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Molecular and Biochemical Analysis of Phenolic Acid Decarboxylase from *Brettanomyces* Yeast Isolates

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MOLECULAR AND BIOCHEMICAL ANALYSIS OF PHENOLIC
ACID DECARBOXYLASE FROM *BRETTANOMYCES* YEAST
ISOLATES

Chad Lawrence Harris

A thesis submitted to the Honors Program
in partial fulfillment of the requirements for
Honors in Biology

UNIVERSITY OF NORTH FLORIDA

HONORS PROGRAM

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Unpublished work © Chad Harris

Dedication

Mom, Dad, Bruce, and Jennifer – thank you for always supporting me, not only financially but emotionally through my college years. I would not have made it this far in my life if it wasn't for all of your support. I love you all dearly.

Acknowledgements

I would like to thank my mentor and research supervisor, Dr. Mike Lentz, for his patience, support, and commitment in transforming me from a naïve student to a student scholar. As I traverse the perils of graduate school, I know I will think of you often – and probably call you frequently. The preparation you provided enables me to approach graduate education with confidence knowing that in a few demanding but short years I will begin my career as a physician.

I would also like to thank Mrs. Louanne Hawkins and Dr. Judith Ochrietor for their conscientious support through the tough times I've faced as a researcher, and guiding me on the path to success.

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Abstract

Many strains of *Brettanomyces* pose a major threat to the commercial brewing industry by producing phenolic off-flavors, while a few strains contribute desirable characteristics in unique beer styles. This genus of yeast possesses a two-step pathway that converts hydroxycinnamic acids (HCAs) to vinyl and ethyl derivatives that add unwanted flavors and aromas to the final beverage. This project focused on analyzing *Brettanomyces* yeast isolates for variation in phenolic acid decarboxylase activity (PAD), which catalyzes the first step in the pathway. Each strain was grown in the three different common HCAs (ferulic acid, *p*-coumaric acid, caffeic acid) to understand each strain's reactivity to the compound. Strains displayed variation in their PAD activity and growth in HCAs. Most good brewing strains were highly sensitive to *p*-coumaric acid, and displayed significant lags in their growth in *p*-coumaric and ferulic acid. All strains were highly resistant to caffeic acid. These data show some trends towards differences in good brewing strains and may lead to easier identification methods for yeast strains that may be good for brewing.

Molecular and biochemical analysis of phenolic acid decarboxylase from *Brettanomyces* yeast isolates

Introduction

Beer ales have been produced and served longer than any other beverage currently produced. Historical records indicate that ales date back to the 5th millennium BC through its depiction in the ancient writings of Egypt and Mesopotamia. The oldest record of an ale beverage dates back 7,000 years to the Iranian region, revealed through chemical analysis of ancient pottery discovered (Mirsky, 2007). It is possible that this beverage was discovered accidentally, in which cereal-containing fluid was spontaneous inoculated with microflora and a flavorful fermented beverage was discovered. Ironically, these techniques using spontaneous fermentation are undergoing a resurgence. The dawn of a new kind of beverage emerged, and most cultures around the world are independently discovering this phenomenon.

Ales were produced on a domestic scale even before the industrial revolution. More sophisticated brewing techniques evolved around the 19th century with the invention of thermometers and hydrometers, and more importantly the discovery of yeast. Today, brewing is a ~300 billion dollar a year industry (Research and Markets).

Historically, different regions all produced different types of beers varying in their organoleptic properties. These properties are specific to the flavor and aroma of the beverage. The properties vary due to the specific cereal used, but contrary to popular belief, are immensely reliant on the genus, species, and strain of the yeast and bacteria used in the inoculation. The domestication of some of these strains, specifically *Saccharomyces cerevisiae* revolutionized commercial fermentation, allowing fermenters of beer and wine to control their process and

produce a more predictable consistent product. There are now hundreds of different strains of yeast available to brewers all used to produce different types of beers, each contributing a unique physiological mechanism to produce variable organoleptic properties. Common protocol requires commercial breweries to inoculate wort with pure strains of yeast as it cools. This ensures production of desired aromas and flavors in the final product (Erten, 2007; Suihko, 1993).

Though commercial fermentation has brought much success to the brewing industry, certain biological complications pose a major threat. *Brettanomyces* yeast have a large impact on the brewing process, and often result in spoilage, leading to significant economic loss (Fugelsang, 1997). Control of this species is a top priority for brewers and winemakers worldwide. The American Vineyard Foundation ranked influence of yeast on chemistry and flavor as a top priority in enology related areas. *Brettanomyces* are ubiquitous in nature and display long-term survival on brewery and winery surfaces such as walls, grape processing equipment, and aging barrels (Woolfit, 2007). There are presently five species recognized within the genus *Brettanomyces*: *B. bruxellensis*, *B. anomalus*, *B. nanus*, *B. naardenensis*, and *B. custersianus*. Teleomorph (spore-forming) forms have been reported for *B. bruxellensis* and *B. anomalus* and are identified as the *Dekkera* genus; these forms are rare and *Brettanomyces* will be used in this manuscript. *Brettanomyces* contribute to spoilage via a two-step pathway, initially involving metabolism of plant derived compounds known as hydroxycinnamic acids (HCAs). HCAs are a ubiquitous plant compound that aid in anti-microbial properties, and are thus present in wine must and beer wort (Faulds, 1999). In this two step pathway, *Brettanomyces* convert HCAs to volatile phenolic compounds that contribute off-flavors and aromas to fermented beverages, often referred to as “Brett taint” (Oelofse, 2008).

Though this genus of yeast has been damaging to the commercial fermentation industry, it has also brought much opportunity, especially to the craft beer industry. Craft brewing is characterized by production of unique small batches of beer. *Brettanomyces* offers a unique quality to fermentation that conventional strains cannot. Though many strains of this yeast contaminate the product, certain strains also offer unique favorable organoleptic properties. By-products of some of these strains have been described as smokey, clove-like, and fruit-like. Craft brewing companies have taken advantage of these unique properties to produce contemporary products. The aforementioned spontaneous fermentation method of brewing also takes advantage of unique species of yeast. In this method, beer wort is left open to the environment to be inoculated by the local environmental flora. In this scenario, uncharacterized strains of yeast and bacteria to metabolize offer distinctly different flavors and aromas to the beverage. Every strain offers something different and unique to the product, and it is largely attributed to the PAD enzyme pathway, but why is that species of yeast using the same biochemical pathway in metabolism produce by-products with notably different organoleptic properties?

Phenolic acid decarboxylase (PAD) is the first enzyme involved in the two-step pathway converting HCAs to vinyl derivatives that are major contributors to phenolic off-flavor in beer and wine, however this enzyme is also responsible for producing organoleptic properties that are considered favorable in some fermentations. Some strains also encode vinyl reductase enzyme that converts the vinyl compounds into ethyl derivatives, also contributing to varying organoleptic properties in fermentation (Figure 1).

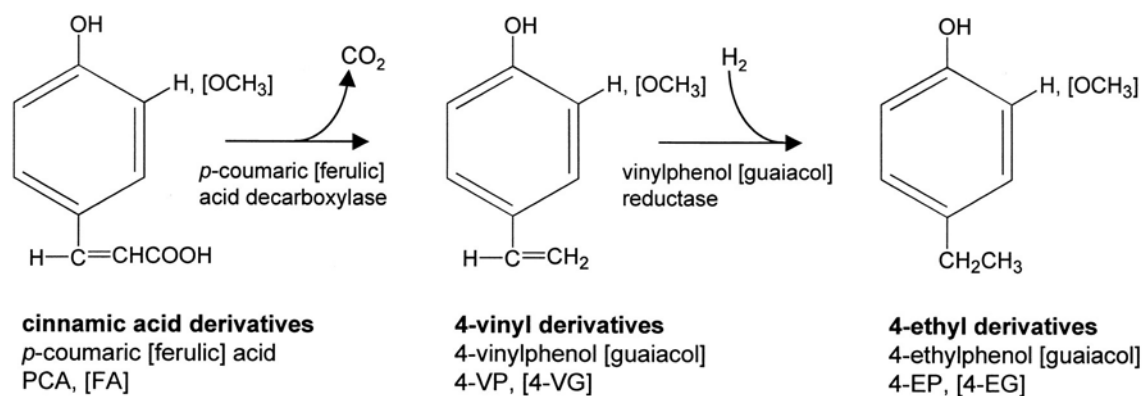


Figure 1 Two-step pathway for metabolism of HCAs in yeast (van Beek, 2000).

This study aims to understand why varying strains with similar biochemistry display vastly differentiated organoleptic properties in fermentation, and to establish trends correlating certain enzymatic behavior to species and or good brewing potential. To accomplish this, we aimed to determine the level of variation of PAD enzyme activity towards three common HCAs among a panel of 12 isolates of local environmental *Brettanomyces* isolates and commercially available strains (Table 1).

Table 1 Strain identification of each species studied

Strain Number	Species	Source	Brewing Potential
1	<i>Brettanomyces bruxellensis</i>	<i>Butia capitata</i> (pindo palm)	Not Good
2	<i>Brettanomyces bruxellensis</i>	<i>Butia capitata</i> (pindo palm)	Not Good
3	<i>Brettanomyces bruxellensis</i>	<i>Butia capitata</i> (pindo palm)	Not Good
4	<i>Brettanomyces anomalus</i>	<i>Celtus sp.</i> (hackberry)	Good
5	<i>Brettanomyces anomalus</i>	<i>Coccoloba uvifera</i> (seagrape)	Not Good
6	<i>Brettanomyces anomalus</i>	<i>Eriobotrya japonica</i> (loquat)	Good
7	<i>Brettanomyces anomalus</i>	<i>Rubus sp.</i> (blackberry)	Good
8	<i>Brettanomyces anomalus</i>	<i>Vaccinium darowii</i> (blueberry)	Not Good
9	<i>Brettanomyces anomalus</i>	Brewery (commerical strain)	Good
10	<i>Brettanomyces bruxellensis</i>	Brewery (commerical strain)	Good
11	<i>Brettanomyces bruxellensis</i>	Brewery (commerical strain)	Good
12	<i>Brettanomyces anomalus</i>	Brewery (commerical strain)	Good

These data will aid in identifying a correlation between substrate specificity and spoilage potential. Strains were also analyzed for the presence of each enzyme in the pathway. It was hypothesized that good brewing strains would display higher PAD enzyme activity, and there would be distinct differences between *B. anomalous* and *B. bruxellensis*.

Materials and Methods

HCA Metabolism

200µL samples of growth medium (MYPG) supplemented with 2 mM HCA (ferulic acid, *p*-coumaric acid, caffeic acid) and were arranged in 96-well microtiter plates. Each well was inoculated with 10^3 cells of each strain. Each experiment was conducted in triplicate for statistical analysis. Plates were incubated at 28°C in a moist environment for 10-14 days, as appropriate. Control (HCA in sterile culture medium) was subjected to an absorbance scan at 200-400nm wavelengths. Peaks are typically 313nm for ferulic acid and caffeic acid, and 287nm for *p*-coumaric acid, however the peak was determined empirically for each experiment. Yeast cells were removed from each sample via centrifugation and supernatant analyzed for absorbance at the peak wavelength. “Percent of original absorbance remaining” was used as a target value since spectral scan curves show low but measurable absorbance at the substrate peak level, even when pure product is analyzed (Figure 2)

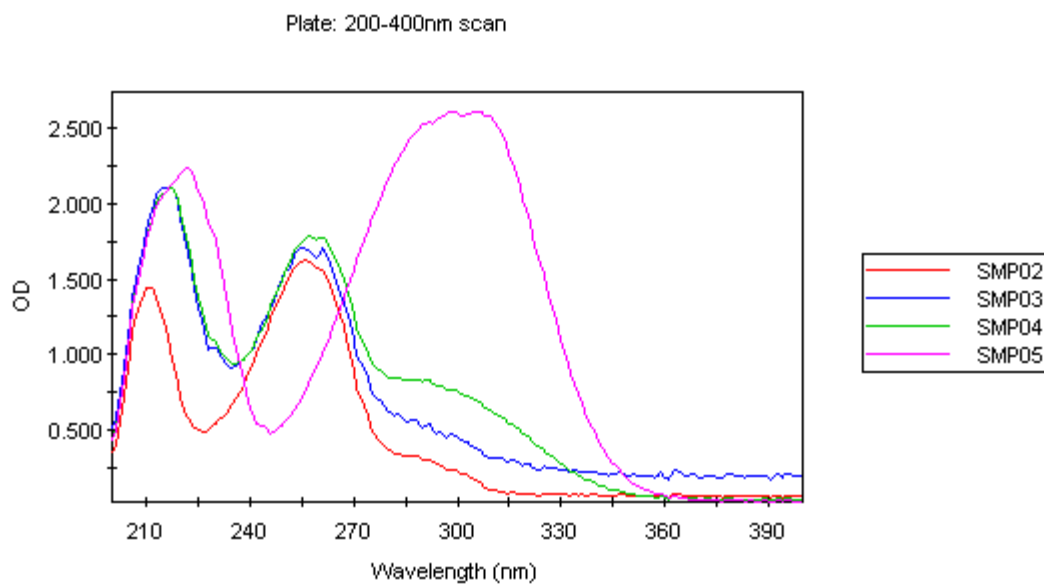


Figure 2 Sample spectral scan of ferulic acid control and yeast supernatant to determine percent of ferulic acid remaining. SMP02, 4-vinyl guaiacol control; SMP03, strain 6; SMP04, strain 7; SMP05, ferulic acid control.

HCA Minimum Inhibitory Concentration

200µL samples of MYPG supplemented with 0-20 mM HCA (ferulic acid, *p*-coumaric acid, caffeic acid) and were arranged in 96-well microtiter plates. Each well was inoculated with 10^3 cells of each strain. Each experiment was conducted in triplicate for statistical analysis. Plates were incubated at 28°C in a moist environment for 48-72 hours. Results were interpreted qualitatively by the observation of growth at each concentration.

Growth in HCA

Strains were grown in 20 mL of MYPG supplemented with 4 mM HCA (ferulic acid, *p*-coumaric acid, caffeic acid). Control cultures contained 1% ethanol, the solvent used for HCA stocks. Each

tube was inoculated with 10^5 cells of each strain. Vials were agitated and incubated at 24°C for 14 days. Absorbance of culture was determined for each sample at 600 nm at regular time points.

Results

Twelve *Brettanomyces* isolates were grown in 2mM HCA. After ten days incubation, HCA remaining was determined by comparing the absorbance of the sample to starting medium at either 287nm (p-coumaric acid) or 313nm ferulic acid and caffeic acid) (Figure 2).

Preliminary analysis of PAD activity in the local isolates of *Brettanomyces* yeast as well as several commercially available brewing strains demonstrated significant variation, particularly towards ferulic acid and *p*-coumaric acid. All strains displayed significantly less PAD activity towards caffeic acid while some strains were unable to metabolize the compound at all (Figure 3).

As can be seen in figure 3, there is wide variation among isolates for metabolism of the different substrates. Some common themes were also observed. All 12 strains utilized caffeic acid least efficiently of the three HCAs tested, ranging from not using this substrate at all, to reducing the peak absorbance by about 50 percent. Ferulic acid and *p*-coumaric acid were metabolized equally efficiently by five of the strains, however the proportion remaining after metabolism ranged from 20 percent to 50 percent within this group. Six strains metabolized ferulic acid more efficiently than *p*-coumaric acid, again with wide strain-to-strain variation. Only one strain used *p*-coumaric acid to a higher degree than ferulic acid.

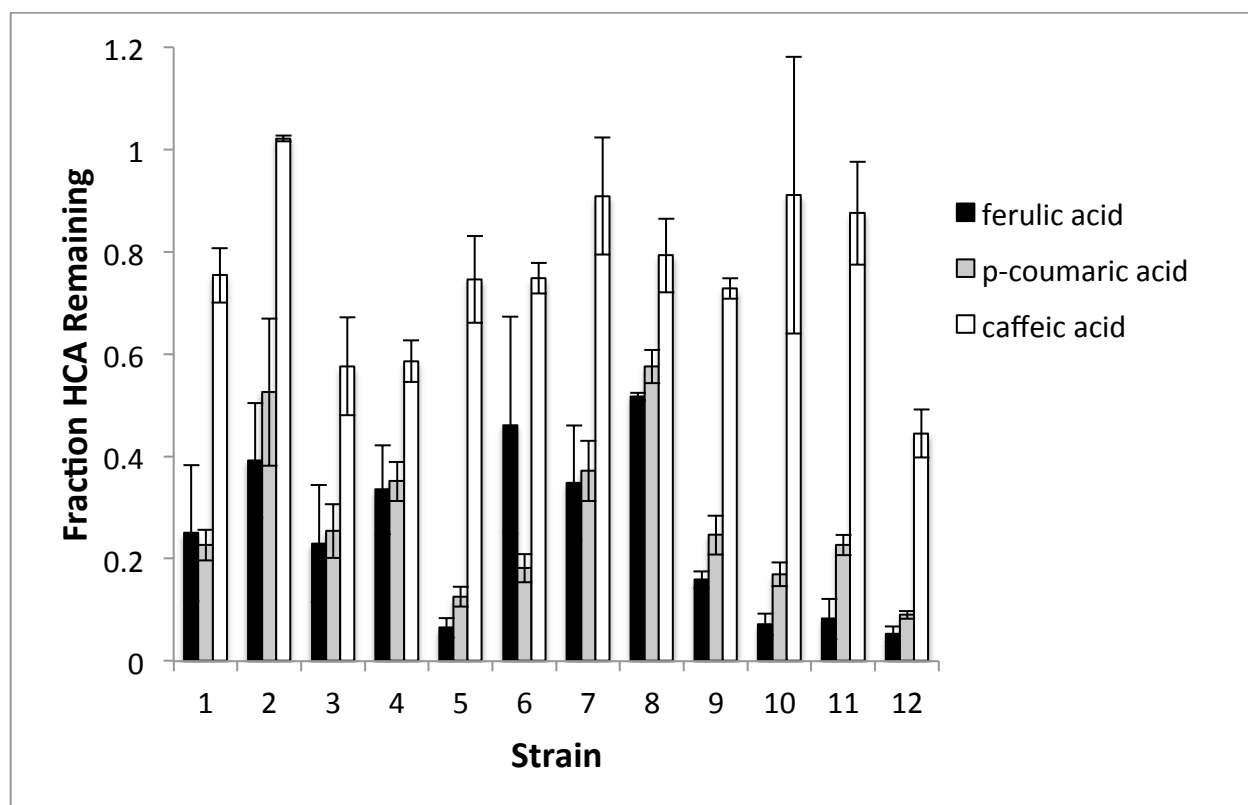


Figure 3 Fraction of hydroxycinnamic acid remaining after 10 days of incubation with each *Brettanomyces* strain.

The minimum inhibitory concentration (MIC) of each HCA to each strain was determined by incubating each strain in MYPG supplemented with 0-20 mM HCA and observing growth after 2-3 days.

All strains grew well in media supplemented with caffeic acid at 20mM, exhibiting no inhibition by this compound. Strains 1, 2, and 7-11 also grew at 20mM p-coumaric acid. These strains represent both *B. bruxellensis* and *B. anomalus*, and brewing and non-brewing strains of each species. Strains 3, 4, 5, 6, and 12 were unable to grow in p-coumaric acid concentrations of 10mM, 12mM, 8mM, 14mM, and 8mM, respectively. Strain 3 is *B. bruxellensis*, while the rest

are *B. anomalus*. Ferulic acid provided the strongest inhibition, with one strain (#4) growing in 14mM, but no others growing at concentrations above 12mM. Strain 4 is an environmental isolate used successfully in brewing. Inhibition of growth above 6mM was observed for three of the strains (3, 5, and 12). Growth at 12mM but not above this concentration was seen for strains 6, 7, 9, 10, and 11, all strains that have been used successfully in brewing. The remaining strains were inhibited by intermediate concentrations of ferulic acid (Table 2).

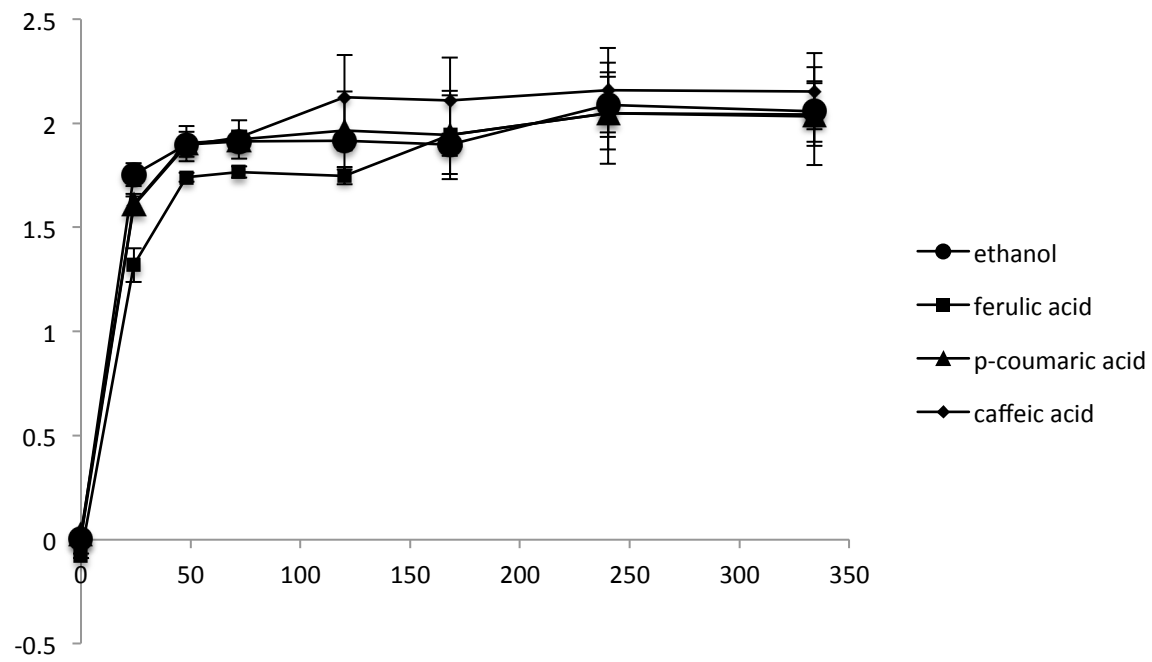
Table 2 Minimum inhibitory concentration of each yeast strain for each HCA* (in mM).

	1	2	3	4	5	6	7	8	9	10	11	12
FA	10	10	8	14	8	12	12	16	12	12	12	8
pCA	20	20	10	12	8	14	20	20	20	20	20	8
CA	20	20	20	20	20	20	20	20	20	20	20	20

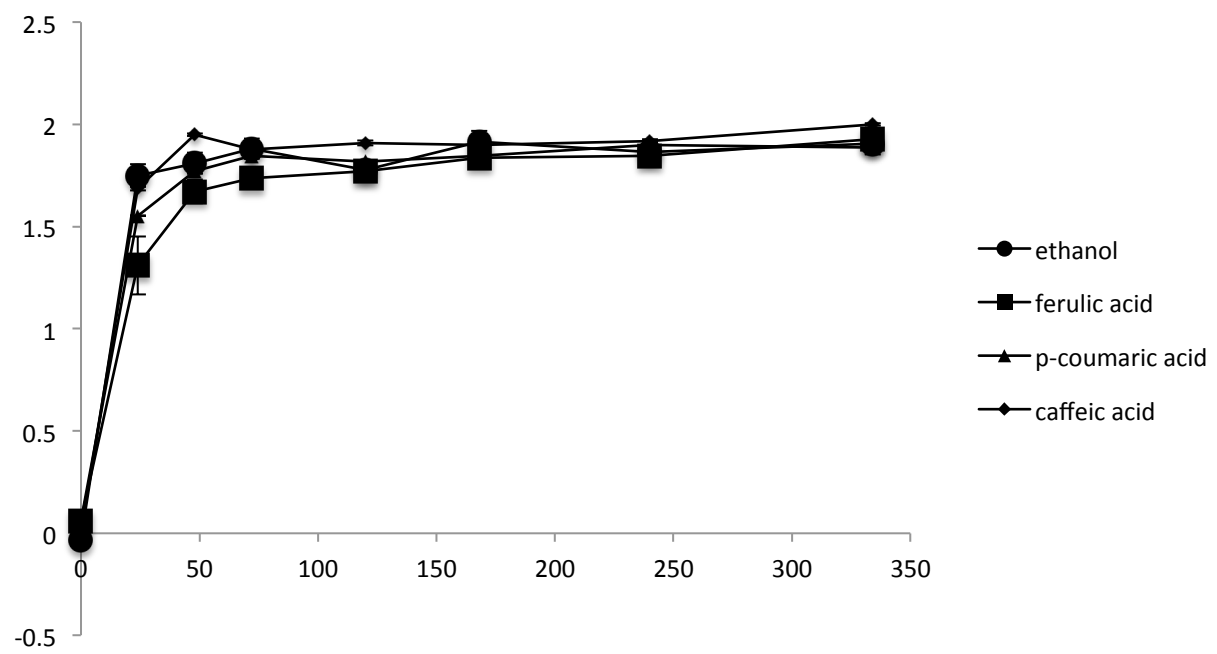
* FA.: Ferulic acid; pCA: p-Coumaric acid; CA: Caffeic acid

Each strain was grown in 4 mM HCA for 14 days. Absorbance readings of cultures were read at regular intervals to generate growth curves (Figure 4). Most strains displayed variation in their growth patterns, however it was noted that some strains display a significant growth lag between ferulic acid and *p*-coumaric acid. This trend was particularly evident in strains with good brewing potential.

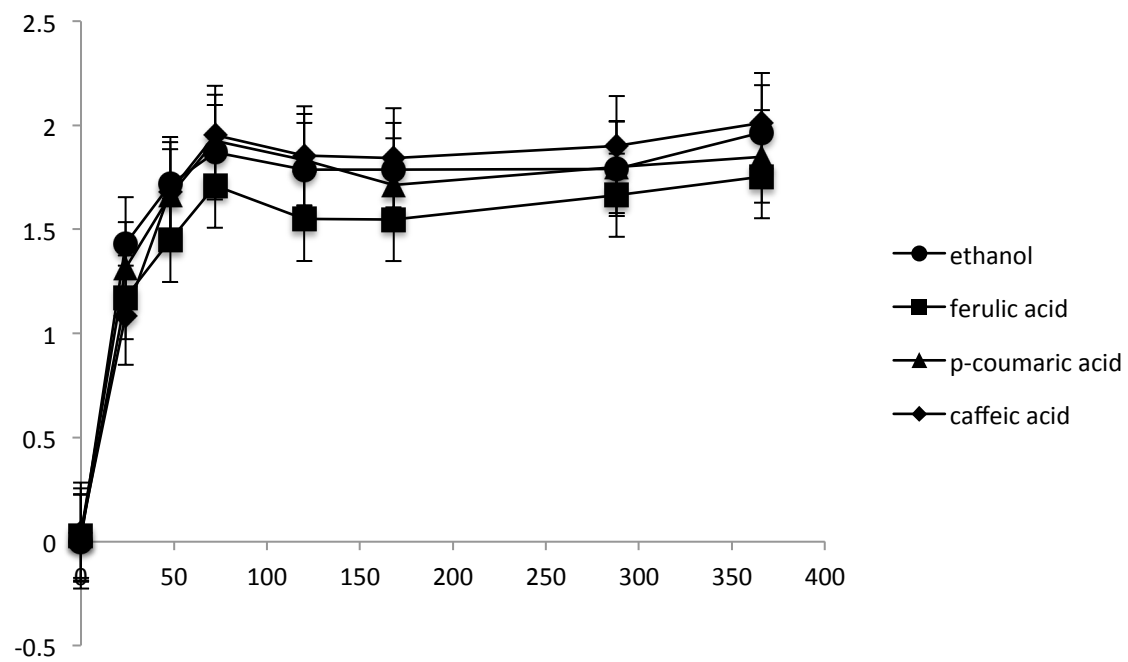
A.)



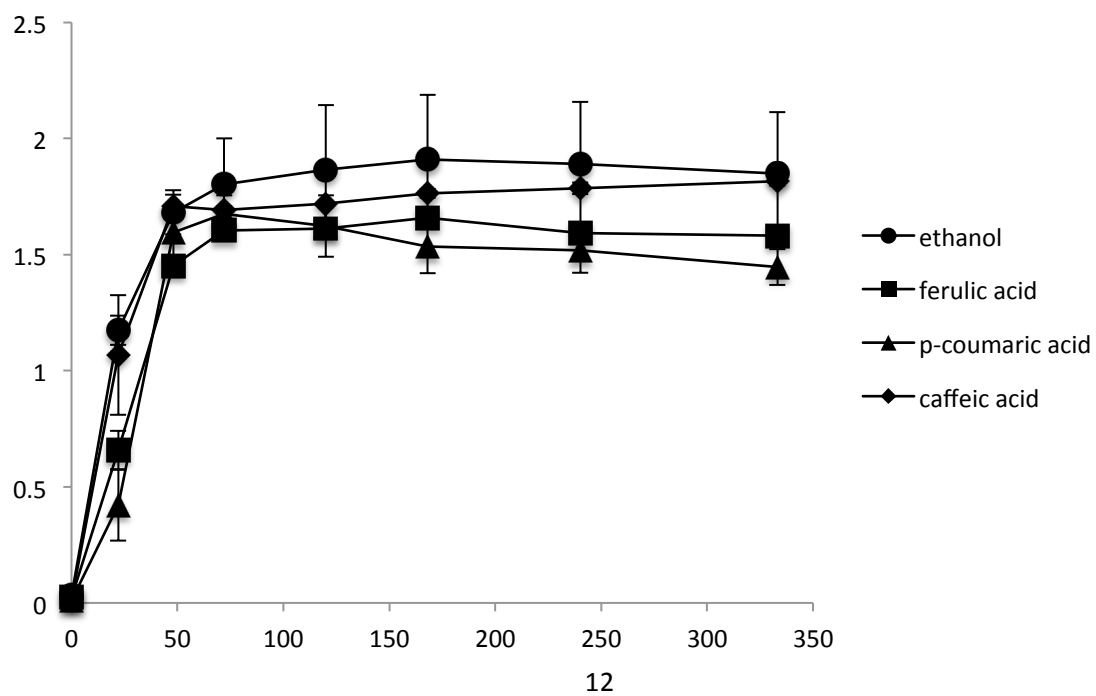
B.)



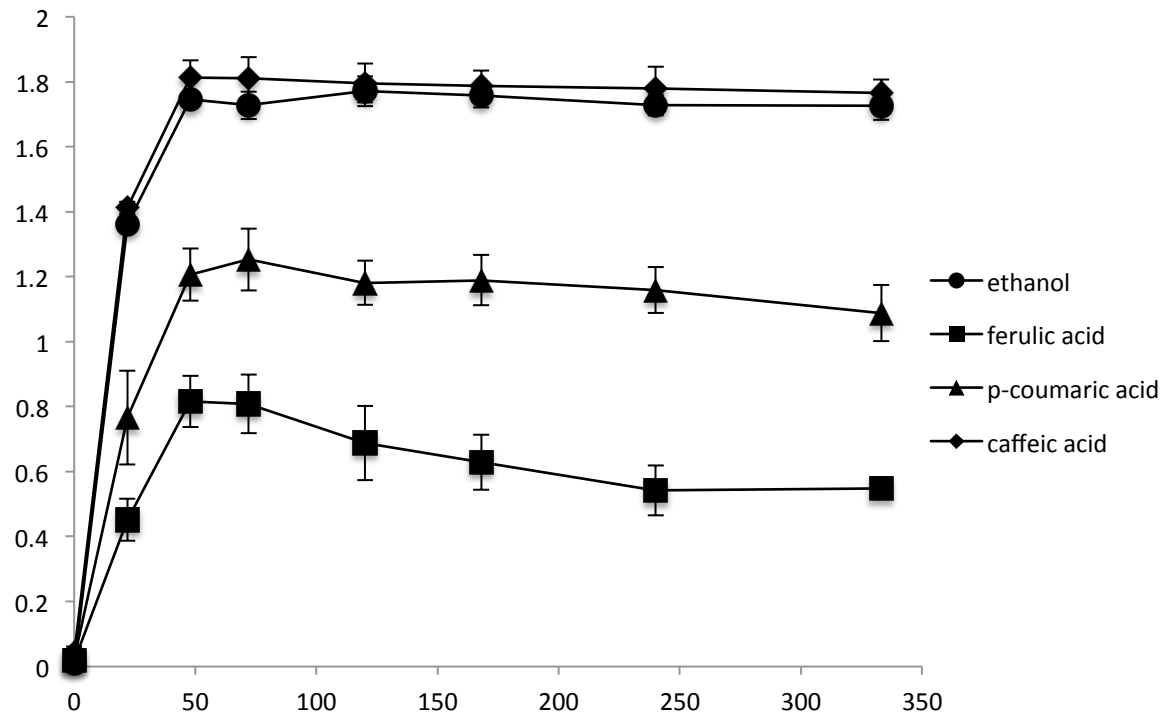
C.)



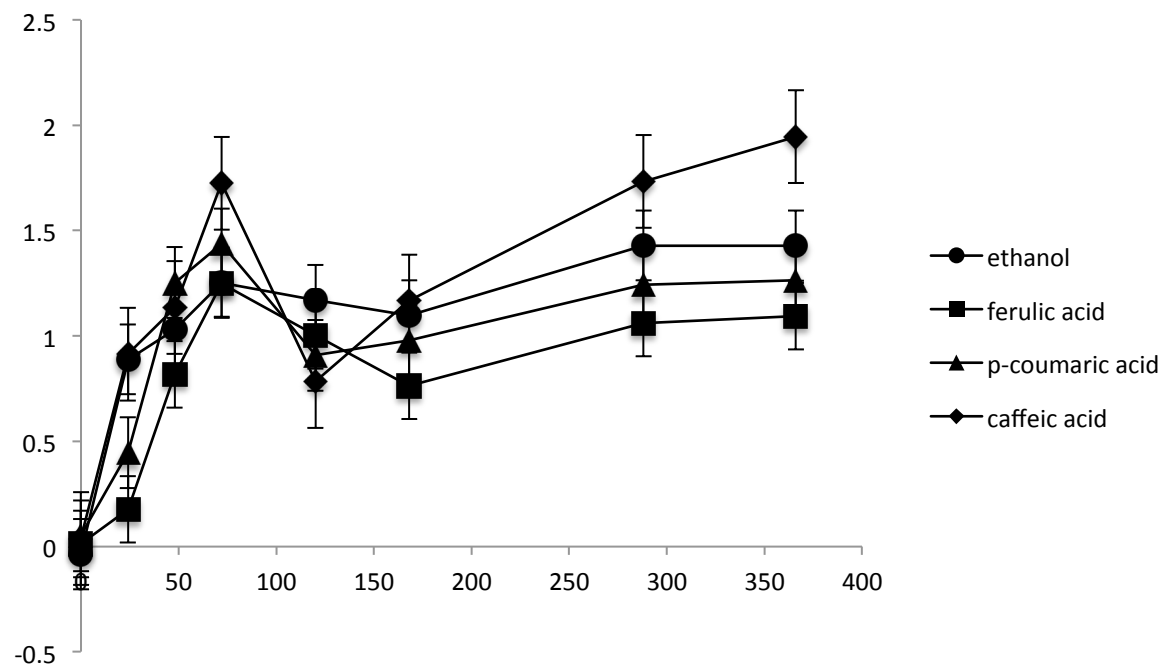
D.)



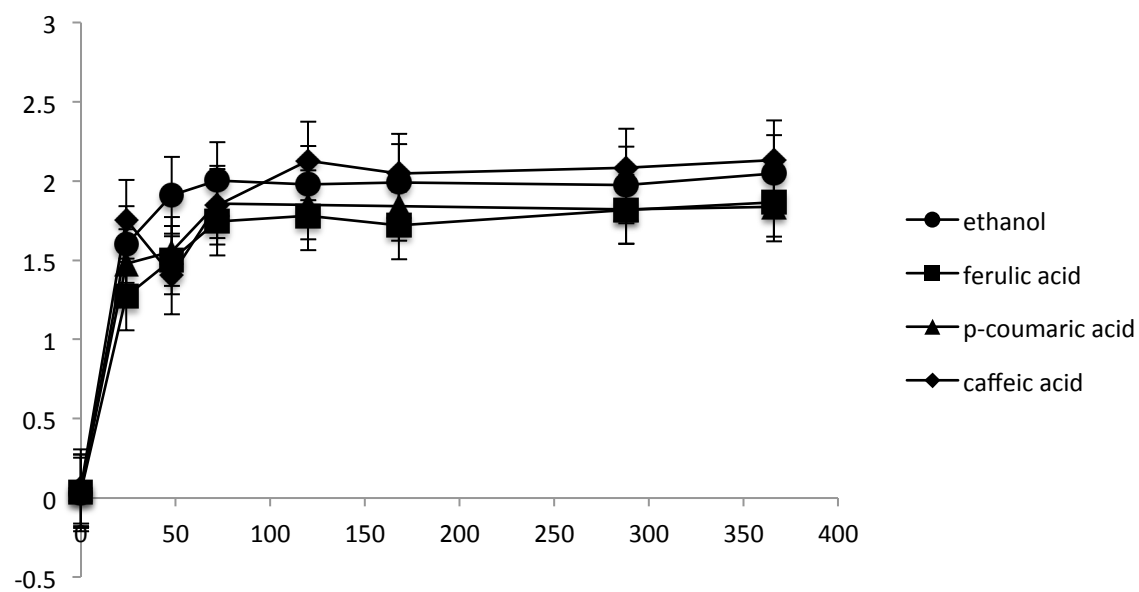
E.)



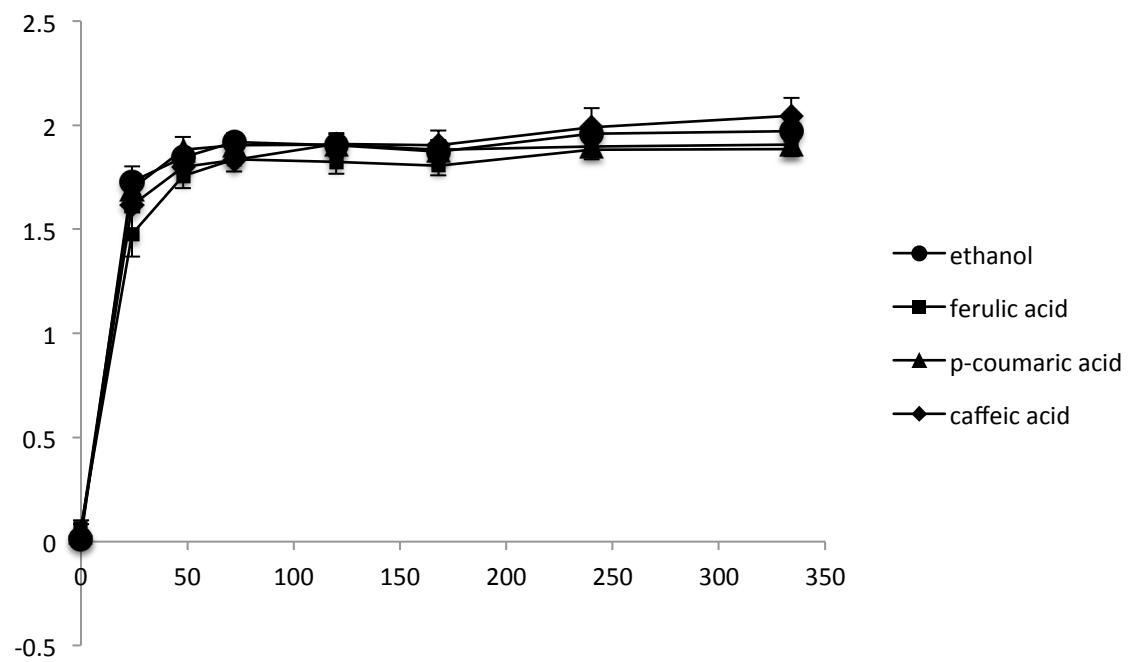
F.)



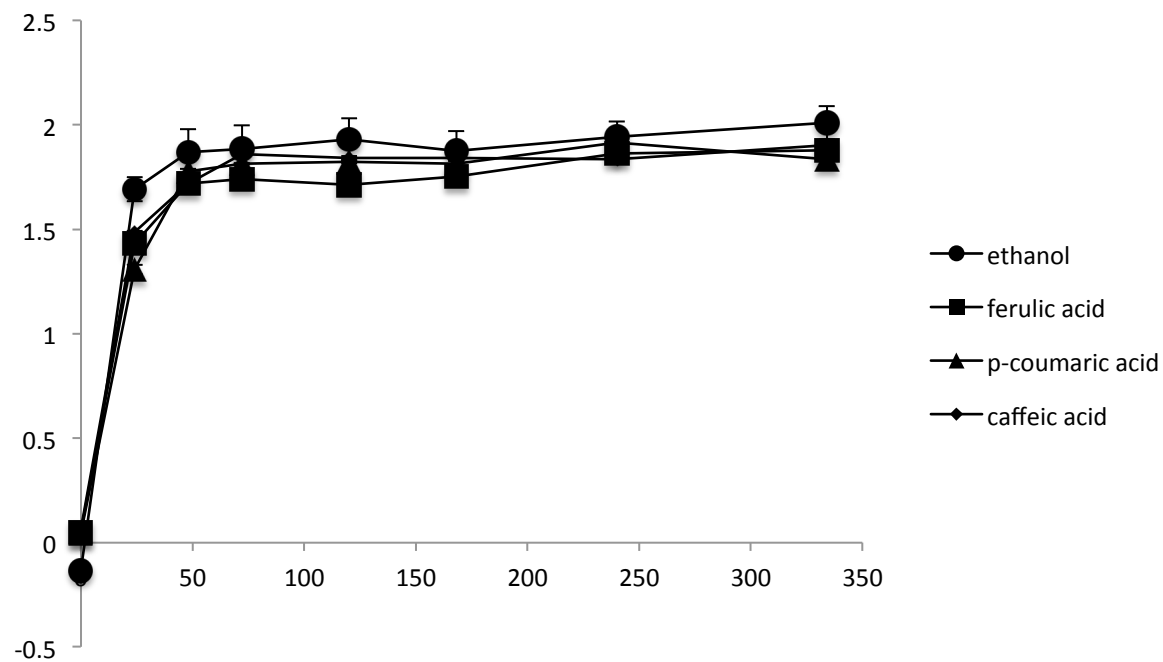
G.)



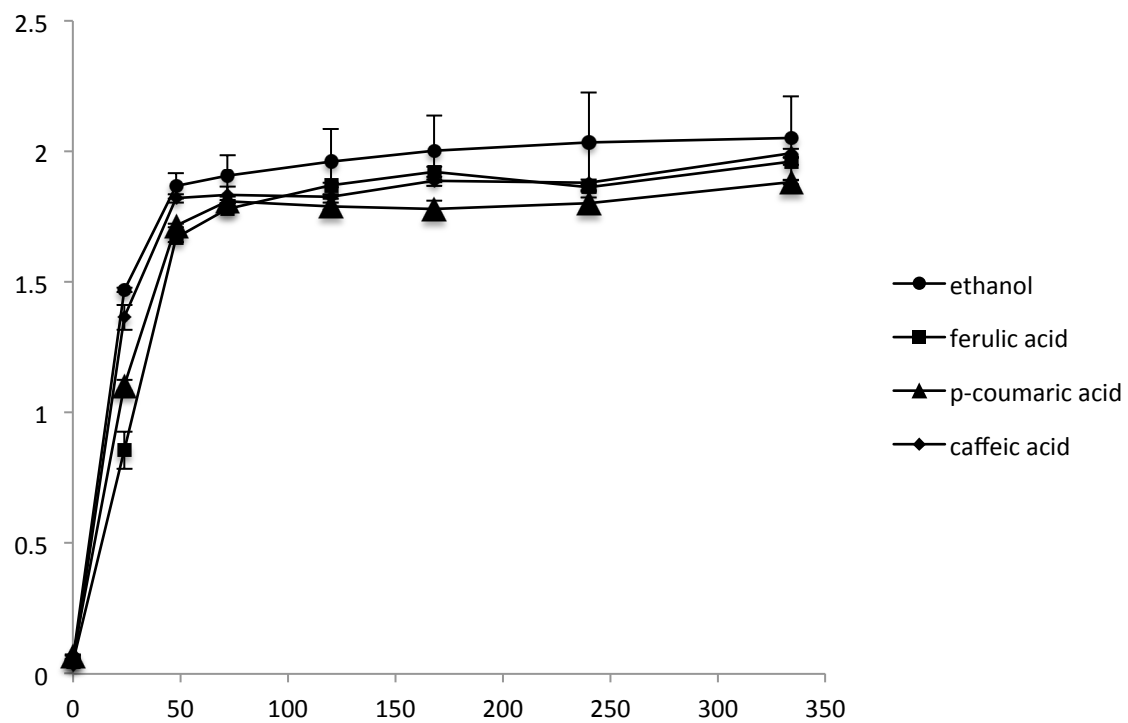
H.)



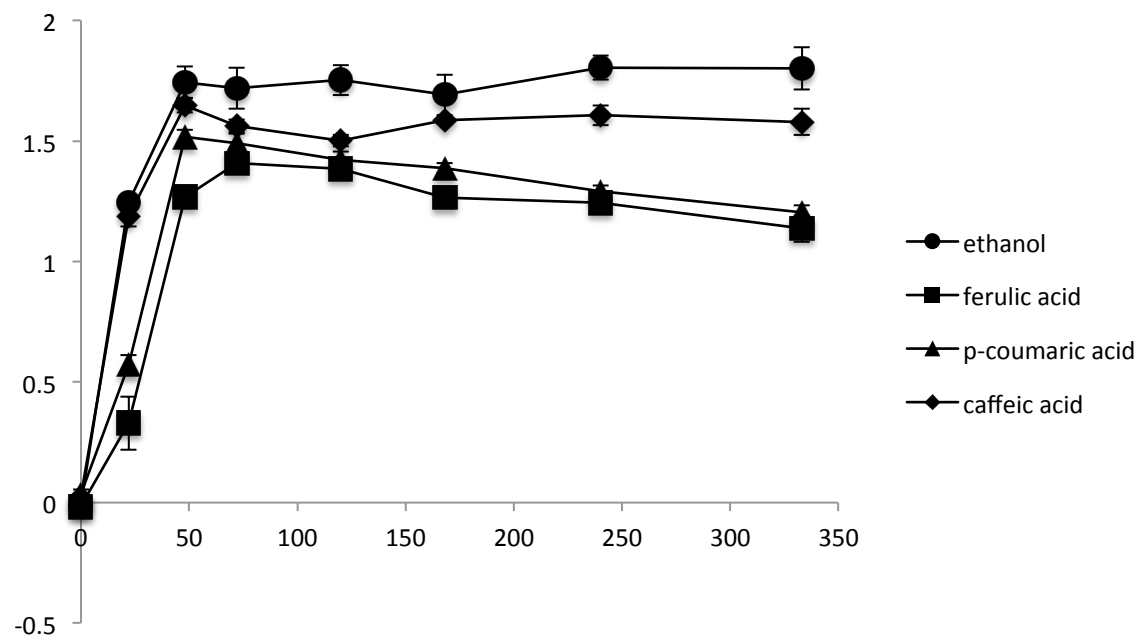
I.)



J.)



K.)



L.)

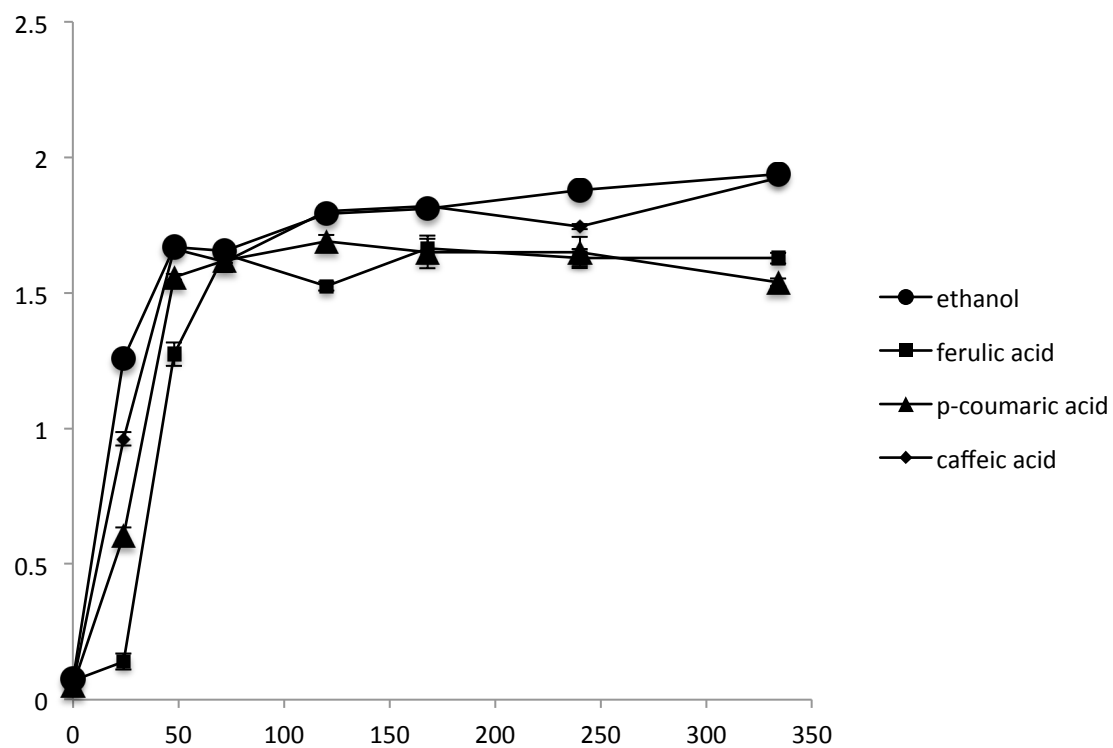


Figure 4 Growth curves of all strains grown in 5 mM HCA and ethanol as a control for 14 days with absorbance readings taken as appropriate. Y-axis: Absorbance; X-axis: hours. A.) Strain 1; B.) Strain 2; C.) Strain 3; D.) Strain 4; E.) Strain 5; F.) Strain 6; G.) Strain 7; H.) Strain 8; I.) Strain 9; J.) Strain 10; K.) Strain 11; L.) Strain 12.

Discussion

A variety of yeast strains can be naturally found on fruits skins, grains, and a range of other environments. In this study, 8 strains were isolated and characterized from several different fruits. Isolates were identified based on RFLP mapping of the 5.8S rDNA region to be either *B. bruxellensis* or *B. anomalus*, but all displayed a variety of different physiological characteristics. Some strains displayed properties appropriate for fermentation while others were deficient in alcohol tolerance (Lentz, 2014). It is well recognized that although yeast are abundant in nearly every environment, only a small amount are likely to possess the physiological characteristics for potential use in commercial brewing. Even fewer of these strains will possess favorable organoleptic properties.

Total contribution of yeast to beverage characteristic is very complex, but a simple biochemical pathway underlies some of a strains contribution to the flavor and aroma of alcoholic beverages. All plant cells contain members of a family of compounds, the HCAs. They perform a structural role in the plant cell wall and also possess antimicrobial properties. Some yeast and bacteria that live on plant matter have developed pathways to metabolize and detoxify HCAs. In *Brettanomyces* and other microbes, a two-step pathway is used to metabolize these compounds. The vinyl and ethyl compounds produced depend on the specific HCAs present in the starting plant material. Barley is rich in ferulic acid, which is known to be metabolized to compounds described as spicy, clove-like, smokey, While *p*-coumaric acid yields compounds described as plastic, bandaid, medicine. In this study *Brettanomyces* yeast isolates were grown in

varying concentrations of ferulic acid, *p*-coumaric acid, and caffeic acid to ascertain trends towards identifying differences in yeast species and good brewing strains versus poor brewing strains. These data uncover several trends towards identifying differences in good brewing strains and not good brewing strains. 12 strains were analyzed, seven of which were characterized as not good for brewing. Good brewing strains displayed trends towards having significant differences between *p*-coumaric and ferulic acid minimum inhibitory concentration. Growth curves demonstrated that good brewing strains also showed a trend towards a significant lag early in growth when exposed to *p*-coumaric and ferulic acid. It was also noted that all strains were highly resistant to caffeic acid, and good brewing strains were highly tolerant to *p*-coumaric acid.

Using UV spectral analysis of HCA containing media before and after exposure to *Brettanomyces*, it is possible to observe the PAD enzyme activity of these organisms by measuring how much of the compound remains. Analysis of each strain using each HCA displayed variation in PAD activity in all strains, however there were some trends noted. In this analysis, a trend was noted in good brewing strains, in which most displayed significant differences between the PAD activity towards ferulic acid and *p*-coumaric acid, with less ferulic acid remaining in the final media (Figure 3). This suggests that the PAD enzyme is much more reactive towards ferulic acid. Ferulic acid is the most abundant HCA in varieties of barley, but all HCAs are present in all plant cells. Ferulic acid exists in barley at a factor of 10 more than *p*-coumaric acid and a factor of 20 times more than caffeic acid, thus it can be suggested that these organisms have evolved an increased ability to metabolize ferulic acid due to its high concentration (Yu, 2001). Since barley is one of the main ingredients in beer wort, and ferulic

acid is in the greatest concentration in the HCA content of plant tissue, these data suggest that these species are more tolerant to ferulic acid and their associated PAD enzyme is much more efficient at breaking it down, making them better candidates for brewing.

There was an early lag in growth curves of good brewing strains noted between ferulic acid and *p*-coumaric acid, and ferulic acid had the most inhibiting effect (Figure 4). These data support Harris (2007) findings that HCAs showed reduced growth and 1mM ferulic acid completely inhibited the growth of some species. Ferulic acid appears to be the most toxic compound to these organisms, while their PAD enzyme seems to break down the compound more efficiently.

All strains displayed significant reduced reactivity toward caffeic acid (Table 2). Growth curves with this added compound showed no statistically significant difference between caffeic acid and the control (Figure 4). Caffeic acid appears to be the least toxic compound to these organisms. Caffeic acid is present in barley in the least abundance, which may be due to their decreased effectiveness as an anti-microbial compound (Yu, 2001).

Data also demonstrated that good brewing strains were highly tolerant to *p*-coumaric acid. This HCA is present in the second most abundance in barley. Thus once again, this may allude to these organisms having a more evolved PAD enzyme that is more efficient at breaking down HCAs, especially those in the highest abundance in barley.

There appears to be one major trend noted in this study: good brewing strains display an increased ability to break down HCAs, *p*-coumaric acid and ferulic acid in particular. These data demonstrate that an increased functionality of PAD enzyme, and thus increased PAD activity,

results in an increased fermentation profile. HCAs may have a more inhibitory effect on not-good brewing strains resulting in fermentation reduction and abnormal phenolic characteristics. It is also possible that the inhibitory effect may alter the enzymatic breakdown of HCAs resulting in off-flavors. Thus our hypothesis was supported, good brewing strains demonstrate an increase in PAD enzyme activity; however, there were no distinct difference between *B. anomalous* and *B. bruxellensis*. These differences have the potential to facilitate the discovery of new strains of yeast with an appropriate fermentation profile.

Future experiments will focus on determining if HCA inhibition is correlated to phenolic off-flavor or bad brewing potential. We have also began cloning the gene to characterize the PAD enzyme. Once an enzyme assay is developed, pH and temperature optima will be determined under a variety of conditions. Protein extracts will be mixed with different HCA substrates and monitored by UV spectroscopy. Kinetic parameters such as K_m and V_{max} will also be determined. Targeted mutations in cloned gene will ultimately be used to understand biochemical details of PAD enzyme structure and function.

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Vita

Chad Harris was born on 1992 in Florida. He was raised by his parents Cynthia and Charles Harris, and step parents Bruce Brandt and Jennifer Sword. Chad attended high school in Melbourne. While at High, he was a member of science research, AP classes, Environmental Club, and science fair.

Chad pursued a Bachelor of Science in Cellular and Molecular Biology at the University of North Florida. Chad was a member of the Dean's list, President's list, Summa Cum Laude honor roll, and served as the Biology Club President and Presidential Envoys Vice President. Chad was awarded a student research grant/scholarship, the Biomedical Bowman scholarship, and an MOACC scholarship two times. In addition to his studies, Chad was very involved in research labs involved in Atomic Force Microscopy, anti-biotic resistance, and yeast enzymatics. Chad completed his Honors Thesis, completed a thesis practicum, volunteered for Community Hospice, the Sultzbacher homeless shelter, and the Hubbard House center for domestic violence. Chad has been accepted to medical school and will be pursuing a medical degree in the fall of 2015.

