Variation in Irc7p activity amongst brewing strains of Saccharomyces cerevisiae

by
Esteban Vega

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented June 3, 2021
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Esteban Vega for the degree of Honors Baccalaureate of Science in Bioengineering presented on June 3, 2021. Title: Variation in Irc7p activity amongst brewing strains of Saccharomyces cerevisiae.

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Abstract approved: _____

Christopher Curtin

Polyfunctional thiols are sulfur containing compounds that are important to the flavor and aroma profile of some foods and beverages. *S. Cerevisiae* has been shown in wine yeast that it is capable of producing these thiols to create a fruity aroma and flavor profile. *IRC7*, a gene that codes the carbon sulfur β-lyase enzyme Irc7p, has shown to be a main contributor to the production of polyfunctional thiols in wine. There has been a gap in research of whether or not beer yeast can reproduce the same results found in Irc7p ability to modulate carbon sulfur activity. This study looked at common mutations between wine and beer strains and if they had the same effect on the activity of *IRC7* through analysis of the frequency of non-synonymous mutations, determination of biochemical activity for 22 brewing strains, and the laboratory-scale wort fermentations to evaluate the relevance of carbon sulfur β-lyase activity in a brewing context. Some of the results have been consistent with *IRC7* activity in wine yeast. However, there was not enough conclusive evidence to suggest that brewing yeast's *IRC7* has the same ability to modulate activity.

Key Words: *Saccharomyces Cerevisiae, IRC7, Beer, Volatile Thiols, Aroma*

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Honors Baccalaureate of Science in Bioengineering project of Esteban Vega presented on June 3, 2021.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Esteban Vega, Author

CONTRIBUTION OF AUTHORS

Devin Tani performed biochemical analyses on selected beer strains and carried out pseudo-wort fermentations. Esteban Vega carried out wort and grape-juice fermentations and mapped genetic variation of the *IRC7* gene for 1011 previously sequenced strains of *Saccharomyces cerevisiae*. Vega also performed statistical analyses and prepared the manuscript draft. Dr. Karen Fortmann contributed to study conception and design, biochemical analyses, and supervised Tani. Dr. Chris Curtin conceived and designed the study, analyzed whole-genome data for selected strains, supervised Vega and edited the manuscript.

Background

Ales and lager beer are produced by fermentation of a grain-based mash (called wort) using yeast of the *Saccharomyces* genus. The style, type, and flavor profile of a beer is usually determined by the yeast selection, grain selection, hop content, length of fermentation, temperature, and pH. The process to make beer begins with the mashing of the grains and mixing with hot water ranging between 62°-78° C for starch to be converted to simpler carbohydrates to be readily available for yeast consumption. The mashed grains, or malt, is then boiled with hops, bitters, and other spices are added to construct a beer with a desired aroma and flavor profile. Those spices and hops are then removed the resulting seasoned grains are cooled into wort (Thesseling et al., 2019). Wort is then inoculated with *S. cerevisiae* and the primary fermentation takes place for four to seven days.

After primary fermentation, the beer is siphoned into a secondary fermenter and hops can be re-added in a process called dry hopping. Hops are added back in with a bag or strainer and fermented for a period between two and four days. It is this secondary fermentation that imparts the most significant hoppy flavor into beer (Thesseling et al., 2019). The strain of *S. cerevisiae* used can greatly dictate the flavor and aroma of the beer by interacting with the malt and hops.

The selected yeast(s) are comprised in a specific recipe by each brewer to create a specific flavor. Brewers will occasionally use a mixed recipe of different yeast strains that can impart unique flavor (Libkind et al., 2011; Bokulich et al., 2012; Steensels et al., 2015). Within *S. Cerevisiae* there are many strains that have effect on producing different flavors. Different strains have enzymes that react with different molecules that are in wort and hops throughout fermentation, and it is in the variation of biochemical reactions during fermentation that allow for truly unique beer flavor and aroma varying yeast recipes (Thesseling et al., 2019). Some *S. cerevisiae* have a disposition to produce volatiles from raw materials such as esters, higher alcohols, fatty acids, and volatile and polyfunctional thiols (Cordente et al., 2012).

Polyfunctional thiols are sulfur containing compounds that are considered important in a variety of beverages. Polyfunctional thiols have been deemed especially important in coffee, tea, wine, and beer (Kumazawa & Masuda, 2003;

Kumazawa & Masuda, 1999; Roland et al., 2011; Takoi et al., 2019). Volatile thiols have been determined as important in brewing for over a century and the key contributor being 3-mercapto-4methylpentan-1-ol (4MMP) being found in hops and in malts. Hops, mainly used to add bitterness to beer, have shown to contain volatile thiol precursors that lead to a fruity aroma and flavor in finished beer (Takoi et al., 2009). Non-volatile thiol precursors have been discovered to exist in hops and malts and throughout the course of fermentation bio transform to polyfunctional thiols to impart citrus aromas and flavors (Cordente et al., 2012).

Free polyfunctional thiol concentrations are negligible in grape juice/must, yet they contribute prominently to varietal character of some wine styles, such as Sauvignon Blanc (Coetzee et al., 2012). During wine fermentation, yeasts are responsible for cleaving the volatile components from cysteine-bound conjugates as shown in Figure 1 (Swiegers et al., 2009). In several studies, non-volatile polyfunctional thiol precursors Cys-4MMP, and cys-3MH, decreased in concentration throughout the course of fermentation (Dubourdieu et al., 2006; Peyrot des Gachons et al., 2002; Tominaga et al., 1998); Swiegers et al., (2007).

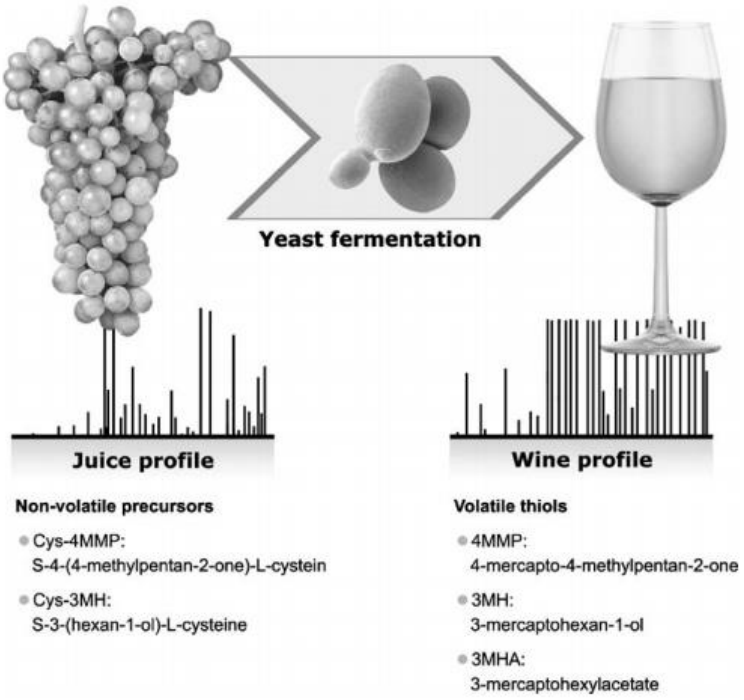

Figure 1: Reproduced figure from Swiegers et al. (2009) describes throughout the course of fermentation non-volatile precursors are biotransformed into volatile thiols. Fermentation produces several volatiles comprising of esters, higher alcohols, carbonyls, volatile fatty acids, and sulfur compounds. During wine fermentation *S. cerevisiae* cleaves the non-volatile cysteine conjugate precursors producing volatile thiols (Swiegers et al., 2009).

Wine strains of *S. cerevisiae* have been shown to have varying ability to impart flavor (Cordente 2012). Volatile thiol production varies with the use of different *S. cerevisiae* suggesting that the type of strain used in fermentation plays a significant role. Swiegers et al. (2009) found that different strains of wine yeast produced different profiles of final concentration of volatile thiols. These differences in volatile thiol concentrations were determined both by chemical analysis and by sensory analyses. In this context, the polyfunctional thiols produced the same effects as other beverages, exhibiting a passionfruit, grapefruit, and guava aromas (Swiegers et al., 2009). The varying amounts of thiol production between strains suggests that there are genetic determinants for the production of volatile thiols.

The specific pathway in which non-volatile cysteinylated are cleaved by *S. cerevisiae* is shown in Figure 2. Cysteinylated precursors (cys-X) can diffuse into the cell by GAP1 and then directly be cleaved by *IRC7*, *STR3,* or enzymes from other genes. Glutathione S-conjugates (GSH-X) can also diffuse into the cell via OPT1 and then be metabolized by DUG1, DUG2, and DUG3 to produce cys-X and cys-gly-X. Leftover GSH-X and cys-gly-X can then be metabolized by CPC and CPY into cys-X to finally be cleaved by *IRC7, STR3,* or enzymes from other genes. The pathways here show that *IRC7* is the final step regardless of the conjugate form (Cordente et al., 2015). It has been shown that glutathionated (GSH-X) polyfunctional thiol conjugates exist in grape juice and beer wort (Cordente et al., 2015).

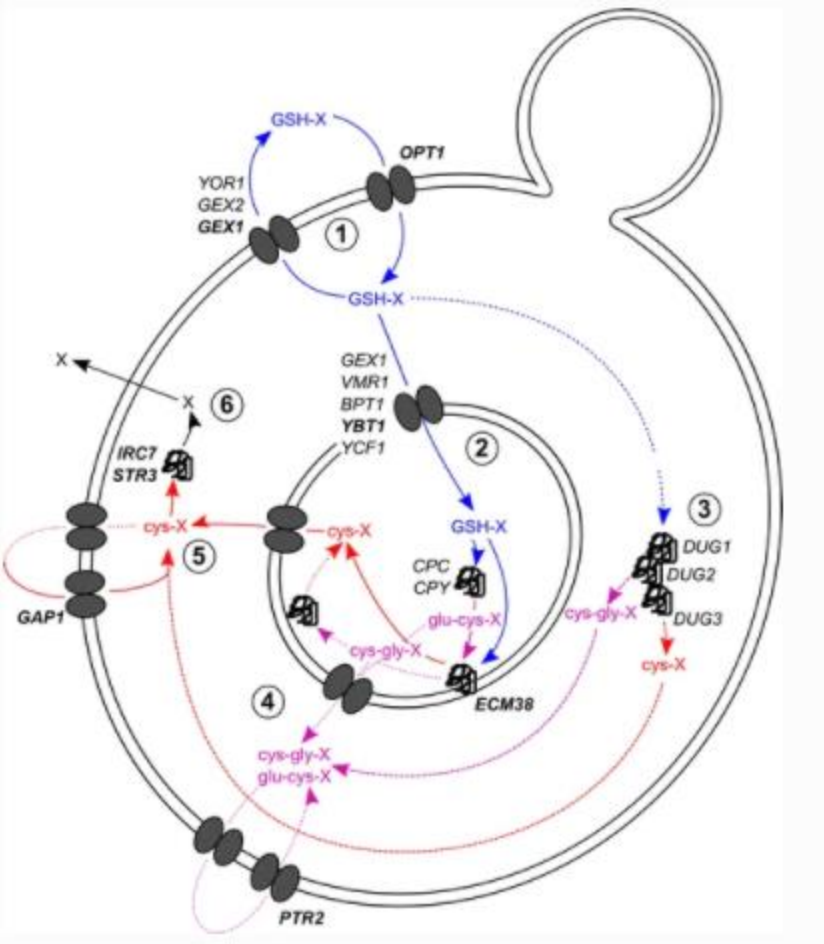

Figure 2: Recreation from Cordente et al. (2015). The pathways in which cysteinylated (cys-X), and glutathione precursors (GSH-X) metabolize in *S. cerevisiae* to produce volatile thiols (X). Glutathione compounds break to cysteinylated precursors which are broken down by *IRC7* (Roncoroni et al., 2011) and *STR3* (Holt et al., 2011).

IRC7 encodes Irc7p, an enzyme that is a main contributor carbon-sulfur β-lyase activity. Irc7p activity against cysteinylated precursors has been mostly explained by the presence or absence of a 38bp deletion in the gene sequence. The short *IRC7* allele is present in the majority of *S. cerevisiae* wine strains (Cordente et al., 2019) and was highly prevalent amongst vineyard strains (Santiago et al., 2015) and has been shown to decrease the β-lyase activity of Irc7p (Santiago et al., 2015). This deletion evolved simultaneously from the *S. cerevisiae* ancestor *S. paradoxus* as shown in Figure 3 (Roncoroni et al., 2011).

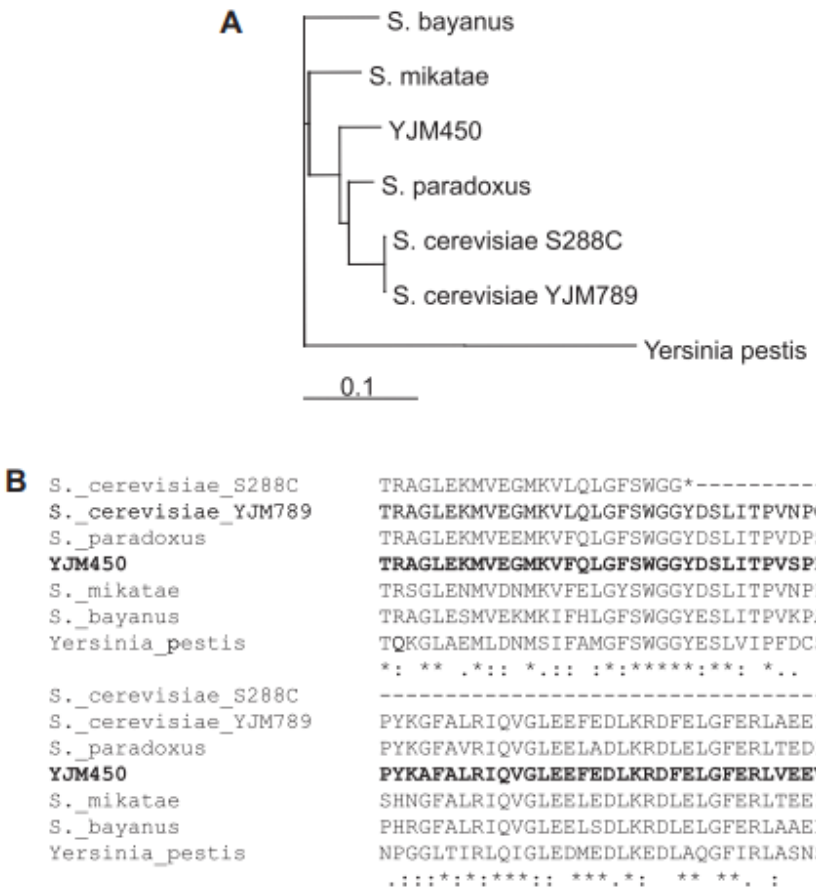

Figure 3. A recreation from Roncoroni et al. (2011) depicting: A. The phylogenetic tree of *S. cerevisiae* and showing the mutation from *IRC7*-long to *IRC7*-short split simultaneously from the evolution of *S. paradoxus* to *S. cerevisiae*. B. The 82 amino acids of the C-terminal of β-lyase proteins.

Cordente et al. (2019) found that *IRC7* allele length alone did not fully explain strain-strain CS-lyase activity variation. However, it was found that there are mutations that have significant effect on Irc7p activity. For example, mutation T185A, proximal to the active site of Irc7p, had a significant effect on the release of 4-MMP and H_2S (Cordente et al., 2019). In wine yeast strains exhibiting the long allele variant of *IRC7* and the T185A mutations showed a 50% decrease in production of 4-MMP than those without the T185A mutation. Mutations G253R, G321D, almost neutralized the activity of Irc7p entirely while mutations K43R, P146R, and E323G showed some residual activity (Cordente et al., 2019).

Irc7p can produce H_2S from cysteinylated precursors or additives (Santiago et al., 2015). In the same study, Santiago et al. (2015) demonstrated that the full-length version of *IRC7* in necessary and solely sufficient for yeasts to grow on sulfur amino acid as a nitrogen source. It was concluded that these strains produce positive and

negative volatile thiols simultaneously and still produce a positive net outcome (Santiago et al., 2015, Roncoroni et al., 2011, Swiegers et al., 2007). The measurement of the negative volatile thiol H_2S can be correlated with the product ion positive volatile thiols 4-MMP and 3MHA.

CS-lyase activity and how Irc7p modulates that activity has been well defined in wine yeast, however the role that it plays in beer fermentation is unknown. This thesis sought to identify how Irc7p, and its polymorphisms can explain CS-lyase activity in beer. A look into relevant mutations and how they affect biochemical activity of *S. cerevisiae* was done in conjunction with laboratory ferments. These experiments were done to seek how *IRC7* effects CS-lyase activity in a brewing context.

Manuscript – Variation in carbon-sulfur lyase activity and *IRC7* gene sequence amongst brewing strains of *Saccharomyces cerevisiae*

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Introduction

Volatile sulfur compounds, especially the presence of 4-mercapto-4-methylpentain-2-one (4MMP) have significant effects in food and beverages on the aroma and flavor of the product. Coffee, despite having thiols in low concentrations, have thiols ranked among the most important contributors to aroma. The compounds found in coffee appear in many other beverages like wine and beer (Sunarharum et al., 2014). Fruits such as grapefruit, guava, grapes, oranges, and others contain volatile sulfur compounds and play a significant role and is an important contributor to the recognized citrus flavor and aroma especially when theses fruits are juiced (Demole et al., 1982). Tea aroma is also significantly influenced by the presence of volatile sulfur compounds. Green tea in particular contains perceivable traces of 4MMP (Kumazawa & Masuda, 1999). Volatile thiol precursors, especially glutathione S-conjugates could be found from anywhere from fruits to hops. The majority of volatile sulfur compounds identified in foods are positive and favorable components of aroma and flavor in these foods. However, in beverages alongside the positive volatile thiols, there is a greater concentration of negative thiols such as hydrogen sulfide. The variation amongst the compounds in beverages is believed to be associated with a β-lyase activity by fermentative yeast (Bonnaffoux et al., 2020).

A major factor that has been heavily researched is the contribution of *Saccharomyces cerevisiae* wine yeast (Swiegers et al., 2007). Hydrogen sulfide (H_2S), which imparts a rotten-egg aroma, is a natural intermediate of the yeast sulfate assimilation pathway (Cordente et al., 2019). Yeasts have also been shown to release

polyfunctional thiols such as 4-mercapto-4-methyl-pentan-2-one (4-MMP) and 3-mercaptohexanol (3MH), which impart positive fruity aromas and flavors, from non-volatile amino acid conjugates present in grape must (Capone et al., 2018).

It is well-established that microorganisms degrade sulfur containing cysteine and methionine to form various VSCs (Dainty et al., 1989; Russell et al., 1995; Bonnarme et al., 2000; Morales et al., 2005). One of the key enzymes involved is cysteine desulfydrase, which catalyzes the release of H_2S from cysteine (Santiago et al., 2015). In *S. cerevisiae*, this activity is associated predominantly with the gene *IRC7* (Cordente et al. 2019). Despite the complexity of pathways associated with release of polyfunctional thiols from conjugated precursors (Cordente et al., 2015) and other genes encoding enzymes with carbon-sulfur beta-lyase (CS-lyase) activity (Dufour et al., 2012), *IRC7* has emerged as the major genetic determinant of 4-MMP and 3-MH release (Cordente et al., 2015; Santiago et. al 2015; Dufour et al., 2012; Roncoroni et al., 2011; Michel et al., 2019).

Roncoroni et al. (2011) established that variation in Irc7p activity against cysteine and cysteine-conjugates was largely explained by the presence or absence of a 38bp deletion in the gene sequence. The short *IRC7* allele is present in the majority of *S. cerevisiae* wine strains (Cordente et al., 2019) and was highly prevalent amongst vineyard strains (Santiago 2015) and has been shown to decrease the β-lyase activity of Irc7p (Santiago 2015). However, Cordente et al. (2019) found that *IRC7* allele length alone did not fully explain strain-strain CS-lyase activity variation. Upon examination of the *IRC7* sequences of 179 wine yeast, non-synonymous polymorphisms present in the long allele were also identified that affect thiol release during wine fermentation. In particular, the T185A mutation, proximal to the active site of Irc7p, had a significant effect on the release of 4-MMP and H_2S (Cordente et al., 2019). In wine yeast strains exhibiting the long allele variant of *IRC7* and the T185A mutations showed a 50% decrease in production of 4-MMP than those without the T185A mutation. Mutations G253R, G321D, G101D almost neutralized the activity of Irc7p entirely while mutations K43R, P146R, and E323G showed some residual activity (Cordente et al., 2019).

CS-lyase activity has been well researched and defined in wine yeast, however the role that this activity, and the *IRC7* gene itself, plays in beer fermentation is unknown. It was recently shown that cysteinylated and glutathionated polyfunctional thiol precursors can be found in malted barley and hops (Michel et al., 2019). In this study, we sought to determine whether mutations in the *IRC7* gene that exist in wine yeast also exist in beer yeast, and to what extent they explain CS-lyase activity. Analysis of the frequency of non-synonymous mutations amongst previously sequenced strains was combined with determination of biochemical activity for 22 brewing strains of S. cerevisiae. Finally, laboratory-scale wort fermentations were performed to evaluate the relevance of CS-lyase activity in a brewing context.

Materials and Methods

Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Yeast Strains and Laboratory Media

Brewing strains of *Saccharomyces cerevisiae* were obtained from White Labs (San Diego, CA, USA) (Table 1) on YPD agar (10 g/L, 20 g/L peptone, 20 g/L D-glucose, 15 g/L agar), and stored in 15% glycerol/YPD broth at -80 °C. Strains from cryogenic storage were regrown onto YPD agar.

Table 1: Identification and origin for the *S. cerevisiae* strains used. With the exception of S288C, all strains are commercially available.

Identifier	PCR identifier	Origin
BE044	[44]	Beer (White-Labs)
BE048	[48]	Beer (White-Labs)
BE053	[53]	Beer (White-Labs)
BE057	[57]	Beer (White-Labs)
BE058	[58]	Beer (White-Labs)
BE060	[60]	Beer (White-Labs)
BE061	[61]	Beer (White-Labs)
BE062	[62]	Beer (White-Labs)
BE064	[64]	Beer (White-Labs)
BE065	[65]	Beer (White-Labs)
BE066	[66]	Beer (White-Labs)
BE067	[67]	Beer (White-Labs)
BE076	[76]	Beer (White-Labs)
BE078	[78]	Beer (White-Labs)
BE079	[79]	Beer (White-Labs)
BE080	[80]	Beer (White-Labs)
BE081	[81]	Beer (White-Labs)
BE083	[83]	Beer (White-Labs)
BE085	[85]	Beer (White-Labs)
BE088	[88]	Beer (White-Labs)
BE089	[89]	Beer (White-Labs)
BE091	[91]	Beer (White-Labs)
S288C	[SC]	Lab Yeast
MaxiThiol	[MT]	Wine
EC1118	-	Wine

Polymerase Chain Reaction of *IRC7* allele length

Single colonies of each yeast strain grown on YPD agar were transferred into 200 μL molecular biology grade water containing 5% w/w (50mg/mL) Chelex for DNA extraction. Following incubation at 95°C for 20min, samples were chilled for 5 minutes on ice and centrifuged at 13,000 g. Supernatants were transferred to fresh tubes. The polymerase chain reaction (PCR) genotyping assay of Roncoroni et al. (2011) was used. Each reaction contained 10 μL EconoTaq Green (2x), 1 μL each primer (PF6 forward primer, PR7 reverse primer), 8 μL Chelex DNA extract. Reactions were according to Roncoroli et al. (2011) using an Eppendorf Mastercycler thermal cycler. *IRC7* allele length was assessed by running 3 μL of each PCR product with Gel red 6x loading dye on 3% TBE agarose gel in 1xTBE buffer at 120 V for 120 minutes. Gels were imaged using the BioRad Gel Doc XR+ Imaging System.

Yeast Starter Cultures

All yeast strains were propagated by transferring a single colony from YPD agar into 150 mL liquid YPD (10 g/L, 20 g/L peptone, 20 g/L D-glucose) in

Erlenmeyer Flasks. The starter cultures were incubated at 28°C in a shaker incubator at 175 RPM for 2 days until turbid. The cell density was determined by hemocytometer counting at 400x magnification using a Leica DM750 microscope. Starter cultures were then centrifuged at 3,900 g for five minutes and resuspended into wort or grape juice according to experimental requirements. The starter cultures were then inoculated into wort at 1 x 10^7 cells/mL or into grape juice to the cell density of 3.00E06 cells/mL.

Wort Preparation

Two different beer worts were prepared. The first was made with 161.55 g/L liquid malt extract, and 100μL Isohop/L. The recipe had the finish specifications of 12°P /25 IBU. 4 mL of 20mg/mL of L-cysteine was added to 200mL of wort. A second wort was prepared that consisted of 23.35 kg of Premium Pilsner Malt (Rahr Malting). This was mashed in water and treated with 1.3 grams of $CaCl_2$, and 2.7 grams of $CaSO_4$, resulting in 158.4ppm of calcium, at 70 °C for 45 minutes with a 3:1 water to malt grist ratio. The wort was at 14.8 °P boiled for 4 minutes then poured into a bucket and stored in a freezer. Wort was thawed, mixed, and poured into 2 L media bottles (VWR, Radnor, USA) and autoclaved.

Beer Fermentation Without L-cysteine Experimental Set Up

Laboratory-scale beer fermentations using yeast strains BE053, BE062, BE064, BE067, BE081, BE085, BE088, BE089, MaxiThiol, S288C had 200 mL of autoclaved wort poured into 250 mL media bottles in triplicate. The inoculation density for each fermenter was 1.00E7 cells/mL. Each fermenter was air-tight, fitted with one-way check-valves and H_2S indicators (Kitagawa Precision Gas Detector Tubes, Japan). The fermenters were incubated at 20°C in a temperature-controlled room and agitated using stir plates and stir-bars stirred at 200 rpm. Fermentation consisted of a 3-day period. Fermentation was observed every 12 hours to record the H_2S production of each strain. Fermentation curves were created using weight loss data.

Beer Fermentation with L-cysteine Experimental Set Up

The wort with added L-cysteine was inoculated with strains BE047, BE053, BE062, BE064, BE077, BE088, BE089, BE091, S288C, and MaxiThiol. The

ferments were inoculated at 1.00E7 cells/mL. Each fermenter was air-tight, fitted with one-way check-valves and H_2S indicators. The fermenters were incubated at 20°C in a temperature-controlled room and agitated. The ferments went for seven days and H_2S were monitored at day 0, 4, and 7.

Grape Juice Preparation

The grape juice used for the wine ferments was Chardonnay grape juice. Grape juice was thawed, mixed, and poured into 2 L media bottles (VWR, Radnor, USA).

Wine Fermentation Experimental Set Up

Laboratory-scale wine fermentations using yeast strains BE088, MaxiThiol, and EC1118 were performed in triplicate at 20°C. The ferments were done using 800 mL of grape juice in 1000 mL Schott media bottles with stir-bars stirred at 200 rpm. The airtight caps were fitted with one-way check valves and H_2S indicators (Kitagawa Precision Gas Detector Tubes, Japan) which were replaced when the indicators reached near capacity, to measure H_2S throughout fermentation. The fermentation progressed through 21 days, and the weight loss and H_2S production. After fermentation, the products were cold settled at 4°C and sent for sugar concentration and ethanol percentage analysis.

In-silico analysis of *IRC7* sequences derived from whole-genome sequencing datasets

A database of 1011 previously sequencing *S. cerevisiae* strains was drawn upon to identify mutations in *IRC7* relative to the standard reference strain S288c. Each strain's *IRC7* sequence was compiled in a multiple sequence alignment in Seaview 5.0.4, and grouped according to results from a neighbor-joining tree. Non-synonymous mutations were recorded in excel and their frequency determined. Each amino acid polymorphism was analyzed against the Provean database (provean.jcvi.org) to predict whether it may have a deleterious effect on protein function. A database of 102 beer strains with various polymorphisms from Gallone et al. (2018) was also analyzed in the same way. A chi-squared analysis was performed using R statistical language (Team, 2013) and the MASS package (Ripley, 2013) to determine the statistical significance for comparisons of allele frequency between

"overall *S. cerevisiae* population" (1011 strains) and "beer *S. cerevisiae*" (102 strains) datasets.

Biochemical Analysis

Carbon-sulfur β-lyase activity for the yeast strains identified in Table 1 were assessed according to the method described in Cordente et al. (2019). The yeast strains were grown over 24 hours in liquid YPD at and a volume of 8 ml of the culture was centrifuged for 2 min at 4,000 g's. Cell were then washed two times with water, then the pellet was resuspended in 400 µL of cold buffer consisting of 100mM HEPES (pH 7.5), 20 µM pyridoxal-5'-phosphate (PLP), 200 µM EDTA, 10% glycerol, and the protease inhibitors leupeptin (2 µg mL^{-1}) and PMSF (1mM). Glass beads were used in the suspension and vortexed for 30 seconds, with 30 seconds of rest, for 10 minutes at 4°C. The suspension was centrifuged for 30 minutes at 16,000 g at 4°C, then the supernatant was sent for enzyme assays. The resulting protein concentrations were measured in a Bio-Rad protein assay (Cat. Number: 5000006), with bovine serum albumin as the standard control.

The assay reactions were performed in a volume of 200 µL in 96-well microplates (UV-Star® UV-Transparent Microplates, Greiner Bio-One), containing 100mM of HEPES buffer pH 7.5, 20µM PLP, 25 µM EDTA, and 2 mM concentration of the substrates L-cysteine or Cys-4-MMP in 30°C. 10 µg of the protein extracts was added to start the reactions. The reaction released pyruvate as a result of β-lyase which can be analyzed with an enzymatic conversion to lactate by L-lactic dehydrogenase enzyme (5U μL^{-1}). Parallel conversion of NADH (400 µM) to NAD^+ by this enzyme was measured by absorbance at 340 nm (ε = 6220 1 mol^{-1}) every 5 minutes for 60 minutes.

Statistical Analysis

Mutations as listed in Table 111, K43R, Y56stop, T72T, G77S. G77G. G101D, G120G, T185A, L190F, H197Q, S202S, P229P, E263G, L269P, G296R, G304D, V312V, F336Y, V348L, A356V, stop360R, K369E, and Allele Deletion (Y341del), were scored with the proportion in which they occur in *IRC7*, with the activity found in the biochemical analysis. Random Forest analysis in R was performed using the randomForest (Liaw & Winer, 2002) and randomForestExplainer

(Paluszynska et al., 2017) packages, to find significant strains in relation to activity. Linear regression using the lm function in R was used to determine correlation between proportion of mutations versus activity. Linear regression using the lm function in R was used to compare variables and find significance in variables.

Results

IRC7 sequence and length polymorphisms amongst brewing strains of S. cerevisiae relative to other strains

IRC7 gene sequences were extracted from two recent large-scale *S. cerevisiae* whole-genome sequencing datasets (Peter et al., 2018.; Gallone et al., 2016) and non-synonymous polymorphisms scored. Fourteen variants that were found in at least 1% of the strains in either study is shown in Table 2, full summary in Appendix 1. There is a significance between the frequency of mutations that occur in beer strains versus all *S. cerevisiae* strains (chi-squared p-values <0.05). Interestingly, the 38-bp deletion appears in only 14% of brewing strains while it occurs in 79% of all *S. cerevisiae* strains (chi-squared p-values <0.05).

Table 2: Summary of prevalent and potentially impactful non-synonymous mutations in *IRC7*. Frequency of each mutation across *S. cerevisiae* genome sequencing datasets was scored, with only those present amongst >1% of either yeast populations included in this table. Provean scores are a measurement of the effect of each mutation relative to the reference sequence for the *IRC7* gene.

Variant	PROVEAN results		Frequency of polymorphism	
	Score	Prediction of impact[1]	Peter et al. (2018)	Gallone et al. (2016)
D3N	-0.189	Neutral	0.4%	1.1%
T5del	-0.86	Neutral	2.8%	N/A
K43R	-0.747	Neutral	5.0%	10.8%
Y56stop	N/A	Deleterious	11.2%	3.2%
G77S	-1.811	Neutral	2.2%	14.0%
G101D	-6.901	Deleterious	0.3%	9.7%
T185A	-4.815	Deleterious	11.1%	32.3%
H197Q	-3.572	Deleterious	N/A	4.3%
M238L	-0.54	Neutral	0.6%	6.5%
E263G	-4.677	Deleterious	0.1%	1.1%
V312F	-4.424	Deleterious	0.3%	2.2%
Y341del	-14.463	Deleterious	47.4%	14.0%
V348L	0.583	Neutral	N/A[2]	21.5%
A356V	-1.834	Neutral	N/A[2]	21.5%

[1] Cut-off for prediction of deleterious impact upon protein function is -2.5

[2] Not applicable because the data in Peter et al. (2018) was mapped against S288c which does not include these positions.

The strains used in the fermentation and biochemical activity assay experiment had a distribution of mutations that exhibited deleterious provean scores and frequent in brewing strains. The main polymorphisms of interest were G101D, T185A, and the allele deletion (Y341del).

Carbon-sulfur lyase activity of brewing *S. cerevisiae* strains

CS-lyase activity against a model substrate was assessed for a subset of brewing strains (Table 1) that represented frequent and potentially deleterious IRC7 mutations. Figure 4 summarizes these data, normalized to the high-activity control (MaxiThiol), and distributed based on the allele variant. The short mutation of the allele has a very low activity except for the BE088 being an outlier having activity above that of MaxiThiol. The median value of CS-lyase activity for strains with the long-allele, heterozygous-allele short-allele of *IRC7* were 0.69 nmol/min/μg, 0.22 nmol/min/μg, and 0.13 nmol/min/μg, respectively. There were 19 long-allele strains, 3 heterozygous-allele strains, and 4 short-allele strains. The average number of replicate measurement of CS-lyase activity for each strain is 5. When excluding the outlier BE088 the *IRC7* double long allele and *IRC7* short show statistical significance in a Dunn test ($p < 0.05$).

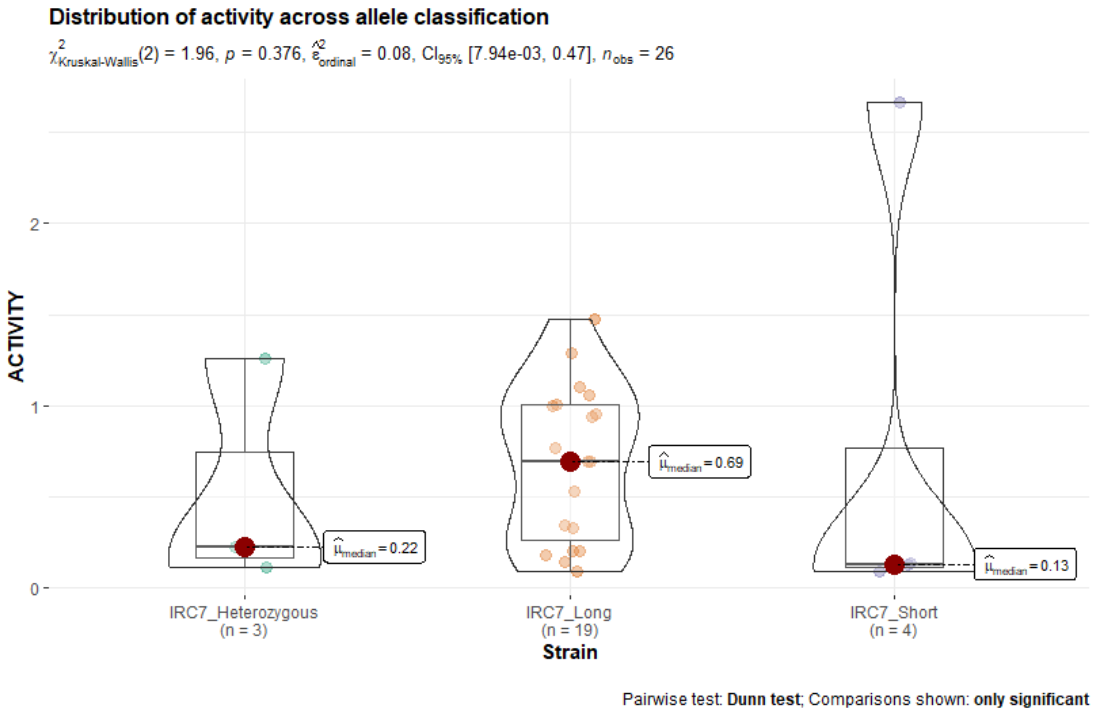

Figure 4: Box plot distribution of the different strains based on the activity of the allele length of *IRC7* and their resulting biochemical activity value. The ggbetweenstats distribution in R compares the activity of the different strains and shows the di between the different strains shown in the brackets above the box plots. *IRC7* long and *IRC7* heterozygous had a wider distribution of biochemical activity than IRC7 short.

Confirmation of IRC7 allele length

Brewing strains that had been assayed for CS-lyase activity were subjected to PCR to confirm the sequencing-based prediction of IRC7 allele length. An example of gel electrophoresis analysis of this PCR is shown in Figure 5, with results summarized in Appendix 2. Illumina short-reads from the original genome assemblies were also mapped against a reference strain that harbors full-length IRC7 (wine strain VL3) and based upon sequence coverage scored as homozygous or heterozygous for the two allele lengths (Appendix Table III).

BE088 despite having high activity is shown by PCR and sequence analysis to have a short allele. The PCR shows that BE062 has a long allele despite the sequencing data showing a short allele. Similarly, the PCR shows BE067 to have only a long allele while sequence analysis shows heterozygosity.

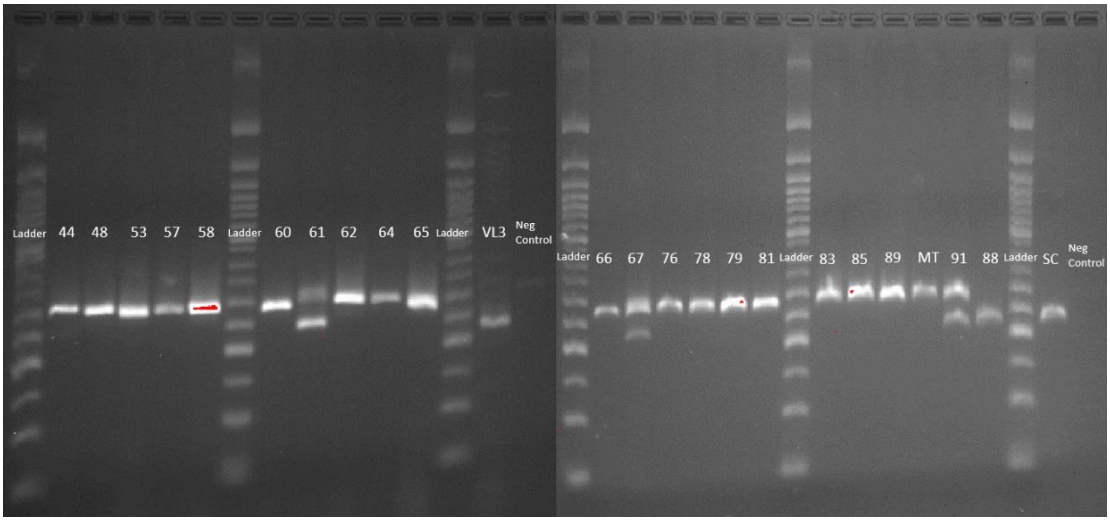

Figure 5: Polymerase chain reaction (PCR) identifying the allele length of the *IRC7* gene in *S. cerevisiae*. The high control MaxiThiol (MT) is consistent with the long allele mutation and the low control S288c shows the short mutation. BE061 (61) shows the heterozygosity, or one allele being long and one allele being short.

Analysis of IRC7 sequence polymorphisms

Because *IRC7* length polymorphism did not fully explain variation in CS-lyase activity, even after correction for heterozygosity, we attempted to relate observed mutations in *IRC7* sequence to activity using random-forest analysis. This machine-learning approach evaluates the impact of predictor variables (mutations) upon a model that solves for CS-lyase activity (Figure 6). Presence or absence of full-length *IRC7* sequence (DEL_SEQ/DEL_PCR) and T185A were highly ranked, along with the beer-strain mutation V348L. V348L exists only in strains without the deletion because amino acid position 348 occurs within the 38 bp gene deletion. Of the mutations Provean scored as deleterious; the deletion, T185A, and G101D are deemed as important and in Cordente et al. (2019), those polymorphisms were most significant in modulating Irc7p activity.

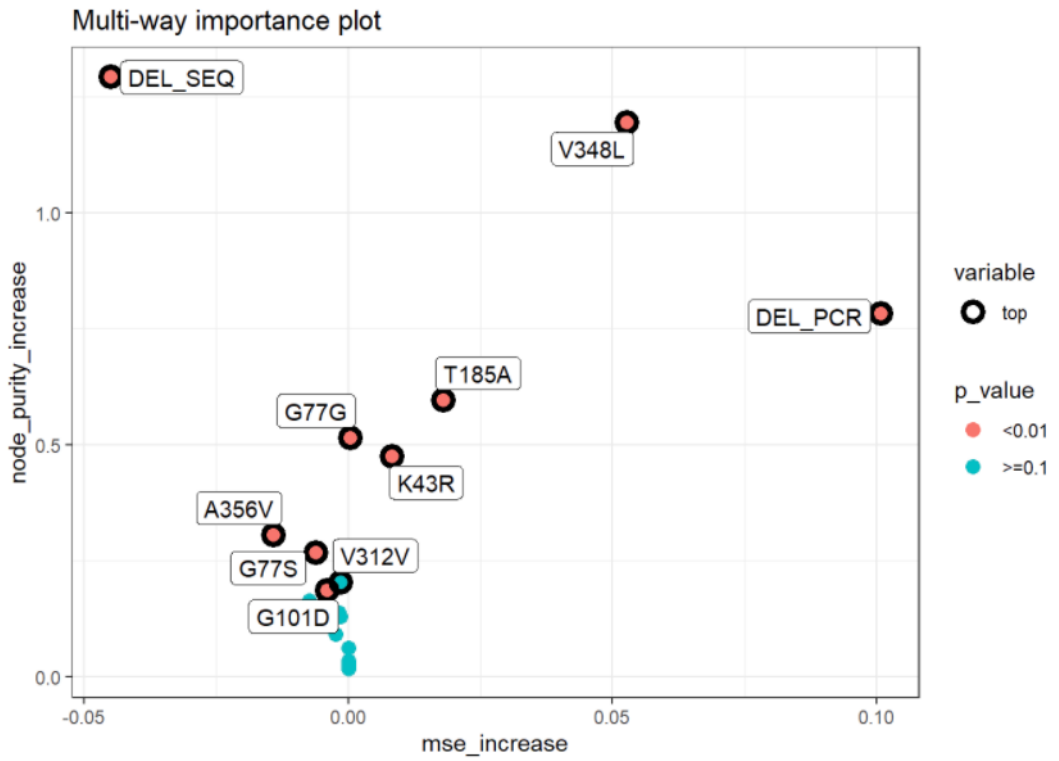

Figure 6: Random Forest Analysis of carbon-sulfur lyase activity and scored mutations/deletions for brewing strains of S. cerevisiae. Random Forest Analysis attempts to link importance from a variable in prediction. MSE_increase and node_purity_increase correlating on the p-value based on the node splits of the analysis should predict mutations that are important. Red points are significant and the more up and to the RIGHT the point the more important Random Forest Analysis ranks the variable.

Despite being ranked as important in the randomForest test and being consistent with Cordente et al. (2019) the different polymorphisms did not test as significant in a linear regression. Each of the deemed important mutations had the proportion of which they occur in each strain tested against activity each producing a $p > 0.05$. Activity was directly related to the deletion of in wine strains, but beer strains appear to be more complicated. To further test the significance of the polymorphism activity H_2S production of ferments will be statistically tested against activity of the strains.

Fermentations of wort using strains with varied CS-lyase activity

While the importance of *IRC7* mutations in determining CS-lyase activity was not fully determined for brewing strains, evaluation of whether the observed differences in activity would translate into relevant impact during fermentation (Figure 7). The low control S288c did not produce as much H_2S as MaxiThiol consistent with the biochemical analysis. BE088 is the only significant outlier

producing less H_2S than MaxiThiol. Overall, higher H_2S production correlated with higher biochemical activity (p-values <0.05, R-squared = 0.64). Correlation between CS-lyase activity and H_2S formation shows that during fermentation of wort there is potential for variation between strains with regards to release of polyfunctional thiols from cysteinylated precursors, as shown previously for wine strains during wine fermentation (Cordente et al., 2019).

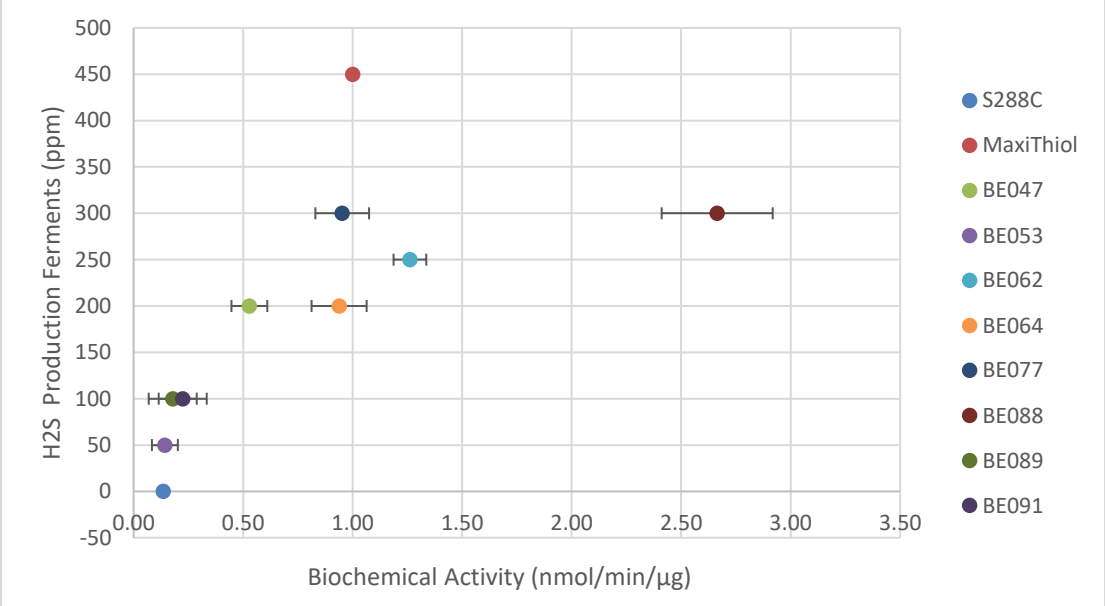

Figure 7: The effect of biochemical activity (nmol/min/µg) on the H_2S production (ppm). The higher the biochemical activity there was a likelihood for higher H_2S production (R-squared = 0.64, p = 0.0062). All biochemical activity was scored against the reference MaxiThiol at 1.00 nmol/min/µg. H_2S production was measured over the course of a 7-day 200 mL ferment with 400mg/L L-cysteine added.

To evaluate whether the production of H_2S via cysteine-desulfydrase activity of brewing strains could be relevant during beer fermentation, additional fermentations were performed in a standard wort without the addition of L-cysteine (Figure 8). Successful ferments were shown in Figure 555 with strains losing between 38 and 55 g/CO_2 L, except S288C which predictably only lost between, 10 and 12 g/CO_2 L. In this experiment, only beer strain BE088 and wine strain MaxiThiol produced detectable H_2S (Figure 9). This showed that high activity strains are capable of producing H_2S even without the addition of cysteine into wort.

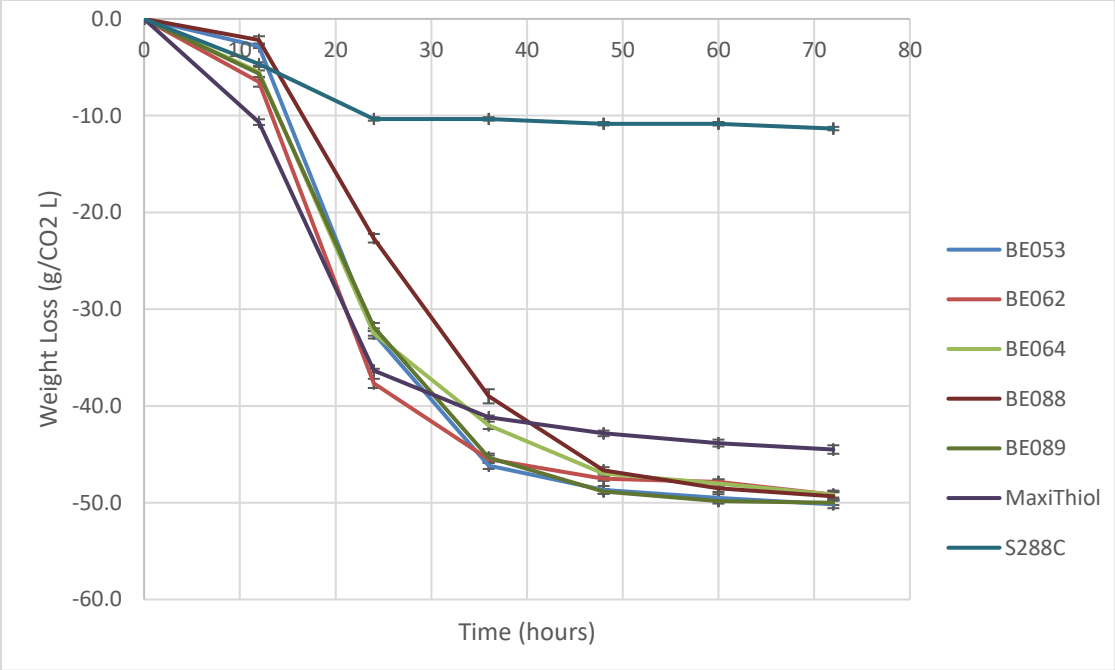

Figure 8: Weight loss of the beer fermentations done without addition of L-cysteine over a 72-hour ferment.

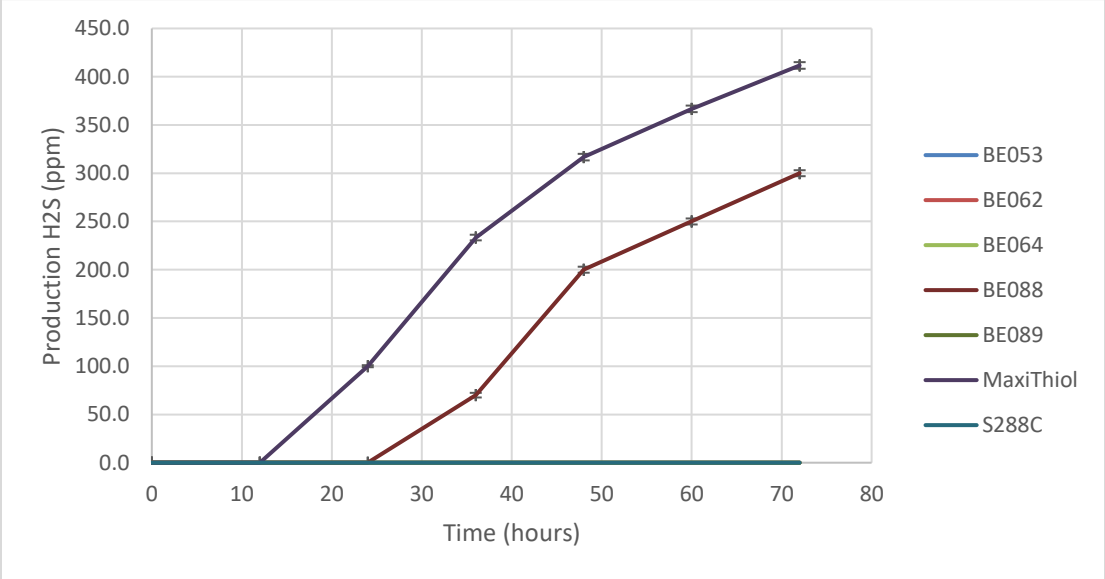

Figure 9: H_2S (ppm) production of the beer fermentations done without addition of L-cysteine over the course of 72 hours.

Fermentations of grape juice using BE088

The brewing strain BE088 exhibited the highest CS-lyase activity and a strong capacity for H_2S production during fermentation of wort, despite being scored as homozygous for the short allele of *IRC7*. Interestingly, BE088 clustered amongst wine yeast according to whole-genome alignments (Gallone et al., 2016). We were,

therefore, interested to see whether this strain could ferment grape juice to dryness and to what extent it would produce H_2S under these conditions. Results of a laboratory-scale fermentation of Chardonnay juice is shown in Figure 10. BE088 failed to ferment as well as the wine strains MaxiThiol and EC1118, but produced significantly more H_2S, as shown in Figure 11. Although BE088 produced less H_2S than MaxiThiol in the wort ferments, it does not reproduce that result in the ferments of grape juice. This is more in line with the prediction of activity found in the biochemical activity assay.

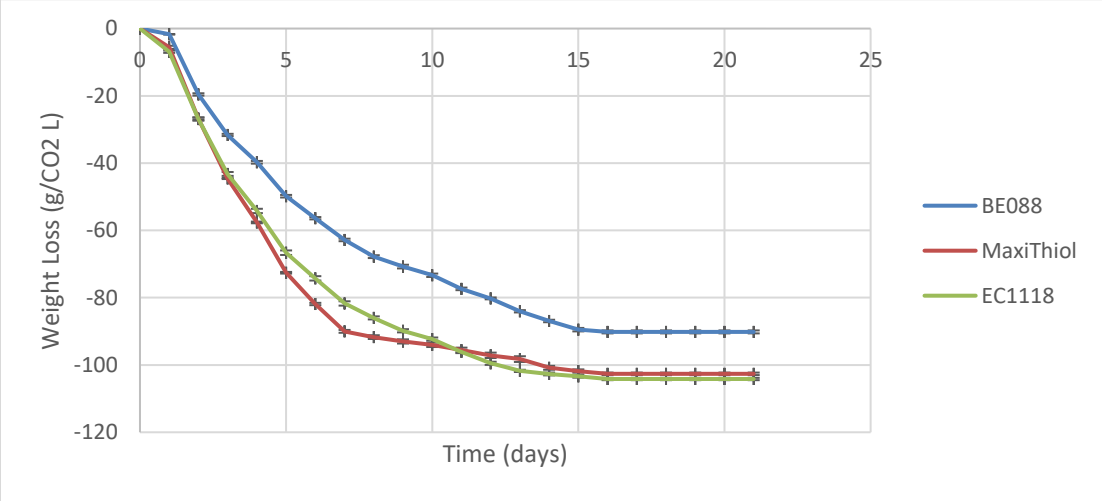

Figure 10: Wine fermentation comparing BE088, MaxiThiol, and EC1118. Examining the weight loss from CO_2 (g/CO_2 L) production over a 21-day period. MaxiThiol and S288C produced enough CO_2 to ferment successfully as a wine and BE088 fell short by 5-10g CO_2/L.

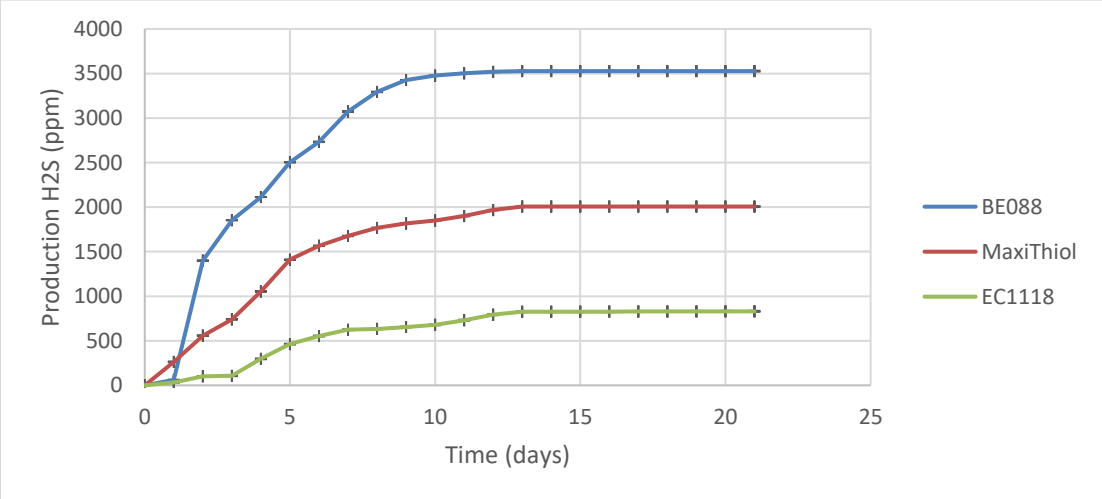

Figure 11: Production of H_2S (ppm) from BE088, MaxiThiol, and EC1118 over the course of a 21-day ferment. BE088 produced significantly more H_2S than the high control MaxiThiol.

Discussion

IRC7 activity in wine has shown that the deletion of the allele, T185A, and G101D greatly reduce the effective activity of Irc7p (Cordente et al., 2019). The distribution of biochemical activity across allele classification as shown in Figure 4 showed that strains with the deletion had low activity, while strains with heterozygous and long activity have a larger range of activity. This is consistent with the analysis done by Cordente et al. (2019). BE088 is an outlier for its high activity strain and the genetic similarities that it has with wine even though it has the allele deletion. The expected polymorphisms T185A, and G101D do not have a direct effect in decreasing of activity as shown due to the limitations of the number of strains analyzed the high variability of activity in versions of *IRC7* with the double long or heterozygous alleles. Furthermore, other genes such as *STR3* could play a larger role in beer than they do in wine (Holt et al., 2011). A further look in the polymorphisms of the beer strains could lead to finding other mutations in *IRC7* that may be more significant when grouped with previously known important mutations that could be more responsible for modulation Irc7p activity.

Biochemical activity was shown to correlate positively to produce H_2S and from established literature should indicate the production of positive volatile thiols (Cordente et al., 2019; Santiago et al., 2015, Roncoroni et al., 2011, Swiegers et al., 2007). Additionally, high activity strains were shown to produce H_2S with non-hopped malt and without any Cysteine added to the wort. Volatile Thiol production is easily feasible in beer strains with active form of *IRC7.*

Final Conclusions and Future Directions

This thesis study some found consistency with previous studies, specifically wine studies, of *IRC7* have an effect on biochemical activity and production of H_2S. Strains with the deletion showed lower activity except for the significant outlier strain BE088, and strains with the long allele and heterozygous alle having much more variable activity. However, the presence of the short-allele or long-allele with the known inactivating mutations could reliably predict a strain's biochemical activity. Analysis of more strains may improve this. Correlation between biochemical activity

and H_2S production from cysteine was consistent with previous literature, suggesting that brewing strains with varied levels of biochemical activity could be good a predictor of varied thiol release potential. Further experiments with more strains and replicates are needed to fully conclude these findings. A sensory analysis of the beers using high and low activity strains would be essential to determine whether the production of the polyfunctional is previable.

These results need to be built upon in future studies to conclude whether the findings here have implications for brewers attempting to incorporate a unique flavor and aroma flavor to their beer. Further fermentations and an assay identifying positive thiol concentrations in before and after fermentation needs to be looked at to confirm the findings in this study. Further studies could look into the oddities that exist in BE088 to identify other pathways for volatile thiol production in yeast. Additionally, research that focuses on glutathione precursors could also be incorporated to find additional ways to produce volatile thiols in beers.

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Appendix 1 – Analysis of mutation frequency and impact

Full summary of prevalent and potentially impactful non-synonymous mutations in *IRC7*. Frequency of each mutation across *S. cerevisiae* genome sequencing datasets was scored, with only those present amongst >1% of either yeast populations included in this table. Provean scores are a measurement of the effect of each mutation relative to the reference sequence for the *IRC7* gene.

Variant	PROVEAN results		Frequency of polymorphism	
	Score	Prediction of impact[1]	Peter et al. (2018)	Gallone et al. (2016)
D3N	-0.189	Neutral	0.4%	1.1%
R4L	-1.492	Neutral	0.1%	N/A
R4H	0.328	Neutral	0.2%	N/A
T5N	0.326	Neutral	0.2%	N/A
T5del	-0.86	Neutral	2.8%	N/A
E6K	-0.812	Neutral	0.5%	N/A
K9R	-0.543	Neutral	0.1%	N/A
R21P	-5.751	Deleterious	0.5%	N/A
R21S	-4.944	Deleterious	0.3%	N/A
Q26K	-2.421	Neutral	0.3%	N/A
S27C	-1.653	Neutral	1.3%	N/A
L42F	4.272	Neutral	0.3%	N/A
K43I	-3.033	Deleterious	0.2%	N/A
K43R	-0.747	Neutral	5.0%	10.8%
L45P	-3.266	Deleterious	0.3%	N/A
S46C	-2.856	Deleterious	0.1%	N/A
D47G	-3.724	Deleterious	0.1%	N/A
R54stop	N/A	Deleterious	0.1%	N/A
Y56stop	N/A	Deleterious	11.2%	3.2%
G60D	-6.463	Deleterious	0.9%	N/A
D65A	-0.313	Neutral	0.1%	N/A
D65E	0.76	Neutral	0.1%	N/A
N69D	0.408	Neutral	0.1%	N/A
G77S	-1.811	Neutral	2.2%	14.0%
A84P	6.229	Neutral	0.1%	N/A
G88S	-1.288	Neutral	0.1%	N/A
G101D	-6.901	Deleterious	0.3%	9.7%
M106I	-0.655	Neutral	0.5%	N/A
D108N	-4.59	Deleterious	1.0%	N/A
A123S	-0.898	Neutral	0.1%	N/A
G126S	-5.443	Deleterious	0.1%	N/A

S135P	-1.367	Neutral	0.1%	N/A
D139A	-1.862	Neutral	0.1%	N/A
D139N	-0.928	Neutral	0.9%	N/A
P146A	-5.648	Deleterious	0.4%	N/A
T149S	-0.208	Neutral	0.1%	N/A
S155I	-4.782	Deleterious	0.4%	N/A
G157D	-6.487	Deleterious	0.1%	N/A
V163I	-0.133	Neutral	0.1%	N/A
N184K	-5.948	Deleterious	0.2%	N/A
T185S	-3.877	Deleterious	0.1%	N/A
T185A	-4.815	Deleterious	11.1%	32.3%
T188A	-4.324	Deleterious	0.1%	N/A
L190F	-3.415	Deleterious	0.2%	N/A
A196S	0.408	Neutral	0.1%	N/A
H197Q	-3.572	Deleterious	N/A	4.3%
D200del	-15.695	Deleterious	0.1%	N/A
V203L	-1.116	Neutral	0.1%	N/A
L210F	-2.727	Deleterious	0.2%	N/A
G211S	-1.181	Neutral	0.1%	N/A
I218L	1.286	Neutral	0.1%	N/A
A221T	-0.203	Neutral	0.1%	N/A
A223T	-3.121	Deleterious	0.1%	N/A
R232Q	-2.656	Deleterious	0.1%	N/A
M238L	-0.54	Neutral	0.6%	6.5%
A244V	-2.231	Neutral	0.2%	N/A
C247Y	-4.414	Deleterious	0.1%	N/A
Q248stop	N/A	Deleterious	0.2%	N/A
R252stop	N/A	Deleterious	0.1%	N/A
G253V	-8.922	Deleterious	0.3%	N/A
T256I	-5.915	Deleterious	0.2%	N/A
stop260R	N/A	Deleterious	0.1%	N/A
E263G	-4.677	Deleterious	0.1%	1.1%
L269P	-6.39	Deleterious	0.3%	N/A
A273V	-1.286	Neutral	0.1%	N/A
V281I	-0.897	Neutral	0.1%	N/A
E282del	-9.865	Deleterious	0.2%	N/A
H286R	-7.785	Deleterious	0.3%	N/A

P293L	-9.325	Deleterious	1.1%	N/A
G294R	-7.478	Deleterious	2.5%	N/A
E296G	-2.439	Neutral	0.4%	N/A
D301Y	-7.656	Deleterious	0.1%	N/A
Y302C	-5.49	Deleterious	0.1%	N/A
G304S	-5.769	Deleterious	0.1%	N/A
G304D	-6.812	Deleterious	3.6%	N/A
G307S	-5.948	Deleterious	0.5%	N/A
L308V	-2.409	Neutral	0.2%	N/A
V312F	-4.424	Deleterious	0.3%	2.2%
L313F	-3.965	Deleterious	0.1%	N/A
G321D	-0.625	Neutral	0.5%	N/A
G328E	-3.064	Deleterious	0.1%	N/A
L332F	5.754	Neutral	0.1%	N/A
F336Y	0.244	Neutral	0.1%	N/A
S337L	-5.904	Deleterious	0.1%	N/A
S337F	-5.87	Deleterious	0.1%	N/A
V348L	0.583	Neutral	N/A	21.5%
A356V	-1.834	Neutral	N/A	21.5%
Y341del	-14.463	Deleterious	47.4%	14.0%

[1] Cut-off for prediction of deleterious impact upon protein function is -2.5

[2] Not applicable because the data in Peter et al. (2018) was mapped against S288c which does not include these positions.

Appendix 2 – Full Summary of *IRC7* allele length

Full Summary table of *IRC7* allele length from PCR and sequence analysis.

Strain	PCR identifier	PCR Alele Length	Sequence Allele Length
BE044	[44]	Homozygous Long	Homozygous Long
BE047	[47]	Homozygous Long	Homozygous Long
BE048	[48]	Homozygous Long	Homozygous Long
BE053	[53]	Homozygous Long	Homozygous Long
BE057	[57]	Homozygous Long	Homozygous Long
BE058	[58]	Homozygous Long	Homozygous Long
BE060	[60]	Homozygous Long	Homozygous Long
BE061	[61]	Heterozygous	Heterozygous
BE062	[62]	Homozygous Long	Homozygous Long
BE064	[64]	Homozygous Long	Homozygous Long
BE065	[65]	Homozygous Long	Homozygous Long
BE066	[66]	Homozygous Long	Homozygous Long
BE067	[67]	Heterozygous	Heterozygous
BE076	[76]	Homozygous Long	Homozygous Long
BE078	[78]	Homozygous Long	Homozygous Long
BE079	[79]	Homozygous Long	Homozygous Long
BE080	[80]	Homozygous Short	Homozygous Short
BE081	[81]	Homozygous Long	Homozygous Long
BE083	[83]	Homozygous Long	Homozygous Long
BE085	[85]	Homozygous Long	Homozygous Long
BE088	[88]	Homozygous Short	Homozygous Short
BE089	[89]	Homozygous Long	Homozygous Long
BE091	[91]	Heterozygous	Heterozygous
BE092	[92]	Homozygous Short	Homozygous Short
S288C	[SC]	Homozygous Short	Homozygous Short
MaxiThiol	[MT]	Homozygous Short	Homozygous Short

Appendix 3 – Strain Biochemical Activity

Biochemical activity of each individual replicate summarized.

Strain	Activity nmol/min/μg	Average Activity nmol/min/μg	Standard Deviation
BE044	0.08	0.21	0.09
BE044	0.22		
BE044	0.22		
BE044	0.14		
BE044	0.33		
BE044	0.25		
BE047	0.59	0.53	0.11
BE047	0.40		
BE047	0.64		
BE047	0.49		
BE048	0.70	1.08	0.98
BE048	0.36		
BE048	0.39		
BE048	0.99		
BE048	0.43		
BE048	3.03		
BE048	1.68		
BE053	0.06	0.14	0.06
BE053	0.16		
BE053	0.16		
BE053	0.19		
BE057	1.21	1.47	0.58
BE057	2.14		
BE057	1.07		
BE058	0.08	0.20	0.09
BE058	0.21		
BE058	0.31		
BE058	0.20		
BE060	0.20	0.34	0.26
BE060	0.29		
BE060	0.23		
BE060	0.24		
BE060	0.64		
BE060	0.12		
BE060	0.14		
BE060	0.84		
BE061	0.05	0.11	0.06

BE061	0.16		
BE061	0.11		
BE062	1.19		
BE062	1.09		
BE062	1.50		
BE062	1.92		
BE062	1.73	1.26	0.45
BE062	1.51		
BE062	0.68		
BE062	1.17		
BE062	0.57		
BE064	1.01		
BE064	1.00	0.94	0.25
BE064	0.58		
BE064	1.16		
BE065	0.28		
BE065	0.10	0.20	0.11
BE065	0.31		
BE065	0.12		
BE066	0.72		
BE066	1.04		
BE066	0.72		
BE066	0.34	1.00	0.76
BE066	2.80		
BE066	0.56		
BE066	0.84		
BE066	1.02		
BE067	0.11		
BE067	0.07	0.09	0.03
BE067	0.12		
BE067	0.06		
BE075	1.81		
BE075	1.32	1.29	0.38
BE075	1.02		
BE075	1.00		
BE076	0.93		
BE076	1.00	1.10	0.24
BE076	1.37		
BE077	0.84		
BE077	1.22	0.95	0.24
BE077	0.68		

BE077	1.07		
BE078	0.15	0.32	0.28
BE078	0.07		
BE078	0.05		
BE078	0.61		
BE078	0.29		
BE078	0.46		
BE078	0.81		
BE078	0.15		
BE079	0.99	1.06	0.36
BE079	1.43		
BE079	1.22		
BE079	0.59		
BE080	0.07	0.12	0.06
BE080	0.19		
BE080	0.11		
BE081	0.83	0.69	0.26
BE081	0.60		
BE081	0.91		
BE081	0.34		
BE081	0.95		
BE081	0.38		
BE081	0.38		
BE081	0.90		
BE081	0.91		
BE083	0.61	0.76	0.65
BE083	0.11		
BE083	0.07		
BE083	1.63		
BE083	1.40		
BE083	0.76		
BE085	0.46	0.69	0.22
BE085	0.57		
BE085	0.64		
BE085	1.03		
BE085	0.76		
BE088	2.15	2.66	1.03
BE088	1.50		
BE088	3.77		
BE088	3.23		
BE089	0.19	0.18	0.06

BE089	0.13		
BE089	0.27		
BE089	0.13		
BE091	0.05	0.22	0.19
BE091	0.31		
BE091	0.08		
BE091	0.46		
BE092	0.14	0.09	0.04
BE092	0.05		
BE092	0.11		
BE092	0.05		
S288C	0.28	0.13	0.10
S288C	0.27		
S288C	0.12		
S288C	0.11		
S288C	0.12		
S288C	0.12		
S288C	0.02		
S288C	0.03		
MaxiThiol	1.00	1.00	0.00
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		
MaxiThiol	1.00		