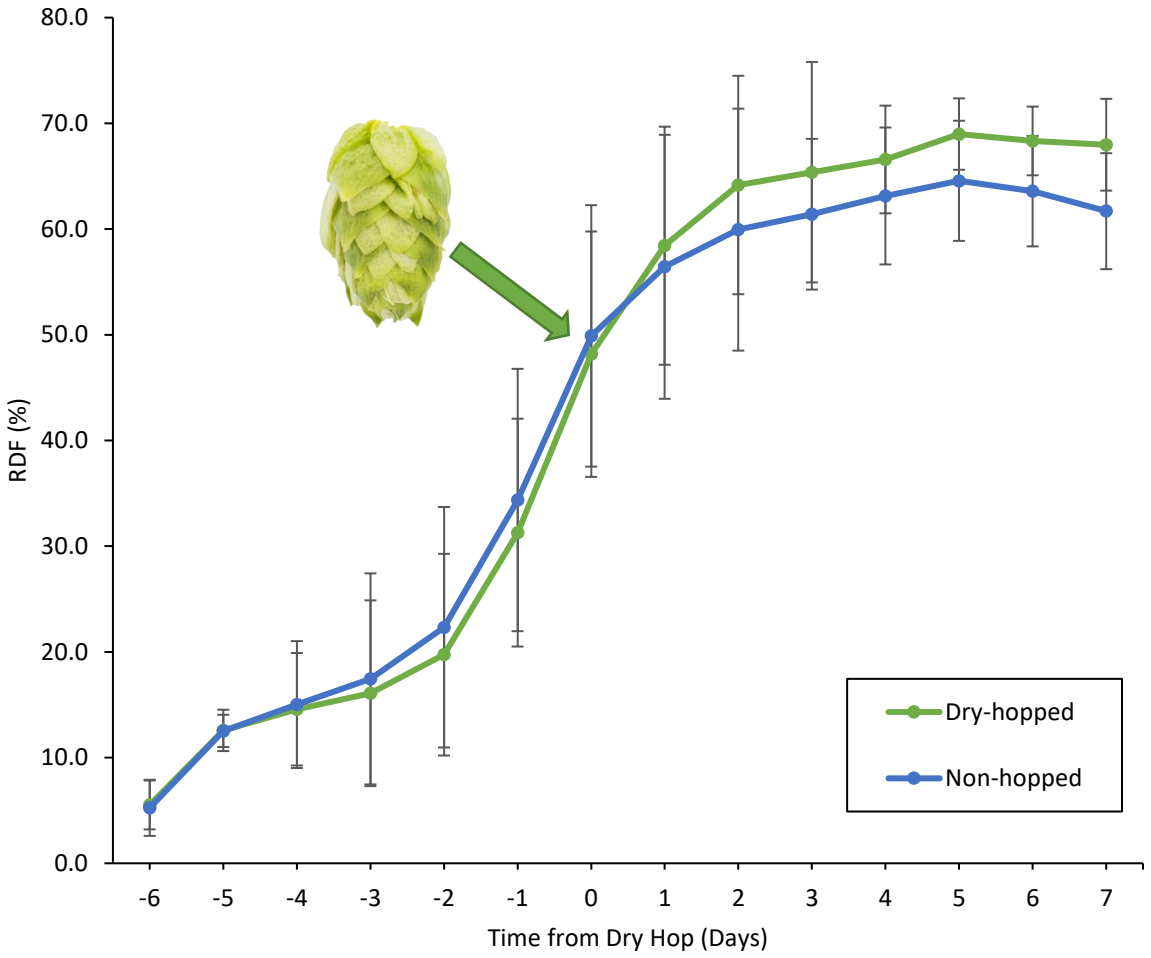

Figure 5.3. Average of the RDF for all yeasts in this study, excluding UCDFST 01-157 and UCDFST 11-510, expressed as a percentage, relative to the days from performing of the dry-hop, with day 0 as dry-hop day. The green hop cone signifies the point of dry-hop, where a clear increase in RDF of the dry-hopped beers is observed. The average RDFs begin to decrease five days after dry-hop due to the relationship between fermentation kinetics and attenuation, meaning if the beer took longer than twelve days to fully ferment, the degree of fermentation tended to be lower overall. Error bars indicate standard deviation

Of additional note, UCDFST 21-101 (*S. cerevisiae* var. *chevalieri*), has been reported as maltose negative, meaning it is not able to hydrolyze maltose and is only able to ferment the available monosaccharides, but contributes great aromas given the non-maltose sugar matrix of wine must [179]. Here, UCDFST 21-101 fermented to 58.7% and 69.4 % RDF in the 40.0 L pilot fermentations, and 70.2% and 71.6% in the corresponding bench top comparisons (*Table 5.4*),

implying that maltose was utilized by the yeast, as it is the main sugar component of the wort medium used here [23,30,249]. Commercial manufacturer guidelines for this strain report full attenuation within 48 hours, and suggest pasteurization and not to re-pitch this yeast to a new fermenter in order to prevent any infection. It is possible that during propagation, the yeast mutated or was out-competed by another *S. cerevisiae* strain. Initial microbiological checks proved no infection when plated on Wallerstein Labs Differential (WLD) media; genetic sequencing is currently being run and results are incoming. Work remains to understand this *S. cerevisiae* variant in a production brewing setting.

2. Bench-Top Fermentations

Most fermentations at bench scale reached terminal gravities lower than the pilot scale counterparts, but not all strains were effective when using the method previously devised to predict the amount of hop creep in unknown fermentations [242,243]. This perhaps help illuminate the inherent flaw of the methods, but still indicates that a bench-top fermentation with dry-hops is a promising tool for assessing the potential extent of hop creep when trialing a new beer or ingredient. It is suggested additional yeast is added to bench top fermentations in order to ensure maximum attenuation; this approach, called an end fermentation measurement, is taken from ASBC method Beer – 16 [43].

Table 5.4. Heat Map of the Real Degrees of Fermentation (RDF), expressed as a percentage expressed as a percentage based on the color values associated to the scale at right, for all paired dry-hopped and non-dry-hopped 100 mL benchtop fermentations. The difference between the samples that were dry-hopped and those that were not is also reported. A negative difference value represents a lower RDF in the dry-hopped treatment when compared to the non-hopped and vice versa.

Yeast Name	Non-Hopped	Dry-Hopped	Difference	RDF (%)
BY881			20.68	
SafAle BE-134			-3.07	
SafAle BE-256			3.25	
SafAle K-97			20.71	
SafAle S-33			5.40	
SafAle T-58			5.16	75%
SafAle US-05*			3.50	
SafLager W-34/70			2.90	
SafŒno BC S103			10.41	
SafŒno CK S102			4.94	
SafŒno HD T18			1.83	
SafSpirit USW-6			3.13	
UCDFST 01-135			4.33	
UCDFST 01-157			-42.97	
UCDFST 01-161			-0.37	
UCDFST 11-510			1.68	65%
UCDFST 11-512			31.85	
UCDFST 11-515			38.13	
UCDFST 21-101			1.49	
UCDFST 77-65			11.23	
UCDFST 96-12			43.33	
WLP001			3.39	
WLP002			2.69	
WLP013			2.43	
WLP030			1.78	
WLP066			1.97	55%
WLP090			4.15	
WLP095			0.37	
WLP351			1.92	
WLP518			9.94	

* indicates this value as the mean of the three biological replicates using that yeast, with a standard deviation in the difference of ± 1.46.

Clear outliers exist in comparisons of pilot vs benchtop fermentations of BY881, SafAle K-97, UCDFST 11-512, UCDFST 11-515, and UCDFST 96-12 on the non-hopped samples, and UCDFST 01-157 on the dry-hopped sample (*Table 5.4*), signifying sluggish fermentations according to the ASBC End of Fermentation method. The lowered RDF seen on the bench fermentations for these strains signal the yeast pitch volume did not meet recommended

guidelines or was low vitality upon initial pitch [193,194], but the pilot fermentations all finished with acceptable RDFs (*Table 5.3*), with only one extended lag period previously discussed with UCDFST 01-157.

Another species of interest, UCDFST 11-161 *S. paradoxus*, showed a slight decrease of -0.37 % RDF in bench top fermentations (*Table 5.4*) and a slight increase of 0.54 % RDF in the pilot scale fermentations (*Table 5.3*). While not an effective yeast for hop creep mitigation, low relative difference between the dry-hopped and non-dry-hopped fermentations, without over or under attenuation like SafAle BE-134 or UCDFST 11-510, show that it may be of interest for future trials. Research remains on the brewing potential and flavor characteristics of *S. paradoxus*, but initial insights from research in this lab to be published at a later date, as well as research from VTT Technical Research Centre in Finland [228] show that this species has great prospects in beer fermentation.

3. Flocculation and Attenuation

Flocculation is defined as the likelihood of yeast cells of a particular strain or species to cluster together during fermentation, forming a multicellular mass that eventually precipitates to the bottom of a lager fermenter or aggregates at the surface of an ale fermentation. It is a complex process that involves several genetic, environmental, and physiological parameters, but it is of great importance as beer is the only fermented beverage whose industry serially re-pitches its yeast [260–262]. Of importance to this study, there is a conventionally-held belief amongst craft brewers that dry-hop creep may be mitigated with a more flocculant yeast strain [252]. Because flocculation involves yeast health variables controlled by the brewer, such as nutrient conditions, dissolved oxygen, pH, fermentation temperature, and yeast handling and storage conditions, it is generally reported by commercial suppliers on a scale of “low” to “high”.

The given supplier references were type converted to numerical values, then visualized against the difference in RDF between the dry-hopped and standard fermentations for each yeast. This study found no correlation between flocculation and hop creep, with an extremely low R^2 value of 0.0001 (*Fig. 5.4*). The Pearson's Correlation Coefficient was also calculated to be -0.0102, suggesting no reliable relationship. As an alternative to the intensity scale type converting the informal flocculation amounts from the yeast supplier catalogues, future research could use the accepted method of the Helm's sedimentation test [263], and relate it to genetic research of the yeasts [261,262] to determine a more accurate flocculation numerical value.

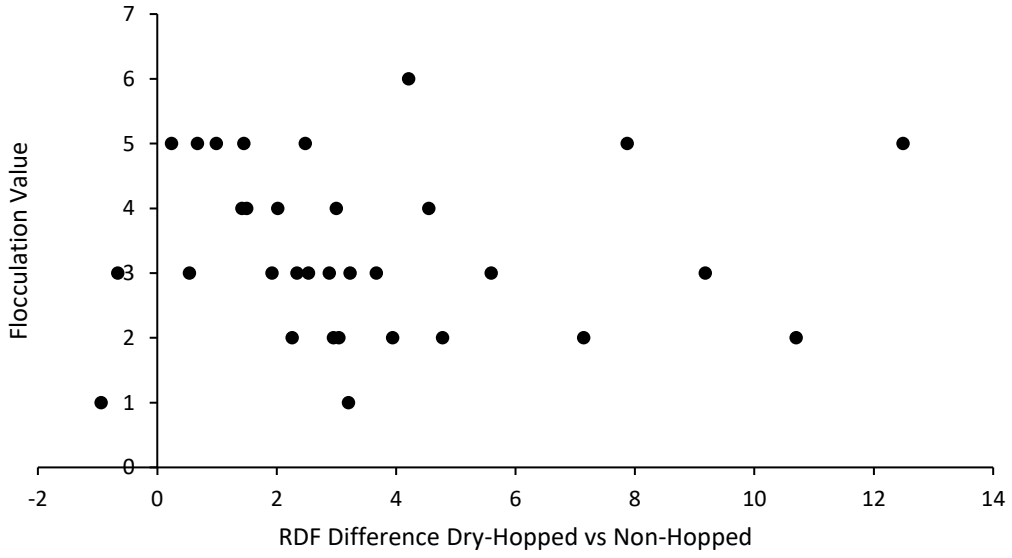

Figure 5.4. Type converted flocculation numerical values plotted against the RDF difference between dry-hopped and standard fermentations for each beer using pilot scale fermentation data. Negative RDF difference indicates lower RDF in dry-hopped fermentations vs non-hopped. A linear regression of these data produces an R^2 value of 0.0001, indicating that the flocculation value is not correlated to the excess attenuation from hop creep.

4. pH and Dry-Hopping

An observed increase in pH following dry-hopping has been shown in several studies [189,244,264]. Following a hop addition of 4.0 g/L (1.0 lbs./BBL) of dry-hop, green beer pH has been shown to increase by 0.10 pH unit. In our study, a similar trend was observed: dry-hopping

increased the pH an average of 0.20 at the dry-hop rate of 10.0 g/L. A clear increase in pH towards the end of fermentation was observed in dry-hopped when compared to standard fermentations for all yeasts studied (*Fig. 5.5*), with the largest separation being observed at four days from dry-hop. Significant differences were shown between the two treatments following dry-hopping, with a *p*-value of < 0.05 one day after dry-hop and a *p*-value of < 0.01 for all subsequent days of fermentation until terminal. No significant difference was shown for days prior to and including the day of dry hopping. Large error bars in *Figure 5* are likely due to the large variability in pH in fermentations in this study, as different yeast strains in this study fermented to variable pH and with different buffering capacities amongst the pilot beers due to variable starting gravities and ion contents.

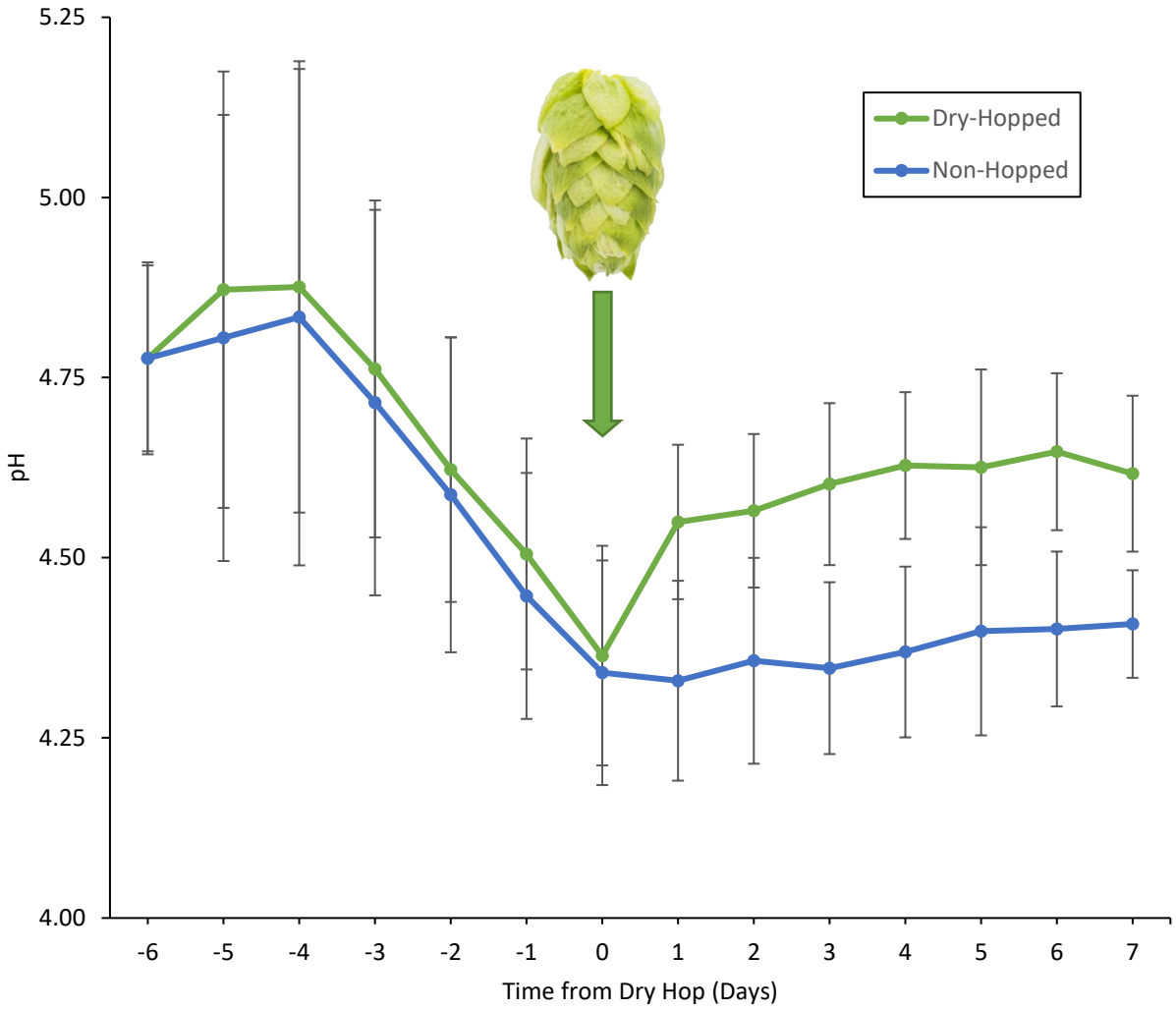

Figure 5.5. Average of the pH for all fermentations in this study relative to the days from b*re*wing each beer. Error bars are ± standard deviation. The green hop cone signifies the point of dry-hop at day 0, where a clear increase in pH of the dry-hopped beers is observed ($p < 0.05$ one day after dry-hop, $p < 0.01$ for all following days).

pH is an important brewing and fermentation parameter, as there are optimal pH levels at which enzymes are active in the mash [9] and that are necessary to control the extraction of polyphenols and tannins in wort [265], as well as pH optimums for yeast during fermentation [266]. There is evidence that *Saccharomyces* species replicate more readily at a pH of 5.0 than at a pH of 4.2 [266,267], which could cause more available biomass to ferment any available sugars. The observed increase in pH from dry-hopping could be creating more yeast to not only ferment the

newly hydrolyzed sugars, but also any residual sugars from the primary fermentation that were not consumed by the lower amount of available yeast. Additionally, amylase enzymes from malt have an optimum pH of 5.4 – 5.5 in the mash [15], and it could be postulated that when the pH of the fermenting beer is increased from the addition of dry-hops, there is increased activity from those similar glucoamylase hop enzymes concurrently.

5. *Amino Analysis* (not included in *Fermentation* publication)

The biological triplicates of SafAle US-05 were chosen for further amino acid analysis at the start of fermentation and three times during the fermentation: directly prior to dry-hop, twenty-four hours post dry-hop, and at terminal gravity. There was a clear downward trend of amino acid content during the first four days of fermentation, at which time US-05 beers were deemed ready for dry-hopping (*Fig 5.6*). With the addition of dry-hops, there was a clear increase in amino acid content, leaving the values statistically different at twenty-four hours post dry-hop ($p < 0.05$) and at terminal gravity ($p < 0.01$). Most of the increase in amino acid content following dry-hop was measured in serine, arginine, glutamate/glutamine, and alanine, with small increases in glycine and histidine (*Fig. 5.7*). A slight increase in glycine was also observed as fermentation concluded in the non-hopped treatment of the yeast (*Fig. 5.8*); glycine is an intermediate product of an amino acid biosynthesis pathway in *Saccharomyces* [268,269], or it may be seen due to interference with glycerol in the reading of ion exchange chromatography. A slight increase in threonine and asparagine/aspartic acid was observed in the middle of fermentation before trending downward, again likely due to biosynthesis during glycolysis.

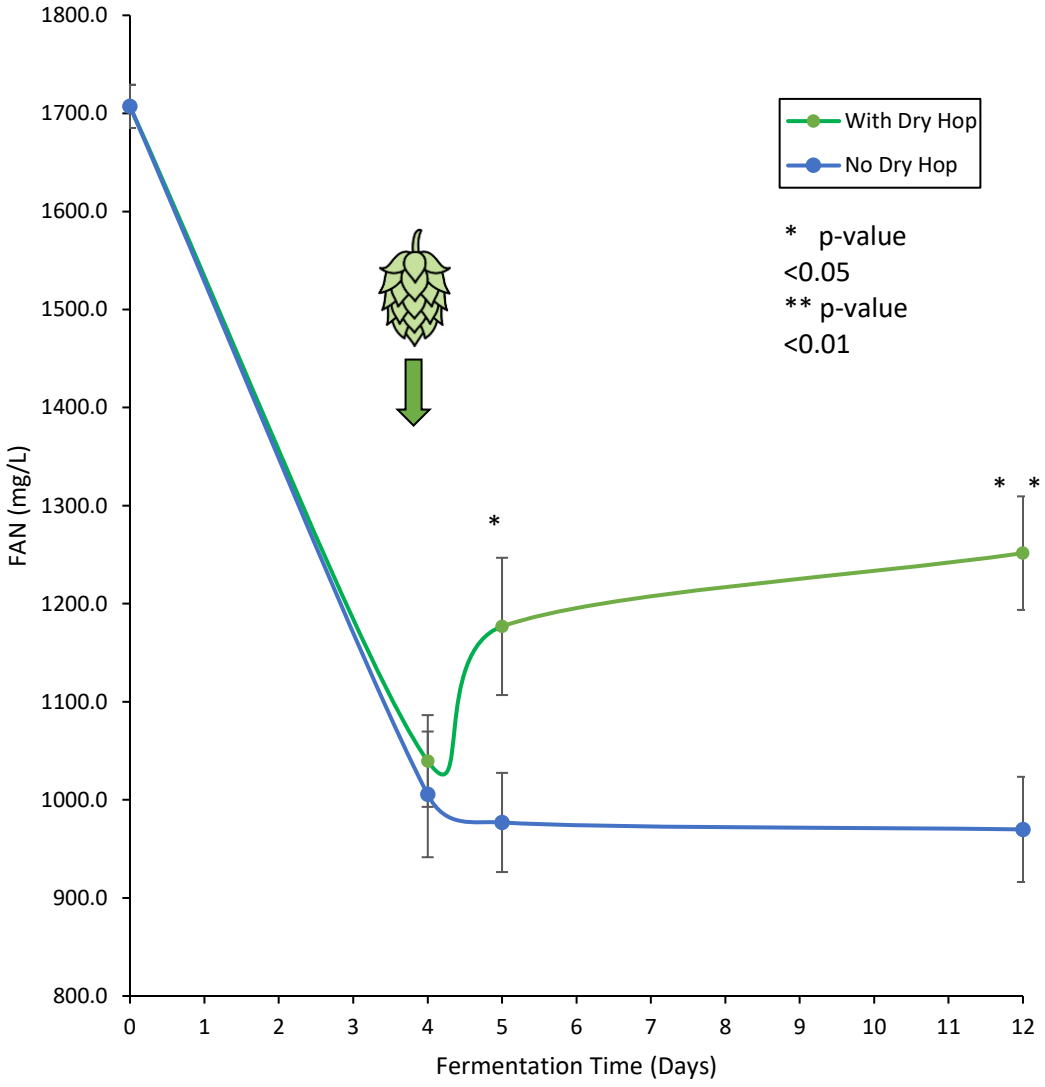

Figure 5.6. Mean amino acid content for biological triplicate fermentations of SafAle US-05 in mg/L. Measured via ion exchange chromatography for dry-hopped and non-hopped treatments at the start of fermentation, on the day of dry-hopping, twenty-four hours after dry-hopping, and at terminal gravity.

In brewing science, amino acids are divided into four classes based on the affinity of *Saccharomyces* yeast to assimilate them during fermentation [270,271]. Amino acids in Class A are readily absorbed by yeast during fermentation; in this study threonine (THR), serine (SER), lysine (LYS), arginine (ARG), asparagine/aspartic acid (ASX), and glutamine/glutamic acid (GLX) were measured. Class B amino acids are intermediately assimilated by yeast during

fermentation; in this study methionine (MET), valine (VAL), isoleucine (ILE), leucine (LEU), and histidine (HIS) were measured. Amino acids in Class C are slowly absorbed during fermentation of *Saccharomyces* yeast; in this study phenylalanine (PHE), glycine (GLY), alanine (ALA), and tyrosine (TYR) were measured. Class D contains just one amino acid, which is not readily assimilated by yeast during fermentation; proline (PRO) was also measured in this study. In the non-hopped treatments for US-05 treatments, it is observed that curves follow the absorption trends outlined, with the exception of leucine in Class B (*Fig. 5.8*).

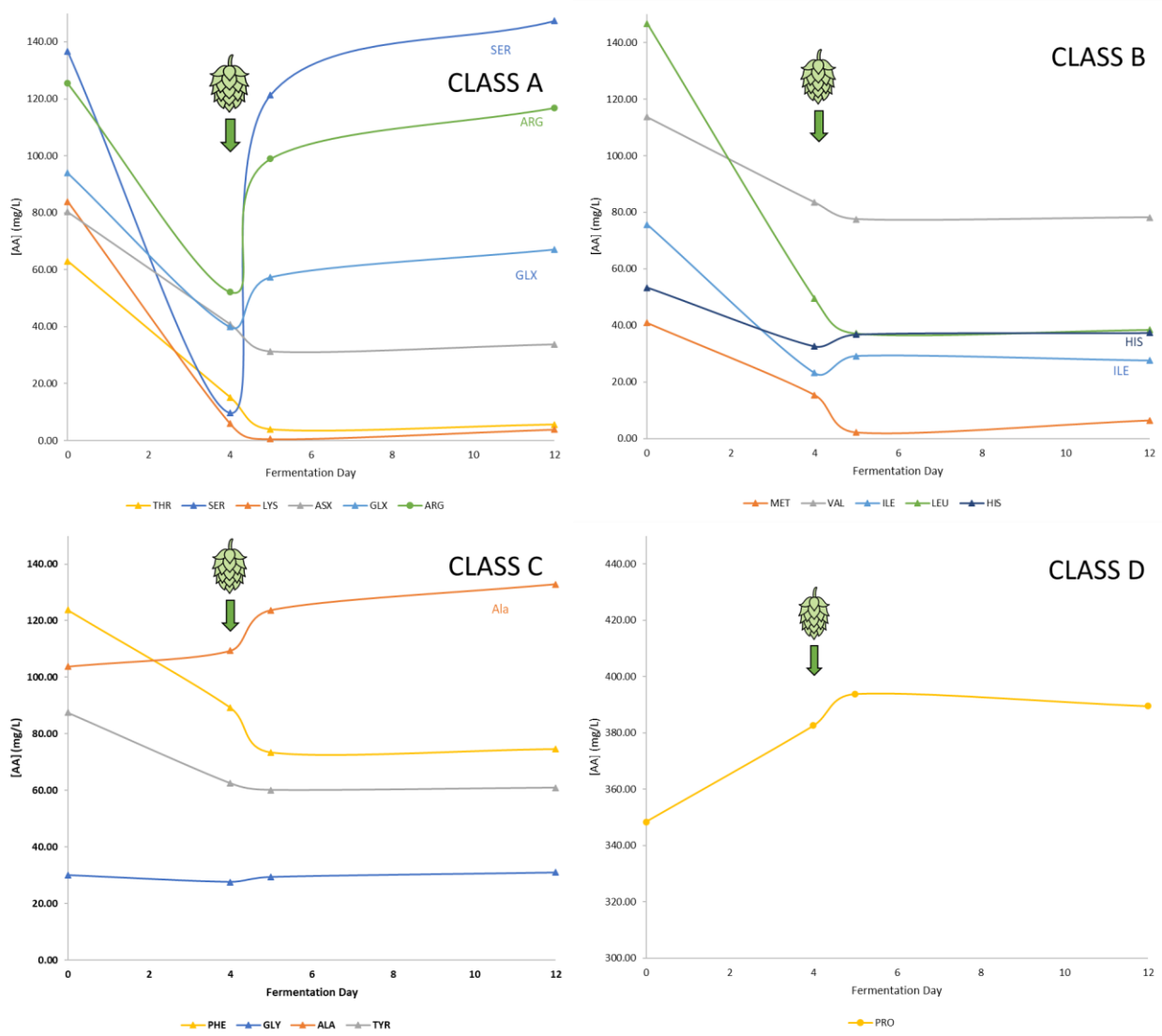

Figure 5.7. Individual amino acid content for biological triplicate fermentations of SafAle US-05 in mg/L. Measured via ion exchange chromatography for the dry-hopped treatment at the start of fermentation, on the day of dry-hopping, twenty-four hours after dry-hopping, and at terminal gravity. It is of note, that the scale for proline concentration is much higher than that of all other amino acids, starting with 300 mg/L at the axis.

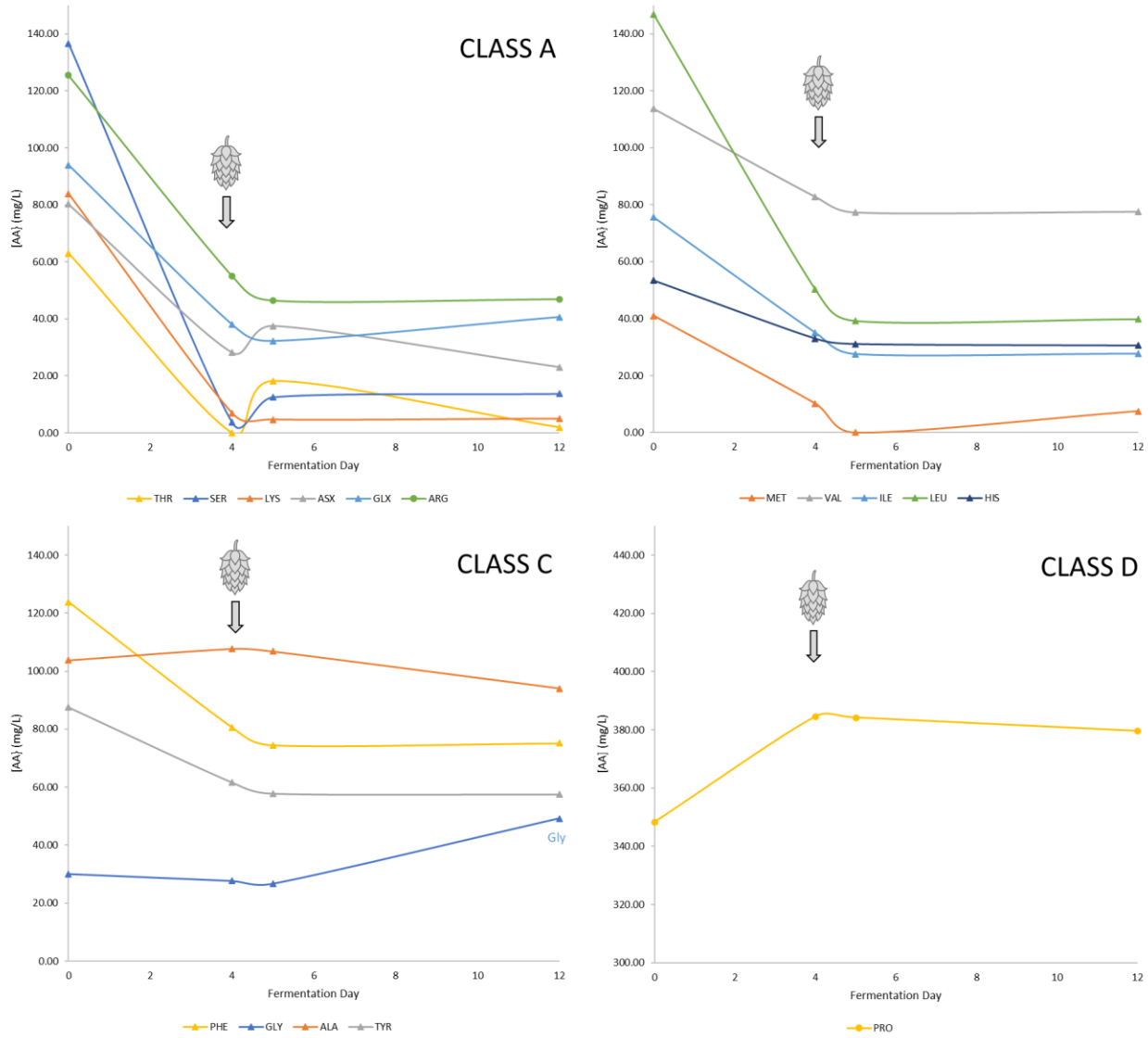

Figure 5.8. Individual amino acid content for biological triplicate fermentations of SafAle US-05 in mg/L. Measured via ion exchange chromatography for the non-hopped treatment at the start of fermentation, day four, day five, and at terminal gravity. It is of note, that the scale for proline concentration is much higher than that of all other amino acids, starting with 300 mg/L at the axis.

The amino acid content of the grist bill (malt blend) and hops used in this study were also measured in order to track the contributions from each ingredient. By mass, asparagine/aspartic acid is the highest amino acid contribution from hops (*Fig. 5.9*). This does not seem to solubilize in the green beer, as the highest increases from the additional dry-hopping were seen in serine,

arginine, and glutamate/glutamine (*Fig 5.7*), which all have greater than 0.5% of the mass (v/v) in the Centennial hops measured here. Proline and glutamate/glutamine contributed the highest mass of amino acids from the grist (Fig 5.9), which did translate to the wort, as proline was the highest concentration at the start of fermentation in all beers. Also of notes is that typical FAN levels are in the 150-400 mg/L range [23], protein concentration of hops is typically 15% [43], and the total protein content of the grist bill was measured as around 10.6% (*Table 5.1*), all fairly different values than what is reported here via ion exchange chromatography. Further analysis of amino acid content across fermentation in dry-hopped beers is required, as this is preliminary data that opens as many questions as it answers and challenges old paradigms.

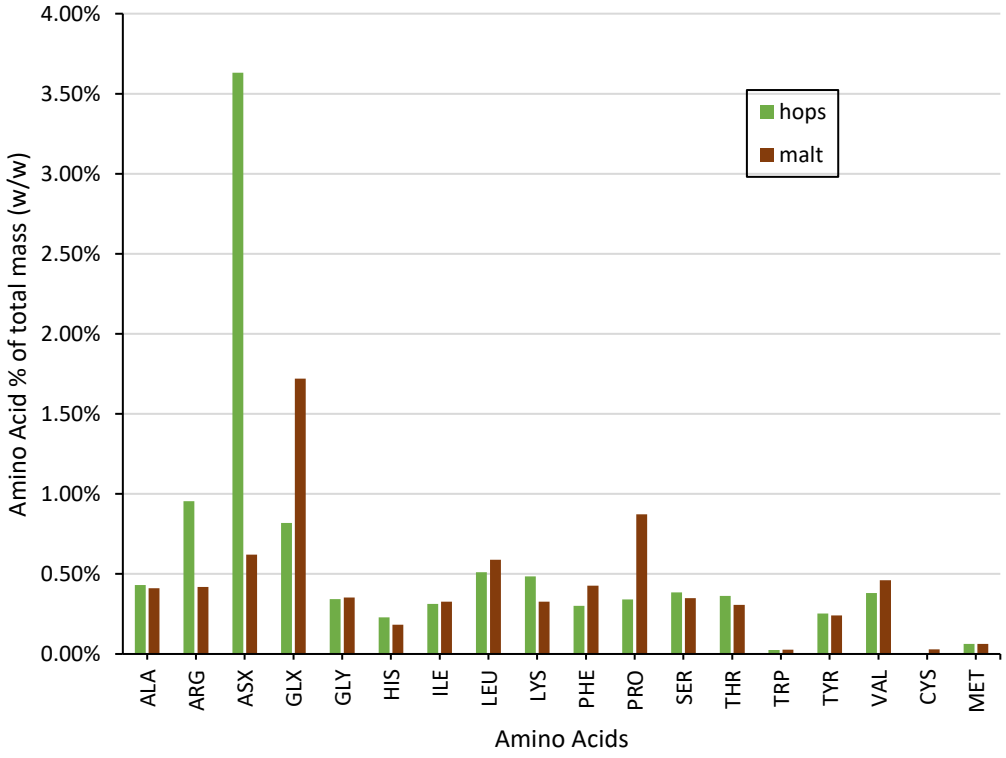

Figure 5.9. Amino acid content as percentage of total mass (w/w) wet basis in the Centennial hops and malt blend used for this study, measured by ion exchange chromatography. ASX and GLX are a combination measurement of asparagine/aspartic acid and glutamate/glutamine respectively, accounting for their much higher relative concentrations.

6. *Diacetyl Content* (not included in *Fermentation* publication)

Twelve of the yeasts were chosen for further diacetyl analysis at three sample points during the fermentation: directly prior to dry-hop, twenty-four hours post dry-hop, and at terminal gravity. Diacetyl is a known byproduct of fermentation, and is one of the primary off-flavors that has been associated with hop creep [272]. The twelve yeasts selected for additional analysis included six standard brewing strains that are ubiquitous in industry, a strain used for bourbon fermentation (USW-6), a wine strain (CK S102), a *S. bayanus* strain used in German Weissbier (WLP351), a Norwegian kveik yeast (WLP518), a Belgian *S. cerevisiae* var. *diastaticus* strain (BE-134), and a genetically modified yeast that has an alpha-acetolactate decarboxylase gene inserted capable of preventing the formation of diacetyl by breaking down the precursor, alpha-acetolactate (BY881). The BY881 strain performed as advertised and kept the amount of diacetyl below aroma threshold for the entirety of fermentation in both dry-hopped and non-hopped fermentations (*Fig. 5.10*). Also of interest are the K-97 kolsch ale strain, the CK S102 wine strain, and the WLP518 kveik strain, as they all had very low relative diacetyl concentrations, but did have values above aroma threshold prior to terminal gravity. A clear increase in diacetyl concentration was seen in seven of the twelve yeasts following the addition of dry-hops.

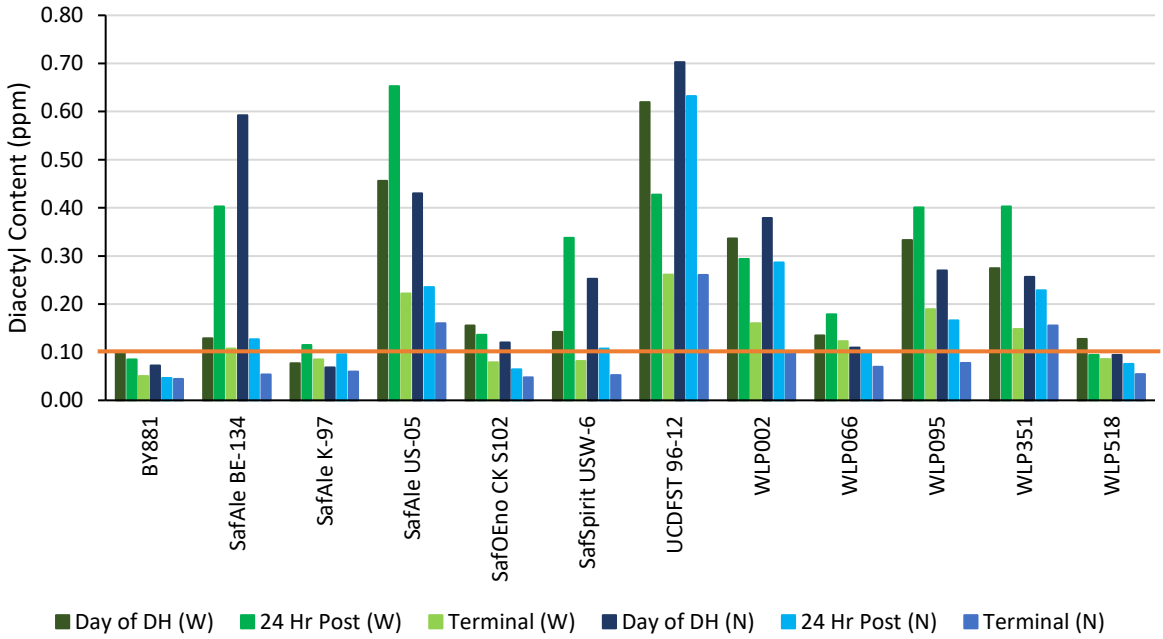

Figure 5.10. Amount of diacetyl measured via GC/MS in ppm for dry-hopped (W) and non-hopped (N) treatments on the day of dry-hopping, twenty-four hours after dry-hopping, and at terminal gravity for twelve yeasts (n = 1). The red line at 0.1 ppm indicates the aroma threshold for diacetyl in beer as reported in literature [15].

7. Biological Replicates

SafAle US-05 is a yeast ubiquitous among the craft beer industry and serves as a form of quality control to assess variation and change from brew to brew in this study. All values reported for this yeast throughout the study are the mean of the three biological replicates from three separate brews, totaling six fermentation using US-05. Variance was within an acceptable range as standard deviation among values remained low. RDF and pH values followed the same trends as all yeasts in this study: A one-tailed *t*-test was performed and showed statistical significance only on days following dry hopping on pH ($p < 0.01$) and RDF ($p < 0.05$) when comparing dry-hopped and non-hopped fermentations.

D. Conclusions

This study found a statistically increased average RDF in the dry-hopped versus non-hopped treatments for all the yeasts in this study, with the exception of two outliers in pilot scale and six bench-top fermentations. SafAle BE-134 *S. cerevisiae* var. *diastaticus* and UCDFST 11-510 *S. mikatae* were the only two yeasts that showed decreased RDF when dry-hopped in pilot scale fermentations, but both have other unique considerations when used for beer fermentation, as described previously. Bench top fermentations proved effective at predictively determining the likelihood of potential hop creep in pilot scale fermentations, but modifications to the method are suggested for increased predictive power. Unfortunately, no yeasts in this study present themselves as effective strategies for mitigation of hop creep, but this research serves as a first look at many yeasts and a new perspective on how the impact of hop creep can vary in different fermentations. Further investigation should be done on the correlation of yeast flocculation and over-attenuation from dry-hop creep. Instead of using a type-correlated intensity scale to correspond to the degrees of flocculation reported in commercial yeast supplier catalogues, future research could use genetic information of the yeasts to determine a quantifiable flocculation value. The observed increase in pH from adding dry-hops implies altered parameters that optimized yeast growth and enzyme activity, both of which may actively contribute to hop creep. It still stands that the best strategies to completely avoid hop creep are to pasteurize after fermentation is complete and denature the diastatic enzymes of the hops while rendering remaining yeast inactive, or to sterile filter the beer and remove all possible fermentative organisms.

E. Acknowledgements

Many thanks to Dr. Kyria Boundy-Mills and Dr. Irnayuli Sitepu from the UC Davis Phaff Collection for advice in yeast selection and subsequent revival of cryogenically stored yeast. Thanks to Kelly Scott and Joe Williams of the UC Davis Food Science and Anheuser-Busch Research Pilot Brewery for guidance and training on equipment in the brewery. Much gratitude to Dr. Nick Harris of Berkeley Yeast, Kara Taylor of White Labs, Anne Flesch and Kevin Lane of Fermentis, and Rebecca Newman of Lagunitas for advice on yeast selection. Additional thanks to Berkeley Yeast, White Labs, Fermentis, and the Phaff Collection for donating the yeast for this study, as well as Hopsteiner for providing the hops. JB thanks the UC Davis Food Science and Technology Department for their financial support, as well as funds from the H.A. Jastro-Shields Research Award, Margrit Mondavi Graduate Fellowship, George F. Stewart Memorial Fund, and Michael J. Lewis Endowment.

General Conclusions

Hop creep was confirmed in the first experiment, with significant increases in alcohol of package product in the presence of yeast and hops. Previous research had determined a statistical difference in the amount of hop creep amongst different cultivars, but this study was unable to confirm. Research was able to develop a bench method for hop creep determination in unknown fermentation that brewers could easily perform. The screening of *Saccharomyces* spp. from the UC Davis Phaff Yeast Culture Collection was successful, but the assimilation of proline should be explored further as it is currently understood that yeast do not utilize this amino acid. Many of the yeasts were expanded to full pilot scale at the Anheuser-Busch InBev Research Pilot Brewery at the Robert Mondavi Institute, several of which have never been used in the fermentation of beers before. Five non-conventional species of *Saccharomyces* and one hybrid of *S. cerevisiae* and *S. bayanus* fermented beer effectively, with *S. mikatae* being a possible candidate for the production of low or non-alcoholic beer. All yeasts displayed spicy notes similar to Belgian or German ale strains, as well as increased fruit, bitterness, and astringency perception with the dry-hopped treatment. These unique characteristics may offer great potential to a very complex and everchanging, consumer driven beer market. These six non-conventional yeasts, as well as several other *S. cerevisiae* and *S. pastorianus* strains were assessed for their hop creep potential; no *Saccharomyces* yeasts presented themselves for the effective mitigation of hop creep.

Analysis of this manner has never been performed before, so much insight was gained into the interactions of hops and yeast during fermentation. More research remains on a correlation between flocculation and dry-hop creep, as flocculation was not measured according to the Helm's sedimentation test in this study. Amino acid and diacetyl analysis on dry-hopped fermentations in comparison to non-hopped beers has never been performed before. Initial

results here prove exciting, but further analysis should be performed, with both analyses being performed on more time points during fermentation and on all yeasts. Secondary metabolites from the fermentation of beer with these unique yeasts should also be measured, especially in the presence of hops, as little is known of the interaction in *S. cerevisiae,* and next to nothing on some of the more unique species studied here. Samples of each fermentation at each day during the process were archived for future analyses. Proteomics and nuclear magnetic resonance (NMR) spectroscopy is currently being planned, allowing for more detailed analysis that has the capabilities to delineate between glutamate and glutamine or asparagine and aspartic acid with amino acids. Diacetyl production, as well as the change in sugar content should be measured for all of these yeasts in this studied, as well as more yeasts common or novel to the commercial brewing industry.

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Appendix A. Posters presented at World Brewing Congress Connect 2020.

Confirmation of Endogenous Enzymes in *Humulus lupulus* and a Proposed Method for Determination of Secondary Refermentation

WBC Connect 2020

Poster #90

UCDAVIS Brewery

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UCDAVIS FOOD SCIENCE AND TECHNOLOGY

ABSTRACT

This study describes two experiments, where firstly confirmation of refermentation in finished beer was possible with added hops [1,2] and secondly, a proposed forced fermentation method to speed up the process to assess refermentation. In our study, lab scale dry-hopping was carried out by adding 10 g/L hops to both a commercially available ale and lager, in the presence of yeast, over the course of 30 days. Extract and alcohol measurements were made at 1, 2, 3, 10, and 30 days in order to confirm refermentation. Four different hop cultivars were used, with one variety from each of the four categories established previously [3], ranking hops based on starch degrading potential. Refermentation due to dry-hopping in the presence of yeast was confirmed. However, the verification of the hierarchical classification by cultivar was inconclusive, as observed results in this study showed similar results across all hop varieties. Further studies should be done to address these conditions, the enzymatic potential of other advanced hop products, as well as the implications this information has on production brewing. Additionally, five different experimental brews with variable malt profiles and yeast selections were performed and forced fermentations done with and without hops. Each forced fermentation with hops showed a significant reduction in residual sugar and increase in alcohol when compared to the non-hopped sample. A simple method is proposed to determine the effect of dry-hopping on secondary fermentation.

Keywords: dry-hopping, hops, *Humulus lupulus*, enzymes, "hop creep", over attenuation, refermentation, laboratory method, forced fermentation

Figure 1. Lab Scale Dry-Hopping (above)
Figure 2. Aseptic Sampling (right)

INTRODUCTION

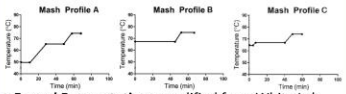
- **Dry-hopping**: the process of adding hops to beer in the fermenting, serving, or conditioning vessel for aroma
- **"Dry-hop creep"**: secondary fermentation of beer following the addition of dry-hops; yeast is reactivated following the hydrolysis of non fermentable oligosaccharides by enzymes in hops
  - Can lead to over-attenuation, high alcohol, and flavor inconsistencies in beer
- Typical mashes in brewing consist of 65-70% fermentable sugars, leaving a fair amount of residual dextrins that contribute to mouthfeel and body of the finished beer
- **Specific gravity**: the density of a beer in relation to a distilled water standard, used to determine alcohol content
- 30 different hop cultivars previously classified into groups based on enzymatic activity[3]
- **Forced fermentation**: a rapid bench scale fermentation of wort that is helpful in determining the final gravity of an unknown beer

HOP CREEP CONFIRMATION

- **Hops** - all BSG (crop year 2018)
  - one from each of the four classes in Ref. 3
  - Amarillo, Centennial, Mosaic, Willamette
- **Yeast** - American Ale Yeast (Fermentis US-05)
- **Beer** - commercially available ale and lager
  - Lagunitas IPA
  - Coors Original
- **Lab-Scale Dry-Hop** - Figure 1
  - 10 g/L hops per sample
  - 710 mL of beer in 1 L flasks
- **Sample Collection** - Figure 2
  - aseptically in laminar flow hood
  - 1-, 2-, 4-, 10- and 30-day samples
  - randomized order based on Random.org
  - stored in freezer until analysis
- **Sample Measurement** - thawed and centrifuged
  - sufficiently degassed after these steps
  - alcohol and specific gravity measured via an Anton Paar Alcolyzer DMA 4100 M

FORCED FERMENTATION METHOD

- **Experimental Beers** - with variable yeast (Table 2)
  - 3 brewed on 2 hL system, 2 on 38 L system
  - 5 different malt profiles, 3 different mash regimes



- **Forced Fermentation** - modified from White Lab method[4]
  - sample 100 mL into 250 mL Erlenmeyer flask after pitch
  - add double dry-hop rate to one flask
    - 20 g/L Willamette used here
  - ferment at room temperature for 48 hours on stir-plate
    - set to 150 rpm
  - centrifuge each sample in 50 mL conical tube
  - test for gravity on Anton Paar alcolyzer and compare

REFERENCES

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2. Kirkendall, J. A.; Mitchell, C. A.; Chadwick, L. R. The Freshening Power of Centennial Hops. *ASBCJ* **2018**, 1–7.
3. Kirkpatrick, K. R.; Shellhammer, T. H. A Cultivar-Based Screening of Hops for Dextrin Degrading Enzymatic Potential. *ASBCJ* **2018**, *76* (4), 247–256.
4. White Labs. Lab Methods and Supplies: Forced Fermentation

RESULTS

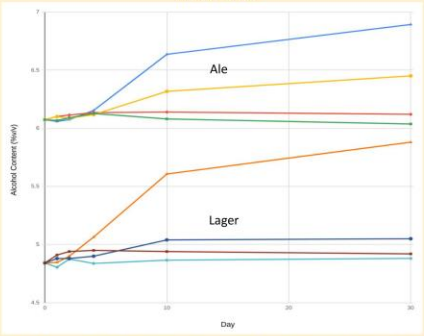

Figure 3. Average of the alcohol content measured as percent volume per volume at 1, 2, 4, 10 and 30 day time points after the addition of hops to the experimental samples. The lower four curves are for the lager, as the upper four curves are for the ale. The bottom two curves on both ale and lager represent the controls with nothing added to the beer and with only hops added. The middle curve on each represents the samples with beer and yeast. The top curve on each beer shows the average across all cultivars of the experimental trials, with hops, yeast, and the beer.

- **Secondary Refermentation of Commercial Beer**
  - Lager Initial: 1.0064 SG and 4.84% ABV
  - Ale Initial: 1.0102 SG and 6.07% ABV
  - changes reported in Table 1 and Figure 3
  - increase from beer/yeast control much less than experimental trials
    - additional sugar from hops and amount refermented from just yeast is still significantly less than change in experimental trials
  - no significant variability based on hop cultivar
- **Proposed Lab Method for Hop Creep Determination**
  - all samples showed comparative increase in alcohol and decrease in specific gravity (Table 2)
  - most saw increase of 0.50% ABV with hops
  - method is successful as performed
  - no variability due to yeast strain
  - only variability with malt and mash profile was with the brew intended to mimic hazy/juicy IPAs in craft beer market

Table 1. Average decrease in specific gravity (SG) and increase in alcohol content %v/v (ABV) with standard deviation for all experimental varieties of hops over 30 days.

	Amarillo	Centennial	Mosaic	Willamette
Lager SG	0.0066 ± 0.00035	0.0064 ± 0.00015	0.0061 ± 0.00035	0.0064 ± 0.00031
Lager ABV	1.02 ± 0.06	1.05 ± 0.02	1.03 ± 0.05	1.06 ± 0.04
Ale SG	0.0053 ± 0.00011	0.0051 ± 0.00005	0.0046 ± 0.00032	0.0053 ± 0.00052
Ale ABV	0.84 ± 0.04	0.78 ± 0.05	0.78 ± 0.06	0.88 ± 0.09

Table 2. Specific gravity and alcohol by volume from forced fermentation samples on five experimental beers, with or without 20g/L of Willamette hops.

		Brew 1A	Brew 2A	Brew 3A	Brew 1B	Brew 2B
SG	No Hops	1.0135	1.0111	1.0112	1.0115	1.0161
	With Hops	1.0111	1.0081	1.0081	1.0092	1.0121
ABV	No Hops	4.37	4.84	4.41	5.05	5.55
	With Hops	4.91	5.35	4.90	5.55	6.27
Yeast Pitched		S-04	K-97	US-05	WLP830	WLP013
Mash Profile		B	C	C	A	B

CONCLUSIONS

Verification of the hierarchical classification by hop cultivar was inconclusive. This may be a result of the enzyme activity variability due to different procedures in growing, processing, and storing hops by different farms. Further studies should be done to address the interactions between hops and yeast. The method proposed to determine the effect of dry-hopping on secondary fermentation is set to be used by local craft breweries in order to test the efficacy of the method and the ease of determination.

Diversity of properties related to brewing in *Saccharomyces* species and strains

Poster #153

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ABSTRACT

The Phaff Yeast Culture Collection at University of California, Davis, allows researchers a unique opportunity to use yeasts that may be unavailable in any other case. The collection is one of the largest public compendiums of wild yeasts in the world, with over 7,500 strains belonging to 1,000 different species and contains upwards of 200 novel species not found in any other public collection, including several "heirloom" brewing strains. This study aimed at screening diverse *Saccharomyces* yeast for growth in laboratory media varied in nitrogen, sugar, and ethanol concentrations, as well as in laboratory wort media. 46 different *Saccharomyces* yeast strains were selected; species were mainly *cerevisiae* or *pastorianus*, but there were also novel examples of *S. bayanus*, *S. paradoxus*, and *S. mikatae*. The yeast cultures were grown in several media, including Yeast Carbon Base (YCB) assessing, Yeast Nitrogen Base (YNB), varying concentrations of ethanol, and wort with and without hops to determine brewing fermentative capacity. 46 different yeast strains were grown in biological duplicates in 96-well plates in microplate shaker incubators for one week. Optical density at 600 nm was measured every 6 hours; MATLAB was used to create heat maps of the maximum optical density measurement. Most yeast grew well under many conditions, with wort media without hops having the most vigorous growth. Unexpectedly, some yeasts consumed proline as the sole nitrogen source. There was significant strain diversity in ethanol tolerance. Further work will be carried out to confirm the ability of the yeasts to consume proline, as well as the high ethanol tolerance of some strains. The Phaff Collection is an untapped resource for research on brewing yeast strains with great value for the industry.

Keywords: fermentation, novel yeast, *Saccharomyces*, *S. cerevisiae*, *S. pastorianus*, *S. bayanus*, *S. paradoxus*, *S. mikatae*

METHODS

- 46 *Saccharomyces* spp. in biological duplicate from Phaff Yeast Collection in 96-well plates representing:
  - 9 commercially available brewing strains
  - 10 strains isolated from beers around the world
  - 4 laboratory strains
  - 8 winery strains
  - 5 strains isolated from fruit
  - 4 distillery strains
  - 1 strain isolated from cherry soda
  - 3 strains isolated from tree sap or bark
  - 1 strain isolated from Japanese soil
  - 1 strain isolated from kefir fermentation
- Media used:
  - Yeast Carbon Base (YCB)
    - assessing assimilation of amino acids as nitrogen source
    - proline or glutamine as the sole nitrogen source
  - Yeast Nitrogen Base (YNB)
    - determine assimilation of sugars as carbon source
    - glucose, maltose, or maltodextrin as carbon source
  - YNB with glucose and varying concentrations of ethanol
    - determine tolerance of ethanol
    - 2%, 5%, 8%, 12% and 16% concentrations
  - Wort media
    - with and without hop extract
    - determine capacity of brewing fermentation
- Optical density at 600 nm
  - measured every 6 hours on spectrophotometer
- MATLAB was used to create heat maps
  - maximum optical density measurement

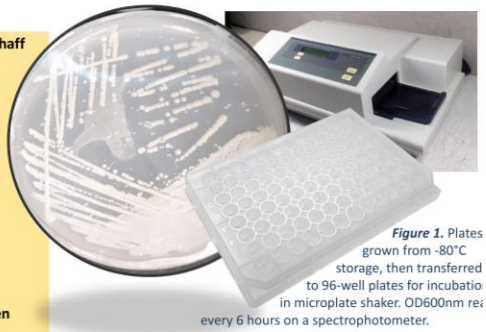

Figure 1. Plates grown from -80°C storage, then transferred to 96-well plates for incubatio in microplate shaker. OD600nm rea every 6 hours on a spectrophotometer.

RESULTS/DISCUSSION

- Most yeast grew well under many conditions
- Wort media w/out hops had the most vigorous growth, but with hops showed incredible growth as well
- Unexpectedly, some yeasts consumed proline as the sole nitrogen source
  - This may signify that the proline used was old batch and may degrade to glutamate. Need a confirmation study.
- Significant strain diversity in ethanol tolerance, as evidenced by the growth recorded at 5%, 8%, and 12%
- Novel strains:
  - *S. bayanus* shows promise at low temperatures
  - *S. paradoxus* and *S. mikatae* should be explored further

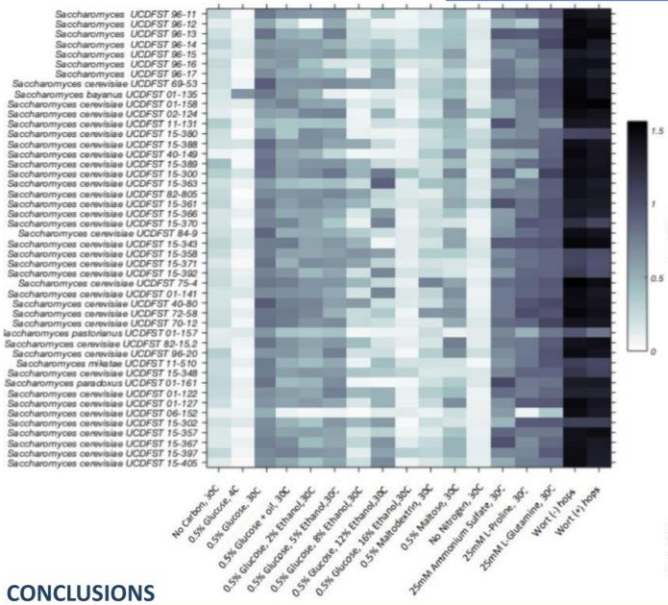

Figure 2. Maximum optical density measurement at 600 nm after 1 week of incubation on a microplate shaker. The darker the color in the square relates to higher absorbance at 600 nm for each optical density reading on the spectro-photometer. All strains grew exceptionally well on the wort media. Most strains did not grow very well in the 4°C incubation setting, which may require additional fermentation time, to mimic a production setting. Two yeast strains showed decent growth on maltodextrin, inferring a possible STA1+ gene in those two.

phaff yeast culture collection

CONCLUSIONS

All the selected strains, as well as many more from the Phaff Yeast Culture Collection (https://phaffcollection.ucdavis.edu/), show great opportunity for use in the brewing industry due to their excellent growth in wort. Further work will be carried out to confirm the ability of the selected yeasts to consume proline as all previous research has indicated that yeast cannot use this amino acid as its nitrogen source. The high ethanol tolerance of some strains also remains a point of interest, as modern brewing seeks to explore extremes. The Phaff Yeast Culture Collection is an untapped resource for research on brewing yeast strains with value for the brewing industry when a lab has the ability to propagate its own yeast.

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